Cytoskeletal Regulation and Morphogen Signaling During Synaptic Outgrowth at the *Drosophila* Larval Neuromuscular Junction: A Dissertation

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CYTOSKELETAL REGULATION AND MORPHOGEN SIGNALING DURING SYNAPTIC OUTGROWTH AT THE DROSOPHILA LARVAL NEUROMUSCULAR JUNCTION

A Dissertation Presented

By

PREETHI RAMACHANDRAN

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester

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Neuroscience
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This body of work would not have been possible without the contributions of many people who have provided immense help and support during various stages of my doctoral career. First and foremost, I would like to thank my advisor, Dr. Vivian Budnik whose continual guidance and mentoring helped me in successfully completing my doctoral program. I also thank all the current and former members of my lab including Dr. James Ashley, Dr. Sean Speese, Dr. Kate Koles, Ceren Korkut, Romina Barria, Yuly Fuentes, Alex Koon, Dr. Bulent Ataman, Dawn Merdaa, and Norberto Gherbesi for many stimulating scientific discussions and for their valuable and timely help with my project. I would also like to express my gratitude to my committee members Dr. Marc Freeman, Dr. Scott Waddell, Dr. Stephen Doxsey, Dr. Patrick Emery, Dr. Michele Jacob, Dr. Tzumin Lee, and Dr. Zheng-Zheng Bao for taking the time out of their busy schedules to attend my TRAC meetings and provide me with critical input about my work and my performance. Additionally, I thank the entire neurobiology department for the cordial and highly stimulating scientific environment and the administrative staff for making the process really smooth and tireless. Last but not the least, I would like to express deep gratitude to my husband Suresh Balasubramanian and my parents Indra Ramachandran and M. Ramachandran, without whose support and encouragement, I would have not been able to pursue this endeavor.
ABSTRACT

Synaptic plasticity, in its broadest sense, can be defined as the ability of synapses to be modified structurally and functionally in response to various internal and external factors. Growing evidence has established that at the very core of these modifications are alterations in the cytoskeletal architecture. This discovery has led to the unearthing of a number of signaling pathways that might be involved in cytoskeletal regulation and also in the regulation of other aspects of synapse development and plasticity. In this regard, polarity proteins and secreted morphogens such as the Wnt proteins, typically involved in embryonic development, are emerging as critical determinants of synaptic growth and plasticity. However, their mechanism of action at synapses needs further investigation. Additionally, not much is known about how these morphogens are secreted or transported across synapses. Using the Drosophila larval NMJ as a model system, I have addressed aspects related to the issues mentioned above in the subsequent body of work. In the first half of my thesis, I have uncovered a role for the aPKC/Baz/Par-6 polarity protein complex in the regulation of the postsynaptic actin cytoskeleton in conjunction with the lipid and protein phosphatase PTEN. In the second half of my thesis, I have contributed to the elucidation of mechanisms underlying the secretion of Wg, the Drosophila Wnt homolog. Our findings suggest that Wnts might be secreted via a previously unidentified mechanism involving the release of exosome like vesicles from the presynapse and this process requires Evi/Wntless (Evi), a protein dedicated to
Wnt secretion. Alterations in signaling pathways and aberrant cytoskeletal regulation lead to a variety of neurological disorders. The body of work in this thesis will provide a deeper understanding of the mechanisms involved in synaptic plasticity and provide a basis for uncovering similar pathways in the context of vertebrate synapses.
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FasII- Fasiclin II (Cell Adhesion Molecule)

EJP- Evoked Junctional Potential – The postsynaptic response obtained by the release of the contents of numerous vesicles, normally triggered by an action potential. It depends on the number of vesicles released from the presynaptic cell as well as the amount of receptors present on the postsynaptic side.

mEJP- mini Evoked Junctional Potential – The postsynaptic response observed upon the binding of individual quanta (neurotransmitter obtained from the spontaneous fusion of a single synaptic vesicle). Considering that the amount of neurotransmitter loaded per vesicle is constant, changes in mEJP usually represent changes either in the amount or kinetics of the postsynaptic glutamate receptors at the Drosophila NMJ.

mini Frequency – The number of synaptic vesicles spontaneously released per second.

GAL4/UAS – Originally identified in yeast, Gal 4 is a transcriptional activator that binds to the UAS (Upstream Activating Sequence), a short section of the promoter region of a gene, and activates transcription. In Drosophila, the Gal4/UAS system is used for tissue specific gene expression. The GAL4 gene is placed under the control of a driver gene, while the UAS controls expression of a target gene. GAL4 is then only expressed in cells or tissues where the driver gene is usually active activates target gene transcription by binding to the UAS region only in those cells or tissues.

GAL80 – Transcriptional repressor of Gal4. Provides additional level of control of gene expression in Drosophila by allowing for more precise spatial and temporal regulation of gene expression.

LexA/lexAop- A mechanism similar to the Gal4/UAS system where LexA binds to lexAop region of a gene and regulates transcription. Used in parallel with the Gal4/UAS system to obtain differential gene expression in different tissues.
PREFACE

The figures presented in Chapter 1 (Introduction) have been included to provide a clear understanding of the larval NMJ model system and the organization of the cytoskeleton at the larval NMJ. These figures have been adapted from the following published works:


In all cases, the corresponding author of the papers, Dr. Vivian Budnik has consented to allow the use of the respective figures in the ‘Introduction’ section of my thesis.

The research work presented in Chapter 2 titled “A Critical Step for Postsynaptic F-actin Organization: Regulation of Baz/Par-3 Localization by aPKC and PTEN” is a reprint from the article published in the journal ‘Developmental Neurobiology’, Volume 69, Issue 9. I am the first author of the paper and was responsible for all the experimental work involving specimen preparation, immunocytochemistry, confocal imaging, and quantification for all the Figures. I also designed and generated all the mutant PTEN constructs required for the Western Blots in Fig 2.6. I also generated antibodies against PTEN and was involved in testing and optimizing antibodies against Phospho Baz. The Western Blots in Fig. 2.3 and Fig 2.6 were performed by Romina Barria and the quantification of the Westerns in Suppl. Fig.2.7 was done by Dr. Vivian Budnik. The electrophysiology experiments in Fig. 2.8 were performed by Dr. James Ashley and the EM experiments and analysis in Suppl. Fig. 2.2A was
done by Norberto Gherbesi and Dr. Vivian Budnik. The text was a collaborative effort between Dr. James Ashley, Dr. Vivian Budnik and me.

The research work presented in Chapter 3 titled “Trans-Synaptic Transfer of Wnt Signals through Release of Evi/Wntless Vesicles and Trafficking of Postsynaptic Frizzled-2 Receptors” is currently in press for publication in the journal, ‘Cell’. I am the third author while the first authorship is shared by Ceren Korkut and Bulent Ataman. Bulent Ataman initiated the project which was taken over by Ceren Korkut. My contribution to this study includes intellectual input and experimental work such as dissection of larval NMJ preparations and brain tissues, immunocytochemistry, confocal imaging, quantification and analysis of data for many of the figures. Additionally, I was responsible for parts of the experiments done using S2 cells. Biochemical and RT-PCR experiments in Fig. 3.1, Fig. 3.4 and Supp. Fig 3.5 were carried out by Romina Barria and live imaging experiments were carried out by Dr. James Ashley. EM experiments in Fig. 3.5 were done by Norberto Gherbesi and Dr. Vivian Budnik. The body of text in this study is a collaborative effort between Ceren Korkut, Dr. Bulent Ataman, Dr. James Ashley, me and Dr. Vivian Budnik.
CHAPTER I

General Introduction
Much of our understanding about the brain comes from the pioneering work of Ramoni Y Cajal and his contemporaries, done in the late 19th and early 20th century. However, the idea that really brought research in neuroscience to the forefront was the notion that the brain was not a continuous web of material, but was composed of individual cells or neurons that communicated with each other through specialized junctions or spaces between them. This hypothesis was proposed by Santiago Ramon y Cajal, which came to be known as the ‘Neuron Doctrine’ (Cajal, 1906; DeFelipe, 2002) This idea of neuronal communication through specialized junctions led to the coining of the term ‘synapse’ in 1897 by Sherrington (Pearce, 2004). Some elegant experiments performed by Otto Loewi and Sir Henry Dale along with the discovery of acetylcholine in the early 1900s, established that the communication between neurons occurred mainly through chemical transmission (Loewi, 1921; Dale, 1938; Fishman, 1972; Zimmer, 2006). A major concept that revolutionized research in the field of neuroscience was the idea that synapses are dynamic and undergo changes in response to activity and that this plastic behavior of synapses may underlie the process of learning and memory. This plastic behavior of synapses as we know today encompasses many different aspects including changes in synaptic function, synaptic structure and formation of new synapses (Engert and Bonhoeffer, 1999; Harris et al., 2003; Matsuzaki et al., 2004). Additionally, current evidence has established that all these changes are tightly controlled by various signaling pathways and rely to a great degree on
precise cytoskeletal modifications (Schubert and Dotti, 2007; Cingolani and Goda, 2008). This idea was in its nascent stage during Cajal’s time and did not achieve prominence until 1949, where in an attempt to correlate behavior and mind with changes in the physiological and biological make up of the brain, Donald O. Hebb in his book *The Organization of Behavior*, postulated that “when axon of cell A is near enough to excite a cell B and repeatedly and persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency as one of the cells firing B is increased” known as the Hebbian rule often paraphrased as ‘neurons that fire together wire together’ (Hebb, 1949; Brown and Milner, 2003). Hebb’s postulate not only led to the emergence of synaptic plasticity as a feasible concept for explaining the mechanisms underlying learning and memory, but also aided in understanding how neural networks were formed and how they regulated behavior.

Perhaps the most striking evidence supporting the Hebbian model was obtained in 1973 by Bliss and Lomo with the discovery of the phenomenon of long term potentiation (LTP) (Bliss and Lomo, 1973; Lomo, 2003). In brief, multiple trains of high frequency tetanic stimulation, when applied to afferent neurons that projected to different parts of the hippocampus, resulted in an enhancement of the excitatory postsynaptic potential and reduced the threshold for eliciting an action potential that lasted for a few hours. This phenomenon was called LTP (Bliss and Lomo, 1973; Lomo, 2003). Subsequent studies identified the NMDA
receptor (N-Methyl-D-Aspartate) as being required for LTP where it was shown to function as a ‘coincidence detector’ requiring the simultaneous depolarization of the postsynaptic cell coupled with the binding of glutamate released by the presynaptic cell to open, thereby meeting the conditions of the Hebbian synapse (Bourne and Nicoll, 1993). On the other hand, repeated low frequency afferent stimulation resulted in the opposite effect characterized by a long lasting decrease in synaptic efficacy, termed long term depression (LTD) (Ito and Kano, 1982). Since then studies have demonstrated that both LTP and LTD are associated with functional as well as structural changes at the synapse. While functional changes include changes in presynaptic neurotransmitter release or a modification in postsynaptic receptor clustering or a composition of the clusters, structural changes include changes in the size and shape of the synaptic terminals and the formation of new synaptic connections (Lang et al., 2004; Leenders and Sheng, 2005; Kessels and Malinow, 2009). Collectively, the results obtained from these studies have lent a great deal of support to Hebb’s model.

Subsequent studies have identified that integral to these functional and structural changes are controlled modifications of the cytoskeletal architecture. For example, both an increase and decrease in dendritic spine size during LTP or LTD is associated with a change in actin organization (Okamoto et al., 2004; Dillon and Goda, 2005). Additionally, a gradually increasing number of signaling pathways are being identified that mediate these synaptic changes either by regulating the cytoskeleton or via other mechanisms (Schubert and Dotti, 2007;
Bramham, 2008). In this context, polarity proteins and morphogens such as Wnts, which play critical roles in tissue patterning during embryonic development, are beginning to emerge as critical determinants of synaptic plasticity and development (Drier et al., 2002; Pastalkova et al., 2006; Speese and Budnik, 2007).

However, how appropriately these models reflect long term changes that occur during learning and memory is still an open question. Most of these models involve artificial stimulation of the afferent neurons in hippocampal slices. Furthermore, the response is obtained from a population of neurons which precludes scientists from detecting definite changes occurring in the cytoskeleton or studying the effects of these signaling mechanisms on synaptic structure and function at the level of single synapses. Additionally, some studies looking at the performance of rats and mice in learning and memory tasks, find that performance results in memory tasks do not necessarily correlate with the occurrence of LTP or LTD demonstrating the need to monitor these changes in the context of an intact organism (Holscher, 1999; Lynch, 2004). All this has led scientists to interpret these results with some caution. Nevertheless, models of LTP and LTD have proven to be extremely valuable in understanding mechanisms of how neural networks are formed and for uncovering the molecular pathways involved in the functioning of these networks.

To overcome the problems imposed by the complexity of the mammalian brain and in an attempt to validate their findings in other systems, scientists have
turned to simpler model systems such as *Aplysia* and *Drosophila* to study long term plasticity (Heisenberg et al., 1985; de Belle and Heisenberg, 1994; Pittenger and Kandel, 2003). While in *Aplysia*, one of the models used to study long term plasticity includes the neural circuitry involved in the sensitization of the gill and siphon withdrawal reflex (Buonomano and Byrne, 1990; Antonov et al., 2003; Pittenger and Kandel, 2003), in *Drosophila* it includes the mushroom body neurons which have been implicated in olfactory memory (Heisenberg et al., 1985; de Belle and Heisenberg, 1994). In these model systems, the ability to map behavior to synaptic changes coupled with easy accessibility to single synapses have validated the use of these model systems in understanding molecular pathways underlying long term plasticity. Indeed, studies using these model systems have demonstrated a remarkable degree of conservation of molecular mechanisms and pathways between these models and mammalian synapses. For example, studies have revealed that signaling proteins belonging to the MAPkinase (Mitogen Activated Protein Kinase) and the CREB (Cyclic AMP Response Element Binding) pathway, critical for LTP in vertebrates, have corresponding homologs in *Aplysia* which have similar functions and are involved in similar steps of LTP formation as seen in vertebrates (Pittenger and Kandel, 2003). Such findings provide greater validity and importance to the results obtained from the mammalian systems.

**The Drosophila Larval NMJ**
Growing evidence suggests that a number of molecules involved in learning based plasticity also function earlier in development, especially during synaptogenesis. For example, molecules such as BDNF (Brain Derived Neurotrophic Factor) and Wnts have been shown to be required for coordinated pre and postsynaptic assembly during the initial stages of synaptic development and progressively for activity dependent modulation of synaptic structure and function during LTP formation (Wang et al., 1995; Korte et al., 1995; Packard et al., 2002; Chen et al., 2006; Speese and Budnik, 2007; Ataman et al., 2008). These findings suggest that similar signaling pathways might be involved in both processes and underscores the need to understand how synapses are assembled and the changes they undergo during the course of development. At the very core of studies involving changes occurring during developmental plasticity, are those that have been done in the *Drosophila* larval neuromuscular junction (NMJ). This system was pioneered in the mid to late 1970s by Y.N. Jan and L.Y. Jan who first described the basic physiological and pharmacological properties of the larval NMJ (Jan and Jan, 1976). Subsequent studies demonstrated that similar to vertebrate synapses, the larval NMJ responds to and can be modified by activity (Budnik et al., 1990; Zhong et al., 1992).

Several reasons exist why researchers, to this date, have continued to exploit this system for studying synaptic development and plasticity. First, the larval NMJ shares a great deal of similarities with the synapses of the vertebrate central nervous system allowing for extrapolation of observations from this
system to the vertebrate system. For example, both the larval NMJ as well as the synapses of the vertebrate central nervous system are glutamatergic i.e. glutamate is used as the main neurotransmitter (Jan and Jan, 1976). More importantly, just like vertebrate synapses, the larval NMJ is highly plastic and is capable of undergoing structural and functional changes in response to activity making it a great system to study both developmental as well as activity dependent plasticity (Budnik et al., 1990; Zhong et al., 1992). Additionally, a great deal of conservation exists between the two systems. Many of the vertebrate synaptic proteins have corresponding Drosophila homologs and in most instances both of them have been shown to have similar functions. For example, scaffolding proteins such as the vertebrate PSD-95 (Postsynaptic Density-95/Discs-Large/Zona Occludens) and its Drosophila homolog, Dlg (Discs Large), are both present at the postsynaptic region have been implicated in the assembly of the postsynaptic apparatus and the recruitment of ion channels such as the Shaker-type K+ channels (Kim et al., 1995; Tejedor et al., 1997). Second, the larval NMJ consists of large, easily accessible synapses made by individual neurons with their respective muscles in a highly stereotypic fashion (Halpern et al., 1991; Sink and Whittington, 1991; Landgraf et al., 1997; Landgraf and Thor, 2006; Prokop, 2006). This allows for observing structural and functional changes in the same synapse in multiple larval preparations. This is unlike the vertebrate nervous system where individual neurons form billions of tiny synaptic connections making it difficult to study changes occurring at a single synapse.
Further compounding this problem is the fact that the sheer number of neurons in the vertebrate nervous system makes it difficult to follow the same neuron or the same sets of neurons in multiple samples. Third, the ability to manipulate the system genetically, pharmacologically, as well as physiologically enables most of the experiments to be done in vivo. Since the *Drosophila* genome has been sequenced, the sequence of virtually every gene is available making it easy to generate mutants and design RNAi or transgenic constructs to manipulate protein levels in vivo (Fjose et al., 2001). Furthermore, the ability to spatially and temporally regulate protein levels using the GAL4/UAS (Brand and Perrimon, 1993), the LexA/lexAop (Lai and Lee, 2006) or the GAL80 (McGuire et al., 2004) systems helps in bypassing the early developmental effects or pleiotropic effects of the respective genetic mutants. In addition to the ease of manipulation, the ability to express fluorophore tagged synaptic proteins and perform live imaging experiments allows us to observe changes in synaptic structure through the cuticle, over a considerable time span during larval development, therefore allowing the NMJ to be used for large scale mutant screens (Parnas et al., 2001). Lastly, the fruit fly larval NMJ is becoming an attractive model for studying neurological diseases (Trotta et al., 2004; Lee et al., 2009). Many of the genes responsible for causing neurodegenerative diseases have homologs in *Drosophila* which when mutated recapitulate many behavioral phenotypes seen in their vertebrate counterparts validating its use as a disease model. For example it is known that mutations in genes such as *spastin* and *atlastin* in
vertebrates result in Hereditary Spastic Paraplegia characterized by progressive weakness and spasticity of the lower extremities. Along the same lines, flies that are mutant for the spastin gene show dramatic weakness in their hind legs and are unable to walk or fly (Sherwood et al., 2004; Trotta et al., 2004). Further investigation revealed that, in spastin and atlastin null flies, NMJ growth and transmission was significantly affected along with impaired MT dynamics (Sherwood et al., 2004; Trotta et al., 2004; Lee et al., 2009). Likewise, similar behavioral and cellular phenotypes are seen in mammals and flies lacking the FMRP protein (Fragile X mental retardation protein), a protein that binds mRNA and functions as a translational repressor (Siomi et al., 1993; Zalfa and Bagni, 2004). In mammals, the behavioral deficits include impaired visuospatial perception and defective motor control while in flies, it is observed as decreased photoreceptor function and an inability to walk or fly. Similarly, cellular deficits in mammals include abnormal neuronal dendritic morphology whereas in flies it includes abnormal NMJ morphology and defective NMJ outgrowth (Zhang et al., 2001; Zalfa and Bagni, 2004). More importantly, the larval NMJ has been used to study the interactions of these genes and identify signaling pathways that might be disrupted in these mutants. For example, further investigation in flies revealed that these proteins exert their effect by directly or indirectly regulating MT dynamics (Zhang et al., 2001; Sherwood et al., 2004; Trotta et al., 2004; Lee et al., 2009).
In my thesis, using the NMJ as my model system, I have investigated the role of polarity proteins in cytoskeletal regulation. Additionally, I have also used the larval NMJ to address questions related to morphogen, in particular Wnt secretion and transport across cells.

**General Anatomy of the Drosophila NMJ.**

Anatomically, a dissected third instar larval NMJ preparation consists of 7 abdominal segments (A1-A7) with each hemisegment composed of 30 skeletal, supercontractile body wall muscles. These muscles, which are easily identifiable based on their location and sites of insertion, are arranged in a highly stereotypic pattern that is repeated in each abdominal segment (Figure 1.1) and are innervated by approximately 36 motoneurons per hemisegment (Landgraf, et al., 1997; Landgraf and Thor, 2006). The motoneurons, whose cell bodies are located in the ventral ganglion, send out axons, which after exiting the CNS navigate and branch all over the body wall musculature in a highly stereotypic fashion (Halpern et al., 1991; Sink and Whittington, 1991). Each motor neuron innervates specific muscle cells with high fidelity and demonstrates a characteristic branching pattern that ends in highly specialized presynaptic terminals (Figure 1.2 A, B). These terminals are arranged in the form of a string of varicosities or synaptic boutons separated by thin stalks or neurites giving it the classic ‘beads on a string’ appearance (Figure 1.2 B) (Landraf, et al., 1997; Yoshihara, et al., 1997; Prokop, 2006)
NMJ growth in larvae is a tightly orchestrated process that depends on the interplay of signals between the muscle and the nerve terminal. By the end of embryogenesis, motor neurons have initiated contact with the muscle at the potential site of synapse formation. With the differentiation of the motor neuron growth cones into synaptic boutons and the initiation of synaptic transmission in these newly formed boutons NMJ formation is virtually complete by the end of embryogenesis (Yoshihara et al., 1997). Subsequently, the period of growth between the first and the third instar larval stage is characterized by a remarkable increase in the size of the NMJ which occurs in response to the needs of a rapidly growing body wall musculature. During this period, body wall muscles undergo a 100 fold increase in size (Figure 1.1). Consequently, to maintain synaptic efficacy and provide adequate synaptic input for normal muscle contraction, there is a concomitant and synchronous expansion of the NMJ (Figure 1.2 B) (Prokop, 2006). This expansion occurs mainly by increasing the size of the boutons, the number of branches and by addition of new boutons to a growing branch which can occur via bouton budding where an existing bouton gives out a new bud that matures into a bouton, bouton division where an existing bouton divided into two or de novo formation of boutons (Zito et al., 1999).

Synaptic boutons can be of varying sizes, have different postsynaptic specializations and contain different neurotransmitters depending on the neuron from which they originate. Based on their sizes and the characteristics of their
neurotransmitter content, they have been classified into three types (Figure 1.2 C) (Anderson et al., 1988; Johansen et al., 1989; Atwood et al., 1993; Gorczyca et al., 1993; Jia et al., 1993; Monastirioti et al., 1995):

**Type I boutons**

The great majority of body wall muscle cells receive innervations from at least two motor neurons that release glutamate as the primary excitatory neurotransmitter, the so-called type I big (type-Ib) and type I small (type-Is) (Figure 1.2C; Figure 1.3), Atwood et al., 1993; Jia et al., 1993; Prokop, 2006). These boutons serve as the primary source of stimulus input for rapid muscle contraction during larval locomotion (Prokop, 2006).

**Ultrastructure and morphology of Type I boutons**

Type I boutons typically have a rounded shape, and in addition to a few mitochondria and endosomes, these boutons contain a large number of clear, rounded neurotransmitter vesicles of around 30-40 nm diameter carrying glutamate (marked ‘v’ in Fig 1.3A), occupying most of the bouton volume (Atwood et al., 1993; Jia et al., 1993). These vesicles increase dramatically in number during larval development forming the readily releasable and reserve pools (Kuromi and Kidokoro, 2003) and are derived from a combination of axonal transport of vesicle precursors and vesicle recycling via endocytosis.
The presynaptic membrane is composed of specialized domains called active zones which are site of neurotransmitter release and can be distinguished from the adjacent perisynaptic membrane by their denser appearance and the presence of electron dense structures referred to as T-bars (marked as 'az' in Figure 1.3B) (Atwood et al., 1993; Jia et al., 1993). T-bars are dense bodies that are anchored to the presynaptic membrane by a pedestal surmounted by a platform (Atwood et al., 1993; Jia et al., 1993) and are surrounded by a higher density of synaptic vesicles that usually fuse to the plasma membrane beneath the T-bars (Jia et al., 1993; Prokop, 1999). They are thought to play a role in tethering vesicles for release as multiunit packages (Prokop and Meinertzhagen, 2006). Furthermore, their association with clusters of calcium channels suggests that they may play a role in inducing local high Ca++ microdomains which are required for synaptic release (Kawasaki et al., 2004; Prokop and Meinertzhagen 2006). Unlike the vertebrate active zone components, not much is known about proteins that make up the T-bar. A recent study demonstrated that Nc82/Bruchpilot, a homologue of CAST/ELKS, is contained in these structures and is required for their structural integrity (Wagh et al., 2006).

The presynaptic membrane is separated from the postsynaptic membrane by a 15-20 nm wide synaptic cleft (Jia et al., 1993). The postsynaptic membrane houses cell adhesion molecules, glutamate receptor complexes situated in direct opposition to the active zones and many other
transmembrane protein complexes. Initially, at the onset of the first instar larval stage, the Type I boutons lie in a shallow groove formed on the muscle surface. As concurrent muscle and synaptic growth occurs and the new boutons penetrate deeper into the muscle, the muscle membrane surrounding the bouton evolves into a highly complex and convoluted system of membranes that completely envelops the bouton. This complex system of folded membranes is known as the subsynaptic reticulum (SSR) (Figure 1.3A, C). The presence of the SSR is a common feature of both the Type Ib and Type Is boutons although the SSR surrounding Type Ib is more voluminous and densely packed than Type Is boutons (Atwood et al., 1993; Jia et al., 1993). Although the exact function of the SSR is not known, it houses many important signaling molecules, cytoskeletal proteins and scaffolding proteins such as Dlg, the Drosophila homolog of PSD-95 (Budnik et al., 1996) (Figure 1.2C). Furthermore, some studies have found evidence of polysomal profiles at the SSR and have identified proteins involved in translational control within the SSR suggesting that it may also be a seat of subsynaptic protein synthesis (Sigrist et al., 2000; Schuster, 2006).

**Type II and Type III boutons**

In addition to type I boutons, two other bouton types have been identified. Type II endings contain small synaptic boutons, but their branches are much longer than those of type I, usually running through the length of the
muscle fibers (Figure 1.2C) (Johansen et al., 1989). These boutons contain both glutamate and octopamine (Johansen et al., 1989; Monastirioti et al., 1995), but their exact function is still poorly understood. Unlike the great majority of motorneurons that give rise to Type I terminals, all Type II terminals arise from just three unpaired motor neurons that lie in the ventral midline of the ventral ganglion. Type II terminals innervate all but 8 muscles in the body wall with each motor neuron having multiple targets (Monastirioti et al., 1995). In contrast to type I boutons that are likely to mediate rapid excitation-contraction, octopamine-containing type II terminals are postulated be more modulatory in nature. Type III boutons are less defined subsets of motor endings containing peptides, which innervate specific muscles in a segment specific fashion (Cantera and Nassel, 1992; Gorczyca et al., 1993). The type III endings in muscle 12, for example, contain an insulin-like peptide, and innervate muscle 12 at abdominal segments 2-5. These endings have elliptical boutons, with branches of intermediate length between type I and II branches (Figure 1.2C) (Gorczyca et al., 1993; Jia et al., 1993)).

**Ultrastructure of Type II and Type III boutons**

Type II and III terminals lack SSR and remain on grooves in the muscle surface covered only by the basement membrane (Jia et al., 1993). While Type II boutons contain a ‘mixed population’ of vesicles with both clear vesicles (similar to Type I boutons) and dense-core vesicles and have no clear synaptic
specialization sites or active zones, Type III boutons are filled mostly with discrete populations of spherical dense-core vesicles with varying size and degree of electron density. In addition to dense core vesicles, Type III boutons also have small clear vesicles which are exclusively concentrated at restricted sites of the presynaptic membrane surrounding putative synapses. In contrast to the synapses of Type I boutons, the synaptic release sites for clear vesicles in Type III boutons appear as darker thickenings of the presynaptic membrane surrounded by translucent vesicles (Jia et al., 1993).

Thus evidence related to the structural and functional changes occurring during larval development clearly demonstrate that the growth and expansion of the neuromuscular junction is a highly complex process that depends on a number of different mechanisms functioning in a coordinated manner. Among these mechanisms, two key events that are critical for normal synaptic growth are synchronized changes in the pre and postsynaptic cytoskeletal architecture and accurate spatiotemporal control of the anterograde and retrograde signaling mechanisms that orchestrate these changes.

**The Cytoskeleton**

At the very core of cell motility, growth, structure, and function are alterations in cytoskeletal architecture involving the actin and microtubule (MT) cytoskeletons. The ability of these cytoskeletal elements to take part in many different cellular processes can be attributed to the distinct mechanical properties and dynamics of their individual filaments that allows them to
undergo rapid changes in length and shape and assume complex arrangements. Both actin and MT filaments are composed of individual subunits that assemble in a head to tail fashion to generate linear arrays. While actin filaments are made up of repeats of monomeric G-actin (globular actin) subunits that give rise to two parallel protofilaments that twist around each other to form F-actin (filamentous actin), MT filaments consist of alpha and beta tubulin heterodimer subunits assembled into linear arrays which associate laterally to form a wide hollow cylinder (Alberts, 2002; Conde and Caceres, 2009; Lowery and Van Vactor, 2009). Both are polarized structures and have characteristic properties that are integral to their functions. Actin filaments are mainly characterized by their ability to undergo treadmilling where continual addition of subunits at the plus end and disassembly of subunits at the minus end allows for filament length to stay constant while ensuring that the individual subunits move forward (Alberts, 2002; Lowery and Van Vactor, 2009). On the other hand, MT filaments are characterized by their ability to undergo cycles of rapid growth and disassembly known as ‘dynamic instability’, thus transitioning between states of ‘catastrophe’ or rapid depolymerization and ‘rescue’ or rapid polymerization (Alberts, 2002; Conde and Caceres, 2009; Lowery and Van Vactor, 2009).

Superimposed on the inherent properties of these filaments is an additional step of regulation by many different proteins and signaling pathways. The actin cytoskeleton is regulated by actin-binding proteins (ABP) which
include proteins that promote nucleation and polymerization of monomeric actin and depolymerization or severing of F-actin (Paavileinen et al., 2004). They also encompass capping proteins that bind to filament ends and modify filament turnover, actin associated motors such as Myosin and crosslinking proteins that can generate distinct actin rearrangements (dos Remedios et al., 2003). These arrangements include filopodia which are finger like projections where actin filaments are bundled or lamellipodia which are sheet like extensions where actin filaments form branched networks (Alberts, 2002; Hall et al., 2005). Microtubule binding proteins (MBPs), on the other hand, include plus end and minus end motors which are involved in cellular transport, microtubule severing and destabilizing proteins, structural Microtubule Associated Proteins (MAPs) which confer stability on MT bundles and plus end tracking proteins that accumulate at the plus end of microtubules and control MT dynamics, growth directionality and link MT to actin associated proteins (Conde and Caceres, 2009). Key signaling complexes that regulate actin and microtubule binding proteins are RhoGTPases that regulate actin rearrangements and actin-MT binding and many different kinases and phosphatases that, by phosphorylating or dephosphorylating, regulate the binding affinity or activity of ABPs or MAPs ultimately resulting in a change in the behavior of actin/MT filaments (Allen et al., 1997; Hall et al., 2005; Schubert and Dotti, 2007; Conde and Caceres, 2009; Lowery and Van Vactor, 2009).
A great deal of our current knowledge about cytoskeletal dynamics and its regulation in the nervous system comes mainly from the study of growth cone behavior. At axon growth cones, actin filaments are contained at the leading edge where they are organized into filopodia or lamellipodia which are involved in exploring the substrate while MT filaments are arranged in the form of a stable bundle in the axon shaft and at the center of the growth cone (Tanaka, et al., 1991; Gungabissoon and Bamburg, 2003; Lowery and Van Vactor, 2009). However, many times single dynamic MT filaments extend out from the core of the axon into the peripheral actin rich region and penetrate into the filopodia, traveling along the actin bundles (Zhou and Cohan, 2004; Lowery and Van Vactor, 2009). Interactions between the actin and microtubule cytoskeleton via ABPs and MAPs allow signals propagated from the growth cone periphery to be transmitted to the dynamic microtubule filaments through the peripheral actin layer (Lee et al., 2004; Ng et al., 2004; Bouquet et al., 2007; Tsvetkov et al., 2007). Together, these mechanisms allow the cytoskeleton to steer the growth cone in a particular direction and therefore determine the course of axonal growth (Lowery and Van Vactor, 2009).

In contrast to growth cones, cytoskeletal changes occurring at synapses during synaptogenesis or synaptic plasticity have been less well understood. At vertebrate synapses, actin has been implicated in many different processes on both sides of the synapse (Dillon and Goda, 2005; Cingolani and Goda, 2008). Within the presynaptic terminal, actin is involved in the sequestration of
vesicles in the reserve pool (Greengard et al., 1994), recruitment of vesicles to the readily releasable pool (Sakaba et al., 2003) synaptic vesicle recycling (Shupliakov et al., 2002), and transfer of vesicles between boutons (Darcy, et al., 2006). Additionally, activity induced polymerization of actin has also been demonstrated to trigger the conversion of silent synapses to functional synapses by restructuring the presynaptic cytomatrix to organize vesicle clusters for efficient release (Shen et al., 2006; Yao et al., 2006). At the postsynapse, studies have identified heterogenous pools of actin that exhibit different structural arrangements and contribute to different postsynaptic mechanisms (Star et al., 2002; Cingolani and Goda 2008; Honkura et al., 2008). While the subplasmalemmal actin network is highly dynamic, has a role in anchoring receptors (Allison et al., 1998; Cingolani and Goda, 2008; Honkura et al., 2008) and has been proposed to be involved in remodeling the spine head during LTP (Fukazawa et al., 2003; Okamoto et al., 2004; Cingolani and Goda, 2008), longitudinal actin filaments that are present along the core of the spine provide stability to the spine and have been proposed to facilitate exo-endocytic trafficking of receptors to regulate the abundance of receptor sub-types (Cingolani and Goda, 2008; Honkura et al., 2008). However, many of these pre- and postsynaptic roles are highly dependent on the synapse type, the state of synaptic activity, giving rise to differing results between studies warranting more detailed analyses using a variety of experimental conditions (Zhang and Benson, 2001; Cingolani and Goda, 2008).
Evidence for the role of MTs and MT regulating proteins at the synapse in vertebrates has been precluded by the inability to detect their presence in dendritic spines. Initial studies demonstrated the presence of MTs and MT regulating proteins in dendrites, but not in dendritic spines (Kaech et al., 2001; Conde and Caceres, 2009). Only recently has the presence of MTs in the spines been acknowledged. Using live confocal imaging and TIRF (Total Internal Reflection Fluorescence) microscopy, recent studies have reported activity dependent invasion of microtubules into dendritic spines (Hu et al., 2008; Conde and Caceres, 2009). Further studies are needed to elucidate the relationship between microtubule dynamics and functional and structural synaptic plasticity in vertebrates.

*Cytoskeleton at the Drosophila NMJ*

Very few studies have demonstrated the presence of actin at the NMJ. Studies looking at the organization of the actin cytoskeleton at the NMJ have been challenged by the inability to label and visualize actin using phalloidin, a toxin that binds F-actin. This is because in addition to synaptic actin, phalloidin also labels the F-actin in the contractile apparatus. Compounded with the fact that the boutons are embedded into the muscle cortex and sit right atop the contractile fibers, this prevents proper visualization of the synaptic actin cytoskeleton. Thus the localization and distribution of actin binding proteins such as spectrin (Pielage et al., 2005; Pielage et al., 2006) have mostly been
used to extrapolate and describe the distribution of actin at the NMJ (Coyle et al., 2004; Pielage et al., 2005; Pielage et al., 2006). Labeling with phalloidin and antibodies to spectrin demonstrate that presynaptically actin/spectrin is present in the nerve and at the periphery of the boutons, along the inner border of the presynaptic membrane (Figure 1.4A) (Pielage et al., 2005). At the postsynapse, actin/spectrin is highly enriched in the SSR region where it shows a meshwork like arrangement and forms a thick rim that completely envelops the bouton (Figure 1.4A) (Ruiz-Canada et al., 2004; Pielage et al., 2006).

On the other hand, with the availability of antibodies against tubulin and Futsch, an MT binding protein, the organization of the MT cytoskeleton and the changes occurring in the MT cytoskeleton during NMJ growth can be easily visualized (Roos et al., 2000; Ruiz-Canada et al., 2004). Indeed, most of the current knowledge about microtubule dynamics during synaptogenesis and synaptic plasticity has been obtained from studies of the larval NMJ. At the presynapse, MTs form a single bundle that traverses the entire length of the nerve and extends out into the boutons (Figure 1.4A; Figure 1.4C, bottom panel). Many times, within the terminal bouton, the MT bundle splays apart into its individual filaments signaling active growth occurring at that region (Ruiz-Canada et al., 2004). In the muscle cell, microtubules radiate from multiple muscle nuclei and form a crisscross network composed of wavy strands that is spread throughout the muscle cortex (Figure 1.4A; Figure 1.4C, bottom panel) (Ruiz-Canada et al., 2004; Lee et al., 2009). However these MT strands stop
right at the boundary defined by the actin/spectrin network and as a result is completely excluded from this region (Figure 1.4A, Figure 1.4C, bottom panel) (Ruiz-Canada et al., 2004). Thus, there appears to be clear cut segregation between the actin/spectrin domain and the MT cytoskeleton at the postsynapse with the actin/spectrin domain preventing direct contact between the MT and the boutons (Figure 1.4A, Figure 1.4C, bottom panel).

**Role of actin at the Drosophila NMJ**

At the NMJ, actin has been demonstrated to be involved in the initial stages of synaptogenesis as well as in mature synapses. Studies have shown that at the time that the axon reaches its target muscle at AEL (After Egg Laying) 16, many actin rich filopodia like structures called 'myopodia' emanate from the target muscle and mainly cluster around the area where the motor neuron growth cone comes in contact with the muscle surface (Ritzenthaler et al., 2000; Ritzenthaler and Chiba, 2003). Application of Cytochalasin B, an actin depolymerizing drug, prevents these filopodia from forming and consequently prevents proper synapse formation. Sometimes these filopodia contact the motor neuron growth cones and convert to lamellopodia thereby increasing the contact area with the growth cone (Ritzenthaler et al., 2000; Ritzenthaler and Chiba, 2003). These observations have resulted in speculations that these structures might be involved in initiating adhesions between the growth cone and the muscle and transferring signals to the growth cone for synapse formation.
Similar, activity induced actin rich filopodial structures called 'synaptopods' are also be observed in mature synapses of third instar larvae, emanating from the nerve branches (Ataman et al., 2008). However the function of these synaptopods is not yet clear.

At mature synapses, the actin cytoskeleton is critical for proper synaptic transmission. At the presynapse, using actin depolymerizing drugs, a study looking at FM-143 dye uptake during endocytosis of synaptic vesicles demonstrated that, similar to vertebrate synapses, actin had a role in recycling synaptic vesicles. In particular, actin was shown to be required for replenishing the reserve pool (RP, vesicle pool present in the center of the bouton that is utilized only during high frequency stimulation) while it was dispensable for the replenishment of the readily releasable pool (RRP, the pool of vesicles close to synaptic sites that are docked and primed for release utilized first and during low frequency stimulation). The same study showed that actin was also involved in recruiting vesicles from the RP to the RRP (Kuromi and Kidokoro, 2003). In support of this role for actin, a study looking at the recovery rate of GFP-fused Synaptotagmin (a synaptic vesicle protein) after photobleaching in the presence of actin polymerizing and depolymerizing drugs revealed that increasing actin polymerization resulted in faster recovery rates while depolymerizing actin resulted in slower recovery rates demonstrating the requirement for actin for synaptic vesicle mobility inside the boutons (Nunes et al., 2006).
At the postsynapse, actin was found to be required for anchoring glutamate receptors complexes through interaction with 4.1 protein Coracle, a protein demonstrated to have a role in linking membrane proteins to the underlying actin cytoskeleton (Chen et al., 2005). The larval NMJ consists of two different types of glutamate receptor complexes having different activation kinetics, each composed of the three subunits (GluRIIC or GluRIII, GluRIID, and GluRIIE) essential for viability and one of two non-essential subunits (GluRIIA or GluRIIB) giving rise to clusters either containing GluRIIA or GluRIIB (DiAntonio, 2006). While depolymerization of postsynaptic F-actin led to a dramatic reduction in GluRIIA clusters at the postsynapse with a corresponding change in the transmission properties, it did not have any effect on GluRIIB clustering demonstrating that the interaction between F-actin and Coracle was specifically required for anchoring GluRIIA clusters (Chen et al., 2005).

While a role for actin cytoskeleton in the structural stability of the synapse is implied based on findings in vertebrate synapses and on the fact that mutations in actin binding proteins show defective synaptic growth and altered bouton morphology at the NMJ (Parnas et al., 2001; Coyle et al., 2004; Pielage et al., 2006), further studies are required to clarify actin’s role in this regard. In the latter case, the challenge essentially lies in the inability to tease apart the direct effect of mutating these proteins on synaptic growth vs those mediated by the misregulation of the actin cytoskeleton. Furthermore, studies looking at the effect of actin depolymerization or effect of mutations in actin
binding proteins on the NMJ show that while certain structural components are disrupted, others are intact (Chen et al., 2005). It is possible that while in the earlier stages of NMJ development, the actin cytoskeleton is critical for maintaining the overall structural stability of the synapse, in mature synapses its role becomes limited to assembling and regulating the function of certain specific synaptic components. Indeed, studies done in vertebrate synapses have demonstrated that while actin depolymerization has significant consequences for newly formed synapses, it does not have any effects on mature synapses with a well established PSD (postsynaptic density) (Zhang and Benson, 2001).

**Role of MTs at the Drosophila NMJ**

Studies at the NMJ have revealed that MTs exhibit complex behaviors and precise changes in MT behavior are crucial for proper synaptic growth. Indeed, applications of MT drugs such as taxol which increases MT stability or nocodazole and vinblastine which destabilize MTs have deleterious effects on synaptic structure and function.

Emerging evidence suggests that the complex behaviors of MTs are brought about by the ability of MTs to bind to Futsch, the *Drosophila* homolog of MAP1B (Microtubule Associated Protein 1B), a MT stabilizing protein (Roos et al., 2000). In vertebrates, MAP1B plays a critical role in stabilizing MTs and organizing their formation into tight bundles (Goold et al., 1999; Conde and
Caceres, 2009). Likewise, at the NMJ, downregulation of Futsch results in fragmented MT bundles with large spots of irregular tubulin immunoreactivity. Furthermore, immunocytochemistry studies using Futsch antibodies show perfect colocalization between Futsch and the MT bundle (Roos et al., 2000; Ruiz-Canada et al., 2004).

At the presynapse, while MTs are generally organized in the form of stable bundles that run the entire length of the nerve and extend into the boutons, they exhibit other rearrangement in certain subsets of boutons. In these boutons, the MT/Futsch bundle either splits into various thinner bundles forming intermingled loops, splay apart into individual strands, or form a single circular loop (Roos et al., 2000; Ruiz-Canada et al., 2004). The presence of single circular MT/Futsch loops usually represent arrested growth whereas the presence of intermingled loops and splayed MT correlate with boutons that undergoing division or budding respectively (Roos et al., 2000). While single circular loops and splayed MTs were normally found in terminal boutons, intermingled loops were found in boutons present at distal branch points. Furthermore, it was observed that the rearrangement of the MT loops correlated with boutons taking on an hour glass shape normally observed prior to bouton division. Indeed, there is a direct correlation between MT/Futsch loop containing boutons and branch bifurcation with 90% of branch points containing an MT/Futsch loop preceding the bifurcation (Roos et al., 2000). Additionally, while manipulations that increase branching demonstrate an
increase in the number of MT/Futsch loops, downregulating Futsch levels completely negates this effect (Roos et al., 2000). Collectively, these data suggest that Futsch is critical for the formation of these loops and that this looped organization of MT/Futsch may be involved in branch bifurcations which arise from the division of branch point boutons into two boutons that elongate independently (Roos et al., 2000).

In boutons that showed splayed MTs, Futsch often showed a diffuse punctuate appearance (Roos et al., 2000; Ruiz-Canada et al., 2004). Analogous to the growth cone where MTs filaments invade filopodial protrusions and get captured resulting in growth cone advancement (Lee et al., 2004; Ng et al., 2004; Zhou and Cohan, 2004; Bouquet et al., 2007; Tsvetkov et al., 2007; Lowery and Van Vactor, 2009), there is some speculation that the splayed MTs in the terminal bouton exhibit a similar behavior where a dynamic MT filament invades into the growing bud. It is important to note that in this context, terminal boutons containing a small nascent bud that has separated from the parent bouton have been observed to contain a single MT filament extending from the parent bouton into the bud. Futsch was found to be specially associated with this MT giving rise to speculations that it might be required to consolidate the new bud (Ruiz-Canada et al., 2004).

Other MT functions identified at the NMJ include include transport of synaptic components in the presynaptic (Wang et al., 2007) and the postsynaptic cell (Ataman et al., 2006). Surprisingly, Futsch does not label MTs
in the muscle cell indicating that muscle MTs are stabilized by other mechanisms (Roos et al., 2000; Ruiz-Canada et al., 2004).

**Proteins regulating the actin cytoskeleton at the *Drosophila* NMJ**

While a number of actin regulating proteins have been identified at the NMJ and while mutations in many of them result in significant disruption of NMJ growth, direct evidence of changes in actin organization in these mutants is lacking owing to the inability to visualize synaptic F-actin clearly using phalloidin staining. At the presynapse, studies have attempted to overcome this hurdle by expressing actin or actin binding proteins fused to GFP (Green Fluorescent Protein) and looking at the localization of GFP (Nunes et al., 2006; Pawson et al., 2008; Seabrooke and Stewart, 2008). While studies that express Actin-GFP in the presynapse have reported the formation of discrete and intense F-actin foci within the bouton (Nunes et al., 2006; Pawson et al., 2008; Seabrooke and Stewart, 2008), studies using actin- binding proteins as a read out for changes in actin organization show a more diffuse distribution (Besse et al., 2007). Furthermore, it is not clear how the distribution of these expressed proteins reflects the distribution of endogenous actin since such foci are not observed within the bouton when stained with phalloidin. However, the functions of many of these regulatory proteins have been characterized in detail in growth cones and in cell culture systems in both vertebrates and invertebrates allowing for their extrapolation to the NMJ.
The family of Wiskott Aldrich Syndrome proteins (WASP) and WAVE/SCAR proteins has been shown to be involved in actin nucleation, polymerization and the formation of branched networks by regulating the activity of downstream protein complexes such as the Actin Related Protein 2/3 (Arp2/3) complex (Higgs and Pollard, 2001; Alberts 2002). These proteins, in turn, are activated by RhoGTPases namely, Rac1, Rho, and Cdc42, whose catalytic function depends on the hydrolysis of GTP (Guanosine Triphosphate). While GTPase Activating Proteins (GAPs) promote the catalytic activity of the GTPase, rendering the protein inactive, Guanine Nucleotide Exchange Factor (GEFs) exchange the GDP for GTP thereby activating the GTPase for its next catalytic cycle (Alberts, 2002; Hall et al., 2005; Schubert and Dotti, 2007). All these regulators are brought together to allow for localized changes in actin organization by adaptor proteins that contain SH3 (Src Homology 3) domains which are capable of binding different combinations of regulators (Alberts, 2002; Coyle et al., 2004). At the NMJ, mutations in WASP, Cdc42 and its adaptor protein, Nervous Wreck (Nwk) all resulted in increased synaptic outgrowth demonstrating that they are negative regulators of bouton proliferation (Coyle et al., 2004; Rodal et al., 2008). These proteins were shown to be highly enriched at the periactive zone (region of the bouton adjacent to the active zone thought to be involved in synaptic growth) implicating them in actin cytoskeleton at these regions (Rodal et al., 2008). At this region, these proteins were also shown to interact with proteins involved in endocytosis such as Dynamin Associated
Protein160 (Dap160) and Dynamin linking the RhoGTPase pathway and actin polymerization to endocytosis (Koh et al., 2004; Marie et al., 2004; Rodal et al., 2008). Furthermore, GEF proteins such as Still Life (SIF) (the *Drosophila* homolog of vertebrate TIAM1 (T-cell lymphoma invasion and metastasis 1) and STEF) and dPIX (the *Drosophila* homolog of vertebrate β-pix), both activators of the Rac1 pathway, have also been identified at the NMJ (Sone, 1997; Sone et al., 2000; Parnas et al., 2001). While SIF was enriched at the periactive zone within the bouton and acted together with the cell adhesion molecule, FasII to regulate bouton outgrowth (Sone et al., 2000), dPIX was enriched at the postsynapse along with *Drosophila* p21 Activating Kinase (dPAK), its downstream effector kinase where it colocalized with glutamate receptor clusters (Parnas et al., 2001). Last but not the least, mutations in the WAVE/SCAR complex also showed alterations in bouton morphology and number although its localization and phenotypes have not been characterized (Schenck et al., 2004).

A novel regulator of actin identified at the NMJ is N-ethylmaleiamide Sensitive Factor 2 (NSF2). NSF2 is an ATPase that was originally identified as a protein required for the disassembly of the SNARE complex, a protein complex required for vesicle fusion (Nunes et al., 2006). At the NMJ, blocking NSF2 activity resulted in a synaptic overgrowth phenotype accompanied by decreased actin polymerization and decreased neurotransmitter release (Nunes et al., 2006). Fluorescence Recovery after Photobleaching (FRAP) experiments
revealed that NSF2 was essential for regulating intrabouton vesicle mobility by controlling actin polymerization (Nunes et al., 2006).

Other proteins involved in presynaptic actin regulation at the NMJ are Basigin, and Moesin. Basigin is an immunoglobulin cell adhesion molecule (IgCAM) and appears to be involved in stabilizing and tethering the presynaptic actin network at the bouton periphery as evidenced by the formation of aggregates of spectrin and other actin binding proteins within the boutons in basigin mutants (Besse et al., 2007). On the other hand, Moesin was identified as a protein involved in linking transmembrane proteins to the actin cytoskeleton (Niggli and Rossy, 2008). Surprisingly, overexpression of Moesin was also able to reverse the phenotypes of dominant negative NSF2 with respect to the NMJ overgrowth phenotype demonstrating that the action of Moesin was antagonistic to NSF2 at least in the context of bouton outgrowth (Seabrooke et al., 2008).

While most of the above proteins have a direct effect on the synaptic cytoskeleton, Mical, a protein belonging to a novel family was shown to affect the synaptic cytoskeleton study by regulating actin organization in the sarcomere (Beuchle et al., 2007).

**Proteins regulating the MT cytoskeleton at the Drosophila NMJ**

Studies in vertebrate systems have shown that most of the genes implicated in a variety of neurological and neurodegenerative disorders, including mental retardation, in humans, are genes whose protein products are involved in
MT regulation (Zhang et al., 2001; Sherwood et al., 2004; Trotta et al., 2004; Jin et al., 2009; Lee et al., 2009). Thus at the very core of many of these disorders are alterations in the organization and behavior of the MT cytoskeleton. A number of these genes have corresponding _Drosophila_ homologs, the functions of many of which are gradually being uncovered at the NMJ. Based on the aspects of MT behavior that they regulate, these proteins can be divided into a number of categories. Proteins such as Spastin and Atlastin which belong to the family of ATPases and GTPases respectively have shown to be negative regulators of microtubule stability at the NMJ (Sherwood et al., 2004; Trotta et al, 2004; Lee et al., 2009). While Spastin is directly involved in microtubule severing protein, Atlastin was suggested to function as an adaptor protein for proper targeting of Spastin to different subcellular locations (Sherwood et al., 2004; Trotta et al, 2004; Lee et al., 2009).

On the other hand, proteins such as the long isoform of Ankyrin (Ankyrin-L) and Dynactin have been shown to be required for stabilizing MTs by linking them to the submembranous actin cytoskeleton at the presynapse. Downregulation of these proteins resulted in considerable increase in unbundled and fragmented MTs at the NMJ. This was further associated with accumulation of Futsch in the presynaptic terminals, reduction in bouton numbers, and significant synaptic retraction characterized by the presence of postsynaptic components such as Dlg without the opposing presynaptic nerve terminal (Eaton et al., 2002; Pielage et al., 2008). Additionally, proteins such as TBCE, a
chaperone protein involved in proper folding of the alpha tubulin subunit, and proteins belonging to the Bone Morphogenic Protein (BMP) pathway have been identified at the NMJ and have shown to be critical for MT assembly (Wang et al., 2007; Jin et al., 2009).

While the above proteins appear to have a more direct effect on MTs, many other proteins regulate MT bundling and stability by regulating the activity of Futsch. Studies looking at the regulation of MAP1B, the vertebrate homolog of Futsch, have shown that MAP1B control of MT behavior is highly dependent on its phosphorylation state (Gong et al., 2000; Goold and Gordon-Weeks, 2001; Goold and Gordon-Weeks, 2004). MAP1B has been identified as a substrate for kinases like Glycogen Synthase Kinaseβ (GSK3β) (Goold and Gordon-Weeks, 2001; Goold and Gordon-Weeks, 2004) and protein phosphatases like Protein Phosphatase 2A (PP2A) (Gong et al., 2000). In vertebrates, phosphorylation of MAP1B by GSK3β results in a decrease in MT stability and bundling (Goold et al., 1999), while dephosphorylation by PP2A enhances it (Gong et al., 2000). Likewise, the *Drosophila* homologs of GSK3β (Shaggy) and PP2A have been shown to function in a similar manner at the NMJ (Franco et al., 2004; Gogel et al., 2006; Viquez et al., 2006) by having antagonistic effects on the phosphorylation state of Futsch (Viquez et al., 2006). While the above proteins regulate the activity of Futsch, FMRP was shown to regulate the levels of Futsch (Zhang et al., 2001). FMRP is a gene product of *fmr1* gene and was initially identified as an RNA binding protein involved in translational repression (Siomi,
et al., 1993; Zalfa and Bagni, 2004). Mutations in this gene cause Fragile X syndrome characterized by severe mental retardation (Zalfa and Bagni, 2004). At the NMJ, dFMRP, the *Drosophila* homolog, was found to bind to Futsch mRNA and suppress translation of Futsch protein (Zhang et al., 2001).

### Proteins regulating both the actin and the MT cytoskeleton at the *Drosophila* NMJ

In contrast to protein complexes that regulate only the actin or MT cytoskeleton, some protein complexes affecting the behavior of both the cytoskeletal elements have also been identified at the NMJ. They are the Formins (Pawson et al., 2008), Spectrins (Pielage et al., 2005; Pielage et al., 2006), and aPKC/Par-3 or Bazooka/Par-6 complex (Ruiz-Canada et al., 2004).

**Formins**

While formins have generally been characterized as actin associated proteins that participate in the nucleation of unbranched actin filaments by associating with their plus end, a sub family of formins, the Diaphanous Related Formins (DRF) have also been shown to bind and regulate MT dynamics (Wallar and Alberts, 2003). *Drosophila* has a single copy of the *diaphanous* gene that encodes for a typical DRF protein. At the NMJ, in addition to being a positive regulator of actin polymerization, Diaphenous (Dia) appeared to be involved in modulating the behavior of a subpopulation of highly dynamic microtubules called ‘pioneer MTs’. Dia dependent modulation of these pioneer MTs was required for
their consolidation into the Futsch labeled MT core found in the axons (Pawson et al., 2008).

**Spectrins**

Spectrin was originally identified in red blood corpuscles as an actin crosslinking protein (Nicolson et al., 1971; Pinder et al., 1975). Each Spectrin molecule consists of two heterodimers composed of alpha and beta subunits (Alberts, 2002). These heterodimers can bind to short actin filaments forming a filamentous network that is associated with the plasma membrane. Labeling with antibodies against the two subunits of Spectrin show that both α and β isoforms of spectrin are present at the NMJ (Pielage et al., 2005; Pielage et al., 2006). While presynaptically they are enriched in the axons and at the bouton periphery, postsynaptically they are enriched in the SSR region and colocalize with Dlg. Surprisingly, contrary to the well established role of Spectrin, in many different cell types, at the NMJ presynaptic Spectrin was shown to regulate MTs (Pielage et al., 2005). Similar to the phenotypes seen in ankyrin and dynactin mutants, loss of presynaptic spectrin resulted in a dramatic reduction in bouton number caused due to excessive synaptic retraction events associated with the withdrawal of MTs towards the nerve and complete loss of Futsch staining at the terminal boutons (Pielage et al., 2005). On the other hand, downregulation of postsynaptic spectrin resulted in a severe reduction in the SSR thickness, and disorganized Dlg (Pielage et al., 2006). These phenotypes were associated with an increase in the levels of glutamate receptor clusters and in the size of the
active zones (Pielage et al., 2006). Based on their findings, the authors speculated that, at the presynapse, Spectrin might be involved in linking the MTs to the underlying cortical actin cytoskeleton whereas at the postsynapse, Spectrin may act with actin to form a hexagonal lattice which could serve as a framework for the assembly of postsynaptic components or it may function independently to anchor various postsynaptic proteins.

*aPKC/Par-3 or Bazooka/Par-6 complex*

A highly conserved protein cassette known to play a critical role in cytoskeletal regulation and cell polarity is the Atypical Protein Kinase C/Par-3/Par-6 complex. Par-3 and Par-6 (Partition Defective) were first identified in *C.elegans* and were found to be required for proper segregation of cell fate determinants during normal asymmetric division of *C.elegans* embryos (Macara, 2004). Additional roles for this protein complex in vertebrate systems include stabilization of the actin cytoskeleton during tight junction formation in epithelial cells (Chen and Macara, 2005; Chen and Macara, 2006) and mobilization of the cytoskeleton in neuronal cells during the establishment of neuronal polarity (Shi, et al., 2003; Nishimura et al., 2005). The corresponding *Drosophila* homologs, daPKC/Bazooka (Baz)/dPar-6 have been demonstrated to be required for neuroblast division in embryos (Wodarz et al., 2000) and Baz in particular, was found to be crucial for the formation of the actin rich zonula adherens in neurectodermal cells (Muller and Wieschaus, 1996). Additionally, the active form
of aPKC, Protein Kinase M (PKM) has been implicated in memory formation in both mammals and in flies (Drier et al., 2002; Pastalkova et al., 2006).

aPKC belongs to the family of atypical PKC isotypes which unlike the conventional PKC isotypes are not activated by diacylglycerol (DAG) or Ca++. Instead, it is activated by lipid molecules such as phosphatidylinositol 3,4,5,-triphosphate (PIP$_3$) and proteins such as PDK1(pyruvate dehydrogenase kinase isoenzyme 1), PI3Kinase (phosphoinositide-3-kinase), downstream effectors of the G-protein coupled pathway (Hirai and Chida, 2003; Suzuki et al., 2003). Additionally, it can also be activated by RhoGTPases such as Rac1 and Cdc42 through Par-6 (Lin et al., 2000; Nagai-Tama et al., 2002). Within its protein sequence, it consists of an N-terminal regulatory domain and a C-terminal kinase domain (Hirai and Chida, 2003).

On the other hand, Par-3/Baz and Par-6 are PDZ (PSD-95/ DlgA/zo-1) domain containing proteins that can bind to each other through their PDZ domains (Nagai-Tamai et al., 2002). While Par3/Baz has three PDZ domains, an N-terminal oligomerization domain, and a C-terminal region containing a conserved aPKC phosphorylation site, Par-6 has one PDZ domain that binds to Par-3/Baz and a semi-CRIB (Cdc42/Rac Interactive Binding) domain which binds activated Rac1 or Cdc42 (Izumi et al., 1998; Lin et al., 2000; Nagai-Tamai et al., 2002; Benton and St Johnston, 2003). A small region present at the N-terminal of Par-6 is required for interaction with aPKC (Nagai-Tamai et al., 2002). Binding partners of Par-3/Baz include Par-6 that binds to PDZ1 of Par-3/Baz (Joberty et
al., 2000; Lin et al., 2000), dPTEN that binds to PDZ 1 and 2 (von Stein et al., 2005; Pinal et al., 2006) and aPKC that binds to PDZ 2 and 3 (Nagai-Tamai et al., 2002).

Based on biochemical and genetic interaction studies done in vertebrates and invertebrates, a model for interaction between the three proteins has been proposed, the end result of which is the regulation of the cytoskeleton (Izumi et al., 1998; Lin et al., 2000; Cox et al., 2001; Hirose et al., 2002; Nagai-Tamai et al., 2002). In an inactivated state, Par-6 is constitutively bound to aPKC and the kinase domain of aPKC is normally inhibited by its regulatory domain and by Par-3. Binding of active Cdc42 or Rac1 to Par-6 relieves the inhibition of the regulatory domain over the kinase region. The active kinase domain, in turn phosphorylates Par-3 which results in the dissociation of the two proteins allowing aPKC to phosphorylate other proteins (Izumi et al., 1998; Lin et al., 2000; Cox et al., 2001; Hirose et al., 2002; Nagai-Tamai et al., 2002). While there is some conservation in the interactions between the three proteins in different cell types, many variants of this model have been observed depending on the cellular context giving rise to a number of open questions. For example, while phosphorylation of Par-3 is required for its dissociation from aPKC, it is not clear what role phosphorylation plays in regulating the localization or the other functions of Par-3. Additionally, although not much is known about their role in synaptic development, the implication of PKM in memory formation purports the
possibility of this protein cassette having interesting roles in synaptogenesis and synaptic plasticity.

At the larval NMJ, this highly conserved cassette was found to be present at both the pre and postsynaptic regions (Figure 1.4B,C, top panel). Presynaptically, aPKC and Par-6 were associated with MT (Figure 1.4B). In the presynapse, aPKC was found to play a major role in regulating MT stability by facilitating its association with Futsch (Ruiz-Canada et al., 2004). While increasing aPKC activity increased the association between MTs and Futsch, decreasing aPKC activity resulted in the opposite effects (Ruiz-Canada et al., 2004). Postsynaptically, aPKC was distributed in the MT rich area in the muscle cortex while it was completely absent from the spectrin/actin rich peribouton area (Figure 1.4C, top panel). On the other hand, at the presynapse, Baz was distributed in a diffuse manner throughout the bouton whereas at the postsynapse Baz coexisted in the muscle cortex with aPKC and was also highly enriched in the peribouton area with Par-6 (Ruiz-Canada et al., 2004). Interestingly, changes in postsynaptic aPKC activity levels resulted in change in the postsynaptic cytoskeletal organization. While increasing aPKC activity led to the expansion of postsynaptic area labeled by the spectrin antibody, decreasing aPKC activity led to a dramatic reduction in the same (Figure 1.4D top and bottom panels). Furthermore, while an expansion of the spectrin staining was associated with retraction of MT, a reduction was associated with a greater degree of MT penetration into the peribouton area (Ruiz-Canada et al., 2004).
Surprisingly, manipulating aPKC activity also resulted in postsynaptic changes in peribouton Baz levels suggesting that Baz levels may be regulated by aPKC dependent phosphorylation and Baz in turn may play a role in regulating the peribouton area (Ruiz-Canada et al., 2004).

Here we show that aPKC’s effect on the postsynaptic spectrin organization is indeed mediated by Baz and this effect is not limited to spectrin alone, but also includes changes to the underlying actin cytoskeleton. We further show that Baz localization and function is highly dependent on its phosphorylation state mediated by aPKC and the dual lipid protein phosphatase PTEN that is highly enriched at the postsynapse. We show that while Baz phosphorylation by aPKC is required for its dissociation from aPKC and targeting to the postsynapse, its dephosphorylation by PTEN is required to retain it at the postsynaptic region where it is involved in regulating the actin cytoskeleton.

**The Wnt Pathway**

Integral to normal synaptic growth and plasticity are both anterograde and retrograde signaling mechanisms that allow for precise temporal and spatial communication between the muscle and the nerve. One such pathway that has proven to be critical for synaptic development over the last few years is the Wnt pathway (Gordon and Nusse, 2006; Speese and Budnik, 2007; Angers and Moon, 2009). Misregulation of this pathway has been linked to cancer, and the pathogenesis of Alzheimer’s and Huntington’s disease (Speese and Budnik, 2007).
Wnts are secreted glycoproteins which were initially discovered in *Drosophila*. Early studies identified Wingless, the *Drosophila* homolog of vertebrate Wnt-1, as being a morphogen and segment polarity gene critical for defining positional information in the embryo and for wing and haltere development during metamorphosis (Sharma and Chopra, 1976; Nusslein-Volhard and Wieschaus, 1980). Since then Wnts have been demonstrated to play important roles in a variety of cellular processes both in invertebrates and vertebrates. Further corroborating this is the evidence for the existence of a multitude of Wnt ligands (19 in mice and 7 in flies) and its cognate receptor, Frizzled (Fz), that are expressed individually or in sets in different cell types and perform a variety of functions depending on the cellular context (Speese and Budnik, 2007).

Growing evidence suggests that Wnt are involved in the activation of many different signaling pathways within the cell (Gordon and Nusse, 2006). The best studied Wnt transduction cascade is the canonical pathway in which secreted Wnt binds to its receptor Frizzled (Fz). This leads to the activation of the PDZ protein, Dishevelled, which is a component of a protein complex containing GSK-3β, Axin, and Adenomatous Polyposis Coli (APC). In the absence of Wg, this complex constitutively phosphorylates β-catenin leading to its degradation. The binding of Wnt to Fz disrupts the β-catenin ‘destruction complex’, resulting in cytoplasmic stabilization of β-catenin and its import into the nucleus where it associates with Lymphoid Enhancer Factor (LEF)
transcription factor (Gordon and Nusse, 2006; Angers and Moon, 2009).

Alternative non-canonical pathways have been identified such as (1) the planar cell polarity pathway where Wnt binding to Fz leads to the activation of Rho GTPase and Jun N-terminal kinase (JNK) pathways that regulate the cytoskeleton. (2) The calcium signaling pathway where Fz activation leads to increased intracellular Ca++ and nuclear import of transcription factor, NF-AT. (3) Divergent canonical pathway for MT regulation where Wnt to Fz inhibits the ‘destruction complex’ through Dishevelled (Gordon and Nusse, 2006; Angers and Moon, 2009). This prevents GSK-3B from phosphorylating microtubule binding proteins such as MAP1B thereby regulating MT stability (Goold et al., 1999; Goold and Gordon-Weeks, 2004). Thus depending on the combination of Wnts expressed in a specific cell, different pathways might be activated in the same cell for different processes.

**Wnts in the nervous system**

Initial discoveries about the role of Wnts in synapse development were made in vertebrates. Current knowledge with respect to Wnt signaling shows that Wnts can be secreted in an anterograde, retrograde, or autocrine manner (Hall et al., 2000; Packard et al., 2002) and activate signaling pathways involved in axonal and growth cone spreading and branching, in the clustering of presynaptic proteins (Lucas et al., 1997; Hall et al., 2000) and postsynaptic receptors (Luo et al., 2002; Wang et al., 2003), and in the regulation of
microtubule stability via the divergent canonical pathway (Hall et al., 2000; Speese and Budnik, 2007).

**Wnt pathway at the Drosophila NMJ**

The initial discovery that Wnts regulate synapse development in invertebrates emerged from studies at the *Drosophila* NMJ. The *Drosophila* Wnt-1 homolog, Wingless, was found to be expressed in the presynaptic terminal whereas its receptor DFrizzled2 (DFz2) was concentrated at the presynaptic as well as the postsynaptic membrane (Packard et al., 2002). Further analysis demonstrated that Wg was secreted from the presynaptic terminal and bound to both presynaptic and postsynaptic DFz2. Moreover, blocking Wg release, using temperature sensitive Wg mutants, during the critical period of bouton proliferation, resulted in a dramatic reduction in bouton number, changes in bouton morphology, and significant presynaptic and postsynaptic alterations (Packard et al., 2002). Presynaptically, Wg mutants showed defects in microtubule organization wherein mutant boutons had a high degree of unbundled microtubules suggesting that MTs remained in an abnormal dynamic state. Postsynaptic alterations included abnormal Dlg and Glutamate receptor localization around the boutons. In many instances, instead of surrounding the boutons and forming a thick postsynaptic rim, Dlg was absent from regions of the bouton periphery. Similarly, instead of showing a discrete and well defined appearance, GluRIIA clusters appeared more diffuse and irregular (Packard et al., 2002). Most strikingly, loss of Wg signaling led to a subset of undifferentiated
boutons called 'ghost boutons' that contained synaptic vesicles but were devoid of active zones, PSDs, SSR and mitochondria (Packard et al., 2002; Ataman et al., 2006; Ataman et al., 2008). All of the above synaptic phenotypes could be rescued by presynaptic expression of Wg, but not by postsynaptic expression of Wg, consistent with the idea that Wg is secreted from the presynaptic terminal (Packard et al., 2002). This idea has been further bolstered by the results of a recent study which demonstrated an activity induced increase in Wg release from the presynaptic terminal (Ataman et al., 2008).

The requirement of Wg for both pre and postsynaptic differentiation coupled with the enrichment of the Wg receptor, DFFz2 on both sides of the synapse raised the possibility that Wg signaling might function not only in an anterograde fashion from the neuron to the muscle, but also in an autocrine fashion within the neuron (Packard et al., 2002; Miech et al., 2008). The presence of the autocrine loop was established by the identification of other players of the Wnt cascade within the neurons. While Dishevelled, and Shaggy (the *Drosophila* homolog of GSK-3β) were present at the presynapse, Armadillo, the *Drosophila* homolog of β-catenin was absent. Furthermore, while mutations in *dishevelled* showed MT phenotypes similar to *wg* mutants, *shaggy* mutants showed the opposite phenotype consistent with the idea that Wg signaling inhibits GSK3β activity. Collectively, this data suggests that at the presynapse, Wg plays a role in regulation of MT stability most likely through the divergent canonical pathway (Miech et al., 2008). On the other hand, the search for the
Wg transduction cascade in the muscle led to the identification of a previously unrecognized pathway in the muscle where the Wg receptor, Dfz2 was internalized and the C-terminal was cleaved giving rise to an 8 KDa fragment (DFz2C) which was imported into the nucleus (Mathew et al., 2005). Within the nucleus, the Dfz2 foci were excluded from areas rich in heterochromatin (Mathew et al., 2005). Further analysis showed that, while Dfz2 internalization and cleavage was constitutive, the nuclear import of Dfz2C was dependent on the binding of Wg to DFz2 raising the possibility that DFz2C was somehow modified in a Wg-dependent fashion before import (Mathew et al., 2005). Additionally, this nuclear import was also shown to be dependent on trafficking of DFz2 from the synapse to the nucleus by the PDZ protein dGRIP (Glutamate Receptor Interacting Protein; Ataman et al., 2006). However the function of Dfz2 in the nucleus is unknown as yet warranting detailed investigation.

While a huge body of evidence is available with regard to the intercellular pathways activated by Wnts, not much is known about how these molecules are secreted and transported. Wnt proteins are highly hydrophobic owing to palmitoyl modifications essential for their biological activity which prevent them from being easily diffusible in the extracellular milieu (Willert et al., 2003; Mikels and Nusse, 2006). Although several mechanisms have been proposed to explain the movement of Wnt molecules from their site of secretion, including their association with glycosaminoglycan-modified proteins at the extracellular matrix (Baeg et al., 2001), the formation of exosome-like vesicles called argosomes
(Greco et al., 2001), extracellular lipoprotein particles (Panakova et al., 2005), and transcytosis (Coudreuse et al., 2006), the processes governing intercellular Wnt transport have been elusive.

Recent studies have identified a novel protein called Evenness Interrupted/Wntless/Sprinter (Evi/Wls/Srt) dedicated to the secretion of Wnts. Evi is a type II multi-pass transmembrane protein, conserved from worms to human and appears to be specifically required in vivo for Wnt secretion in epithelial cells of flies and human cultured cells (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Further analysis has suggested that Evi functions as a Wnt cargo receptor during trafficking from the Golgi to the plasma membrane, and recycled back to the Golgi through the retromer complex (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008).

In Chapter 3, we show that Evi is localized at both the presynapse as well as the postsynaptic SSR region and its function is critical for proper transduction of the Wg pathway. We further demonstrate that presynaptic Evi is involved in Wg secretion from the bouton and its transport across the synapse which occurs through a previously unknown mechanism that involves the release of Evi containing exosome like vesicles. Further, that Evi also plays a role in the signal-receiving postsynaptic muscle cell where it is required for the proper trafficking of the Wg receptor DFz2, through actions that involve the DFz2-interacting protein dGRIP (Mathew et al., 2005; Ataman et al., 2006).
Thus in my thesis, I have dealt with two critical events required for proper synaptic proliferation: actin regulation at the postsynapse mediated by the aPKC/Baz/Par-6 polarity complex and Wnt signaling across the synapse. While previous studies made assumptions about the effect of modulating upstream actin regulators on the postsynaptic actin cytoskeleton, ours is the first study to actually demonstrate these changes. Furthermore, growing evidence suggests that downstream effectors of the Wnt pathway might be involved in aPKC activation indicating a potential link between the two pathways. However the significance of these links at the NMJ and their relevance to activity dependent synaptic plasticity still remain to be explored.
Figure 1.1. (Adapted from Gorczyca et al., 2006) **Body wall muscles of the Drosophila larvae.**

Third instar wandering stage (left) and young first instar (right) larval body wall muscle preparations labeled using FITC-conjugated phalloidin. Note that while the number of muscle fibers remains the same, there is nearly a 100 fold increase in the size of the muscle fibers. Abdominal segments 1-7 are labeled A1-A7. Up is anterior. In these preparations the lateral muscles correspond to the dorsal musculature (arrowheads = dorsal midline), whereas the ventral muscles run along the midline separating the left and right hemisegments (arrow at the bottom of the preparation = ventral midline).
Figure 1.1

Gorczyca et al., 2006

Figure reprinted from International Review of Neurobiology, Appendix: Anatomy of the Larval Body Wall Muscles and NMJs in the third instar larval stage, 75, 2006, 367-373 with permission from Elsevier.
Figure 1.2. Anatomical Structure of the larval NMJ and the organization of the cytoskeleton.

(A) Illustration showing the organization of the larval NMJ. The nerves exit out of the ventral ganglion and synapse on the muscle. Muscle fibers are shown in green and the ventral ganglion and nerves are shown in red.

(B) Confocal image of a first instar larval NMJ (top) and a third instar larval NMJ (bottom) labeled with HRP demonstrating the dramatic increase in bouton number from the first instar larval period to the third instar larval period. Confocal image of an arbor stained with HRP, a presynaptic marker in red (Adapted from Gorczyca et al., 2006).

(C) Preparation demonstrating type-I big, type-I small, type-II and type-III bouton types at the NMJ stained with antibodies against the presynaptic marker HRP (red) and the scaffolding protein Dlg (green). Note that Dlg is highly enriched at the postsynaptic region immediately surrounding type-I big boutons and can also be seen around type-I small boutons. However it is almost non-existent around type-II and type-III boutons (Adapted from Guan et al., 1995).
Figure 1.2

A

B

Gorczyca et al., 2006

C

Guan et al., 1995

Figure 1.2
Figure 1.3. (Adapted from Gorczyca et al., 2006) **Ultrastructural view of type 1b boutons.**

(A) Midline cross section through a type 1b bouton. b = bouton, v = vesicles, mi = mitochondria, bl (white arrow) = basal lamina, SSR = subsynaptic reticulum, m = muscle. Arrows point to T-bar active zones. Arrowhead denotes an endocytic coated pit.

(B) High magnification view of a synaptic area, showing a T-bar active zone, and a coated pit (p).

(C) Longitudinal cross section through a type 1b NMJ showing two boutons (b) joined by a neurite process.

Calibration bar is 0.8 μm in A, 0.3 μm in B, and 2.5 μm in C.
Figure 1.3

Figure reprinted from International Review of Neurobiology, Appendix: Anatomy of the Larval Body Wall Muscles and NMJs in the third instar larval stage, 75, 2006, 367-373 with permission from Elsevier.
Figure 1.4. (Adapted from Ruiz Canada et al., 2004) **Organization of the postsynaptic cytoskeleton and the effect of altering muscle aPKC levels on postsynaptic spectrin levels.**

(A) Confocal image of an NMJ branch showing the organization of the postsynaptic cytoskeleton. The arbor has been stained with antibodies against HRP (blue), Spectrin (green) and Microtubules (red). Note that Spectrin is highly enriched at the postsynaptic region and there is clear cut segregation between the postsynaptic Spectrin rich area and the microtubule network of the muscle.

(B) Low mag image of a wild type NMJ branch to show the localization of aPKC. The arbor has been stained with antibodies against aPKC (green) and HRP (red). Note that aPKC is present in the presynapse (arrow) and shows a punctuate distribution in the muscle (arrowhead).

(C) Confocal image of a branch at a higher magnification showing the localization of aPKC in greater detail. The arbor has been stained with antibodies against aPKC (green) in combination with either HRP (blue, top image) or tubulin (red, bottom image). Note that aPKC is associated with the microtubule network in the muscle and the nerve terminal, but is completely absent from the actin/spectrin area (arrow).

(D) Confocal image of an NMJ branch from an aPKC mutant stained with antibodies with HRP (blue), Spectrin (green) and tubulin (red). Note that downregulation of aPKC activity in the muscle causes a dramatic reduction in the levels of postsynaptic spectrin which is accompanied by penetration of the muscle MTs (red) closer to the bouton (blue, arrow).
Figure 1.4

Figures A and D also reprinted from International Review of Neurobiology, Synaptic Cytoskeleton at the Neuromuscular Junction, 75, 2006, 217-236 with permission from Elsevier
CHAPTER II

A Critical Step for Postsynaptic F-Actin Organization: Regulation of Baz/Par-3 Localization by aPKC and PTEN
Abstract

Actin remodeling has emerged as a critical process during synapse development and plasticity. Thus, understanding the regulatory mechanisms controlling actin organization at synapses is exceedingly important. Here we used the highly plastic Drosophila neuromuscular junction (NMJ) to understand mechanisms of actin remodeling at postsynaptic sites. Previous studies have suggested that the actin-binding proteins Spectrin and Coracle play a critical role in NMJ development and the anchoring of glutamate receptors most likely through actin regulation. Here we show that an additional determinant of actin organization at the postsynaptic region is the PDZ protein Baz/Par-3. Decreasing Baz levels in postsynaptic muscles has dramatic consequences for the size of F-actin and spectrin domains at the postsynaptic region. In turn, proper localization of Baz at this site depends on both phosphorylation and dephosphorylation events. Baz phosphorylation by its binding partner, atypical Protein Kinase C (aPKC), is required for normal Baz targeting to the postsynaptic region. However, the retention of Baz at this site depends on its dephosphorylation mediated by the lipid and protein phosphatase PTEN. Misregulation of the phosphorylation state of Baz by genetic alterations in PTEN or aPKC activity has detrimental consequences for postsynaptic F-actin and spectrin localization, synaptic growth, and receptor localization. Our results provide a novel mechanism of postsynaptic actin regulation through Baz, governed by the antagonistic actions of aPKC and PTEN. Given the conservation of
these proteins from worms to mammals, these results are likely to provide new insight into actin organization pathways.

Introduction

Reorganization of the actin cytoskeleton plays pivotal roles during the establishment of cell polarity (Wodarz, 2002) and the formation of cellular junctions (Chen and Macara, 2005). In the nervous system, actin remodeling underlies axon pathfinding, synaptogenesis, and synaptic plasticity (Meng et al., 2003; Kalil and Dent, 2005). However, the underlying processes and pathways that regulate actin organization are still poorly understood.

A central property of synapses is their ability to undergo structural and functional changes, such as those occurring during learning and memory (Matsuzaki et al., 2004; Alvarez and Sabatini, 2007). These changes are known to be accompanied by modifications in the actin cytoskeleton (Dillon and Goda, 2005; Lynch et al., 2007). For example, synaptic plasticity in the mammalian brain is accompanied by structural changes in dendritic spines, which involve actin-based cytoskeletal dynamics (Tada and Sheng, 2006).

An important regulator of actin and microtubule cytoskeleton is a conserved cassette of proteins, the Par-3/Par-6/aPKC complex. This complex is essential for the establishment of oocyte and epithelial cell polarity in flies, worms, and mammals (Watts et al., 1996; Lin et al., 2000; Cox et al., 2001; Benton and St Johnston, 2003a; Benton and St. Johnston, 2003b), and the formation and maintenance of actin-based cell junctions in mammals (Ohno, 2001; Hirose et al.,
Par-3 and Par-6 are PDZ proteins that bind to and inhibit or activate atypical Protein Kinase C (aPKC) respectively as indicated by studies in primary cell cultures (Lin et al., 2000; Ohno, 2001). These studies also suggest that binding of the small G-proteins, Rac1 or Cdc42, to Par-6 activates aPKC, which in turn phosphorylates Par-3. This phosphorylation leads to the dissociation of Par-3 from aPKC and lifts the inhibition imposed by Par-3 on aPKC kinase activity (Nagai-Tamai et al., 2002). Additionally, the phosphorylation of Par-3 has been implicated in its localization and in modulating actin organization in mammalian epithelial cells (Izumi et al., 1998; Chen and Macara, 2005), but this role is poorly understood.

In the mammalian nervous system, this complex is also involved in establishing neuronal cell polarity (Shi et al., 2003) and in myelination (Chan et al., 2006). Alterations in aPKC activity are associated with defects in synaptic and behavioral plasticity in both mammals and flies (Drier et al., 2002; Pastalkova et al., 2006). At the fly neuromuscular junction (NMJ), aPKC regulates the dynamics of microtubules at presynaptic compartments, and actin-microtubule boundaries at postsynaptic sites, and this regulation is important for synaptic growth, glutamate receptor localization, and normal synaptic function (Ruiz-Canada et al., 2004). However, the exact molecular mechanisms by which aPKC influences postsynaptic actin organization has remained unresolved.

Previous studies have implicated the actin-binding proteins Spectrin and the Band 4.1 homolog Coracle in the regulation of NMJ development and clustering
of glutamate receptors (GluR) (Chen et al., 2005; Pielage et al., 2005; Pielage et al., 2006). Here we report that the size of postsynaptic F-actin and spectrin domains also depends on the localization of Baz at the postsynaptic region. In turn, normal Baz localization at the F-actin/spectrin rich area depends on its phosphorylation state. Baz phosphorylation by aPKC allows the translocation of Baz from the muscle cortex to the postsynaptic area. However, a stable localization of Baz at this postsynaptic area requires its dephosphorylation by the lipid and protein phosphatase PTEN, which has also been demonstrated to interact with and is recruited by Baz during the establishment of photoreceptor polarity in flies (von Stein et al., 2005; Pinal et al., 2006). Thus, our results provide a novel molecular mechanism by which postsynaptic F-actin organization is regulated by the phosphorylation state of Baz through the opposing actions of aPKC and PTEN.

Results

**aPKC activity regulates the organization of the postsynaptic F-actin and spectrin area**

The *Drosophila* larval NMJ is innervated by glutamatergic motorneurons that branch over the body wall muscles in a stereotypic fashion. These terminals are composed of synaptic boutons containing synaptic vesicles and vesicle release sites (Prokop, 2006). Synaptic boutons at the NMJ are completely surrounded by the muscle membrane, which forms a highly folded structure, the subsynaptic reticulum (SSR) (Fig. 2.1A, C, G). This specialized postsynaptic region is highly enriched in F-actin, as determined by labeling with fluorescently labeled phalloidin (Coyle et al.,
2004; Chen et al., 2005; Nunes et al., 2006) (Fig. 2.1B, arrowhead), and the actin-binding protein spectrin (Ruiz-Canada et al., 2004; Pielage et al., 2006). Three-dimensional reconstructions of confocal Z-series using Volocity software showed that this postsynaptic F-actin domain appeared separate from the muscle contractile apparatus (Fig. 2.1B, two-way arrow; distance is 2.5±0.16 µm; N=23 arbors). This was also supported by visualization of the terminals at muscle 12 by electron microscopy, showing that myofibrils are separated from the SSR (Fig. 2.1C-F, G; red two-way arrows; distance is 1.6± 0.4, N=15 boutons). Beyond this F-actin/spectrin-rich postsynaptic area there is a microtubule network at the muscle cortex which radiates from the nuclei, and which terminates at the boundary of the F-actin/spectrin-rich area (Ruiz-Canada et al., 2004) (Fig. 2.1A). Previous studies demonstrated that aPKC is localized both inside synaptic boutons and at the microtubule-rich cortical muscle area, but it is absent from the postsynaptic F-actin/spectrin regions (Ruiz-Canada et al., 2004). Decrease in aPKC protein levels led to a disruption in the presynaptic microtubule cytoskeleton by reducing the interaction between microtubules and the presynaptic MAP1B-related protein Futsch, while increase in aPKC activity increased this interaction (Ruiz-Canada et al., 2004). Surprisingly however, mutations in *dapkc* altered the extent of the postsynaptic spectrin region. This observation was surprising, as aPKC is absent from this area (Ruiz-Canada et al., 2004). As an initial attempt to understand the mechanisms by which aPKC could regulate the postsynaptic spectrin region, we first determined if the decrease in spectrin levels upon decreasing aPKC activity was
accompanied by changes in the postsynaptic F-actin domain. We used rhodamine-conjugated phalloidin to label F-actin, and expressed aPKC-RNAi only in the muscles, which reduced aPKC levels in muscle by 50-60% (Suppl. Fig. 2.1A, B). As previously reported, F-actin was found enriched around synaptic boutons (Coyle et al., 2004; Chen et al., 2005; Nunes et al., 2006), in exact colocalization with α-spectrin (Fig. 2.2A, D; arrows) and showed a diffuse meshwork-like appearance (which here is referred to as “F-actin meshwork”). Reducing aPKC levels in muscles alone by expressing aPKC-RNAi using the muscle specific Gal4 driver C57 (Budnik et al., 1996) (aPKC-RNAi-post), was sufficient to substantially reduce spectrin around synaptic boutons (Fig. 2.2B1, B3 arrows; H, I) and this was accompanied by a similar reduction in postsynaptic F-actin (Fig. 2.2B2-3, E arrows; H, I). Thus, spectrin is exactly localized to the F-actin-rich postsynaptic area, and decreasing aPKC activity leads to a concomitant reduction in both F-actin and spectrin. This reduction in F-actin and spectrin was not due to changes in the extent of the SSR, as demonstrated by electron microscopy (Suppl. Fig. 2.2A) and staining with the SSR markers DLG and Scrib (Suppl. Fig. 2.2B, C).

The decrease in F-actin at the postsynaptic region observed upon aPKC downregulation might result from a reduction in spectrin, as spectrin is known to crosslink F-actin microfilaments and facilitate the formation of a network/meshwork-like arrangement (Cohen et al., 1980). If this was the case, then downregulation of spectrin alone should mimic the phenotype observed upon expressing aPKC-RNAi in postsynaptic muscles. This possibility was examined in both β-spectrin mutants
(Featherstone et al., 2001) and by expressing a β-spectrin-RNAi (β-spec-RNAi; (Pielage et al., 2006)) in muscles. Both α- and β-spectrin were virtually eliminated in heterozygote β-spec_{em6}^{+/+} (Fig. 2.2C1) (Featherstone et al., 2001) and upon expressing β-spectrin RNAi in body wall muscles (Suppl. Fig. 2.1C, D) (Pielage et al., 2006). This virtual elimination of α- and β-spectrin in β-spec_{em6}^{+/+} heterozygous animals is likely due to a dominant-negative effect of a truncated β-spectrin product generated in the β-spec_{em6} mutation (Featherstone et al., 2001). In both the β-spec_{em6}^{+/+} heterozygotes as well as the β-Spectrin-RNAi-post, F-actin lost its meshwork-like organization around synaptic boutons and became organized into short and thin wisps/spikes (Fig. 2.2C2-3, F, G). These wisps/spikes appeared quite different from the F-actin phenotype observed upon downregulation of aPKC in muscles. Similar F-actin wisps have previously been shown to be associated with the loss of Fodrin, a non-erythrocyte type spectrin (Sato et al., 2004). Thus while loss of spectrin changes the configuration of postsynaptic F-actin from meshwork-like to wisps, aPKC downregulation influences the size of the postsynaptic F-actin/spectrin domain.

**The aPKC substrate Baz regulates the size of the postsynaptic F-actin-rich area**

The above results suggest that spectrin is critical for postsynaptic F-actin organization, but does not seem to be the sole determinant. To search for additional regulators of F-actin and spectrin that might underlie the effects of aPKC at the
postsynaptic region, we focused on a known aPKC substrate, Baz/Par-3, which is also present at the NMJ (Nagai-Tamai et al., 2002; Ruiz-Canada et al., 2004). Baz has been implicated in the formation of actin-based cellular junctions in mammalian epithelial cells (Lin et al., 2000; Munro, 2006). At the larval NMJ, Baz is highly enriched at the postsynaptic F-actin region (Ruiz-Canada et al., 2004), but it is also present in a punctate pattern at microtubule-rich areas (Fig. 2.3A) where aPKC is localized (Ruiz-Canada et al., 2004). Therefore, we determined if the regulation of postsynaptic F-actin and spectrin by aPKC could involve Baz. Consistent with this view, decreasing aPKC activity in muscle alone, by expressing aPKC-RNAi in muscles resulted in a reduction in Baz localization at the postsynaptic region in exact correspondence with the reduction in F-actin area (Fig. 2.3B, I). This decrease in Baz, spectrin, and F-actin at the postsynaptic region was quite specific, as it was not accompanied by a significant decrease in the levels or localization of other proteins present at this region, such as the scaffolding proteins DLG and Scrib (Suppl. Fig. 2.2B, C). Thus, aPKC might regulate postsynaptic F-actin in part through Baz.

The above possibility was examined by determining if downregulating Baz in muscles mimicked the consequences of decreasing aPKC with respect to NMJ structure and localization of synaptic proteins. Null mutations in baz result in embryonic lethality due to the role of this protein in establishing cell polarity (Izumi et al., 1998; Lin et al., 2000; Nagai-Tamai et al., 2002). Therefore, in order to downregulate Baz we expressed Baz-RNAi in the muscle using the UAS-Gal4 system. To support the idea that the effects observed were not due to off target
RNAi effects, we used two different RNAi lines in which the RNAi was directed against two different regions of the Baz mRNA, as well as Baz-RNAi in a baz/+ heterozygous background. In all of the above genotypes Baz immunoreactivity was significantly decreased (Fig. 2.3C, D; Suppl. Fig. 2.3). Further, downregulating baz in all of the genotypes resulted in phenotypes that completely overlapped with those observed by aPKC downregulation in muscles. First, the volume and intensity of the spectrin and F-actin rich postsynaptic region was drastically reduced (Fig. 2.3E-H, J, K). Second, synaptic boutons were significantly reduced in number and had an abnormal morphology as well as a significant increase in volume, similar to aPKC-RNAi-post (Fig. 2.3L, M). Similar reductions in synaptic bouton number have been observed in other conditions that alter the synaptic cytoskeleton (Pielage et al., 2006; Koch et al., 2008). This reduction in bouton number was not seen in the UAS line or the muscle specific Gal4 line alone showing that these insertions by themselves do not affect NMJ morphology or bouton number (Suppl Fig. 2.4). Thus, downregulation of baz or dapkc result in the same phenotypes.

**Phospho-Baz is excluded from the F-actin/spectrin rich postsynaptic region**

The above results raised a number of important questions. For example, endogenous aPKC is not localized at the postsynaptic F-actin rich area, but rather is present in puncta outside this area (Ruiz-Canada et al., 2004) (see Fig. 2.1A). Therefore, if aPKC regulates F-actin by phosphorylating Baz, then this event must occur outside the F-actin-rich postsynaptic area. This possibility was addressed by
examining the localization of phosphorylated Baz, and the dependency of Baz phosphorylation on aPKC activity. Phospho-specific Baz antibodies were raised against the conserved peptide containing the serine residue (S980 in Baz) known to be phosphorylated by aPKC in mammals and flies (Nagai-Tamai et al., 2002; Benton and St Johnston, 2003b). Surprisingly, we found that in postsynaptic muscles phospho-Baz immunoreactivity was present in a punctate pattern in the muscle (Fig. 2.4A) and was actually excluded from the F-actin/spectrin-rich area (Fig. 2.4D). In addition phospho-Baz was present inside presynaptic boutons (Fig. 2.4D). This immunoreactivity was specific and depended on Baz phosphorylation by aPKC as demonstrated by four lines of evidence. First, expressing a constitutively active aPKC form, PKM, in muscles increased the overall size and intensity of the phospho-Baz immunoreactive muscle puncta (Fig. 2.4B, F). Second, decreasing aPKC levels specifically in muscle by expressing aPKC-RNAi resulted in substantial decrease in the size and intensity of the puncta (Fig. 2.4E, F). Third preincubating the antibody with the phosphopeptide used as immunogen completely eliminated immunoreactivity (Suppl. Fig. 2.5A, B). Finally, phospho-Baz immunoreactivity was significantly reduced in baz/+; Baz-RNAi-post larvae (Fig. 2.4C, F).

To determine if endogenous P-Baz puncta in the muscle cortex colocalized with a subset of endogenous Baz puncta, we carried out sequential immunolabeling (incubations: P-Baz primary → wash → FITC-secondary → wash → Baz primary → wash → TxB-secondary → wash), as both anti-P-Baz and anti-Baz antibodies were made in rabbit, precluding the simultaneous double-labeling
technique (Fig. 2.4G). To ensure that the application of the first round of rabbit secondary (FITC-secondary) completely saturated binding sites in the P-Baz antibody, we performed a control, in which samples were incubated with TxR-secondary after the FITC-secondary antibody (incubations: P-Baz primary → wash → FITC-secondary → wash → TxR-secondary → wash) (Fig. 2.4I). No labeling in the red channel was found under these conditions, demonstrating complete saturation of the anti-P-Baz antibody binding sites after the FITC-secondary incubation (Fig. 2.4I2). We found that every P-Baz puncta exactly colocalized with a Baz puncta (Fig. 2.4G, arrows). However, only a subset of Baz puncta colocalized with P-Baz puncta (Fig. 2.4G, arrowheads), showing that not every Baz puncta becomes endogenously phosphorylated. This result was confirmed by expressing a GFP-tagged Baz transgene (Baz-GFP) in wild type muscles, which colocalized to a subset of Baz puncta (Suppl. Fig. 2.6 A; yellow arrowheads). When the Baz-GFP-post samples were double labeled with the P-Baz antibody, we observed a subset of puncta which were positive for both GFP as well as phospho-Baz immunoreactivity (Suppl. Fig. 2.6B; yellow arrowheads). To determine if this colocalization was the result of simply random coincidence between puncta in the Baz, P-Baz and GFP channels, we rotated the GFP channel at a 90° angle. If the colocalization was simply coincidence, then this rotation should not affect the number of puncta that are colocalized. However, upon 90° rotation there was little colocalization between the GFP and Baz or P-Baz puncta (Suppl. Fig. 2.6C, D), demonstrating that Baz-GFP and P-Baz or Baz-GFP and Baz show a true colocalization. Thus, Baz is phosphorylated at S980
by aPKC outside the postsynaptic F-actin/spectrin-rich region, and therefore the Baz protein observed at the postsynaptic F-actin/spectrin-rich area most likely corresponds to dephosphorylated Baz.

The ability of aPKC to phosphorylate Baz was also demonstrated in Schneider-2 (S2) cells single or double transfected with aPKC and/or Baz. Baz is endogenously expressed in S2 cells but at low levels, and therefore a very faint phospho-Baz band was observed in untransfected cells (Fig. 2.4H), most likely due to the activity of endogenous aPKC. Ratiometric comparison of phospho-Baz levels to Baz levels in different lanes showed that cells transfected with Baz alone or with PKM alone showed a weak increase in the intensity of this band (Fig. 2.4H; Suppl. Fig. 2.7A). However, this increase was not related to the absolute levels of Baz and was due in part to the presence of endogenous proteins. In contrast, cells transfected with Baz and PKM showed a drastic enhancement (100% increase) in the intensity of the phospho-Baz band (Fig. 2.4H; Suppl. Fig. 2.7A). This effect was reversed by treating the Western blots with lambda phosphatase, which eliminated the increase in the P-Baz band levels in Baz and PKM double transfected cells (Fig. 2.4J).

PTEN is a phosphatase localized at the F-actin rich postsynaptic area and is required for postsynaptic Baz localization

The observations described above, suggest that Baz is phosphorylated by aPKC outside the postsynaptic F-actin/spectrin-rich area. Further, they indicate that
Baz protein at the postsynaptic F-actin/spectrin-rich region represents **dephosphorylated** Baz. This raised the question of why a decrease in aPKC activity, which results in a decrease in the phosphorylation of Baz, actually leads to a decrease in Baz at the F-actin/spectrin-rich area. A potential model is that Baz phosphorylation is required for proper transport or targeting of Baz to the F-actin/spectrin-rich area, but that the retention of Baz at this site requires a dephosphorylation event (Fig. 2.5A). To test this model, we used a candidate approach to identify the putative phosphatase mediating Baz dephosphorylation.

Recent studies in flies have demonstrated that Baz interacts with the lipid and protein phosphatase PTEN (von Stein et al., 2005; Pinal et al., 2006) and that Baz is required to recruit PTEN to regions of actin remodeling (Pinal et al., 2006). Therefore, an attractive Baz phosphatase candidate was PTEN. Three predictions of our hypothesis (Fig. 2.5A) are that (1) PTEN should be localized to the F-actin/spectrin-rich postsynaptic area, where dephospho-Baz is localized, (2) that mutations in pten should decrease the amount of dephospho-Baz localized at the F-actin/spectrin-rich region, and (3) that mutations in PTEN should increase the amount of P-Baz at the muscle cortex (outside of the F-actin/spectrin-rich area). To test these predictions we generated an anti-peptide PTEN antibody recognizing all PTEN splice variants (PTEN1-3 (Pinal et al., 2006); Suppl. Fig. 2.5C, D). As predicted, in wild type larvae, PTEN was highly enriched at the F-actin/spectrin region where it exactly colocalized with Baz as determined by its precise colocalization with F-actin at this area (Fig. 2.5D, F). The PTEN immunostaining was
specific, as expressing PTEN-RNAi, which knocks down all PTEN isoforms, in muscles of wild type (PTEN-RNAi-post) or in a pten/+ heterozygous background, resulted in a drastic reduction in both postsynaptic as well as muscle cortical PTEN levels (Fig. 2.5E, B, C). The pten mutant utilized in this study (see methods) has been previously identified as a null allele of pten resulting from an F-element insertion that completely eliminates the phosphatase domain, and which leads to embryonic lethality (Gao et al., 2000). Furthermore, preincubating the antibody with the peptide used as immunogen virtually eliminated immunoreactivity from the muscle cortex (Suppl Fig. 2.5C, D).

The colocalization between Baz and PTEN, as well as the previously reported interactions between the two proteins placed PTEN as a strong candidate for mediating the dephosphorylation of Baz at the F-actin/spectrin-rich postsynaptic area, and according to our model, for the retention of Baz at this area. Therefore, we next examined if downregulating PTEN would result in changes in the localization of Baz at the peribouton area. As predicted, downregulating PTEN resulted in a drastic reduction of Baz localization at the postsynaptic F-actin rich area (Fig. 2.6A, B, K) and a similar reduction was observed in both the spectrin (Fig. 2.6C, D, I, J) as well as the F-actin area (Fig. 2.6I, J). Other markers of the F-actin/spectrin area, such as DLG and Scrib were not changed (Supp. Fig. 2.2B, C).

Consistent with our hypothesis, downregulating PTEN in muscles also resulted in a significant increase in muscle P-Baz puncta outside the postsynaptic F-actin rich area (Fig. 2.6E, F, L). Together, these results provide compelling evidence
that PTEN induces the dephosphorylation of Baz at S980, and that phosphorylated Baz is not retained at the postsynaptic region. Interestingly, downregulating Baz also resulted in a decrease in PTEN localization at the peribouton area (Fig. 2.6G, H) consistent with previous observations in photoreceptor cells that Baz has a role in recruiting PTEN to the membrane (Pinal et al., 2006).

The possibility that PTEN could be involved in dephosphorylating Baz was also tested in S2 cells single or double transfected with Baz and PTEN variants. Given that Baz has been shown to specifically interact with PTEN2 and not with other isoforms of PTEN (von Stein et al., 2005; Pinal et al., 2006), wild type and mutated PTEN2 cDNAs were used to transfect S2 cells. Transfection with Baz showed that Baz was endogenously phosphorylated in S2 cells as revealed with the P-Baz antibody (Fig. 2.6M; Suppl. Fig. 2.7B). In contrast, in cells double transfected with Baz and PTEN2, the level of P-Baz phosphorylation was drastically reduced (Fig. 2.6M; Suppl. Fig. 2.7B), demonstrating that PTEN is involved in the dephosphorylation of Baz. Additionally, mutating the PDZ binding domain of PTEN2, responsible for its interaction with PDZ2-3 of Baz, completely prevented this dephosphorylation (Fig. 2.6M; Suppl. Fig. 2.7B), suggesting that the direct interaction between Baz and PTEN2 is required for Baz dephosphorylation. This observation further raises the possibility that PTEN might directly dephosphorylate P-Baz.

PTEN has both lipid and protein phosphatase enzymatic activity (Lee et al., 1999). Therefore, we next tested which of these activities was required for the
dephosphorylation of P-Baz. In particular, we generated a PTEN2 variant, PTEN2\textsuperscript{C132A}, in which a conserved cysteine (C124 in mammals, C132 in flies) is required for both protein phosphatase and lipid phosphatase activity (Cai et al., 2005), was mutated to alanine. In addition, we generated a PTEN2 variant, PTEN2\textsuperscript{G137E}, in which a conserved glycine (G129 in mammals and G137 in flies), required for the lipid phosphatase activity alone (Cai et al., 2005), was mutated to glutamate. As expected, blocking lipid phosphatase activity in PTEN2\textsuperscript{G137E}, did not affect the ability of PTEN2 to dephosphorylate P-Baz (Fig. 2.6M; Suppl. Fig. 2.7B). In contrast, blocking both the protein and lipid phosphatase activity in PTEN2\textsuperscript{C132A} completely blocked P-Baz dephosphorylation (Fig. 2.6M; Suppl. Fig. 2.7B). Together with the observation that normal localization of Baz at the peribouton area depends on PTEN, that PTEN colocalizes with dephospho-Baz, the above results are consistent with the model that dephosphorylation of Baz by PTEN is required to retain Baz at the postsynaptic region, that Baz dephosphorylation requires its direct interaction with PTEN and depends on the protein phosphatase activity of PTEN.

**Downregulation of PTEN partially mimics mutations in baz and both genes interact**

In addition to the changes in Baz, Spec, and F-actin upon PTEN downregulation, which were identical to those observed by reducing Baz or aPKC in muscles, we found that other common phenotypes were also present. For example, in both \textit{pten}/+; PTEN-RNAi-post or PTEN-RNAi-post alone there was a significant
decrease in the number of synaptic boutons (Fig. 2.7A). In addition, bouton volume was significantly increased in PTEN-RNAi post larvae (Fig. 2.7B).

To further support the notion that PTEN functions in the same developmental pathway at the NMJ as Baz, we used a classical genetic test in null mutant transheterozygotes (Anholt and Mackay, 2004; Greenspan, 2006; Pawson et al., 2008). Heterozygote pten/+ or baz/+ larvae had NMJs that were indistinguishable from wild type (Fig. 2.7C). However, the transheterozygotes displayed a significant reduction in the number of synaptic boutons, strongly suggesting the presence of genetic interactions between pten and baz (expected reduction in synaptic bouton number for purely additive interactions is 19% reduction vs. 30% reduction found in the transheterozygote; Fig. 2.7C).

**Downregulation of aPKC, PTEN and Baz have similar effects on synaptic transmission and glutamate receptor localization**

Previous studies demonstrated that mutations in aPKC led to a drastic increase in glutamate receptor IIA (GluRIIA) levels and a substantial increase in the amplitude of spontaneous events (Ruiz-Canada et al., 2004). Therefore we next examined if GluR localization and synaptic transmission was similarly altered by down regulating baz and pten postsynaptically. We found that the volume, mean intensity, and total intensity of GluRIIA clusters were significantly increased in baz/+; Baz-RNAi-post and pten/+; PTEN-RNAi-post NMJs (Fig. 2.8A, C, D, G), and this increase was similar to that observed by downregulating aPKC in muscle cells (Fig.
In addition to the increase in GluRIIA, other glutamate receptors such as GluRIIB and GluRIII were also increased (Suppl. Fig. 2.8A, B). The increase in GluR cluster size and intensity was consistent with the observation that the amplitude of miniature excitatory junctional potentials was also increased in all three mutants (Fig. 2.8F, H).

While the changes in mEJP amplitude were similar upon downregulating aPKC, Baz, and PTEN, other functional defects differed among genotypes. For example, the amplitude of nerve evoked excitatory junctional potentials (EJP) was significantly increased when Baz and aPKC were downregulated, no such increase in EJP amplitude was observed upon PTEN downregulation (Fig. 2.8E, J). In addition, while there was a significant increase in the frequency of mEJPs upon downregulating Baz and PTEN, no such change was observed in aPKC-RNAi-post (Fig. 2.8I). Thus, while the distribution of GluRs and mEJP amplitude is likely to operate using a common pathway involving aPKC, Baz and PTEN, there are other functional aspects of synaptic transmission in which the function of each protein might be independent. Alternatively, compensatory mechanisms observed at these synapses (DiAntonio et al., 1999) might be activated to different extents in the three mutant backgrounds.

Discussion

Previous studies have implicated the actin-binding proteins spectrin and Coracle in NMJ development and in the selective clustering of GluR subunits most likely through the regulation of pre- and postsynaptic F-actin (Chen et al., 2005;
Pielage et al., 2006). Here we identify an additional novel mechanism by which postsynaptic F-actin/spectrin is regulated during NMJ expansion. Previously, we reported that the aPKC-Baz-Par-6 complex is present at the NMJ and that aPKC was required for normal NMJ expansion and presynaptic microtubule stability (Ruiz-Canada et al., 2004). Here, we show that aPKC and Baz in conjunction with the lipid and protein phosphatase PTEN play an additional role in regulating postsynaptic F-actin/spectrin through a novel mechanism. We propose that Baz is required to stabilize the postsynaptic F-actin/spectrin meshwork, and this process involves changes in its phosphorylation state (Fig. 2.9). Our evidence suggests that proper targeting of Baz to the postsynaptic region requires its phosphorylation by aPKC, but its retention at this region may requires its dephosphorylation by PTEN (Fig. 2.9).

Several lines of evidence support this model. First, reduction of aPKC, Baz and PTEN levels in the muscles result in a similarly dramatic reduction in postsynaptic F-actin. Second, Baz localization at the postsynaptic F-actin-rich region depends on its phosphorylation state. While phosphorylated Baz is excluded, dephosphorylated Baz is concentrated at this site. Third, aPKC is also excluded from the F-actin-rich region and a decrease in Baz phosphorylation by aPKC results in a decrease in Baz at the F-actin rich area. This suggests that aPKC-dependent phosphorylation of Baz occurs outside the F-actin-rich region and that this phosphorylation may be required for proper targeting but not for retention of Baz at the actin-rich postsynaptic region. Fourth, PTEN colocalizes with dephospho-Baz at the actin-rich postsynaptic region and reducing PTEN levels results in a reduction in
Baz at this site, and in an increase in phospho-Baz levels in the surrounding area. Thus, the retention of Baz at the F-actin-rich area requires its dephosphorylation, which is likely mediated through PTEN. Fifth, there is a significant overlap in NMJ phenotypes upon downregulating Baz, aPKC and PTEN, and Baz and PTEN interact genetically suggesting that they might function in the same pathway during NMJ expansion.

Studies in mammalian cells have implicated Par-3 in the maintenance of actin-based junctions, such as the tight junction (Chen and Macara, 2005; Nishimura et al., 2005). Baz is an aPKC substrate, and our studies at the NMJ show that it is in exact colocalization with F-actin. Collectively, this indicates that the effects of aPKC on F-actin/spectrin are mediated most likely through Baz, and that Baz is required for the normal organization of the postsynaptic F-actin/spectrin meshwork. Any reduction in postsynaptic Baz levels as seen by decreasing aPKC, Baz, or PTEN resulted in a decrease in the density and thickness of the postsynaptic F-actin cytoskeleton.

Several potential mechanisms by which Par-3/Baz might regulate F-actin have been suggested, including dephosphorylation of Cofilin through the inhibition of LIM kinase (Chen and Macara, 2006), and regulation of the Rac Guanine nucleotide exchange factor, Tiam1 (Chen and Macara, 2005; Nishimura et al., 2005). Although, Drosophila LIMK and Tiam1 are absent from the postsynaptic region (Sone et al., 1997; Eaton and Davis, 2005), Baz might regulate dPIX, another Rho-GEF that is enriched postsynaptically (Parnas et al., 2001) and its downstream effector dPAK.
Another potential mechanism could involve an interaction between the PDZ2 domain of Baz and membrane phospholipids (Wu et al., 2007). By binding to phospholipids, Baz might bring together a number of actin regulators to the membrane to remodel postsynaptic F-actin.

Cell culture studies demonstrate that Par-3 is phosphorylated by aPKC, which results in the dissociation of the two proteins and targeting and stabilization of phospho-Par-3 at sites of tight junction formation (Hirose et al., 2002; Nagai-Tamai et al., 2002). We hypothesize that phosphorylation of Baz at S980 is similarly required to target Baz to the postsynaptic region. We determined that phospho-S980-Baz was distributed in puncta at the muscle cortex, but excluded from the postsynaptic F-actin-rich area. Consistent with previous studies (Nagai-Tamai et al., 2002), altering muscle aPKC levels resulted in the respective increase or decrease in phospho-Baz levels in the muscle. Thus, our immunocytochemical results, supported by our biochemical assays, strongly validate the idea that Baz is phosphorylated by aPKC in the muscle. To our knowledge, this is the first study to demonstrate the phosphorylation of Baz by aPKC in the context of an intact organism, and during NMJ development. We suggest that, as in mammals, phosphorylation of Baz might disrupt the binding between Baz and aPKC allowing phospho-Baz to be mobilized to the postsynaptic region. However, unlike previous studies which hypothesized that phospho-Par-3 was stabilized at the apical region (Nagai-Tamai et al., 2002), our studies showed that phospho-Baz was absent from the postsynaptic region. Instead, we found that Baz at this region was present in a
dephosphorylated state. Thus we postulate that while targeting Baz to the postsynaptic region requires aPKC phosphorylation, its actual retention at this site necessitates its dephosphorylation.

Studies in epithelial cells show that Baz interacts with the lipid and protein phosphatase PTEN through binding between the PDZ2-3 domain of Baz and the PDZ binding motif in PTEN2 (von Stein et al., 2005; Pinal et al., 2006). Further, these studies demonstrated that Baz was required for the recruitment of PTEN to regions of actin remodeling (Pinal et al., 2006). These observations made PTEN a prime candidate for Baz dephosphorylation. Confirming this model, we found that PTEN was colocalized with dephospho-Baz at the postsynaptic region. Moreover, reducing PTEN activity resulted in a decrease in dephospho-Baz at the postsynaptic region and an increase in phospho-Baz in muscle suggesting that PTEN-dependent dephosphorylation of Baz is necessary to retain Baz at the postsynaptic region. Additionally, our biochemical assays indicate that this dephosphorylation requires the PDZ interaction between PTEN and Baz and is mediated by the protein phosphatase activity of PTEN. PTEN has been shown to directly dephosphorylate many different proteins such as FAK kinase (Tamura et al., 1998), but might also function indirectly through the activation of other protein phosphatases (Traweger et al., 2008). Notably, we found that the localization of Baz and PTEN was interdependent for their mutual localization at the postsynaptic region. Consistent with results of previous studies (Pinal et al., 2006), Baz downregulation also
decreased postsynaptic PTEN supporting the idea that Baz is required for PTEN localization at postsynaptic sites.

Several studies have implicated PTEN in regulating synaptic structure and function (Fraser et al., 2008), neurotransmitter receptors (Ning et al., 2004; Ji et al., 2006), hippocampal LTD (Wang et al., 2006), neuronal arborization, and social interactions in mice (Kwon et al., 2006). Our studies demonstrate for the first time that PTEN is involved in inducing the dephosphorylation of Baz and the regulation of the postsynaptic F-actin cytoskeleton. Thus, these studies reveal a potential molecular mechanism for PTEN function in the nervous system.

A previous study in embryonic NMJs demonstrated a role for postsynaptic F-actin in the proper clustering of GluRIIA-, but not of GluRIIB-receptors through the fly Band 4.1 homolog, Coracle (Cora), which appears to interact directly with the C-terminal tail of GluR1IA (Chen et al., 2005). In cora mutants, GluR1IA-, but not GluR1IB-cluster size and function was reduced (Chen et al., 2005). Pharmacological disruption of F-actin using Lantrunculin mimicked this mutant phenotype, leading the authors to suggest that Cora might function to directly anchor GluR1IA subunits to F-actin (Chen et al., 2005). In our studies and Ruiz-Canada et al. (2004) we found that GluR1IA-, GluR1IB-, and GluR1II-cluster volume and intensity was significantly increased upon downregulating either aPKC, Baz, or PTEN, and that this phenotype was accompanied by an increase in the amplitude of mEJPs. However, in our studies the postsynaptic F-actin region was not completely disrupted, but significantly reduced in size. This raises the interesting possibility that beyond
anchoring receptors on the postsynaptic membrane, the F-actin and spectrin domain might act as a barrier to the diffusion and clustering of receptors at the postsynaptic area or may affect the recycling of receptors. In this regard, it is important to note that an increase in GluRIIA, GluRIIB, and GluRIII size was also observed in another study where spectrin was downregulated exclusively in the larval muscles using RNAi (Pielage et al., 2006). Furthermore, this study also found an increase in the mEJP amplitude upon downregulation of spectrin in the muscle. These studies implicated spectrin in regulating active zone size and spacing, as well as synaptic efficacy.

While some of the phenotypes reported upon spectrin elimination in the muscle, were similar to those examined here upon downregulating aPKC, Baz, and PTEN, others were quite different. For example, spectrin elimination resulted in disrupted SSR and abnormal localization of DLG. The loss of SSR, in addition to being a result of disorganized Dlg, as suggested by the study (Pielage et al., 2006), could also be due to a change in postsynaptic F-actin configuration from meshwork to wisps seen in spec mutants and β-Spec-RNAi-post. In contrast, we found that the SSR was intact upon expressing aPKC RNAi in muscles, and SSR markers such as DLG and Scrib were not affected upon downregulating aPKC, Baz, and PTEN. It was suggested that either the F-actin/spectrin network might be responsible for the stabilization of GluRs, the spectrin-actin hexagonal lattice might serve as a framework that constrains the size of active zones and postsynaptic receptors, or that changes in GluR size and spacing could be a secondary consequence of the disruption of the SSR (Pielage et al., 2006). Our findings that the SSR in aPKC-
RNAi-post and DLG distribution upon downregulating aPKC, Baz, and PTEN is not affected, but that GluR clusters are increased in size suggest that the size of GluR cluster is not necessarily dependent on the SSR or DLG. Together with the studies of Chen et al. (2005) demonstrating that disruption of F-actin does not alter the formation of GluRIIB-clusters, the results of Pielage et al. (2006) and ours are consistent with the idea that the F-actin/spectrin network might restrict GluR-cluster size.

Downregulating aPKC, Baz, or PTEN levels in the muscle, besides decreasing postsynaptic F-actin localization, also result in a substantial increase in synaptic bouton size and a decrease in NMJ expansion. As the larval NMJ arbors are completely surrounded by the muscle membrane, NMJ expansion and bouton size are likely regulated by forces involving a balance between cytoskeletal extension at the presynaptic arbors and cytoskeletal retraction at the postsynaptic sites. Not surprisingly, interfering with this balance leads to misregulation of NMJ extension and bouton size. Interestingly, our previous studies suggest that aPKC might play different roles in the regulation of the presynaptic versus postsynaptic cytoskeletons. At the presynapse, aPKC is associated with microtubules and regulates microtubule stability through interactions with the presynaptic MAPB-related protein Futsch (Ruiz-Canada et al., 2004). In muscles, aPKC is also associated with microtubules, but regulates both postsynaptic F-actin/spectrin through Baz (this report) and postsynaptic microtubules (Ruiz-Canada et al., 2004). The exact mechanisms for the regulation of postsynaptic microtubules by aPKC are
not known. This regulation might involve the modulation of an as yet unknown muscle microtubule-associated protein, or might result from indirect regulation by the stabilization of F-actin, which might exclude microtubules. In summary, our study reveals a novel mechanism by which the postsynaptic F-actin cytoskeleton is regulated during NMJ growth. In this mechanism F-actin organization at the postsynaptic region depends at least in part on Baz function. In turn, proper localization of Baz at this F-actin region depends on the opposing actions of aPKC and PTEN. The conservation of the aPKC-Baz-PTEN interaction across different cell types argues in favor of common mechanisms for cytoskeletal regulation by this complex in many tissues. Further, our studies establish a mechanism by which the dynamics of the postsynaptic actin cytoskeleton might be regulated during plasticity in the brain.

**Materials And Methods**

**Fly strains**

Flies were reared at 25°C in standard media unless otherwise specified. The following strains were used: wild type (Canton-S), baz^{815-8} (Benton and St Johnston, 2003a) (referred to as baz mutants in the text), pten^{dij189FRT40A} (Gao et al., 2000) (referred to as pten mutant in the text), and spec^{em6} (Dubreuil et al., 2000). We also used the UAS strains UAS-Baz RNAi-1 (see below for generation of this strain), UAS-Baz RNAi (VDRC; referred here to as UAS-Baz-RNAi-2), UAS-PTEN RNAi (Bloomington Stock Center), UAS-aPKC RNAi (see below for generation of this
strain), UAS-β-spectrin RNAi (Pielage et al., 2006), UAS-PKM (Drier et al., 2002), and UAS-Baz-GFP (Benton and St Johnston, 2003a). To express transgenes in muscles we used the C57 and BG487 Gal4 strains (Budnik et al., 1996). RNAi crosses were raised at 29˚C unless otherwise specified.

**Generation of RNAi strains**

The aPKC-RNAi transgene was designed against exons 6 and 7 of the *dapkc* genomic region. The Baz-RNAi transgene was designed against exons 3 and 4 of the *baz* genomic region. RNAi constructs were made as in (Kalidas and Smith, 2002). Transgenes were subcloned into the pUAST vector and sent for germ-line transformation to Genetic Services Inc. (Cambridge, MA).

**Immunocytochemistry**

Body wall muscles from third instar larvae were dissected in Ca++-free saline (Jan and Jan, 1976) and fixed for 30 minutes with 4% paraformaldehyde or 15 min with non-alcoholic Bouin’s. Anti-Baz (Wodarz et al., 2000) (1:500), anti-PTEN (1:200; see below), anti-phospho-Baz (1:100; see below), anti-α-spectrin (1:30; Developmental Studies Hybridoma Bank [DSHB]), anti-GluRIIA (1:10; DSHB), anti-GluRIIB and anti-GluRIII (Marrus et al., 2004) (1:500 and 1:200 respectively), anti-DLG_PDZ (Koh et al., 1999) (1:20,000), and anti-Scribble (Roche et al., 2002) (1:1000), anti-aPKC (1:1000, Sigma, Santa Cruz, CA), Texas Red or Cy5-conjugated anti-HRP (1:200; Jackson Immunoresearch, West grove, PA) were used as primary antibodies. Postsynaptic and muscle actin was labeled using rhodamine-conjugated phalloidin (1:50-1:100; Molecular Probes). Glutamate receptor staining
was performed with an additional round of amplification with anti-FITC (1:800; Sigma, Santa Cruz, CA). As secondary antibodies, FITC-, TxRed-, and Alexa 647-conjugated antibodies (Jackson Immunoresearch, West grove, PA) were used at 1:200.

**Generation of the PTEN and Phospho-Baz antibodies**

The affinity purified phospho-Baz antibody was raised in rabbits against the peptide CLGRRSISEK with the phosphorylation on the first serine residue (CLGRRpSISEK) by Biosource (also known as QCB; Hopkinton, MA). Affinity purified antibodies against PTEN were raised against two different peptides, CIRNVVSKKRIRYKEKGYD and CISVLHDSATENAKPDRLK, which were coinjected in rabbits by QCB. The specificity of the antibodies was tested by expressing specific RNAi transgenes in muscles and with transfected S2 cells using both immunocytochemistry as well as Western blot analysis.

**Confocal microscopy and morphological quantification**

Images were acquired using a Zeiss (Oberkochen, Germany) confocal microscope using a 63X objective (1.4 numerical aperture). For comparison between genotypes, confocal stacks were collected from samples that were processed simultaneously and imaged using identical confocal acquisition parameters. Quantification of fluorescence signal intensity was performed by volumetric measurements of the raw data through confocal stacks using Volocity 4.0 Software (Improvision, Waltham, MA). For measurement of postsynaptic volume (V) and intensity (I) single boutons were selected and analyzed as three-dimensional
volumes in Volocity. The labeled region around the boutons was segmented by intensity thresholding based on the difference in the intensities at the NMJ vs background intensity. Volume and fluorescence intensity represent the sum of the volumes or intensities of each of the voxels that fell above the threshold value. Presynaptic volume or intensity was measured by calculating the volume or intensity occupied by the label of interest that overlapped with the volume occupied by the anti-HRP label (presynaptic bouton volume). To obtain postsynaptic volume or intensity, the anti-HRP volume was subtracted from the total volume or intensity. Postsynaptic volumes and intensities were expressed as $V_{\text{post-vol or int}} / V_{\text{bouton}}$. For quantification of phospho-Baz, aPKC, or PTEN signal intensity in muscles, a specific region of muscle 6 was chosen and the puncta were quantified based on size and intensity. Once puncta were segmented, measurements were run for average intensity and size. For GluRIIA, GluRIIB and GluRIII quantification, the volume, mean intensity, and total intensity of individual clusters were calculated using Volocity software. Data were normalized to wild type samples. For F-actin, confocal imaging and quantification was done at muscle 12/13 where the distance between postsynaptic F-actin and the F-actin within the contractile apparatus is relatively large (see Fig. 2.1C), thus allowing the acquisition of images of NMJ F-actin without interference from the strong myofibril background. All other quantifications were done at muscles 6/7. For determination of the number of type I boutons, body wall muscle preparations were double stained with anti-HRP and anti-Dlg. Measurements were taken from muscles 6 and 7, abdominal segment 3. In all experiments,
quantifications of mutant phenotypes were normalized to wild type control samples dissected and imaged using the confocal microscope in the same experimental session. For statistical analysis, unpaired Student’s t-tests were run for comparisons between the experimental samples and the wild type controls, which were processed simultaneously. If the variance between the samples was significant, an unpaired t-test with Welch’s correction (Frank and Althoen, 1994) was performed. Numbers in histograms represent mean ± SEM. *=p< 0.05, **=p< 0.001, ***=p< 0.0001.

**Western Blots**

Full-length Baz, PKM and dPTEN2 cDNAs were subcloned into the pAcV5.1/HisA or -B vector (Invitrogen, Carlsbad, CA) for transfection into *Drosophila* Schneider-2 (S2) cells. Mutations in the dPTEN2 construct eliminating the lipid (G137E) and protein (C132A) phosphatase activity were carried out using the QuikChange II Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA). Cell cultures were centrifuged at 1000g X 5min, the pellet lysed in lysis buffer (20mM Tris HCL pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% NP-40, 25µM MG132, protease inhibitor cocktail (Thomas et al., 1997), and phosphatase inhibitor I and II cocktail (Calbiochem, San Diego, CA) for the kinase assay, and without the phosphatase inhibitor for the PTEN dephosphorylation assay, and then boiled with sample buffer containing 10% DTT and 5% β-mercaptoethanol. Proteins were separated on 5% SDS PAGE gels, transferred onto a PVDF or nitrocellulose membrane and sequentially immunoblotted with anti-phospho-Baz (1:5000), anti-Baz (1:2000) and anti-α-spectrin (1:1000). For the lambda phosphatase assay,
proteins were transferred onto a nitrocellulose membrane and incubated in Tris Buffer Saline (TBS) containing 1% BSA, 0.1% Triton X-100, 2 mM MnCl₂ and 400 µl/ml of lambda phosphatase (Upstate, Lake Placid, NY) for 1 hour at room temperature, following which they were washed with TBS with 0.1% Tween20 and sequentially probed with anti-phospho-Baz (1:5000) and anti-Baz (1:2000). Bands were visualized by chemoluminiscence methods (Amersham, Piscataway, NJ). Normalized P-Baz/Baz band intensities were measured using Image J. After adjusting for loading (spectrin) control and subtracting the background provided by non-transfected controls, values were normalized to Baz or P-Baz from Baz-transfected cells, and the ratio P-Baz/Baz calculated.

**Transmission Electron Microscopy**

Body wall muscles were prepared for TEM as in (Jia et al., 1993; Packard et al., 2002). Analysis of SSR thickness was done as (Budnik et al., 1996). For analysis of the distance between the outer SSR boundary and the myofibrils at muscle 12, 15 boutons from 3 preparations were used. Comparisons of SSR thickness in wild type and aPKC-RNAi-post animals was done from 3 wild type (9 boutons measured at the bouton midline) and 3 aPKC-RNAi-post animals (6 boutons measured at the bouton midline). For statistical analysis, unpaired Student’s t-tests were run for comparisons between the experimental samples and the wild type control.

**Electrophysiology**
Larval body wall muscle voltage recordings were performed as in (Ashley et al., 2005). Briefly, recordings were collected from muscle 6 of wandering 3rd instar larvae, and only those muscles with resting potentials between –60 mV to –63 mV were analyzed. Larva were dissected in HL-3 saline (Stewart et al., 1994) containing 0.3 mM Ca\(^{2+}\) and recorded in HL-3 saline containing 0.5 mM Ca\(^{2+}\). Recordings collected using an Axoclamp 2A (Molecular Devices, Union City, CA) were digitized using an Instrutech (Port Washington, NY) ITC-16 computer interface, processed using Pulse software (HEKA Electronik, Lambrecht/ Pfalz, Germany) and measured using Mini Analysis software (Synaptosoft, Decatur, GA.). For statistical analysis, unpaired Student’s t-tests were run for comparisons between the experimental samples and the wild type controls.
Figure 2.1. Arrangement of postsynaptic F-actin and the SSR in relationship to the contractile apparatus.

(A) Diagram of the NMJ showing the relative positions of post-synaptic Factin/spectrin, aPKC, microtubules, the SSR and the contractile apparatus. N= nucleus.

(B) 3-D rendered confocal images of a wild type arbor at muscle 12 labeled with anti-HRP (blue) and rhodamine-phalloidin (red) in (left panel) a X-Y-Z 3-D isosurface rendering showing a single NMJ branch containing synaptic boutons (blue) surrounded by postsynaptic Factin-rich domain (red) sitting above the contractile apparatus (red) of the muscle. In the right panel, the same image is now shown in a Y-Z plane, demonstrating that postsynaptic F-actin (red) surrounding synaptic boutons (blue) appears separate from the F-actin at the contractile apparatus (distance marked by a two-way arrow). Arrow and arrowhead point to the postsynaptic actin.

(C) Transmission electron micrograph through an NMJ branch at muscle 12 (m12) showing several boutons (lb, ls, III) and a region of the contractile apparatus near the NMJ branch-point. Red two-way arrows show the distance between the SSR and the myofibrils of the contractile apparatus in these hypercontractile muscles.

(D-F) High magnification views of myofibril regions showing (D) perforated Zbands, (E) the myosin-actin lattice and the sarcoplasmic reticulum (sr), and(F) the dyads composed of the t-tubule (tt) and sr compartments.

(G) View of the nerve as it runs from muscle 13 (m13) to muscle 12 and the NMJ branch-point at muscle 12. At this region the distance between the SSR and muscle contractile apparatus (two-way arrow) is larger. N= nucleus; Z= perforated Z band; mi =mitochondria; ssr= subsynaptic reticulum. Calibration bar is 3.5 μm in B, 0.4 μm in C, G and 80 nm in D-F.
Figure 2.1
Figure 2.2. Localization of Spectrin and F-actin at NMJs of wild type, aPKC RNAi post and $\beta$-$spec^{em6}$/+ mutants.

(A, B, C) Single confocal slices of third instar NMJ branches at muscles 12/13 in preparations, triple stained with anti-HRP (blue), antic-spectrin (green), and rhodamine-phalloidin (red) in (A), wild type, (B) larvae expressing aPKC RNAi in muscles, (C) $\beta$-$spec^{em6}$/+ mutants.

(D, E, F) are high magnification views of synaptic boutons in the above genotypes stained with anti-HRP (blue) and rhodamine-phalloidin (red) showing the distribution of F-actin around the presynaptic compartment labeled by anti-HRP.

(G) Extended view of an NMJ at muscle 12/13 in a $\beta$-$spec^{em6}$/+ heterozygote larval body wall muscle preparation stained with anti-HRP (blue) and rhodamine-phalloidin (red) showing the organization of F-actin into wisps/ spikes. Arrows point to the F-actin-rich postsynaptic area.

(H, I) Quantification of the (H) normalized volume and (I) normalized fluorescence intensity of postsynaptic $\alpha$-spectrin (white bars; n= 8 boutons for wild type and 12 boutons for aPKC RNAi) and F-actin (black bars; n= 15 boutons for both genotypes) in the indicated genotypes. Data was normalized to wild type controls.

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001.

Calibration bar is 7 μm for A, B, C, 2 μm for D, E, F, and 10 μm for G.
Figure 2.2
**Figure 2.3** Baz is enriched in the F-actin region and reduction in Baz levels phenocopy aPKC downregulation at the NMJ.

Single confocal slices of third instar larval NMJs,

(A-B) Triple stained with anti-HRP (blue), anti-Baz (green) and rhodamine phalloidin (red) in (A) wild type or (B) aPKC-RNAi-Post (muscle 12).

(C-D) Double stained with anti-HRP (blue) and anti-Baz (green) in (C) wild type and (D) baz/+; Baz RNAi-post (muscle 6 or 7).

(E-H) Double stained with anti-HRP (blue) and (E-F) rhodamine-phalloidin (red), or (G-H) anti-α-spectrin (green) in (E,G) wild type (F, H) baz/+; Baz RNAi-post (muscles 12/13 for F-actin and muscles 6/7 for spectrin)

(I-M) Quantification of (I) normalized postsynaptic Baz intensity (n= 8 boutons for both genotypes) (J) normalized postsynaptic F-actin (black bars, muscles 12/13; n= 18 boutons for wild type, 15 for Baz-RNAi-1-post, 25 for Baz-RNAi-2-post, 22 for baz/+; and 26 for baz/+; Baz RNAi-post), and spectrin (white bars, muscles 6/7; n=18 boutons for wild type, 20 for Baz-RNAi-1-post, 18 for Baz-RNAi-2-post 23 for baz/+ and 10 for baz/+; Baz RNAi-Post) intensity, (K) normalized postsynaptic volume (n is the same as in J), (L) bouton number (n= 11 arbors for wild type, 12 for aPKC-RNAi-post, 13 for Baz-RNAi-1-post, 12 for Baz RNAi-2-post, and 12 for baz/+;Baz RNAi-Post), and (M) bouton volume (n= 16 boutons for wild type, 20 for aPKC-RNAi-post, 50 for Baz-RNAi-1-post, 35 in Baz-RNAi-2-post, and 12 for baz/+;Baz RNAi-post) in the indicated genotypes.

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001; *=p<0.05. Calibration bar is 15 μm for A, B, and 10 μm for C-H.
Figure 2.3
Figure 2.4 Baz is phosphorylated by aPKC in muscles and is absent from the postsynaptic F-actin-rich area.

(A-E) Third instar muscle regions and NMJs at muscles 6 or 7 in preparations labeled with anti-phospho Baz (green) and anti-HRP (blue) in (A, D) wild type, (B) PKM-post, (C) baz/+; Baz-RNAi-post and (E) aPKC-RNAi-post. Arrow in D points to the postsynaptic F-actin rich area outlined by a white line, which is devoid of phospho-Baz.

(F) Quantification of the volume and intensity of phospho-Baz puncta in muscles of wild type, aPKC-RNAi-post, and PKM-post. Data was normalized to wild type values (n=12 muscles for wild type, 16 for aPKC-RNAi-post, 16 for PKM-post and 16 for baz/+;Baz-RNAi-post).

(G, I) Confocal image of a muscle region in a preparation (G) sequentially labeled with antibodies to P-Baz (green) and Baz (red) showing that each P-Baz puncta colocalizes to a Baz puncta (arrows), and that only a subset of Baz puncta contains phosphorylated P-Baz (arrowheads point to Baz puncta devoid of P-Baz immunoreactivity), and (I) treated similar to G, but in an experiment were the anti-Baz antibody was omitted to ensure that the first secondary antibody (FITC 2nd) saturated all anti-P-Baz sites observed in I2 by the lack of signal upon incubation with the TxR-2nd antibody.

(H, J) Western blots of S2 cell extracts transfected with the constructs indicated above the blots and probed sequentially with the antibodies indicated at the left, showing (H) the phosphorylation of Baz by PKM and (J) the lambda phosphatase assay. Untrans=untransfected cells.

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001.

Calibration bar is 14 μm for A, B, C, E, 5 μm for D, and 2 μm for G.
Figure 2.4
**Figure 2.5 PTEN is localized at the postsynaptic F-actin-rich area.**

**(A)** A model of Baz trafficking at the NMJ. See text for details.

**(B-C)** Quantification of (B) normalized postsynaptic PTEN fluorescence intensity (black bars) and normalized volume (white bars) in the indicated genotypes (n represents number of boutons) and (C) normalized cortical PTEN intensity in the indicated genotypes (n=12 muscles for wild type, and 10 for *pten/+;PTEN-RNAi-post*)

**(D-E)** Third instar NMJs at muscles 6 or 7 in preparation double stained with anti-PTEN (green) and anti-HRP (red) in (D) wild type and (E) *pten/+;PTEN-RNAi-post*.

**(F)** Third instar NMJ from wild type triple labeled with rhodamine-phalloidin (red), anti-PTEN (green) and anti-HRP (blue).

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001; *=p<0.05.. Calibration bar is 14 μm for D-F.
Figure 2.5
Figure 2.6. PTEN induces dephosphorylation of Bazooka and affects the postsynaptic actin/spectrin area.

(A-B) Third instar NMJs at muscles 6 or 7 double stained with anti-HRP (red) and anti-Baz (green) in (A) wild type, (B) PTEN-RNAi-post. Note the reduction in postsynaptic Baz in PTEN-RNAi-post compared to wild type as indicated by arrows in A3, B3.

(C-F) Third instar NMJs stained with anti-HRP (red) and either (C,D) anti-α-spectrin (green), or (E,F) anti-phospho-Baz (green) in (C,E) wild type and (D,F) PTEN-RNAi-Post.

(G-H) NMJs stained with anti-HRP (red) and anti-PTEN (green) in (G) wild type and (H) baz/+; Baz-RNAi-post.

(I, J) Quantification of (I) normalized volume and (J) normalized intensity of α-spectrin (black bars; muscles 6/7; n= 12 boutons for wild type, 30 for pten/+; 11 for PTEN-RNAi-post and 25 for pten/+;PTEN-RNAi-post) and F-actin (white bars, muscle 12; N=14 boutons for wild type, 33 for pten/+, 14 for PTEN-RNAi-post and 18 for pten/+;PTEN-RNAi-post) in the indicated genotypes. n is the same for I and J.

(K) Quantification of normalized postsynaptic Baz volume (black bars) and normalized postsynaptic Baz fluorescence intensity (white bars) in the indicated genotypes (n=13 boutons for wild type and 15 for PTEN-RNAi-post).

(L) Quantification of normalized phospho-Baz fluorescence intensity in the indicated genotypes. (n=13 muscles for wild type, 12 for PTEN-RNAi post and 14 for pten/+; PTEN-RNAi-post).

(M) Western blot of S2 cell extracts from cells transfected with the genes shown above the blots and probed sequentially with the antibodies indicated at the left, showing the dephosphorylation of Baz by PTEN.

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001; *=p<0.05. Calibration bar is 7 μm for A1-2, B1-2, C-H and 3.5 μm for A3, B3.
Figure 2.6
Figure 2.7 Downregulation of PTEN affects bouton number and bouton volume.

(A-C) Quantification of (A, C) bouton number in the indicated genotypes (for A, n= 12 arbors for wild type, 10 for PTEN-RNAi-post and 12 for pten/++; PTEN-RNAi-post. For C, n=16 arbors for wild type, 19 for baz/+, 19 for pten/+, and 20 for baz/+; pten/) and (B) normalized bouton volume (n=25 boutons for wild type and 26 for PTEN-RNAi-post. Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001; *=p<0.05.
Figure 2.7
Figure 2.8. Downregulation of aPKC, PTEN and Baz have similar effects on synaptic transmission and glutamate receptor localization.

(A-D) Third instar larval NMJs labeled with HRP (blue) and GluRIIA (red) in (A) wild type, (B) aPKC-RNAi-post, (C) baz/+;Baz-RNAi-post, and (D) pten/+;PTEN-RNAi-post.

(E, F) Electrophysiological traces of (E) EJPs and (F) mEJPs in the indicated genotypes.

(G, H, I, J) Quantification of (G) GluRIIA cluster volume (black bars), mean intensity (blue bars), and total intensity (red bars) (n=157 clusters for wild type, 172 for aPKC-RNAi-post, 160 for baz/+;Baz-RNAi-post and 168 for pten/+;PTEN-RNAi-post), (H) mEJP amplitude, (I) mini EJP frequency, and (K) EJP amplitude in the indicated genotypes. In (H-J) n=6 animals for all genotypes.

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001; *=p<0.05. Calibration bar is 7 μm for A-D.
Figure 2.8
Figure 2.9. Proposed model for the regulation of the synaptic cytoskeleton by the aPKC-Baz-Par-6 complex at the larval NMJ.

(A) At the presynaptic compartment, aPKC regulates microtubule stability by facilitating an interaction between the MAP1B-related Futsch and bundled microtubules (see (Ruiz-Canada et al., 2004)). At the postsynaptic compartment, aPKC regulates both F-actin and microtubules (Ruiz-Canada et al., 2004). Here we propose that the regulation of the F-actin cytoskeleton is carried out through the opposing functions of aPKC and PTEN by modulating the phosphorylation state of Baz (see text for details).

In (B) the synaptic bouton has been subdivided into 4 regions, each representing a different genotype as indicated, and the consequence of each genotype on the F-actin-rich postsynaptic region (see text for details).
Figure 2.9
Supplementary Figure 2.1. Comparison of muscle aPKC and postsynaptic spectrin levels between wild type and aPKC-RNAi-post or β-spectrin-RNAi-post respectively.

(A-B) Low magnification images of third instar larval NMJs at muscles 6 and 7 stained with anti-HRP (red) and anti-aPKC (green) in (A) wild type and (B) aPKC-RNAi-post showing the decrease in aPKC levels in the muscle.

(C-D) High magnification images of third instar larval NMJs stained with anti-HRP (red) and anti-α-spectrin (green) in (C) wild type and (D) β-spectrin-RNAi-post. Calibration bar is 40 μm for A, B and 9 μm for C and D.
Supplementary Figure 2.1
Supplementary Figure 2.2. SSR size and postsynaptic DLG and Scrib levels and volume.

(A) Quantification of SSR thickness in the indicated genotypes (n=9 boutons measured at the bouton midline from 3 wild type larvae and 6 boutons measured at the bouton midline from 3 aPKC-RNAi-post larvae)

(B, C) Quantification of postsynaptic normalized intensity (white bars) and volume (black bars) of (B) DLG and (C) Scrib in the indicated genotypes (In B, n=31 boutons in wild type, 27 in aPKCRNAi-post, 21 in baz, Baz-RNAi-post, and 18 in pten, PTEN-RNAi-post. In C, n=20 boutons in wild type, 23 in aPKC-RNAi-post, 19 in baz/+, Baz-RNAi-post, and 18 in pten, PTEN-RNAi-post).

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001; *=p<0.05.
Supplementary Figure 2.2
Supplementary Figure 2.3. Baz levels in the genetic variants used in this study.

Histogram shows normalized postsynaptic Baz fluorescence intensity (black bars) and volume (white bars) in the indicated genotypes (n= 23 boutons in wild type, 20 in Baz-RNAi-1-post, 16 in Baz-RNAi-2-post, 24 in baz/+, and 13 in baz/; Baz-RNAi-1-post).

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001; *p<0.05.
Supplementary Figure 2.3
Supplementary Figure 2.4. Number of synaptic boutons in controls.

Histogram shows the total number of synaptic boutons at muscles 6 and 7 in heterozygous animals as indicated. N= 8 arbors for all the indicated genotypes.

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001; *=p<0.05.
Supplementary Figure 2.4
Supplementary Figure 2.5 Peptide preincubation controls for the P-Baz and PTEN antibodies.

(A-D) Images of NMJs and regions of the muscle cortex at muscles 6 and 7 in preparations double labeled with anti-HRP (blue) and (A, B) anti-P-Baz (green) or (C, D) anti-PTEN (green) anti-peptide antibody. In (B, D) the antibodies were preincubated with the peptide antigens, which almost completely eliminated immunoreactivity.

Calibration bar for A-D is 18 μm.
Supplementary Figure 2.5
Supplementary Figure 2.6. P-Baz colocalized with Baz immunoreactive puncta at the muscle cortex.

(A, B) Views of the muscle cortex in larvae expressing Baz-GFP in preparations double labeled with antibodies to GFP and antibodies to (A) Baz, and (B) PBaz. Note that Baz-GFP localizes to many (yellow arrowheads), but not all (white arrows) Baz puncta. Also note that many P-Baz puncta (yellow arrowheads) are colocalized with Baz-GFP puncta.

(C, D) In these panels the GFP channel has been rotated 90° in relationship with the red channel, to show that puncta colocalization observed in (A, B) is not simply due to random coincidence.

Calibration bar for A-D is 3 μm.
Supplementary Figure 2.6
Supplementary Figure 2.7. Quantification of signal in Western Blots.

(A) Normalized P-Baz/Baz band ratios in Western blots of S2 cell extracts transfected with Baz, PKM, or Baz+PKM cDNAs.

(B) Normalized P-Baz/Baz band ratios in Western blots of S2 cell extracts transfected with Baz, Baz + PTEN, Baz + PTEN in which the PDZ domain has been mutated (PTEN_{PDZ(-)}), Baz + PTEN_{G137E}, and Baz + PTEN_{C132A}.

Data was obtained from at least three independent experiments.

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001; *=p<0.05
Supplementary Figure 2.7
Supplementary Figure 2.8 GluRIIB and GluRIII receptor cluster volume and intensity in Baz and PTEN variants.

Histograms show the volume (black bars) and intensity (white bars) of

(A) GluRIIB and

(B) GluRIII clusters in the indicated genotypes

In A, n= 203 clusters for wild type, 103 for aPKC-RNAi-post, 199 for baz/+, Baz-RNAi-post and 238 for pten/+, PTEN-RNAi-post. In B, n= 214 clusters for wild type, 121 for aPKC-RNAi-post, 217 for baz/+, Baz-RNAi-post, and 187 for pten/+, PTEN-RNAi-post.

Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001; *= p<0.05.
Supplementary Figure 2.8
CHAPTER III
Trans-Synaptic Transfer of Wnt Signals Through
Release of Evi/Wntless Vesicles and Trafficking of
Postsynaptic Frizzled-2 Receptors
Abstract

Wnts play pivotal roles during development and in the mature nervous system. However, the mechanism by which Wnts traffic between cells has remained elusive. Here we demonstrate a novel mechanism of Wnt transmission through release of exosome-like vesicles containing the Wnt-binding protein Evenness Interrupted/Wntless/Sprinter (Evi/Wls/Srt). We show that at the *Drosophila* larval neuromuscular junction (NMJ), presynaptic vesicular release of Evi is required for the secretion of the Wnt, Wingless (Wg). We also show that Evi acts cell-autonomously in the postsynaptic Wnt-receiving cell to target dGRIP, a Wg-receptor-interacting protein, to postsynaptic sites. Upon Evi loss of function, dGRIP is not properly targeted to synaptic sites, interfering with postsynaptic Wnt signal transduction. These findings uncover a previously unknown cellular mechanism by which a secreted Wnt is transported across synapses by Evi-containing vesicles, and reveal novel trafficking functions of Evi in both the Wnt-producing and the Wnt-receiving cell.

Introduction

Members of the Wnt family of morphogens orchestrate a myriad of developmental processes in all metazoan organisms studied to date (Siegfried and Perrimon, 1994). These include the establishment of cell identity during pattern formation, control of cell proliferation and migration, and cytoskeletal remodeling. Wnts are also known to coordinate major aspects of the nervous system from early development to adult function, in which they regulate neural
stem cell proliferation, axon pathfinding, synapse differentiation and plasticity, as well as learning (Zhao et al., 2005; Speese and Budnik, 2007; Ataman et al., 2008; Salinas and Zou, 2008). Not surprisingly, alterations in Wnt signaling in humans have been linked to a number of cognitive disorders, such as schizophrenia and Alzheimer’s disease (De Ferrari and Moon, 2006).

Wnts activate a variety of intracellular signal transduction pathways that regulate gene expression and cytoskeletal organization events (Gordon and Nusse, 2006; Salinas and Zou, 2008). The best understood signaling pathway is the canonical Wnt pathway, in which Wnt ligands bind to the Frizzled (Fz) family of serpentine receptors. Receptor activation in turn stabilizes cytoplasmic β-catenin, which enters the nucleus and regulates gene expression. In a divergent canonical pathway, GSK3-β operates through a non-genomic mechanism, by phosphorylating microtubule-associated proteins, thereby regulating microtubule stability. Alternative signal transduction mechanisms activated by Wnt ligands include the planar cell polarity (PCP) pathway, and the Wnt/Ca²⁺ pathway. Recent studies at the Drosophila neuromuscular junction (NMJ) and in the developing mammalian nervous system have uncovered a novel transduction mechanism, in which Wnt receptors themselves are cleaved and translocated into the nucleus (Mathew et al., 2005; Lyu et al., 2008). These non-exclusive transduction cascades provide alternative mechanisms for cells to regulate diverse processes in different spatio-temporal contexts.
While considerable progress has been made in elucidating the signaling pathways activated by Wnts, much less is known about how Wnts are secreted and transported to distant locales. At the *Drosophila* imaginal wing disc, the Wnt-1 homolog Wingless (Wg) is secreted by a discrete row of Wg-producing cells. Secreted Wg forms a long-range gradient expanding many cell diameters away from the source of Wg secretion (Neumann and Cohen, 1997). The mechanisms by which Wg is transported from its site of secretion to distant target cells have remained poorly understood. Wnt proteins are highly hydrophobic and tightly associated to cell membranes owing to palmitoyl modifications essential for biological activity (Willert et al., 2003). Thus, unescorted Wnt molecules are not easily diffusible in the extracellular milieu. Several mechanisms have been proposed to explain the movement of Wnt molecules from their site of secretion, including their association with glycosaminoglycan-modified proteins at the extracellular matrix (Baeg et al., 2001), the formation of exosome-like vesicles called argosomes (Greco et al., 2001), extracellular lipoprotein particles (Panakova et al., 2005), transcytosis (Coudreuse et al., 2006), or a combination of the above. However, the exact mechanism employed during intercellular Wnt transport has remained elusive.

Recent studies have identified a type II multi-pass transmembrane protein called Evenness Interrupted/Wntless/Sprinter (Evi/Wls/Srt), which appears to be specifically required *in vivo* for Wnt secretion in epithelial cells of flies and human cultured cells (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al.,
In the wing epithelium of *Drosophila*, Wg cannot be secreted from the evi mutant cells and this leads to the accumulation of Wg within these cells. In contrast, the secretion of other morphogens, such as Hedgehog (Hh), remains unaffected, suggesting that Evi is dedicated to the secretion of Wnt proteins. Further analysis has suggested that Evi functions as a Wnt cargo receptor during trafficking from the Golgi to the plasma membrane, and recycled back to the Golgi through the retromer complex (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008).

In the nervous system, Wnts are released by pre- or postsynaptic cells and function in either a retrograde or anterograde manner (Speese and Budnik, 2007; Salinas and Zou, 2008). Similar to other cell types, the mechanisms by which Wnts are transported between synaptic compartments are principally unexplored. Considering that Wnt-1 is released from synapses in an activity-dependent manner (Ataman et al., 2008), and the substantial short and long-term effects of Wnt signaling on neurons, elucidating the mechanisms by which Wnt secretion/transport is regulated in the nervous system remains an important problem.

Here we have addressed this key question by using the glutamatergic synapses of the *Drosophila* larval NMJ, where Wnt-1/ Wingless (Wg) is secreted from motorneurons. We report that Evi is localized at these synapses and its function is indispensable for proper Wg secretion and signaling. We also demonstrate a novel mechanism for transport of the Wg signal across the
synapse through the release of Evi-containing exosome-like vesicles. Further, we show that Evi is required for the proper trafficking of the Wg receptor DFrizzled-2 (DFz2), through actions that involve the DFz2-interacting protein dGRIP, a PDZ protein required for the transport of internalized DFz2 vesicles towards the nucleus (Mathew et al., 2005; Ataman et al., 2006).

Results

**Evi is required for Wg secretion at the NMJ**

Previous studies have suggested that Evi is required for Wg secretion in non-neuronal cells (Banziger et al., 2006; Bartscherer et al., 2006). Since Wg is secreted from motorneurons at the fly NMJ (Packard et al., 2002; Ataman et al., 2008) we first examined the distribution of Wg at the NMJ of *evi* null mutants, which survive to the third instar larval stage (Bartscherer et al., 2006). We found that secreted Wg levels were substantially reduced at postsynaptic muscles in *evi* mutants (Fig. 3.1A, C, E). However, this reduction was not limited to the postsynaptic compartment, but was also observed in the presynaptic boutons as determined by volumetric quantifications of the Wg signal inside the presynaptic bouton demarcated by anti-HRP staining (Fig. 3.1A, C, E). A similar decrease was observed when Evi was downregulated using Evi-RNAi expressed in neurons using the elav-Gal4 driver (Suppl. Fig. 3.1A, B). These results could indicate that Evi might be required for the stability or synthesis of Wg in motorneurons. However, we did not observe any changes in Wg levels in the nervous system (Fig. 3.1F). Thus, at the NMJ, Evi is required for the transport
and/or secretion of Wg in presynaptic terminals. A prediction of this hypothesis is that Wg should accumulate in the cell bodies or axons of motorneurons. We found that there was a substantial increase in Wg immunoreactivity levels in motorneuron cell bodies and longitudinal axons within the neuropil (Suppl. Fig. 3.1C-E).

The above model was further tested by rescue experiments. Expressing an Evi-GFP transgene in the motorneurons of evi mutants, by using the Gal4 driver C380, completely rescued the low levels of Wg in both the pre- and postsynaptic compartments (Fig. 3.1B, E). Notably, however, expression of the Evi-GFP transgene in postsynaptic muscles, by using the Gal4 strain BG487, did not (Fig. 3.1D, E). Thus, Evi is required in motorneurons for normal Wg transport and/or secretion.

We also observed that mutations in evi mimicked synaptic phenotypes previously observed in mutations affecting Wg signaling (Packard et al., 2002; Mathew et al., 2005; Ataman et al., 2006). As muscle fibers grow in size, the Drosophila larval NMJ continuously expands by adding new synaptic boutons. This expansion is critically dependent on Wg signaling (Packard et al., 2002). Wg appears to be secreted by motorneurons, and suppressing Wg secretion substantially reduces synaptic bouton proliferation. Further, in wg mutants many boutons are misshapen, and some remain in an undifferentiated state (ghost boutons), lacking active zones and postsynaptic apparatus. Conversely, increasing Wg secretion by overexpressing Wg in motorneurons enhances
formation of synaptic boutons. In the presynaptic cell Wg activates a divergent canonical pathway that regulates microtubules (Franco et al., 2004; Ataman et al., 2008; Miech et al., 2008). In the postsynaptic muscle cell Wg initiates an atypical pathway in which the DFz2 receptor itself is cleaved and a fragment imported to the nucleus (Mathew et al., 2005; Ataman et al., 2006; Ataman et al., 2008). In evi mutants the total number of synaptic boutons was decreased by over 50%, without any change in muscle size, and the boutons had an aberrant morphology being large and deformed (Fig. 3.2A-E; Suppl. Fig. 3.2). In addition, evi NMJs had a significantly higher number of ghost boutons (Fig. 3.2D arrows, F). The decrease in bouton number was only partially rescued by expressing Evi in either the pre- or postsynaptic cell (Fig. 3.2E). However, it was completely rescued by simultaneously expressing Evi in both cells (Fig. 3.2E). In the case of ghost boutons, expressing the Evi transgene in motorneurons or in both motorneurons and muscles completely rescued the abnormal increase in ghost boutons in evi mutants (Fig. 3.2F). Expressing Evi in muscles using the weaker Gal4 driver BG487-Gal4 did not rescue the increase in ghost boutons, but this phenotype was completely rescued by using the stronger muscle Gal4 driver C57-Gal4 (Fig. 3.2F). Thus, although Evi is required only in motorneurons for proper Wg transport and/or secretion, Evi is needed both in neurons and muscles for normal synaptic growth.

The similarity in the synaptic phenotypes between evi and wg mutants at the NMJ, together with previous evidence suggesting that both proteins establish
biochemical interactions (Banziger et al., 2006), raised the question of whether there were genetic interactions between evi and wg during NMJ growth. This was addressed by analysis of transheterozygotes. The number of boutons was normal in heterozygotes, but there was a supra-additive reduction in the number of boutons in the transheterozygote (expected decrease in bouton number by simple additivity in wg/+; evi/+ is 14.2% vs. 27% observed in wg/+; evi/+ transheterozygotes; Fig. 3.2G), suggesting that evi and wg genetically interact during synaptic bouton proliferation.

**Evi is localized both pre- and postsynaptically and is transferred transsynaptically from the pre- to the postsynaptic compartment**

To examine the synaptic localization of Evi, we generated two antibodies directed either to a predicted extracellular (Evi-Nex) or intracellular (Evi-Cin) region of the Evi protein (Fig. 3.3A; Suppl. Fig. 3.3A, B). Both antibodies strongly labeled the NMJ in similar patterns (Fig. 3.3B, C). This immunoreactivity was specific, as it was severely decreased in evi mutants (Fig. 3.3D, E). Immunoreactive Evi label was observed both in pre- and postsynaptic compartments at the NMJ, as determined by double labeling with anti-HRP, which defines the boundary of the presynaptic compartment. However, Evi was particularly enriched at the postsynaptic junctional region (Fig. 3.3B, C), the same region occupied by secreted Wg, and its receptor DFz2 at the NMJ
(Packard et al., 2002). In this region Evi immunoreactivity was present in a punctate pattern presumably reflecting vesicular structures (Fig. 3.3B, C).

The rescue experiments suggested that Evi functions both pre- and postsynaptically during synaptic bouton proliferation, but that it is required solely presynaptically for Wg transport and/or secretion. To further determine the requirement of Evi in the pre- and postsynaptic side, we expressed Evi-RNAi with the cell-specific Gal4 drivers. Surprisingly, expressing Evi-RNAi in the motorneurons (Evi-RNAi-pre) led not only to a reduction in Evi immunoreactivity inside presynaptic terminals, but also substantially reduced the label at the postsynaptic region (Fig. 3.3F, I). The observation that presynaptic knockdown of Evi has a trans-synaptic effect on Evi levels in muscle was quite unexpected. Such a phenomenon is not observed when knocking down other well-known synaptic proteins such as dGRIP (Ataman et al., 2006) or spectrin (Pielage et al., 2005). This observation was also not due to a leaky Gal4 driver, as C380-Gal4 expresses Gal4 in motorneurons and not in muscles (Budnik et al., 1996; Sanyal et al., 2003). Further, expressing a nuclear LacZ (UAS-LacZ-NLS) with C380-Gal4 resulted in strong labeling of neuronal but not muscle nuclei (Suppl. Fig 3.3D, E), and expressing myristylated-mRFP (myr-mRFP) using C380-Gal4 did not result in postsynaptic myr-mRFP signal (Suppl. Fig. 3.3C). These observations suggest that postsynaptic Evi is at least partly derived from the presynaptic motorneurons. The possibility that Evi could be transferred from the pre- to the postsynaptic compartment was tested by expressing Evi-GFP in
motorneurons (Evi-GFP-pre). Notably, GFP was observed both in presynaptic boutons as well as at the postsynaptic junctional region (Fig. 3.3G), supporting the notion that Evi could be transferred from pre- to postsynaptic compartments. This transfer was unlikely to result from Evi overexpression, as when the Evi-GFP transgene was expressed presynaptically in an evi mutant background, at levels similar to endogenous levels (Suppl. Fig. 3.3F), a similar distribution of the GFP label in the postsynaptic side was observed (Fig. 3.3H; Suppl. Fig. 3.3F). This transfer of presynaptic Evi was also clearly observed in vivo in samples expressing myr-mRFP and Evi-GFP in motorneurons and imaged live (Suppl. Fig. 3.3C).

Given that Wg is secreted by presynaptic boutons and that Evi is required for normal Wg secretion, we next examined if presynaptically derived Evi colocalized with endogenous secreted Wg observed at postsynaptic sites. For these experiments we expressed Evi-GFP in motorneurons and examined both the Wg and Evi-GFP labels at the postsynaptic compartment. We found that there was substantial colocalization between Wg and Evi-GFP distal to the bouton rim, right outside the HRP label (Fig. 3.3J) consistent with the idea that secreted Evi vesicles contain Wg.

In contrast to the expression of Evi-RNAi in motorneurons, expressing Evi-RNAi in the muscles (Evi-RNAi-post), although significantly reducing the levels of Evi protein in the postsynaptic compartment, did not change the levels of Evi in presynaptic boutons (Fig. 3.3K, I). These results demonstrate that Evi is
expressed by both motorneurons and muscles, but that there is a unidirectional transfer of Evi from presynaptic boutons to the postsynaptic region.

Considering that Evi is a multipass transmembrane protein, two possible scenarios might account for the above transfer of Evi from the pre- to the postsynaptic region. One possibility is that an extracellular region of Evi is cleaved, as is the case for other membrane receptors (Selkoe et al., 1996) (Fig. 3.3N). However, this possibility is highly unlikely, as in the Evi-GFP transgene the GFP tag is fused to the intracellular C-terminal region of Evi, and thus the transfer must include the intracellular domain. An alternative possibility is that the entire Evi protein could be transported in the form of a vesicle from the pre- to the postsynaptic compartment (Fig. 3.3O), as has been previously suggested with argosomes, vesicular structures that can transport Wg from cell to cell (Greco et al., 2001). To address this possibility, we took advantage of the Evi-Nex antibody, which recognizes an epitope localized at the first extracellular loop of Evi (Fig. 3.3A; red region in Fig. 3.3N, O), and which is separated from the C-terminal GFP tag by 7 transmembrane domains. For these experiments, we expressed Evi-GFP in motorneurons in an evi null mutant background and determined whether the postsynaptic GFP signal colocalized with the Evi-Nex and Evi-Cin immunoreactivity. We found that anti-Evi-Nex and anti-Evi-Cin immunoreactivities were exactly colocalized with GFP at the postsynaptic region (Fig. 3.3L, M; Suppl. Fig. 3.3G, H). Thus, these results support the notion that intact Evi is transferred across the synapse likely in a vesicle.
We also examined *Drosophila* Schneider-2 (S2) cells transfected with the Evi-GFP construct. We found that untransfected S2 cells in contact with Evi-GFP-transfected cells often contained Evi-GFP positive puncta within their cytoplasm (Fig. 3.4A, arrowheads). To verify that this was due to transfer of Evi-GFP from transfected to non-transfected cells, Evi-untransfected cells were separately transfected with mCherry and mixed with the Evi-GFP transfected cells. Again, we found that mCherry-positive (Evi-untransfected) cells had GFP puncta within their cytoplasm (Fig. 3.4B), suggesting that Evi-transfected cells transferred Evi to nearby cells.

We also found that Evi-GFP puncta were observed in the medium, suggesting the secretion of Evi vesicles into the medium (Fig. 3.4A, arrow). To determine if the Evi vesicles that were transferred to adjacent cells contained Wg, we cotransfected S2 cells with Evi-GFP and Wg. We found that the Evi vesicles transferred to adjacent cells or to the medium contained Wg (Fig. 3.4C, D, arrowhead). Interestingly, in these double transfected cells Wg localized to varicosities within filopodia (arrows in Fig. 3.4D). These filopodia were also present in untransfected cells as seen with phalloidin staining to label endogenous F-actin (Suppl. Fig. 3.4C). Two other membrane proteins, DFz2 and rCD2-mRFP, which also become localized to filopodia were not observed to be secreted (Suppl. Fig. 3.4A, B). We also carried out a Western blot analysis of the S2 cells and the culture medium. We found that indeed the culture medium contained full-length Evi protein, suggesting that Evi was secreted to the medium.
The above observation was directly visualized by time-lapse imaging the Evi-GFP fluorescence. We found that Evi-GFP puncta trafficked within highly dynamic filopodia-like structures in the S2 cells and that some of these puncta were secreted to the media in a time-frame of several minutes (Fig. 3.4F). Thus, release and trans-cellular transfer of Evi vesicles to adjacent cells is a common biological mechanism utilized by both neuronal and non-neuronal cells.

**Evi is present in multiple compartments at the NMJ**

To determine the subcellular localization of Evi within pre- and postsynaptic compartments we next carried out immunoelectron microscopy studies with the Evi antibodies. For these experiments 1.4 nm gold conjugated secondary antibodies followed by silver intensification were used to mark sites of Evi-antibody binding using the pre-embedding technique. Consistent with our immunofluorescence studies, Evi was found to be localized in several pre- and postsynaptic structures.

At the postsynaptic junctional region, Evi was found within the subsynaptic reticulum (SSR), a system of muscle-derived membrane folds that completely surrounds synaptic boutons (Fig. 3.5A; the presynaptic bouton highlighted in pink overlay). Within the SSR, silver-intensified gold particles were observed in close association with the membrane folds (Fig. 3.5E, arrows). Notably, gold particles were also found inside approximately 200 nm in diameter membranous vesicles within the SSR (Fig. 3.5A, B; arrows and insets). In summary, at the postsynaptic
region, Evi is present in association with SSR membranes and with novel postsynaptic vesicles.

Evi was also associated with the pre- and postsynaptic membrane (Fig. 3.5F, arrows) and sometimes the signal was observed at the synaptic bouton cleft (Fig. 3.5H, arrow and inset). Within the presynaptic bouton, Evi was observed in large multi-membrane structures (Fig. 3.5G, arrowhead). Thus, Evi is present in multiple structures at synapses, including pre-and postsynaptic vesicular structures, the SSR, and synaptic membranes.

To determine if these vesicles were endocytosed from the muscle surface, we next conducted an internalization assay. These experiments were facilitated by the finding that the Evi-Nex antibody can bind to surface Evi in vivo (Suppl. Fig. 3.3A). For these studies, unfixed and unpermeabilized body wall muscles were incubated with the Evi-Nex antibodies in the cold, washed, and brought to room temperature for 30 min prior to fixation. Then, samples were permeabilized and incubated with the gold-conjugated secondary antibody, followed by preparation for EM. Interestingly we found that the Evi label was found at SSR membranes as well as inside the large SSR vesicles (Fig. 3.5C, arrows and insets), suggesting that at least a subset of these postsynaptic vesicles are derived from the endocytosis of postsynaptic surface Evi.

To verify that Evi was transferred from presynaptic boutons to the postsynaptic SSR at the ultrastructural level, GFP-tagged Evi was expressed in motorneurons using the C380 Gal4 driver, and the NMJ was examined by
immunoelectron microscopy using an anti-GFP antibody. We found that the GFP label was found not only within synaptic boutons (Fig. 3.5D, arrowhead), but throughout the postsynaptic SSR membrane (Fig. 3.5D). We also expressed Evi-GFP in the motorneurons of evi mutants and immunolabeled Evi with the Evi-Cin antibody using the postembedding technique. Again, we found the label both in the presynaptic compartment (Fig. 3.5I, arrowhead), at the synaptic bouton cleft (Fig. 3.5J, arrowhead) and the postsynaptic SSR region (Fig. 3.5I, K, arrows). Thus, Evi is transferred trans-synaptically as expected from the observations at the light level.

**Postsynaptic Evi is required for the trafficking of DFz2 through the DFz2-interacting protein dGRIP**

Given that presynaptic Evi alone is not sufficient for normal NMJ development we predicted that Evi was also endogenously expressed in muscle. To test this prediction we carried out real time PCR experiments from body wall muscle mRNA. We found that there were significant levels of Evi mRNA in muscles and that these levels were substantially decreased upon expressing Evi-RNAi (Suppl. Fig. 3.5). What is the cell-autonomous role of Evi in the postsynaptic target cell? To address this issue we examined postsynaptic Wg signaling while downregulating Evi selectively in the muscle using Evi-RNAi. Previous studies suggest that Wg is secreted by presynaptic boutons (Packard et al., 2002), and unraveled a novel postsynaptic Wg signal transduction pathway in
the postsynaptic muscles, the Frizzled Nuclear Import (FNI) pathway (Speese and Budnik, 2007), which is also shared by other Wnt receptors (Lyu et al., 2008). In this pathway, the Wg receptor, DFz2, is internalized from the postsynaptic muscle membrane and back-transported from the synapse to the nucleus through a mechanism that requires an interaction between the PDZ-binding C-terminal tail of DFz2 and the PDZ4-5 domain of the 7-PDZ protein dGRIP (Ataman et al., 2006). The entire cytoplasmic domain of DFz2 (DFz2-C) is then cleaved and imported into the nucleus (Mathew et al., 2005).

In muscles expressing Evi-RNAi we found that DFz2 was localized normally at the postsynaptic region of the NMJ. However, the postsynaptic levels of DFz2 were substantially increased (Fig. 3.6A, B, E). In contrast, no such increase in DFz2 levels was observed in the presynaptic cell upon expression of Evi-RNAi in motorneurons (normalized presynaptic DFz2 intensity in wild type is 1.0 ± 0.07 vs 0.93±0.07 in Evi-RNAi-pre). The same phenotype has been previously observed when the transport of DFz2 from the synapse to the nucleus is prevented by interfering with dGRIP function in muscles (Ataman et al., 2006). Interestingly, a similar accumulation of Wg at the postsynaptic region was observed upon downregulating Evi in muscle (Fig. 3.6C, D, E), consistent with the notion that Wg is trafficked with its receptor (Gagliardi et al., 2008). To determine if the increase in DFz2 at synapses of Evi-RNAi-post larvae was due to a defect in the internalization and/or trafficking of DFz2, we carried out DFz2 internalization assays. In these experiments we used an anti-DFz2-N antibody
that binds to the extracellular domain of the receptor in vivo, allowing us to follow
the fate of internalized DFz2. Dissected third instar body wall muscles were
incubated with the DFz2-N antibody at 4˚C in vivo, and after washing the excess
antibody, samples were brought to room temperature and fixed at 5 and 60
minutes after the antibody-binding step. To determine the fraction of DFz2 that
remained at the surface, samples were then incubated with Alexa 647-
conjugated secondary antibody in the absence of detergent permeabilization as
previously reported (Mathew et al., 2005; Ataman et al., 2006). To determine the
amount of internalized DFz2, the above procedure was followed by
permeabilization and incubation with an FITC-conjugated secondary antibody.

As in previous studies (Mathew et al., 2005; Ataman et al., 2006), in wild
type samples, surface DFz2 was internalized and observed near synaptic
boutons at 5 min after the antibody-binding step (Fig. 3.6G2, F). However, at 60
min after the antibody-binding step, internalized DFz2 was significantly reduced
at the NMJ as a result of its trafficking away from the synapse (Fig. 3.6H2, F;
(Mathew et al., 2005; Ataman et al., 2006)). In contrast, upon expressing Evi-
RNAi in muscles, no decrease in internalized synaptic DFz2 was observed at 60
min (Fig. 3.6I2, J2, F). No significant changes were observed in surface DFz2 in
both genotypes (Fig. 3.6G1-J1, F), suggesting that only a small pool of the DFz2-
antibody complexes become internalized. Thus, similar to alterations in dGRIP, a
decrease in Evi function in muscles appears to interfere with the trafficking of
DFz2 away from the synapse. This conclusion was further supported by
examination of the levels of DFz2-C imported into the muscle nuclei. Previous studies show that the C-terminal region of DFz2 is cleaved and imported into the nucleus, where it is observed in the form of discrete immunofluorescent puncta (Fig. 3.6K; (Mathew et al., 2005)). In evi mutants and upon expressing Evi-RNAi in muscles alone, nuclear DFz2-C puncta were almost completely abolished (Fig. 3.6K, L, M), in agreement with the model that in the absence of Evi function, DFz2 is not properly transported to the muscle nucleus. Furthermore, a complete rescue of the DFz2-C nuclear spots to wild type levels was observed in the evi mutant by expressing the Evi transgene in the muscle alone. In contrast, expressing Evi in motorneurons provided only a partial rescue of the nuclear DFz2-C foci (Fig 3.6M). Therefore, we conclude that muscle Evi is involved in the trafficking of DFz2 to the nucleus.

Given the substantial similarities between the phenotypes observed upon knocking down evi and dgrip in postsynaptic DFz2 trafficking as well as in the synaptic morphology and NMJ growth (Ataman et al., 2006), we next examined whether interfering with Evi function could be disrupting the postsynaptic function of dGRIP. For these studies we examined the localization of dGRIP in larvae expressing Evi-RNAi in muscles. In wild type, dGRIP is present in small trafficking vesicles highly concentrated at postsynaptic sites (Fig. 3.7A, arrows; (Ataman et al., 2006)), as well as in Golgi bodies in juxtaposition to the cis-Golgi marker Lavalamp (Lva; Fig. 3.7A; arrowheads (Ataman et al., 2006)). Notably, we found that upon knocking-down Evi specifically in muscles, dGRIP was
substantially reduced from postsynaptic sites as well as from Golgi bodies in muscle (Fig. 3.7B, C). In addition, dGRIP was localized throughout the muscle submembrane region in a diffuse manner (Fig. 3.7B, C). Thus, Evi controls dGRIP localization at the postsynaptic muscle region and in the absence of Evi function dGRIP is not normally localized to the Golgi and synapses, likely disrupting postsynaptic DFz2 trafficking. A prediction of this model is that overexpressing dGRIP in muscles should overcome some of the defects arising from the lack of Evi in muscles. To test this model we overexpressed dGRIP in muscles while downregulating Evi in these cells. We found that the DFz2 accumulation at the postsynaptic region of Evi-RNAi-post was completely rescued, and indeed the postsynaptic levels of DFz2 became significantly lower than wild type (Fig. 3.7D). In addition, both the number of synaptic boutons and nuclear DFz2C spots were partially rescued by overexpressing dGRIP in evi mutants (Fig. 3.7E, F).

An additional prediction is that a population of Evi vesicles should traffic with dGRIP vesicles. To determine if this was the case we performed time lapse imaging of muscles expressing both Evi-GFP and dGRIP-mRFP. We found that in many instances Evi and dGRIP vesicles colocalized and followed the same trajectory. These results demonstrate that Evi, in addition to its important role in the Wg-secreting cell, has a critical function in Wg-target cells, as it mediates the transport of the downstream Wg signaling component, dGRIP.
Discussion

Here we show that the multipass transmembrane protein Evi has a critical role in trans-synaptic Wnt-1/Wg transport through vesicular structures. To our knowledge, this is the first report to identify trans-synaptic communication through a vesicular structure. Further, our studies highlight a novel mechanism by which secreted factors can be transmitted from cell to cell. We propose that presynaptic Evi is required for trafficking Wg from the cell body to the presynaptic terminals, and across the synaptic cleft, to present Wg to postsynaptic DFz2 receptors (Fig. 3.7G). On the other hand, postsynaptic Evi is required to transport dGRIP to postsynaptic sites. At the postsynaptic region dGRIP interacts with postsynaptic DFz2 receptors and participates in the trafficking of DFz2 to the nucleus, where its C-terminal tail is cleaved, and imported to the nucleus (Fig. 3.7G).

Previous studies had implicated Evi only in the secretion of Wnts in Wnt-expressing cells (Banziger et al. 2006; Bartscherer et al., 2006; Coudreuse et al., 2006; Goodman et al., 2006; Belankaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008). However, endogenous Evi was also found in Wnt-target cells (Port et al., 2008), where it plays an as yet unidentified role. Our studies here identify an unprecedented role for Evi in Wg receiving cells in trafficking the Wg receptor DFz2 through the regulation of the synaptic targeting of the DFz2-interacting protein dGRIP, which was previously shown to function in transporting DFz2 receptors from the postsynaptic
membrane to the muscle nucleus (Ataman et al., 2006). These studies unravel new processes and cellular mechanisms by which Evi functions as an essential component of synaptic Wnt signaling.

**Trans-synaptic signaling in the nervous system: role of Wnts**

Intercellular communication in the brain is primarily accomplished through the exocytosis of neurotransmitter-laden vesicles or by direct current conduction through gap junctions. Pre- and postsynaptic partners also release factors important for cell survival, synapse development, synapse maintenance, and synaptic plasticity (reviewed in Lu and Figurov, 1997; reviewed in Marques, 2005). Among these are neurotrophins such as Bone Derived Neurotrophic Factor (BDNF) and Nerve Growth Factor (NGF), members of the Bone Morphogenetic Protein (BMP) family and Wnts. These molecules are released from pre- or postsynaptic terminals and they function in retrograde or anterograde manners to influence synaptic growth, function and plasticity. At the *Drosophila* larval NMJ, continuous coordination of synaptic growth in relationship to muscle size requires the release of a retrograde signal of the BMP family which acts on BMP receptors in the presynaptic cell (Marques, 2005). This correlated synaptic growth is also controlled by the release of Wg, which is thought to act on DFz2 receptors in both the pre- and postsynaptic cells where it initiates alternative transduction pathways (Franco et al., 2004; Ataman et al., 2008; Miech et al., 2008).
A major gap in our understanding of how Wnts function is the mechanism by which they reach their destination once released. Despite the presence of charged amino acid residues in the primary sequence of Wnts, Wnts are hydrophobic molecules tightly bound to cell membranes due to the addition of palmitate moieties during maturation (Willert et al., 2003; Zhai et al., 2004). This hydrophobic nature of Wnts argues against the simple model of passive diffusion in the extracellular milieu. The studies presented here suggest that one mechanism for this transport is the association of Wg with Evi-containing vesicles, which are released from presynaptic boutons and become localized to postsynaptic sites. This model is supported by several lines of evidence. (1) Downregulating Evi in the presynaptic motorneurons (Evi-RNAi-pre) led not only to a reduction in Evi immunoreactivity inside presynaptic terminals, but also substantially reduced the label at the postsynaptic region. (2) Expressing Evi-GFP in motorneurons (Evi-GFP-pre) led to the localization of the GFP label in the postsynaptic junctional region in the form of puncta which colocalized with both the Evi-Cin and Evi-Nex antibodies, and which showed substantial colocalization with secreted Wg at the postsynapse. (3) Evi-GFP could be transferred from transfected to untransfected S2 cells, and S2 cell culture medium contained full-length Evi protein, suggesting that Evi was also secreted in cultured cells. In addition, the secreted and transferred Evi vesicles contained Wg. This trans-synaptic transfer of a synaptogenic signal through specialized vesicles containing
a dedicated membrane protein is a novel signaling mechanism in the nervous system that might be used for a number of secreted signaling factors.

The release of endosomal vesicles, called exosomes, has been reported in a variety of tissues, including cultured neurons (Fevrier and Raposo, 2004; Faure et al., 2006; van Niel et al., 2006). These exosomes are released by the fusion of MVBs with the plasma membrane and are thought to be involved both in the removal of cellular debris as well as in intercellular communication. For example, in the immune system integrin- and MHC-containing exosomes are used for antigen presentation, and they are able to prime T lymphocytes in vivo (van Niel, et al., 2006). In cultured cortical neurons the release of exosomes containing the cell adhesion molecule L1, the GPI-anchored prion protein and the GluR2/3 subunit of glutamate receptors has been reported, in a process that is regulated by membrane depolarization (Faure et al., 2006). Our finding that exosome-like vesicles containing a synaptogenic factor are released at synapses provides a novel mechanism for trans-synaptic communication.

The mechanism by which Evi-containing vesicles are released from the presynaptic cell is not known, but a few potential possibilities are depicted in Fig. 3.3O. For example Evi might be transported within the presynaptic cell in multivesicular bodies (MVB) that fuse with the plasma membrane thus releasing the Evi vesicle. In turn, after presentation of Wg to DFz2 receptors, the vesicle might fuse with the postsynaptic membrane. Interestingly, we found that Evi in presynaptic terminals was present in multimembrane compartments. Similarly, in
Wg-secreting wing disc epithelial cells, Evi (Franch-Marro et al., 2008) and Wg (van den Heuvel et al., 1989) have been shown to be localized within MVBs. We also found that Evi label was found in association with postsynaptic SSR membranes and in the form of approximately 200 nm vesicles in the SSR. Our internalization assays suggest that these vesicles are endocytosed from the postsynaptic membrane.

**Cell-autonomous role of Evi in Wg-target muscles**

Besides its involvement in transporting the Wg signal across the synapse, we also found that Evi had a cell-autonomous function in the postsynaptic target cell, as revealed by specifically downregulating Evi in muscle. In this case both Wg and DFz2 accumulated at the postsynaptic region. In addition, DFz2 did not traffic normally from the NMJ and the nuclear import of DFz2-C was largely abolished. These findings suggest that Evi, beyond the regulation of Wnt secretion, organizes further downstream signaling events in the Wg-target cell.

The phenotypes observed upon downregulating Evi in muscle cells were highly reminiscent of those observed upon loss of dGRIP function. Further, decreasing Evi levels led to the virtual elimination of synaptic and Golgi dGRIP. The evidence relating Evi to dGRIP function is further supported by the findings that Evi and dGRIP are often observed trafficking in the same vesicles and that overexpressing dGRIP in either evi mutants or Evi-RNAi-post elicited partial rescue of phenotypes resulting from evi loss of function. Thus, Evi
appears to be required for the trafficking of dGRIP to synaptic sites where dGRIP binds DFz2 receptors and functions to traffic them towards the nucleus. The elimination of dGRIP from the Golgi complex might arise from a defect in its recycling to the Golgi due to its abnormal targeting to synapses. In the absence of postsynaptic dGRIP, DFz2 is not trafficked towards the nucleus leading to its accumulation at postsynaptic sites. Interestingly, Evi has been demonstrated to be involved in trafficking Wg from the Golgi to the plasma membrane in Wg-secreting cells (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Yang et al., 2008). Our studies showing that Evi is required for the trafficking of dGRIP to postsynaptic sites suggest that Evi might have a role not solely in transporting Wnts, but also in trafficking components associated with Wnt pathways.

Although this study identifies a pre- and postsynaptic role for Evi, it is clear that both roles are not completely independent. For example, we found that restoring Evi levels only in the motorneurons of evi mutants was sufficient for a complete rescue of the ghost bouton phenotype and resulted in a partial rescue in the number of nuclear DFz2C spots. These results were surprising given that in the absence of postsynaptic Evi, dGRIP does not traffic normally, and thus interferes with postsynaptic Wnt signaling. A potential explanation is that the transferred presynaptic Evi can partially compensate for the lack of Evi in the postsynaptic cell.
In conclusion, our studies identify a novel mechanism by which the Wnt-1/Wg signal is transmitted across the synapse, through the use of an Evi vesicle, and find an additional cell-autonomous role of Evi in Wnt-receiving cell, the synaptic recruitment of dGRIP, which functions in transporting the signal to the muscle nucleus.

Materials And Methods

Fly Strains
Flies were reared in standard Drosophila media at 25°C unless otherwise stated. (See Suppl. Materials for fly strains.) RNAi crosses and controls were performed at 29°C. The wgts flies were tested at the restrictive temperature (25°C).

Cytochemistry
Third instar larvae were dissected in Ca²⁺-free saline and fixed in either 4% Paraformaldehyde or non-alcoholic Bouin’s fixative (see Suppl. Methods for antibodies and Hoechst conditions).

Image Quantification
Confocal images were acquired using a Zeiss Pascal Confocal Microscope. Preparations from different genotypes were processed simultaneously and imaged using identical confocal acquisition parameters. Fluorescence signal intensity was quantified by volumetric measurements of confocal stacks using Volocity 4.0 Software (see Suppl. Materials). Measurements were taken from muscles 6 and 7, abdominal segment 3. A Student’s t test was performed for
pair-wise comparisons between each genotype and controls. Error bars in the histograms represent mean±SEM, where ***= p<0.0001; **= p<0.001; *=p<0.05.

**Schneider-2 (S2) cell cultures**

S2 cells were transfected as described in Suppl Materials. For Evi-GFP transfer experiments, pAc-Evi-EGFP (Bartscherer et al., 2006) transfected S2 cells were washed 24 hours after transfection and mixed with pAc-mCherry transfected S2 cells. For co-transfection experiments we used, pAc-Wg (Bartscherer et al., 2006), pAc-Evi-EGFP, pAc-rCD2-RFP and pAc-DFz2-myc (Mathew et al., 2005). Cells were then grown for 24-48 hours and processed for immunocytochemistry.

**Live Imaging**

Live imaging of transfected S2-cells and body wall muscles was performed using an Improvision Spinning Disk confocal microscope as described in Suppl. Materials.

**Western blots**

Western blots were performed as in (Mendoza-Topaz et al., 2008). For examination of Evi in S2 cells and the culture medium, transfected cells were washed with fresh medium 24 hours after transfection, and 24 to 48 hours later cells and medium were harvested for immunoblotting (Suppl. Materials).

**Immunoelectron microscopy**

For the pre-embedding technique, third instar body wall muscles were fixed and incubated with anti-Evi-Nex (1:100) or anti-GFP (1:300) followed by anti-rabbit IgG-1.4 nm-nanogold (1:50; Nanoprobes, NY), and intensification
using HQ silver reagents (Nanoprobes, NY). The EM Internalization assay was performed as above, except that 1.4 nm nanogold secondary antibody was used after permeabilization. For the post-embedding technique, samples were fixed and then embedded in LR White resin followed by antibody staining on grids with secondaries conjugated to 18nm gold (1:75; Jackson). Transmission electron microscopy analysis was performed as described by (Torroja et al., 1999). See Suppl. Materials for further details.

Supplementary Materials And Methods

Fly Strains
The following fly strains were used for these studies: wild type (Canton-S), evi\textsuperscript{2} (Bartscherer et al., 2006) (referred to as evi in the text), wg\textsuperscript{fs} (wg\textsuperscript{IL114}; (Nusslein-Volhard et al., 1985)), UAS-myr-mRFP (Bloomington Stock Center), UAS-Evi-EGFP (Bartscherer et al., 2006), UAS-Evi-RNAi (stock 5215; Vienna Drosophila RNAi Center www.vdrc.at/), UAS-dGRIP-mRFP (Ataman et al., 2006), UAS-LacZ-NLS and the Gal4 drivers C380, BG487 and C57 (Budnik et al., 1996), Elav-Gal4 (Luo et al., 1994), and Wg-Gal4 (gift of Dr. S. Cohen).

Immunocytochemistry and cytochemical staining.
The following primary antibodies were used: anti-Wg (1:300 (Packard et al., 2002)), anti-DLG\textsubscript{PDZ} (1:20,000 (Koh et al., 1999)), anti-Evi-Nex (1:100; see below), anti-Evi-Cin (1:100; see below), anti-DFz2-N (1:600 (Packard et al., 2002)), anti-DFz2-C (1:100 (Mathew et al., 2005)), anti-GFP (1:200; Molecular Probes), anti-dGRIP (1:300 (Ataman et al., 2006)), anti-Wg monoclonal (1:2,
clone 4D4; Developmental Studies Hybridoma Bank (DSHB)), anti-elav (1:50; clone 9E8A10; DSHB), anti-myc (1:200; Roche), mouse anti-GFP (1:200; Molecular Probes), Rabbit anti-GFP (1:300; MBL International), anti-β-gal (1:1000; Organon Teknika Corporation), Texas red (TxR) conjugated anti-HRP (1:200; Sigma), FITC conjugated anti-HRP (1:800; Sigma). FITC-, TxR-, and Alexa 647-conjugated secondary antibodies (Jackson Immunoreasearch) were used at 1:200. Rhodamine conjugated Phalloidin (Molecular Probes) was used at 1:200. Hoechst nuclear stain (33342, Invitrogen) was used at 10 µg/ml for 15 minutes at room temperature.

**Quantification of immunoreactivity levels and morphometric analysis**

Images were acquired using a Zeiss (Oberkochem, Germany) Pascal Confocal Microscope with a 63X (1.4 numerical aperture) objective. Preparations from different genotypes were processed simultaneously and imaged using identical confocal acquisition parameters for comparison. Fluorescence signal intensity was quantified by volumetric measurements of confocal stacks using Volocity 4.0 Software (Improvision, Waltham, MA). For measurement of pre- and postsynaptic intensity, single boutons were selected and analyzed as three-dimensional volumes in Volocity. The labeled region around the boutons was segmented by intensity thresholding based on the difference in the intensities at the NMJ vs background intensity. Fluorescence intensity represents the sum of the intensities of each of the voxels that fell above the threshold value. Presynaptic intensity was measured by calculating the volume occupied by the label of
interest that overlapped with the volume occupied by the anti-HRP label (presynaptic bouton volume) and measuring the total intensity within that volume. To obtain postsynaptic intensity, the volume occupied by the anti-HRP label was subtracted from the total volume of the labeled region resulting in the postsynaptic volume. The total intensity of the label within the postsynaptic volume was computed to obtain the postsynaptic intensity. Both pre and postsynaptic intensities were expressed as $V_{\text{post-int}} / V_{\text{bouton}}$ and normalized to wild type controls dissected and processed in the same experimental session. For determination of the number of type I boutons and ghost boutons, body wall muscle preparations were double stained with anti-HRP and anti-DLG. Measurements were taken from muscles 6 and 7, abdominal segment 3. For measurements of Wg accumulation in the brains of wild type and evi mutant larvae, regions of the brain that included either the motoneuron cell bodies or the neuropil were selected from both genotypes and mean intensity of Wg in that region were measured. A Student’s t test was performed for pair-wise comparisons between each genotype and its simultaneously processed wild type control samples.

**Schneider-2 (S2) cell cultures**

*Drosophila* Schneider (S2) cells were cultured in SFX (Hyclone) medium containing 10%FBS, penicillin (100 U/μl) and streptomycin (100 μg/μl) (SFX-SPS). 3x2ml-wells/sample of 60-80% confluent S2 cells were transfected with 1.0 μg DNA using Cellfectin and Serum Free Medium (Invitrogen). For Evi-
GFP transfer experiments, pAc-Evi-EGFP (Bartscherer et al., 2006) transfected S2 cells were washed 24 hours after transfection and mixed with pAc-mCherry transfected S2 cells. For co-transfection experiments, pAc-Wg (Bartscherer et al., 2006) was used along with pAc-Evi-EGFP. In addition pAc-rCD2-RFP and pAc-DFz2-myc (Mathew et al., 2005) constructs were used for transfections. Cells were then grown for 24-48 hours and processed for immunocytochemistry.

**Live Imaging**

Live imaging of cells was performed on transfected S2 cells grown on 22mm coverslips, and then mounted over cell culture medium, and sealed in place with dental wax. These slides were then imaged using an Improvision Spinning Disk confocal microscope using a 40X Zeiss 1.2 NA objective. Z-stacks were taken once every 5 seconds. Live imaging of larval muscles was performed basically as in Ataman et al. (2008). Briefly, larvae were dissected under 0.1mM CaCl₂ HL-3 saline, covered with a coverslip and imaged under the Improvision Spinning Disk confocal microscope. Due to two color live imaging, Z-stacks were taken once every 15 seconds.

**Western blots**

Larval brains were dissected in ice cold Ca²⁺-free saline and homogenized at 4°C in RIPA buffer containing protease inhibitors as previously described (Mendoza-Topaz et al., 2008). Proteins were separated on an 8% SDS PAGE gels, transferred onto nitrocellulose membranes and sequentially immunoblotted with anti-Wg (1:3000) and anti-tubulin (1:5000). For examination
of Evi in S2 cells and the culture medium, the culture medium of S2 cells transfected with pAc-Evi-EGFP was changed 24 hours after transfection. Cells were then harvested the next day and spun down at 4,000rpm to collect the cellular pellet and the supernatant. The supernatant was subsequently centrifuged at 12,000rpm. Cell lysates and supernatants were separated on 8% SDS PAGE gels and immunoblotted as above with anti-GFP (1:5000; Abcam) and anti-Lamin C (1:300; LC28.26, Developmental Studies Hybridoma Bank) antibodies. Signal was detected using chemiluminescence reagents (Amersham).

**Extraction and Isolation of RNA and cDNA synthesis**

Larvae were homogenized in trizol reagent using a pellet pestle (Kimble-Kontes, Vineland, NJ). Total RNA was treated with DNAse and eluted with the RNeasy Micro Kit (Qiagen, CA). RNA was quantified by UV spectrophotometry using a NanoDrop 2000c spectrophotometer (Thermo Scientific). cDNA synthesis was performed using a SuperScript III cDNA synthesis kit from 1 µg of eluted total RNA (Invitrogen, CA). Expression of Evi mRNA was analyzed by Real time PCR with Taqman Gene expression assays, house keeping: GapDH [assay ID: Dm01841185_m1 gpdh], Target: Evi [assay ID:Dm 01802231_g1 wls] using the ABI 7000 SDS software, Applied Biosystems and analyzed via the delta-delta Ct method. Absence of DNA contamination in total RNA was confirmed by real-time qRT-PCR using -RT as a control.

**Generation of Evi antibodies**
Affinity purified antibodies anti-Evi-Nex and anti-Evi-Cin, were raised in rabbits using the peptides TIDMRLAYRNKGDNP and SHKQHTMHSDETTQSN as immunogens (Biosource).

**Internalization assay**

The internalization assay was performed as in (Mathew et al., 2003). Wandering third instar larvae were dissected in 0.1 mM Ca\(^{2+}\) HL-3 saline and incubated in saline containing DFz2-N antibody for 2 hr at 4°C. Samples were then washed and shifted to room temperature for 5 or 60 min, fixed, and labeled with Alexa 647-conjugated secondary antibody under nonpermeabilized conditions to label surface DFz2. To label internalized DFz2, samples were permeabilized and labeled with an FITC-conjugated secondary antibody.

**Immunoelectron microscopy**

For the pre-embedding technique, third instar body wall muscles were dissected in 0.1 mM CaCl\(_2\) HL-3 saline and fixed using 0.1% Glutaraldehyde in Trump’s fixative with 2mM MgCl\(_2\) for 1 hour at room temperature. The samples were then washed and incubated with anti-Evi-Nex (1:100) or anti-GFP (1:300) antibody for 1 hour at room temperature. The secondary antibody was anti-rabbit IgG-1.4 nm-nanogold (1:50; Nanoprobes, NY). The samples were then washed, rinsed with deionized water and the signal intensified by using silver enhancement using HQ silver reagents (Nanoprobes, NY) in the dark for 4 minutes. Samples were then post-fixed for 1 hour at 4°C. Boutons from muscles 6 and 7 at segments A3 were serially sectioned and photographed at 19,500X and 66,000X using a TEM. For
the internalization assay, samples were dissected as above and incubated in saline containing anti-Evi-Nex for 1 hr at 4°C. Samples were then washed and shifted to room temperature for 0 or 60 min and fixed in Trump's fixative containing 0.1% glutaraldehyde and 2 mM MgCl₂ for 1 hr at room temperature. After washing, samples were incubated with 1.4 nm nanogold conjugated anti-rabbit secondary (1:50; Nanoprobes, NY) prior to processing for TEM as above.

For the post-embedding technique, samples were fixed in Trump's fixative containing 0.1% glutaraldehyde and 2 mM MgCl₂ for 2.5 hours, and then embedded in LR White resin. Thin sections were captured on nickel grids, treated with glycine to quench free aldehyde groups, blocked, and incubated with anti-Evi-Cin (1:100), and anti-rabbit IgG antibody conjugated to 18 nm gold (1:75; Jackson). Samples were then stained and visualized by TEM.
Figure 3.1. Wg localization at the NMJ is regulated by presynaptic Evi.

(A-D) Single confocal slices of 3rd instar larval NMJs at muscles 6 or 7, labeled with anti-HRP (blue) and anti-Wg (red) in (A) Wild type, (B) an evi mutant expressing transgenic Evi in motorneurons to determine if it rescues the Wg decrease (evi, Evi-pre), (C) an evi mutant, and (D) an evi mutant expressing transgenic Evi in muscles to determine if it rescues the Wg decrease (evi; Evipost). Note that Wg levels were decreased at NMJs of evi mutants, and this phenotype could be rescued by expression of Evi in motorneurons but not in muscles.

(E) Normalized Wg levels inside synaptic boutons (black bars; pre-), and at the postsynaptic region (white bars; post-) in the indicated genotypes (see methods for details on the volumetric quantification). Number of samples is 33 for wild type, 11 for evi mutants, 13 for evi, Evi-pre, and 11 for evi, Evi-post. Bars in the histogram represent mean±SEM. ***= p<0.0001; **= p<0.001.

(F) Western blot of larval brain extracts showing that Wg levels do not change in evi mutants, but they are enhanced when Evi is expressed in the presynaptic motorneurons. Numbers at the right of the blot represent molecular weight in KDa.

Calibration bar is 7 μm.
Figure 3.1
Figure 3.2. Mutations in evi mimic abnormal synaptic phenotypes observed in wg mutants.

(A-D) Confocal images of 3rd instar larval NMJs at muscles 6 and 7, labeled with anti-HRP (red) and anti-DLG (green) in (A, C) wild type, and (B, D) an evi mutant. (A, B) are low magnification projections of an entire NMJ at muscles 6 and 7, while (C, D) are high magnification single confocal slices of NMJ branches. In evi mutants the number of synaptic boutons is drastically reduced, and boutons have an abnormal shape (arrowheads in C, D). These mutants also have an abnormally high number of undifferentiated boutons (ghost boutons; arrows in D).

(E-G) Quantification of the number of (E, G) boutons and (F) ghost boutons at the 3rd instar larval stage at muscles 6 and 7, abdominal segment A3 in the indicated genotypes. Number of samples in E and F is 26 for wild type; 12 for evi mutants, 14 for evi, Evi-pre; 13 for evi, Evi-post-BG487; 15 for evi, Evi-post-C57; and 13 for evi, Evi-pre + post-C57. Number of samples in G is 13 in wild type, 11 in wgts/+, 14 in evi/+, and 10 in wg/+;evi/+.

Bars in the histograms represent mean±SEM. ***= p<0.0001; **= p<0.001; *=p<0.05. Calibration bar is 30 μm for A, B and 13 μm for C, D.
Figure 3.2
Figure 3.3. Evi is localized pre- and postsynaptically at the NMJ and it is transported trans-synaptically as an intact protein.

(A) Diagram showing the predicted structure of Evi and the protein regions (underlined) used for generation of the Evi-Nex and Evi-Cin antibodies.

(B-E) Single confocal slices of NMJs at muscles 6 or 7 double stained with anti-HRP (red) and antibodies to(green) (B,D) Evi-Nex and (C, E) Evi-Cin, in (B,C) wild type, (D,E) evi mutant. Both the Evi-Nex and Evi-Cin antibodies label the NMJ, and this immunoreactivity is severely decreased in evi mutants.

(F-H) Confocal slices stained with Evi-Nex (green) and HRP (red) in (F) Evi-RNAi-pre, (G,H) Single confocal slices of NMJs at muscles 6 or 7 of (G) Evi-GFP-pre triple stained with anti-GFP (green), anti-HRP (red) and anti-Dlg (blue) or (H) evi;Evi-GFP-Pre stained with anti-GFP (green), anti-HRP (red).

(I) Quantification of normalized pre- (black bars) and postsynaptic (white bars) Evi levels in the indicated genotypes. Number of samples is 17 for wild type, 10 for Evi-RNAi-Pre and 12 for Evi-RNAi-Post.

(J) Single confocal slices at (J1-J3) low magnification and (J4-J6) high magnification in Evi-GFP-Pre stained with anti-GFP (green), anti-Wg (red) and anti-HRP (blue) showing that secreted Evi-GFP colocalizes with secreted Wg at the postsynaptic region.

(K) Confocal images of NMJ from Evi-RNAi-Post stained with (K1, K2) Evi-Nex (green) and (K2) HRP (red).

(L,M) Confocal images of NMJs from evi;Evi-GFP-Pre triple stained with GFP (green), anti-HRP (blue) and antibodies to red (L2,L3) Evi-Nex or (M2, M3) Evi-Cin. Note that postsynaptic GFP is colocalized with both anti-Evi-Nex and anti-Evi-Cin suggesting that Evi is transferred trans-synaptically as an intact vesicle (see text for details).

(N, O) Models on the potential mode of Evi trans-synaptic transfer. Note that Evi is fused to GFP at the C-terminal tail and that the Evi-Nex antibody binds to the first extracellular loop (red) of Evi. In (N) an extracellular region of Evi is cleaved
and transported to the postsynaptic compartment. In (O) Evi is transferred as an intact protein through the use of vesicular compartments. 1 (red arrows) and 2 (black arrows) represent potential vesicular pathways. In 1, an Evi-containing vesicle is released from the presynaptic membrane through a multivesicular body (MVB), which either fuses with the postsynaptic membrane or is taken up as an intact vesicle in an MVB. In 2, a presynaptic Evi vesicle is released from the presynaptic membrane and either fuses with the postsynaptic membrane or is taken up as an intact vesicle in an MVB.

Bars in the histograms represent mean±SEM. *** = p<0.0001; ** = p<0.001. Calibration bar is 2 μm for Fig.3.3E5-8 and 6 μm for the rest of the panels.
Figure 3.3
**Figure 3.4. Evi is transferred from cell to cell and to the medium.**

(A, B) Single confocal slice through a mixture of S2 cells (A) either untransfected (outlined by white circles) or transfected with Evi-GFP (green) and (B) either transfected with mCherry (red) or Evi-GFP (green). Note that in either case, Evi-GFP puncta are observed in the Evi-negative cells (arrowheads) and in the media (arrow).

(C) Evi-GFP and Wg are transferred together into an untransfected cell (arrowheads).

(D) Wg localizes with Evi into punctuate structures within filopodia (arrows), as well as in the medium (arrowhead).

(E) Western blot of lysates and media from Evi-GFP transfected (+) S2 cells. Note the presence of GFP signal in the growth medium from transfected cells, indicating the secretion of Evi to the medium.

(F) Time-lapse imaging of an S2 cell transfected with Evi-GFP and showing the shedding of an Evi-GFP vesicle to the medium (arrows).

Calibration bar is 3 μm for panel D and 8 μm for the rest of the panels. Time points in F are (in minutes).
Figure 3.4
**Figure 3.5. Evi is localized to pre- and postsynaptic vesicular structures as well as pre- and post-perisynaptic membranes.**

(A-K) Electron micrographs of synaptic bouton regions in preparations labeled with antibodies to Evi-Nex or GFP, followed by 1.4 nm gold-conjugated secondary and silver intensification, or antibodies to Evi-Cin labeled with 18 nm gold-conjugated secondary. In these micrographs the presynaptic compartment has been overlayed in pink. Insets are high magnification views of the structures indicated by the arrows. In (A, B, D-H) samples were fixed and permeabilized followed by primary and secondary antibody incubation. In (C) samples were processed for an internalization assay (see text for details). (I-K) Samples were stained post-embedding with anti-Evi-Cin.

(A, B) Immunoreactive vesicles found at the SSR region.

(C) Internalized Evi is found in postsynaptic SSR vesicles. (D, K) Localization of label at SSR membranes.

(E-H) Evi label at the perisynaptic region of pre- and postsynaptic membranes. Arrowheads in (F) mark the active zone.

(I) Evi localization at a presynaptic multimembrane body.

(J) Evi-immunoreactive gold particles at the presynaptic region and the synaptic cleft.

Calibration bar is 0.6μm in A, C, D, K; 0.3μm in B, E-H; 0.2μm in the insets of A; 0.15μm in the inset of B, and 0.1 μm in the inset of C.
Figure 3.6. Evi downregulation in muscle results in postsynaptic Wg and DFz2 accumulation and alterations in the Frizzled Nuclear Import Wg pathway.

(A-D) Single confocal slices through NMJs at muscles 6 or 7 triple stained with antibodies to HRP (red), DLG (blue) and (A, B) DFz2 (green) or (C, D) Wg (green) in (A, C) wild type and (B, D) Evi-RNAi-Post. Note that both Wg and DFz2 accumulate at the postsynaptic region of NMJs expressing Evi-RNAi postsynaptically.

(E) Quantification of Wg (black bars) and DFz2 (white bars) immunoreactivity levels at the postsynaptic region of the indicated genotypes. Number of samples is 10 for wild type, and 10 for Evi-RNAi-post.

(F) Intensity of surface and internalized DFz2 at 5 and 60 min after the antibody-binding step in wild type (black bars) and Evi-RNAi-post (white bars). Number of samples is 13 and 10 for wild type at 5 and 60 min; 11 and 10 for evi mutants at 5 and 60 min.

(G-J) Single confocal slices of branches form NMJs subjected to the internalization assay, showing (G1-J1) surface DFz2 (blue) and (G2-J2) internalized DFz2 (green) (G, I) at 5 min and (H, J) 60 min after the antibody binding step, in (G, H) wild type, and (I, J) Evi-RNAi-post. Note that in Evi-RNAi-post internalized DFz2 remains at high levels at the NMJ at 60 min.

(K, L) Confocal slices of muscle nuclei in preparations stained with anti-DFz2-C (green) and Hoechst (blue) in (K) wild type and (L) evi mutants showing the drastic decrease in intranuclear DFz2-C in the mutants.

(M) Normalized number of DFz2-C nuclear spots in the indicated genotypes. Number of nuclei quantified is 159 for wild type, 154 for evi mutants, 115 for Evi-RNAi-post, 163 for evi, Evi-GFP-post, and 92 for evi, Evi-GFP-pre. Bars in the histograms represent mean±SEM. **= p<0.001; *=p<0.05 Calibration bar is 10 μm for panels A-H1-2, I-L, 5 μm for panels A-D3-4.
Figure 3.6
Figure 3.7. Downregulating Evi in postsynaptic muscles alters the localization of dGRIP, and proposed function of Evi in the pre- and postsynaptic compartment.

(A, B) Single confocal slices through NMJs at muscles 6 or 7 in preparations triple labeled with antibodies to HRP (blue), dGRIP (green) and Lavalamp (Lva; red) in (A) wild type, and (B) Evi-RNAi-post. Arrows in A point to synaptic dGRIP, Arrowheads in A and B point to dGRIP positive and dGRIP negative Golgi bodies (marked with lava lamp) respectively. Note the decrease in synaptic and Golgi immunoreactivity and the diffuse appearance of dGRIP in the muscle cortex.

(C) Quantification of dGRIP levels at the postsynaptic junctional region, Golgi bodies, and muscle cortex, in wild type (black bars) and Evi-RNAi-post (white bars), showing that dGRIP is significantly reduced from postsynaptic sites and Golgi, but increased at the muscle cortex in Evi-RNAi-post. Number of samples is 10 for wild type and 10 for Evi-RNAi-post.

(D) Quantification of postsynaptic DFz2 levels in wild type, Evi-RNAi-post and Evi-RNAi-post, dGRIPpost. Number of samples is 10 for wild type, 10 for Evi-RNAi-Post and 16 for Evi-RNAi-post, dGRIP-Post.

(E) Quantification of bouton number in wild type, evi mutants, and evi mutants expressing dGRIP-post. Number of samples is 26 for wild type, 12 for evi mutants, and 8 for evi mutants expressing dGRIP-post.

(F) Quantification of DFz2C spots in wild type, evi mutants, and evi mutants expressing dGRIP-post. Number of samples is 205 for wild type, 154 for evi mutants, and 136 for evi mutants expressing dGRIP-post.

(G) Proposed model for Evi function in the pre- and postsynaptic compartment (see text for details).

Bars in the histograms represent mean±SEM. ***= p<0.0001; **= p<0.001; *=p<0.05. Calibration bar is 6 μm for A-B1-3, and 3 μm for A-B4-6.
Figure 3.7

[Image of a figure with panels labeled A1 to B6, C, D, E, F, and G. Each panel contains graphical data and images.]
Supplementary Figure 3.1. Reduction of postsynaptic Wg by expressing Evi-RNAi in motorneurons.

(A, B) Single confocal slices through muscles 6 or 7 in preparations double labeled with antibodies against HRP (blue) and Wg (red) in (A) wild type, and (B) in larvae expressing Evi-RNAi in motorneurons.

(C, D) Single confocal slices through the ventral ganglion of (C) wild type and (D) an evi mutant stained with Wg (green) and Elav (red).

(E) Quantification of Wg levels either in the neuropil or the motor neuron region of the indicated genotypes. Number of samples is 8 for wild type and 10 for the evi mutant.

Calibration bar is 7µm for panels A, B and 90µm for C,D.
Supplementary Figure 3.2. Muscle surface area in different genotypes used in this study.
Number of samples is 20 for wild type, 13 for evi mutants, 6 for Evi-RNAi-post, 11 for Evi-RNAi-pre, 6 for Evi-RNAi/+.
Supplementary Figure 3.2
Supplementary Figure 3.3. Localization of the Evi antibody epitopes to extra and intracellular sites, and *in vivo* shedding of Evi-containing vesicles.

(A,B) Single confocal slices of unpermeabilized NMJs at muscles 6 or 7 double stained with antibodies against HRP (red) and (A) Evi-Nex (green) or (B) Evi-Cin (green). Note that only the Evi-Nex antibody stains the NMJ in unpermeabilized samples, consistent with the idea that the Evi-Nex epitope is localized extracellularly and the Evi-Cin epitope likely intracellularly.

(C) Single confocal slice of an NMJ imaged live, in a larva expressing myr-mRFP (red) in motorneurons to label the presynaptic compartment and Evi-GFP (green) in motorneurons to show that presynaptic Evi-GFP vesicles are shed to the postsynaptic compartment.

(D,E) Confocal image of a larva expressing LacZNLS with the Gal4 driver C380 showing (D) the ventral ganglion —note the clear nuclear localization, and (E) the NMJ —note the lack of signal in muscle nuclei (arrows).

(F) Volumetric quantification of Evi signal in either wild type or presynaptic rescue of *evi* mutants.

(G-H) Confocal slices and colocalization measurements between presynaptically expressed Evi-GFP and (G) anti-Evi-Nex or (H) anti-Evi-Cin.

Calibration bar is 5 µm for A-B and G-H, 7µm for C and 160µm for D and 13µm for E.
Supplementary Figure 3.3
Supplementary Figure 3.4. S2 cells do not transfer DFz2 or rCD2-mRFP to the medium.

(A-C) Confocal images of S2 cells transfected with (A) DFz2, (B) rCD2-mRFP, and (C) untransfected. Note that the transfected cells are not transferring transmembrane proteins, as previously observed with Evi-GFP. Also, the formation of filopodia is independent of transfection as they are observed in untransfected cells as well.

Calibration bar is 5μm.
Supplementary Figure 3.4
Supplementary Figure 3.5. Real-time PCR analysis of Evi mRNA levels in body wall muscles.

Normalized Evi mRNA levels in wild type and Evi-RNAi-post.
Supplementary Figure 3.5
CHAPTER IV

Discussion
Two fundamental processes governing normal synaptic proliferation are proper cytoskeletal regulation and precise anterograde and retrograde signaling. Studies have shown that coordinated changes in the actin and microtubule cytoskeleton are required for normal synaptic outgrowth and function. Additionally, precise bidirectional signaling across the synapse is required to maintain a balance between the increasing muscle size and bouton outgrowth. In the first part of my thesis, I have attempted to describe some of the intracellular events that lead to the regulation of the postsynaptic actin cytoskeleton by the aPKC/Baz/Par-6 polarity protein cassette in conjunction with PTEN. The role of these proteins has been elucidated quite well in other cell types with respect to cytoskeletal regulation (Cox et al., 2001; Hirose et al., 2002; Chen and Macara, 2005). However, their function in the context of the synapse outgrowth has not been investigated. Here I have demonstrated a mechanism by which aPKC regulates postsynaptic actin through its substrate Baz. My study also demonstrates that phosphorylation of Baz by aPKC changes its localization and consequently enables it to regulate the actin cytoskeleton. These results provide some understanding into the long standing question of the role of phosphorylation in the regulation of Par-3 function. Additionally, my study has demonstrated that PTEN is highly enriched at the NMJ and has identified a novel role for PTEN in mediating Baz dephosphorylation and postsynaptic actin regulation. Lastly, I have demonstrated that changes in the cytoskeleton not only
affect synaptic proliferation and morphology, but also affect postsynaptic
neurotransmitter receptor abundance and synaptic transmission.

In the second part of my thesis, I have contributed to a study attempting
to elucidate the mechanisms by which *Drosophila* Wingless (Wg), a member of
the Wnt family of secreted proteins, is secreted from the pre- to the postsynaptic
terminal. We demonstrate that Evenness/Wntless (Evi), a protein dedicated to
the transport of Wnts from the TGN to the plasma membrane in different cell
types (Banziger et al., 2006; Bartscherer et al., 2006), is required for the
secretion of Wg at the NMJ. We further show that Evi is secreted along with Wg
across the synapse in exosome-like vesicles. Additionally, Evi plays a role in the
postsynapse in transducing the Wg transduction pathway by regulating the
trafficking of GRIP (Ataman et al., 2006), a downstream component of this Wg
pathway, to the synaptic membrane.

Based on our findings in Chapter 2, we propose that aPKC
phosphorylation of Baz targets it to the postsynapse while PTEN mediated
dephosphorylation of Baz retains it at the postsynapse where Baz regulates the
actin cytoskeleton. A caveat of our study is that since null mutants of these genes
are lethal, we had to rely mostly on RNAi based knockdown of protein levels to
draw conclusions about the function of the proteins. Additionally, overexpression
of these proteins with or without a tagged construct using the UAS-Gal4 system
resulted in protein mislocalization, leading to a dominant negative effect
precluding us from attempting rescue experiments or live imaging experiments.
However, we have tried to circumvent these issues by using two different RNAi lines or available hypomorphs for each of the genes examined to ensure that the phenotypes we observe are specific to the knockdown of the protein of interest.

While presynaptic actin regulation has been quite well studied at the NMJ, (Coyle et al., 2004; Nunes et al., 2006; Rodal et al., 2008) very few proteins have been identified that regulate postsynaptic actin. The reason for this is that the inability to visualize post synaptic actin using phalloidin or by expressing Actin-GFP in the muscle has effectively limited the ability to detect changes in actin organization. To alleviate this problem, we have focused on the Type Ib synaptic terminals on Ms 12 and 13. Unlike Ms 6 and 7 or Ms 4 which are typically used for quantification or detection of phenotypes, the distance between the boutons and the contractile apparatus in Ms 12 and 13 is quite extensive making it easy to separate synaptic actin from sarcomeric actin upon staining with phalloidin.

Our results that the loss of the postsynaptic actin envelope does not lead to the loss of other synaptic proteins such as Dlg and Scribble (another PDZ protein that interacts with Dlg) (Mathew et al., 2002) or to the disruption of the SSR was a little surprising to us considering that at vertebrate synapses, results obtained by using actin depolymerizing drugs have demonstrated that actin is required for the stability of the Post Synaptic Density (PSD). However, this function appears to depend largely on the state of the synapse as well as the concentration of drugs used. While PSDs in newly formed synapses have a high propensity to undergo disassembly when exposed to actin depolymerizing drugs,
PSDs in more mature synapses are highly resistant to these drugs (Zhang and Benson, 2001; Cingolani and Goda, 2008). Additionally, the vertebrate postsynapse has been shown to have multiple pools of actin having different postsynaptic functions that are vulnerable to different concentrations of actin depolymerizing drugs (Cingolani and Goda, 2008; Honkura et al., 2008). Unlike these studies where application of depolymerizing drugs may have resulted in a partial or complete dismantling of the postsynaptic actin cytoskeleton, in our study, there is still some amount of actin remaining at the postsynapse. It is possible that these low levels of actin may be sufficient to stabilize the postsynaptic apparatus or alternatively, like vertebrate studies, perhaps proteins or structures that are associated with Dlg such as Scribble and GluRIIB (Mathew et al., 2002; Chen and Featherstone, 2005) are highly stable even in the absence of actin in mature boutons. In keeping with this idea, a study looking at the anchoring of glutamate receptors at the NMJ, reported that while treatment with Lantranculin, a drug that prevents actin polymerization, or Cofilin, that severs actin filaments, resulted in the loss of GluRIIA receptors, it did not have any effect on GluRIIB receptors clusters (Chen et al., 2005). Indeed, previous studies have reported a role for Dlg in stabilizing GluRIIB clusters (Chen and Featherstone, 2005) further lending credibility to the idea that Dlg associated complexes may be more resistant to the disruption of the postsynaptic cytoskeleton. Alternatively, the results of the above study can also be explained
by the idea that postsynaptic actin might be organized into different pools with different levels of stability that are involved in specific postsynaptic functions.

**Model for cytoskeletal regulation and function during synaptic growth**

*Potential Regulation and Functions of Presynaptic Actin*

Based on the distribution of proteins required for neurotransmitter release versus transmembrane adhesion and signaling proteins that regulate the actin cytoskeleton, it has been proposed that the plasma membrane of the presynaptic terminal is subdivided into two distinct domains, the active zone where neurotransmitter release occurs and the periactive zone which lies adjacent to the active zone where active synaptic growth occurs (Sone et al., 1997; Sone et al., 2000). Previous studies have described a cortical network of actin at the presynapse close to the membrane (Coyle et al., 2004; Rodal et al., 2008). It is possible that while at the active zones, the actin cytoskeleton is involved in regulating neurotransmitter release by altering vesicle mobility and fusion (Nunes et al., 2006), at the periactive zone, it is involved in bouton budding and increase in bouton size.

Studies focusing on presynaptic actin regulation have proposed a model for how actin might be regulated during bouton growth and budding based on actin dynamics in axonal growth cones (Collins and DiAntonio, 2004). According to this model, while bouton enlargement occurs in a manner similar to the leading edge of the translocating lamellopodia, bouton budding is akin to the formation of exploratory filopodia. Thus during bouton enlargement, F- actin is primarily
arranged in the form of a branched network along the bouton periphery, and the activity of regulatory proteins that stimulate F-actin branching is enhanced. On the other hand, during bouton budding, a localized rearrangement of actin in the region of budding results in the formation of filopodia. The actin filaments in these filopodia are arranged in the form of linear bundles which continuously elongate and in the process, push and expand the membrane to form a bud. Likewise, during this process, the activity of regulatory proteins that stimulate F-actin bundling is enhanced (Collins and DiAntonio, 2004). One can speculate that a gradual maturation of the bud is accompanied by the formation of a new cortical actin meshwork that while providing stability to the overlying plasma membrane also plays an active role in increasing the size of the bud. Furthermore, at some point during bud maturation, dynamic MT filaments that penetrate these buds could be captured and linked to the actin filaments which would increase the stability of the bud and also allow for the transport of various presynaptic components into the bud ensuring its maturation into a bouton. Indeed studies have shown that the inability to link presynaptic MTs to the cortical actin cytoskeleton at the presynapse results in the withdrawal of MTs and subsequent synaptic retraction (Eaton et al., 2002; Pielage et al., 2005; Pielage et al., 2008).

However additional roles for actin, particularly in the area of MT regulation, can be envisioned at the presynapse based on emerging evidence from growth cone studies. Growth cone navigation is mainly characterized by periods of substrate exploration associated with growth cone pausing or idling alternating
with periods of active movement and growth cone advancement. While net polymerization of actin and increase in length of actin filaments at the leading edge allows for growth cone advancement in the forward direction, growth cone idling is mainly characterized by actin treadmilling and retrograde actin flow which keeps the length of the actin filaments constant (Lowery and Van Vactor, 2009). During exploration of the substrate, dynamic MT filaments extend out into the filopodia and explore the periphery by following the trajectories laid out by F-actin bundles (Letourneou, 1983; Gordon-Weeks, 2004; Zhou and Cohan, 2004). However recent studies have shown that the dynamic nature of MTs in these filopodia depends largely on whether or not these MTs are coupled to the filopodial actin bundles. While MTs not coupled to actin bundles can explore growth cone sides more frequently, the penetration of actin coupled MTs into the filopodia is inhibited (Burnette et al., 2007; Lee and Suter, 2008). Furthermore, in cases where the MTs are coupled to the actin filaments, the processes of actin treadmilling and retrograde actin flow is proposed to cause these MTs to form loops because the growing plus ends of MTs are transported back towards the centre of the growth cones by their linkage to F-actin retrograde flow (Purro et al., 2008; Lowery and Van Vactor, 2009). Indeed many proteins have been identified that have been demonstrated to regulate this process. While proteins like LIS1(Lisencephaly) (Grabham et al., 2007) and APC (Purro et al., 2008) are involved in uncoupling of dynamic MTs from actin retrograde flow and promoting MT dynamic instability, proteins such as CLASP (Cytoplasmic Linker Protein-
associated protein) (Tsvetkov et al., 2007) and MAP1B (Bouquet et al., 2007) have been proposed to couple MTs and actin, enhancing the formation of loops. Upstream regulators of these proteins that have been identified to regulate loop formation include Wnt3A and GSK3β (Trivedi et al., 2005; Lowery and Van Vactor, 2009).

Drawing parallels to growth cone dynamics, it is very tempting to speculate that MT loops observed in boutons might follow a similar pattern of actin-MT interactions and the *Drosophila* counterparts of the above proteins might be involved in regulating these interactions. While many proteins like APC and CLASP have yet to be identified at the *Drosophila* NMJ, preliminary evidence suggests that this could indeed be the case. Consistent with studies in growth cones, loss of Wg at the NMJ results in unbundling of presynaptic MTs and decreases the formation of loops (Packard et al., 2002). Similarly, it has been established that Futsch is required for the formation of these loops (Roos et al., 2000). It is possible that in addition to enhancing bundling within MTs, Futsch could also mediate bundling between actin and MTs thereby aiding in the formation of these loops. The functions of these proteins in MT regulation have been established; however their roles in actin regulation have yet to be explored.

**Potential Regulation and Functions of Postsynaptic Actin**

While it is challenging to propose a model for postsynaptic actin regulation based on the results of a single study, one can speculate that at the postsynapse, the
actin/spectrin envelope while possibly functioning as a framework for the assembly of postsynaptic components, also has other functions. Owing to its ability to be more flexible than MTs, it can act as a pliable and elastic cushion for boutons to bud and grow into as well as for protection against the mechanical stress experienced during muscle contraction while providing enough resistance to maintain normal bouton size. An important finding of our study was that downregulation of aPKC, Baz, or PTEN in the muscle led to a significant reduction in the thickness of the actin/spectrin envelope. However, in these loss of function lines, the actin envelope continued to show a meshwork like appearance very similar to wild type larvae. Furthermore, this reduction was associated with a decrease in bouton number associated with an increase in the size of the boutons. A potential mechanistic explanation of these changes is that, during bouton enlargement, the pressure exerted by the growing bouton can result in the actin/spectrin envelope exhibiting some sort of elastic sponge like behavior where it is capable of undergoing compression and relaxation probably by undergoing myosin mediated contractions. On the other hand, it can also result in a continuous de- and repolymerization followed by crosslinking at the immediate area surrounding the bouton and the actin-MT interface, thereby exhibiting a form of treadmilling, which while keeping the thickness of the actin/spectrin envelope constant, will allow boutons to continually increase in size. In theory, while the former mechanism would primarily be dependent on
actin crosslinking proteins and Myosin, the latter mechanism would also need actin polymerizing and depolymerizing proteins.

In contrast, during the formation of a bud, it can be conceived that an area of localized depolymerization of postsynaptic actin, brought about by regulatory proteins, allows the bouton membrane to protrude in that particular region giving rise to a bud which then develops its own postsynaptic actin meshwork and continues to mature into a bouton. Thus, it is possible that the ability of the bouton to sense the difference in the pressure exerted by adjacent regions of the postsynaptic actin cytoskeleton determines whether boutons should enlarge or undergo budding. In keeping with this model, in mutants where the thickness of the actin/spectrin envelope is dramatically reduced, the ability to generate this pressure differential is probably lost leading to a continuous increase in bouton size and a decreased propensity of bouton budding resulting in fewer but larger boutons.

Additionally, the postsynaptic actin envelope might also play a role in regulating the abundance of postsynaptic proteins, especially neurotransmitter receptors. Studies in hippocampal neurons and other cell culture systems have reported roles for the actin cytoskeleton in regulating neurotransmitter receptor abundance in dendritic spines by modulating receptor stabilization, and by controlling receptor trafficking to the postsynapse. While actin has been shown to play a role in GluR1IA receptor stabilization at the NMJ (Chen et al., 2005), much remains to be understood about the role of actin in other modes of regulation.
It is well known that receptor trafficking and transport is a coordinated process requiring MT based trafficking for long range transport from the center of the cell body to the periphery or the dendrites and actin based trafficking for localized transport at the periphery (Rodionov et al., 2003). In both cases, the transport of cargo is enabled by motor proteins that bind directly to MTs or actin and travel along their respective tracks (Karcher et al., 2001; Ally et al., 2008). MT motor proteins include proteins belonging to the Kinesin family which are involved in anterograde/ plus end transport (Vale et al., 1985a; Vale et al., 1985b) and proteins belonging to the Dynein family which are involved in retrograde/minus end transport (Schnapp, 1989; Ross et al., 2008), whereas Myosin V, in general, has been implicated in transport of cargo towards the plus end on actin tracks (Karcher et al., 2001; Ross et al., 2008). Once the cargo reaches its destination, the motor proteins are phosphorylated resulting in the dissociation of the cargo (Karcher et al., 2001; Ally et al., 2008).

An important regulator of this process is CAM Kinase II (CamKII). CamKII has an established role in LTP induction owing to its ability to remain active for long periods of time in the absence of Ca++ by autophosphorylation (Merrill et al., 2005). In the context of protein trafficking, CamKII has been shown to phosphorylate both MyosinV (Karcher et al., 2001), and KIF3, a member of the kinesin family, which results in the dissociation of the NMDA receptor subunit, NR2B from these motor (Ally et al., 2008). Furthermore, CamKIIβ interacts and bundles actin filaments (Okamoto et al., 2007; Sanabria et al., 2009). At the NMJ,
CamKII is highly enriched at the SSR region and localized at the interface of the actin-MT boundary and is actively involved in regulating synaptic growth and function (Koh et al., 1999).

In the context of the larval NMJ, GluRIIA clusters require actin for their stabilization (Chen et al., 2005) and GluRIIB clusters require Dlg for their stabilization (Chen and Featherstone, 2005). Additionally, recruitment of GluRIIA to the synapse is believed to be enabled by trafficking from extrasynaptic cell wide pools within the muscle (Rasse et al., 2005). Similar to vertebrate synapses, trafficking of these receptors from cell wide pools to the postsynapse could be enabled by initial transport on the muscle MT network via the Kinesin family of motor proteins until the actin/spectrin boundary followed by transport on actin filaments in the postsynaptic meshwork via the *Drosophila* homolog of Myosin V. In this regard, studies looking at the mechanism of navigation of these motor proteins within the cytoskeletal network have demonstrated that the greater the numbers of intersections between cytoskeletal filaments, the greater are the chances that motor proteins might stall at these intersections (Ali et al., 2007). Based on this evidence, it is possible that the thickness of the actin envelope might indirectly determine postsynaptic receptor abundance at the NMJ. While a decrease in the extent of the actin network might reduce the number of intersections, resulting in increased trafficking to the postsynapse, an increase in the thickness of the actin envelope might have the opposite effect. Indeed, in our mutants where the thickness of the F-actin/spectrin network was dramatically
reduced, a significant increase in all the glutamate receptor subtypes was observed. Additionally, owing to its localization, CamKII might be a likely candidate to phosphorylate both MT and actin motors allowing for the potential release of GluRIIA cargo from the MTs at the actin-MT interface as well as from actin filaments at the postsynaptic membrane.

Studies have shown that the sarcomeric actin bundles are highly stable whereas cytoplasmic actin structures are highly dynamic with a rapid turnover rates (Alberts, 2002). Future studies looking at synaptic actin regulation can take advantage of this by enforcing a high degree of temporal control on transgenic expression of actin. For example, expressing fluorophore-tagged transgenic actin constructs for very short periods of time during the third instar larval stage might prevent the incorporation of these monomers to the sarcomeric actin, while being incorporated into the synaptic actin. Additionally, combining these with Fluoroscence Resonance Energy Transfer (FRET) techniques will help in understanding the dynamics of actin polymerization (Okamoto et al., 2004).

**Role of the aPKC/Baz/PTEN pathway in activity dependent NMJ growth**

The presence of the aPKC/Baz/PTEN pathway in the postsynapse and its role in synaptogenesis as evidenced by the reduction in bouton number and changes in the level of the neurotransmitter receptor levels raises the interesting possibility that this pathway might also play contribute to activity dependent synaptic outgrowth at the larval NMJ. In general, while the role of this protein
complex in activity induced synaptic plasticity has yet to be explored
hippocampal slice culture studies and studies looking at olfactory memory in
Drosophila have showed that expression of PKMzeta, the constitutively active
form of aPKC is induced during and required for the maintenance of late LTP in
hippocampal synapses and for long term memory formation in Drosophila
mushroom bodies (Sacktor et al., 1993; Drier et al., 2002; Pastalkova et al.,
2006; Sacktor, 2008). However the function of PKMzeta at a more cellular level
with respect to its ability to regulate the shape and structure of dendritic spines or
Drosophila mushroom body synapses is yet to be examined.

The Drosophila larval NMJ is an ideal model system to explore these
issues. Similar to vertebrate synapses, synaptic outgrowth at the NMJ is highly
responsive to changes in activity. Early studies demonstrated an increase in
bouton number and changes in bouton morphology in mutants with enhanced
neuronal activity compared to their third instar wild type counterparts (Budnik et
al., 1990; Zhong et al., 1992; Jia et al., 1993; Schuster et al., 1996). To further
dissect the sequence of steps involved in activity induced synaptic outgrowth,
recent studies have combined live imaging techniques with various stimulation
paradigms to observe morphological changes that occur in response to acute
changes in neuronal activity. Studies looking at synaptic plasticity as an outcome
of larval crawling activity termed as ‘experience dependent potentiation” have
proposed that plasticity occur in different phases (Schuster, 2006). Changes
occurring during phase I and II observed after 40-80 or 90-120 minutes
respectively of high crawling activity at 25°C included alterations in synaptic transmission such as an increase in mEJP and eEJP which are mediated primarily mediated by presynaptic events that regulate the amount of neurotransmitter released (Steinert et al., 2006). On the other hand, Phase III observed after 4-5 hours involved an increase in GluRIIA protein synthesis and clustering at the postsynaptic membrane resulting in an increase in the number of postsynaptic densities (Sigrist et al., 2000; Rasse et al., 2005). This increase in PSD’s was followed by an increase in the number of active zones resulting in an increase in the total number of synapses per bouton (Sigrist et al., 2003; Ataman et al., 2008). Lastly, Phase IV which can be observed after 6 hours of high crawling denotes the onset of morphological changes, new bouton formation and maturation which continues for the next 24 - 48 hours (Zito et al., 1999; Sigrist et al., 2003). Subsequently, using a combination of simulation paradigms such as spaced K+ depolarization and neuronal stimulation using Channel Rhodopsin, Ataman et al., (2008) showed that activity can enhance the formation of immature synaptic boutons, called ‘ghost boutons’ which carry synaptic vesicles, but lack active zones and postsynaptic apparatus (Ataman et al., 2006; Ataman et al., 2008). While a small percentage of these boutons get stabilized and develop PSDs, a large number of them disappear (Ataman et al., 2008). Thus they demonstrated that the formation of the presynaptic terminal is initiated earlier than the postsynaptic terminal. However, in contrast to the ‘experience dependent potentiation’ paradigm, the increase in the number of ‘ghost boutons’
in stimulated versus unstimulated samples in this study could be seen in just 2 hours after the start of the stimulation paradigm (Schuster, 2006; Ataman et al., 2008). While many of these structures were observed to disappear within the time scale of hours, the ones that remained appeared to stabilize and developed glutamate receptor clusters and active zones approximately 10-12 hours after their formation corroborating the results of the ‘experience dependent potentiation’ study (Zito et al., 1999; Schuster, 2006; Ataman et al., 2008; Fuentes-Medel et al., 2009). Thus, while bouton formation can be initiated in a relatively short period of time, assembly of the postsynaptic apparatus and bouton maturation occurs over a long time.

In our study, considering that the aPKC/Baz/PTEN pathway in the muscle seemed to affect the postsynaptic glutamate receptor levels and postsynaptic actin organization, it is conceivable that this pathway is activated primarily in Phase III and Phase IV of ‘experience dependent potentiation’. Consistent with our results, vertebrate studies have shown that PKM induced during late LTP plays an important role in potentiating AMPA receptor mediated synaptic transmission by regulating the trafficking of GluR2 receptors from the extrasynaptic region to the synaptic region (Ling et al., 2006; Yao et al., 2008). This function of PKM appeared to depend on its interaction with NSF2 (Yao et al., 2008) a protein involved in membrane vesicle fusion (Whiteheart et al., 1992). While there is no evidence of PKM regulation of actin dynamics, based on the finding that AMPA receptors interact with and are anchored to the actin
cytoskeleton (Allison et al., 1998), PKM mediated alteration in actin organization has been proposed as an additional mechanism of AMPA receptor regulation during LTP (Vlachos et al., 2008).

To further explore the postsynaptic role of the aPKC/Baz/Par6 protein complex in activity dependent synaptic growth, a number of interesting experiments could be performed. First, fluorophore tagged actin constructs could be expressed and the dynamics of actin polymerization over time could be by studied by simulating larvae expressing Channel Rhodopsin for different time periods, imaging at different time periods and comparing these changes to unstimulated larvae. Similarly, these experiments could be performed in larvae mutant for the polarity proteins to identify the activity dependent postsynaptic processes regulated by these proteins. Lastly, using a combination of FRAP (Fluorescence Recovery After Photobleaching) and FRET (Fluorescence Resonance Emission Tomography) techniques, the dynamics of interaction between the different proteins in the complex and their response to activity can be teased apart. For example, following expression of fluorophore tagged Baz constructs, photobleaching experiments could be carried our and the recovery time could be monitored in stimulated and unstimulated samples and under conditions where other proteins of the pathway such as aPKC and PTEN are downregulated to understand he effects of activity and the role of phosphorylation vs dephosphorylation on postsynaptic Baz dynamics. Similarly, by tagging aPKC and Baz with different fluorophores, FRET experiments can be
performed to determine the dynamics of interaction between aPKC and Baz in stimulated and unstimulated samples.

**Potential upstream and downstream components of the aPKC/ Baz/Par-6 polarity cassette**

Growing evidence has indicated that the aPKC/Par-3/Par-6 cassette performs numerous functions which are highly dependent on the cellular context and are brought about by a variety of complex protein-protein interactions (Suzuki and Ohno, 2006; Assemat et al., 2008). For example, in *Drosophila* epithelial cells, the aPKC/Baz/Par-6 cassette is localized at the adherens junctions and in neuroblasts, at the apical tip (Rolls et al., 2003; Assemat et al., 2008). While in epithelial cells it is required for segregation of the other polarity complexes (Humbert et al., 2006), in neuroblasts it is required for the basal localization of many cell fate determinants like Miranda (Atwood and Prehoda, 2009). In our study, we found that Baz was critical for the regulation of the postsynaptic actin cytoskeleton at the NMJ. A potential mechanism of actin regulation by Baz could be through the activation of RhoGTPases such as Rac1 and Cdc42. Studies in mammalian epithelial cells and hippocampal neurons have revealed a role for Par-3 in the recruitment of the Rac GEF, TIAM1 for tight junction formation and for axonal polarization respectively (Chen and Macara, 2005; Nishimura et al., 2005). In both cell types, Par-3 recruitment of TIAM1 was required for spatially restricted activation of Rac GTPase and modulation of actin
dynamics (Chen and Macara, 2005; Nishimura et al., 2005; Mertens et al., 2006). Additionally, while interaction between aPKC and Par-3 was required for the recruitment of TIAM1 by Par-3 during axonal polarization, in epithelial cells, this function was independent of aPKC (Chen and Macara, 2005; Nishimura et al., 2005). In both cases, activation of Rac in turn was found to enhance the activation of aPKC and Par-6 (Chen and Macara, 2005; Nishimura et al., 2005). A similar mechanism has been implicated in the formation of dendritic spines based on the finding that depletion of Par-3 resulted in a more widespread and aberrant activation of Rac1 preventing the stabilization of dendritic spines (Mertens et al., 2006; Zhang and Macara, 2006). While the constitutively active form of aPKC, PKM has been implicated in LTP (Drier et al., 2002; Pastalkova et al., 2006) its relationship to Par-3 and role in dendritic spine formation has not been examined.

In our study, we found that phosphorylated Baz was enriched at both the pre and the postsynapse as identified by our phospho-peptide antibody specific to the aPKC phosphorylation residue suggesting that its localization at both sides of the synapse might be aPKC dependent. An interesting scenario at both the pre- and the postsynapse is that while aPKC is involved in regulating MTs stability, it mediates actin regulation through Baz by phosphorylating and targeting Baz to actin rich areas. The mechanism of targeting may involve MT based transport of Baz to actin rich areas via interactions with MT motor proteins. Indeed, studies in hippocampal neurons have demonstrated an interaction
between Par-3, members of the Kinesin family (Nishimura et al., 2004) and APC (Shi et al., 2004) which is required for targeting Par-3 to axonal growth cones. In keeping with this idea, it is possible that at the presynapse, following phosphorylation by aPKC, Baz might be targeted to the periactive zones where it might be involved in recruiting SIF, the *Drosophila* homolog of TIAM1. Further support for this model comes from the fact that both SIF and exogenously expressed Cdc42 are enriched at these zones of the presynapse and SIF has been shown to activate Rac1 and Cdc42 in vitro (Sone et al., 2000; Rodal et al., 2008). While SIF is absent at the postsynapse, dPIX another RacGEF is enriched at the postsynapse (Parnas et al., 2001). Thus it is possible that at the presynapse, Baz may activate Rac or Cdc42 by recruiting SIF and at the postsynapse by recruiting dPix or other GEFs not yet identified at the NMJ. Moreover, studies at the NMJ have demonstrated that downstream effectors of Rac and Cdc42 such as WASP (Coyle et al., 2004) are highly enriched at the NMJ. However their role in actin regulation during synaptic growth has not yet been investigated.

Studies have demonstrated that in addition to aPKC, a number of other proteins regulate Par-3/Baz. Par-1 has been shown to phosphorylate Baz at Serine 151(Serine 144 in mammals) and Serine1085 (Serine 885 in mammals) (Benton and St Johnston, 2003b). Phosphorylation by Par-1 enhances the binding of 14-3-3 or Leonardo, the *Drosophila* homolog of vertebrate Par-5, to Par-3/Baz thereby disrupting its interaction with aPKC (Benton and St Johnston,
On the other hand, while in mammals, PP1 (protein phosphatase 1) has been shown to dephosphorylate Par-3 on the two Par-1 sites as well as the aPKC site (Serine 827 in mammals) (Traweger et al., 2008), in Drosophila, PP2A has been shown to dephosphorylate only the C-terminal Par-1 site (Serine 1085) (Krahn et al., 2009). While our study suggests that PTEN is required for the dephosphorylation of the aPKC site of Baz (Serine 980) in Drosophila, the phosphatase required for the dephosphorylation of the N terminal Par-1 site (Serine 151) is not yet known. It is tempting to speculate that in addition to dephosphorylation of S980, both phosphorylation of Serine 151 and Serine 1085 by Par-1 might be required for Baz to be anchored at the postsynapse. On the other hand, dephosphorylation of Baz by PP2A at the Par-1 site, might remove Baz at the postsynaptic region. Studies have shown that while Par-1 and PP2A are enriched in the SSR region along with Baz (Viquez et al., 2006; Zhang et al., 2007). Leonardo is enriched in the presynapse, but absent from the postsynapse (Broadie et al., 1997). Thus the coordinated activities of Par-1 and PTEN may be required in retaining Baz at the postsynapse. However, this function may not involve Leonardo at the postsynapse.

In mammalian epithelial cells, retention of Par-3 at tight junction has been shown to depend on its ability to oligomerize by N-terminal interactions (Benton and St Johnston, 2003a) and its ability to bind to junctional adhesion molecule (JAM) (Ebnet et al., 2001) and phosphoinositides (Wu et al., 2007). Similar interactions with cell adhesion molecules such as FasII or phosphoinositide
moeities might be required to anchor Baz at the SSR region. In this regard, recruitment of PTEN by Baz to the postsynaptic region might play an important role in altering the balance of phosphoinositides. Additional binding partners of Par-3 in vertebrates include Par-6 with which it interacts directly through the first PDZ domain (Lin et al., 2000; Nagai-Tamai et al., 2002). At the NMJ, Par-6 distribution can be seen as punctuate spots both within the bouton and in the muscle (Ruiz-Canada et al., 2004). Additionally it is also enriched at the SSR region along with Baz (Ruiz-Canada et al., 2004); however the significance of its interaction with Baz has not yet been investigated.

In the context of aPKC regulation, the best characterized mechanism in vertebrates involves activation of aPKC through Par-6 bound to active Cdc42 or Rac GTPase through its semi-CRIB domain (Lin et al., 2000; Ohno, 2001; Nagai-Tamai et al., 2002). Although the roles of these proteins have yet to be characterized at the NMJ, it is possible that signaling at the synapse could result in the activation of small RhoGTPases which might bind Par-6 enriched at the SSR. This complex might be transported outside the actin/spectrin region into the MT region of the muscle where it might activate aPKC. Activated aPKC could then phosphorylate and release Baz. In addition to RhoGTPase mediated activation, other downstream components of receptor tyrosine kinase pathways such as PI3K, PDK1 and PIP₃ have also been demonstrated to activate aPKC (Hirai and Chida, 2003; Suzuki et al., 2003).
Downstream effectors of aPKC mainly include other polarity proteins and proteins involved in MT regulation. With respect to polarity proteins, aPKC has been shown to phosphorylate Par-1 (Hurov et al. 2004; Suzuki and Ohno, 2006), and Lgl (Henrique and Schweisguth, 2003; Plant et al., 2003). In epithelial cells and in embryos phosphorylation of Par-1 and Lgl by aPKC causes these proteins to be excluded from the apical region of the cell (Henrique and Schweisguth, 2003; Macara, 2004; Suzuki and Ohno, 2006; Assemat et al., 2008). At the NMJ, Par-1 is present at the SSR region (Zhang et al., 2007). However it is not clear if similar mechanisms regulate their localization.

At the presynapse, aPKC activity has been shown to positively regulate MT bundling by enhancing the association of Futsch, with MTs (Ruiz-Canada et al., 2004). However the exact mechanism behind this regulation is not clear. Studies in *Drosophila* and mammals have shown that phosphorylation of Futsch and MAP1B respectively by GSK3β prevents it from bundling MTs (Roos et al., 2000; Goold and Gordon-Weeks, 2001; Franco et al., 2004; Trivedi et al., 2005; Gogel et al., 2006; Miech et al., 2008). GSK3B in turn has been shown to be inhibited by aPKC phosphorylation in mammals (Etienne-Manneville and Hall, 2003; Macara, 2004). Thus increased binding of Futsch to MTs could be enabled either by direct phosphorylation of Futsch by aPKC at specific sites or through the inhibition of GSK3B. Additionally, studies have shown that aPKC is also involved in MT stabilization by regulating the activity of APC (Etienne-Manneville and Hall, 2003; Macara, 2004; Etienne-Manneville et al., 2005). Likewise, in the
muscle aPKC might be involved in mediating the transport of phospho Baz to the postsynaptic actin/spectrin region on MTs by regulating the activity of these proteins.

Novel roles for aPKC/Par-3/Par-6 complex have been recently identified which include roles in vesicle trafficking, exocytosis via interactions with RalA, a small GTPase, and members of the exocyst complex (Lalli, 2009) and in Cdc42 and Arp2/3 mediated endocytosis of tight junction components (Georgiou et al., 2008). More research is required to determine if similar mechanisms exist at the NMJ.

The complexity of interactions between different proteins in the polarity pathway and their interdependency on each other for their localization makes it highly challenging to delineate the functions of individual proteins at the NMJ. More often than not, the loss of different proteins gives rise to similar phenotypes making it difficult to order them in a pathway. Live imaging experiments combined with techniques like FRAP will further help in elucidating the relationship between these proteins and clarify their role in synaptic development.

**Potential PTEN pathways at the NMJ**

PTEN was initially identified as a lipid phosphatase with the ability to dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$) to PtdIns(4,5)P2 (PIP$_2$) Analysis of PTEN domains revealed an N-terminal
phosphatase domain, a C2 domain which is required for promoting membrane recruitment, and a C-terminal tail that carries a number of phosphorylation sites required for its stability and activity and a PDZ binding domain required for its interactions with scaffolding proteins (Lee et al., 1999; Wu et al., 2000). While phosphorylation of the C-terminal tail of PTEN renders it inactive, dephosphorylation of the tail region activates PTEN (Das et al., 2003). Growing evidence suggests the interesting possibility that PTEN can function as a dual phosphatase, capable of both lipid and protein phosphatase activity (Ji et al., 2006). In line with the novel function of PTEN as a protein phosphatase, the results of our western blot show that the protein phosphatase activity of PTEN is required for mediating the dephosphorylation of Baz. Our findings are supported by results from past studies that have demonstrated a role for the protein phosphatase activity of PTEN in mediating the dephosphorylation of proteins like FAK (Focal Adhesion Kinase) (Tamura et al., 1998), Src family kinases (Dey et al., 2008), and neurotransmitter receptors such as 5-HT2CR (Serotonin 2C) receptors (Ji et al., 2006). Additionally, studies have demonstrated an interaction between PTEN and NR1/NR2B complexes of NMDA (N-methyl D-aspartic acid) receptors and showed that the protein phosphatase activity of PTEN was required for maintaining extrasynaptic receptor levels although the mechanism of regulation was not described (Ning et al., 2004).

Recent studies have demonstrated a role for PP1 in dephosphorylating mouse Par-3 at the aPKC phosphorylation site (Ser827 incorrectly mentioned in
the paper as Ser824) (Traweger et al., 2008). While we are tempted to believe that PTEN is directly involved in the dephosphorylation of Baz at the aPKC phosphorylation site, we cannot completely exclude a role for PP1. A yet unidentified mechanism could exist where the protein phosphatase activity of PTEN may be required for activating these downstream phosphatases which might enter into a complex with Baz and dephosphorylate it. Indeed studies have shown that dephosphorylation of PP1 is required for its activation. On the other hand, it could result from a difference in protein-protein interactions between the mammalian and *Drosophila* homologs of Par-3. For example, mouse Par-3 was shown to interact with PP1 and was dephosphorylated by it at both the N and C terminal Par-1 phosphorylation sites (Ser144 and Ser 885) as well as the aPKC phosphorylation site (Ser 827) (Traweger et al., 2008). Surprisingly, no interactions were found between mouse Par-3 and PP2A (Traweger et al., 2008). By contrast, Baz was shown to interact with PP2A and was dephosphorylated at the C-terminal Par-1 phosphorylation site (Ser1085) by PP2A (Krahn et al., 2009). Alternatively, it is possible that the aPKC phosphorylation site of Baz can be dephosphorylated by more than one phosphatase. For example, studies have shown that Ser144 in Baz/Par-3 can be phosphorylated by both Par-1 and RhoKinase (Benton and St Johnston, 2003b; Nakayama et al., 2008). Indeed, while the activation mechanisms of kinases have been well characterized, not much is known about the mechanisms of activation of protein phosphatases.
While the protein phosphatase activity might be important for the retention of Baz at the synapse, the lipid phosphatase activity of PTEN might be required for other functions. Results from a number of studies have established that PTEN is required for converting PIP3 to PIP2 (Das et al., 2003). Increased levels of PIP3 are generated as a result of PI3K activation, a downstream effector of receptor tyrosine kinase pathways. PIP3 in turn recruits Akt (Protein Kinase B) and PDK1 to the membrane where they bind PIP3 via their Pleckstrin Homology (PH) domain (von Stein et al., 2005). This leads to the phosphorylation and activation of Akt by PDK1 resulting in the activation of a number of downstream pathways that are positive regulators of cell growth (Gao et al., 2000; Alberts, 2002). By dephosphorylating PIP3 to PIP2, PTEN suppresses Akt and inhibits cell growth (Gao et al., 2000). Additionally, PIP2 is also involved in enhancing the activity of actin regulating proteins such as Arp2/3 and N-Wasp (Yin and Janmey, 2003). In support of a role for PTEN in actin regulation, studies have shown that PTEN mutant embryos in *Drosophila* show a severely disorganized actin cytoskeleton resulting in early embryonic lethality (von Stein et al., 2005; Pinal et al., 2006).

At the NMJ, enrichment of PTEN at the SSR regions suggests that PTEN might be involved in maintaining a balance between PIP3 and PIP2 levels at the actin/spectrin area. A likely scenario might exist where a local increase in PIP3 in the SSR region might result in the activation of aPKC either by Cdc42/Rac-Par-6 dependent on independent mechanisms which then targets Baz to the SSR region. Baz on the other hand, recruits PTEN to the SSR region where in addition
to retaining Baz, PTEN might also be involved in increasing the levels of PIP₂ thereby inhibiting Akt and mediating changes in the actin cytoskeleton.

Antibodies and mutants are available for almost all of the proteins that are part of the PTEN pathway. Additionally the levels and subcellular localization of PIP₂ and PIP₃ can be determined by expressing fusion constructs comprising the PH domains of PIP₂ and PIP₃ binding proteins tagged to fluorophores (von Stein et al., 2005). Visualizing the localization of these proteins and observing the phenotypes associated with the mutants of these proteins will be an initial step towards studying their function in synaptic outgrowth.

**Wingless (Wg) Secretion and Transport**

While research about the intracellular pathways transduced by Wnts in the Wnts receiving cells has received a great deal of attention, very little has been described about the mechanisms behind their secretion and transport from the expressing cells. Recent studies identified Evi, a multipass transmembrane protein, which was required for transporting Wnts from the trans-golgi network (TGN) to the plasma membrane and loss of Evi led to the accumulation of Wnts in the TGN preventing their release into the extra cellular milieu (Banziger et al., 2006; Bartscherer et al., 2006; Bartscherer and Boutros, 2008). In light of the limited understanding about the mechanisms of Wnt secretion, the above finding has been a highly significant one. However a number of other questions such as the mode and mechanism of intracellular Wg transport by Evi, or the process by which Wnts are transported outside the cell over large distances have yet to be
answered. At the NMJ, we observed that Evi is required for the secretion and transport of Wg from the presynapse to the postsynapse through a previously undescribed mechanism, involving the transfer of vesicle like structures resembling exosomes.

In our study, immuno EM experiments demonstrated that Evi was present in large multi-membrane structures inside the bouton. The findings of our study that Evi and Wg colocalize within the bouton coupled with the results of previous studies that Evi and Wg exist in a single complex entertains the possibility that Wg might be present along with Evi in these large multi membranous structures. Analogous to our results, previous studies have demonstrated that a fraction of Wntless (Franch-Marro et al., 2008) and Wg, in Wg expressing cells, were found to exist in similar multimembranous structures resembling multi vesicular bodies (MVBs) (Pfeiffer et al., 2002). These structures have been identified in polarized and non polarized cells as structures typically thought to be involved in lysosomal degradation, although recent studies have confirmed a more active role in extracellular transport of signaling molecules for these structures (Piper and Katzmann, 2007; Lakkaraju and Rodriguez-Boulan, 2008).

During endocytosis, the region of the plasma membrane containing integral membrane proteins that need to be internalized buds and pinches off into the cell and these proteins are initially incorporated into early endosomes. Maturation from early to late endosomes involves the removal of proteins and lipids via tubo- vesicular elements that sort components back to the TGN and
plasma membrane. Receptors that have not been recycled and soluble lumenal materials are instead directed to late endosomes where they are incorporated into intraluminal vesicles (ILV), which are formed by the inward budding of the limiting membrane of the late endosome, giving rise to MVBs which are targeted for lysosomal degradation. Recent studies have demonstrated that these MVBs can also fuse to the plasma membrane releasing the ILV into the extracellular milieu. These released vesicles are called exosomes (Piper and Katzmann, 2007; Lakkaraju and Rodriguez-Boulan, 2008; Simpson et al., 2008). The fact that in our model system, Evi is present in these multi-membrane endosomes and that Wg and Evi are secreted together in vesicle like structures from the presynapse strongly suggests that these secreted vesicles might be exosomes. Although our findings strongly support the idea of intracellular transport of Evi and Wg through the formation of MVBs and their transsynaptic transport through exosomes, additional experiments are required to validate this hypothesis. Studies have shown that while the molecular content of MVBs and exosomes are specific to the cell type, most MVBs and exosomes carry a common set of proteins conserved across species. MVBs typically carry proteins such as Rab 7 GTPase, Alix, and proteins belonging to the Endosomal Sorting Complex Required for Transport (ESCRT) complex, all of which are involved in MVB biogenesis (Simpson et al., 2008). Exosomes on the other hand, in addition to containing most of the MVB associated proteins, also contain cytoskeletal proteins, heat shock proteins and chaperons and proteins belonging to the
tetraspanin family (Simpson et al., 2008). Demonstrating that these proteins are present at the NMJ, they colocalize with Evi and Wg, and localize to these presynaptic multimembrane bodies and/or exosomes would lend strong support for our hypothesis. Additionally, available mutants of these genes can be tested to verify if manipulating the levels of these proteins affects Wg secretion. Alternatively, the *Drosophila* S2 cell culture system can be used to isolate these exosomes, perform mass spectrometric analysis and identify their components. However, the drawback in this case might be the fact that it may not exactly represent exosomal components found at the NMJ. Nevertheless, the results of these experiments will significantly extend the knowledge in the field about the mechanisms involved in morphogen secretion.

In addition to the ‘exosome’ model, other models have been proposed for Wg transport in *Drosophila*. These models purport that different mechanisms underlie short range signaling versus long range signaling of Wg (Hausmann et al., 2007; Bartscherer and Boutros, 2008). According to this model, the hydrophobic nature of Wg causes it to attach to the phospholipid membrane and accumulate close to the expressing cell once it is transported by Evi from the TGN to the membrane. This results in a high concentration of Wg in the area close to the secreting cells which provides for short range signaling by binding to the receptors of the neighboring cells. However some of this Wg is endocytosed and incorporated into vesicles containing lipoproteins which are also endocytosed from the extracellular matrix. These vesicles then fuse with the
plasma membrane resulting in Wg associated lipoprotein particles, previously termed as ‘argosomes’ being released in the extracellular region (Greco et al., 2001; Vincent and Magee, 2002). Thus while accumulation on the membranes of Wg expressing cells is required for short range signaling, its association with lipoproteins is required for long range signaling and formation of a concentration gradient. Consistent with a role of lipoprotein mediated transport, loss of Lipophorin, the *Drosophila* lipoprotein resulted in a reduction in the range of Wg signaling (Panakova et al., 2005; Bartscherer and Boutros, 2008). Although our studies suggest that Wg incorporation into MVB like structures seem to be the primary mode of transport at the NMJ, mechanisms involving the direct release of Wg onto the cell membrane or its transport via association with lipoproteins cannot be excluded. It is possible that the former mechanism could be involved in the autocrine Wg signaling cascade identified at the presynapse while the latter mechanism could be involved in transfer from the presynapse to the postsynapse.

The idea that the release of exosome like vesicles carrying Evi and Wg from the presynapse might be responsible for transducing the Wg pathway in the muscle raises important questions about how these exosomes interact with the muscle cell. Studies in immune cell systems have demonstrated that exosomes interact with their target cell by fusion or adhesion to the target cell plasma membrane, or by direct binding with receptors on the surface of the membrane (Lakkaraju and Rodriguez-Boulan, 2008). Our study has shown that Evi is not
only expressed at the presynapse, but also in the postsynaptic cell where it is
enriched at the SSR region along with the Wg receptor, Frizzled2 (Fz2) (Packard
et al., 2002). In this regard, it is tempting to speculate that ‘exosomal’ Evi might
interact with postsynaptic Evi and help in targeting the exosome to the region of
the plasma membrane housing Fz2. Evi consists of multiple transmembrane
domains with a long extracellular loop between the first and the second
transmembrane domain and a long intracellular C-terminus. However, these
regions do not appear to contain any conserved domains and not much is known
about the function or binding partners of this region (Banziger et al., 2006;
Bartscherer et al., 2006). One possibility is that these extracellular loop regions
might be involved in the dimerization of ‘exosomal’ and postsynaptic Evi resulting
in proper targeting of the presynaptically released exosomes. This can be
confirmed both in vivo and in cell culture systems by biochemical interaction and
co-immunoprecipitation studies. Additionally, using the Gal4/Lex A system
(Brand and Perrimon, 1993; Lai and Lee, 2006), two different populations of Evi
each tagged with one half of the GFP molecule could be expressed in the neuron
and muscle and larvae could be examined for the detection of GFP fluorescence
at the synapse which will result from the interaction between the two Evi
molecules. Similarly Evi tagged with two different fluorophores could be
expressed in the neuron and muscle respectively and assayed for FRET
(Fluorescence Resonance Energy Transfer) at the synaptic region. A positive
FRET with energy transfer would indicate that pre and postsynaptic Evi
molecules interact. One drawback of this experimental technique might be that insertion of fluorophore molecules in the intracellular loop of the protein might interfere with the proper folding of the protein. Nevertheless, additional biochemical interaction studies to identify the binding partners of the intracellular and extracellular regions of Evi/Wntless will provide more insight into how Evi might function in Wg transport.

In the postsynaptic cell, Evi is highly enriched at the SSR region and also shows a punctuate distribution in the muscle, although its subcellular localization in the muscle still remains a mystery. Additionally, it is thought to play a role in trafficking GRIP, a Frizzled interacting protein (Ataman et al., 2006), to the plasma membrane based on the finding that downregulation of Evi in the muscle led to increased GRIP staining in the cytoplasm and reduced GRIP staining at the golgi and the synaptic region. While it is clear that the concerted efforts of pre and postsynaptic Evi are required for proper transduction of the Wg pathway, it is not clear what happens to postsynaptic and presynaptically released Evi after the binding of Wg to Fz2. Based on Evi localization in the TGN and at membranes in other tissues, a potential scenario at the postsynapse is that Evi is endocytosed along with Wg, Fz2 and dGRIP and following sorting in the early endosome, while the Fz2-dGRIP complex is directed to the nucleus, Evi could be recycled back to the golgi or sent to the lysosome for degradation. In favor of the ‘recycling’ model, collective results of studies done in Drosophila larval wing discs and in C. elegans have demonstrated that recycling of Evi from the
endosomes to the Golgi, following Wg delivery to the cell surface, requires the action of the retromer complex, a protein complex composed primarily of three proteins namely Vps35, Vps26, and Vps29 (Belankaya et al., 2008; Yang et al., 2008). Further evidence supporting a role for the retromer complex in Evi recycling includes a direct interaction between Vps35 and Evi/Wntless and loss of Evi from the TGN in Vps35 mutants resulting in loss of both short range and long range Wg signaling (Coudreuse et al., 2006; Belenkaya et al., 2008; Port et al., 2008; Yang et al., 2008). These studies demonstrated that in the absence of the retromer complex, instead of accumulating at the Golgi, the recycling Evi continued to be localized in endosomes or got incorporated into MVBs headed for lysosomal degradation, resulting in a gradual decrease in overall Evi levels.

Analogous to its role in other *Drosophila* tissues, if the retromer played a role in Evi recycling at the NMJ, downregulation of the proteins of the retromer complex would result in a decrease in Golgi Evi levels, decreased Wg secretion, reduction in bouton number, and other phenotypes observed upon downregulating Evi. However, a study by Korolchuk et al., 2007, demonstrated that Vps35 mutants surprisingly had more boutons and unlike *evi* mutants did not demonstrate any change in postsynaptic Fz2 levels. On the contrary, Vps35 was involved in the BMP pathway and in actin regulation through Rac1 (Korolchuk et al., 2007). The results of this study indicate that Vps35 might not be involved in Evi recycling leading us to consider the possibility that following the internalization of Wg and Fz2, Evi might be targeted and degraded in the
lysosomes. Internalization assays or immuno EM studies to compare the internal pools of Evi in the presence or absence of lysosomal inhibitors will provide further answers to questions about the fate of internalized Evi.

**Wnt signaling and actin regulation**

While previous studies have established a role for Wg signaling in MT regulation at the presynapse through the divergent canonical pathway involving Dishevelled and GSK3β (Miech et al., 2008), it is not known whether Wg plays any role in regulation of synaptic actin. However, evidence from other systems has identified many possible links between Wnt signaling and actin regulation which could function at the synapse. One potential mechanism could involve Wnt mediated activation of aPKC. Recent studies in hippocampal and commissural neurons have revealed that aPKC can be activated by exposure to certain Wnt ligands through Dishevelled (Dsh), a downstream component of the Wnt pathway (Zhang et al., 2007; Wolf et al., 2008). These studies demonstrated that Dsh was involved in stabilizing aPKC and preventing its degradation. In contrast to these vertebrate studies, studies looking at *Drosophila* embryos did not find any significant changes in aPKC activity in *dsh* mutants. However, GSK3β, another component of the Wg pathway, was found to negatively regulate aPKC activity by phosphorylating it and targeting it for degradation. On the other hand, aPKC also phosphorylates GSK3β (Etienne-Manneville and Hall, 2003) and inhibits its activity suggesting that both of them regulate each other’s activities. Although
different components of the Wnt pathway are involved in aPKC activation in vertebrates and in Drosophila embryos, these pathways could function together at the NMJ. Wg dependent activation of Dsh could not only lead to the inhibition of GSK3β, but could also lead to sequestering of aPKC, preventing its degradation due to phosphorylation by GSK3β. Activation of aPKC could activate actin regulators resulting in changes in actin organization. Additionally, Wnts have also shown to activate RhoGTPases directly through Dsh, a pathway typically involved in planar cell polarity (PCP) (Strutt et al., 1997; Speese and Budnik, 2007).

Another potential mechanism by which Wg could regulate actin is through APC. APC was initially characterized as a component of the Wnt pathway involved in the degradation of β-catenin by linking GSK3β phosphorylated β-catenin with ubiquitinating enzymes (Li et al., 2005; Angers and Moon, 2009). Additionally, APC itself has been demonstrated to be phosphorylated by GSK3β (Zumbrunn et al., 2001). Parallel studies have demonstrated a role for APC in cytoskeletal regulation (Smith et al., 1994; Rosenberg et al., 2008). Cell culture studies have revealed that APC normally accumulates as clusters at growing ends of MT (Nathke et al., 1996; Mimori-Kiyosue et al., 2000). However APC is also associated with the plasma membrane and this association requires the presence of actin (Rosen-Arbesfeld et al., 2001). Furthermore, APC binds directly to actin (Moseley et al., 2007) and also interacts with many different actin regulators such as RhoGTPases like Rac1 and Cdc42 and IQGAP1, a protein
with a role in crosslinking actin (Watanabe et al., 2004; Akiyama and Kawasaki, 2006; Rosenberg et al., 2008). While phosphorylation by GSK3β causes APC to dissociate from MTs (Zumbrunn et al., 2001), it is not known if the same applies to interactions with actin. Furthermore, at the mammalian NMJ, APC has shown to be enriched at the postsynaptic region where it has been demonstrated to regulate the clustering of acetylcholine receptors (Wang et al., 2003). It would be interesting to observe if APC is distributed in a similar manner at the Drosophila NMJ and activated by the Wg pathway and if the role of APC in actin regulation is conserved from vertebrates to Drosophila.

Studies have shown that, at the NMJ, Dishevelled is expressed primarily in the presynapse and appears to have a presynaptic role in bouton proliferation as a component of the Wg pathway (Miech et al., 2008). Considering that all the pathways mentioned above require Dishevelled function, these pathways may function primarily in the presynapse. In the muscle, Dishevelled is absent and Wg transduction occurs through the FNI (Frizzled Nuclear Import) pathway (Packard et al., 2002; Mathew et al., 2005; Ataman et al., 2006). Although not much is known about how this pathway regulates synapse assembly, it is possible that one of the outputs of this pathway might be the regulation of the postsynaptic actin cytoskeleton. Further investigation is required to determine if this is indeed the case.

The conservation of the ‘synapse’ as the basic structural and functional unit of interneuronal communication replete with its components and pathways
that regulate it across species strongly validates the use of invertebrate model systems for neuroscientific research. Additionally, the ability to apply findings pertaining to events occurring during synaptic development, obtained from these simpler species, to vertebrates makes them indispensable to the field of neuroscience. In my thesis, using the fly larval NMJ as a model system, I have described some aspects of the pathways involved in the regulation of two very critical synaptic events required for normal synaptic growth namely proper cytoskeletal regulation and proper synaptic signaling. The conservation of mechanisms of synaptic regulation across species suggests that these mechanisms might play a role in shaping the structure and function of dendritic spines in vertebrates. Future studies should focus on linking these mechanisms and their effect on synaptic development with behavior.
**Figure 4.1 Potential presynaptic cytoskeletal modifications during synaptic growth.**

Actin (red), MTs (green), active zones (purple), Futsch (Brown).

**(A)** In a bouton that is increasing in size, the cortical actin cytoskeleton (red) in the periactive zones is arranged in the form of a meshwork at the bouton periphery.

**(B)** During bouton budding, the cortical actin network at the periactive zone transforms into filopodia like structures. At the same time dynamic MTs (green) are penetrating into the bud attempting to explore the surrounding.

**(C)** If the dynamic MT gets captured and linked to the actin, the treadmilling action of actin in the bud transforms the MT into a loop like structure.

**(D)** MT not linked to actin is free to penetrate into the bud and explore the substratum.

**(E)** In buds where MT loops are formed, these loops are stabilized by Futsch as the bouton matures and bouton growth is arrested.

**(F)** In buds where the MT is not linked to actin, the MTs are splayed and dynamic allowing the boutons to undergo budding.
Potential Presynaptic Cytoskeletal Modifications During Synaptic Outgrowth.

Figure 4.1

A. INACTIVE BOUTON

B. BOUTON THAT IS BUNNING

C. MT LINKED TO ACTIN FORMS LOOP

D. MT NOT LINKED TO ACTIN IS FREE TO EXPLORE

E. BOUTON GROWTH IS ARRESTED

F. BOUTON IS FREE TO GIVE RISE TO BUDS

ACTIVE ZONE

CORTICAL ACTIN NETWORK

MICROTUBULES

FUTSCH
Fig. 4.2. Potential mechanism of postsynaptic actin remodeling during synaptic outgrowth.

 Postsynaptic actin (pink), Active zones (purple) and MTs (green).

(A) During bouton enlargement, the actin can either undergo compression relaxation (depicted by the dashed line in A1) or undergo a process where depolymerization in the area close to the bouton (shown in white) is accompanied by polymerization at the actin/spectrin area-MT interface (shown in red) in order to allow the bouton to gradually increase in size.

(B) During bouton budding, a localized area of actin depolarization at the postsynapse provides room for protrusion of the presynaptic membrane resulting in the formation of a bud.
Potential Postsynaptic Actin Remodeling During Synaptic Outgrowth

Figure 4.2
Fig 4.3. Potential pre and postsynaptic signaling pathways of the aPKC/Baz/Par-6 polarity complex functioning at the larval NMJ.

Presynaptic mechanism: Bazooka phosphorylated by aPKC is transported to the periactive region of the bouton on MTs by binding with kinesin, a MT motor that can also be activated by aPKC. At the periactive region, Baz recruits SIF, a Rac1 GEF which can activate Rac allowing it to modulate actin dynamics.

Postsynaptic mechanism:

(A) aPKC phosphorylates Bazooka in the muscle. Bazooka phosphorylated at S980 is transported to the postsynapse along MT by binding to Kinesin. At the postsynapse, it has to be dephosphorylated by PTEN in order to be retained at the actin/spectrin area. Additionally, it might also need to be phosphorylated by Par-1 at two sites for its retention at that site. Bazooka enriched at the actin/spectrin area could interact with dPIX that is enriched at that region or an unknown GEF thereby regulating actin.

(B) Removal of Baz from the actin/spectrin area could be mediated by dephosphorylation of Baz at the Par-1 phosphorylation sites.

(C) Additionally, Par-6 that is enriched at the actin/spectrin area could bind to active Cdc42, be transported out of the actin/spectrin area into the muscle where it could activate aPKC.
Potential Pre and Postsynaptic Pathways of the aPKC/Baz or Par-3/Par-6 Polarity Complex

Figure 4.3
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