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EGFR Signaling Regulates Synaptic Connectivity Via Gurken

By

Sarah Ann Naylor

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
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of Doctor of Philosophy

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December, 2011

ABSTRACT OF THE DISSERTATION

EGFR Signaling Regulates Synaptic Connectivity Via Gurken

by

Sarah Ann Naylor

Doctor of Philosophy in Biological and Biomedical Sciences

(Developmental Biology)

Washington University in St. Louis, 2011

Professor Aaron DiAntonio, Chair

The synapse is the essential unit of neural function. It is critical to understand how synapses form during development, how they are maintained throughout the life of an organism, and how their structure and function are affected by neural activity. An understanding of these aspects of synapses will likely provide insight into the etiology of neurodevelopmental disorders such as autism, mental retardation or epilepsy. To identify novel regulators of synaptic development, I screened for mutants with defects in synaptic morphology and growth at the *Drosophila* larval neuromuscular junction (NMJ). My screen identified several mutants with defects in various aspects of synaptic development. I pursued a more in depth analysis of an identified mutant, *happyhour*, with defects in synaptic target selection. Synaptic target selection is critical for establishing functional neuronal circuits. The mechanisms regulating target selection remain incompletely understood. I describe a role for the EGF receptor and its ligand Gurken in target selection of octopaminergic Type II neurons in the *Drosophila* neuromuscular system. Mutants in *happyhour*, a regulator of EGFR signaling, form ectopic Type II

neuromuscular junctions. These ectopic innervations are due to inappropriate target selection. I demonstrate that EGFR signaling is necessary and sufficient to inhibit synaptic target selection by these octopaminergic Type II neurons, and that the EGFR ligand Gurken is the post-synaptic, muscle-derived repulsive cue. These results identify a new pathway mediating cell-type and branch-specific synaptic repulsion, a novel role for EGFR signaling in synaptic target selection, and an unexpected role for Gurken as a muscle-secreted repulsive ligand.

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Introduction

Organisms must receive, process and respond to many streams of information at all times. The nervous system is a complex combination of multiple cell types and connections that have been designed for such a purpose. One of the most critical components of this network is the connection between the cells themselves known as synapses. Synaptic transmission is critical for every activity of the nervous system and slight changes in synaptic properties can greatly influence nervous system function.

These synapses can change dramatically throughout the life of an organism. Early in development there are often more synaptic connections than are necessary and synaptic pruning will occur to refine these networks. While this pruning is taking place, still other synapses are being maintained and often strengthened. This strengthening is can be neuronal activity dependent and is the basis for nervous system functions like learning and memory. In addition to these changes to existing synapses, new synapses can be formed during the entire lifespan of an organism and in response to injury (Waites, Craig, and Garner 2005).

Despite extensive research, the fundamental processes of learning and memory have not been satisfactorily explained. To truly grasp these higher processes and how neurological and psychiatric diseases disrupt these processes, it is critical to understand neurons at their most simple functional level, the synapse. By working to understand how synapses form, grow, and how their structure and function are modulated by neuronal activity, we can further our understanding of the function of the brain at its most basic level.

Structure of the Synapse

To begin to understand complex changes in the synapse, first we must define the structure of the chemical synapse. A chemical synapse is an asymmetric cellular junction between neurons and their target cells, which can be other neurons, or muscles. A synapse requires close apposition between the presynaptic terminal and the postsynaptic terminal. The primary synapses in the central nervous system are chemical synapses, meaning that signaling between the terminals is carried out via a chemical messenger known as neurotransmitter, released presynaptically that binds to receptors on the postsynaptic cell. Neurotransmitters are packaged into vesicles that cluster around an electron dense region termed the active zone where the machinery for vesicle release is assembled. The postsynaptic terminal contains neurotransmitter receptors, ion channels, and other scaffolding proteins that cooperate to transduce the chemical signal. Cell adhesion molecules help to maintain a close apposition between pre and postsynaptic cells to ensure efficient transmission of the neurotransmitter signal.

Drosophila larval Neuromuscular Junction as a Model Synapse

In the mid-1970s the *Drosophila* larval NMJ was first described as a potential model system to study synaptic development (L. Y. Jan and Jan 1976). The ultimate goal of many neuroscientists is to gain understanding of central synapses in humans. To reach this goal, a model with molecular simplicity, accessibility and genetic flexibility would be a great tool. *Drosophila* has become a valuable model for studying synaptic development and it has been shown that vertebrate homologs of fly genes often exhibit comparable function in the vertebrate system.

The NMJ is the junction of the axon terminal of a motoneuron that projects from the ventral nerve cord and makes contact with the body wall muscle membrane. The nerve terminal contacting the muscle develops specialized varicosities called boutons that contain hundreds of individual synapses (Atwood, Govind, and Wu 1993). These boutons are enveloped by a post-synaptic specialization, the subsynaptic reticulum. The subsynaptic reticulum consists of membrane folds surrounding the boutons, and is believed to be the site of clustered postsynaptic receptors (Lahey et al. 1994). In the past, studies have referred to this entire nerve terminal as a synapse. It is more common to refer to this entire structure as the nerve terminal or the NMJ and individual pairings of presynaptic release machinery and postsynaptic receptor clusters as synapses.

Drosophila has an extensive history as a genetic organism, and as a consequence, a well-stocked genetic toolbox to identify molecular components of synaptic development. Forward genetic screens facilitate identification of genes responsible for a phenotype of interest. The function of genes can be tested via reverse genetic techniques such as RNAi or dominant negative transgenes. The Gal4/UAS system refines such techniques by providing tissue specific expression of transgenes that can modulate a gene's function (Brand and Perrimon 1993). This tool allows study of essential genes. Tissue specific Gal4 drivers allow the study of synaptic function of a gene separately in the neuron or muscle. Also of great value is the large collection of readily available mutants to facilitate testing for genetic interactions.

The *Drosophila* 3rd instar larva is composed of repeating segments each with a stereotyped pattern of muscles and innervations of those muscles (Lahey et al. 1994).

This strict patterning and repetition allows us to quantify subtle changes in the development of this pattern and also increases the power of our observations by providing a larger number of sample NMJs in each individual larva (Bate and Broadie 1995). The *Drosophila* NMJ is a glutamatergic innervation similar to those found in the mammalian central nervous system. The NMJ shows developmental and activity dependent plasticity, which is also similar to mammalian synapses.

There are four types of motoneurons that innervate the *Drosophila* body wall musculature: Type Ib, Is, II and III (Prokop 2006). The Type Ib and Is motoneurons are glutamatergic and responsible for muscle contraction. Type II motoneurons are octopaminergic and perform a neuromodulatory role. Type III neurons secrete insulin-like peptide and sparsely innervate the body wall. Each of these neurons forms synapses with body wall muscle cells. The Type I motoneurons are the most well studied. My work is focused on the Type II neurons.

Type II octopaminergic motoneurons have long been observed but relatively little has been done to uncover the physiological role of these neurons. Initially, octopamine was known to function as a neurotransmitter/modulator/hormone with a number of functions in other invertebrates. For example, octopamine can participate in neuromodulation of the escape response of crayfish, and excitatory modulation of somatic and visceral muscles. Immunohistochemistry revealed that octopamine was localized to a number of specific areas (midline of ventral ganglion, peripheral fibers that innervate most of the body wall muscles) in *Drosophila* larvae (Monastirioti et al. 1995). These Type II neurons can be labeled with several specific reagents: SSB antibody (Budnik and Gorczyca 1992), Octopamine antibody (Monastirioti et al. 1995), Vesicular

Monoamine Transporter (necessary to package octopamine into synaptic vesicles) antibody (Greer et al. 2005), Tyrosine Beta-Hydroxylase (enzyme necessary to produce octopamine) antibody (Koon et al. 2011) as well as the Tyrosine Decarboxylase-2 Gal4 line (Tdc2-Gal4) which expresses specifically in these Type II neurons.

The role of these neurons in *Drosophila* is still being established. Recent work has established a role for these neurons in synaptic and behavioral plasticity. Octopamine is known to play a role in appetitive behaviors and locomotion. Upon starvation, *Drosophila* larvae exhibit an increased rate of locomotion that may be modulated by Type II octopaminergic synapses. These larval Type II innervations generate dynamic filopodia like extensions in response to the starvation stimulus. This change in locomotive behavior requires these octopaminergic neurons (Koon et al. 2011). This represents a situation in which Type II neurons are modulating both synaptic and behavioral plasticity to a given stimulus. Both Type I and Type II NMJs express octopamine receptors on their surface and both respond to octopamine with synaptic growth. This is yet another mechanism by which Type II neurons can influence synaptic plasticity.

Guidance and Target Selection of Larval Motoneurons

All motoneurons begin development as neuronal cell bodies in the ventral cord. These neurons must extend an axon out of the ventral nerve cord and project to the posterior of the animal. These axons encounter a large number of guidance molecules from their initial exit from the ventral nerve cord until they reach their specific muscle target. This whole process takes only 6 hours from the first extension of the axons until

functional NMJs are formed (J. Johansen, Halpern, and Keshishian 1989). Some examples of known guidance molecules include netrins and their receptors DCC or Unc5, slits and their receptor robo, ephrins and eph receptors, semaphorins and plexins. Most of these molecules can act as attractants or repellants depending on the context and the expression of receptors. In addition to these major guidance players there are several other known factors that influence axon guidance, developmental morphogens such as BMP, Wnt, hedgehog and FGF. Lastly, extracellular matrix proteins and adhesion molecules can play a large role in guiding the extending growth cones by providing permissive or non-permissive substrates for innervation.

Once axons utilize a complement of these pathways to extend to the proper segment and approach their target muscle field, another more specific type of guidance must occur to provide synaptic specificity. These axons must specifically choose their synaptic partners reproducibly and specifically direct synapse formation onto subcellular compartments of their synaptic partners. One mode of target selection is mutual attractive recognition through cell adhesion or cell surface molecules. This mode of target identification involves direct recognition and homotypic interactions between cell adhesion molecules on the extending neuron and its target. In *Drosophila*, neurexin/neurologin, Eph/ephrin, and SynCAM are capable of inducing specific synaptic connections.

In addition to attractive cues, there are also repulsive cues to refine synaptic target selections. While there are significantly fewer of these pathways delineated, repulsive guidance is an important complement to the attractive pathways. One example of this in *Drosophila* is Wnt4. In the *Drosophila* body wall there are two neighboring muscles M12

and M13 which are innervated by MN12s and RP1/4 neurons, respectively. Wnt4 is expressed by M13 muscles but not M12 muscles. Wnt4 loss of function mutants exhibit inappropriate synapse formation of MN12s neurons onto M13 muscles and ectopic expression of Wnt4 in M12 muscle inhibited MN12s neuron synapses (Inaki et al. 2007). This repulsive pathway acts in conjunction with attractive cues to refine synaptic specificity between two adjacent muscle fibers.

Gradients of cues can guide axons outward to their target muscle field but they can also play a role in generating synaptic specificity. In *Drosophila*, olfactory receptor neurons (ORNs) that express a specific olfactory receptor all project their axons to the same glomerulus in the brain. Projection neurons (PNs), the postsynaptic partners of ORNs, independently project their dendrites in a spatially defined map to the antennal lobe before the ORNs innervate those areas. Graded expression of Semaphorin1a by the PNs is necessary for proper dendritic targeting of the ORN projections (Komiya et al. 2007).

One last example of synaptic specificity control involves control at the level of transcription. At the *Drosophila* NMJ, the transcription factor Tey regulates synaptic specificity by repressing the expression of repulsive cell surface molecule Toll (Inaki et al. 2010). Tey is specifically expressed in M12 muscles but not M13 muscles resulting in the down-regulation of Toll expression in M12 muscles but not M13 muscles. Loss of Tey leads to upregulation of Toll on M12 and inhibition of synapse formation. Conversely, misexpression of Tey in M13 causes ectopic innervation on M13 muscles. There are a number of converging pathways that govern synaptic specificity and within these pathways are a number of mechanisms critical for providing such specificity.

Synaptic Components

A chemical synapse comprises pre-synaptic and post-synaptic components. Pre-synaptically, neurotransmitter-filled vesicles cluster at and are released from specializations called active zones (Inaki et al. 2007). This specialization involves the clustering of several different proteins, which contribute to the calcium-dependent fusion of the synaptic vesicles (Fejtova and Gundelfinger 2006). These active zones must have post-synaptic clusters directly opposite in order to maintain the fidelity of signaling. This very coordinated and precise apposition of active zone and post-synaptic receptor clusters is essential for rapid and efficient synaptic transmission and determines the efficacy of the particular synapse (R J Kittel, Hallermann, et al. 2006).

Each of the individual synapses at the synaptic terminals of the NMJ contains a presynaptic release site referred to as the active zone. Two known proteins present at the pre-synaptic active zone in *Drosophila* synapses are Bruchpilot (Brp) and Cacophony (Cac). Brp is a member of the ERC/CAST family of proteins, which localizes to the active zones of various synapses and binds to other active zone proteins (Deguchi-Tawarada et al. 2004; Ko et al. 2008; Ohtsuka et al. 2002; Y. Wang et al. 2002). Additionally, Brp is a direct component of the T-bar (Fouquet et al. 2009), an electron dense structure that may be associated with increased release probability at the active zone.

One of the primary components of the post-synaptic density is the glutamate receptor subunit DGluRIII (Marrus et al. 2004), a necessary subunit of the glutamate receptor complex. In cases of limiting DGluRIII levels, these receptors cluster

preferentially opposite the largest active zones, which have a higher release probability (Marrus and DiAntonio 2004). This supports a model of activity dependent correlation between the post-synaptic density and the size of the apposed active zone. It is possible that neurotransmitter release promotes the clustering of DGluRIII, and that active zones with higher release probability accumulate more DGluRIII. Alternatively, there could be a retrograde specification of presynaptic release properties.

A large number of genes are necessary to regulate and influence the process of a neuron extending its axon and growing toward its specific target and subsequently forming a functional synapse onto its target. Therefore, it is not surprising that there are many mutants altered synaptic development in the *Drosophila* larvae. The following portion will focus on specific genes that play a role in synaptic development.

Happyhour is a MAP4k with many functions

The gene *happyhour* (*hppy*) has recently been discovered for its role in the ethanol response of *Drosophila*. When exposed to ethanol, *hppy* mutant flies are resistant to intoxication (Corl et al. 2009). *Hppy* is predicted to encode protein with an N-terminal serine/threonine kinase domain and a C-terminal citron-homology domain. The closest mammalian homologs of *Hppy* are members of the Germinal Center Kinase-1 (GCK-1) family of Ste20-related kinases, including GLK (Germinal Center-like Kinase) and GCK itself (Dan, Watanabe, and Kusumi 2001; Findlay et al. 2007). GCK-1 family members have been shown to act as mitogen-activated protein kinases (MAPKs) upstream of the Jun N-terminal kinase (JNK) signaling pathway (Chen and Tan 1999). *Hppy* has also been shown to be a pro-apoptotic kinase through its activation of JNK (Lam et al. 2010).

While generally it is known that *hppy* is a MAP4K, there is still very little known about this kinase and its role in vivo.

Epidermal Growth Factor Receptor (EGFR)- a molecule with many roles throughout development

The *Drosophila* EGFR is the single member of the EGFR/ErbB family in the fly genome and is very similar to mammalian ErbB family members in its structure. Extracellularly, it has the typical four domains including two cysteine-rich domains required for ligand binding. There are at least 30 known roles for EGFR signaling during *drosophila* development (Shilo 2003). These roles range from early embryogenesis and patterning to much later patterning in the nervous system and appendages.

In order to regulate such diverse signaling events, EGFR has five different ligands, four activating and one inhibitory. Three of the ligands, Spitz, Keren and Gurken are produced as transmembrane precursors. The primary activating ligand is Spitz which is responsible for activation in most tissues (Rutledge et al. 1992). The activated form of Spitz is produced by tightly regulated cleavage of the membrane bound precursor (Schweitzer et al. 1995). A ligand, Keren, that is structurally related to Spitz has recently been identified (Reich and Shilo 2002; Sinisa Urban, Lee, and Freeman 2002). A fourth activating ligand, Vein, is a secreted ligand that has an inherently weaker activation capacity than the other 3 activating ligands (Schnepp et al. 1996). In some tissues, Vein functions as the main ligand; for example, Vein induces muscle cell attachment fate (Yarnitzky, Min, and Volk 1997). It can also be used as positive feedback reinforcement to initial activation of EGFR by another ligand. Lastly, Argos functions as a secreted

ligand that binds the receptor but inhibits activation by competing with the activating ligands (M Freeman et al. 1992). It is induced in response to activation of the EGFR and plays a major role in restricting the activation range of the activating ligands (Golembo et al. 1996).

Ligand processing is a key regulatory step in EGFR signaling regulation. This processing has been shown to modify the Spitz ligand and was subsequently found to also be valid for processing the other membrane tethered ligands. Spitz is produced as an inactive membrane precursor and is ubiquitously expressed (Schweitzer et al. 1995). It has a relatively high turnover rate in order to prevent nonspecific cleavage from taking place within the cell (J R Lee et al. 2001). The regulation of Spitz processing was further informed upon finding mutations in two genes that give rise to similar phenotypes as loss of function of *spitz*. These genes are *Star* and *rhomboid* (Mayer and Nüsslein-Volhard 1988). *Star* regulates the trafficking of Spitz from the endoplasmic reticulum to the Golgi compartment though its function as a type II transmembrane protein that functions as a cargo receptor for Spitz (Kolodkin et al. 1994).

The second gene responsible for ligand processing of Spitz, Keren, and Gurken is *rhomboid*. Rhomboid is a seven-transmembrane protein that functions as the protease for Spitz cleavage (S Urban, Lee, and Freeman 2001). The catalytic domain of Rhomboid is within its conserved transmembrane domains which leads to regulated intramembrane proteolysis (Brown et al. 2000). This cleavage takes places within the Golgi rather than on the plasma membrane. Rhomboid expression is extremely dynamic and synonymous with EGFR activation (Golembo, Raz, and Shilo 1996; Sturtevant, Roark, and Bier 1993). Expression of Rhomboid is the limiting step in EGFR activation.

EGFR appears to provide a relatively short-range signaling pathway between cells. It also can occasionally participate in some short range signaling several cells away from the source. In most cases, activation of EGFR serves as a binary switch either in the adjacent cells or the ligand secreting cell itself. There are three identified negative feedback circuits that have been identified to be critical to maintaining this tight restriction of EGFR activation. As mentioned previously, the Argos ligand is a component of the negative feedback that is induced upon ligand binding. Argos is induced in the cells receiving high levels of EGFR ligand and can exert influence up to several cells away from the source (Golembo et al. 1996). Argos acts as a competitive inhibitory ligand on the source cell and on cells further away. The distribution profiles of the activating ligand and Argos are likely inverse, with activating ligand highest at the source cell and Argos highest several cells away maintaining this refined EGFR activation.

The other feedback circuits are cell autonomous and less universally used than Argos. Kekkón is a transmembrane protein that binds EGFR and prevents receptor dimerization and consequently, activation of EGFR (Ghiglione et al. 1999). Sprouty is an intracellular protein that can interfere with EGFR signaling at several levels (Casci, Vinós, and Freeman 1999). Sprouty interacts with signaling elements that are shared by other receptor tyrosine kinases. Sprouty activity can therefore disrupt the function of multiple signaling pathways. The inhibitors Yan and DCbl are not transcriptional targets of EGFR and are not activated in response to EGFR signaling. Yan competes with Pointed which is a transcription factor that is triggered by EGFR activation preventing activation of negative feedback via Argos, and activation of other genes (Gabay et al.

1996). DCbl exerts its inhibition by facilitating endocytosis of the EGFR and subsequent degradation (Pai, Barcelo, and Schüpbach 2000).

The main intracellular signaling pathway activated by EGFR is the Ras/MAPK pathway. Mutations in the intracellular components of the Ras/MAPK pathway phenocopy loss of EGFR or ligands (Diaz-Benjumea and Hafen 1994). Activated Ras can mimic EGFR gain of function phenotypes (Brand and Perrimon 1994). However, there is an exception to Ras/MAPK activation being the only output of the EGFR. In most cases, transcriptional activation based on the average of EGFR activation around the cell is the final read out. In cases of cell migration, the location of the receptor that has been activated is critical and the final response is likely to be local rather than transcriptional (Duchek et al. 2001). This suggests that in the case of cell migration EGFR is acting through a different yet unknown signaling pathway.

Gurken is a regulator of oocyte development

A homolog of transforming growth factor-alpha (TGF-alpha) encoded by the *gurken* gene can initiate polarity along both the dorsal ventral axis and the anterior posterior body axis (González-Reyes, Elliott, and St Johnston 1995; Roth et al. 1995). Briefly, in midoogenesis the nucleus of the oocyte is positioned posteriorly and produces the *gurken* signal that activates a program in the nearest follicle cells by activating the EGFR receptor *torpedo*, that instructs them to acquire a posterior fate. These posterior cells then signal back to the oocyte to induce polarization of the oocyte's anterior-posterior axis. Later, the oocyte nucleus moves to an anterior corner of the oocyte and again produces a *gurken* signal that activates the nearest follicle cells via EGFR and they

take on a dorsal identity. These dual roles of Gurken were quite surprising as many developmental biologists at the time assumed that each of the axes were determined by separate signaling pathways and the specification was not interdependent. Gurken is tightly regulated at several levels including localized transcription as well as regulatory sequences that restrict gurken mRNA localization and concentration of Gurken protein. Gurken has only been shown to be localized to the developing oocyte and a role for Gurken outside the oocyte has never been described.

Summary

While many studies have examined various regulators of synaptic development, the full repertoire of processes responsible have not yet been discovered. In the following chapters, I perform a forward genetic screen to identify novel regulators of synaptic development. I characterize the role of EGFR signaling in regulating synaptic target selection of Type II neurons via its ligand Gurken. These findings have contributed to our understanding the pathways involved in synaptic target selection and have highlighted an unusual and unexpected role for Gurken in this process. In addition, this work has laid the foundation for future studies by identifying other genes that also play an important role in synaptic development.

Chapter 2

Genetic Analysis of Synaptic Development

SAN directed and performed the majority of this work. SAN was assisted by Dominic Berns on the screen.

A portion of this work appears in the manuscript:

Valakh, V., Naylor, S.A., Berns, D.S., DiAntonio, A. Large-scale RNAi screen reveals novel regulators of synapse development. *Developmental Biology*: In Revision

Abstract

The synapse is the essential unit of neural function. It is critical to understand how synapses form during development and are maintained throughout the life of an organism as well as how their structure and function are affected by neural activity. An understanding of these underpinnings of synapse development will provide insight into the etiology of neurodevelopmental disorders such as mental retardation or epilepsy. In order to uncover these mechanisms, I undertook a forward genetic screening approach to identify mutants with defects in synaptic development. I took advantage of the tight apposition between presynaptic release machinery and postsynaptic receptors and stained for markers of each of these structures, Bruchpilot and DGluRIII respectively. These markers allow examination of a number of phenotypes in addition to synaptic apposition. We utilized these antibodies to screen several collections of mutants including EMS mutants, piggyBac insertional mutants and transgenic RNAi lines. From these lines I have performed further analysis on three independent mutants. piggyBac insertional mutant 141-604 was found to have a defect in synaptic apposition. With genetic analysis, I was able to confirm that this phenotype was due to a second site mutation in the *bruchpilot* gene. I also performed genetic mapping on an EMS-induced mutation provisionally named MA3.5 which exhibited extensive axonal clogs of Bruchpilot protein. I found that this phenotype is due to a mutation in *Beta-Spectrin*. Lastly, I have characterized a hit from the RNAi screen, *happyhour*, in more detail. This analysis of *happyhour* is the focus of Chapter 3.

Introduction

A chemical synapse is composed of pre-synaptic and post-synaptic components. Pre-synaptically, neurotransmitter-filled vesicles cluster at and are released from specializations called active zones (Chen and Tan 1999). This specialization involves the clustering of several different proteins, which contribute to the calcium-dependent fusion of the synaptic vesicles (Fejtova and Gundelfinger 2006). These active zones must directly oppose post-synaptic clusters in order to maintain the fidelity of the signal. This very coordinated and precise apposition of active zone and post-synaptic receptor clusters is essential for rapid and efficient synaptic transmission and determines the efficacy of the particular synapse (R J Kittel, Hallermann, et al. 2006). To uncover what genes are necessary to establish and maintain this apposition, we undertook a forward genetic screen.

Our forward genetic screen used the easily accessible and genetically malleable *Drosophila melanogaster* NMJ. Terminals called boutons contain hundreds of individual synapses (Atwood, Govind, and Wu 1993). The *Drosophila* 3rd instar larva is composed of repeating segments each with a stereotyped pattern of muscles and innervations of those muscles (Collins and DiAntonio 2007). This strict patterning and repetition allows us to quantify subtle changes in the development of this pattern and also increases the power of our observations by providing a larger number of sample neuromuscular junctions (NMJs) in individual larvae (Bate and Broadie 1995).

Each of the individual synapses at the synaptic terminals of the NMJ contains a presynaptic release site referred to as the active zone. Two known proteins present at the

pre-synaptic active zone in *Drosophila* synapses are Bruchpilot (Brp) and Cacophony (Cac). Brp is a member of the ERC/CAST family of proteins, which in other organisms including *drosophila* has been shown to localize to the active zones of various synapses and to bind to other active zone (Deguchi-Tawarada et al. 2004; Ko et al. 2008; Ohtsuka et al. 2002; Y. Wang et al. 2002). Brp is a direct component of the T-bar (Fouquet et al. 2009), an electron dense structure that may be associated with increased release probability at the active zone. One of the primary components of the post-synaptic density is the essential glutamate receptor subunit DGluRIII (Marrus et al. 2004). The schematic in Figure 2.1A illustrates the spatial relationship between these two proteins.

The precision of apposition can be exploited in mutagenesis screens to identify molecules involved in active zone assembly and synaptic development. Previously, the lab performed a forward genetic screen using a library of p-element insertional mutants to look for mutants that disrupt the synaptic alignment. These screens were performed by staining individual mutant lines with an antibodies specific to Brp and DGluRIII and examining the larvae for any defects in expression (Figure 2.1B). These screens were successful in identifying several different genes critical to synapse development (Wairkar et al. 2008; Wairkar et al. 2009; Graf et al. 2009). While these previous screens did identify some critical and previously unknown contributors to active zone alignment, they have been limited by technical restrictions. One limiting factor is the insertional mutant library itself. P-elements have an insertion preference near the 5' end of genes, which limits the number of genes that are actually disrupted by the insertion. Additionally, the P-element collection has not reached saturation on the second and third chromosomes.

A new library of insertional mutants

To address these deficiencies in the P-element collection, a new collection of insertional mutants has been made by the Luo lab at Stanford (Schuldiner et al. 2008). This library takes advantage of a different transposable element, the piggyBac. The piggyBac transposon was originally isolated from the cabbage looper moth. This element acts similarly to a P-element in that in the presence of its transposase it is able to move about within the genome. Differing from P-elements, these piggyBac insertions excise cleanly and do not have the same insertion preference for the 5' end of genes. The Luo lab has created a modified version of the piggyBac transposon that has been altered to enhance its mutagenic capabilities. This version contains splice acceptors followed by stop codons in all three frames and in both orientations of the transposon. This will enhance mutagenicity by splicing into transcripts and terminating them rather than by inserting lengthy sequence. It also carries a marker, DsRed, driven by an eye promoter (3XP3) to provide an easily visualized live marker of the transposon.

EMS and RNAi knockdown as additional screening tools

In addition to these pBac mutants, I also generated a collection of EMS mutants to screen for synaptic development defects. EMS creates random point mutations throughout the genome. These EMS mutations provide a set of mutants that are divergent from other insertional mutants that have insertional bias. I've also taken advantage of the collection of RNAi transgenic flies from the VDRC stock center. I screened a specific collection of RNAi lines, by expressing the RNAi and Dicer2 (known to enhance the efficacy of RNAi knockdown) specifically in post-mitotic neurons using

the ElavGal4 driver. These lines allowed me to test genes that might have caused severe developmental defects if disrupted in the entire animal.

These three collections of mutants provide a new and untapped resource to uncover mutations that disrupt the establishment and maintenance of the active zone and post-synaptic density in *Drosophila*. By adapting a screen that has proven fruitful in other mutant collections, I have been able to uncover a collection of new mutants critical to the active zone and its apposition to the post-synaptic density. It is likely that multiple signaling pathways may be involved in this complex process and this collection may provide means to uncover their roles. There are likely a great number of proteins involved in these processes and so far only a handful have been found and examined, suggesting that there are a great number left to be found. By delineating the pathways involved in establishing the active zone and guiding synaptic development, we are enabling ourselves to more clearly identify situations where the synapse is improperly formed or functioning, which may be a leading cause of neurodevelopmental dysfunction. This will begin to uncover how we think, learn and remember throughout our lives.

Materials and Methods

Fly Stocks

Flies were maintained at 25°C on standard fly food. Wild type (WT) flies were Canton S (CS) unless specified otherwise. The following flies were obtained from the Bloomington StockCenter: $w^{118}; P\{hs-hid\}2$, wg^{Sp-1}/CyO , $w^{118}; P\{hs-hid\}3$, $Dr^1/TM6B$, Tb^1 , $al[1]dp[ov1] b[1] pr[1] c[1] px[1] sp[1], y[1]cv[1]v[1]f[1]car[1], t[1]v[1]m[74f]f[1]$. The

pBac collection was supplied by the Kyoto Drosophila Genome Resource Center (DGRC) who maintains the pBac insertional library made by the Luo Lab. The collection of KK RNAi lines were supplied by the Vienna DGRC.

Immunohistochemistry

Third-instar larvae were dissected in PBS and fixed in either Bouin's fixative for 5 min. at room temperature (RT) or for 30 min on ice. Larvae were washed with PBS containing 0.1% Triton-X-100 (PBT) and blocked in 1% NGS in PBT for 30 min. This was followed by overnight incubation in primary antibodies in 1% NGS in PBT and three washes in PBT. Washes were followed by incubation in secondary antibodies in 1% NGS in PBT for 45 min, three final washes in PBT, and equilibration in 70% glycerol in PBS. Samples were mounted in Vectashield (Vector, Burlingame, CA). The following primary antibodies were used: mouse anti-nc82 (Brp) (Wagh et al. 2006) 1:250, rabbit anti-DGluRIII (Marrus et al. 2004) 1:2500, rabbit anti-Pak 1:2000. Goat Cy3- and FITC conjugated secondary antibodies against mouse and rabbit IgG were used at 1:1000 and were obtained from Jackson ImmunoResearch. Antibodies obtained from the Developmental Studies Hybridoma Bank were developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences of the University of Iowa, Iowa City, IA.

Imaging and Analysis

Samples were imaged with a Nikon D-Eclipse C1 confocal microscope using 60X oil

objective. Shown images are z projections of confocal stacks acquired from serial laser scanning.

Results and Discussion

In order to identify new pathways involved in synaptic development and synaptic apposition, we performed a forward genetic screen on a library of piggy-Bac insertional mutants. We dissected third-instar larvae from each line and stained for a marker of the presynaptic active zone Bruchpilot (Brp) (Wagh et al. 2006) and the essential glutamate receptor subunit DGluRIII (Marrus and DiAntonio 2004). We identified lines with defects in synaptic development including axon transport defects, synaptic targeting defects, active zone defects, and NMJ morphology defects.

The pBac library included 1795 independent insertion lines. Of these lines, 344 exhibited larval lethality and were therefore not screened for synapse defects. 22 lines were found to have a synapse relevant phenotype in a number of categories. These categories are listed in Table 1.

Synaptic Apposition, Active Zone number-1
Overall Size of NMJ - 5
Bouton Size, Number, or Shape - 5
Axon Transport - 4
Axon Structure and Ventral Nerve Cord - 3
Other - 4

Table 1. Phenotypic categories of hits from piggyBac screen. Numbers indicate individual mutant lines exhibiting that phenotype

Of these mutants, we chose the mutant 141-604 from the category “Synaptic Apposition, Active Zone number” to investigate further. We chose this mutant because it has a synaptic apposition and active zone phenotype without any general defect of the NMJ (Figure2.2A). The phenotype is specific to active zone development and less likely to be

due to a developmental defect. The other categories of mutants have more general overall defects and may be due to genes that are involved in general development rather than specific to active zone development.

We found 141-604 due to a decrease in overall Brp levels as well as a decrease in the number of Brp puncta. In addition to this phenotype, the DGluRIII staining is more diffuse in mutants. We quantified several aspects of these phenotypes. We found significant changes in three categories of measurement (density of Brp puncta per DGluRIII area, average area of Brp puncta, average intensity of Brp puncta Figure 2.2B-D). Due to the decrease in Brp puncta number we observed a defect in synaptic apposition in which DGluRIII clusters were unapposed by presynaptic Brp. To determine whether this defect is truly a defect in apposition and not a consequence of the change in DGluRIII localization we described, we examined a second postsynaptic marker. The kinase Pak is also a marker of the postsynaptic density. We also found a defect in apposition between Brp and Pak (Figure 2.3A,B) this indicates that the apposition defect is not a consequence of a change in glutamate receptors.

A defect in apposition can lead to defects in synaptic transmission. To test whether 141-604 exhibits a defect in synaptic transmission we performed electrophysiological recordings. Miniature excitatory junction potentials (mEJPs) and evoked responses were examined, Figure 2.4 A&B are representative mEJP traces. 141-604 homozygous larvae have an increased frequency and amplitude of mEJPs. In Figure 2.4C I have grouped mEJPs into bins by their amplitude. There is a shift toward mEJPs with a larger amplitude. The evoked response of 141-604 mutants was comparable to wildtype (data not shown). Together, these data indicate that there is a decrease in quantal

content ($p=.01$). Quantal content is the measure of the number of vesicles released per stimulation event (Figure 2.4D). This significant decrease in quantal content and increased mEJP amplitude suggests that there may be changes on both the pre-synaptic and post-synaptic sides or that there may be larger synaptic vesicles released. In addition to the previous recordings, we also tested the mutant for paired pulse facilitation by delivering two subsequent stimuli with a short inter-stimuli period. A test for paired pulse facilitation would measure whether there is an increased probability of release upon consecutive stimuli as observed in wild-type. If the mutant has a higher response than wild-type to the second stimuli, we can conclude that the release probability in the mutant is lower than wild-type. Conversely, if we find the second response to be smaller in amplitude, we can conclude that the release probability is higher in the mutant than wild-type and we are seeing vesicle depletion in the decreased second response. We found the facilitation response to be comparable to wildtype indicating that the pre-synaptic release probability is normal. The decrease in number of vesicles released and the normal release probability is consistent with the anatomical data of decreased number and intensity of Brp puncta. Fewer Brp puncta indicates that there are fewer sites with a high release probability correlating with the decrease in number of vesicles released. This electrophysiology differs from the *Brp* mutant where the evoked amplitude is significantly decreased and the QC is decreased by ~80% (Kittel, Wichmann, et al. 2006). However, Kittel et al. have not addressed the probability of release in the Brp mutant.

Stock 141-604 has a putative piggyBac (pBac) insertion between two genes on the second chromosome. In order to determine which gene was disrupted in 141-604 we

undertook several approaches. We first tested several mutants in the genes surrounding the pBac insertion. We found that the phenotype we observed in 141-604 was not due to disruption of the genes immediately adjacent to the pBac insertion site. It is possible that this pBac insertion line also carries a second site mutation that is responsible for the active zone phenotype we observed. In order to test this possibility we allowed genetic recombination to occur between the pBac insertion carrying chromosome and a wild-type chromosome. If there is a second site mutation causing the phenotype, a separation between the marked pBac insertion and the phenotype should be possible. We tested a large number of recombinants and isolated a single recombinant chromosome lacking the pBac insertion (termed 43) but exhibiting the observed active zone phenotype. This low frequency of recombination between the pBac insertion and the mutation indicates that the mutation of interest is near the pBac insertion.

Genetic mapping of allele 43

In order to gain more information about the location of the mutation I performed recombination mapping. By calculating the recombination frequency between known phenotypic markers and the isolated 43 chromosome, a genetic map distance between the mutation and the given marker can be determined. I found that the recombination rate between the phenotype and the marker *artistaless* (*al¹*) was 39.8%. The recombination rate between the phenotype and the marker *plexus* (*px*) was 37.8%. This indicates that our phenotype is due to a mutation relatively far from the ends of the second chromosome as these markers are close to the left and right ends of the chromosome respectively. I also

recorded the recombination rate between the phenotype and the marker *curved* (c^1) which was 18.8%.

I next began deficiency mapping of the region (43-48) I narrowed down by recombination mapping. Genetic deficiencies are lines carrying a defined deletion of a particular area of a chromosome. If you generate a transheterozygote of a mutant chromosome and the deficiency chromosome and find that it has the same or worse phenotype as a homozygous mutant it is likely that the mutation is within that deficiency region. I tested deficiencies in the region 43-48 on the second chromosome. I generated transheterozygotes of the mutation and the genetic deficiencies and tested them for the active zone phenotype. From this analysis, I determined that the mutation was in the region 45C4-45F4. In this region there was a very obvious candidate gene to test, *bruchpilot*, a component of the pre-synaptic active zone. To test whether the mutation isolated from the 43 line was an allele of *Brp*, I generated a transheterozygote of the isolated mutation and an allele of *Brp* (*Brp*⁶⁹). These larvae exhibit a decrease in Brp levels and a decrease in the number of Brp puncta observed in homozygous *Brp* mutants. This data indicates that the mutation isolated from line 43 is an allele of *bruchpilot* (Figure 2.5).

We decided not to pursue further study of this allele of *bruchpilot* for several reasons. There are many labs specifically studying *bruchpilot* and its function. The goal of the screen was to identify novel regulators of synaptic development yet to be characterized. Therefore, I continued to pursue genetic screening and analysis of other mutants.

Screening of EMS mutants

In addition to screening the pBac library, we also generated a library of EMS mutants on the third chromosome. We generated 975 individual EMS mutagenized lines. We found that 235 of these lines carried larval lethal mutations and did not screen these lines. We found that 126 lines were sterile. We were able to screen 612 independent lines and found 15 with synaptic phenotypes. We did not choose any of these mutants to pursue further as none of the phenotypes justified the time investment of genetic mapping. We did however, carry out genetic mapping on an EMS mutant from a previous screen in the lab.

Genetic Mapping of allele MA3.5

MA3.5 is an EMS mutant found in a previous EMS screen in the lab in which the X chromosome was mutagenized. This mutant exhibited extensive axon transport defects with very large accumulations of Brp occurring in the nerves (Figure 2.6). This phenotype is very unusual. In order to determine the genetic lesion responsible for this phenotype in MA3.5 mutants I began by performing recombination mapping. By calculating the recombination frequency between known phenotypic markers and the MA3.5 phenotype, a genetic map distance between the mutation in MA3.5 and the given marker can be determined. I found that the recombination rate between MA3.5 and markers *yellow* (y^1) position 1A5 and *crossveins* (cv^1) position 5A13 was approximately 50%. This indicates that the MA3.5 lesion is far from these two markers. I also measured the recombination rate between MA3.5 and *forked* (f^1) position 15F4-15F7, which was approximately 4.4%. This indicates that MA3.5 is close to the forked locus.

From this recombination data, I narrowed down a region to test with genetic deficiencies, 14B-17F.

To determine the identity of the gene mutated in MA3.5 I generated transheterozygotes with several overlapping deficiencies covering the area of interest I identified with recombination mapping. Combining MA3.5 with a deficiency in the same region resulted in lethality. Using this lethality I was able to reduce my region of interest to between 16B10 and 16C1. Within this region, there were 11 genes. One gene in particular, *Beta-Spectrin*, seemed a likely candidate to be the gene mutated in MA3.5. To test whether the lesion in MA3.5 is a mutation in *Beta-Spectrin* I generated a transheterozygote of MA3.5 and an allele of *Beta-Spectrin*. Trans-heterozygous larvae exhibited an interesting phenotype. These larvae's tails were flipped upward while they were in culture. This tail flip phenotype is associated with axon transport defects (Hurd and Saxton 1996). These transheterozygotes also exhibited the same axon transport phenotype as MA3.5 heterozygotes. Together, this data suggests that MA3.5 is an allele of *Beta-Spectrin*.

We decided not to pursue further analysis of this allele of *Beta-Spectrin*. *Beta-Spectrin* has been well studied in synaptic development in *Drosophila*. Recently, overexpression of *Beta-Spectrin* alleles known to cause symptoms in human patients results in severe axon transport defects very similar to what we observed in the *Beta-Spectrin* allele MA3.5 (Lorenzo et al. 2010). Having no other novel phenotypes to explore, we proceeded to screen with an RNAi collection.

Screening a subset of the Vienna RNAi collection

In addition to the screens already described, we also performed our Brp/DGluRIII staining screen on a collection of transgenic RNAi lines acquired from the Vienna DGRC. This collection consists of 500 independent transgenic lines whose target genes include neuron in their gene ontology description. This collection also features a common insertion site for every RNAi transgene. This single insertion site can help control for expression level effects often observed when transgenes are inserted in various places in the genome. To examine the effect of knockdown of these genes in neurons, I expressed these RNAi transgenes under the control of the post-mitotic pan-neuronal driver *Elav-gal4*. In addition to expressing the RNAi construct I also expressed the gene *Dicer-2* known to enhance the efficiency of RNAi processing.

From this collection, I found 40 lines that exhibited a synaptic phenotype when the RNAi was expressed in neurons. To test whether the phenotype is specifically caused by RNAi knockdown of that particular gene, we acquired mutants of as many of the 40 hits as possible. We have been able to confirm several of these RNAi hits with true mutations of the gene. Many of the RNAi lines were isolated as having a phenotype of synaptic retraction. Synaptic retraction is a condition where a branch or an entire pre-synaptic neuron has disassembled leaving behind a post-synaptic “footprint” as the post-synaptic clusters disassemble much more slowly than the pre-synapse. We found that none of the genes suspected to have retractions actually exhibited retractions in the mutant lines. This is an example of one of the caveats of working with RNAi knockdown. There is a potential for spurious phenotypes due to off target effects of the RNAi or general effects of the genetic background of these transgenic flies.

From this collection of confirmed RNAi hits we elected to pursue further study of the gene *happyhour*. These studies are detailed in Chapter 3.

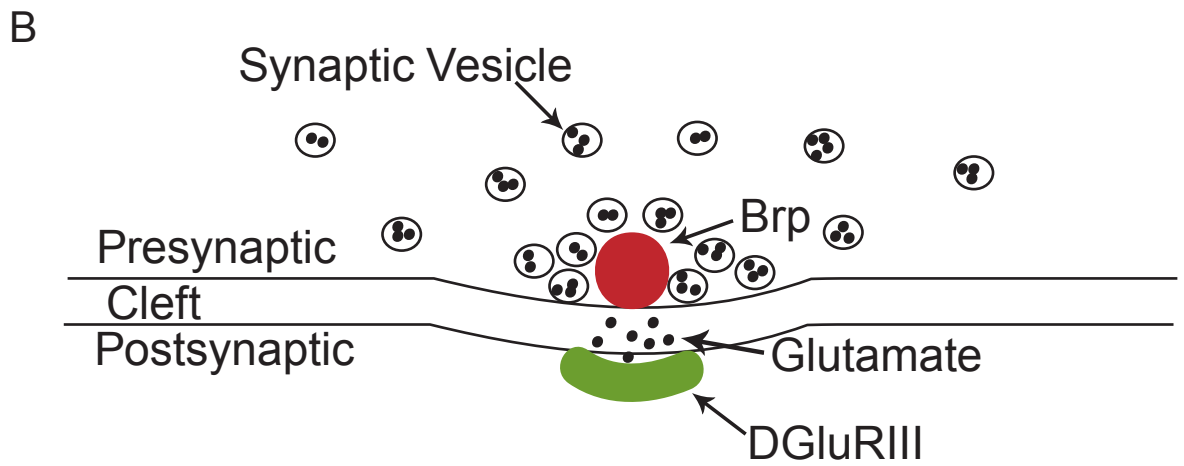
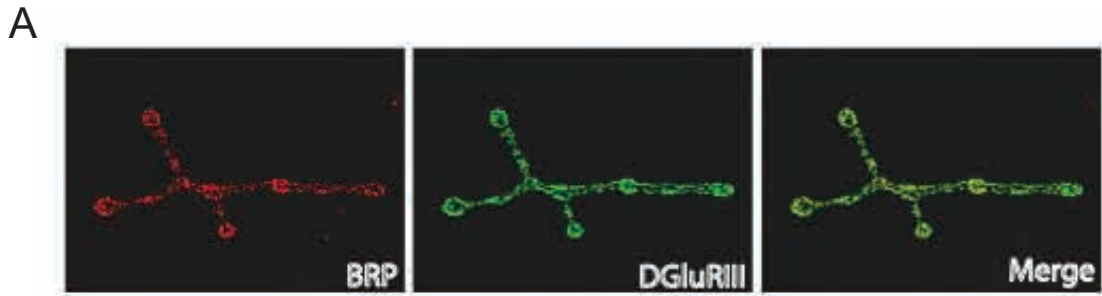


Figure 2.1. Bruchpilot (BRP) and DGLuRIII are tightly apposed at synapses in the Drosophila NMJ

A) Images of 3rd instar larval muscle 4 NMJ immunolabeled with anti Brp (red) and anti-DGLuRIII (green)

B) Schematic of a single active zone. The presynaptic release machinery is labeled by Bruchpilot (Brp) staining indicated by the red circle. This active zone actively clusters synaptic vesicles to the membrane to facilitate release. Upon fusion of the vesicles, neurotransmitter is released into the synaptic cleft and binds to postsynaptic receptors. Directly opposite the presynaptic release machinery is the postsynaptic receptor cluster. This is indicated in green and is labeled by staining for the essential glutamate receptor subunit DGLuRIII.

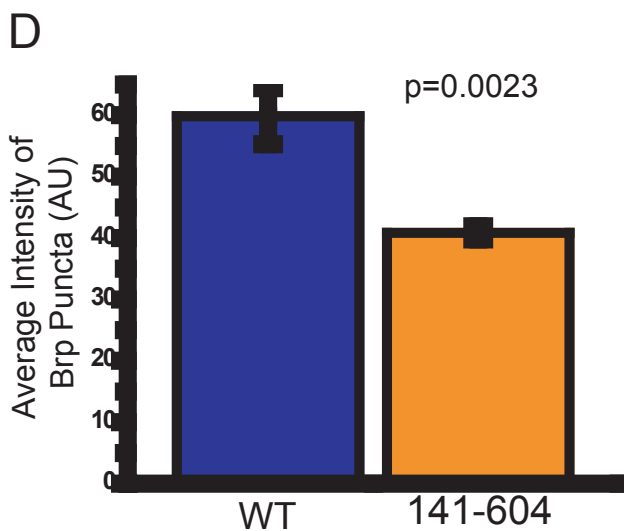
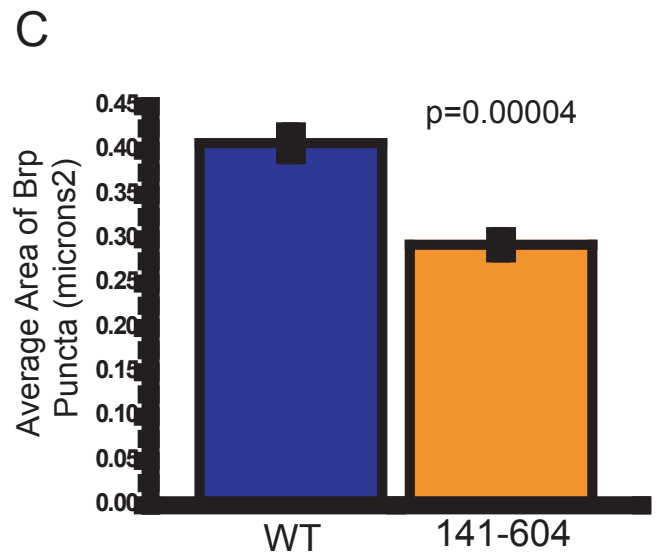
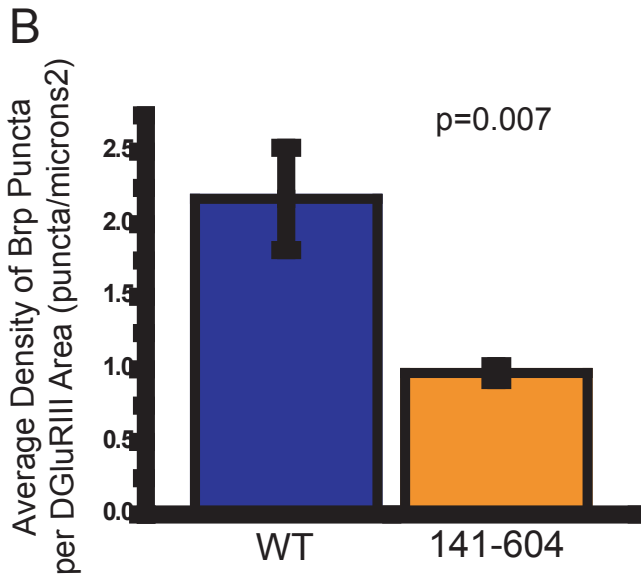
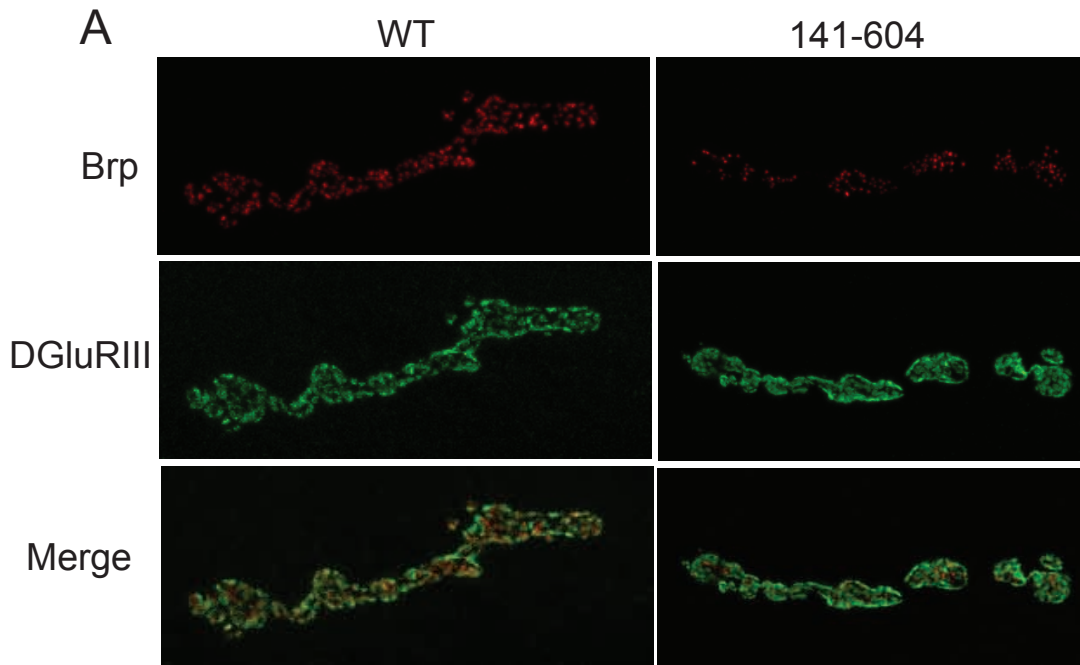


Figure 2.2 Bruchpilot is significantly affected in 141-604

A. Images of 3rd instar larval muscle four NMJs immunolabeled with anti-Brp (red) and anti-DGluRIII (green).

B. Graph shows quantification of average density of Brp puncta per DGluRIII area (puncta/microns²). The mutant line 141-604 has a significant decrease in Brp puncta density (WT- 2.17 ± 0.35 , 141-604- $.97 \pm 0.05$; $p=0.007$, student's t-test, two-tailed) Error bars represent standard error of the mean.

C. Graph shows quantification of the average area of Brp puncta (microns²). 141-604 has a significant decrease in the area of Brp puncta (WT- 0.41 ± 0.01 , 141-604- 0.29 ± 0.009 ; $p=0.00004$, student's t-test) Error bars represent standard error of the mean.

D. Graph shows quantification of the average intensity of Brp puncta (Arbitrary Units). 141-604 exhibits a significant decrease in intensity of Brp puncta (WT- 59.8 ± 4.6 , 141-604- 40.8 ± 1.07 ; $p=0.0023$, student's t-test). Error bars represent standard error of the mean.

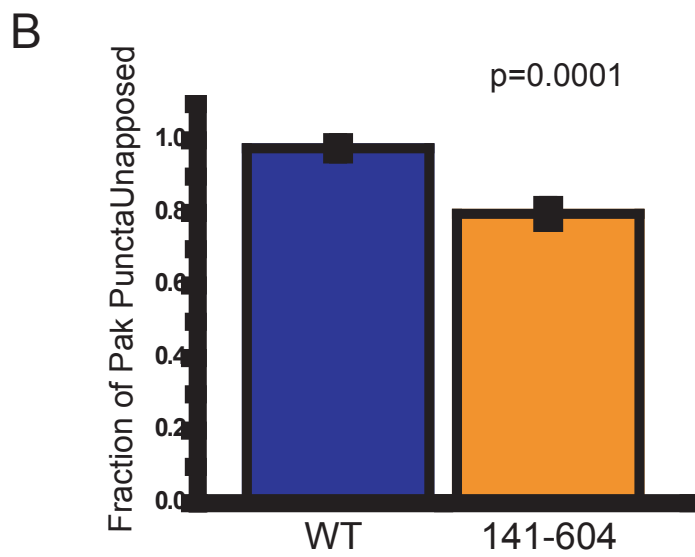
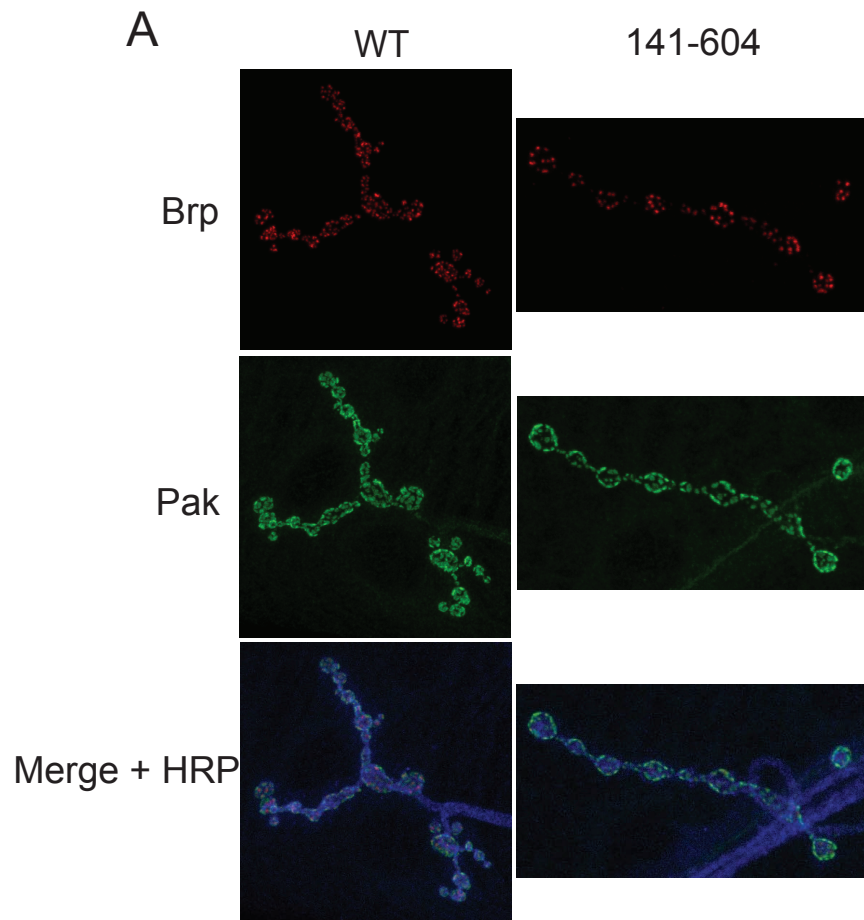


Figure 2.3. Synaptic apposition is defective in 141-604

A. Images of 3rd instar larval muscle 4 NMJs immunolabeled with anti-Brp (red), anti-Pak (green) and anti-HRP (blue).

B. Graph shows quantification of apposition between Brp puncta and Pak clusters (fraction of Pak clusters unapposed). There is a significant decrease in synaptic apposition in 141-604 (WT- 0.98 ± 0.02 , 141-604- 0.79 ± 0.02 ; $p = 0.0001$, student's t-test). Error bars represent standard error of the mean.

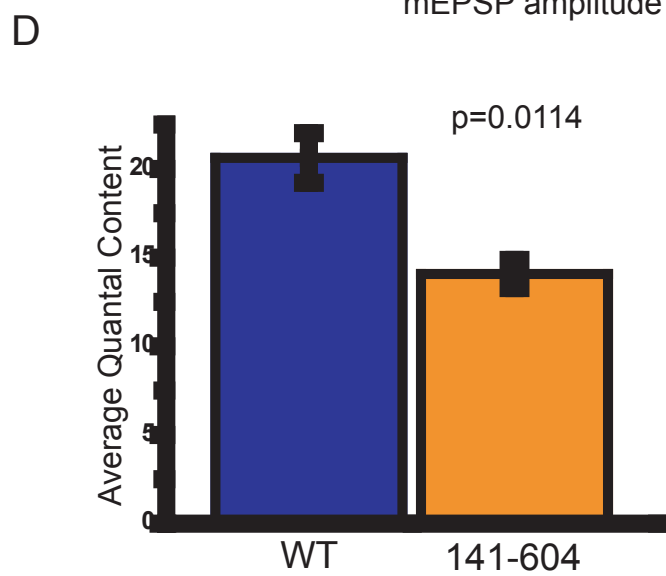
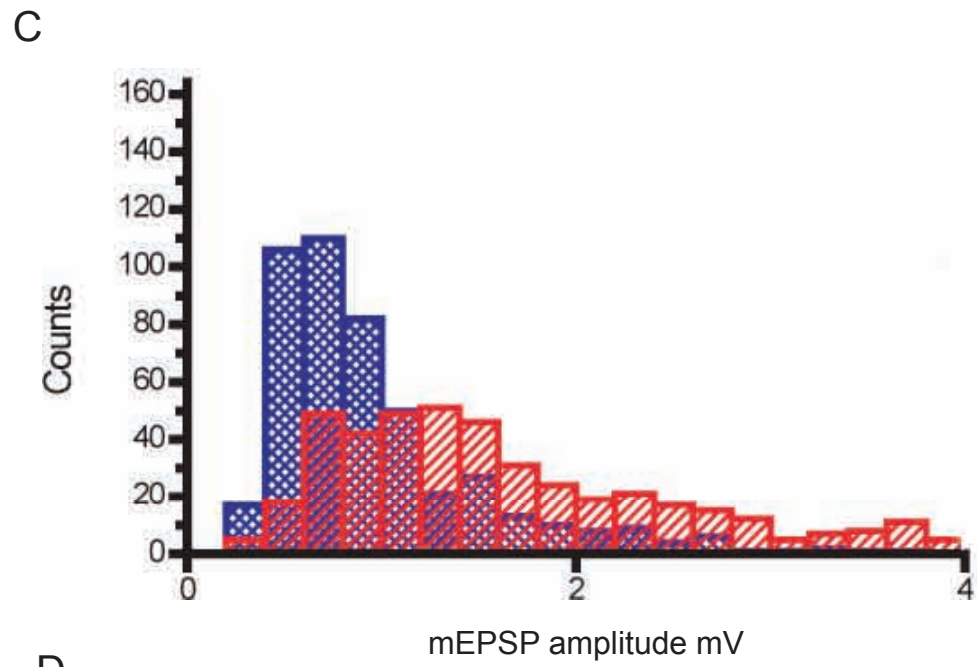
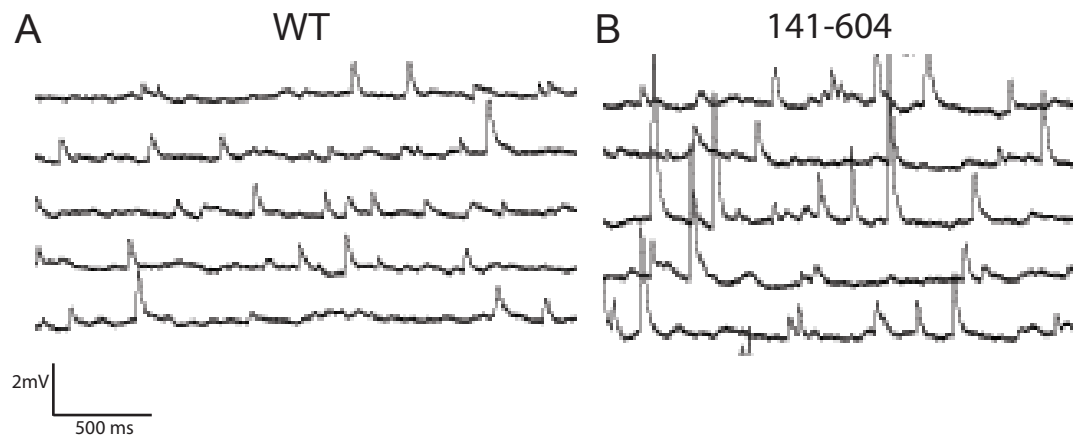


Figure 2.4. 141-604 exhibits a defect in synaptic transmission

A&B. Muscle cell (post-synaptic) mEJP recordings from WT (A) and 141-604 (B). An mEJP is a measure of the response of the postsynaptic cell to spontaneous fusion of a single synaptic vesicle.

C. Representation of mEJP amplitudes and their relative sizes. There is a shift in the 141-604 (orange) mEJPs toward a larger amplitude as compared to the wild-type (blue). An increase in mEJP amplitude is likely due to a change in the responding muscle cell.

D. Histogram representation of quantal content, a measure of the number of vesicles released at the synaptic cleft per pre-synaptic stimulation event. The number is significantly decreased in 141-604. ($p=0.0114$)

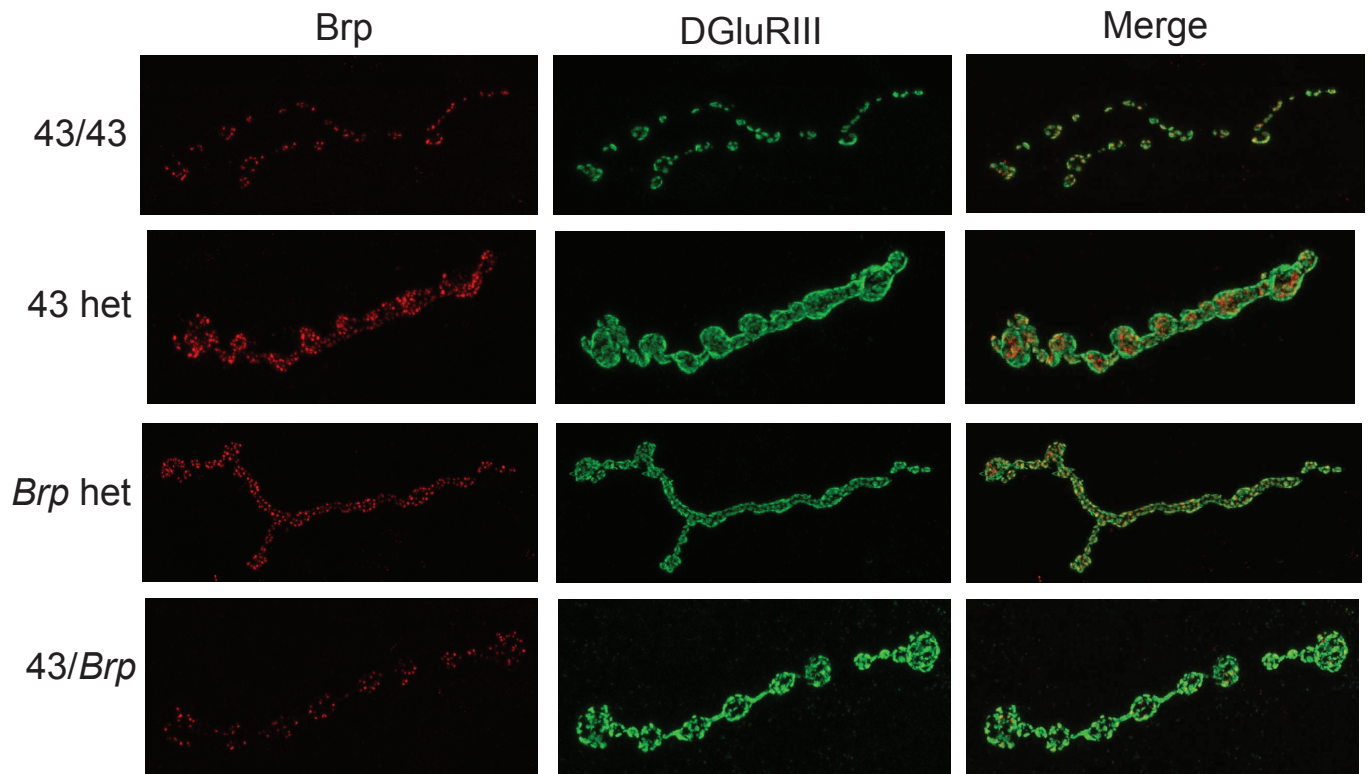


Figure 2.5 43 is an allele of *Bruchpilot* (*Brp*)

Images of third instar larval muscle 4 NMJs immunolabeled with anti-Brp (red) and anti-DGluRIII (green). A transheterozygote of 43 and *Brp* exhibits a phenotype very similar to homozygous 43 mutants. These data indicate that mutant 43 is an allele of *Brp*.

MA3.5

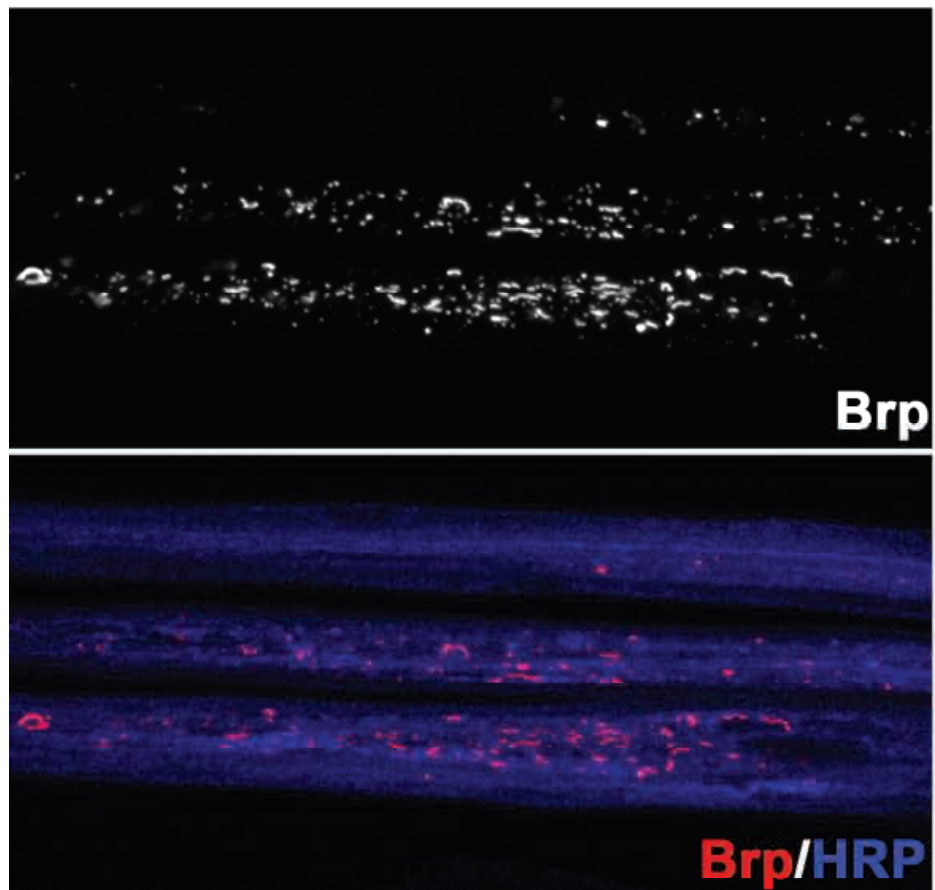


Figure 2.6 MA3.5 exhibits a severe axon transport defect. Images of third instar larval nerves immunolabeled with anti-Brp (red) and anti-HRP (blue). MA3.5 mutants exhibit extensive large clogs of Brp in their nerves. Brp is virtually undetectable in wildtype nerves.

Chapter 3

EGFR signaling modulates synaptic connectivity via Gurken

This chapter includes the manuscript:

Naylor SA, DiAntonio A. EGFR signaling modulates synaptic connectivity via Gurken,
Developmental Neurobiology (In Press)

Abstract

Synaptic target selection is critical for establishing functional neuronal circuits. The mechanisms regulating target selection remain incompletely understood. We describe a role for the EGF receptor and its ligand Gurken in target selection of octopaminergic Type II neurons in the *Drosophila* neuromuscular system. Mutants in *happyhour*, a regulator of EGFR signaling, form ectopic Type II neuromuscular junctions. These ectopic innervations are due to inappropriate target selection. We demonstrate that EGFR signaling is necessary and sufficient to inhibit synaptic target selection by these octopaminergic Type II neurons, and that the EGFR ligand Gurken is the post-synaptic, muscle-derived repulsive cue. These results identify a new pathway mediating cell-type and branch-specific synaptic repulsion, a novel role for EGFR signaling in synaptic target selection, and an unexpected role for Gurken as a muscle-secreted repulsive ligand.

Introduction

Neurons form synapses with appropriate partners to generate complex circuits during development. These circuits are critical for regulating behavior, homeostasis, learning and memory. Many different processes guide neurons to their target including cell migration, axon guidance, and the localization of pre- and post-synaptic components (Tessier-Lavigne and Goodman 1996). Most neurons recognize their specific targets in spite of encountering many other cells en route to their destination (Shen and Scheiffele 2010). While there are many examples of finely tuned target selection, the molecular mechanisms that govern this process remain poorly defined. A better understanding of these wiring mechanisms is crucial to appreciate circuit assembly and to begin to understand the consequences of miswiring and its implications in neurological disorders.

The *Drosophila* larval neuromuscular system is formed by identified pre- and postsynaptic cells and the wiring pattern is invariant, making it a powerful system to study synaptic connectivity. The primary innervations are glutamatergic, are responsible for fast synaptic transmission, and are subdivided into Type Ib and Is based on the innervation pattern and bouton morphology (J. Johansen et al. 1989). The second major class of neuromuscular junctions (NMJs) are termed Type II, and are octopaminergic and neuromodulatory (Monastirioti et al. 1995). Type II NMJs are present on fewer muscles of the larva and have a distinctive morphology. Neurons forming Type Is NMJs innervate multiple muscle targets in a given hemisegment (Landgraf et al. 1997) whereas Type II neurons innervate a different complement of muscle targets in the same region (Monastirioti et al. 1995). These two types of neurons encounter the same muscle field

and yet make divergent target selection choices, making this an interesting system in which to study synaptic target selection.

One advantage of the *Drosophila* system is the ability to unravel mechanisms of target selection genetically (Collins and DiAntonio 2007). Many of the most striking targeting phenotypes result from gain-of-function rather than loss-of-function studies (Ghose and Van Vactor 2002). For example, overexpression of muscle-derived Fasciclin II (FasII) potently promotes the formation of ectopic synapses that are functional and stable (Davis, Schuster, and Goodman 1997). Loss-of-function studies may find less dramatic phenotypes because targeting decisions are complex and involve the integration of a number of positive and negative cues (Winberg, Mitchell, and Goodman 1998). Indeed, when their normal muscle targets are ablated, motoneurons retain the ability to synapse on alternate muscles (Cash, Chiba, and Keshishian 1992). This suggests that the target selection of neurons is a malleable process that requires multiple simultaneous cues. In addition to molecular cues, decreases in neuronal activity via mutations in sodium channel subunits also lead to the formation and stabilization of ectopic synapses (Jarecki and Keshishian 1995). Recent studies have highlighted the important role of electrical activity and semaphorin signaling in shaping synaptic connectivity in this system (Carrillo et al. 2010; Koon et al. 2011). Despite this progress, it is still not clear how Type II octopaminergic neurons distinguish between appropriate and inappropriate targets, and more generally, how neurons navigate to their appropriate targets to form synaptic connections.

To address this question, we undertook a genetic screen to identify mutants with defects in synaptic targeting. From this screen we identified a role for EGFR signaling in

Type II neuron target selection. We find that EGFR signaling inhibits synaptic target selection in a cell-type and branch-specific manner. The inhibitory cue is provided by a muscle-derived EGFR ligand, Gurken. This is a novel function for Gurken, which was previously suggested to function exclusively in the developing oocyte (Nilson and Schüpbach 1999). This work defines EGFR and Gurken as an important receptor/ligand pair contributing to the array of signals shaping the development of neural circuits.

METHODS

Fly Stocks

Flies were maintained at 25°C on standard fly food. Wild type (WT) flies were Canton S (CS) unless specified otherwise. The following flies were obtained from the Bloomington Stock Center: P{SUPor-P}*hppy*^{KG05537}, *Df(2R)Exel6069*, *Tdc2-Gal4* (Koon et al. 2011), *UAS-CD8-GFP* (T. Lee and Luo 1999), *rl¹* (Biggs et al. 1994), *UAS-DCR2* (Y. S. Lee et al. 2004), *G7-Gal4*, *MI2-Gal4*, *UAS-EGFR* (2nd chromosome) (Carmena et al. 1998), *gurken^{2B}* (Neuman-Silberberg and Schüpbach 1993), *UAS-EGFR_{DN}*, and *UAS-Rhomboid* (Guichard et al. 1999).

UAS-hppy was a gift from Ulrike Heberlein (UCSF, CA). *UAS-Gurken RNAi* (36251 & 101701) was from the Vienna Drosophila Genome Resource Center (Vienna, Austria).

UAS-Gurken was a gift of Bruce Edgar (Jiang and Edgar 2009).

Immunohistochemistry

Third-instar larvae were dissected in PBS and fixed in either Bouin's fixative for 5 min. or 4% paraformaldehyde for 30 min on ice. Larvae were washed with PBS containing 0.1% Triton-X-100 (PBT) and blocked in 1% NGS in PBT for 30 min, followed by overnight incubation in primary antibodies in 1% NGS in PBT, three washes in PBT, incubation in secondary antibodies in 1% NGS in PBT for 45 min, three final washes in PBT, and equilibration in 70% glycerol in PBS. Samples were mounted in Vectashield (Vector, Burlingame, CA). The following primary antibodies were used: rabbit α -DGluRIII (Marrus et al. 2004) 1:2500 in Bouin's, mouse α -FasII (clone 1D4) 1:5 in PFA, 1:100 in Bouin's, rabbit α -Vmat (Greer et al. 2005) 1:200 in PFA, mouse α -

Brp (Wagh et al. 2006) 1:200 in Bouin's, mouse α -Futsch 1:100 in Bouin's, rabbit α -Synaptotagmin1 (Mackler et al. 2002) 1:2500 in Bouin's, rat α -mCD8a (Invitrogen) 1:100 in Bouin's, rabbit α -TBH (Koon et al. 2011) 1:400 in Bouin's, rabbit anti-GFP conjugated to Alexa Fluor-488, 1:200 (Invitrogen). Goat Cy3-, Alexa-633 and FITC conjugated secondary antibodies against mouse and rabbit IgG were used at 1:1000 and were obtained from Jackson ImmunoResearch. Chicken Cy3 conjugated secondary antibody against rat IgG was used at 1:200. Antibodies obtained from the Developmental Studies Hybridoma Bank were developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences of the University of Iowa, Iowa City, IA.

Imaging and Analysis

Samples were imaged with a Nikon D-Eclipse C1 confocal microscope using 60X oil objective. Shown images are z projections of confocal stacks acquired from serial laser scanning except for the Tdc2-GFP cell bodies which are presented as single confocal sections. Quantification was performed to determine presence or absence of FasII and Vmat positive projections on muscles 6,7,12, 13, and 4. These muscles were examined in segments A3-A5. Values were analyzed for significance using the Fisher's exact test and two-tailed P value tests. Type I boutons were counted on muscles 6 and 7 of segment A3 and analyzed with a two-sample t-test. Type Is bouton presence was examined on muscles 12, and 13 in segments A3-A5 and analyzed with Fisher's exact test and two tailed P value tests. Length of Type II synapses in segments A3-A5, was quantified using ImageJ and significance was calculated with a two-sample t-test.

RESULTS

To identify pathways and mechanisms that govern synaptic growth and development, we performed a genetic screen of several collections of *Drosophila* mutants including p-element insertions from the BDGP project (Bellen et al. 2004). We dissected third-instar larvae from each line and stained with a marker of the presynaptic active zone Bruchpilot (Brp) (Wagh et al. 2006) and the essential glutamate receptor subunit DGluRIII (Marrus and DiAntonio 2004). We identified lines with defects in synaptic development including synaptic targeting defects, active zone defects, and NMJ morphology defects. From this screen we identified an allele of *happyhour* (*hppy*), $P\{SUPor-P\}^{KG05537}$, which has a defect in synaptic targeting of the octopaminergic motoneurons that form Type II NMJs.

These octopaminergic motoneurons face an interesting problem: how do they choose their multiple synaptic targets and bypass other potentially suitable locations to form a synapse? The normal innervation pattern of the Type II motoneuron MNSNb/d-II and the Type Is motoneuron MNSNb/d-Is is shown in Fig 3.1A. Motoneuron MNSNb/d-Is forms three branches that innervate the cleft between muscles 6 and 7, muscle 13 and muscle 12 respectively. In contrast, the axon from motoneuron MNSNb/d-II extends under muscles 6 and 7 without forming a synapse, and then branches twice to form NMJs onto muscles 13 and 12. In *hppy* mutants, however, ectopic Type II NMJs are more likely to form on muscles 6 and 7 than in wild type (Fig 3.1B, 3.1C). We conclude that these ectopic NMJs are Type II endings because, like wild type Type II NMJs, they 1) are long

and thin with very small synaptic boutons and 2) express the vesicular monoamine transporter (VMAT)(Greer et al. 2005), which exclusively labels octopaminergic Type II NMJs in the periphery. In the higher magnification panels, colocalization between FasII and VMAT occurs in wild type Type II NMJs as well as the ectopic Type II NMJs in *hppy* mutants.

It is possible that these ectopic Type II NMJs may represent immature or defective Type II synapses. To investigate this possibility we labeled Type II synapses in wildtype and *hppy* mutants by expressing *UAS-CD8-GFP*, a membrane targeted GFP, with *Tdc2-Gal4*, which in the periphery expresses exclusively in octopaminergic neurons (Cole et al. 2005). We find that the ectopic Type II synapses in *hppy* exhibit expression of tyrosine beta-hydroxylase (TBH) (Fig 3.1E). TBH is necessary to synthesize octopamine and is necessary for mature Type II NMJ function. To determine if the ectopic Type II NMJs contain machinery for synaptic release, we examined the localization of the active zone component Bruchpilot (Brp). Brp is present in the ectopic Type II NMJs in *hppy* mutants (Fig 3.1F). In addition, the MAP1B-related protein Futsch and the synaptic vesicle protein Synaptotagmin-1 are also present in the ectopic Type II NMJs (Fig 3.1G, H). Together, these data indicate that the ectopic Type II endings on muscles 6 and 7 in *hppy* mutants have the anatomical hallmarks (Koon et al. 2011) of mature Type II NMJs.

The establishment of Type I innervation occurs before Type II innervation and is critical to guide the Type II neurons to their appropriate locations (Halpern et al. 1991). To test the possibility that the ectopic Type II innervation is due to a primary defect in Type I innervation, we examined Type Ib and Is innervations on muscles 6, 7, 12, and 13. We find no difference in the number of Type I boutons on muscles 6 and 7 in the *hppy*

mutant (WT= 79.1± 3.0; *hppy*= 87.5±6.8 p=0.29) or detectable differences in their morphology. Type I boutons are present on muscles 12 and 13 both in wild type and *hppy* mutants (p=1 for muscles 6/7,12 and13). Hence, Type I innervation appears to be unaffected in *hppy* mutants and so is unlikely to contribute to the formation of ectopic Type II NMJs.

To assess whether this synaptic phenotype is due to loss of *hppy*, we generated transheterozygotes for *hppy*^{KG05537} and a deficiency chromosome, *Df(2R)Exel6069*, that deletes *hppy* and adjacent genes. We find no significant difference in the presence of ectopic Type II synapses between *hppy* and *hppy/df* (p> 0.05) (Fig 3.1C). Hence, this phenotype is not due to a second site mutation on the *hppy*^{KG05537} chromosome. In addition, since the *hppy*^{KG05537} phenotype was not exacerbated in combination with a definitive null allele, the *hppy*^{KG05537} allele is behaving as a genetic null or a very strong hypomorph. This phenotype is not restricted to muscles 6 and 7—*hppy* mutants also have a significant increase in the likelihood of ectopic Type II innervation on muscle 4 (WT 18% ± 0.6% n=60; *hppy* 50%± 0.8% n=60 p= 0.0005). Type II synapses are found on muscles 12 and 13 in wild-type animals (Fig 3.1C). Potentially, the ectopic Type II synapses on muscles 6 and 7 in *hppy* mutants are being formed at the expense of normal Type II innervation onto muscles 12 and 13. In this scenario, these Type II synapses would be significantly smaller or absent altogether. To investigate this possibility, we measured total Type II synaptic span on muscles 12 and 13 and found that Type II synapses are unaffected in *hppy* mutants (M12; WT 296 ± 25 μm, *hppy* 301 ± 46 μm p=.92, M13; WT 371 ± 45 μm, *hppy* 308 ± 44 μm p=.33 n=10) (Fig 3.1D). In addition, we observed no missing type II NMJs on these muscles (n = 20). This demonstrates that

the Type II synapses at muscles 6 and 7 in *hppy* mutants are not misplaced synapses that should be located on muscles 12 and 13, but are ectopic synapses formed in addition to the normal complement of Type II synapses.

To investigate the cellular basis for these ectopic synapses, we analyzed the cell number and projection pattern of octopaminergic motoneurons. In wild-type larvae, there are clusters of three octopaminergic (Type II) neurons in the ventral nerve cord corresponding to each segment of the animal. These neurons each have one process that bifurcates within the ventral nerve cord forming bilateral projections to each hemisegment. These projections then branch in the periphery and form Type II NMJs at appropriate locations within the hemisegment. In light of this anatomy, there are three potential explanations as to the source of the ectopic synapses: 1) *hppy* mutants have an increase in octopaminergic cell number 2) existing octopaminergic neurons form additional primary branches within the nerve cord or 3) the existing octopaminergic neuronal projections are forming ectopic branches in the periphery. To distinguish among these possibilities, we counted octopaminergic neuron cell bodies and their primary projections in wild-type and *hppy* mutant larvae. In order to count these neurons, we expressed *UAS-CD8-GFP*, a membrane targeted GFP, with *Tdc2-Gal4*. In wild-type larvae there are clusters of three octopaminergic neurons in each segment of the animal (Fig 3.2A). In *hppy* larvae there are also three octopaminergic neurons per segment (WT-3; *hppy-3*; n=10; p=1). To assess the number of primary projections formed by these neurons, we counted the number of labeled axons innervating each hemisegment and determined that the number of projections was the same (WT-3; *hppy-3*; n=10; p=1) in *hppy* and wild-type (Fig 3.2B). Following these GFP positive projections to their targets,

we confirmed that the ectopic synapses in *hppy* mutants form from the extensions of the TDC2 positive neurons (Fig 3.2C). These ectopic synapses in *hppy* mutants can originate from extensions of MNSNb/d-II, the Type II neuron that normally innervates muscles 12 and 13, or occasionally from MNISN-II whose projection runs in the intersegmental nerve and that normally innervates more lateral targets. Hence, the wiring defects in the *hppy* mutant are due to ectopic branching of octopaminergic projections within their muscle target field.

To test whether this phenotype is due to loss of *happyhour* in octopaminergic neurons, we expressed *UAS-hppy* with *Tdc2-Gal4* in the *happyhour* background in an effort to rescue the ectopic synapse phenotype. Expression of *happyhour* in octopaminergic neurons reduces the frequency of ectopic synapses in *hppy* mutants (Fig 3.2D&E) ($p < .05$). While our rescue is statistically significant, it does not fully recapitulate wild-type innervation, potentially due to the strength or timing of expression from *TDC2-gal4*. Overexpression of *hppy* in a wild-type background does not show a significant change in either Type II NMJ structure or target selection (data not shown).

hppy regulates EGF receptor (EGFR) signaling that can modulate downstream activation of ERK, known in *Drosophila* as *rolled* (*rl*) (Corl et al. 2009). We investigated whether or not this same signaling pathway is responsible for the ectopic synapse phenotype. To test whether overactivation of *rolled* downstream of *happyhour* is responsible for the ectopic Type II synapses, we made a double mutant of *happyhour* and a hypomorphic allele of *rolled* (*rl^l*) (Biggs et al. 1994). We find that homozygous *rl^l* mutants do not have any changes in Type II innervation in an otherwise wild type background (Fig 3.3A). However, the *rl^l* mutant potently suppresses the ectopic Type II

synapses in *hppy* mutants (Fig 3.3A). This suppression is essentially complete (Fig 3.3B) ($p < .005$).

Suppression with *rl^l* suggests that the phenotype is due to an overactivation of the EGFR pathway, however, such overactivation often leads to strong negative feedback. Such feedback ensures that strong activation of the pathway is quickly and effectively turned off (Avraham and Yarden 2011). Indeed, baseline levels of p-ERK are significantly decreased in *hppy* mutants, indicating that the pathway is turned down (Corl et al. 2009). Hence, while the findings with *hppy* and *rl* implicate EGFR signaling in the restraint of ectopic synapse formation, they are unable to distinguish the directionality of the signal. To investigate the role of EGFR directly, we manipulated the receptor in octopaminergic neurons. We expressed a dominant negative EGFR construct, *UAS-EGFR-DN* (M Freeman 1996), with the *Tdc2-Gal4* driver (Fig4A). This inhibition of EGFR function in octopaminergic neurons results in a significant increase in the number of ectopic Type II NMJs on muscles 6 and 7 (Fig 3.4B) ($p < .05$). This suggests that EGFR acts within octopaminergic neurons to inhibit inappropriate innervation. If so, then overexpression of EGFR in octopaminergic neurons may overactivate this repulsive signal and may lead to the loss of Type II innervation onto muscles 12 or 13, the normal synaptic partners of these neurons. To investigate this hypothesis, we expressed wild-type EGFR (*UAS-EGFR*) with the *Tdc2-Gal4* driver (Fig 3.4C). This led to a significant decrease in the proportion of muscle 12 cells with Type II NMJs (Fig 3.4D) ($p < .005$). Taken together, these loss- and gain-of-function studies demonstrate that EGFR signaling is necessary and sufficient to inhibit the capacity of octopaminergic motoneurons to form NMJs with targets.

Since EGFR signaling mediates a branch-specific repulsive signal in motoneurons, a muscle-derived ligand is likely the inhibitory cue. There are several known ligands of the EGFR, including Vein, Spitz, Keren and Gurken (Schnepp et al. 1996) (Rutledge et al. 1992) (Reich and Shilo 2002) (Neuman-Silberberg and Schüpbach 1993). To test whether any of these is the repulsive cue, we knocked down expression of each ligand via RNAi expressed under the control of a muscle specific driver, *G7-gal4* (Y. Q. Zhang et al. 2001). Knockdown of Vein, Spitz and Keren has no synaptic phenotype. In contrast, knockdown of Gurken recapitulates the dominant negative EGFR phenotype (Fig 3.5A). Expression of an RNAi targeting Gurken (VDRC36251) leads to a significant increase in ectopic Type II innervations on muscles 6 and 7 (Fig 3.5A,B) ($p < .05$). Similar results were obtained with a second Gurken RNAi (VDRC101701) ($p < .05$) that shares no common sequence with VDRC36251 as well as the homozygous *gurken*^{2B} mutant (Neuman-Silberberg and Schüpbach 1993), which has ~25% ectopic Type II NMJs at muscles 6 and 7 ($p < .05$) (Fig 3.5A,B).

As a second test of this model, we asked whether overexpression of Gurken is sufficient to inhibit octopaminergic NMJ formation at the normal target. We expressed *UAS-Gurken* and its processing enzyme *UAS-Rhomboid* with *M12-Gal4*, which expresses in muscle 12, a normal synaptic target for MNSNb/d-II (Fig 3.6A). Overexpression of Gurken and Rhomboid in muscle 12 is sufficient to inhibit the formation of Type II NMJs at their normal targets (Fig 3.6B). These loss- and gain-of-function studies strongly support the model that Gurken is an inhibitory cue for octopaminergic synaptogenesis. To shape target selection, however, the model predicts that Gurken expression should be localized to muscles that do not receive octopaminergic input, such as 6 and 7.

Unfortunately, we are unable to confirm this aspect of the model via *in situ* or antibody staining, as we have been unable to detect endogenous gurken transcript or protein in any muscles. Although it cannot speak to muscle specific expression, the modEncode RNA-seq data does demonstrate that Gurken is enriched in body wall muscle (Chintapalli, Wang, and Dow 2007), supporting our genetic findings that Gurken functions outside of the developing oocyte.

DISCUSSION

Synaptic target selection is a critical step in establishing functional neural circuits. The molecular mechanisms governing this selection have not yet been fully explored. We have taken our observation that *hppy* mutants have an increased frequency of ectopic octopaminergic Type II NMJs and identified a pathway critical for synaptic target recognition in these neurons. We find that the EGFR signaling pathway is required to prevent the development of inappropriate synaptic contacts. This inhibitory signal is mediated by muscle-derived EGFR ligand, Gurken, working through the EGF receptor in type II motoneurons. This mechanism sculpts the neuronal wiring pattern in a cell-type and branch-specific manner.

EGF as a guidance cue

There are many signaling pathways that influence the innervation pattern of *Drosophila* motor neurons. Our findings identify a novel role for EGFR signaling in mediating a repulsive guidance cue to Type II neurons. EGF has previously been demonstrated to regulate axon growth and guidance. For example, EGF positively regulates Sema-3a levels in the cornea (Ko et al. 2008) and interacts with NCAM-180 to promote neuritogenic activity (Povlsen, Berezin, and Bock 2008). However, to our knowledge our data is the first to demonstrate that an EGF receptor and ligand provide a synaptic targeting signal.

We find ectopic Type II NMJs in *hppy* mutants, as well as when we express a dominant negative EGFR in Type II neurons or knock down its ligand Gurken in the muscle targets. This presents an apparent contradiction. Hppy is described as a negative regulator of EGFR (Corl et al. 2009), and its phenotype in this system is suppressed by a hypomorphic mutation in *rolled* (ERK), consistent with Hppy functioning as a negative regulator of EGFR signaling. Why then does loss of *hppy* have the same phenotype as inhibition of the receptor or ligand? We posit that the well-described strong negative feedback (Avraham and Yarden 2011) induced by EGFR signaling may be the explanation. We propose a model in which activation of the EGFR pathway mediates a signal that inhibits the formation or stabilization of Type II NMJs. In *hppy* mutants, however, loss of negative regulation would allow for excess activation of the EGFR that would induce a quick, strong and long-lasting negative feedback activity early in development, essentially turning off EGFR signaling in cells expressing *happyhour*. The result would be loss of the synaptic inhibitory signal mediated by EGFR at the stage when these Type II neurons are extending to their targets and the promotion of ectopic synapse formation. We appreciate that this model is speculative, and that an alternative is that *hppy* and *rolled* are regulating a pathway that is distinct from the EGFR/*gurken* pathway.

EGFR and synapse formation

By what mechanism does EGFR signaling affect synapse formation in Type II neurons? Presumably, there is a molecular program downstream of EGFR activation that modifies the Type II neuron such that it does not form and/or maintain an NMJ with an

inappropriate target. These changes could occur at the level of the cell body or locally within individual branches. It is unlikely that cell body changes are central to the phenotype because such neuron-wide mechanisms could not easily be translated into branch-specific behavior. In contrast, local effects of EGFR signaling within neurites could explain such specificity. We do not know the cell biological mechanism mediating the branch-specific inhibition. Possibilities include alterations in the local translation or membrane insertion of synaptogenic molecules, local modulation of cytoskeletal dynamics, or failure to properly prune Type II connections.

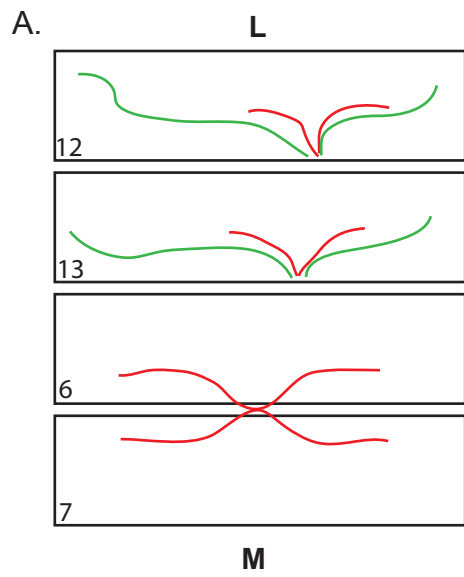
Not only is the EGFR mediating a branch-specific effect, but it is also cell-type specific. As described above, the Type II motoneuron MNSNb/d-II and the Type Is motoneuron MNSNb/d-Is travel together and presumably encounter the same cues across the hemisegment, however they generate different innervation patterns. This implies that these two types of neurons have developed cell-type specific repertoires of receptors or signaling pathways that shape their target choices.

While our data indicate a role for EGFR signaling in Type II synaptic target selection it is also likely that Type II target selection has multiple components. Our phenotypes occur at a relatively low penetrance, so it is likely that complementary and combinatorial guidance cues function with the EGFR pathway to shape target selection of Type II neurons.

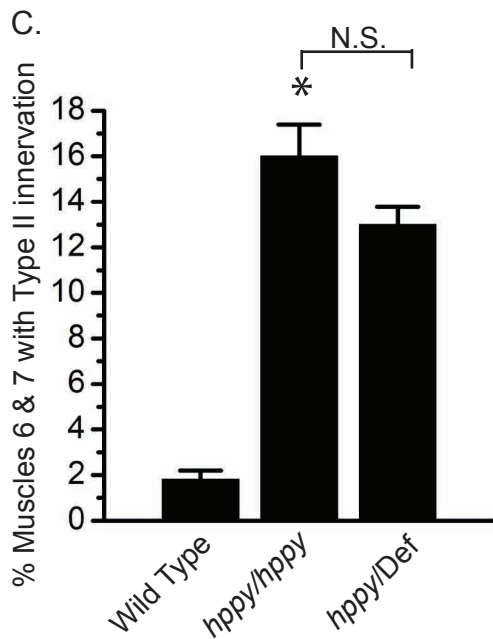
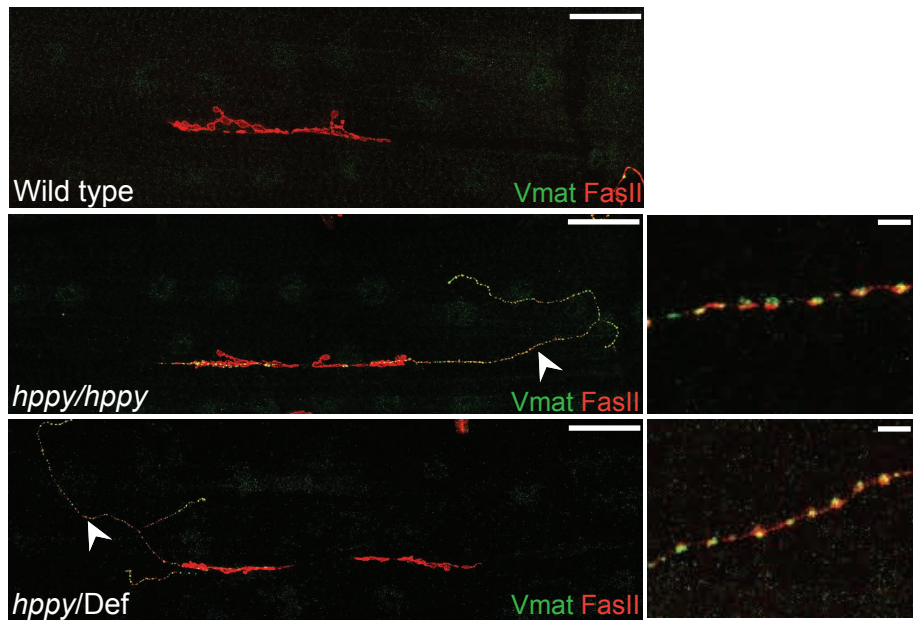
A novel role for Gurken

Gurken has been studied exclusively in the developing oocyte (Schüpbach 1987) (Neuman-Silberberg and Schüpbach 1993) (González-Reyes, Elliott, and St Johnston

1995) and, to our knowledge, has no known roles in other tissues. Hence, it is surprising that Gurken conveys the repulsive signal from muscle to the octopaminergic Type II neurons. In support of a function in muscle, the modEncode RNA-seq project has found that Gurken transcript is enriched 3.5 fold in larval body wall muscles (Chintapalli, Wang, and Dow 2007). While Gurken may be secreted from all muscles, we prefer a model in which localized expression in a muscle subset shapes the branching pattern of the innervating motoneuron. In this model, Gurken released from muscles 6 and 7, as well as other targets that should not be innervated, would locally inhibit synaptogenesis, blocking the formation of ectopic connections while allowing for the normal innervation at muscles 12 and 13. This model is consistent with our findings that knockdown of Gurken in muscle results in ectopic NMJs while localized overexpression in the normal target cell inhibits formation of appropriate NMJs. While these functional data are strong, our model must remain speculative because we have been unable to determine the localization of Gurken using currently available reagents. Future studies will investigate how this Gurken/EGFR pathway is integrated with the recently defined semaphorin- and activity-dependent mechanisms that also play an important role in shaping synaptic target selection in these neurons (Carrillo et al. 2010).



B. Muscles 6 & 7



D. Muscles 12 & 13

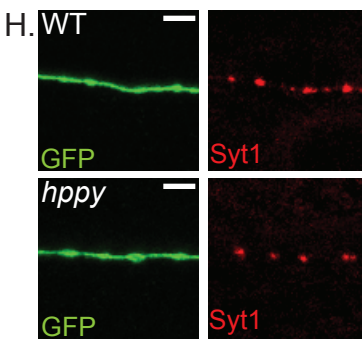
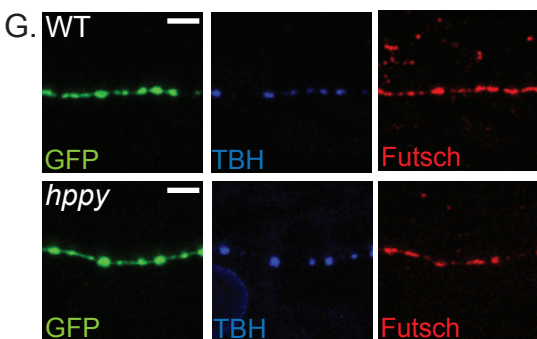
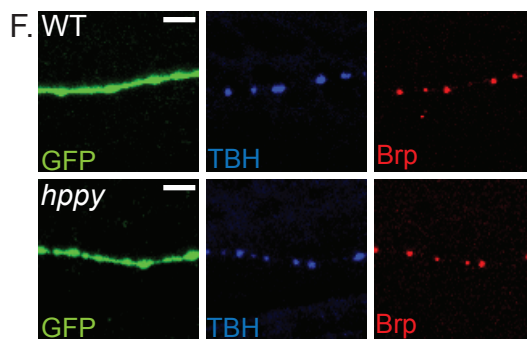
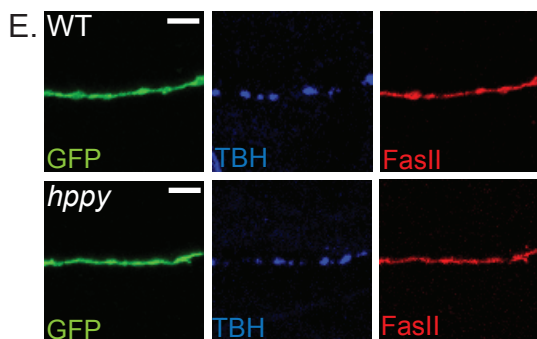
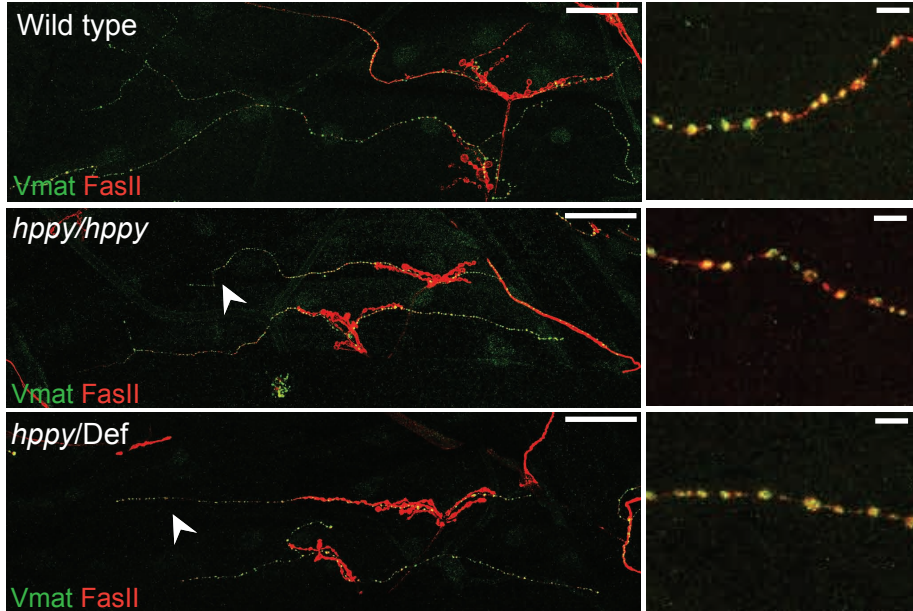


Figure 3.1. *hppy* mutants have ectopic Type II synapses

A. Schematic detailing the innervation pattern of a wild-type larval ventral muscles. Red represents Type I glutamatergic innervations. Green represents Type II octopaminergic innervations. Muscles are identified by number. L corresponds to lateral and M corresponds to medial.

B. Images of 3rd instar larval muscles 6 and 7 with neurons immunolabeled with anti-FasII (red) and anti-Vmat (green). Arrowheads indicate ectopic Type II innervations positive for Vmat. Scale bars represent 50 μ m.

C. Histogram shows quantification of the presence of Type II innervation on muscles 6 and 7 (percent of muscles innervated by Type II synapses). *hppy* larvae have a significant increase in the presence of Type II innervation on muscles 6 and 7 (WT 1.8% \pm .4% n=54; *hppy* 16% \pm 1.4% n=54; WT:*hppy* p<.05 Fisher's exact test). *hppy/def* flies also have a significant increase in the presence of Type II innervation on muscles 6 and 7 (*hppy/def* 13% \pm .8%; n=92; WT:*hppy/def* p< .05 Fisher's exact test). Error bars represent 95% confidence interval.

D. Images of muscles 12 and 13, Arrowheads indicate normal Type II innervations that are unaffected in *hppy* mutants. Scale bars represent 50 μ m.

E-H. Images of wild-type (WT) Type II synapses on muscle 12 and ectopic Type II synapses in *hppy* mutants on muscles 6 and 7. Synapses are labeled with markers of mature Type II innervation. Scale bars represent 1 μ m.

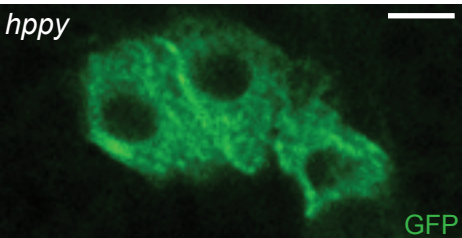
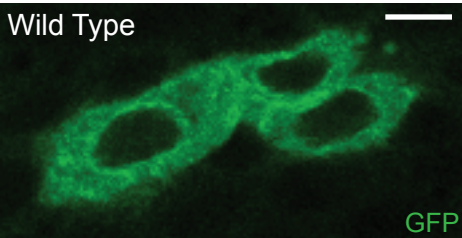
E. Type II NMJs immunolabeled with anti-mCD8 (Tdc2-GFP)(green), anti-TBH (blue) and anti-FasII (red).

F. Type II NMJs immunolabeled with anti-mCD8 (Tdc2-GFP)(green), anti-TBH (blue) and anti-Brp (red).

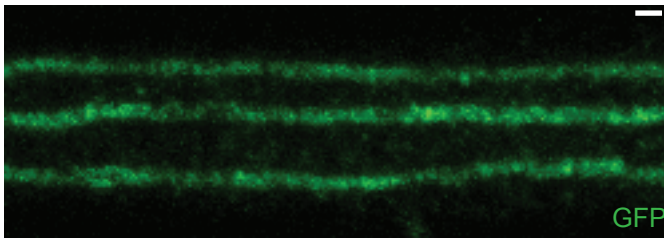
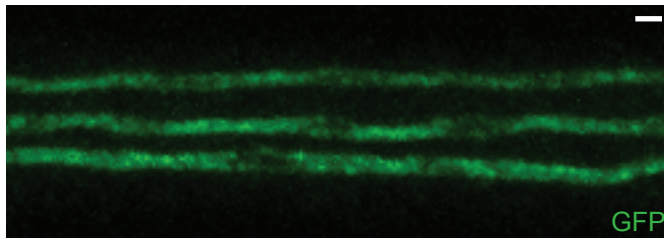
G. Type II NMJs immunolabeled with anti-mCD8 (Tdc2-GFP)(green), anti-TBH (blue) and anti-Futsch (red).

H. Type II NMJs immunolabeled with anti-mCD8 (Tdc2-GFP)(green), and anti-Syt1 (red).

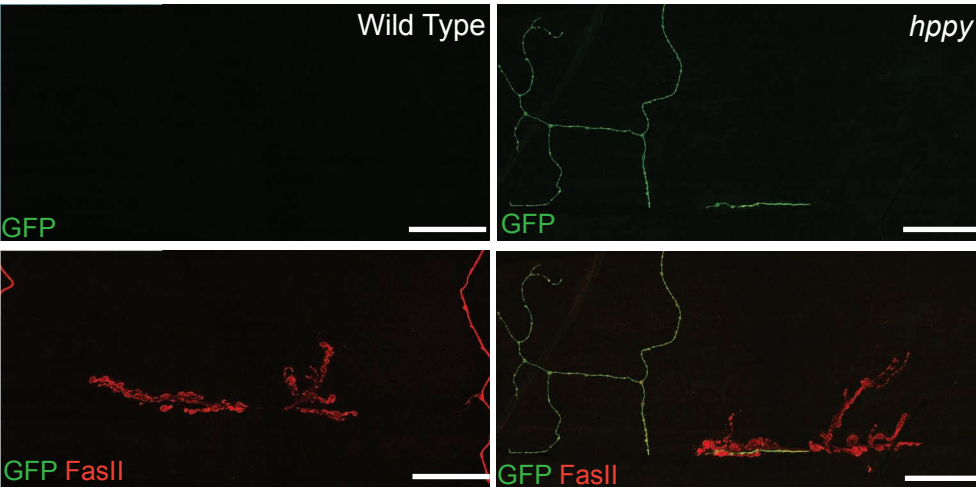
A. Cell Bodies



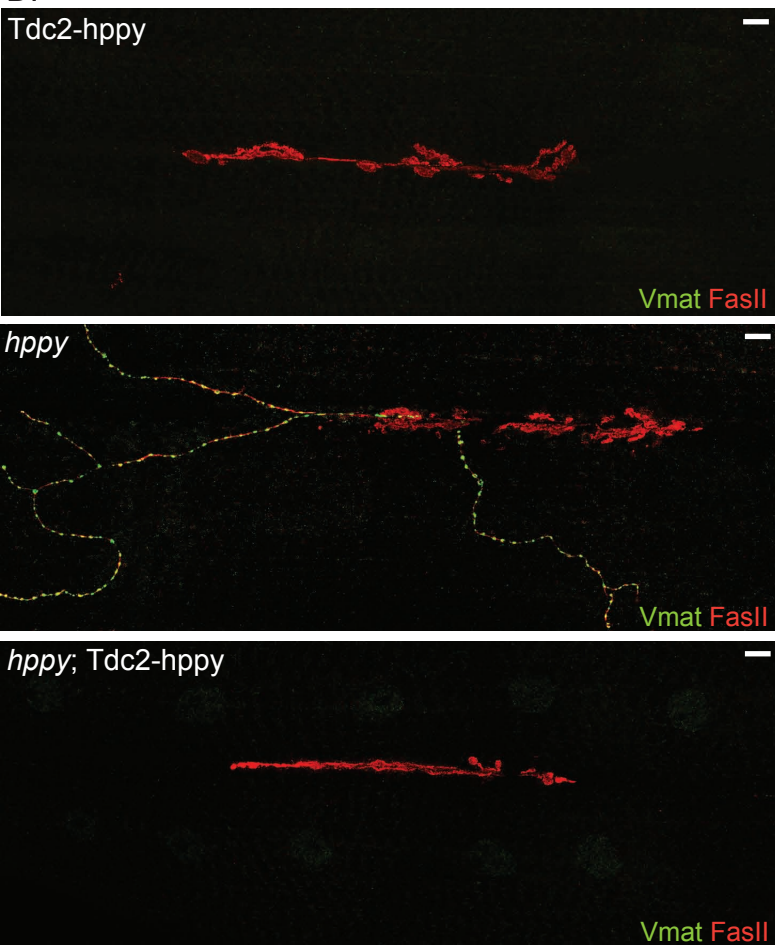
B. Axonal Projections



C.



D.



E.

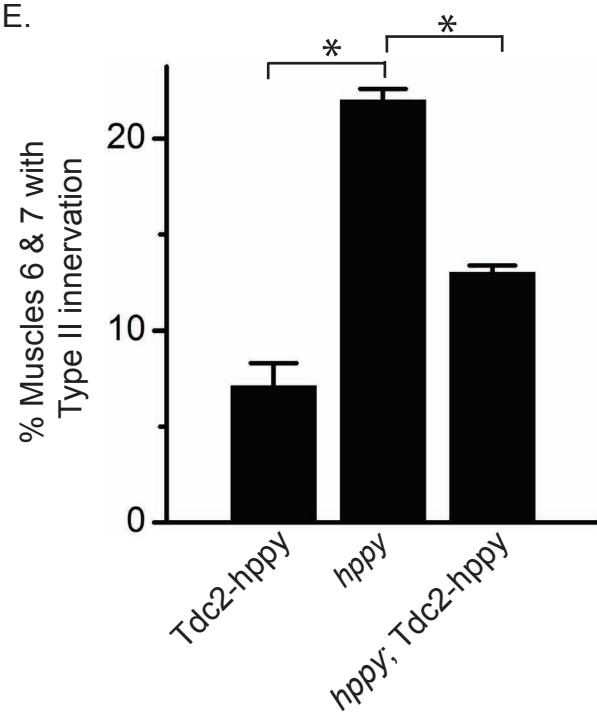


Figure 3.2. Ectopic synapses result from a targeting defect of the existing Type II neurons

A. Single confocal sections of Type II neuronal cell bodies expressing *UAS-CD8-GFP* under *Tdc2-Gal4* control. *hppy* mutants do not exhibit an increased number of Type II neurons. (WT-3; *hppy*-3; n=10; p=1) Scale bars represent 5 μ m.

B. Images of projections expressing *UAS-CD8-GFP* under *Tdc2-Gal4* control in Type II neurons. *hppy* mutants do not exhibit an increased number of projections from Type II neurons. (WT-3; *hppy*-3; n=10; p=1) Scale bar represents 1 μ m.

C. Images of projections expressing *UAS-CD8-GFP* under *Tdc2-Gal4* control. The *Tdc2-GFP* projections form the ectopic Type II synapses in *hppy* mutants. FasII (red) labels neuronal membranes. Scale bar represents 5 μ m.

D. Images of larval muscles 6 and 7, neurons immunolabeled with FasII (red) and Vmat (green). Ectopic Type II innervations found in *hppy* mutants are rescued by expression of *UAS-hppy* with *Tdc2-Gal4* (Type II neuron specific). Scale bar represents 10 μ m.

E. Quantification of the presence of Type II innervation on muscles 6 and 7. Type II neuron specific rescue of *hppy* mutants significantly suppressed ectopic Type II innervations on muscles 6 and 7. *Tdc2-hppy* 7.1% \pm 1.2% n=42; *hppy* 22% \pm .6% n=150; *hppy*; *Tdc2-hppy* 13% \pm .4% n=150) (*hppy* : *hppy*; *Tdc2-hppy* p < .05 Fisher's exact test). Error bars represent 95% confidence interval.

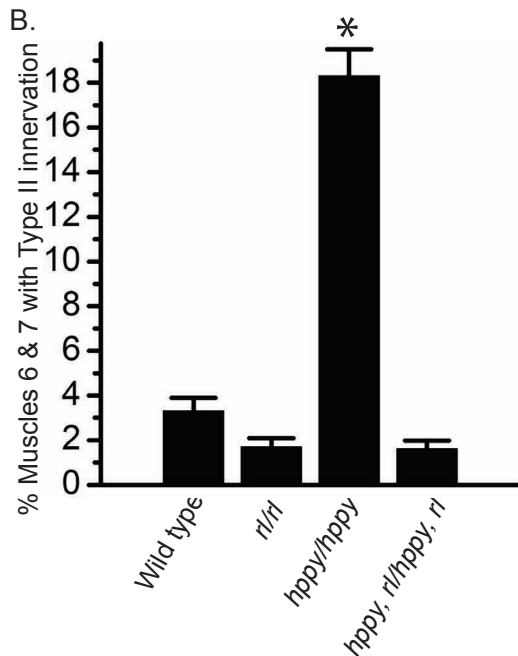
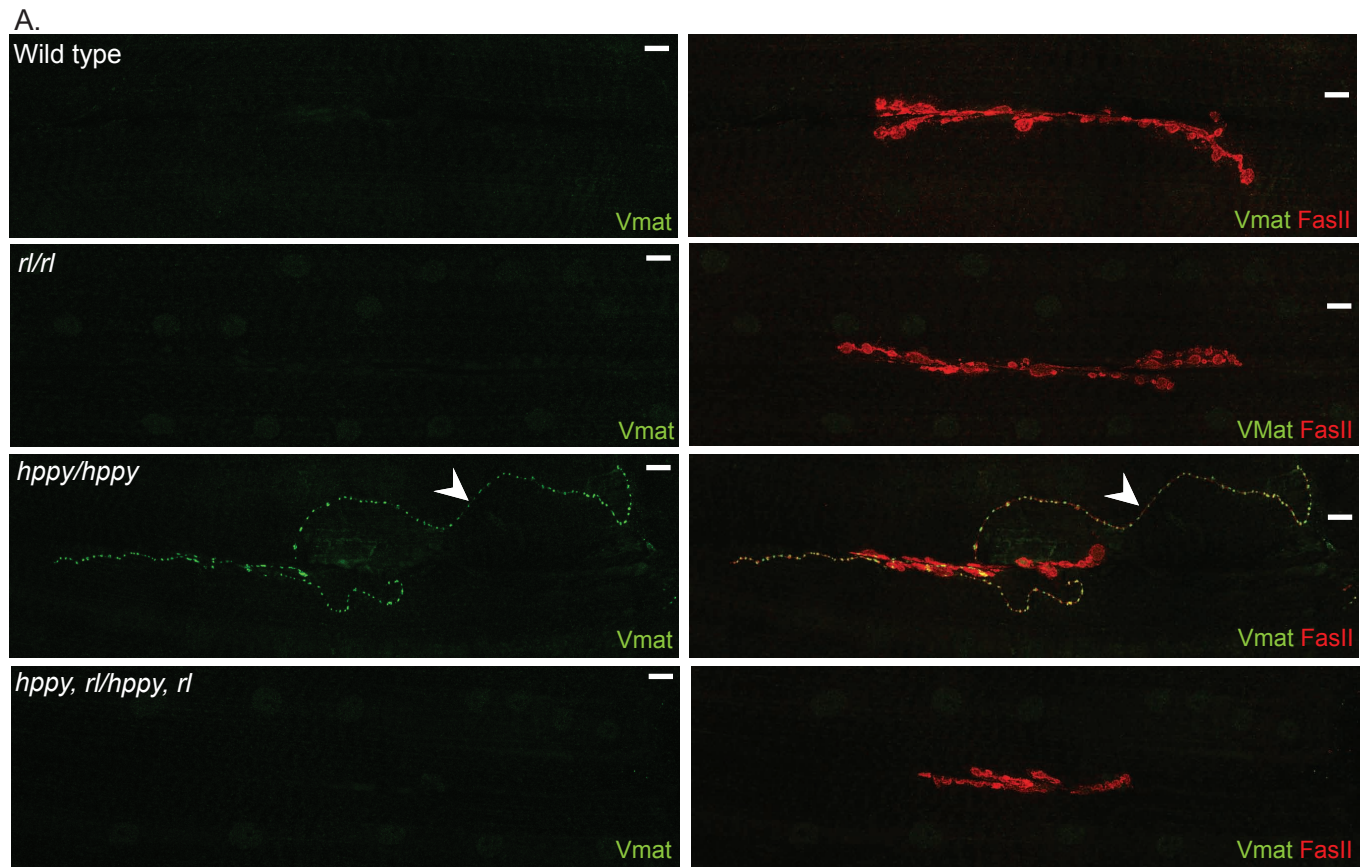


Figure 3.3 Overactivation of *rolled* downstream of *happyhour* is responsible for the ectopic synapses

A) Images of larval muscles 6 and 7, neurons immunolabeled with anti-FasII (red) and anti-Vmat (green). Arrowheads indicate the presence of ectopic Type II innervation. Scale bar represents 10 μ m.

B) Quantification of the presence of Type II innervations on muscles 6 and 7. A double mutant of *hppy* and *rolled* significantly suppresses the *hppy* phenotype (WT 3.3% \pm .59% n=60; *rl/rl* 1.7% \pm .4% n=59; *hppy* 18.3% \pm 1.2% n=60; *hppy,rl/hppy,rl* 1.6% \pm .4%) (*hppy*: *hppy,rl/hppy,rl* p < .005 Fisher's exact test). Error bars represent 95% confidence interval.

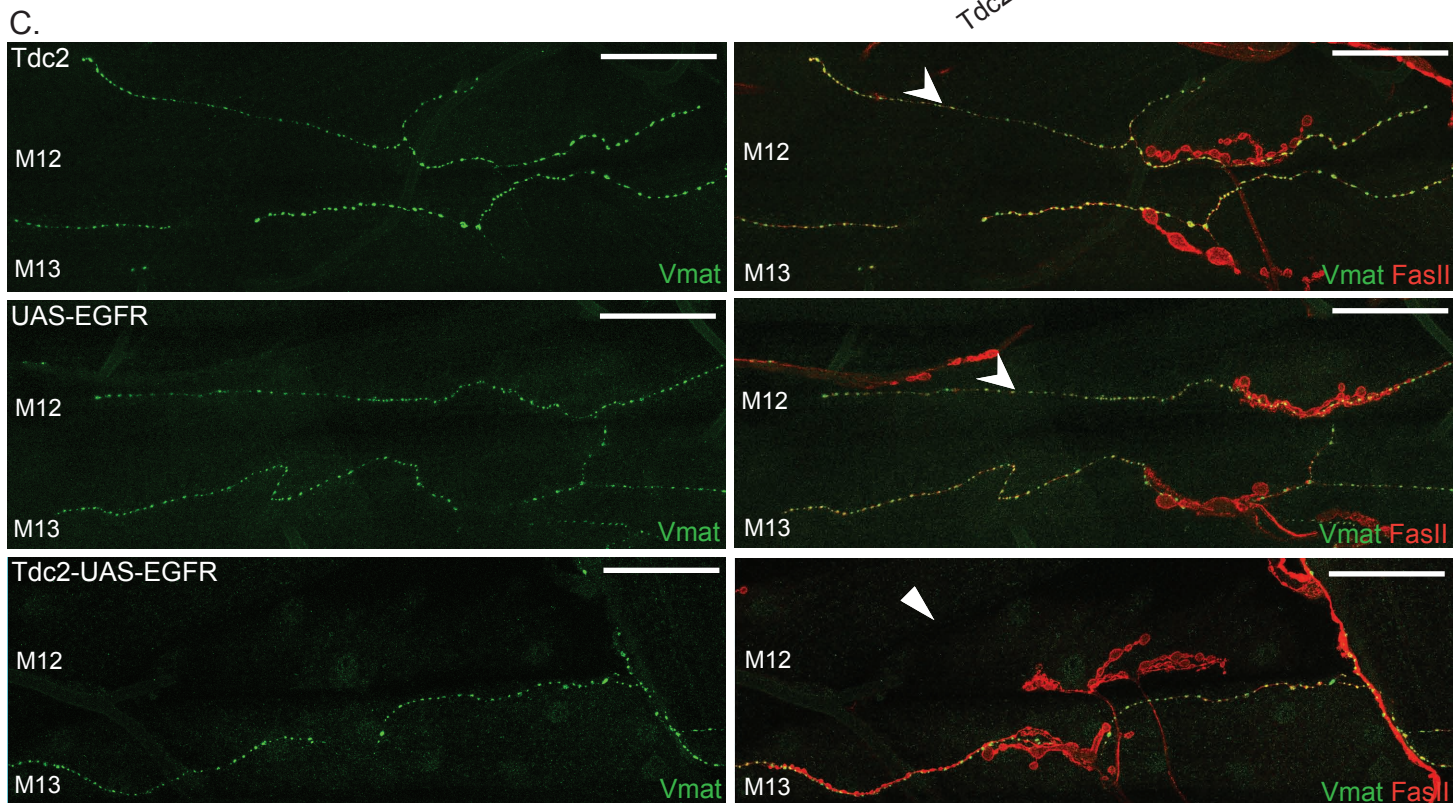
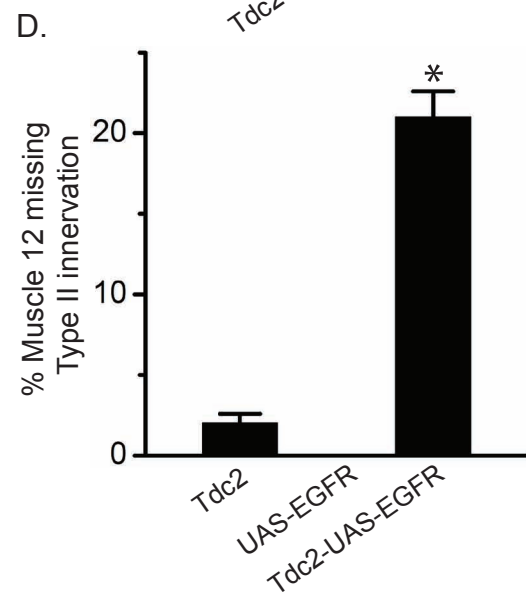
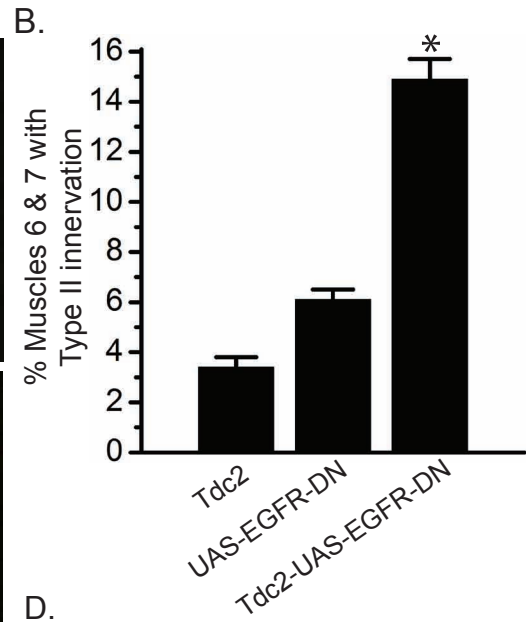
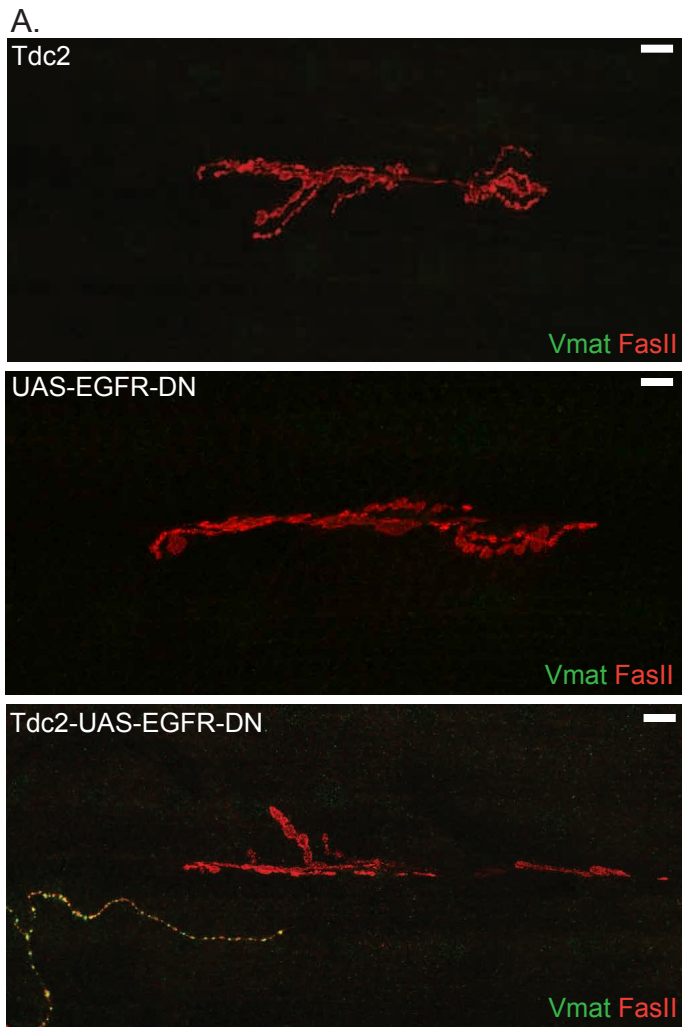


Figure 3.4. EGFR is necessary and sufficient to inhibit Type II innervation

A. Images of muscles 6 and 7 immunolabeled with anti-Vmat (green) and anti-FasII (red). Loss of EGFR signaling in Type II neurons results in ectopic Type II innervation on muscles 6 and 7.

B. Graph shows quantification of the presence of Type II innervation on muscles 6 and 7. Loss of EGFR signaling results in a significant increase in Type II innervation on muscles 6 and 7 (*Tdc2/+* 3.4% ± .4% n=100; *UAS-EGFR-DN* 6.1% ± .4% n=100; *Tdc2-UAS-EGFR-DN* 14.9% ± .8% n=100) (*Tdc2/+* : *Tdc2-EGFR-DN* p< .05; *UAS-EGFR-DN*:*Tdc2-UAS-EGFR-DN* p< .05) Fisher's exact test). Error bars represent 95% confidence interval.

C. Images of muscles 12 and 13 immunolabeled with anti-Vmat (green) and anti-FasII (red) M12 and M13 notations indicate location of muscles. Arrowhead indicates normal Type II innervation found on Muscle 12. Arrow indicates absence of Type II innervation on Muscle 12 when *UAS-EGFR* is overexpressed with *Tdc2-Gal4*.

D. Graph shows quantification of the absence of Type II innervations on Muscle 12. Overexpression of wild-type EGFR with a Type II neuron specific driver results in a significant number of muscles 12 that are not innervated by Type II synapses (*Tdc2/+* 2% ± .6% n=50; *UAS-EGFR/+* 0% n=50; *Tdc2-UAS-EGFR* 21% ± 1.6% n=50) (*Tdc2/+*:*Tdc2-UAS-EGFR* p< .005; *UAS-EGFR/+*:*Tdc2-UAS-EGFR* p= .0005 Fisher's exact test). Error bars represent 95% confidence interval.

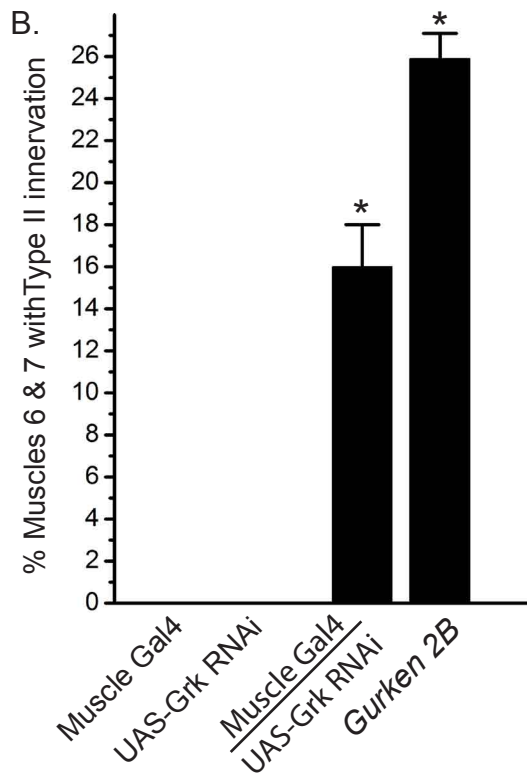
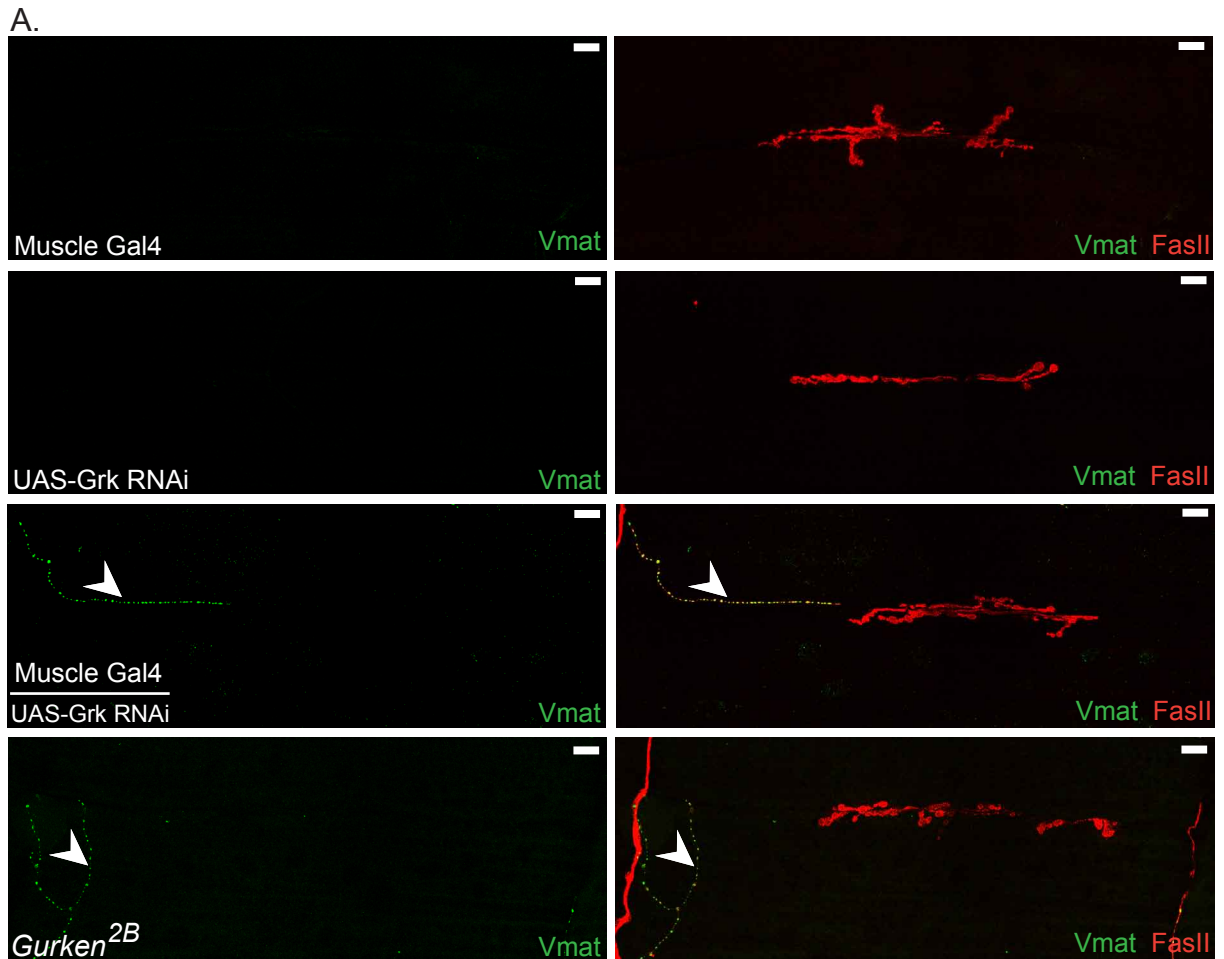


Figure 3.5. Gurken ligand is necessary to mediate a repulsive signal through EGFR

A. Images of muscles 6 and 7 immunolabeled with anti-Vmat (green) and anti-FasII (red). Knockdown of Gurken in the muscle results in ectopic Type II synapses on muscles 6 and 7. Gurken2B mutants also exhibit ectopic Type II synapses on muscles 6 and 7. Scale bars represent 10 μ m.

B. Graph shows quantification of the presence of Type II innervation on muscles 6 and 7. Knockdown of Gurken in the muscle results in a significant increase in Type II innervation on muscles 6 and 7 (UAS-Dcr2;G7gal4/+ 0% n=36; UAS-Grk RNAi 0% n=36; UAS-Dcr2;G7gal4/UAS-Grk RNAi 16% \pm 2% n=36; Gurken2B 25.9% \pm 1.2% n= 77) (UAS-Dcr2;G7gal4/+ : UAS-Dcr2;G7gal4/UAS-Grk RNAi $p < .05$ Fisher's exact test) (UAS-Dcr2;G7gal4/+ : Gurken2B $p < .0005$ Fisher's exact test). Error bars represent 95% confidence interval.

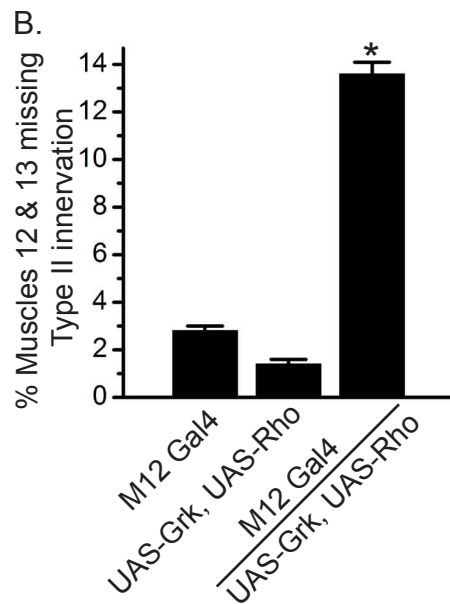
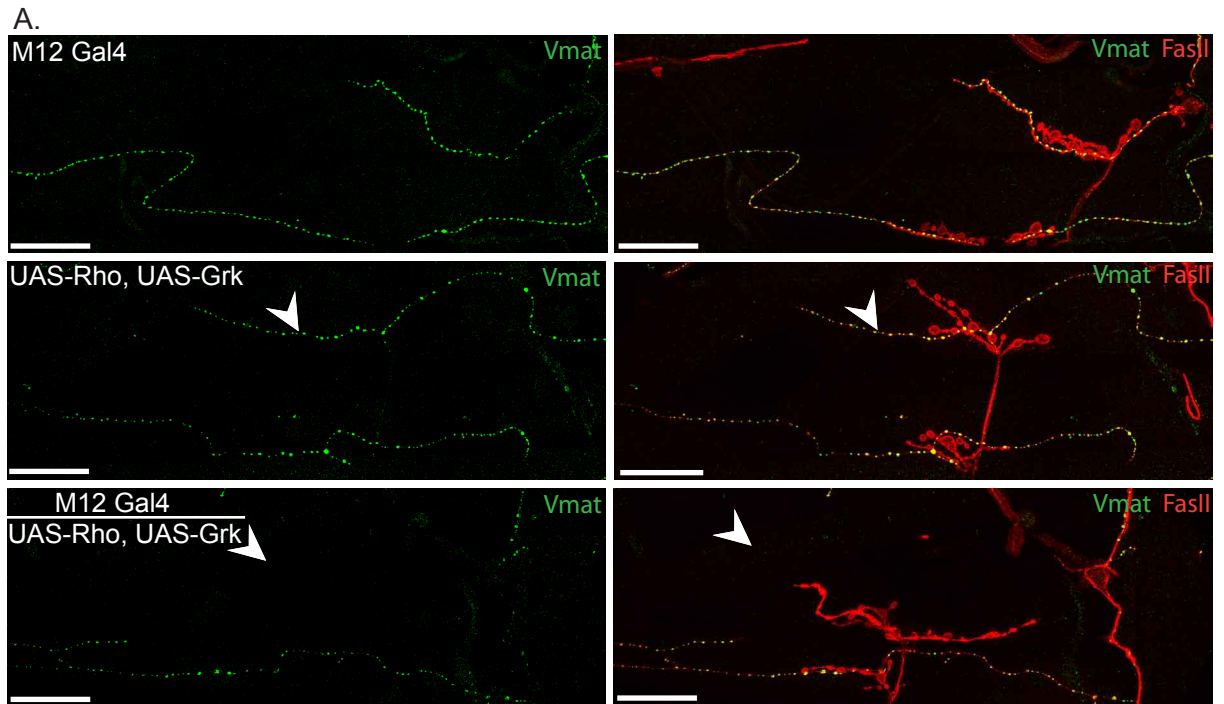


Figure 3.6. Gurken ligand is sufficient to inhibit Type II innervation through EGFR

A. Images of muscles 12 and 13 immunolabeled with anti-Vmat (green) and anti-FasII (red). Expression of UAS-Gurken and UAS-Rhomboid with the muscle 12 specific driver M12-Gal4 results in a loss of Type II synapses on muscles 12 and 13. Scale bars represent 50 μ m.

B. Graph shows quantification of the loss of Type II innervation on muscles 12 and 13. (M12/+ 2.8% ± .2% n=70; UAS-Gurken, UAS-Rhomboid/+ 1.4% ± .2% n= 70; M12/UAS-Gurken, UAS-Rhomboid 13.6% ± .5% n=66) (M12/+ : M12/UAS-Gurken, UAS-Rhomboid p= 1; UAS-Gurken, UAS-Rhomboid/+ : M12/UAS-Gurken, UAS-Rhomboid p<.05 Fisher's Exact Test). Error bars represent 95% confidence interval.

Chapter 4

Discussion

Discussion

In this dissertation, I have performed a forward genetic screen of multiple collections of mutants in order to uncover new genes involved in synaptic development. I have isolated many mutants with a variety of phenotypes ranging from general growth defects, to specific defects in the components of the presynaptic release machinery. I have characterized three of these mutants in more detail with the in depth characterization of *happyhour* leading to the publication in Chapter 3. Here I will discuss the results and implications of my thesis work more in depth.

Forward genetic screening for defects in synaptic development

Forward genetic screening is an unbiased approach to uncovering novel regulators of synaptic development. By screening three divergent collections of mutants or RNAi lines, I have been able to generate a diverse list of mutants and RNAi lines which affect synaptic development. While time constraints have precluded study of all of these lines, many of these genes have since been published by other groups and many remain to be studied in the lab. For example, I found the gene *srpk79D* to have a defect in axonal transport with large aggregates of Brp clogging the axons. It has since been shown to be a kinase that regulates the assembly of Brp molecules and normally acts to prevent their assembly until they reach the NMJ (Nieratschker et al. 2009). While my screens did identify several genes that already have known roles in synaptic development, the identification of several novel genes demonstrates the utility of this sort of approach.

Bruchpilot mutant phenotypic variance

The allele of Bruchpilot that we identified as a second site mutation in a piggy-Bac insertional mutant is a great example both of the constantly evolving genomes of flies in the lab and the variability of phenotype for any given gene. The published phenotypes for Bruchpilot mutants include: decrease in immunoreactivity of nc82 antibody, decreased number of T bar structures at active zones, reduction in evoked response, reduced quantal content, and increased miniature epsp amplitude, and mislocalization of presynaptic calcium channels (Wagh et al. 2006; Robert J Kittel, Wichmann, et al. 2006). The allele that I isolated appeared to be a very weak allele as there was a slight decrease in the number of Brp puncta and the intensity of those puncta. We also observed an increase in miniature epsp amplitude and the concomitant decrease in quantal content. Our allele however, also exhibited some post-synaptic defects in that the receptor clustering was diffuse. These slight variances and relative weakness of our allele along with the piggyBac insertion originally led us to believe our allele was that of another gene. It is interesting to note that our allele did have a slight variance in the phenotype from other published alleles. It is possible the mutation in our allele affected only a small domain of *bruchpilot*. It is also possible that the variation we saw in the post-synaptic receptors was merely a genetic background effect and was not influenced by bruchpilot at all.

Beta-Spectrin in disease

Spinocerebellar ataxia type 5 (SCA5) is an autosomal dominant neurodegenerative disorder caused by mutations in *Beta-Spectrin*. When SCA5 mutation containing transgenes are expressed in *Drosophila*, larvae exhibit posterior paralysis, reduced synaptic terminal growth and axonal transport defects (Lorenzo et al. 2010). We identified an allele of Beta-Spectrin, MA3.5, in our forward genetic screen. MA3.5 has a significant axonal transport defect but does not exhibit any of the other known phenotypes of Beta-Spectrin mutants such as posterior paralysis or smaller synaptic terminals. It is interesting that our allele most closely resembles the overexpression of spino-cerebellar ataxia (SCA5) causing mutations. It is possible that the MA3.5 allele has increased expression of Beta-Spectrin and is causing the axonal transport defects. It is also possible that overexpression is occurring and there is also a mutation affecting the function of Beta-Spectrin. MA3.5 represents a possible model for SCA5 without introducing transgenic overexpression of disease constructs.

Happyhour's influence at the NMJ

Happyhour is a Ste20 kinase about which relatively little is known. It is known to function in neurons to modulate responsiveness to alcohol (Corl et al. 2009). My work corroborates these data in that *hppy* modulates EGFR signaling in Type II neurons specifically. It is possible that *happyhour's* primary role in neurons is to modulate EGFR signaling for a particular purpose. It is not known whether its primary function is to modulate synaptic target selection, modulate alcohol sensitivity or another as yet undiscovered function. It is also possible that *hppy* is meant to modulate all processes

requiring EGFR in the neuron and this explains the pleiotropy we find in *hppy* mutants. It would be interesting if this is the case because it is yet another mechanism to refine EGFR signaling in addition to negative and positive feedback loops. It also helps to prevent any “slop” in the system from causing any changes that affect cell fate or specification. Future work may be able to delineate whether *happyhour* is a general modulator or a modulator of specific aspects of EGFR signaling.

EGFR as a guidance pathway

There are many known pathways that influence axonal and synaptic guidance. My work is the first that indicates a role for EGFR in synaptic target selection. It is not surprising that there are targeting pathways that have yet to be identified. Providing guidance and specificity to so many neurons with so many targets should require a complex combinatorial code of guidance cues in order to generate such specificity. EGFR signaling is well suited to this sort of task due to its refined response capabilities to activate a downstream signaling pathway in a single cell without influencing nearby neighbor cells. The relatively low penetrance of the phenotype I observe in Type II target selection suggests that there are other pathways also influencing this particular target selection event. It is also possible that EGFR is involved other target selection events other than those I’ve described in the Type II neurons.

Gurken outside the oocyte

It was very surprising to find that Gurken is the EGFR ligand responsible for mediating target selection of Type II neurons. Most of the literature strongly asserts that

Gurken plays a role only in the developing oocyte. It is possible that this body of research has been focused so narrowly that a role in later development could have been missed entirely. I have been unable to demonstrate Gurken expression in the larval muscles myself, which suggests that observing this expression at the exact time when it must be occurring is not trivial. It is possible that Gurken is secreted from a select group of muscles for a very short time window as all of the axon extension and target selection happens within six hours. To generate synaptic specificity many pathways must be employed, in this context it is less surprising that an alternative ligand that has already performed its primary patterning functions is employed.

Future Directions

There are a number of questions as yet unanswered about the role of EGFR signaling in target selection. It will be critical to determine if the EGFR negative feedback mechanism is activated in *hppy* mutants. It will also be interesting to determine if activity of the Type II neurons can modulate the ectopic synapse phenotype. Activity has been shown to modulate synaptic outgrowth and maintenance (Koon et al. 2011; Carrillo et al. 2010). More broadly, the complete mechanism contributing to synaptic development is still a mystery. It is possible that some of the other mutants I have found will contribute to delineating other molecules and pathways critical for synaptic development.

Conclusions

In conclusion, synaptic development is a complex and critical process necessary for establishing the proper connectivity and function of neural circuits. These neural

circuits are the foundation of the nervous system and the basis of learning and memory and general brain function. This work has advanced the field by screening for novel regulators of synaptic development and uncovering a novel pathway necessary for synaptic target selection.

Appendix I.

Electron Microscopic analysis of autophagosomal development in *Ema* mutants

This work appears in the manuscript:

Kim S, Naylor SA, DiAntonio A. *Ema* promotes autophagosomal growth via Golgi membrane trafficking. *Developmental Cell* (In Revision)

SAN performed electron microscopy, quantification and data analysis.

Autophagy is a cellular process of self-digestion during which cytosolic components are engulfed in double membrane autophagosomes and digested by lysosomal acidic hydrolases (C. He and Klionsky 2009). While many genes are known to be conserved in the formation, maturation and fusion of autophagosomes, there are still many processes such as regulation of the size of autophagosomes, that remain to be described. Our lab previously described a novel gene *Ema* that promotes endosomal maturation. We hypothesized that *ema* may also play a role in autophagosomal maturation.

To investigate *Ema*'s role in autophagy we took advantage of the *Drosophila* fat body whose cells undergo extensive autophagy in response to starvation stimuli. While many antibodies and transgenic fluorescently tagged molecules are available to investigate autophagy in flies, to definitively determine autophagosome number and size electron microscopy (EM) is necessary. I contributed to this project by performing transmission EM on fed and starved fat body cells from wild type and *ema* mutant *drosophila* larvae.

Methods

For ultrastructural analysis of the *Drosophila* fat cells, samples were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM phosphate buffer, pH 7.2 overnight at 4°C. Samples were washed in phosphate buffer and postfixed in 0.5% osmium tetroxide (Polysciences Inc., Warrington, PA)/0.08% potassium ferricyanide (Electron Microscopy Sciences, Fort Washington, PA)/100 mM phosphate buffer for 1 hr at room temperature, and subsequently in 1% tannic acid

(Electron Microscopy Sciences, Fort Washington, PA)/100 mM phosphate buffer for 1 hr. Samples were then rinsed extensively in dH₂O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 hr. Following several rinses in dH₂O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA). Electron micrographs were also taken on a transmission electron microscope (H-7500; Hitachi). For the analysis of the size of autophagic structures, one image per section from wild type (n=8) and mutant (n=12) cells were taken randomly at a magnification of 10,000X. The length along the longest axis of autophagic structures was measured using imageJ.

Results

From our EM analysis, we found that both wild type and *ema* mutant fat cells contain lipid droplets, glycogen deposits, membrane tubules, and cellular organelles including mitochondria and ER (Fig A). Consistent with other markers tested in these fat cells, very few profiles that could be endosomes or lysosomes could be detected under fed conditions. Upon starvation, wild type fat cells exhibit extensive vesicular and vacuolar autophagic structures, many containing remnants of mitochondria, ER and glycogen particles (arrows Fig B). However, in the starved *ema* mutant fat cells, autophagosomes are present but are significantly smaller than those in wild type. To quantify this we measured the length of autophagic structures along their longest axes. Histogram and cumulative probability plots illustrate that autophagic structures rarely reach 5 μ m in

length in the *ema* mutant but can reach sizes up to 10 μm in wild type (Fig D &E). On average, autophagic structures in *ema* mutants are approximately two times smaller than wild type (Fig C). This change in length suggests an approximately eight-fold decrease in autophagosome volume. This EM data corroborates our light level data indicating that *ema* mutants have significantly smaller autophagosomes.

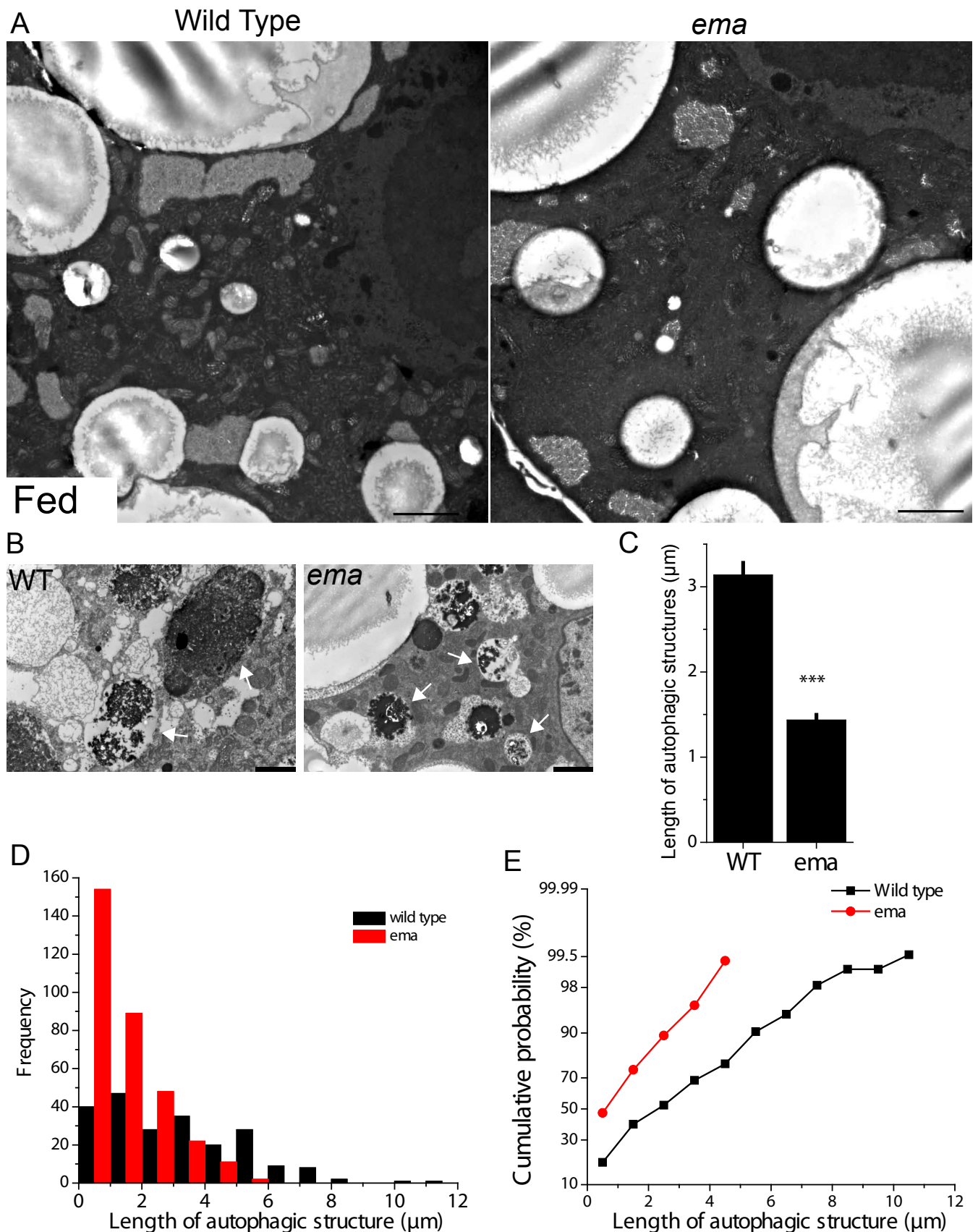


Figure AI-1.

(A) Transmission electron micrograph of fed *Drosophila* fat cells. Scale bars = 2 μm . (B) Transmission electron micrograph of starved *Drosophila* fat cells. Autophagic structures (arrows) contain many remnants of rough ER and mitochondria. Scale bars= 2 μm . (C) Histogram of quantification of the length of autophagic structures in wild type and *ema* mutant fat cells n = 219 autophagic structures from 8 sections from wild type; n = 326 from 12 sections from *ema* mutant. Error bars represent SEM. t-test; *** ($p < 0.001$) (D and E) Histogram (D) and cumulative probability plot (E) of the length distribution of autophagic structures in starved fat cells.

Appendix II.

Electron Microscopic analysis of Taxol-induced neurodegeneration

This work appears in the manuscript:

Bhattacharya, M.R.C., Gerdts, J., Naylor, S.A., Royse, E., Ebstein, S.Y., Sasaki, Y.,
Milbrandt, J., DiAntonio, A., A model of toxic neuropathy in *Drosophila* reveals a
requirement for MORN4 in executing axonal degeneration. *Journal of Neuroscience* (In
Revision)

SAN performed electron microscopy, quantification and data analysis.

Chemotherapeutic drugs have greatly advanced the treatment of many types of cancer. Despite their great value in fighting cancer, these drugs are still associated with dose-limiting and debilitating side effects including peripheral neuropathy. Our lab is investigating the molecular mechanisms responsible for axon degeneration in response to chemotherapeutic agents. A post-doc in the lab has developed an assay to deliver a chemotherapy agent, Taxol, to developing drosophila larvae through their food. These larvae can then be dissected and axonal degeneration can be observed. This model serves as a powerful genetic tool to understand axonal degeneration.

In order to pursue studies to find genes and treatments that abrogate axonal degeneration, we first had to establish the metrics of axonal degeneration in the drosophila larval nerves. While changes in a genetically encoded GFP that labels class IV sensory neurons can be observed at the light level (Figure A-C), electron microscopy is necessary to ascertain that these nerves are degenerating in response to Taxol. I contributed to this project by performing TEM analysis on vehicle and Taxol treated larval nerves.

Methods

For ultrastructural analysis of the *Drosophila* nerves, samples were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM phosphate buffer, pH 7.2 overnight at 4°C. Samples were washed in phosphate buffer and postfixed in 0.5% osmium tetroxide (Polysciences Inc., Warrington, PA)/0.08% potassium ferricyanide (Electron Microscopy Sciences, Fort Washington, PA)/100 mM phosphate buffer for 1 hr, and subsequently in 1% tannic acid (Electron Microscopy

Sciences, Fort Washington, PA)/100 mM phosphate buffer for 1 hr. Samples were then rinsed extensively in dH₂O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 hr. Following several rinses in dH₂O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 100 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate. Electron micrographs were taken on a transmission electron microscope (H-7500; Hitachi). Quantification of axon number was performed on 10 non-consecutive sections of nerves from at least three independent larvae per treatment condition. Statistical analysis was performing using Student's t-test with two tails.

Results

EM analysis of vehicle and taxol treated nerves revealed a striking loss of axons in the taxol condition. There is an approximately two-fold decrease in the number of axons per nerve after taxol treatment. Nerves of taxol-treated animals do not exhibit the tight packing of axons found in vehicle treated nerves. There are large gaps that often contain debris including organelles such as mitochondria, from the degenerating axons that once filled the nerve. These data indicate that the response to taxol in our *Drosophila* model is comparable to observed responses in mammals.

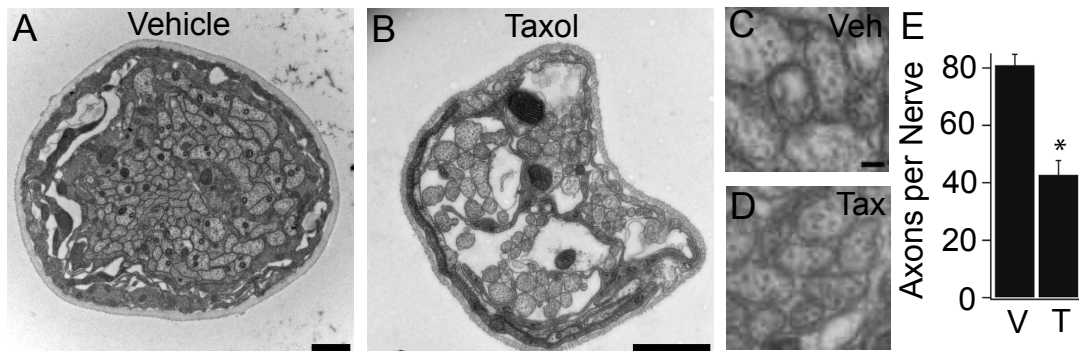


Figure All-1. Taxol exposure leads to axon loss in *Drosophila* larval nerves

A) Transmission electron micrograph of vehicle (DMSO) treated *drosophila* larval nerve cross-section. Axons are tightly packed with little empty space within the nerve. Scale bar 1 micron. B) Transmission electron micrograph of 30 mM Taxol treated *drosophila* larval nerve cross section. There is a significant loss of axons and an increase in empty space within the nerve. Scale bar 1 micron C&D) Higher magnification of vehicle and Taxol treated *drosophila* larval nerve cross sections. These images indicate that the remaining nerves appear to be intact. Scale bar 200nm E) Histogram shows quantification of number of axons per nerve in vehicle or Taxol treatment. Taxol treatment causes a significant loss of axons. (Vehicle 80.9 ± 3.9 $n=10$, Taxol 42.7 ± 5.1 $n=10$ $p < .0001$) Error bars represent SEM.

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