Analysis of Myosin VI in *Drosophila melanogaster* synaptic function and development

by

Marta Kisiel

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Cell and Systems Biology
University of Toronto

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Abstract

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2013

Myosin VI, encoded by *jaguar (jar)* in *Drosophila melanogaster*, is the only member of the myosin superfamily of actin-based motor proteins known to move towards the minus ends of actin filaments. *In vitro* studies demonstrate that Myosin VI has the ability to perform distinct functions as a cargo transporter and anchor in the cell, however which of these roles Myosin VI plays in the nervous system has yet to be determined. A locomotor defect, observed as sluggish movement in severe *jar* mutant larvae, was confirmed by behavioural assays. As this can indicate problems at the neuromuscular synapse, microscopy and electrophysiology were used to investigate neuromuscular junction (NMJ) structure and function in *jar* loss of function mutants of varying severity. Confocal imaging studies revealed a decrease in NMJ length, a reduction in bouton number per NMJ, alterations to the microtubule cytoskeleton and mislocalization of the synaptic vesicle protein Synaptotagmin in *jar* mutant boutons. FM dye labeling was consistent with the immunostaining data revealing vesicles endocytosed following electrical stimulation occupy the bouton centre in *jar* mutants. The data is indicative of a function for Myosin VI in maintaining proper peripheral vesicle localization. Electrophysiological experiments revealed a role for Myosin VI in basal synaptic transmission, with a reduction in low frequency nerve-evoked responses and spontaneous release in severe *jar* mutants. Changes in short-term synaptic plasticity were also observed in Myosin VI mutants by using both paired-pulse experiments to
examine release probability and high-frequency stimulation paradigms to recruit vesicles from different functional pools. Taken together, the data suggest that Myosin VI functions as an anchor to peripherally localize vesicles within the bouton enabling their efficient release during nerve stimulation. Synaptic vesicles are mobile at the *Drosophila* NMJ; thus if Myosin VI is acting as a vesicle tether, it would normally be expected to restrain vesicle mobility at the synapse. FRAP analysis revealed a significant increase in synaptic vesicle mobility in *jar* mutant boutons. This study elucidates novel roles for Myosin VI function in the nervous system via regulation of the synaptic microtubule architecture and localization of synaptic vesicles within the nerve terminal.
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List of Drosophila Stocks

*Df(3R)crb87-5*.................................................................deficiency removing Myosin VI

*elav*\(^{3A}\)*Gal4*.............................................................pan-neuronal Gal4 driver

*elav*\(^{C155}\)*Gal4*..........................................................pan-neuronal Gal4 driver

*jar*\(^{322}\)...........................................................................loss of function of Myosin VI

*OregonR (OreR)*.................................................................used as a control line

*UASactinGFP*.........................................................allows tissue specific expression of GFP-tagged actin

*UASSynaptotagminGFP*.............................GFP-tagged Synaptotagmin to mark synaptic vesicles

*yw*.............................................................................used as a control line
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<tbody>
<tr>
<td>Brp</td>
<td>Bruchpilot</td>
</tr>
<tr>
<td>CBD</td>
<td>cargo binding domain</td>
</tr>
<tr>
<td>Dab2</td>
<td>Disabled 2</td>
</tr>
<tr>
<td>EJP</td>
<td>evoked junctional potential</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FM 1-43</td>
<td>N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>G-actin</td>
<td>globular actin</td>
</tr>
<tr>
<td>GIPC</td>
<td>GAIP interacting protein, C terminus</td>
</tr>
<tr>
<td>IRP</td>
<td>immediately releasable vesicle pool</td>
</tr>
<tr>
<td>jar</td>
<td>jaguar loss of function mutant</td>
</tr>
<tr>
<td>mEJP</td>
<td>miniature Excitatory Junctional Potential</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>PTP</td>
<td>post-tetanic potentiation</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RP</td>
<td>reserve pool of vesicles</td>
</tr>
<tr>
<td>RRP</td>
<td>readily releasable pool of vesicles</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble NSF Attachment Protein Receptor</td>
</tr>
</tbody>
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Chapter 1: General Introduction

Synaptic transmission

The nervous system receives and processes internal and external sensory cues to maintain homeostasis and to mediate appropriate motor responses. It is also responsible for higher order functions such as learning and memory. Information is transmitted in the nervous system as electrochemical signals and communication between neuronal and other effector cells occurs at synapses, which may be electrical or chemical. At electrical synapses, current passes through channels connecting the presynaptic cell to the postsynaptic cell; thereby directly eliciting a voltage change in the postsynaptic cell. Chemical synaptic transmission requires the release of neurotransmitter, which is packaged into vesicles at the presynaptic nerve terminal. Upon release, neurotransmitter diffuses across the synaptic cleft that separates the presynaptic and postsynaptic cells. The neurotransmitter then binds to receptors in the postsynaptic cell membrane and this in turn induces a voltage change in the postsynaptic cell. Proper synaptic development is essential to maintaining rapid and accurate communication within the nervous system.

An understanding of the nature of neurotransmission began to form over 150 years ago when nerves were shown to be electrically excitable and able to generate currents (Krnjevic, 1974). In 1863, Krause deduced that some form of electrical discharge led to the excitation of the muscle membrane at a neuromuscular junction (NMJ) (Krnjevic, 1974). A decade later, du Bois-Reymond first proposed that the source of this electrical discharge was the secretion of a chemical from a nerve ending, although he incorrectly asserted the nerve terminal was inserted directly into the muscle. In the early 1900s, Langley began to develop and test the idea that receptors at the effector cell could respond to transmitter substances released from the nerve terminal (Bennett, 2000). The junctional hypothesis of neurotransmitter release into a cleft
between the nerve ending and the target cell failed to gain popularity until Loewi demonstrated that acetylcholine mediated chemical neurotransmission in cardiac muscle in 1921 (Bennett, 2000). In the 1940s, Hodgkin and Huxley were able to provide the first direct evidence of a cell’s negative resting potential and demonstrate that an action potential results from a reversal of this resting potential by inserting microelectrodes into the squid giant axon (Hodgkin and Huxley, 1945). Further insights into synaptic physiology would be gleaned from the work of del Castillo and Katz on frog NMJs, where they observed miniature end plate potentials. They believed that this represented the least unit of the end plate potential and that neurotransmitter was released in discrete quantal units (Del Castillo and Katz, 1954). These quantal units were thought to represent the neurotransmitter content of a single vesicle, which was supported by ultrastructural studies that showed a relationship between quantal electrophysiology and vesicular exocytosis (Heuser et al., 1979).

In the 1980s, attention shifted to uncovering the molecules that mediated synaptic transmission. Some of the earliest proteins to be identified were members of the Soluble NSF Attachment Protein Receptor (SNARE) complex. These include syntaxin and SNAP-25, which are bound to the target membrane, and synaptobrevin, which is bound to the vesicular membrane. Vesicle exocytosis requires the fusion of phospholipid bilayers and must overcome the repulsive forces between their respective negative charges. SNARE proteins complex to form a bundle of 4 α-helices surrounded by hydrophobic amino acids providing the energy for this fusion event to occur (Antonin et al., 2002). Following synaptic vesicle fusion, N-ethylmaleimide-sensitive factor functions to disassemble the SNARE complex through its ATPase activity allowing the component proteins to participate in future fusion events (Littleton et al., 2001). The calcium binding protein, Synaptotagmin, functions as a calcium sensor during synaptic transmission to initiate calcium-dependent vesicle fusion (Yoshihara and Littleton,
2002). At the postsynaptic membrane, receptors bind neurotransmitter mediating the transmission of information across the synapse. Synaptic response is plastic and can either strengthen or depress based on previous activity.

**Drosophila as a Model Organism**

Over a hundred years ago, embryologist Thomas H. Morgan began to work with *Drosophila* in an attempt to decipher the principles of genetics. During the course of his work, Morgan came across a fly with white eyes and this laboratory mutant set the stage for subsequent investigations into the nature of heredity (Morgan, 1910). *Drosophila* has remained a popular choice for genetic, developmental and behavioral studies because of the low cost of maintaining stocks, its short generation time and the large number of offspring produced. The fruit fly has a small genome, consisting of 13,667 genes distributed on 3 pairs of autosomes and 1 pair of sex chromosomes (Celniker and Rubin, 2003). The small number of chromosomes simplifies the process of mapping mutations and the availability of balancer chromosomes prevents the loss of these mutations through recombination (Celniker and Rubin, 2003). A high level of evolutionary conservation between genes that regulate basic developmental processes means that these genes in humans often have counterparts in *Drosophila* (Bier, 2005). Thus, investigations can be conducted into a variety of biological processes in the fruit fly that can in turn provide an understanding of the genetic basis of these processes in humans. As an invertebrate, however, there are certain limitations to working with *Drosophila* as a tool for understanding human genetics as it cannot be used to study certain vertebrate specific processes such as the synthesis of bone (Bier, 2005). Nevertheless, *Drosophila* has proven to be a useful model in the study of many diseases with 61% of identified human disease genes having an ortholog in the *Drosophila* (Rubin et al., 2000).
In the 1970s, the *Drosophila* larval NMJ became the focus of studies aimed at investigating the mechanisms of synaptic physiology (Jan and Jan, 1976a; Jan and Jan, 1976b). *Drosophila* has since become a favourite model organism for the study of synaptic assembly, function and plasticity. NMJs are chemical synapses between a presynaptic neuronal cell and a postsynaptic muscle fiber, which in *Drosophila melanogaster* are glutamatergic. Glutamate is an excitatory neurotransmitter and serves as the most common neurotransmitter in the vertebrate central nervous system. *Drosophila* NMJs are similar to vertebrate synapses at the molecular level, with homologous ionotropic glutamate receptors, and thus, they provide a model system for the study of synaptic development and function (Keshishian et al., 1996). Insights can be gained into fundamental questions about synapses with the practical advantages of working with an invertebrate, wherein genetic methods can be used to directly manipulate molecules of interest (Keshishian et al., 1996). Additionally, *Drosophila* larval NMJs develop in a readily identifiable, stereotyped pattern that is accessible for imaging and electrophysiological experiments (Zhang, 2003).

During embryonic development, motor axons leave the ventral nerve cord through three major nerve tracts; the intersegmental nerve, the segmental nerve and the transverse nerve. Axonal growth cones from these nerves extend out towards their synaptic targets directed by a variety of molecular cues. The neuromusculature in the developing embryo consists of repeating hemisegments (A2-A7) of 30 muscles, which will be innervated by 35 motor neurons that exit from the central nervous system. Synaptogenesis proceeds over a four hour period that can be visualized in real time (Ritzenthaler and Chiba, 2003). Neuromuscular synapses begin to form through the interaction between filopodia from the axonal growth cone with filopodia from the target muscle allowing for the precise pairing required between pre- and post-synaptic partners for proper synaptic function (Ritzenthaler and Chiba, 2003). Small synaptic currents are detected
at this stage suggesting that neurotransmitter release modulates synaptic development (Kidokoro and Nishikawa, 1994). Filopodia next transition into prevaricosities, which appear as large terminal swellings that are later constricted to form mature varicosities or boutons (Yoshihara et al., 1997). Boutons are specialized pre-synaptic sites of neurotransmitter release and recycling. As muscle growth continues during the larval stages, NMJs simultaneously expand by the budding of new boutons from existing boutons or de novo formation either at the end of the string of boutons or between two boutons (Zito et al., 1999). During larval development, synaptic connectivity is maintained via the homophilic cell adhesion molecule Fasciculin II, which is localized both pre- and post-synaptically (Schuster et al., 1996). By the third larval instar, the boutons are surrounded by muscle membrane, which undergoes a series of infoldings to form the subsynaptic reticulum (Schuster et al., 1996).

Many of the proteins involved in synaptic transmission are conserved and homologues of *Drosophila* proteins are found in higher organisms demonstrating the usefulness of this system in studying neural function. Although the basic features of synaptic transmission are shared between invertebrates and vertebrates, there are some key differences to note at the neuromuscular synapse. In *Drosophila*, larval muscles are polyneuronally innervated, whereas the muscle fibers of mammalian skeletal muscle receive innervation from a single neuron. Larval body wall muscles are super contractile and can contract to 50% of their resting length, in contrast to the length-tension relationship of vertebrate skeletal muscles (Peron et al., 2009). Another difference is that unlike in their mammalian counterparts, voltage-gated sodium channels are not expressed in *Drosophila* larval muscles (Hong and Ganetzky, 1994). Instead, excitation-contraction coupling occurs via voltage-gated calcium channels (Sullivan et al., 2000). Increases in stimulation lead to increases in graded postsynaptic potentials, unlike the all-or-none responses generated at the mammalian muscle. Despite these difference, the underlying
molecular mechanisms that regulate synaptic transmission are conserved between vertebrates and invertebrates.

**The Myosin Superfamily**

Myosins are a superfamily of actin-based motor proteins that use energy derived from ATP hydrolysis to move along actin filaments (Cramer, 2000). The evolution of these motor proteins is concurrent with that of eukaryotes; *Saccharomyces cerevisiae* being the simplest organism in which Myosins are present (Syamaladevi et al., 2012). Phylogenetic analyses indicate that there are 37 Myosin types that vary in their protein domains and taxonomic distribution; for example, there are 12 classes of Myosins encoded by approximately 40 genes in humans (Richards and Cavalier-Smith, 2005). Myosins are divided into classes based on sequence similarities in the head and tail domains, which are related to the conserved biological functions of those classes (Berg et al., 2001). All Myosins are thought to have evolved from three ancestral Myosin classes that differed primarily in the tail region (Richards and Cavalier-Smith, 2005). Of the 18 known classes of Myosins, only two, classes V and XI, appear in lower eukaryotes as well as plants and animals, suggesting they represent the earliest Myosin lineages (Berg et al., 2001).

Myosin motors are comprised of three functional domains, including a catalytic head domain, a neck domain that binds calmodulin or light chains and a tail domain, which has a cargo binding subdomain (Sellers, 2000). The head domain of Myosin motors contains both the nucleotide binding and actin binding sites (Hartman et al., 2011). Distal to the head is the converter subdomain that transmits movements to the lever arm, which consists of calmodulin light-chain binding domains (Spudich and Sivaramakrishnan, 2010). In the neck domain, light chains or calmodulin bind to the IQ motif and the number of IQ motifs present varies among Myosins (Sellers, 2000). The greatest variability among Myosin motors is observed at the tail
region, which may have a coiled-coil domain for dimerization as well as a cargo-binding domain (Spudich and Sivaramakrishnan, 2010). The variable tail determines the cellular function of the Myosin motor through interactions with adaptors and cargo (Hartman and Spudich, 2012). The conversion of chemical energy by Myosins into mechanical work has been described by the swinging lever arm hypothesis. According to the swing lever arm hypothesis, Myosin motion along an actin filament is coupled to conformational changes in the catalytic domain during ATP hydrolysis, which are in turn amplified by the converter domain to the lever arm directing the motion of the motor (Hartman et al., 2011). Myosins do vary, however, in their motor properties with respect to length of time spent attached to the actin filament, processivity, amount of force generated during movement and velocity (Krendel and Mooseker, 2005).

Through their molecular properties, Myosins are able to participate in a diverse range of cellular functions. Perhaps, the best known role is that of the conventional class II of Myosins, which are responsible for producing contractions in muscle cells and can complex to form thick bipolar filaments (Hartman and Spudich, 2012). Other unconventional Myosins have been shown to play a role in generating plasma membrane protrusions by maintaining membrane-cytoskeleton adhesions and through a transporter role by delivering regulatory cargo (Nambiar et al., 2010). They may also be responsible for generating the force required to direct cell movement (Hartman et al., 2011). Other biological processes that Myosins are known to be important for include cytokinesis, organelle transport, cell polarization, transcription and signal transduction (Syamaladevi et al., 2012). In addition to transport functions along actin tracks, Myosins can alter the structure and dynamics of actin filaments through interactions with actin regulatory binding partners (Hartman et al., 2011). The regulation of Myosin function in different cell processes can occur through a variety of mechanisms such as phosphorylation, calcium-dependent activation, dimerization or cargo binding (Hartman et al., 2011).
**Myosin VI Structure: A unique motor protein**

Myosin VI, first identified in *Drosophila melanogaster*, shares the well-conserved basic structural conformation of other Myosin proteins (Kellerman and Miller, 1992). The crystal structure of the Myosin VI N-terminal motor domain, which includes the nucleotide and actin-binding sites, is similar to that of the actin plus-end moving motor, Myosin V (Menetrey et al., 2005). The C-terminal tail sequence consists of four different subdomains: the 70 residue proximal tail, the 70 residue medial tail, the 50 residue distal tail and the 250 residue cargo-binding domain (CBD) (Spink et al., 2008). The medial tail was previously predicted to contain a coiled-coil region (Park et al., 2006); however using a variety of imaging techniques, (Spink et al., 2008) showed that it in fact is comprised of a 10 nm single α-helix. This α-helix has a repeating sequence of four negatively charged glutamic acid residues and four positively charged amino acids, arginines and lysines, known as an ER/K motif, whose interactions stabilize the medial tail (Spudich and Sivaramakrishnan, 2010). Interestingly, molecular dynamic simulations and Fluorescence Imaging with One-Nanometer Accuracy suggest that the medial tail region is capable of dimerization (Kim et al., 2010). Spontaneous self-association of the medial tail is accompanied by a vertical shift that enables electrostatic interactions to occur between oppositely charged residues (Kim et al., 2010). The CBD, which is composed of four β strands and six α helices, recognizes and binds cargo (Yu et al., 2009). This domain has been highly conserved throughout the evolution of Myosin VI molecules and no homologous sequences are present in any other proteins (Yu et al., 2009).

Myosin VI is the only myosin known to move towards the minus or pointed ends of actin filaments (Menetrey et al., 2007). The reverse directionality of Myosin VI is due to a unique insert between the converter and the IQ motif of the motor domain, insert 2, which orients the lever arm towards the minus end of the actin filament (Menetrey et al., 2007). The proximal
region of insert 2 wraps around the converter domain (Menetrey et al., 2005). In its distal region, insert 2 forms a previously uncharacterized calmodulin-binding domain and the bound calmodulin at this insert is thought to act as structural component (Bahloul et al., 2004). Crystal structures reveal that the interactions between the insert and the converter produce 180° redirection of the lever arm to the minus end of the actin filament (Menetrey et al., 2005). Motility assays confirm that Myosin VI constructed truncated without the complete sequence of unique insert 2 show plus-end directed motion (Bryant et al., 2007). Another unique insert near the nucleotide-binding pocket of the motor domain, insert 1, regulates Myosin VI kinetics by restricting the accessibility of ATP to the nucleotide-binding pocket (Menetrey et al., 2007). This slows ADP release and results in Myosin VI's weak affinity for ATP, which in turn leads to slow the dissociation of Myosin VI from actin (Menetrey et al., 2007). These kinetic properties suggest that Myosin VI may function as an anchor that can bind cellular components to the actin cytoskeleton, in addition to functioning as a cargo transporter (Naccache and Hasson, 2006). Using optical trapping, Altman et al. (2004) demonstrated that applying a backward load to Myosin VI dimers increased ADP association to the trailing head allowing Myosin VI to remain bound to actin, identifying a possible mechanism for regulating its anchoring function. Naccache and Hasson (2006) employed molecular techniques to show that phosphorylation at threonine 406 in the motor domain may also be involved in switching Myosin VI from a transport to an anchoring function.

Although in vitro motility assays indicate that Myosin VI dimers move processively along actin filaments (Rock et al., 2001), the mechanism of Myosin VI transport has yet to be fully resolved, as it is often purified as a monomer from native tissues (Sweeney and Houdusse, 2007). Myosin VI expressed using the baculovirus system was eluted as a monomer that exhibited non-processive attachment to actin in in vitro motility assays (Lister et al., 2004).
Single-headed Myosin VI has, however, been shown to move processively upon cargo binding by detaching then reattaching to actin (Iwaki et al., 2006). Myosin VI has been shown to dimerize in vitro using electron microscopy and functional assays under conditions where monomers are clustered in close proximity (Park et al., 2006). Cargo binding has also been implicated in initiating Myosin VI dimerization and the resulting dimers have been observed to move by hand-over-hand steps (Park et al., 2006; Phichith et al., 2009). Nuclear magnetic resonance and X-ray crystallographic studies confirm that dimerization can occur in the tail domain of Myosin VI when it is bound to a cargo (Yu et al., 2009). Fluorescence resonance energy transfer of labeled Myosin VI molecules bound to endocytic vesicles in human retinal cells indicates they are positioned in close proximity, potentially enabling cargo-mediated dimerization (Altman et al., 2007).

Interestingly, artificial dimers have revealed a large step size of 36 nm for Myosin VI, which has only two light chains, that is comparable to Myosin V, which has six light chains. The large step size of Myosin VI can be partially explained by conformational changes in the converter region which lead to the 180° rotation of its lever arm (Spudich and Sivaramakrishnan, 2010). Spink et al. (2008) have used gliding filament and optical trapping assays to support the hypothesis that the medial tail, composed of a long, single α-helix, might lengthen the lever arm extending the reach of Myosin VI heads during processive stepping. Alternatively, Liu et al., (2011) used steered molecular dynamics stimulations to lend support to their theory that in the Myosin VI dimer the medial tails form a tight complex and the lever arm is lengthened by extension of the three-helix bundle that makes up the proximal tail.
Diverse cellular functions of Myosin VI

Myosin VI has been shown to be important in regulating the localization and function of various organelles within the cell. In mammalian cells, Myosin VI has been shown to localize to the nucleus, where it regulates transcription through a complex with RNA polymerase II (Vreugde et al., 2006). Myosin VI also plays a role in regulating the transport of mitochondria in Drosophila primary neurons. When Myosin VI levels are depleted using RNA interference, retrograde mitochondrial movement is enhanced (Pathak et al., 2010). Myosin VI is required for efficient vesicle transport from the endoplasmic reticulum to the Golgi and when Myosin VI was knocked down, the rate of cargo transport visualized using a GFP-tagged reporter molecule was significantly reduced (Bond et al., 2011b). Myosin VI also co-localizes with optineurin at the Golgi complex where they complex to mediate transport in the Golgi secretory pathway (Sahlender et al., 2005). Specifically, total internal reflection fluorescence microscopy experiments used to image vesicle fusion events at the plasma membrane showed that a reduction in Myosin VI expression was accompanied by a reduction in fusion events (Bond et al., 2011a). This was visualized as a large proportion of vesicles remaining docked at the membrane, which is suggestive of a role for Myosin VI in the final stages of secretion (Bond et al., 2011b). Similarly, live imaging of the vesicle cycle in mammalian cells indicates a role for Myosin VI in the process of endocytosis (Aschenbrenner et al., 2004).

In Drosophila, Myosin VI is encoded by the gene Jaguar and it has been implicated in numerous functions during development. During embryogenesis, Myosin VI interacts with the cell adhesion molecule Echinoid to regulate the morphology of epithelial cells involved in proper dorsal closure (Lin et al., 2007). Knockdown of Myosin VI expression revealed it was also required for maintaining follicle cell morphology and regulating the migration of follicle cells during oogenesis (Deng et al., 1999). A reduction in Myosin VI in migratory cells was
accompanied by a reduction in the expression of the cell adhesion molecules E-cadherin and Armadillo (Geisbrecht and Montell, 2002). This is consistent with in vitro mammalian cell studies that show Myosin VI is recruited to E-cadherin cell-cell adhesions and that Myosin VI regulates these contacts by stabilizing the underlying actin cytoskeleton through its interaction with the actin regulator vinculin (Maddugoda et al., 2007). In Drosophila spermatogenesis, Myosin VI is required to stabilize the actin cone responsible for cytoplasmic expulsion in spermatid individualization (Noguchi et al., 2006).

In human subjects, a loss of Myosin VI has been associated with various disease pathologies. Genetic analysis reveals that a missense mutation in the Myosin VI gene results in cochlear hearing loss and cardiac abnormalities (Mohiddin et al., 2004). Likewise, the Snell's waltzer mouse, a Myosin VI loss of function mutant, experiences deafness associated with a loss of Myosin VI expression within the sensory hair cells of the cochlea (Avraham et al., 1995). In contrast to wild-type mice, Myosin VI loss of function mutants have disorganized hair cell bundles with fused stereocilia, which ultimately leads to cellular degeneration in the inner ear (Hertzano et al., 2008). These Myosin VI null mice also exhibit defects at the intestinal epithelium with electron microscopy highlighting the importance of Myosin VI for tethering the membrane to the underlying actin cytoskeleton (Hegan et al., 2012). In addition, Myosin VI has been found to play a role in the migratory activity of various types of cancer cells. Immunostaining shows that while Myosin VI is generally absent from the ovarian epithelium, a high level of Myosin VI was expressed in ovarian carcinomas (Yoshida et al., 2004). In vitro human cell studies and in vivo mouse models show that when Myosin VI was inhibited using RNA interference, the spread of cancer cells was impeded (Yoshida et al., 2004). Similarly, in leukemic cell lines, down regulation of Myosin VI expression resulted in reduced migration accompanied by an overall reduction in filamentous actin (Jbireal et al., 2010). Microarrays of
prostate cancer cell lines show Myosin VI expression to be upregulated and knockdown of Myosin VI accompanied by a reduction in protein secretion (Puri et al., 2010). The exact mechanism by which Myosin VI is involved in the progression of these cancers is yet unknown. However, Myosin VI expression has been shown to be mediated by DNA damage through tumor suppression protein p53-dependent activation of the promoter of the Myosin VI gene, which is associated with a reduction in DNA damage-induced apoptosis (Jung et al., 2006).

**Myosin VI function in the nervous system**

Although Myosin VI has received intense attention as a result of its unique directionality and its ability to perform distinct functions as a cargo transporter and anchor in the cell, little is known regarding the role of Myosin VI in the nervous system (Osterweil et al., 2005). In the vertebrate nervous system, the expression of Myosin VI has been confirmed by immunostaining and western blot analysis (Osterweil et al., 2005; Suter et al., 2000). In cultured chick neurons, Myosin VI is found at growth cones, where it is enriched in the microtubule-rich central domain, however, its function therein remains unclear (Suter et al., 2000). In Myosin VI mutant mice, electron microscopy of hippocampal preparations reveals a decrease in the number of synapses, a decrease in dendritic spine length and an increase in the number of astrocytes (Osterweil et al., 2005). Myosin VI mutant mouse hippocampal neurons also exhibit defects in the internalization of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptor (AMPAR), responsible for fast glutamatergic transmission, suggesting Myosin VI normally plays a role in AMPAR endocytosis (Osterweil et al., 2005). In addition, basal synaptic transmission is reduced in Myosin VI deficient mouse hippocampal slices compared to wild-type controls (Yano et al., 2006). Electrophysiological experiments also indicate that Myosin VI mediates glutamate release induced by brain-derived neurotrophic factor, which is known to modulate synaptic transmission and plasticity in the mammalian central and peripheral nervous
system (Yano et al., 2006). Recent work in cultured mouse cortical neurons suggests a novel function for Myosin VI in the enrichment of transmembrane proteins to the axonal surface by increasing the relative rate of endocytosis of axonal proteins from dendritic compartments (Lewis et al., 2011). In rat cells used to model undifferentiated peripheral neurons, Myosin VI has been found to interact with Dock7, an atypical guanine nucleotide exchange factor, and this interaction is thought to regulate actin dynamics in these neuronal cells (Majewski et al., 2012). At the neuromuscular junction in the rat hindlimb skeletal muscle, imaging and mutant studies show Myosin VI localization at the postsynapse and suggest a contribution to neurotransmission via an as yet unidentified postsynaptic effect (Karolczak et al., 2012).

In Drosophila, Myosin VI localization in the embryonic nervous system has been observed using the Gal4/UAS system to express a Myosin VI-GFP fusion protein in a similar pattern to native Myosin VI (Millo and Bownes, 2007). During Drosophila embryonic nervous system development, Myosin VI has a known role in the asymmetric division of neural progenitor cells, called neuroblasts. Myosin VI functions in the proper asymmetric localization of cell fate determinants during neuroblast division via its interaction with the adaptor protein, Miranda (Petritsch et al., 2003). Fluorescence recovery after photobleaching (FRAP) analysis indicates that Miranda moves to the basal crescent of the dividing neuroblast by diffusion, where it is retained and experiences decreased mobility likely through its interaction with Myosin VI (Erben et al., 2008). Additionally, a genetic study from our lab identified a potential role for Myosin VI at the NMJ as a suppressor of the overgrowth phenotype induced by a dominant negative mutation of N-ethylmaleimide sensitive factor 2, an ATPase shown to function in the disassembly of the soluble NSF attachment receptor complex of proteins (Peyre et al., 2006). Altogether the results of these studies suggest Myosin VI may function at the Drosophila NMJ.
Thesis Aims and Objectives

My thesis investigates a novel role for Myosin VI in synaptic development and function. I hypothesize that Myosin VI functions as an anchor at the NMJ that tethers synaptic vesicles and thereby, ensures their proper localization within the nerve terminal. Through this vesicle tethering function, I propose that Myosin VI may function to restrict synaptic vesicle mobility ensuring vesicles are properly distributed for efficient release during synaptic transmission.

To test these hypotheses, the following specific aims will be explored:

1. Investigating the role of Myosin VI in synaptic vesicle localization within the nerve terminal.

2. Examining the relationship between vesicle localization and synaptic transmission in Myosin VI mutants using electrophysiological techniques.

3. Describing the means by which changes in synaptic plasticity can be achieved through Myosin VI regulation of specific synaptic vesicle populations.

4. In exploring a role for Myosin VI as a vesicle tether, this work will also investigate the regulation of vesicle mobility within the nerve terminal.
Chapter 2: Myosin VI mutants exhibit altered synaptic morphology at the *Drosophila* neuromuscular junction

Excerpts from Chapter 2 have been previously published in BMC Neuroscience:


The experiments were conceived and designed by Marta Kisiel and Bryan Stewart. Marta Kisiel performed all experiments described, analyzed the data, and assembled figures. Debolina Majumdar and Shelagh Campbell performed initial observations of *jar* mutant phenotypes.

Myosin VI is an actin-based motor protein with a unique directionality, moving towards the minus or pointed ends of actin filaments. The kinetic properties of Myosin VI allow it to function as either a cargo transporter or an anchor in the cell. In *Drosophila*, *jar* was previously uncovered in a screen for genes that modify NMJ development. To further investigate the role of Myosin VI in the nervous system, a genetic analysis of the effect of Myosin VI in synaptic morphology was conducted using loss of function *jar* alleles. Confocal imaging of *Drosophila* third-instar larvae revealed a decrease in NMJ length and a reduction in Ib bouton number at *jar* mutant NMJs. Loss of function *jar* mutants also exhibited disruptions to the microtubule architecture suggesting that Myosin VI may normally regulate the synaptic cytoskeleton to ensure proper NMJ development. Visualization of Synaptotagmin staining in *jar* mutant boutons showed altered synaptic vesicle distribution. In contrast to the characteristic doughnut-shaped pattern of Synaptotagmin localization present in wild-type Ib boutons, the majority of *jar* mutant Ib boutons exhibited diffuse Synaptotagmin localization throughout the bouton centre. This is suggestive of a tethering function wherein Myosin VI acts as an anchor maintaining vesicles at the bouton periphery.
**Introduction**

Fast neurotransmission requires close apposition of the pre- and postsynaptic elements, with precise arrangement of their protein components. The presynaptic active zone is defined as the site of synaptic vesicle docking, priming and release, which is visualized at the ultrastructural level as distinct morphological specializations consisting of electron dense membrane. The adjunct postsynaptic density contains neurotransmitter receptors for signal reception and other proteins for further integration. The *Drosophila* NMJ is composed of higher order presynaptic specializations known as boutons and an average NMJ has 20 to 50 synaptic boutons along its length (Collins and DiAntonio, 2007). Each bouton in turn consists of 10 to 20 synapses that are characterized by presynaptic active zones opposing postsynaptic glutamate receptors. *Drosophila* active zones are associated with a characteristic T-bar structure, which is thought to enhance vesicle release, and they contain orthologs of the major vertebrate active zone proteins, with the exception of the two largest scaffolding proteins, Piccolo and Bassoon (Wairkar et al., 2009; Wichmann and Sigrist, 2010). At the postsynapse, the glutamate receptor subunits, GluRIIA and GluRIIB, are functionally redundant for viability, but exhibit different physiological properties with GluRIIA subunits producing larger currents (Schmid et al., 2008).

In *Drosophila*, there are four types of boutons that differ in their morphological and chemical properties (Hoang and Chiba, 2001). Of interest for this study were the largest synaptic boutons found at type I axon terminals. Boutons at type I terminals are classified as either type Ib (big type I), which are about 3 to 6 µm and are found at all NMJs of mature larvae, or as type Is (small type I), which are slightly smaller (Zhang, 2003). These boutons are thought to innervate all muscles and mediate glutamatergic synaptic transmission. Type II boutons are small, about 1 to 2 µm, and exhibit reactivity to octopamine (Monastirioti et al., 1995). Finally, type III boutons only innervate muscle 12 and occasionally muscle 13. Type III boutons exhibit immunoreactivity
to an insulin-like peptide and possess unique physiological properties mediated by a fast-gating potassium current (Gorczyca et al., 1993; Martinez-Padron and Ferrus, 1997). During the four day period of larval growth, pre-existing boutons spread out to accommodate new bouton addition, which is not restricted to any particular location (Zito et al., 1999). Rather, new boutons are added evenly along the synapse, with 61% of these additions occurring between existing boutons and the rest added to the ends of the NMJ (Zito et al., 1999).

Although Myosin VI is known to be important for Drosophila embryonic nervous system development, a function for Myosin VI at the NMJ has yet to be described. Myosin VI loss of function mutants of varying severity were used to examine changes to NMJ morphology in third-instar larvae. These studies revealed a reduction in NMJ size and Ib bouton number that was accompanied by changes to the microtubule cytoskeleton. In addition, synaptic vesicles were visualized by immunolabeling of Synaptotagmin to assess their localization within synaptic boutons. In wild-type Ib boutons, Synaptotagmin is localized in a characteristic torus pattern, present in an outer ring and excluded from the middle of the bouton (Stimson et al., 2001). In contrast, Synaptotagmin staining was present throughout the entire bouton area in the majority of jar mutant boutons. A similar phenotype was observed at the NMJs of Drosophila larvae lacking Synapsin, a phosphoprotein that reversibly associates with vesicles. Synapsin mutants exhibited bouton centre occupancy with FM1-43 loading under low frequencies (Akbergenova and Bykhovskaia, 2007). EM analysis confirmed that in Synapsin knockouts there was a spread of vesicles into the bouton centre, accompanied by a reduction in the size of the reserve pool of vesicles (Akbergenova and Bykhovskaia, 2010). In addition, extra vesicles formed upon intense stimulation, which are normally directed to the reserve pool in wild-type boutons, were immediately available for release in Synapsin knockouts (Akbergenova and Bykhovskaia, 2010; Akbergenova and Bykhovskaia, 2009). Thus, Synapsin functions in maintaining the peripheral
distribution of vesicles in Ib boutons, as well as in the formation and maintenance of the reserve pool (Akbergenova and Bykhovskaia, 2010). Similarly, changes to the vesicle localization in jar mutants would indicate that Myosin VI also plays a role in maintaining proper peripheral vesicle localization at the Drosophila NMJ.
Results

Quantification of Myosin VI loss of function alleles

Myosin VI protein levels were quantified by western blot of third-instar larval brains and body walls, excluding the mouth parts. The resulting blot revealed reduced Myosin VI levels in all jar loss of function mutants tested (Figure 1), with virtually no Myosin VI present in the most severe jar\textsuperscript{322}/Df(3R)crb87-5 zygotic and maternal null allelic combination. Myosin VI levels were compared between genotypes by measuring band density for Myosin VI staining and normalizing it to the intensity of the loading control, β-tubulin. This confirmed a reduction in Myosin VI levels for two different heterozygote combinations, jar\textsuperscript{322}/+ and Df(3R)crb87-5/+, as compared to the control in blots of both brain and body wall samples, although this was less obvious than for the null mutants on the blot (Figure 2). These data are representative of three separate trials of this experiment and serve to confirm that the Myosin VI alleles used for this study were indeed loss of function. The results are consistent with previous descriptions of these alleles (Morrison and Miller, 2008).
Figure 1. Quantification of loss of function jaguar alleles.

Western blotting of third-instar larval brains and body walls revealed a reduction in Myosin VI levels in jar loss of function mutants. Lanes for both blots are numbered as follows: 1-ladder, 2-OreR, 3- jar\textsuperscript{322}/+, 4- Df(3R)crb87-5/+, 5- jar\textsuperscript{322}/Df(3R)crb87-5 and 6- jar\textsuperscript{322}/Df(3R)crb87-5 MN. The loading control used was β-Tubulin. Myosin VI levels were reduced in jar\textsuperscript{322}/+ and Df(3R)crb87-5/+ larvae compared to the control in blots of both brain and body wall samples. No bands were observed for either brain or body wall samples for jar\textsuperscript{322}/Df(3R)crb87-5 and jar\textsuperscript{322}/Df(3R)crb87-5 MN larvae indicating the absence of Myosin VI expression in these mutants at the third-instar larval stage.
Figure 2. Loss of function jaguar mutants exhibit a reduction in relative Myosin VI proteins levels measured in larval brain and muscle tissue. 

Fluorescence intensity of protein bands was first normalized to the level of β-tubulin, the loading control, and then to the OreR control genotype to quantify relative Myosin VI protein levels in the jar loss of function mutants. A significant reduction in Myosin VI levels was observed for all jar mutants analyzed compared to the control (Two-way ANOVA, ****=p<0.0001). Bars represent mean ± SEM.
Myosin VI loss of function mutants exhibit altered NMJ morphology

To characterize the role of Myosin VI in *Drosophila* NMJ development, NMJ morphology on ventral longitudinal muscles 6 and 7 was visualized in *jar* loss of function mutants by staining neuronal tissues with an FITC-conjugated anti-HRP antibody. Muscles 6 and 7 are innervated by two motor neurons, the type Ib motor neuron (MN6/7-Ib) and the type Is motor neuron (MNSNb/d-Is), which originate from segmental nerve branches b and d (Hoang and Chiba, 2001). Third-instar larval NMJs exhibited a significant decrease in length for all of the *jar* loss of function mutants studied compared to both the *yw* and *OreR* control (Dunn’s Multiple Comparison Test, p<0.05) (Figure 3). The shortest mean NMJ length was observed for *jar*^322/*Df(3R)crb87-5 larvae, 171.56 ± 2.29822 μm (mean ± SE, n = 24 NMJs), compared to the *yw* control, 293.87 ± 12.9877 μm (n = 66 NMJs). In addition, a significant decrease in Ib bouton number was observed for *jar* loss of function mutants at muscle 6/7 NMJs (Dunnett's Multiple Comparison Test, p < 0.05) (Figure 4). On control NMJs from muscle 6/7, a mean number of 32.78 ± 1.3025 boutons (n = 58 NMJs) were observed; whereas these synapses in *jar*^322/*Df(3R)crb87-5 larvae had a mean number of 21.02 ± 0.60573 boutons (n = 58 NMJs).
**Figure 3. jaguar loss of function mutants display reduced muscle 6/7 NMJ length.**

Representative NMJs on ventral longitudinal muscles 6 and 7 from third-instar *Drosophila* larvae of the yw control (A) and a jar loss of function mutant, *jar*<sup>322</sup>/Df(3R)crb87-5 (B). All images were acquired at the same magnification using a LSM510 confocal laser microscope. Muscle 6/7 NMJ lengths for the jar loss of function genotypes studied were significantly shorter than the yw and *OreR* control (Dunn’s Multiple Comparison Test, ***=p<0.001) (C). Number of NMJs analyzed were: *OreR* n=42, yw n=66, *jar*<sup>322</sup>/+ n=68, Df(3R)crb87-5/+ n=70, *jar*<sup>322</sup>/Df(3R)crb87-5 n=66 and *jar*<sup>322</sup>/Df(3R)crb87-5 MN n=56. NMJs used for this study were visualized at muscle 6/7 in segments A3, A4 and A5. A maximum of two NMJs from the same larva were used in this analysis. Bars represent mean ± SEM.
Figure 4. *jaguar* loss of function mutants exhibited a reduction in *Ib* bouton number.

A significant decrease in *Ib* bouton number was observed for all *jar* loss of function mutants studied at muscle 67 NMJs in segments A3, A4 and A5 compared to the control (Dunnett's Multiple Comparison Test, *p*<0.05, **=p<0.01, ***=p<0.001). Number of NMJs from which bouton number was analyzed for each genotype were: *yw* n=54, *jar322/+* n=40, *Df(3R)crb87-5/+* n=26, *jar322/Df(3R)crb87-5* n=58 and *jar322/Df(3R)crb87-5 MN* n=14. A maximum of two NMJs from the same larvae were used in this analysis. Bars represent mean ± SEM.
Synaptic vesicles are mislocalized in Myosin VI loss of function mutants

Ultrastructural studies show vesicles are localized to the periphery of boutons, with the centre void of vesicles and partially occupied by mitochondria (Atwood et al., 1993). Surprisingly, visualization of Synaptotagmin staining in jar mutant boutons revealed a disruption in proper vesicle localization. The majority of jar^{322}/Df(3R)crb87-5 Ib boutons exhibited diffuse Synaptotagmin localization across the entire bouton area. The differences in Synaptotagmin distribution are illustrated in Figure 5 by a representative control yw bouton and mutant jar^{322}/Df(3R)crb87-5 bouton (A and B), shown with their corresponding plot profiles. The characteristic plot profile corresponding to yw boutons has two maxima consistent with a peripheral distribution of vesicles. In contrast, a single maxima is observed extending across the entire bouton area for jar^{322}/Df(3R)crb87-5 mutant larvae indicating a diffuse distribution of Synaptotagmin labeled vesicles. Differences in Synaptotagmin localization were quantified by calculating the relative difference in fluorescence intensity across a bouton’s plot profile [(max intensity-min intensity)/max intensity]. Relative difference in fluorescence intensity was significantly lower across jar^{322}/Df(3R)crb87-5 mutant boutons compared to yw control boutons (Unpaired T-test, p<0.001) (Figure 5C). However, there was no significant difference in mean fluorescence intensity over total bouton area for both genotypes (Unpaired T-test, p>0.05) (Figure 5D). Interestingly, diffuse jar^{322}/Df(3R)crb87-5 boutons were found to be significantly larger than yw control boutons and jar^{322}/Df(3R)crb87-5 boutons with a normal, torus-shaped distribution of Synaptotagmin (Dunn’s Multiple Comparison Test, p<0.001) (Figure 6). Diffuse jar^{322}/Df(3R)crb87-5 boutons had an average area of 13.81 ± 0.6156 μm^2 (mean ± SE, n = 53 boutons) whereas the yw control boutons had an average area of 8.81 ± 0.3199 μm^2 (n = 64 boutons).
To quantify the population of boutons displaying abnormal Synaptotagmin distribution, Ib boutons were scored as normal or diffuse for control and jar mutant muscle 6/7 NMJs in third-instar larvae. This protocol revealed an increase in the percentage of diffuse Ib boutons for jar mutant NMJs compared to control NMJs (Figure 7). A Chi Square goodness of fit test revealed that all jar mutants significantly differed from the control in number of normal and diffuse Ib boutons ($p < 0.05$). Taken together these data indicate that Myosin VI contributes to proper synaptic development. Specifically, the unexpected diffuse Synaptotagmin staining over the Ib bouton centre observed in jar mutants suggests that Myosin VI has a function in maintaining normal peripheral vesicle localization.
Figure 5. Synaptic vesicles are mislocalized at *jaguar* mutant boutons as revealed by immunostaining against Synaptotagmin.

A representative control Ib bouton and mutant *jar*^{322}/*Df(3R)crb87-5* Ib bouton are shown with their corresponding plot profiles (A and B). There was a significant reduction in relative difference in fluorescence intensity across *jar*^{322}/*Df(3R)crb87-5* mutant boutons (n=13) compared to the control boutons (n=25; Unpaired T-test, \(*\ast\ast\ast=p<0.001\) (C). Mean fluorescence intensity did not differ significantly between the mutant (n=48 boutons) and the control (n=64 boutons; Unpaired T-test, \(p>0.05\)) (D). Boutons used for this study were from muscle 6/7 NMJs in segments A3, A4 and A5. Bars represent mean \(\pm\) SEM.
Figure 6. Ib bouton area was increased in diffuse jaguar mutant boutons.

Diffuse \(jar^{322}/Df(3R)crb87-5\) boutons (n=53) were significantly larger than \(yw\) control boutons (n=64) and \(jar^{322}/Df(3R)crb87-5\) boutons with a normal, doughnut-shaped distribution of Synaptotagmin (n=27, Dunn’s Multiple Comparison Test, ***=p<0.001) Boutons used for this study were from muscle 6/7 NMJs in segments A3, A4 and A5. Bars represent mean ± SEM.
A) yw

B) jar^{322}/Df(3R)crb87-5

C) Bar graph showing the Ib bouton phenotype (% of total) for different genotypes:
- yw
- jar^{322}/+
- Df(3R)crb87-5/+
- jar^{322}/Df(3R)crb87-5
- jar^{322}/Df(3R)crb87-5 MN

Legend:
- Normal
- Diffuse
Figure 7. Boutons exhibiting vesicle mislocalization increased corresponding to the severity of jaguar loss of function.

Immunostaining against Synaptotagmin of NMJs on muscles 6/7 of control and jar\textsuperscript{322}/Df(3R)crb87-5 third-instar larvae revealed different proportions of boutons exhibiting normal and diffuse Synaptotagmin staining (A and B). Images were taken at different magnifications to visualize the entire NMJ. Open arrows indicate the normal, doughnut-shaped pattern of Synaptotagmin localization and closed arrows indicate the diffuse pattern of Synaptotagmin localization. Scoring the Synaptotagmin distribution phenotype revealed a reduction in the percentage of normal boutons and an increase in the percentage of diffuse boutons in jar loss of function mutants compared to the control (C). Number of boutons analyzed were: yw n=48, jar322/+ n=38, Df(3R)crb87-5/+ n=28, jar\textsuperscript{322}/Df(3R)crb87-5 n=58 and jar\textsuperscript{322}/Df(3R)crb87-5 MN n=18. Boutons used for this study were from muscle 6/7 NMJs in segments A3, A4 and A5. Bars represent mean ± SEM.
**Myosin VI mutants exhibit defects in the synaptic cytoskeleton**

Given that interactions with the cytoskeleton are thought to contribute to synaptic vesicle localization and mobilization, the distribution of actin and microtubules at the nerve terminals of *jar* mutants was examined. Actin-GFP was imaged at NMJs on muscle 6/7 of control and *jar* mutant larvae. Actin-GFP visualized in control, *UAS-Actin-GFP/+; elav³⁴-GAL4/+*, and *jar* mutant, *UAS-Actin-GFP/+; jar³²²/elav³⁴-GAL4, and UAS-Actin-GFP/+; jar³²²/elav³⁴-GAL4, Df(3R)crb87-5*, nerve terminals was dynamic and appeared similar, as non-uniform bright puncta (Figure 8A,B,C). Supplementary movies 1, 2 and 3 are sample movies that demonstrate actin dynamics in control and Myosin VI mutant NMJs. In addition, phalloidin staining was used to image and quantify F-actin levels in the third-instar larval brain. A comparison of F-actin levels between *yw* control and *jar³²²/Df(3R)crb87-5* mutant larvae revealed no significant difference in average fluorescence intensity between the samples (T-test, p>0.05) (Figure 9).

Microtubule morphology at control and *jar* mutant nerve terminals was examined by staining for Futsch, which is a microtubule-associated protein that colocalizes with the neuronal microtubule population and is not present in the underlying muscle tissue (Roos et al., 2000). Futsch is thought to participate in synaptic growth by regulating the formation and rearrangement of microtubule-based loops present in dividing boutons (Roos et al., 2000). Double labelling with Synaptotagmin and Futsch of NMJs on muscle 6/7 are shown in representative images for the *OreR* control and *jar³²²/Df(3R)crb87-5 MN* mutant (Figure 10A, B). All of the boutons on an NMJ were counted and scored to determine whether or not microtubule staining extended into them. A Chi Square goodness of fit test revealed that all *jar* mutants significantly differed from the control, exhibiting a higher proportion of boutons without microtubule staining within a given NMJ (p<0.05). Next, the number of microtubule loops per NMJ were quantified. A significant reduction in the number of microtubule loops was observed
in the two most severe jar mutants compared to the control (Dunn’s Multiple Comparison Test, p<0.001) (Figure 11). At control NMJs on muscle 6/7, there were an average of 3.074 ± 0.3771 microtubule loops (mean ± SE, n = 27 NMJs). In contrast, the jar322/Df(3R)crb87-5 MN mutant had an average of 1.045 ± 0.2415 microtubule loops (n = 22 NMJs) per NMJ on muscle 6/7.
A) UAS-Actin-GFP/+ ; elav^{TA}-GAL4/+,
B) UAS-Actin-GFP/+; jar^{s22}/elav^{sA}-GAL4
C) UAS-Actin-GFP/+: jar^{122}/elav^{5A}-GAL4, Df(3R)crb87-5

\[ t = 0 \text{ s} \]

\[ t = 160 \text{ s} \]

\[ t = 320 \text{ s} \]
Figure 8. Live imaging of actin-GFP in control and jaguar mutant nerve terminals revealed a similar punctate appearance.

Representative images shown every ~40 seconds from time 0 to 10 minutes demonstrate the dynamic actin-GFP puncta at NMJs on muscles 6/7 of $UAS$-$Actin$-$GFP$/+; $elav^{3A}$-$GAL4$/+; $UAS$-$Actin$-$GFP$/+; $jar^{322}$/elav$^{3A}$-$GAL4$, and $UAS$-$Actin$-$GFP$/+; $jar^{322}$/elav$^{3A}$-$GAL4$, $Df(3R)crb87$-5 third-instar larvae (A, B and C). Coloured arrows point to puncta whose movement can be followed in subsequent frames and which were seen to appear then disappear or vice versa.
C) **Average Fluorescence Intensity of the Larval Brain (a.u.)**

![Graph showing average fluorescence intensity comparison between yw and jar322/Df(3R)crb87-5 genotypes.](image)
Figure 9. Control and jaguar mutants exhibited similar levels of F-actin in the larval brain. Immunostaining against phalloidin of yw control (A, n=6) and jar^{322}/Df(3R)crb87-5 (B, n=5) third-instar larval brains revealed no significant difference in average fluorescence intensity measured from three areas of each brain sample (T-test, P> 0.05). Bars represent mean ± SEM.
Figure 10. Proportion of boutons exhibiting a lack of microtubule staining increased corresponding to the severity of jaguar loss of function.

Immunostaining against Synaptotagmin (green) and Futsch (red) of NMJs on muscles 6/7 of OreR control and jar^{322}/Df(3R)crb-87-5 MN third-instar larvae revealed different proportions of boutons with or without microtubule staining (A and B). Imaging parameters were kept the same to allow for comparative analysis. Scoring the NMJs for boutons with or without microtubule staining revealed an increase in the percentage of boutons lacking Futsch immunolabeling that corresponded to the severity of Myosin VI loss of function (C). Number of NMJs analyzed were: OreR n=27, jar^{322}/+ n=28, Df(3R)crb-87-5/+ n=25, jar^{322}/Df(3R)crb87-5 n=30 and jar^{322}/Df(3R)crb87-5 MN n=23. NMJs used for this study were from muscle 6/7 in segments A3, A4 and A5. A maximum of two NMJs from the same larva were used in this analysis. Bars represent mean ± SEM.
Figure 11. A reduction in microtubule loops was observed at jaguar mutant NMJs.

Representative images of microtubule loops in the OreR control and the jar^{322}/Df(3R)crb87-5 MN mutant boutons (A and B). Immunolabeling of Futsch (red) and Synaptotagmin (green) at control and jar mutant revealed a significant decrease in microtubule loop number for the two most severe jar loss of function mutants studied (Dunnett's Multiple Comparison Test, ***=p<0.001). Number of NMJs analyzed were: OreR n=27, jar^{322}/+ n=28, Df(3R)crb87-5/+ n=25, jar^{322}/Df(3R)crb87-5 n=30 and jar^{322}/Df(3R)crb87-5 MN n=22. Microtubule loops were counted for muscle 6/7 NMJs in segments A3, A4 and A5. Bars represent mean ± SEM.
Discussion

Myosin VI mutants exhibit changes in the microtubule cytoskeleton at the presynapse

Imaging of synaptic morphology revealed that Myosin VI is important for NMJ development. A reduction in NMJ length and a decrease in bouton number were observed at jar loss of function mutant nerve terminals. The changes to synaptic morphology observed in the Myosin VI mutants may be attributable to underlying defects in the synaptic cytoskeleton. Actin is present throughout the presynaptic terminal either in monomeric G-actin form or as filamentous F-actin (Bleckert et al., 2012). Imaging studies reveal a similar localization of actin surrounding synaptic vesicle clusters across a variety of preparations; however, the precise function of actin at the nerve terminal remains disputed (Dillon and Goda, 2005). A number of roles have been attributed to actin at the presynapse and the function of the actin cytoskeleton likely varies depending on the synapse type. For example, live imaging of GFP-tagged actin in rat hippocampal neurons reveals that chemical disruption of actin does not directly impair the synaptic vesicle cycle, rather it reduces the concentration of important regulatory molecules suggesting actin functions as a scaffold (Sankaranarayanan et al., 2003). In contrast, ultrastructural studies in the lamprey spinal cord indicate that F-actin disruption is associated with impaired vesicle recycling; accompanied by an increase in clathrin-coated intermediates and a failure to complete vesicle endocytosis (Shupliakov et al., 2002). Actin does appear, however, to share a common function among synapses during synaptic development, wherein dynamic actin is responsible for the growth and guidance of neuronal processes (Dillon and Goda, 2005).

In mammalian neurons, actin is the major cytoskeletal protein underlying dendritic spine architecture (Hotulainen and Hoogenraad, 2010; Lang et al., 2004), and there is mounting experimental evidence to suggest that reorganization of actin underlies the structural changes seen at dendritic spines associated with synaptic plasticity (Hotulainen and Hoogenraad, 2010;
Lang et al., 2004). Likewise, in *Drosophila*, mutant studies have revealed that the presynaptic actin cytoskeleton is required for proper synaptic morphogenesis (Pawson et al., 2008). In addition, depletion of F-actin at the *Drosophila* NMJ reveals that it is important for maintaining vesicle cycling under high-frequency stimulation (Kuromi and Kidokoro, 1998).

In *Drosophila*, Myosin VI has already been shown to function in regulating the actin cytoskeleton during the process of spermatid individualization, likely by acting to tether actin regulatory proteins at the front edge of the actin cone, and during nuclear divisions in the syncytial blastoderm (Isaji et al., 2011; Mermall and Miller, 1995). If *jar* mutants exhibited disruptions to the actin cytoskeleton, the defects observed in the morphology of *jar* mutant NMJs could have been attributable to these changes. To establish a role for Myosin VI in regulating the actin cytoskeleton at the NMJ, live imaging was used to assess the distribution of actin in the synaptic boutons of third-instar larvae. In control genotypes, there was a punctate distribution of actin-GFP within nerve terminal boutons and these punctate structures were constantly moving in living preparations (Nunes et al., 2006b). Likewise, in *jar* mutant nerve terminals, dynamic actin puncta were observed. In addition, F-actin levels were found to be similar in brain tissue of control and *jar* mutants. These experiments indicate that there are no major defects in the actin cytoskeleton in the nervous system of *jar* mutants. Thus in contrast to other cellular process that rely on Myosin VI mediation of the actin cytoskeleton, Myosin VI function at the NMJ is not related to a role in actin regulation.

Another possibility is that Myosin VI has a function in regulating the morphology of the microtubule cytoskeleton at the nerve terminal. Disruptions observed in microtubule structure may instead result in the defects in NMJ morphology observed in *jar* mutants. Microtubules are important for the formation and maintenance of neuronal processes, as well as the transport of materials throughout the nerve terminal (Yan and Broadie, 2007). In *Drosophila* NMJs,
microtubule dynamics exhibit both a stable component with slow turnover of tubulin subunits and a more rapid component (Yan and Broadie, 2007). Disruption of the microtubule cytoskeleton is associated with synapse destabilization and is thought to underlie neurological disease in Drosophila models for Fragile X Syndrome and Hereditary Spastic Paraplegia (Zhang et al., 2001; Trotta et al., 2004).

In Drosophila embryos, Myosin VI has been shown to co-immunoprecipitate with D-CLIP-190, the Drosophila homologue of CLIP-170, which is a mammalian microtubule plus-end binding protein (Lantz and Miller, 1998). Myosin VI and D-CLIP 190 colocalize to the axonal processes of neurons (Lantz and Miller, 1998). During embryogenesis, defects in D-CLIP-190 and Myosin VI localization to the posterior pole following actin depolymerization suggest that they form a complex that may be a direct link between microtubules and actin (Lantz and Miller, 1998). In addition, affinity chromatography and co-immunoprecipitation have identified Cornetto, a microtubule binding protein important for apical protein targeting during Drosophila asymmetric neuroblast division, as a binding partner for Myosin VI (Finan et al., 2011; Bulgheresi et al., 2001). If Myosin VI is interacting with the microtubule cytoskeleton at the NMJ, a reduction in Myosin VI expression may result in alterations to microtubule morphology at jar mutant nerve terminals. At the presynaptic bouton, microtubules and associated proteins can form distinct loops. Staining against Futsch revealed a reduction in the number microtubule loops observed in boutons of the null and maternal null jaguar mutants. Futsch colocalizes with microtubules and regulates synaptic growth through its interaction with the microtubule cytoskeleton (Roos et al., 2000). Futsch stabilizes microtubule loops, which are thought to be indicative of stable synaptic boutons (Roos et al., 2000). Thus, severe Myosin VI mutants with fewer loops have fewer stable synaptic boutons, which would account for the reduction in bouton number observed at these synapses. Rearrangement of these loops is important for bouton
division and ultimately, nerve terminal growth (Roos et al., 2000). Double labeling with Synaptotagmin showed a reduction in the proportion of boutons that contained microtubules in **jar** mutants compared to control NMJs. Thus, a reduction in Myosin VI expression alters microtubule architecture at the NMJ. These changes are consistent with the reduction in NMJ size and bouton number observed in **jar** mutant, which could result from the failure of proper bouton division associated with disruption to the microtubule cytoskeleton.

**Myosin VI regulates proper peripheral vesicle localization within synaptic boutons**

The accumulation and clustering of vesicles at the nerve terminal is important for effective neurotransmission, as the neurotransmitter contents of these vesicles are ultimately released to generate a postsynaptic response. Transport of synaptic vesicles from the neuronal cell body to the terminal is mediated by a *Drosophila* member of the kinesin-3 family of molecular motors (Pack-Chung et al., 2007). At hour fourteen of *Drosophila* embryogenesis, Synaptotagmin is first observed at the presynaptic terminal (Siechen et al., 2009). Live imaging of *Drosophila* embryos reveals that axonal tension plays a critical role in initial vesicle clustering and without this tension, vesicles would disperse throughout the nerve terminal (Siechen et al., 2009). Electron microscopy and immunostaining for a synaptic vesicle marker have consistently shown that the bouton centre is generally devoid of vesicles (Atwood et al., 1993; Kuromi et al., 2004; Denker et al., 2009). Thus far, Synapsin has been the only protein identified to participate in maintaining this peripheral vesicle organization in *Drosophila* Ib boutons (Akbergenova and Bykhovskaia, 2010).

A role for Myosin VI in regulating the synaptic vesicle localization has been implicated in mammalian cells, where Myosin VI has been shown to associate with endocytic vesicles following clathrin uncoating and to subsequently transport these uncoated vesicles through the
actin-rich periphery to the early endosome (Aschenbrenner et al., 2004). Different tail splice variants of Myosin VI in human and mammalian tissues can also mediate Myosin VI localization to clathrin-coated pits/vesicles (Buss et al., 2001). Myosin VI recruitment to clathrin-coated pits appears to be modulated by Disabled Homolog 2 (Dab2), an adaptor protein known to be important in endocytosis (Dance et al., 2004). To determine if Myosin VI has a function in regulating synaptic vesicle localization at Drosophila NMJs, vesicles within jar loss of function mutant and control boutons were labeled and visualized to assess their distribution within the nerve terminal. Visualization of Synaptotagmin staining revealed a surprising mislocalization of synaptic vesicles in jar mutant boutons (Kisiel et al., 2011). An increasing number of jar mutant boutons, corresponding to the severity of Myosin VI loss of function, were found to exhibit diffuse staining over the entire bouton area as opposed to the torus-shaped staining pattern present in control boutons (Kisiel et al., 2011). The unexpected diffuse Synaptotagmin staining over the Ib bouton centre observed in jar mutants suggests that Myosin VI plays a role in maintaining normal peripheral vesicle localization. This may be achieved through a Myosin VI anchoring function wherein Myosin VI could act as a tether restricting vesicles to the bouton periphery.
Chapter 3: Myosin VI contributes to basal synaptic transmission and short-term synaptic plasticity at the Drosophila neuromuscular junction

Excerpts from Chapter 3 have been previously published in BMC Neuroscience:


The experiments were conceived and designed by Marta Kisiel and Bryan Stewart. Marta Kisiel performed all behavioural and electrophysiological experiments, analyzed the data, and assembled figures. Kris Mckenzie performed the active zone staining experiments and quantified active zone number.

A locomotor defect, observed as sluggish movement in severe jar mutant larvae, was confirmed by behavioural assays. As this can indicate problems at the neuromuscular synapase, microscopy and electrophysiology were used to investigate neuromuscular junction (NMJ) structure and function in jar loss of function mutants of varying severity. Electrophysiological experiments revealed a role for Myosin VI in basal synaptic transmission, with a reduction in low frequency nerve-evoked responses and spontaneous release in severe jar mutants. Changes in short-term synaptic plasticity were also observed in Myosin VI mutants using paired-pulse stimulation as well as high-frequency stimulation paradigms to recruit vesicles from different functional pools. In addition, a decrease in the number of active zones, which are the sites of vesicle release, was observed by staining against Bruchpilot at jar mutant synapses. As Bruchpilot loss of function is associated with a reduction in evoked nerve response, this is consistent with impaired synaptic function observed in jar mutants. Taken together, the data suggest that Myosin VI may function as a synaptic vesicle tether to ensure proper vesicle localization for effective neurotransmission and to regulate vesicle recruitment from different functional pools during an evoked response.
Introduction

To sustain repeated neurotransmitter release, vesicles undergo recycling at the nerve terminal. Following calcium triggered exocytosis, vesicles undergo endocytosis and are recycled. Two mechanisms have been proposed to regulate vesicle recycling, a clathrin-independent pathway and a clathrin mediated pathway; evidence exists to support both (Rizzoli and Jahn, 2007). The highly debated "kiss and run" hypothesis postulates that vesicles may be reacidified and refilled locally without undocking (Sudhof, 2004). In this scenario, a transient fusion pore is formed to release neurotransmitter and fusion between the vesicle and plasma membrane is partial. Loss of the presynaptic protein Endophilin at the Drosophila NMJ results in a block on clathrin-mediated endocytosis, however these mutants can sustain a low level of release during high-frequency stimulation consistent with a rapid retrieval mechanism (Verstreken et al., 2002). Alternatively, synaptic vesicles may undergo conventional clathrin-dependent endocytosis before being refilled with neurotransmitter and may pass through an endosomal intermediate (Sudhof, 2004). The process involves full fusion of the vesicular and plasma membranes with recycling occurring via membrane invagination, formation of a clathrin coat, scission of the coated vesicle from the plasma membrane and then coat disassembly. Interestingly, photoinactivation of clathrin light chain at the Drosophila NMJ results in a failure of vesicles to reform, providing support for conventional recycling at this nerve terminal (Heerssen et al., 2008). In polarized mammalian cells, Myosin VI has been found to localize to clathrin coated pits, where it has been implicated in mediating clathrin-dependent endocytosis (Altman et al., 2007; Buss et al., 2001).

At the Drosophila NMJ, three pools of vesicles with differential release properties have been identified using FM1-43 staining loaded by various stimulation protocols ((Kuromi and Kidokoro, 1998), reviewed by (Rizzoli and Betz, 2005)). The immediately releasable pool (IRP),
representing approximately 1% of all vesicles at the NMJ, consists of vesicles docked and primed at active zones for immediate release and experiences rapid depletion within a few stimuli (Rizzoli and Betz, 2005; Delgado et al., 2000). The readily releasable pool (RRP), making up 14 to 19% of all vesicles at the NMJ, is mobilized by moderate stimulation of \( \leq 3 \) Hz and maintains exocytosis/endocytosis at these stimulation frequencies (Delgado et al., 2000). The reserve pool (RP) represents the vast majority of vesicles, 80 to 90%, and is mobilized upon depletion of the RRP (Delgado et al., 2000). Recruitment from the RP occurs with high-frequency stimulation of \( \geq 10 \) Hz (Kuromi and Kidokoro, 2000). These different vesicle pools were previously thought to be spatially segregated, with the RRP localized to the periphery of the bouton and the RP occupying the bouton centre (Kuromi and Kidokoro, 1998). Subsequent EM studies of wild type boutons, however, revealed the reserve and readily releasable pool to be thoroughly intermixed with the bouton centre unfilled (Denker et al., 2009).

Evoked, calcium dependent neurotransmitter release and spontaneous, calcium independent neurotransmitter release have both been observed at all synapses, with membrane depolarization mediating the switch to synchronized release. Evidence suggests that spontaneously released vesicles that generate evoked miniature potentials are regulated independently from vesicles participating in evoked synaptic release. In rat hippocampal neurons, spontaneously released vesicles are members of a resting pool that does not normally participate in neurotransmission (Fredj and Burrone, 2009; Sara et al., 2005). At the *Drosophila* NMJ, electrophysiology of Synaptobrevin mutants shows the persistence of spontaneous release suggesting a possible distinct mechanism of fusion differing from that of the pool of vesicles participating in evoked response (Deitcher et al., 1998). However, conflicting reports do exist indicating that miniature activity may in fact be supported by the same population of synaptic vesicles involved in evoked neurotransmission (Groemer and Klingauf, 2007).
Neurotransmission has long been recognized to be a plastic process, able to increase or decrease in response to stimuli. Short-term synaptic plasticity lasts milliseconds to minutes and can result in an enhancement or reduction in synaptic strength. In response to repeated stimulation, a synapse may experience short-term depression due to depletion of the readily releasable pool of vesicles or due to a reduction in calcium influx owing to calcium channel inactivation (Fioravante and Regehr, 2011). Alternately, synaptic facilitation may occur, which is associated with a transient increase in neurotransmitter release probability, owing to residual calcium remaining at the nerve terminal or enhanced presynaptic calcium influx (Fioravante and Regehr, 2011). Sustained high-frequency stimulation may induce post-tetanic potentiation lasting several seconds to minutes due to increased calcium accumulation at the nerve terminal or an increase in the size of the RRP (Balakrishnan et al., 2010). Long-term potentiation is characterized by a lasting increase in synaptic efficacy in response to high-frequency stimulation and is attributed to changes in postsynaptic receptor sensitivity as well as presynaptic alterations that enhance release probability (Blundon and Zakharenko, 2008). In contrast, long-term depression refers to long-lasting depression in synaptic transmission and is also associated with persistent pre- and postsynaptic changes (Collingridge et al., 2010). It may arise from desensitization of postsynaptic receptors or retrograde signaling to inhibit neurotransmitter release (Collingridge et al., 2010).

Given the function of Myosin VI in proper vesicle localization at the nerve terminal, it is possible that Myosin VI may participate in the regulation of synaptic vesicle mobilization during synaptic transmission. Indeed, the sluggish behaviour of Myosin VI maternal null larvae is suggestive of problems at the neuromuscular synapse. Basal electrophysiology as well as synaptic plasticity were studied to determine the effect of Myosin VI loss of function on these parameters. This revealed changes to both basal physiology and paired-pulse response in the
most severe jar mutants. In addition, enhanced potentiation and depression were observed in response to high-frequency stimulation at null jar mutant synapses in normal and high calcium saline respectively. To assess active zone morphology, the active zone protein, Bruchpilot, was visualized at the NMJ and a reduction in Brp was observed within jar boutons. Together, these studies elucidate the contribution of Myosin VI to the maintenance of proper synaptic physiology.
Results

Larval behaviour is altered in Myosin VI loss of function mutants

To characterize any locomotor defects present in *jar* mutant larvae, larval path length testing was performed on the most severe *jar* loss of function mutant, *jar*<sup>322</sup>/Df(3R)crb87-5 MN, and a control strain. Larval path length was measured over 5 minutes on both a nutritive and non-nutritive substance. A significant reduction in path length was observed for *jar*<sup>322</sup>/Df(3R)crb87-5 MN larvae (mean ± SE, 1.36 ± 0.19 cm, n = 40) compared to the *OreR* control larvae (5.16 ± 0.55 cm, n = 40) on a nutritive yeast substrate (Two-Way ANOVA, p < 0.0001) (Figure 12). Similarly, path length was significantly shorter for *jar*<sup>322</sup>/Df(3R)crb87-5 MN larvae (5.43 ± 0.75 cm, n = 20) than *OreR* control larvae (16.61 ± 1.30, n = 20) on an agar substrate (Two-Way ANOVA, p < 0.0001) (Figure 12).
Figure 12. *jaguar* mutants exhibit general larval locomotor defects.

Third-instar *jar*$_{322}/Df(3R)crb87-5$ MN larvae exhibited a significant decrease in path length on both a nutritive and non-nutritive substrate compared to the *OreR* control (ANOVA, ***=*p*<0.0001). 40 larvae of each genotype were tested on the nutritive yeast substrate and 20 larvae of each genotype were tested on the non-nutritive agar substrate. Bars represent mean ± SEM.
**Myosin VI contributes to the maintenance of proper basal synaptic transmission**

As the localization of synaptic vesicles is known to be important in basal synaptic function (Rodesch and Broadie, 2000), the vesicle mislocalization phenotype observed at *jar* mutant synapses may be associated with impaired synaptic transmission. To determine if Myosin VI is important for proper synaptic transmission at *Drosophila* NMJs, electrophysiological studies were performed to compare evoked response and spontaneous release between *jar* loss of function mutants and wild-type controls. Mean EJP amplitude was calculated from 16 recordings of EJP amplitude at 1 Hz stimulation. Only *jar* mutants lacking a maternal contribution of Myosin VI, *jar* 

\[
\text{mean} \pm \text{SE}, 21.34 \pm 2.4572 \text{ mV, n=16 NMJs}
\]

compared to the *OreR* control (35.47 ± 2.2311 mV, n=12 NMJs; Dunnett's Multiple Comparison Test, p<0.001) (Figure 13 A,B). Recordings of spontaneous vesicle release measured for 1 to 2 minutes were used to calculate mEJP amplitude and frequency. Average mEJP amplitude was found to be approximately 1 mV for all genotypes studied (ANOVA, p>0.05) (Figure 13 D). A reduction in mEJP frequency was observed in the more severe *jar* loss of function mutants, *jar* 

\[
2.12 \pm 0.238, \text{ n=17 NMJs and } 1.79 \pm 0.2104, \text{ n=16 NMJs}
\]

compared to the *OreR* control (3.73 ± 0.2092; Dunnett's Multiple Comparison Test, p<0.001) (Figure 13 C,E).
Figure 13. *jaguar* loss of function mutants exhibited defects in basal synaptic transmission.

Electrophysiological recordings from muscles 6/7 of third-instar larvae revealed EJP amplitude was significantly reduced in the most severe *jar* mutant, *jar*\(^{322}/Df(3R)crb87-5 MN*, compared to the *OreR* control (Dunnett's Multiple Comparison Test, ***=*p*<0.001) (A). Sample traces of EJP recordings taken at 1 Hz, shown in the same order as listed for the graphs (B). mEJP frequency was significantly lower for *jar*\(^{322}/Df(3R)crb87-5* and *jar*\(^{322}/Df(3R)crb87-5 MN* larvae compared to the control larvae (Dunnett's Multiple Comparison Test, ***=*p*<0.001) (C). There was no significant difference in mEJP amplitude between *jar* mutants and the control (ANOVA, *p*>0.05) (D). Sample traces of electrophysiological recordings from muscles 6/7 over 2 seconds, shown in the same order as listed for the graphs (E). Number of NMJs analyzed were: *Ore* n=27, *jar*\(^{322}/+* n=15, *Df(3R)crb87-5/+* n=17, *jar*\(^{322}/Df(3R)crb87-5* n=17 and *jar*\(^{322}/Df(3R)crb87-5 MN* n=14. NMJs used for this study were from muscle 6/7 in segments A3, A4 and A5. A maximum of two NMJs from the same larva were used in this analysis. Bars represent mean ± SEM.
**Myosin VI mutants exhibit enhanced paired-pulse facilitation in low calcium saline**

Paired-pulse facilitation experiments were performed to examine changes in release probability at Myosin VI mutant synapses. Muscles were stimulated by two pulses separated by a 20 ms interval, which was repeated every 10 s for a total of 16 cycles. A low calcium concentration of 0.5 mM was used during paired-pulse stimulation to reproduce conditions of low release probability, which lead to enhanced facilitation in jar\( ^{322}/Df(3R)crb87-5 \) mutant larvae (Figure 14, Dunnett's Multiple Comparison Test, *\( =p<0.05 \)). The average percent increase for the second pulse relative to the first for OreR control larvae was 1.602 ± 0.0571 % (mean ± SE, n = 24 NMJs) compared to 2.237 ± 0.308 % for jar\( ^{322}/Df(3R)crb87-5 \) mutants (n = 15 NMJs). Although not significant, the trend towards greater facilitation following the second stimulation pulse was also present in maternal null jar\( ^{322}/Df(3R)crb87-5 \) mutants. This is reflected in the representative traces for these mutants (Figure 15). At a higher calcium concentration of 1 mM, this enhanced facilitation was no longer observed for the jar mutants (Figure 16, ANOVA, p>0.05). The representative traces for the control and jar mutant EJPs reveal similar levels of facilitation following the second stimulation pulse in 1 mM calcium saline (Figure 17).
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ratio of Second EJP to First in the Paired-Pulse</th>
</tr>
</thead>
<tbody>
<tr>
<td>OreR</td>
<td>0</td>
</tr>
<tr>
<td>jar322/+</td>
<td>1</td>
</tr>
<tr>
<td>Df(3R)crb87-5/+</td>
<td>2</td>
</tr>
<tr>
<td>jar322/Df(3R)crb87-5</td>
<td>3</td>
</tr>
<tr>
<td>jar322/Df(3R)crb87-5 MN</td>
<td>*</td>
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Figure 14. Severe Myosin VI mutants show enhanced paired-pulse facilitation in 0.5 mM calcium saline.

A significant enhancement in facilitation following the second pulse was observed in the null mutant, jar\textsuperscript{322}/ Df(3R)crb87-5 compared to the control (Dunnett’s Multiple Comparison Test, p<0.05). No significant differences were observed in EJP amplitude of the second pulse for any of the other jar mutants compared to the control (ANOVA, p> 0.05). Number of NMJs analyzed were: OreR n=20, jar322/+ n=14, Df(3R)crb87-5/+ n=14, jar\textsuperscript{322}/Df(3R)crb87-5 n=15 and jar\textsuperscript{322}/Df(3R)crb87-5 MN n=6. NMJs used for this study were from muscle 6/7 in segments A3, A4 and A5. A maximum of two NMJs from the same larva were used in this analysis. Error bars represent standard error. Bars represent mean ± SEM.
Figure 15. Representative EJP traces for control and jaguar mutants during paired-pulse stimulation in 0.5 mM Ca\textsuperscript{2+} saline.

Representative EJP traces following paired-pulse stimulation in 0.5 mM Ca\textsuperscript{2+} saline from muscles 6/7 of third-instar larvae revealed a significant enhancement in facilitation following the second pulse for the \textit{jar}\textsuperscript{322}/\textit{Df(3R)crb87-5} mutant compared to the \textit{OreR} control (Dunnett's Multiple Comparison Test, p<0.05). No significant differences in facilitation were observed between the control and other \textit{jar} mutants (ANOVA, p>0.05). Sample traces of EJP recordings shown are an average of 16 traces recorded every ten seconds following stimulation by two pulses separated by a 20 ms interval. Note that the scale for the null and maternal null \textit{jar}\textsuperscript{322}/\textit{Df(3R)crb87-5} mutants differ from the control to reflect their smaller initial EJPs.
Figure 16. Myosin VI mutants do not exhibit changes in paired-pulse facilitation in 1 mM calcium saline.

No significant changes in EJP amplitude of the second pulse were observed for any of the jar mutants compared to the control (ANOVA, p > 0.05). Number of NMJs analyzed were: OreR n=15, jar322/+ n=13, Df(3R)crb87-5/+ n=16, jar322/Df(3R)crb87-5 n=15 and jar322/Df(3R)crb87-5 MN n=. NMJs used for this study were from muscle 6/7 in segments A3, A4 and A5. A maximum of two NMJs from the same larva were used in this analysis. Bars represent mean ± SEM.
Figure 17. Representative EJP traces for control and *jaguar* mutants during paired-pulse stimulation in 1 mM Ca^{2+} saline.

Representative EJP traces following paired-pulse stimulation in 1 mM Ca^{2+} saline at from muscles 6/7 of third-instar larvae revealed no significant differences in facilitation between control and *jar* mutants (ANOVA, p>0.05). Sample traces of EJP recordings shown are an average of 16 traces recorded every ten seconds following stimulation by two pulses separated by a 20 ms interval. Note that the scale for the *jar^{322}/Df(3R)crb87-5* maternal null mutant differs from the control to reflect its smaller initial EJP.
Loss of Myosin VI alters synaptic response to high-frequency stimulation

To examine if jar loss of function mutants differ in synaptic response to high-frequency stimulation, a baseline recording of 16 EJPs at 1 Hz was taken, followed by a 10 minute recording at 10 Hz and completed with a 10 minute recording at 0.1 Hz. Recordings were performed in 1 mM calcium saline to reflect physiological calcium levels (Seabrooke and Stewart, 2011). Changes in EJP amplitude were analyzed as a percent of the average of the first 16 EJPs for each recording. No significant differences in depression at the onset of high-frequency stimulation were observed (ANOVA, p>0.05) (Figure 18). The enhancement in EJP amplitude following the initial depression was significantly greater for jar^{32}/Df(3R)crb87-5 larvae than OreR control larvae (mean ± SE, 124.14 ± 5.97126%, n=11 and 107.49 ± 2.1921%, n=10 respectively, Dunnett's Multiple Comparison Test, p<0.001) (Figure 19). There were no significant differences in maximum EJP amplitude for the jar loss of function heterozygotes relative to the control (Figure 19).

Following high-frequency stimulation, post-tetanic potentiation (PTP) is observed as an increase in EJP amplitude followed by a steady decline for recordings at 0.1 Hz. PTP is presynaptic in origin, caused by an increase in neurotransmitter quanta release, which is attributed to calcium release from intracellular stores that was accumulated during high-frequency stimulation (Zucker et al., 1991; Zucker and Regehr, 2002). No significant difference was found in maximum EJP amplitude at 0.1 Hz for the genotypes studied (ANOVA, p>0.5) (Figure 18). In addition, there was no significant difference in the amplitude of last EJP measured at 0.1 Hz stimulation between genotypes (ANOVA, p>0.5) (Figure 18).

To further challenge the synapse, the same high-frequency stimulation protocol was carried out in 10 mM (supraphysiological) Ca^{2+} saline. These conditions were used to induce synaptic depression as vesicle release will exceed the maximal rate of vesicle recycling.
(Dickman et al., 2005). Indeed, these stimulation conditions produced a rapid depression in EJP amplitude at the onset of 10 Hz simulation, likely due to vesicle depletion in response to high calcium concentrations (Zucker and Regehr, 2002). The initial depression was followed by a slight recovery and then a continuing decline in EJP amplitude for the remainder of the 10 Hz stimulation. The initial depression in EJP amplitude measured relative to EJP amplitude at the onset of high-frequency stimulation was significantly greater for jar^{322}\text{/}Df(3R)crb87-5 larvae than the control larvae (0.63 ± 0.04, n = 12 and 0.45 ± 0.02, n = 8 respectively; ANOVA, p < 0.01) (Figure 21). There were no significant differences in initial depression among the other genotypes tested (ANOVA, p > 0.05). Recovery of EJP amplitude at 10 Hz stimulation was quantified as a percent increase from the lowest EJP amplitude during the initial depression to the largest EJP amplitude observed during the recovery period. There was no difference in recovery among control and jar loss of function larvae following 10 Hz stimulation (ANOVA, p > 0.05) (Figure 20). Additionally, no difference in recovery of EJP amplitude was found among genotypes at 0.1 Hz stimulation (ANOVA, p > 0.05) (Figure 20).
Figure 18. High-frequency stimulation in 1 mM Ca$^{2+}$ saline revealed enhanced potentiation in jar$^{322}$/Df(3R)crb87-5 mutants.

Data is shown as a percent of the average of the first 16 evoked junctional potentials (EJPs) recorded at 1 Hz. This baseline recording of 16 EJPs at 1 Hz was followed by a stimulation of 10 Hz recorded over 10 minutes and terminated with a 10 minute recording at 0.1 Hz. Following the first 25 seconds from the onset of 10 Hz stimulation, every tenth recording is shown. A significant enhancement in EJP amplitude was observed for jar$^{322}$/Df(3R)crb87-5 larvae during the first three minutes of the high frequency protocol compared to the OreR control (Dunnett’s Multiple Comparison Test, p<0.001).
Figure 19. Representative EJP traces for control and jaguar mutants at various times during 10 Hz stimulation in 1 mM Ca\(^{2+}\) saline.

High-frequency stimulation in 1 mM Ca\(^{2+}\) saline at 10 Hz from muscles 6/7 of third-instar larvae revealed EJP amplitude a significantly greater potentiation in the jar\(^{322}/\text{Df}(3R)\text{crb87-5}\) mutant compared to the OreR control (Dunnett's Multiple Comparison Test, ***=p<0.001) There were no significant differences in maximum EJP amplitude among the control and the jar loss of function heterozygotes. Sample traces of EJP recordings are shown for time 0 minute, 2 minutes and 8 minutes during the high-frequency stimulation protocol.
Figure 20. High-frequency stimulation in 10 mM Ca$^{2+}$ saline revealed enhanced depression in jar$^{322}$/Df(3R)crb87-5 mutants.

Data is shown as a percent of the average of the first 16 evoked junctional potentials (EJPs) recorded at 1 Hz. This baseline recording of 16 EJPs at 1 Hz was followed by a stimulation of 10 Hz recorded over 10 minutes and terminated with a 10 minute recording at 0.1 Hz. Following the first 25 seconds from the onset of 10 Hz stimulation, every tenth recording is shown. The initial depression in EJP amplitude measured relative to EJP amplitude at the onset of high-frequency stimulation was significantly greater for jar$^{322}$/Df(3R)crb87-5 larvae than the control larvae (ANOVA, p<0.01).
Figure 21. Representative EJP traces for control and jaguar mutants at various times during 10 Hz stimulation in 10 mM Ca$^{2+}$ saline.

High-frequency stimulation in 10 mM Ca$^{2+}$ saline at 10 Hz from muscles 6/7 of third-instar larvae revealed EJP amplitude a significantly greater depression in the jar$^{322}$/Df(3R)crb87-5 mutant compared to the OreR control (Dunnett's Multiple Comparison Test, ***=p<0.001) There were no significant differences in maximum level of depression during stimulation among the control and the jar loss of function heterozygotes. Sample traces of EJP recordings are shown for time 0 minute, 2 minutes and 8 minutes during the high-frequency stimulation protocol.
Active zone number is reduced at Myosin VI mutant synapses

Active zones are defined as the sites of synaptic vesicle docking, priming and fusion (Zhai and Bellen, 2004). At the light microscope level, active zones in the Drosophila bouton can be visualized by staining against Bruchpilot (Brp), which localizes specifically to the presynaptic active zones and is an ortholog of the mammalian active zone scaffolding protein CAST (Wagh et al., 2006). To investigate changes in active zone number per Ib bouton in jar mutants, Brp puncta at control and jar mutant synapses were imaged and quantified. A significant decrease in active zone number per Ib bouton was found for all jar mutant genotypes tested compared to the OreR control (Dunnett's Multiple Comparison Test, p<0.05) (Figure 22). At control Ib boutons, 18.7 ± 1.313 active zones per bouton (n=60) were observed compared to 13.2 ± 0.506 active zones per bouton (n=93) at jar^{322/Df(3R)crb87-5 MN} Ib boutons (Dunnett's Multiple Comparison Test, p<0.001).
**Genotype**

- Number of Active Zones per Bouton

- OreR
- jar322/+
- Df(3R)crb87-5/+ 
- jar322/Df(3R)crb87-5
- jar322/Df(3R)crb87-5 MN

**A)** yw

**B)** jar\textsuperscript{322}/Df(3R)crb87-5 MN

**C)**

- Bar graph showing the number of active zones per bouton for different genotypes.
- OreR, jar322/+, Df(3R)crb87-5/+, jar322/Df(3R)crb87-5, jar322/Df(3R)crb87-5 MN.
- Statistically significant differences indicated by asterisks (*, ***, ***).

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Figure 22. A significant reduction in active zone number per bouton was observed at jar mutant synapses.

Staining against Bruchpilot was used to quantify active zone number in Ib boutons of NMJs on muscles 6/7 for OreR control and jar mutant third-instar larvae. Representative images of Bruchpilot staining in Ib boutons of the control (A) and the most severe jar loss of function mutant, jar$^{322}$/Df(3R)crb87-5 MN (B). A significant reduction in active zone number per Ib bouton was found for all jar mutant genotypes tested compared to the control (C, Dunnett's Multiple Comparison Test, *p<0.05, ***=p<0.001). Number of boutons analyzed were: OreR n=60, jar$^{322}$/+ n=96, Df(3R)crb87-5/+ n=91, jar$^{322}$/Df(3R)crb87-5 n=69 and jar$^{322}$/Df(3R)crb87-5 MN n=93. Boutons used for this study were from muscle 6/7 NMJs in segments A3, A4 and A5. Bars represent mean ± SEM.
Discussion

Myosin VI plays a role in the modulation of basal synaptic transmission

Alterations to larval locomotor behaviour can be attributed to disturbances within the muscles, motor neurons or the CNS. The locomotor defects observed at jar mutant synapses could therefore be related to underlying problems in synaptic transmission at the NMJ. The peristaltic longitudinal muscles examined in this study are innervated by segmental nerves, which can be severed and stimulated to record motor neuron activity. Muscle fibers 6 and 7 are innervated by two motor neurons that differ in bouton size, type Ib and type Is (Kurdyak et al., 1994). Intracellular recordings from these muscles, thus, show two different sizes of synaptic potentials with the type Is terminal contributing to the larger of these potentials (Kurdyak et al., 1994). This has been attributed to a greater probability of vesicle release in response to an action potential and a larger vesicle volume, resulting in a larger quantal size, at type Is terminals (Atwood et al., 1997; Karunanithi et al., 2002). A composite EJP can be generated by recruiting these two motor simultaneously. Indeed, neuronal deficits at the NMJ were observed in Myosin VI loss of function mutants in both spontaneous and evoked response.

Even in the absence of presynaptic stimulation, synapses exhibit a low rate of spontaneous vesicle fusion that can be observed electrophysiologically as miniature postsynaptic currents (Sankaranarayanan et al., 2003). mEJP frequency was reduced in more severe jar loss of function mutants (Kisiel et al., 2011). However, mEJP amplitude, which reflects the release of a quantum of neurotransmitter, remained unaltered indicating a presynaptic function for Myosin VI (Roche et al., 2002). To study evoked response, nerves were stimulated at 1 Hz to mobilize the RRP. The 1 Hz stimulation protocol revealed a reduction in EJP amplitude in the jar maternal null mutant (Kisiel et al., 2011). This impaired synaptic response may be due to a reduction in the probability of RRP vesicle release or in the size of the RRP. If Myosin VI
functions to anchor synaptic vesicles, it may act on the RRP to ensure vesicles are localized in
manner that makes them readily available for release. Thus, in jar maternal null mutants, a
significant number of vesicles may be displaced from areas of higher probability release. In
addition, active zone number per bouton, assessed by Brp staining, has been shown to be reduced
in jar mutants. In vivo imaging revealed a correlation between release probability from each
active zone and its levels of Brp (Peled and Isacoff, 2011). Interestingly, mechanistic modeling
of Brp null mutant synapses themselves showed both a decreased probability of vesicle release
and defective vesicle trafficking (Hallermann et al., 2010a). Thus, the reduction in Brp puncta at
jar mutant boutons is consistent with the impaired synaptic transmission observed at the
synapses of severe jar mutants. In the most severe maternal null jar mutant, the decrease in
evoked response observed may be attributable to both the decrease in active zone number, which
is associated with a decrease in vesicle release probability, and an increase in the proportion of
boutons exhibiting vesicle mislocalization. The larger proportion of boutons exhibiting normal
vesicle localization at the NMJs of less severe jar loss of function mutants may account for the
fact that their evoked response at 1 Hz was indistinguishable from the control. In addition,
release probability mapping using a postsynaptic calcium sensor shows that there are only a
small number of high probability release sites at each NMJ that contribute primarily to basal
synaptic transmission (Peled and Isacoff, 2011). Within a bouton, high probability release sites
neighbor a majority of active zones with such low release probabilities that they are considered
functionally silent (Peled and Isacoff, 2011). Thus, although all jar mutants exhibit a reduction in
active zone number, it could be that the remaining sites consist of these minority high probability
zones, which would also explain the ability of less severe mutants to maintain normal evoked
response. With increasing severity of jar loss of function, an increasing proportion of boutons
exhibited vesicle mislocalization culminating with the most severe maternal null jar mutant, in
which basal synaptic physiology was impaired. This suggests that Myosin VI function in maintaining proper vesicle localization may be important for effective synaptic transmission.

**Myosin VI participates in regulating synaptic plasticity**

Different synaptic vesicle pool properties, such as rate of recruitment from the RP in response to high frequency stimuli, may translate to changes in short-term synaptic plasticity (Rodesch and Broadie, 2000). Based on the findings that Myosin VI mutants exhibited defects in basal synaptic transmission, further electrophysiological investigations of jar mutant synapses were performed to examine synaptic plasticity. Paired-pulse facilitation is a type of short term synaptic plasticity resulting from residual calcium remaining at the nerve terminal following the first evoked potential (Rivosecchi et al., 1994). Subsequent stimulation shortly thereafter leads to enhanced vesicle release from the RRP and a second evoked potential of larger amplitude (Rivosecchi et al., 1994). For a given pair of evoked responses, if the initial release probability is high, then less facilitation occurs following the second pulse (Zucker and Regehr, 2002). However, if initial release probability if low, then a greater facilitation is observed during the second pulse (Zucker and Regehr, 2002). In conditions of low calcium, enhanced facilitation was observed in jar^{322}/Df(3R)crb87-5 mutant larvae. In addition, the trend towards greater facilitation following the second stimulus pulse was observed in the other Myosin VI mutants. This is consistent with lower initial release probability from the RRP in these mutants compared to the control. In contrast, at a higher calcium concentration where release probability is increased to approximately 50% in the RRP (Hallermann et al., 2010a), this facilitation was no longer observed as depression is occurring simultaneously during stimulation. This suggests that under conditions of low calcium, the mislocalization of vesicles at Myosin VI mutant nerve terminals contributes to low initial release probabilities.
To further challenge the Myosin VI mutant NMJ, high-frequency stimulation was used to recruit vesicles from the RP. The high-frequency stimulation protocol carried out in 1 mM Ca\(^{2+}\) saline produced a typical pattern of synaptic response, beginning with rapid depression, likely due to the depletion of the RRP (Zucker and Regehr, 2002; Zucker, 1999). This was followed by an increase in EJP amplitude, corresponding to mobilization of the RP due to the presence of residual Ca\(^{2+}\) in the neuronal cytoplasm (Zucker, 1999; Ryan, 1999), and a subsequent steady decline with continuing high-frequency stimulation. Interestingly, \textit{jar}\textsuperscript{322}/\textit{Df(3R)crb87-5} synapses experienced a significantly greater increase in EJP amplitude compared to the control and \textit{jar} loss of function heterozygotes (Kisiel et al., 2011). The increase in EJP amplitude may be due to enhanced mobilization of the RP at 10 Hz stimulation for \textit{jar}\textsuperscript{322}/\textit{Df(3R)crb87-5} NMJs (Akbergenova and Bykhovskaia, 2010). Filamentous actin has been implicated in RP mobilization as cytochalasin D, an inhibitor of actin polymerization, has been shown to reduce RP dynamics (Kuromi and Kidokoro, 1998). This suggests translocation from the RP to the RRP may be mediated by an actin-based myosin motor protein. If Myosin VI functions as a synaptic vesicle tether to regulate recruitment from the RP pool, RP vesicles would be more readily mobilized and transitioned into the RRP upon high-frequency stimulation in \textit{jar} loss of function mutants. If RP vesicles are more rapidly incorporated into the RRP, greater depression would be expected during high-frequency stimulation in 10 mM Ca\(^{2+}\) saline. Indeed, consistent with this hypothesis, \textit{jar}\textsuperscript{322}/\textit{Df(3R)crb87-5} synapses experienced a significantly greater depression in EJP amplitude compared to the control and \textit{jar} loss of function heterozygotes in 10 mM Ca\(^{2+}\) saline (Kisiel et al., 2011).

Taken together, the data suggest that Myosin VI mediates synaptic transmission and short-term plasticity by regulating the mobilization of synaptic vesicles from different functional pools. In mammalian cells, Myosin VI mediates vesicle endocytosis and has been shown to...
transport uncoated vesicles from the cellular periphery to the early endosome for further sorting (Aschenbrenner et al., 2004). Our experiments, however, indicate that endocytosis is not affected at jar mutant synapses. Typically, endocytotic mutants are unable to maintain synaptic transmission in response to high-frequency stimulation (Dickman et al., 2005), whereas our Myosin VI loss of function mutants exhibited enhanced EJP amplitude observed at 10 Hz stimulation in 1 mM Ca\(^{2+}\) saline. With the morphological data, this electrophysiological evidence lends further support to a tethering function for Myosin VI at the nerve terminal that ensures proper localization of vesicles for effective neurotransmission.
Chapter 4: Myosin VI mediates vesicle mobility at the synaptic terminal

Marta Kisiel performed the FRAP experiments as well as the second set of FM dye experiments, analyzed the data, and assembled figures. Kris Mckenzie performed the first set of FM dye experiments.

At the Drosophila NMJ, synaptic vesicles are mobile; however, the mechanisms involved in regulating vesicle traffic at the nerve terminal are not fully understood. Myosin VI has been shown to be important for proper synaptic morphology and physiology at the NMJ, likely by functioning as a vesicle tether. FM dye loading following high-frequency stimulation was used to visualize the vesicle cycle in jar mutants in vivo. These studies revealed a differential distribution of vesicles at the jar mutant nerve terminal, with the newly endocytosed vesicles observed throughout the bouton as opposed to the peripheral localization visualized at control NMJs. This finding is consistent with a role for Myosin VI in restraining vesicle mobility at the synapse to ensure proper localization. To further investigate the regulation of vesicle movement by Myosin VI, FRAP analysis was used to follow GFP labeled synaptic vesicles within individual boutons. FRAP revealed that synaptic vesicle mobility was enhanced at jar mutant boutons, which was indicated by a more rapid recovery of fluorescence following photobleaching. Within jar mutants, the reduced level of Myosin VI expression corresponds to a reduction in the number of synaptic vesicles that are anchored in the correct position. This lack of tethering by Myosin VI means that these vesicles would experience greater freedom in movement at the synapse. This data provides insights into a novel role for Myosin VI in mediating synaptic vesicle dynamics at the nerve terminal.
Introduction

Although vesicles are known to be transported to the nerve terminal from the cell body along microtubule tracks, little is known about the regulation of vesicle traffic within the nerve terminal itself. It was previously believed that upon delivery to the nerve terminal, synaptic vesicles remained static until they were mobilized for neurotransmitter release (Henkel et al., 1996; Kraszewski et al., 1996). This brief period of free mobility was attributed to disassembly of the actin cytoskeleton, which was thought to otherwise cage the vesicles when the synapse was at rest (Miyamoto, 1995). However, recent work has shown that even at rest, synaptic vesicles are mobile at the synaptic bouton. Fluorescently tagged synaptic vesicles in unstimulated Drosophila NMJs exhibited rapid recovery times following photobleaching (Nunes et al., 2006a). Likewise, synaptic vesicles at goldfish ribbon synapses were shown to be highly mobile and this mobility is not related to changes in calcium concentration or the actin cytoskeleton (Holt et al., 2004).

Two in vivo imaging methods, FM dye labeling and fluorescence recovery after photobleaching (FRAP), were used to investigate intrabouton synaptic vesicle mobility at the Drosophila third-instar larval NMJ. FM 1-43 (N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide) dye has a hydrophilic polar head group and a hydrophobic tail that can interact with the plasma membrane, with the central region responsible for fluorescence. Electrical stimulation or chemical stimulation by high K+ concentration can be used to evoke exocytosis. During endocytosis, the FM dye, which does not fluoresce in an aqueous medium, binds to the outer leaflet of the plasma membrane and is internalized within the synaptic vesicles (Verstreken et al., 2008). Interaction with the lipid membrane causes the dye to fluoresce making it possible to follow vesicle trafficking using optical imaging (Verstreken et al., 2008).
Synaptic vesicle mobility within the nerve terminal can further be studied using FRAP. Following photobleaching, recovery of fluorescence into the bleached region is monitored over time (Phair et al., 2004). If the molecule studied is mobile, there will be an exchange of unbleached molecules with bleached ones as they move through the ROI (Phair et al., 2004). Therefore, the rate of fluorescence recovery serves as an indicator of the mobility of the observed molecule (Sprague and McNally, 2005). Thus, the rate of fluorescence recovery following bleaching of labeled vesicles would reflect the rate of intrabouton vesicle mobility.

The disruption in vesicle localization, taken together with the defects in synaptic transmission present in *jar* mutants, suggest that Myosin VI may participate in mediating synaptic vesicle mobility at the synaptic bouton. FM dye studies were used to characterize vesicle localization following electrophysiological stimulation. If Myosin VI is functioning to tether vesicles then it is expected that in mutants with reduced Myosin VI expression the FM labeled vesicles will not traffic to their proper peripheral location in the bouton following high-frequency stimulation. Indeed, like the vesicle mislocalization phenotype observed in Myosin VI loss of function mutant synapses at rest, the FM dye labeled vesicles exhibited diffuse distribution throughout *jar* mutant boutons. FRAP was used to further investigate the impact of Myosin VI on vesicle dynamics at the third-instar larval NMJ. The resultant FRAP curves reveal that a reduction in Myosin VI expression corresponds to an increase in synaptic vesicle mobility. Interestingly, stimulated emission depletion (STED) microscopy studies of rat hippocampal neurons show that although vesicles exhibit dynamic movement, their displacement is restricted within certain areas of the nerve terminal (Westphal et al., 2008). This is consistent with the FM dye and FRAP data in *Drosophila* boutons lending strong support for an anchoring function of Myosin VI, which may act to restrict vesicle displacement at the NMJ ensuring proper vesicle localization and trafficking.
Results

Vesicle distribution is altered in Myosin VI mutants following high-frequency stimulation

FM dye labeling was used to examine synaptic vesicle distribution in vivo following a high-frequency stimulation electrophysiological protocol. Boutons were labeled with a concentration of 4 µM and 10 µM FM 1-43 dye in 1.5 mM and 2 mM Ca\(^{2+}\) saline respectively. Differences in FM dye uptake were quantified by calculating the relative difference in fluorescence intensity across a bouton’s plot profile [(max intensity-min intensity)/max intensity]. For the first labeling protocol using a lower concentration of dye and calcium saline, relative difference in fluorescence intensity was significantly lower across \(jar^{322}/Df(3R)crb87-5\) mutant boutons (n=7) compared to OreR control boutons (n=6; Unpaired T-test, \(p<0.001\)). The characteristic plot profile corresponding to OreR boutons has two maxima indicating a peripheral distribution of FM labeled vesicles (Figure 23A). In contrast, a single maxima is observed extending across the entirety of the bouton for \(jar^{322}/Df(3R)crb87-5\) mutant larvae indicating a diffuse distribution of FM labeled vesicles (Figure 23B). There was no significant difference in mean fluorescence across the entire bouton between OreR controls and \(jar^{322}/Df(3R)crb87-5\) mutant larvae (Unpaired T-test, \(p>0.05\)). To improve labeling, a higher concentration of dye and calcium solution were used with the same 10 Hz stimulation protocol. Like the Synaptotagmin labeling, this protocol revealed that the \(jar^{322}/Df(3R)crb87-5\) mutant larvae exhibited a greater proportion of boutons with diffuse FM dye labeling throughout the bouton area in contrast to OreR control boutons (Figure 24A, B). Measuring relative difference in fluorescence intensity under these experimental conditions also revealed a significantly lower level across \(jar^{322}/Df(3R)crb87-5\) mutant boutons (n=34) compared to OreR control boutons (n=28; Unpaired T-test, \(p<0.05\)). Again, this is consistent with the majority of \(jar^{322}/Df(3R)crb87-5\) mutant larvae.
boutons exhibiting the diffuse synaptic vesicle distribution phenotype observed as a single maxima when a plot profile is made from the bouton area (Figure 24C).
Figure 23. FM1-43 dye labeling reveals differential synaptic vesicle distribution in Myosin VI mutant boutons.

Two representative images showing the distribution of FM labeled synaptic vesicles with their corresponding plot profiles for control OreR (A) and jar^{322}/Df(3R)crb87-5 mutant larvae (B). Arrows highlight an Ib bouton in the control with peripheral FM dye localization and an Ib bouton in the jar mutant with diffuse FM dye localization. There was a significant reduction in relative difference in fluorescence intensity across jar^{322}/Df(3R)crb87-5 mutant boutons (n=7) compared to the control boutons (n=6; Unpaired T-test, ***p<0.001). Boutons used for this study were from muscle 6/7 NMJs in segments A3, A4 and A5.
A) *OreR*

B) *jar*\(^{322}/Df(3R)crb87-5*

C)
Figure 24. Further FM1-43 dye labeling highlights diffuse synaptic vesicle distribution in Myosin VI mutant boutons.

Two representative images showing the distribution of FM labeled synaptic vesicles with their corresponding plot profiles for control OreR and jar<sup>322</sup>/Df(3R)crb87-5 mutant larvae. Arrows highlight an Ib bouton in the control with peripheral FM dye localization and an Ib bouton in the jar mutant with diffuse FM dye localization. A significant reduction in relative difference in fluorescence intensity was observed across jar<sup>322</sup>/Df(3R)crb87-5 mutant boutons (n=34) compared to the control boutons (C; n=28; Unpaired T-test, ***p<0.001). Boutons used for this study were from muscle 6/7 NMJs in segments A3, A4 and A5. Error bars represent mean ± SEM.
Myosin VI mutants exhibit enhanced synaptic vesicle mobility

To examine vesicle dynamics at jar mutant synapses, synaptic vesicles were visualized in vivo using a Synaptotagmin-GFP fusion protein and vesicle mobility was assessed using FRAP. Using image acquisition parameters previously established for FRAP analysis (Nunes et al., 2006a), FRAP recovery curves were compared following double normalization revealing a significant increase in synaptic vesicle mobility in the boutons with proper peripheral vesicle localization of heterozygous jar mutants. First, a comparison of fits tests was used to determine whether a single phase association or two phase association exponential curve represented a better fit for each of the FRAP curves generated. For boutons that exhibited normal vesicle distribution, it was found that both the Myosin VI mutants, \( \text{elav}^{C155}\text{Gal4}; \text{UAS-Synaptotagmin-GFP/}\); \( \text{jar}^{322/+} \) (n = 18 boutons) and \( \text{elav}^{C155}\text{Gal4}; \text{UAS-Synaptotagmin-GFP/}; \text{Df(3R)crb87-5/+} \) (n = 18 boutons), as well as the control \( \text{elav}^{C155}\text{Gal4}; \text{UAS-Synaptotagmin-GFP/} \) (n = 25 boutons) were best fit with a two association curve (Comparison of fits, Figure 25). However, when comparing the control boutons to the diffuse boutons of the heterozygous jar mutants, a two phase association curve was a better fit for the control \( \text{elav}^{C155}\text{Gal4}; \text{UAS-Synaptotagmin-GFP/} \) FRAP curve (n= 25 boutons). In contrast, \( \text{elav}^{C155}\text{Gal4}; \text{UAS-Synaptotagmin-GFP/}; \text{jar}^{322/+} \) (n = 20 boutons) and \( \text{elav}^{C155}\text{Gal4}; \text{UAS-Synaptotagmin-GFP/}; \text{Df(3R)crb87-5/+} \) (n = 15 boutons) mutant FRAP curves were better represented by a single phase association curve (Comparison of fits, Figure 26). Supplementary movies 1, 2 and 3 are sample movies demonstrating vesicle dynamics in control and Myosin VI mutant boutons, both with normal and diffuse vesicle localization.
The protocol was adjusted by employing a greater number of bleach iterations (increased from 9 to 12 iterations) and using more rapid image acquisition to capture additional information about fluorescence recovery in the first part of the curve. This protocol confirms the findings of the initial FRAP studies with the FRAP curve for the control, \( \text{elav}^{C155} \text{Gal4}; \ UAS-\text{Synaptotagmin-GFP}/\langle \) (n = 35 boutons), better fit with a double phase association curve; whereas the FRAP curves for the diffuse boutons of the \( \text{jar} \) heterozygotes, \( \text{elav}^{C155} \text{Gal4}; \ UAS-\text{Synaptotagmin-GFP}/\langle; \ \text{jar}^{322}/+ \) (n = 15 boutons) and \( \text{elav}^{C155} \text{Gal4}; \ UAS-\text{Synaptotagmin-GFP}/\langle; \ \text{Df(3R)crb87-5}/+ \) (n = 16 boutons), were better fit with single phase association curves (Comparison of fits, Figure 27).

In these data, the bleach depth was not as great in the mutant genotypes compared to the control for all FRAP protocols used, which is consistent with faster vesicle movement in the mutants. Bleach depth was significantly greater for \( \text{elav}^{C155} \text{Gal4}; \ UAS-\text{Synaptotagmin-GFP}/\langle \) boutons than both the boutons with proper peripheral vesicle localization (Figure 28A) and the diffuse boutons of heterozygous \( \text{jar} \) mutants immediately following 9 bleach iterations (Figure 28B, Dunnett's Multiple Comparison Test, \( p<0.001 \)). Similarly, following 12 bleach iterations, bleach depth was significantly greater for control \( \text{elav}^{C155} \text{Gal4}; \ UAS-\text{Synaptotagmin-GFP}/\langle \) boutons compared to diffuse boutons of \( \text{jar} \) loss of function heterozygotes (Figure 28C, Dunnett's Multiple Comparison Test, \( p<0.001 \)).
A) 8 sec  10 sec  120 sec

C155SytGFP

C155SytGFP/<; jar322/+

B)

- Red: C155SytGFP/<
- Green: C155SytGFP/<; jar322/+ 
- Blue: C155SytGFP/<; Df(3R)crb87-5/+ 

Fraction of Initial Fluorescence vs. Time after Photobleaching (s)
Figure 25. Synaptic vesicle mobility was enhanced in normal jaguar mutant boutons.

Representative images acquired during FRAP experiments for control and jar mutant boutons with proper peripheral vesicle localization revealed more rapid recovery of labeled vesicles into the bleach area of the jar mutant bouton (A). Images shown correspond to the time before bleaching (8 sec), immediately after bleaching (10 sec) and at the end of image acquisition (120 sec). The bleached region is indicated by a white arrow. Curve fitting of FRAP recoveries was performed using double exponential curves and statistical differences were tested using nonlinear regression (B). The heterozygous jar mutants, elav^{C155}Gal4; UAS-Synaptotagmin-GFP/<; jar^{322}/+ (n = 18 boutons) and elav^{C155}Gal4; UAS-Synaptotagmin-GFP/<; Df(3R)crb87-5/+ (n = 18 boutons), exhibited significantly enhanced vesicle mobility compared to the control elav^{C155}Gal4; UAS-Synaptotagmin-GFP/< (n = 25 boutons; Comparison of Fits, p<0.0001). Boutons used for this study were from muscle 67 NMJs in segments A3 and A4. Error is represented as the 95% confidence interval of the curve.
A) 8 sec  10 sec  120 sec

C155SytGFP

C155SytGFP/<; jar322/+  

B)

Fraction of Initial Fluorescence

0.4  0.5  0.6  0.7  0.8  0.9  1.0

0  20  40  60  80  100  120

Time after Photobleaching (s)

C155SytGFP/<
C155SytGFP/<; jar322/+  
C155SytGFP/<; Df(3R)crb87-5
Figure 26. Diffuse *jaguar* mutant boutons exhibited a significant increase in vesicle mobility.

Representative images acquired during FRAP experiments for control and *jar* mutant boutons with diffuse vesicle localization revealed more rapid recovery of labeled vesicles into the bleach area of the *jar* mutant bouton (A). Images shown correspond to the time before bleaching (8 sec), immediately after bleaching (10 sec) and at the end of image acquisition (120 sec). The bleached region is indicated by a white arrow. Curve fitting of FRAP recoveries was performed using double exponential curves and statistical differences were tested using nonlinear regression (B). Vesicle mobility was significantly greater in the diffuse boutons of the heterozygous *jar* mutants, *elav*<sup>C155</sup>*Gal4; UAS-Synaptotagmin-GFP/♂; jar<sup>322</sup>/+ (n = 20 boutons) and *elav*<sup>C155</sup>*Gal4; UAS-Synaptotagmin-GFP/♂; Df(3R)crb87-5/+ (n = 15 boutons), compared to the control *elav*<sup>C155</sup>*Gal4; UAS-Synaptotagmin-GFP/♂ (n = 25 boutons; Comparison of Fits, p<0.0001). Boutons used for this study were from muscle 6/7 NMJs in segments A3 and A4. Error is represented as the 95% confidence interval of the curve.
A) 4 sec 5 sec 45 sec

C155SyGFP

C155SyGFP/<; jar322/+  

B)  

Fraction of Initial Fluorescence

C155SyGFP/<  
C155SyGFP/<; jar322/+  
C155SyGFP/<; Df(3R)crb87-5/+
Figure 27. FRAP curves for diffuse jaguar mutant boutons have greater initial fluorescence recovery than control boutons.

Representative images acquired during FRAP experiments for control and jar mutant boutons with diffuse vesicle localization revealed more rapid recovery fluorescence into the bleach area of the jar mutant bouton (A). Images shown correspond to the time before bleaching (4 sec), immediately after bleaching (5 sec) and at the end of image acquisition (45 sec). The bleached region is indicated by a white arrow. Curve fitting of FRAP recoveries was performed using double exponential curves and statistical differences were tested using nonlinear regression (B) Rapid fluorescence recovery observed for the FRAP curves of the diffuse boutons of the heterozygous jar mutants, elav$^{C155}$Gal4; UAS-Synaptotagmin-GFP/; jar$^{322}$/+ (n = 15 boutons) and elav$^{C155}$Gal4; UAS-Synaptotagmin-GFP/; Df(3R)crb87-5/+ (n = 16 boutons), indicates enhanced vesicle mobility compared to the control elav$^{C155}$Gal4; UAS-Synaptotagmin-GFP/ (n = 35 boutons; Comparison of Fits, p<0.0001). Boutons used for this study were from muscle 67 NMJs in segments A3 and A4. Error is represented as the 95% confidence interval of the curve.
A) ***

B) ***

C) ***
Figure 28. Control boutons exhibit a significantly greater bleach depth than jar mutant boutons.

Bleach depth was significantly greater for elav\textsuperscript{C155}Gal4; UAS-Synaptotagmin-GFP/\< boutons than both the boutons with proper peripheral vesicle localization (A) and the diffuse boutons of heterozygous jar mutants immediately following 9 bleach iterations (B, Dunnett's Multiple Comparison Test, ***\=p<0.001). Similarly, following 12 bleach iterations, bleach depth was significantly greater for control elav\textsuperscript{C155}Gal4; UAS-Synaptotagmin-GFP/\< boutons compared to diffuse boutons of jar loss of function heterozygotes (C, Dunnett's Multiple Comparison Test, ***\=p<0.001). Boutons used for this study were from muscle 6/7 NMJs in segments A3 and A4. Bars represent mean ± SEM.
Discussion

FM dye distribution is altered in Myosin VI mutant boutons following high-frequency stimulation

Ultrastructural studies have reliably shown that at *Drosophila* synapses type Ib boutons have peripherally distributed vesicles and that the centre of the bouton is free of vesicles (Roche et al., 2002). FM1-43 dye loading at low frequency stimulation to mobilize the recycling pool of vesicles has been consistent with the EM data in showing peripheral vesicle localization following endocytosis (Akbergenova and Bykhovskaia, 2009). Centrally localized vesicles can be observed in Ib boutons following FM1-43 dye staining at 10 Hz followed by a 10 minute resting period, which is attributed to the formation of extra vesicles in response to intense stimulation (Akbergenova and Bykhovskaia, 2009). However, when vesicle localization was visualized immediately following stimulation with no rest period, vesicles were found to occupy a smaller, peripherally restricted area of the bouton (Akbergenova and Bykhovskaia, 2009). Thus, the redistribution of extra vesicles to the bouton centre occurs during the rest period.

In Myosin VI mutants and controls, FM1-43 dye uptake was induced through a similar high frequency protocol using two different dye concentrations. Imaging of vesicle distribution immediately following electrical stimulation revealed in both cases that vesicles remained restricted to the bouton periphery in wild-type controls. In contrast, vesicles were found throughout the bouton area in *jar* loss of function mutant boutons. Given that FM dye labeling was visualized immediately following high frequency loading and that total bouton fluorescence did not differ between mutants and controls, the central localization of vesicles in *jar* mutants cannot be attributed to an increase in vesicle abundance. Rather, it is suggestive of an active role of Myosin VI in peripheral vesicle clustering at the nerve terminal. Optically, fluorescence intensity of styryl dye uptake can be used to confirm changes in vesicle abundance, with a
reduction in brightness expected if vesicle number is decreased or alternately, an increase in brightness corresponding to an increased number of vesicles. Thus, Myosin VI does not appear to play a direct role in determining vesicle number as no differences in total fluorescence intensity were observed between mutants and controls. Synaptotagmin staining data indicates that Myosin VI is important for proper vesicle localization within the bouton at rest. FM labeling reveals an additional requirement for Myosin VI in maintaining peripheral vesicle distribution following nerve stimulation.

**Myosin VI loss of function mutants exhibit enhanced vesicle mobility at the synaptic terminal**

Synaptic vesicles are mobile at the *Drosophila* NMJ (Nunes et al., 2006b; Seabrooke et al., 2010); however, the mechanisms regulating vesicle dynamics at the nerve terminal have yet to be fully resolved. Understanding the mechanisms by which vesicle movement is mediated within nerve terminals can provide insights into the mobilization of vesicles for participation in exocytosis. Vesicles may move within the synaptic bouton by simple diffusion or via active transport; although the relative contribution of these processes in vesicle mobility is unknown. In frog motor nerve terminals, vesicle motion appears to be mediated by simple diffusion and is not affected by disruption of the actin cytoskeleton (Gaffield et al., 2006). However, other studies indicate that a myosin motor moving along actin tracks may be important for regulating vesicle mobility (Seabrooke et al., 2010; Jordan et al., 2005).

If Myosin VI functions in proper synaptic vesicle localization to the bouton periphery by acting as a tether, it should normally restrain vesicle movement at the synapse. FRAP analysis revealed that in boutons exhibiting normal vesicle distribution recovery of fluorescence occurs in two phases. In such cases, the fast diffusive component of recovery occurs first, followed by recovery due to exchange at binding sites resulting in a curve that can be separated into two
phases (Sprague and McNally, 2005). As Myosin VI contributes to ensuring proper vesicle localization, the two phase recovery curve for boutons with proper vesicle localization could be explained by attributing the slow portion of fluorescence recovery to binding interactions between vesicles and Myosin VI. The more rapid recovery phase of this FRAP curve may in turn reflect simple diffusion of vesicles not bound to Myosin VI. Binding interactions can delay the recovery of the a FRAP curve for a given molecule in comparison to if it's mobility was represent by diffusion alone (Sprague and McNally, 2005). In contrast, with the diffuse vesicle localization phenotype of boutons in the Myosin VI loss of function mutants, Myosin VI regulation of vesicle localization is presumably absent. Thus, the FRAP recovery curve can be fit to one phase wherein vesicle motion is attributed to diffusion within the bouton. In addition, the fact that bleach depth is greater in the control than in the mutants is consistent with fluorescence recovering during the actual bleaching procedure (McNally, 2008), which supports the findings of great vesicle mobility in Myosin VI loss of function mutants. These results are consistent with a function for Myosin VI as a vesicle tether because jar mutants have a lower level of Myosin VI expression and therefore, a reduced ability to anchor vesicles resulting in their enhanced mobility at the synapse. The FRAP data is important because it provides the mechanism of Myosin VI function at the synapse. Myosin VI may maintain proper synaptic physiology by tethering vesicles to ensure their proper localization for efficient release.
Chapter 5: General Discussion

The purpose of this research was to provide insights into neural development and function. Specifically, the regulation of vesicle populations within the nerve terminal was investigated. Given the great degree of conservation in both the general features of synaptic transmission and the regulation of the synaptic vesicle cycle, the *Drosophila* system provides a means of examining mutant neural morphology and physiology that can have implications for higher organisms. *Drosophila* NMJs are chemical synapses that have been recognized as a useful model for investigating human neural disorders (Bilen and Bonini, 2005). Human populations exhibit phenotypic and genotypic diversity; this heterogeneity can make isolating factors involved in a disease process difficult (Mackay and Anholt, 2006). To move towards a cure for neural illnesses, a clear understanding of the mechanisms regulating nervous system function is needed and can be gleaned from the *Drosophila* system, which allows for regulation of environmental conditions and background genotype not possible in human studies. Loss of Myosin VI is associated with various disease pathologies in humans (Mackay and Anholt, 2006). This study elucidates the function of Myosin VI within the nervous system providing support for a vesicle tethering function at the nerve terminal. By anchoring vesicles to the bouton periphery, Myosin VI regulates vesicle mobility and release during neural stimulation.

**Myosin VI contributes to synaptic morphology and physiology**

Synapses are specialized junctions that allow neurons to signal to other neurons or effector cells. Proper synaptic development is critical to ensure effective neuronal connectivity and transsynaptic signal relay. During synaptogenesis, the actin and microtubule cytoskeleton are essential during axonal differentiation and extension towards a target tissue. Upon establishment of a synaptic connection, the cytoskeleton continues to play an important role in both synaptic expansion and retraction. Regulation of the presynaptic actin cytoskeleton by adaptor proteins is
essential for restraining synaptic growth at the larval NMJ (Khuong et al., 2010). Reorganization of the microtubule cytoskeleton by budding from terminal boutons or insertion between existing boutons is associated with NMJ growth during larval development (Ruiz-Canada and Budnik, 2006). In addition, actin and microtubules form tracks along which motor proteins can move to transport cargo to and within the nerve terminal (Ruiz-Canada and Budnik, 2006).

Although Myosin motors move along actin tracks, evidence exists for an interaction between Myosin VI and the microtubule cytoskeleton through adaptor proteins. Assays to detect protein interactions show that Myosin VI binds to the microtubule associated protein Cornetto (Finan et al., 2011). In addition, Myosin VI has been shown to colocalize with the microtubule-binding protein CLIP-190 in the *Drosophila* embryonic nervous system (Lantz and Miller, 1998). Staining against Futsch, a microtubule associated protein believed to be important for stabilization of the microtubule cytoskeleton, revealed that there was a reduction in microtubule extension throughout Myosin VI loss of function mutant NMJs. In addition, there was a reduction in the number of boutons containing microtubule loops, which are normally associated with mature, stable boutons, in these mutants. Together, these changes in the synaptic microtubule cytoskeleton could account for the reduction in NMJ length and Ib bouton number observed in Myosin VI mutant larvae. Indeed, Futsch loss of function mutants themselves exhibit a significant reduction in NMJ size and bouton number associated with the disruptions these mutants experience to their microtubule cytoskeleton (Roos et al., 2000). In contrast, increased expression of Futsch has been shown to correspond to increased NMJ branching and bouton number (Zhang et al., 2001). This report provides the first evidence of a role of Myosin VI in maintaining microtubule architecture at the synaptic terminal.

Interestingly, live imaging of actin at the NMJ and immunolabeling of actin in the larval brain revealed no changes to the actin cytoskeleton of Myosin VI loss of function mutants.
During spermatid individualization in *Drosophila*, disruptions to the actin cytoskeleton were observed when Myosin VI expression levels were reduced (Noguchi et al., 2006). Myosin VI has been shown to colocalize with actin polymerization regulatory proteins during spermatogenesis (Rogat and Miller, 2002). In Myosin VI loss of function mutants these proteins failed to localize correctly suggesting a possible mechanism for Myosin VI regulation of actin dynamics (Rogat and Miller, 2002). Additional studies using a variety of truncated Myosin VI constructs support the conclusion that Myosin VI is functioning to tether regulatory molecules at the actin growth cone during spermatid individualization (Isaji et al., 2011). In contrast, at the larval NMJ no alterations to the actin cytoskeleton were found to correspond to any level of Myosin VI loss of function. Thus, Myosin VI mediation of cytoskeletal structures appears to vary among different cellular processes.

Within the *Drosophila* larval synaptic terminal, different functional pools of vesicles are spatially intermixed and restricted to the bouton periphery (Denker et al., 2009). Correct vesicle organization is important for subsequent steps in neurotransmitter release following neural stimulation. Initially, Synapsin, a phosphoprotein that can reversibly associate with synaptic vesicles, had been the only protein identified to regulate vesicle localization to the bouton periphery. However, this report provides data demonstrating a function for Myosin VI in maintaining proper intrabouton vesicle distribution. In Synapsin null larvae, vesicles fail to cluster peripherally and spread through the bouton centre (Akbergenova and Bykhovskaia, 2010). In addition, a greater proportion of vesicles participate in recycling in these mutants suggesting that Synapsin may function to specifically tether vesicles within the reserve pool (Denker et al., 2011). Visualization of Synaptotagmin staining at Myosin VI mutant boutons also revealed an unexpected diffuse distribution of vesicles throughout the bouton centre suggesting that it too may function in maintaining peripheral vesicle localization.
Myosin VI has previously been shown to interact with vesicles during endocytosis through both adaptor proteins and phospholipids in the vesicular plasma membrane of mammalian cells. In early endocytosis, Myosin VI is targeted to clathrin-coated pits through its binding partner, Dab2 (Dance et al., 2004). Dab2 is a clathrin-associated sorting protein that interacts with clathrin as well as clathrin adapter protein 2 and contributes to vesicle formation during endocytosis (Dance et al., 2004). Myosin VI binding to Dab2 has shown in vitro through a yeast two-hybrid screen and co-sedimentation assay (Inoue et al., 2002). GST-pull down experiments and mammalian two-hybrid screens using a Luciferase reporter were also performed to confirm the interaction between Myosin VI and Dab2 (Morris et al., 2002). Binding is abolished between Myosin VI and Dab2 when a single amino acid is altered from a tryptophan to a leucine at amino acid sites 1183 to 1185 in the C-terminal tail, which in turn prevents proper targeting of Myosin VI to clathrin structures (Spudich et al., 2007). The vesicle-associated protein GAIP interacting protein, C terminus (GIPC) /Synectin has also been shown to bind with the tail domain of Myosin VI in yeast two-hybrid screens (Naccache et al., 2006). GIPC is important for recruiting of Myosin VI to vesicles following uncoating (Naccache et al., 2006). Within the Myosin VI C-terminal tail, amino acids 1107 to 1109 (arginine-arginine-leucine) mediate binding between Myosin VI and GIPC (Spudich et al., 2007; Naccache et al., 2006). Additionally, Myosin VI fails to coimmunoprecipitate with a GIPC construct lacking a function PDZ domain (Naccache et al., 2006). However, pull down experiments demonstrate that ligand binding to the PDZ domain enhances GIPC and Myosin VI binding, indicating that this domain is not directly responsible for interaction with Myosin VI (Naccache et al., 2006). Kermit, the *Drosophila* orthologue of GIPC, has also been shown to directly bind Myosin VI through affinity chromatography (Finan et al., 2011). Thus, as in mammalian cells, the interaction between Myosin VI and vesicles may be mediated by a protein present on the vesicular membrane at the
*Drosophila* nerve terminal. In addition, Myosin VI was been shown to bind phosphatidylinositol 4,5-bisphosphate (PIP$_2$)-containing liposomes in human HeLa cells using Fluorescence resonance energy transfer (Spudich et al., 2007). When a Myosin VI protein with a mutated PIP$_2$ binding site was expressed in transfected cells, defects were observed in proper Myosin VI targeting to endocytic vesicles (Spudich et al., 2007). Thus, it is also possible that Myosin VI interaction with vesicles at the NMJ may occur via a binding interaction with PIP$_2$ present in the vesicular membrane.

Imaging of synaptic vesicles immediately following FM1-43 dye labeling is consistent with the Synaptotagmin staining, revealing vesicle mislocalization throughout the bouton centre in Myosin VI mutant larvae. Typically, bouton centre occupancy is only observed with FM dye studies following intense stimulation and a rest period prior to imaging, with these changes found to be associated with an increase in vesicle number (Akbergenova and Bykhovskaia, 2009; Denker et al., 2009). In contrast, the alterations in vesicle distribution in Myosin VI mutant nerve terminals do not correspond to changes in the number of vesicles present within the boutons. No changes to overall fluorescence intensity were observed when measured following immunostaining of a synaptic vesicle marker or FM1-43 dye uptake indicating that there are no alterations to total vesicle number at Myosin VI loss of function mutant synapses compared to controls. Thus, the imaging data shows that a loss of Myosin VI is associated with a failure of vesicles to organize peripherally within a bouton but this alteration is not accompanied by a change in vesicle pool size. These findings indicate a novel function for Myosin VI in regulating proper vesicle localization at the bouton periphery.

Synaptic vesicle localization and availability are crucial in maintaining basal synaptic function and regulating changes in the strength of synaptic transmission. Thus, the vesicle mislocalization phenotype taken together with sluggish behaviour observed in mutant larvae
prompted further investigation of synaptic physiology in Myosin VI loss of function mutants. *In vivo* imaging studies of synaptic activity using a variety of vesicle labeling techniques indicate that in freely moving, unstressed larvae only a small proportion of vesicles corresponding to the RRP participate in synaptic transmission (Denker et al., 2011). Mobilization of the RRP of vesicles in Myosin VI mutants showed they experienced relatively mild changes to basal synaptic physiology that were associated with the most severe reductions in Myosin VI expression levels. This may be accounted for by the fact that even at Myosin VI mutant NMJs not all boutons exhibit vesicle mislocalization. Thus, those boutons with properly localized vesicles may be able to compensate for changes to release probability within mutant boutons and maintain efficient basal release.

Changes to synaptic plasticity at the presynapse can occur through modulation of vesicle fusion machinery and the mobilization of synaptic vesicles at the active zones. Through a vesicle tethering function at the nerve terminal, Myosin VI may affect synaptic plasticity by regulating vesicle mobilization during neural stimulation. Traditionally, mammalian studies have shown that Myosin VI participates mainly in vesicle endocytosis. FRAP of GFP-tagged Myosin VI at clathrin-coated pits in mammalian cell cultures revealed it exhibited rapid turnover similar to its binding partner Dab2, which is more consistent with a role in vesicle transport rather than long-term anchoring (Bond et al., 2012). In retinal cells, Myosin VI was found to be important for efficient trafficking of uncoated vesicles through the actin rich regions of the cell periphery (Aschenbrenner et al., 2004). Myosin VI also plays a role in cargo trafficking from the early endosome, where transferrin receptor build up was found to associated with Myosin VI loss of function reflecting a defect in the endocytotic pathway (Chibalina et al., 2007). A recent study using cells derived from the rat adrenal medulla supports a tethering function for Myosin VI in exocytosis, wherein Myosin VI recruits secretory granules to the cortical actin network near the
plasma membrane and thereby ensures their availability for release upon stimulation (Tomatis et al., 2013).

In *Drosophila*, endocytotic mutants are characterized by a significant reduction in their ability to sustain release in response to intense stimulation (Verstreken et al., 2002). Contrary to expectations based on most mammalian studies, Myosin VI mutants were not found to experience changes in neurophysiology consistent with endocytotic defects. Rather enhanced release was observed at Myosin VI mutant NMJs in response to high-frequency stimulation. The rate at which vesicles can be recruited to docking sites limits the amount of synaptic vesicles that can fuse in response to intense stimulation. Without Myosin VI present at mutant nerve terminals, vesicles are untethered enabling them to transition more rapidly between the RP and the RRP and thereby, to participate in synaptic transmission. The electrophysiological data indicates that Myosin VI function in vesicle clustering at the periphery may be important for synaptic plasticity by regulating transitions between different functional pools of vesicles.

Although synaptic vesicles have been found to be mobile within the nerve terminal, the mechanisms regulating vesicle dynamics are not fully known. Another Myosin motor, Nonmuscle Myosin II, has been shown to impact vesicle dynamics at the bouton in an expression dependent manner (Seabrooke et al., 2010). FRAP of Myosin VI mutant boutons revealed a novel role for Myosin VI in restraining vesicle movement. Loss of Myosin VI expression corresponded to an increase in vesicle mobility at the nerve terminal. The FRAP data is consistent with the imaging and physiological data, supporting a vesicle anchoring function for Myosin VI. This study describes a novel role for Myosin VI in restricting intrabouton vesicle mobility.
Future Work

**Myosin VI may participate in the regulation of active zone assembly**

Interestingly, given that the *Drosophila* NMJ has long been a favourite model of the synapse, little is known about the regulation of protein composition at the *Drosophila* active zones (Wairkar et al., 2009). Electron micrographs of *Drosophila* active zones are associated with a characteristic T-bar structure, which is thought to enhance vesicle release (Wichmann and Sigrist, 2010). The discovery of the *Drosophila* active zone protein Bruchpilot (Brp), an ortholog of the mammalian active zone protein CAST, generated intense interest. CAST is a scaffolding protein important for organizing the molecular structure at the active zone cytomatrix and is part of a network of protein-protein interactions with other active zone proteins (Ohtsuka et al., 2002; Takao-Rikitsu et al., 2004). *In vivo* imaging showed that Brp arrived at an advanced stage of active zone assembly and was required for continued clustering of calcium channels at the active zone (Fouquet et al., 2009). As the mechanisms of active zone assembly remain poorly understood (Giagtzoglou et al., 2009), these studies have raised questions about the regulation of Brp localization to the active zones. Given that reduction in Myosin VI corresponds to reduction in Brp, it is possible that Myosin VI plays an additional role at the NMJ in active zone assembly.

In addition to immunostaining, active zones can be visualized using electron microscopy as electron dense membrane thickenings in *Drosophila* boutons (Zhai and Bellen, 2004). Interestingly, in a Rab3 loss of function mutant that exhibited a significant reduction in Brp puncta number, ultrastructural analysis revealed only a mild reduction in active zone number per micron of membrane (Graf et al., 2009). Thus, EM analysis can be used as a means to further investigate active zone morphology in Myosin VI mutants. Additionally, EM has demonstrated that Brp is a component of the presynaptic electron dense T-bar, which is thought to facilitate vesicle release (Fouquet et al., 2009). Active zones typically possess either no T-bars or one T-
bar; very rarely are two T-bars observed (Graf et al., 2009). Ultrastructural studies would make it possible to assess T-bar number and structure at jar mutant boutons. For a recently described Brp hypomorph, brp<sup>nude</sup>, EM micrographs revealed a significant reduction in vesicle clustering at the T-bars, while other features of the cytomatrix including T-bar morphology and vesicle number remained grossly normal (Hallermann et al., 2010b). This impairment in vesicle tethering at the electron dense bodies was accompanied by enhanced depression following paired-pulse stimulation (Hallermann et al., 2010b). It is possible that Myosin VI plays a role in proper active zone assembly, and contributes to proper vesicle tethering at the T-bars. Ultrastructural analysis of Myosin VI loss of function mutant boutons would reveal if they also exhibit fewer vesicles specifically localized to T-bars.

Given that Brp is required for continued clustering of the calcium channel subunit Cacophony at mature active zones, the reduction in Brp puncta at Myosin VI mutant synapses may be accompanied by changes in Cacophony density at the active zones (Fouquet et al., 2009). Electrophysiological and FM-dye studies of a temperature sensitive Cacophony mutant have demonstrated the importance of this calcium channel in regulating exocytosis during synaptic transmission (Kuromi et al., 2004; Kuromi et al., 2004; Kawasaki et al., 2000). To examine Cacophony expression in Myosin VI mutants, Cacophony could be imaged in vivo at the boutons of third-instar larvae by co-expressing the UAS-cac<sup>1-EGFP</sup> transgene with control and jar mutant genotypes. As a decrease in Cacophony density would diminish vesicle release probability, if Cacophony expression is reduced at jar mutant synapses this would be consistent with the impaired synaptic physiology of these mutants. At active zones, vesicle docking sites and calcium channels are proximal to each other to facilitate efficient release and thus, increasing distance results in decreased vesicle release probability (reviewed by (Neher, 1998)). In Myosin VI mutants even subtle reductions in Cacophony expression may be important as the effect of
these changes might be enhanced by the vesicle mislocalization phenotype of these mutants.

Bath application of a membrane permeable version of the calcium buffer EGTA has been used to measure relative suppression of evoked release in a manner that reflects the spatial relationship between vesicle release sites and calcium channels (Neher, 1998; Kittel et al., 2006). EGTA binding kinetics are not fast enough to buffer calcium and suppress neurotransmission when release sites are close to the calcium channels (Neher, 1998). If indeed the mislocalization of vesicles in Myosin VI mutants results in an increased distance from calcium channels, a greater reduction in evoked response at *jar* mutant synapses would be expected compared to control synapses. These studies could give additional insights into whether Myosin VI participates in the process of *Drosophila* active zone composition.

**Additional factors that may contribute to Myosin VI regulation of synaptic transmission**

At wild-type synapses, presynaptic and postsynaptic elements are aligned for efficient neural communication, which requires precise localization of their protein constituents (Collins and DiAntonio, 2007). Thus, the reduction in presynaptic active zones in Myosin VI mutants may be accompanied by changes to postsynaptic glutamate receptor cluster morphology and number. To assess gross changes to postsynaptic morphology in *jar* mutants, the postsynapse can be labeled by staining for essential glutamate receptor subunit DGluRIII. Additionally, at the *Drosophila* postsynapse, there are two glutamate receptor subunits, GluRIIA and GluRIIB, that are functionally redundant for viability, but exhibit different physiological properties with the GluRIIA subunits producing larger currents (Schmid et al., 2008). In Brp mutant synapses, a shift in glutamate receptor subunit composition was observed to include a higher level of GluRIIA subunits (Schmid et al., 2008). Given that Brp puncta number is reduced in the *jar* mutant, it is possible that, like in the Brp mutant itself, glutamate receptor subunit composition is also altered in the *jar* mutant. If changes in glutamate receptor subunit composition are present in
the Myosin VI mutant larvae, it may give additional insights into the synaptic physiology of these mutants as the subunits have differing physiological properties.

Interestingly, less severe Myosin VI mutants did not experience significant changes to their basal synaptic physiology, which can be accounted for by the fact that they possess a larger proportion of boutons exhibiting normal vesicle localization. With increasing severity of jar loss of function, an increasing proportion of boutons exhibited vesicle mislocalization culminating with the most severe maternal null jar mutant, in which basal synaptic physiology was impaired. To assess whether synaptic activity is specifically reduced at boutons exhibiting vesicle mislocalization in jar mutant nerve terminals, focal recordings can be used to study the synaptic release properties of a single bouton rather than that of the entire population of boutons at the NMJ (Zhang and Stewart, 2010).

**Conclusions**

My study of Myosin VI at the Drosophila NMJ will contribute to a growing understanding of the regulation of synaptic morphology and function. This research has shown the Myosin VI is important for proper NMJ development, likely via an interaction with the synaptic microtubule cytoskeleton. Within the nerve terminal, optical experiments revealed a novel role for Myosin VI in restricting vesicle localization to the bouton periphery. These morphological changes are accompanied by alterations in basal physiology and synaptic plasticity in Myosin VI loss of function mutants. A lack of proper vesicle tethering corresponding to reduction in Myosin VI expression could account for the physiological defects observed. Indeed, vesicle mobility has been shown to be enhanced at Myosin VI mutant synapses, suggesting that Myosin VI normally anchors vesicles in position restraining their movement. This work contributes to a broader understanding of the regulation of vesicle
dynamics within the nerve terminal and the importance of proper vesicle localization for maintaining synaptic efficacy.
Chapter 6: Materials and Methods

Materials and Methods for Chapter 2

Fly Strains and Genetics.

All fly strains and crosses were maintained on Bloomington standard medium (http://flystocks.bio.indiana.edu/bloom-food.htm) supplemented with yeast paste at room temperature. During handling, flies were temporarily anesthetized using CO$_2$. The Myosin VI loss of function alleles used in this study were $jar^{322}$ and $Df(3R)crb87-5$ (maintained as stocks over $Tm3, Sb Ser GFP$). $jar^{322}$ is a null allele that deletes the entire Myosin VI coding region and some of the neighboring gene, CG5706 (Morrison and Miller, 2008). When homozygous, $jar^{322}$ is lethal in first or second instar larvae due to the loss of CG5706 function (Morrison and Miller, 2008). $Df(3R)crb87-5$ is a deletion that removes most of the amino acid coding sequences of the $jar$ gene, through to exon 13 of 17 (Morrison and Miller, 2008). $jar^{322}/Df(3R)crb87-5$ animals were produced by crossing together $jar^{322}/Tm3, Sb Ser GFP$ and $Df(3R)crb87-5/Tm3, Sb Ser GFP$ flies, and then selecting against the GFP balancer chromosomes. Maternal $jar$ mRNA and Jar protein persist in zygotic $jar^{322}$ homozygous embryos until stage 16 (Petritsch et al., 2003). Myosin VI maternal null animals, from herein designated $jar^{322}/Df(3R)crb87-5$ MN, were generated by crossing $jar^{322}/Df(3R)crb87-5$ females to $jar^{322}/Tm3, Sb Ser GFP$ males, and then selecting non-fluorescent larvae. Larvae from $yw$ flies and from Oregon R ($OreR$) flies served as wild-type controls. As $yw$ flies were generated in an $OreR$ background, they are phenotypically equivalent. For in vivo actin imaging, larvae expressing $UAS-Actin-GFP$ transgene under the regulation of the presynaptic neural driver $P(GAL4-elav.L)3A4$ (from herein abbreviated as $elav^{3A}$-GAL4) were used.
Western Blots.

Protein extracts were prepared in ice-cold homogenization buffer with proteinase inhibitor. Four body walls or ten brains from third-instar larvae per genotype were used. The larval preparations were manually homogenized and pulsed in the centrifuge. This was followed by the addition of 0.5 M Dithiotreitol and 2x loading buffer. Larval preparations were heated at 100°C for 5 minutes prior to loading. Samples were run on a 6% SDS PAGE gel using modified procedures from Sambrook (2001). Gels were run for 1.5 hours at 70 V, followed by electropheretic transfer to polyvinylidene difluoride membrane at 350 mA for 55 minutes. Membranes were incubated with 1:20 mouse anti-Myosin VI antibody (gift from Kathy Miller) and 1:1000 anti-tubulin antibody (Hybridoma Bank, University of Iowa). HRP-conjugated goat anti-mouse antibody (BIORAD) was used at a dilution of 1:3000. Resultant bands were visualized using STORM Scanner Control (Molecular Dynamics). The stained bands were now visible for further analysis using Image J. Relative protein levels were normalized to the intensity of the loading control, β-tubulin, quantified and compared.

Antibody Staining and Confocal Microscopy.

For all immunostaining, third-instar larvae were dissected in hemolymph-like saline (HL3) (Stewart et al., 1994), fixed in 4% EM grade formaldehyde in phosphate buffered saline (PBS) for 1 hour and then transferred to an Eppendorf tube with 5 to 7 other larvae. Larval fillets were blocked with Normal Goat Serum and then washed with 10% PBS Tween®20 (BIORAD) for 1 hour, 3 times at 20 minutes each. For NMJ visualization, larvae were stained with 1:1000 FITC-conjugated goat anti-HRP antibody (Stewart et al., 2002). For synaptic vesicle labeling, larvae were stained with 1:1000 rabbit polyclonal anti-Synaptotagmin antibody, followed by the secondary antibody, AlexaFluor® 488 goat anti-rabbit IgG, at a 1:1000 dilution as outlined by Nunes et al. (2006b). To visualize actin in the larval brain, tissue was stained with
1:1000 TRITC-conjugated anti-phalloidin. Microtubule morphology at the NMJ was visualized with 1:100 goat anti-22c10 (Futsch) antibody, followed by the secondary antibody, AlexaFluor® 546 goat anti-mouse IgG, at a 1:1000 dilution. In between primary and secondary antibody staining, another wash was performed with 10% PBS Tween®20 (BIORAD) for 1 hour, 3 times at 20 minutes each. Likewise, at the end of staining for all experiments, the washing protocol was conducted for a final time. Larval fillets or tissues were then mounted onto slides in Vectashield® Mounting Medium for Fluorescence (Vector Laboratories).

Images were obtained using a LSM510 (Carl Zeiss) confocal laser microscope with a 200 mW Argon laser or 1.5 mW HeNe laser depending on the fluorescent probe used to visualize the tissue. To image NMJs, Z-sections were collected for NMJ 6/7, 2 per larvae, at 1 μm intervals through the 20x air lens at 2 times magnification. These images were projected onto a single plane and NMJs were measured (scale: 2.223 pixels/μm) using the computer software, Image J. For Synaptotagmin staining, images for individual boutons were obtained using a 40×/NA 1.4 oil immersion lens under constant imaging parameters, including gain, zoom, pinhole size and laser output, to allow for proper comparative analysis. To analyze Synaptotagmin staining in individual boutons, a rectangular cross section of the bouton was taken and a plot profile was generated for this region using Image J. Phalloidin staining was visualized using the 20x air lens at 2 times magnification using the same imaging parameters for all genotypes. For Futsch and Synaptotagmin double labeling, images were taken through the 20x air lens at 2 times magnification. The resultant images were merged onto a single plane in Image J.

**Actin Imaging.**

Actin dynamics were visualized in vivo by co-expressing a UAS-Actin-GFP transgene with control and jar mutant genotypes. Larvae resulting from crosses of UAS-Actin-GFP to the presynaptic neural driver elav^3A^-GAL4 served as the controls. Larvae varying in severity of
Myosin VI loss of function were made using the following stocks: *UAS-Actin-GFP; jar<sup>322</sup>/Tm3SerGFP*, and *elav<sup>3A</sup>-GAL4, Df(3R)crb87-5/Tm3SbSerGFP*. Actin-GFP signals at NMJ 6/7 of third-instar larvae were imaged using a using a 60x water immersion lens on a Nikon E600FN fluorescent microscope fitted with a Hamamatsu Orca CCD camera. The microscope filter was set for GFP emission and excitation. Image acquisition occurred every 15 seconds following a 1 second exposure using SimplePCI software. These images were converted into movie files in Image J with a maximum of two movies taken per larvae.

**Statistic Analysis.**

All statistical analyses were performed in GraphPad Prism Software 5. A significance level of p<0.05 was used for all experiments.
Materials and Methods for Chapter 3

Fly Strains and Genetics.

The Myosin VI loss of function Drosophila stocks used for the behavioural, electrophysiological and imaging experiments have been described in detail in the Materials and Methods for Chapter 2. Briefly, the Myosin VI loss of function alleles used in this study were jar^{322} and Df(3R)crb87-5 (maintained as stocks over Tm3, Sb Ser GFP). OreR flies served as wild-type controls. During handling, flies were temporarily anesthetized using CO₂.

Behavioural analysis.

Larval path length testing was used to assess changes in general larval locomotion in jar^{322}/Df(3R)crb87-5 MN and OreR control larvae. The protocol was performed as described by Pereira et al. (1995). Individual third-instar larvae (96 ± 2 h posthatching) were placed onto a layer of yeast paste in a circular well. Each well was covered with a Petri-dish lid and after 5 minutes, the paths traveled by the larvae were traced onto the Petri lids. These path lengths were used to calculate the mean path length in ImageJ for the mutant and control larvae, which were tested concurrently. Similarly, path length was tested on a non-nutritive substrate, 0.4% agar. Total distance traveled on agar was measured by placing individual third-instar larvae (96 ± 2 h posthatching) in the center of agar-coated Petri-dishes, which were then covered with the Petri-dish lids. After 5 minutes, the path traveled by the larvae was traced onto the Petri lids and quantified as described above.

Electrophysiology.

Wandering third-instar larvae were dissected in HL3 saline to which calcium was added for a final concentration of 1 mM. Intracellular electrophysiological recordings for muscle 6 or 7 of segments A3 to A5 were taken using a reference electrode filled with 3M KCl. An AxoClamp
2B amplifier (Axon Instruments) was used to make recordings. Input offset was adjusted until the potential across the reference electrode was zero. All muscles selected for analysis had initial resting potentials between −60 and −75 mV. Baseline synaptic transmission was characterized by recording mEJPs for 1 to 2 minutes and 16 EJPs stimulated at 1 Hz. Muscle resistance was recorded for both protocols to ensure it was within normal range, 5 to 10 mΩ (Sullivan W, Ashburner M, Hawley RS, 2000).

For paired-pulse facilitation experiments, larvae were bathed in either 0.5 mM Ca\(^{2+}\) HL3 or 1 mM Ca\(^{2+}\) HL3. The nerve innervating muscle 6 and 7 was then stimulated by two pulses 20 ms apart. This was repeated every 10 s for a total of 16 cycles. As with basal recordings, the evoked potentials were recorded intracellularly from muscle 6 or 7 of segments A3 to A5 using a reference electrode filled with 3M KCl. To determine the amount of EJP amplitude increase that occurred following the second pulse, a ratio of the second EJP amplitude to the first was calculated and an average of the 16 cycles was taken for further statistical analysis.

For high-frequency stimulation, 16 EJPs at 1 Hz were recorded, followed by 10 Hz stimulation for 10 minutes, and concluded with 0.1 Hz stimulation for 10 minutes. The protocol was performed in both 1 mM and 10 mM calcium saline. The 10mM calcium solution was a modified HL3 made with the following ingredients (in mM): NaCl 70, KCl 5, MgCl\(_2\) 10, NaHCO\(_3\) 10, CaCl\(_2\) 10, sucrose 115, trehelose 5, HEPES 5 (pH 7.2) (Dickman et al., 2005). Recordings in which one or both of the motor neurons that innervate muscles 6 and 7 failed were excluded from analysis. This can be seen as two distinct populations of EJPs with different amplitudes, rather than a single EJP amplitude observed when both motor neurons are successfully recruited together. In all experiments, a maximum of two recordings were used per larvae and a minimum of 8 larvae were used.
Analysis of electrophysiological recordings was performed in Clampfit 10.0 (Molecular Devices). To calculate mEJP amplitude and frequency, the template search option was used followed by manual inspection to remove any doublet mEJPs or background noise that was included in the measurement. The same template was used for all mEJP recordings across all genotypes. Maximum EJP amplitudes for basal recordings, paired-pulse experiments and high-frequency stimulations were also measured in Clampfit 10.0 and exported for further analysis in Graphpad Prism 4.

Antibody Staining and Confocal Microscopy.

Third-instar larvae were dissected in HL3 saline and fixed in 4% EM grade formaldehyde in phosphate buffered saline (PBS) for 1 hour. Following transfer to an Eppendorf tube with 5 to 7 other larvae, the larval fillets were blocked with Normal Goat Serum and washed with 10% PBS Tween®20 (BIORAD) for 1 hour, 3 times at 20 minutes each. To label active zones at Drosophila boutons, larvae were stained with 1:20 mouse monoclonal nc82 (Bruchpilot) antibody, followed by the secondary antibody, FITC goat anti-mouse IgG, at a 1:1000 dilution. In between primary and secondary antibody staining, larvae were washed again with 10% PBS Tween®20 (BIORAD) for 1 hour, 3 times at 20 minutes each. Likewise, at the end of staining, the washing protocol was conducted a final time. Larval fillets were then mounted onto slides in Vectashield® Mounting Medium for Fluorescence (Vector Laboratories).

Images were obtained using a LSM510 (Carl Zeiss) confocal laser microscope with a 200 mW Argon laser. Individual boutons were imaged using a 40×/NA 1.4 oil immersion lens under constant imaging parameters, including gain, zoom, pinhole size and laser output, to allow for proper comparative analysis. For analysis of Bruchpilot staining, quantification of Bruchpilot puncta was performed in Image J for all Ib boutons of an NMJ on muscles 6/7, with a maximum of two NMJs used per larvae.
Statistic Analysis.

All statistical analyses were performed in GraphPad Prism Software 5. A significance level of $p<0.05$ was used for all experiments.
Materials and Methods for Chapter 4

Fly Strains and Genetics.

The Myosin VI loss of function Drosophila stocks used for the FM dye and FRAP, experiments have been described in detail in the Materials and Methods for Chapter 2. Briefly, the Myosin VI loss of function alleles used in this study were jar$^{322}$ and Df(3R)crb87-5 (maintained as stocks over Tm3, Sb Ser GFP). OreR flies served as wild-type controls. During handling, flies were temporarily anesthetized using CO$_2$. To visualize Synaptotagmin dynamics in vivo, control elav$^{C155}$Gal4; UAS-Synaptotagmin-GFP flies were used and were crossed to jar mutant genotypes. elav$^{C155}$Gal4; UAS-Synaptotagmin-GFP stocks (from herein abbreviated to C155SytGFP) contain the nervous system Gal4 driver, elav$^{C155}$Gal4, and a UAS-Synaptotagmin-GFP transgene in the same fly (Brand and Perrimon, 1993).

FM Dye Labeling.

To visualize FM dye uptake during electrophysiological stimulation, experiments were performed on a rig equipped with a fluorescent stereomicroscope. Wandering third-instar larvae were dissected in HL3 solution on Sylgard lined glass dissections plates. Larvae were glued to plates using a topical tissue adhesive (GLUture, Abbott Laboratories). Intracellular recordings were taken from muscles 6 and 7 using a reference electrode eluted with a 3M solution of KCl. An AxoClamp 2B amplifier (Axon Instruments) was used to make recordings. All muscles used for analysis had a resting potential of at least -50 mV. To ensure release of the reserve pool of vesicles, high-frequency stimulation was performed at 10Hz for 10 minutes. During the first stimulation protocol, larval fillets were bathed in HL3 solution with 1.5 mM Ca$^{2+}$ containing the FM1-43 dye at a concentration of 4µM. During the second stimulation protocol, larval fillets were bathed in 2 mM Ca$^{2+}$ containing with the FM1-43 dye at a concentration of 10 µM. FM dye
labeling at NMJ 6/7 of third-instar larvae was imaged using a using a 60× water immersion lens on a Nikon E600FN fluorescent microscope fitted with a Hamamatsu Orca CCD camera. The microscope filter was set for GFP emission and excitation.

**Fluorescence Recovery after Photobleaching.**

Experiments were performed on third-instar larval fillets glued to Sylgard-coated glass slides in HL3 saline within 2 hours from the time of dissection. Larvae were glued to plates using a topical tissue adhesive (GLUture, Abbott Laboratories). Recordings of vesicle dynamics were taken from type Ib boutons on NMJ 6/7, with a maximum of 6 boutons from the same larvae used. For the first FRAP protocol, a total of 60 images were taken over a period of 2 minutes, with a 1 second delay between image acquisition. Images were captured as 512 × 512-pixel frames with the 488-nm line of the argon laser on an LSM510 (Carl Zeiss) confocal laser microscope. These images consisted of four baseline images taken prior to bleaching at 5% of full laser power, followed by the fifth scan consisting of 9 rapid iterations of bleaching at 97% of full laser power in the selected region of interest (ROI). The remaining 56 scans were then completed at 5% of full laser power. Another fast FRAP protocol was performed to capture additional data points for fluorescence recovery immediately following bleaching. In this protocol, a total of 45 images were taken over a period of 45 seconds with no delay between image acquisition. Images were captured as 256× 256-pixel frames with the 488-nm line of the argon laser on an LSM510 (Carl Zeiss) confocal laser microscope. Again, these images consisted of four baseline images taken prior to bleaching at 5% of full laser power, followed by the fifth scan consisting of 12 rapid iterations of bleaching at 97% of full laser power in the selected region of interest (ROI). The remaining 41 scans were then completed at 5% of full laser power. Constant imaging parameters were maintained between samples and the size of the ROI was kept constant at 24 x 30 pixels in both FRAP protocols.
Fluorescence intensity was measured in Image J for the bleached region, the background and the total bouton area using the Time Series Analyzer plug-in. To account for photobleaching during the experiment and the movement of bleached molecules, recovery curves were generated following double normalization of the raw fluorescence intensity (Phair et al., 2004). Average fluorescence intensity was measured at each time point during imaging for the bleached region (ROI), the whole bouton (WB) and the a region outside the bouton (BG). First, background fluorescence lost during imaging was corrected for. Fluorescence signal lost during intentional photobleaching of the ROI cannot be recovered to prebleach levels as a portion of the cellular fluorescence has been permanently removed. Thus, loss of fluorescence due to bleaching in the ROI was also measured to account for the movement of bleached molecules out of the ROI. This double normalization makes it possible to assess changes to the fluorescence signal within the bleached region itself. This calculation is summarized in the following equation:

**Equation 1. Double normalization of FRAP data.**

\[
\frac{(WB_{\text{prebleach}} - BG) (ROI_{\text{time}} - BG)}{(WB_{\text{time}} - BG) (ROI_{\text{prebleach}} - BG)}
\]

Nonlinear regression was performed to conduct curve fitting analysis on the normalized FRAP data. Differences between recovery curves were assessed by comparing the Goodness of Fit for one phase association and two phase association curves with the data.

**Statistic Analysis.**

All statistical analyses were performed in GraphPad Prism Software 5. A significance level of p<0.05 was used for all experiments.
References


Seabrooke S, Stewart BA (2011) Synaptic transmission and plasticity are modulated by nonmuscle myosin II at the neuromuscular junction of Drosophila. J Neurophysiol.


