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Abstract:

The *Drosophila* neuromuscular junction (NMJ) is capable of rapidly budding new presynaptic varicosities over the course of minutes in response to elevated neuronal activity. Using live imaging of synaptic growth, we characterized this dynamic process and demonstrate that rapid bouton budding requires retrograde BMP signaling and local alteration in the presynaptic actin cytoskeleton. BMP acts during development to provide competence for rapid synaptic growth by regulating the levels of the Rho GEF *trio*, a transcriptional output of BMP-Smad signaling. In a parallel pathway, we find that the BMP type II receptor Wit signals through the effector protein LIM domain kinase1 (Limk) to regulate bouton budding. Limk interfaces with structural plasticity by controlling the activity of the actin depolymerizing protein Cofilin. Expression of constitutively active or inactive Cofilin in motor neurons demonstrates that increased Cofilin activity promotes rapid bouton formation in response to elevated synaptic activity. Correspondingly, overexpression of Limk, which inhibits Cofilin, inhibits bouton budding. Live imaging of the presynaptic F-actin cytoskeleton reveals that activity-dependent bouton addition is accompanied by formation of new F-actin puncta at sites of synaptic growth. Pharmacological disruption of actin turnover inhibits bouton budding, indicating local changes in the actin cytoskeleton at preexisting boutons precede new budding events. We propose that developmental BMP signaling potentiates NMJs for rapid activity-dependent structural plasticity that is achieved by muscle release of retrograde signals that regulate local presynaptic actin cytoskeletal dynamics.

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Chapter 1

Structural Synaptic Plasticity and Bone Morphogenic Protein Signaling

Introduction

Modification of existing synaptic connections between neurons is of critical importance to the regular operation of an organism's nervous system. Activity-dependent changes in synaptic structure and function play an important role in the developmental wiring of the nervous system and in the storage and maintenance of memory (Silva, 2003; Tessier and Broadie, 2009). Synaptic plasticity is therefore a fundamental component of identity and adaptability. Owing to the genetic conservation of synaptic components among various organisms and the plethora of biological tools and techniques for analyzing synapses, the study of synaptic plasticity has been a successful approach in understanding how the brain works (Littleton and Ganetzky, 2000; Kandel, 2001). Indeed, the goal of understanding how the brain functions is one of the great aims of modern biology, and due to the essential role of synaptic connections in neuronal networks, achieving this goal is likely to be impossible without a comprehensive understanding of the mechanisms of synaptic plasticity.

Retrograde signaling from the postsynaptic to the presynaptic neuron is one cellular communication paradigm involved in many forms of regulated synaptic plasticity. Presynaptic signals are received and interpreted by the postsynaptic cell, which in turn may respond with retrograde signals that then direct the presynaptic cell to elicit changes in synaptic function (Fitzsimonds and Poo, 1998; Regehr et al., 2009). In this way, flow of information across synapses is bidirectional. Some retrograde signaling pathways are triggered downstream of

neuronal activity and rapidly initiate changes in presynaptic function in a synapse-specific manner (Bonhoeffer, 1996; Hartmann et al., 2001). As such, some forms of retrograde synaptic signaling represent molecular mechanisms for stimulus-dependent modification of neuronal network excitability, i.e. learning and memory.

The *Drosophila* neuromuscular junction as a model synapse

The neuromuscular junction (NMJ) of *Drosophila* larvae has emerged as a popular model for mammalian central synapses and is especially well suited to the study of synaptic plasticity. This synapse is glutamatergic, like the majority of excitatory central mammalian synapses, and glutamate receptors at *Drosophila* NMJs are homologous to mammalian ionotropic glutamate receptors (Jan and Jan, 1976b; Petersen et al., 1997). Additionally, many other components of synaptic function and development are conserved (Featherstone and Broadie, 2000; Littleton and Ganetzky, 2000). The chief strengths of this model synapse are its simplicity, accessibility, inherent robust plasticity, and tractability

Larval motorneurons are individually specified and display invariant connectivity in terms of target selection (Johansen et al., 1989; Landgraf et al., 1997; Hoang et al., 2001). Thirty-two motorneurons of three types innervate 30 stereotypically patterned target muscles in each abdominal hemisegment and are easily identified. Additionally, larval body wall muscles, in which synaptic terminals are embedded, are large and relatively accessible by filet dissection to physiological recording and microscopy (Jan and Jan, 1976a). The NMJs of live

intact animals expressing fluorescently tagged synaptic proteins can also be repeatedly imaged through the cuticle over the course of days (Zito et al., 1999). Combining these techniques allows for observation of a multitude of short- and long-term changes in synaptic structure and function.

Motorneuron axonal path finding is completed by the end of embryogenesis, and at this point NMJs contain pre- and postsynaptic transmission machinery and are capable of synaptic transmission (Broadie and Bate, 1993; Prokop et al., 1996). The presynaptic terminal is organized into a limited number of en passant swellings known as boutons which are surrounded postsynaptically by specialized foldings of muscle membrane known as the subsynaptic reticulum (SSR) (Guan et al., 1996; Schuster et al., 1996a). As larvae grow over the course of ~4 days, muscle surface area expands up to 100-fold, requiring a concomitant increase in the strength of motorneuron inputs. This is achieved by an up to 10-fold increase in the number of presynaptic boutons, each housing approximately 10 to 30 active zones in third instar larvae, and a corresponding increase in neurotransmitter release such that excitatory junction potentials maintain steady amplitudes throughout larval life (Zito et al., 1999; Li et al., 2002). The speed and scale at which synaptic terminals expand is indicative of a strong inherent synaptic plasticity. Not surprisingly, there are many mutations that dramatically affect growth of the larval NMJ over the course of larval development (Menon et al., 2013). One class of mutations that affect synaptic growth are those that change motorneuron excitability, indicating that synaptic growth is regulated by neuronal activity (Budnik et al., 1990; Zhong et

al., 1992). The *Drosophila* NMJ has strong inherent plasticity that is sensitive to neuronal activity and is therefore a good model for studying activity-dependent plasticity that underlies learning and memory. In fact, a number of *Drosophila* mutants that were isolated from learning assays harbor NMJ phenotypes (Zhong et al., 1991; Broadie et al., 1997; Rohrbough et al., 1999).

Perhaps the most appealing feature of the *Drosophila* larval NMJ as a model synapse is the extensive repertoire of genetic tools available. Through the use of the Gal4-UAS system, tissue-specific expression of exogenous transcripts is straightforward and easily achieved (Brand and Perrimon, 1993). Recent advances in genetic technology have also made sophisticated site-specific mutagenesis possible (Beumer and Carroll, 2014). However, a critically important feature of *Drosophila* as a model organism is its ability to be used in unbiased forward genetic screening. One such screen by the Goodman laboratory, which isolated mutations affecting NMJ growth, first identified a role for Bone morphogenic protein (BMP) signaling in regulating synapse size (Aberle et al., 2002).

We have identified an additional role for BMP signaling in rapid activity-dependent synaptic growth. Our initial investigations into rapid synaptic plasticity indicated that a muscle to motorneuron retrograde signal was involved and we subsequently found a requirement for canonical BMP signaling. BMP signaling is a major regulator of synaptic growth and plasticity at *Drosophila* NMJs and it has been the focus of many investigations into synaptic plasticity using the fruit fly

model. The following section summarizes the results of these investigations. The accompanying figure diagrams the pathway and its regulators.

Bone Morphogenic Protein signaling at *Drosophila* NMJs

Bone morphogenic proteins belong to the transforming growth factor β (TGF-β) superfamily of signaling proteins. The TGF-β superfamily consists of three classes of signaling molecules: TGF-β, activins, and BMPs. The *Drosophila* genome encodes 3 BMP ligands and 4 activin ligands, but no TGF-β ligands. These proteins play diverse roles during organism development and during adult life and function ubiquitously. Roles for TGF-β superfamily proteins include morphogenesis, cell cycle regulation, cell fate specification, apoptosis, and synaptic plasticity (Cohen, 2003).

The first described role for TGF-β in synaptic plasticity came from a transcription based screen in the sea slug *Aplysia*, in which long-term memory for defensive responses is correlated with synaptic facilitation (Frost et al., 1985; Cleary et al., 1998). Long-term sensitization training resulted in an increase in mRNA levels of tolloid/BMP1 in sensory neurons. BMP1 is an activator if TGF-β proteins (Wozney et al., 1988; Liu et al., 1997). Additional work revealed that direct application of *Aplysia* TGF-β1 to pleural-pedal ganglia was sufficient to specifically enhance excitatory postsynaptic potentials at these synapses (Zhang et al., 1997; Chin et al., 1999).

In *Drosophila*, three BMP ligand molecules were identified through screens based on their functions in dorsal-ventral embryo patterning,

specification of embryonic gut, and imaginal disk patterning and proliferation (Raftery and Sutherland, 1999; Parker et al., 2004). A role for BMP signaling in synaptic growth and plasticity was then uncovered through a large-scale genetic screen. *Drosophila* larvae expressing a fluorescent synaptic marker were imaged through the intact cuticle in order to assess gross synaptic size at NMJs (Aberle et al., 2002). Animals with severely undergrown NMJs harbored mutations in the gene *wishful thinking* (*wit*), encoding a BMP type II receptor (Fig. 1). Wit mutant animals also showed defects in synaptic ultrastructure and synaptic transmission including decreased evoked excitatory junctional potential (EJP) amplitudes and miniature excitatory junctional potential (mEJP) amplitudes and decreased mEJP frequency (Aberle et al., 2002; Marqués et al., 2002). *Wit* mutants survive through larval stages with appropriately wired nervous systems but do not reach adulthood, indicating that BMP signaling through Wit is not required for embryonic nervous system development but rather for synaptic maintenance (Aberle et al., 2002; Marqués et al., 2002). Hyperexcitable animals with mutations in potassium channel genes exhibit synaptic overgrowth, which was eliminated in the *wit* mutant background even though neuronal hyperexcitability persisted; indicating *wit* is required for activity-dependent synaptic growth (Budnik et al., 1990; Berke et al., 2013). These defects could be rescued by motorneuron expression of *wit* in the *wit* mutant background, indicating that Wit functions presynaptically at NMJs to control synaptic growth, structure, and transmission.

Since the identification of a role for the BMP type II receptor *wishful thinking* at *Drosophila* larval NMJs, rapid progress was made in characterizing the broader signaling pathway (Keshishian and Kim, 2004; Marqués, 2005). The BMP ligand Glass Bottom Boat (Gbb) is released from muscle and binds to the presynaptic BMP type I receptors Thickveins (Tkv) and Saxophone (Sax) and type II receptor Wit (Fig. 1) (Haerry et al., 1998; Ray and Wharton, 2001; Rawson et al., 2003; McCabe et al., 2003, 2004). Ligand-receptor binding causes constitutively active type II receptors to associate with and phosphorylate type I receptors that in turn phosphorylate a cytoplasmic Smad, *mothers against decapentaplegic* (*mad*) (Rawson et al., 2003; Shi and Massagué, 2003). The *Drosophila* genome encodes two Smads, *mad* and *smad2/smox*, a single co-SMAD, *medea* (*med*), and a single inhibitory Smad, *daughters against decapentaplegic* (*dad*). Mad phosphorylation causes Mad-Med heterodimerization and eventual translocation into the nucleus to act as a transcription factor together with DNA-binding cofactors (Raftery and Sutherland, 1999; Shi and Massagué, 2003). Mutations in *gbb*, *tkv*, *mad*, and *med* have similar NMJ phenotypes compared to *wit* mutants. Blocking retrograde axonal transport, including transport of ligand-receptor complexes, also phenocopies mutations the pathway (Eaton et al., 2002; McCabe et al., 2003). BMP receptor activity mediated by all three BMP ligands converge on the common Smad, Mad, and the functional consequences of this signaling integration are still under investigation (Lee-Hoeflich et al., 2005; Fuentes-Medel et al., 2012).

Many genes that are required for activity-dependent synaptic growth at *Drosophila* NMJs appear to require BMP signaling for their effects, likely because of a requirement for genes transcribed downstream of Smad signaling (Zhong et al., 1992; Davis et al., 1996; Schuster et al., 1996a, b; Davis and Goodman, 1998; Sanyal et al., 2002, 2003; Berke et al., 2013). In addition to acting upstream of Smad signaling, *wit* has also been implicated in cytoskeletal regulation and regulation of homeostatic neuronal signaling plasticity. The picture that has emerged in recent years suggests that canonical BMP signaling (BMP signaling activating cytoplasmic Smad) occurring throughout larval development potentiates synapses for a variety of plasticity-related functions including activity-dependent synaptic growth.

Canonical BMP signaling

Canonical BMP signaling occurs in motorneurons throughout larval life as evidenced by staining for phosphorylated Mad (P-Mad) at synaptic terminals and motorneuron cell bodies and nuclei (Marqués et al., 2002, McCabe et al., 2003; Collins et al., 2006; Wang et al., 2007). Only recently have insights into the timing requirements for BMP signaling in its various synaptic functions been elucidated, including in synaptic growth and synaptic transmission. By expressing an inducible *gbb* transgene in *gbb* mutants, Berke et al. (2013) were able to show that BMP signaling is only required during the first larval stage (L1) to fully rescue synaptic growth. P-Mad staining, which is lacking in *gbb* mutants, was restored when the *gbb* transgene was expressed, indicating that Smad signaling was

restored. A similar timing requirement was found for Wit activity. Additional experiments also suggest that previously described forms of activity-dependent synaptic growth require BMP signaling during the L1 stage, establishing a critical period for activity-dependent growth (Budnik et al., 1990; Berke et al., 2013). In contrast, BMP signaling was required continuously throughout larval life to support normal synaptic transmission manifested in EJP and mEJP amplitude and mEJP frequency (Berke et al., 2013).

From postsynaptic ligand production to presynaptic transcription of target genes, canonical BMP signaling involves numerous cellular and molecular processes and there is ample evidence for regulation of the signaling pathway at many of these steps. Many laboratories have conducted forward genetic screens for modifiers of NMJ growth, and investigations into the molecular mechanisms governing synaptic growth have led to regulators of BMP signaling in many cases.

Postsynaptic and extracellular regulation of BMP signaling

In the course of characterizing a postsynaptic activin signaling pathway that is required for normal synaptic growth, Ellis et al. (2010) showed that the NMJ undergrowth phenotype of *daw* mutants (an activin ligand) could be rescued by increasing canonical BMP signaling via presynaptic overexpression of a constitutively active Tkv receptor. This result indicates that synaptic undergrowth in activin mutants is mediated by downstream canonical BMP signaling in motorneurons (Fig. 1). Furthermore, *gbb* transcript levels were reduced by ~50%

in muscle in *daw* mutants and muscle overexpression of *gbb* in a *daw* mutant background rescued synaptic undergrowth defects (Ellis et al., 2010). A recent report focused on a glial-derived activin ligand reached similar conclusions and demonstrated that glial cells associated with NMJs can act as a source of activin ligand (Fuentes-Medel et al., 2012). These results provide compelling evidence that postsynaptic activin signaling regulates *gbb* transcription.

Two independently isolated mutants in postsynaptically expressed genes shed some light on how Gbb release from muscle is regulated. *Drosophila* Cdc42 Interacting Protein 4 (dCIP4) was found to restrict synaptic growth by inhibiting Gbb secretion from muscles. dCIP4 controls the postsynaptic localization of Wsp, and by binding Cdc42 and Wsp, promotes Cdc42-dependent Wsp activation (Fricke et al., 2009; Nahm et al., 2010a). Postsynaptic knockdown of dCIP4 increased extracellular levels of Gbb and presynaptic P-Mad staining. Additionally, synaptic overgrowth in dCIP4 mutants was attenuated in a *wit* mutant background, indicating that functional BMP signaling is required for synaptic overgrowth (Nahm et al., 2010a). Alternatively, animals lacking *drich*, a fly orthologue of mammalian Rich/Nadrin proteins, have undergrown NMJs and drich is thought to act in the same postsynaptic Cdc42-Wsp pathway to control Gbb secretion (Nahm et al., 2010b). Mammalian Rich was previously shown to be a Rho family GTPase activating protein (RhoGAP) specific for Cdc42 that promotes Ca^{2+}-dependent exocytosis in neurons (Harada et al., 2000; Wells et al., 2006). Because there are many cellular regulators of actin dynamics that respond to diverse cues, these studies support the notion that Gbb secretion

from muscles could be regulated by extracellular cues upstream of actin regulators and potentially by neuronal signaling in a Ca^{2+}-dependent manner.

Regulation of BMP signaling also occurs in the extracellular space. A series of studies in larval imaginal discss demonstrate that two heparan sulfate proteoglycans (HSPGs) regulate BMP signaling by acting as BMP co-receptors (Jackson et al., 1997; Fujise et al., 2003; Belenkaya et al., 2004; Dejima et al., 2011). Additionally, an RNAi-based candidate screen of *Drosophila* genes regulating glycan modification revealed that many glycan genes regulate synaptic growth or neuronal transmission at NMJs (Dani and Broadie, 2012; Dani et al., 2012). Mutations in two glycan modification proteins cause an increase in levels of synaptic HSPGs and an increase in levels of synaptic Gbb and phosphorylated Mad as detected by antibody staining. Knockdown and null mutations in glycan genes that down regulate HSPGs ultimately cause synaptic overgrowth by increasing BMP ligand levels and upregulating BMP signaling (Dani et al., 2012).

Presynaptic regulation of BMP signaling

Cytoplasmic inhibitory Smads (I-Smads) are responsible for inhibiting canonical Smad signaling through multiple pathways and are therefore critical regulators of BMP signaling. I-Smads bind directly to BMP type I receptors to inhibit phosphorylation of R-Smads (Kamiya et al., 2008). Additionally, I-Smads interact with ubiquitin ligases to down regulate levels of type I receptors and R-Smads through proteosome-dependent degradation (Kavsak et al., 2000; Ebisawa et al., 2001; Murakami et al., 2003). I-Smad expression is also induced

downstream of BMP signaling to act as a negative feedback mechanism. The *Drosophila* genome encodes a single I-Smad called *daughters against decapentaplegic* (*dad*), which is expressed in motorneurons (Tsuneizumi et al., 1997; Inoue et al., 1998; Dudu et al., 2006; Kamiya et al., 2008). Mutants for *dad* exhibit enhanced BMP signaling resulting in synaptic overgrowth (Sweeney and Davis, 2002). Conversely, neuronal overexpression of *dad* results in synaptic undergrowth and a reduction in synaptic P-Mad staining (Eaton and Davis, 2005). These data indicate that overall synaptic growth can be up regulated or down regulated by a reduction or an increase in Dad levels, respectively. By directly inhibiting Mad phosphorylation, Dad regulates canonical BMP signaling at an early step in the presynaptic signal cascade.

Endocytotic regulation of signaling receptors and receptor-ligand complexes is an important form of regulation for cell signaling. Endocytosis can control receptor availability at the plasma membrane and after ligand-receptor complexes are endocytosed, additional sorting steps control signaling duration and receptor fate. Receptors can be sorted back to the plasma membrane through sorting endosomes or lysosomally degraded (Sorkin and Zastrow, 2009). Many genes that regulate synaptic growth at *Drosophila* NMJs have been shown to regulate endocytosis of BMP receptor-ligand complexes or regulate the fate of these complexes (Sweeney and Davis, 2002; Coyle et al., 2004; Koh et al., 2004; Marie et al., 2004; Dickman et al., 2006; Wang et al., 2007; O'Connor-Giles et al., 2008; Kim et al., 2010; Rodal et al., 2011; Nahm et al., 2013; Liu et al., 2014). The pattern that has emerged from these studies is that negative regulators of

endocytosis promote excessive synaptic growth. The mechanism of synaptic overgrowth is persistent BMP signaling from ligand-receptor complexes that are normally dissociated or degraded in wild-type synapses. Mutations in BMP pathway genes, including receptors *wit* and *tkv* as well as *mad*, rescue synaptic overgrowth in the background of endocytic gene mutations. Many mutants in these genes also display a characteristic type of synaptic overgrowth: the formation of satellite boutons. Each synaptic bouton normally has two adjacent boutons or at most three when a bouton lies at a branch point. Additional boutons are attached to a parent bouton are referred to as satellite boutons. In addition to being supernumerary they are often undersized and typically exist on branch terminal boutons. Satellite boutons also occur in *dad* mutant animals, indicating that satellite boutons are a result of enhanced canonical BMP signaling (Sweeney and Davis, 2002; Bayat et al., 2011; Menon et al., 2013). The formation of satellite boutons in endocytic mutants and *dad* mutants demonstrates that in certain cases BMP signaling can generate synaptic structures not typically observed at wild-type NMJs.

Extensive regulation of canonical BMP signaling also occurs through post-translational modification of Smad proteins (Ross and Hill, 2008). Mutants in the neuronally expressed *nemo kinase* exhibit synaptic undergrowth similar to BMP pathway mutants. Closer examination revealed that *nemo* mutants have reduced levels of cell body/nuclear P-Mad, although synaptic levels of P-Mad were increased. These observations indicate that the undergrowth observed in these animals is likely due to reduced canonical BMP signaling (Merino et al.,

2009). Nemo and Mad interact *in vitro* where Nemo phosphorylates a specific site on Mad that is separate from the BMP type I receptor phosphorylation site (Zeng et al., 2007). Phosphorylation of Mad at this site is necessary for normal synaptic growth (Merino et al., 2009). Interestingly, while *nemo* is required for normal levels of nuclear P-Mad and normal synaptic growth, neuronal signaling was unchanged (in contrast to BMP pathway mutants). One conclusion that can be drawn from this data is that loss of nuclear P-Mad is sufficient to induce synaptic undergrowth but not defects in synaptic transmission.

A screen for mutants defective in synaptic transmission identified that *importin-β11* is necessary for normal synaptic growth and normal synaptic transmission. Null *importin-β11* mutants resemble BMP pathway mutants in that they have undergrown synapses and reduced synaptic transmission including reduced EJP amplitude and mEJP frequency. However, *importin-β11* mutants exhibit statistically significantly greater synaptic growth and synaptic signaling than *wit* mutants. Based on phenotypic resemblance, Higashi-Kovtun et al. (2010) tested for a role for BMP signaling in *importin-β11* phenotypes. They found that Importin-b11 functions presynaptically and interacts genetically with multiple BMP pathway genes and that synaptic P-Mad is reduced in *importin-β11* mutants. Interestingly, nuclear P-Mad staining was normal, whereas both synaptic and nuclear P-Mad is eliminated in *wit* mutants (Marqués et al., 2002; Higashi-Kovtun et al., 2010). Synaptic size could be rescued in *importin-β11* mutants by expression of constitutively active Tkv and Sax receptors, which also rescued synaptic P-Mad levels. At this time it is not clear how Importin-β11

regulates levels of synaptic P-Mad (Higashi-Kovtun et al., 2010). These data indicate that Importin-β11 likely functions by modulating BMP signaling and that phenotypes associated with BMP pathway mutants are less severe in *importin-β11* mutants because only synaptic but not nuclear P-Mad is reduced. The presence of synaptic signaling defects in a mutant with reduced synaptic but not nuclear P-Mad indicates that synaptic P-Mad may have distinct signaling roles from nuclear P-Mad.

These studies demonstrate that BMP signal transduction is regulated at numerous steps and that misregulation at any of these steps is sufficient to cause changes in synaptic growth (Fig. 1). In most cases, changes in synaptic growth can be attributed to changes in the amount of nuclear P-Mad transcription factor, resulting in abnormal levels of transcriptional targets.

Transcriptional targets of canonical BMP signaling and their effects

Despite numerous lines of evidence suggesting that SMAD signaling downstream of Wit is a major determinant of synaptic growth and synaptic transmission, only a limited number of transcriptional target genes have been characterized (Yang et al., 2004, Kim and Marqués, 2010). However, in combination, the transcriptional targets that have been identified account for the phenotypes observed in BMP pathway mutants in modest measure. It is worth noting that many of the ontology terms associated with genes that are differentially expressed in *wit* mutants are not obviously related to observed *wit* phenotypes, and a significant fraction are transcription factors (Yang et al., 2004;

Kim and Marqués, 2010). This observation suggests that BMP pathway mutants are unlikely to be exhaustively characterized despite more than a decade of close scrutiny. The difficulty in characterizing BMP signaling is due in part to the fact that it plays various roles in different tissues throughout development and during adult life (Edlund et al., 2002; Allan et al., 2003; Shi and Massagué, 2003; Sanyal et al., 2004).

One verified transcriptional target of canonical BMP signaling is the Ly-6 family gene *target of wit* (*twit*). *twit* shares the same expression pattern as *wit*; expression in motorneurons throughout larval development (Marqués et al., 2002). Expression of *twit* requires intact BMP signaling and nuclear P-Mad (Kim and Marqués 2012). Mutants for *twit* display normal synaptic growth and normal EJP amplitude but reduced mEJP frequency, recapitulating a single *wit* mutant phenotype. Transgenic expression of *twit* in a *wit* mutant background rescues mEJP amplitude but does not increase synaptic growth (Kim and Marqués, 2012). Based on the function of homologous genes and protein characteristics, it is predicted that Twit functions in synaptic vesicle recycling.

Numerous lines of evidence indicate that BMP signaling may regulate synaptic growth via regulation of the presynaptic microtubule (MT) cytoskeleton. Synaptic growth at *Drosophila* NMJs is reduced in mutants for the MAP1B homolog *futsch*, which is required for proper MT stabilization (Roos et al., 2000). *tkv* mutants show a reduction in α-tubulin and acetylated α-tubulin in distal boutons (Wang et al., 2007). Double heterozygotes for *futsch* and BMP pathway components including *tkv* show significant synaptic undergrowth, whereas

synaptic growth is normal in individual heterozygous backgrounds (Nahm et al., 2013). This observation is consistent with BMP signaling and *futsch* acting in a common pathway to regulate synaptic growth. Additionally, synaptic overgrowth observed in *dad* mutants is reduced by feeding low concentrations of the microtubule-severing drug vinblastine to these animals. This suggests that synaptic overgrowth that occurs due to enhanced BMP signaling is achieved in part through a MT-dependent mechanism (Nahm et al., 2013). It is thought that BMP signaling may regulate MT stability by regulating Fragile-X Mental Retardation (dFMR) protein levels. dFMR restrains synaptic growth at NMJs by repressing *futsch* expression (Zhang et al., 2001; Coffee et al., 2010). Like *dad* mutants, *dFMR* mutants exhibit synaptic overgrowth in the form of satellite boutons (Coffee et al., 2010). Furthermore, *dFMR* interacts genetically with *dad* and overexpression of *gbb* or *mad* decreases dFMR protein levels (Nahm et al., 2013). These data suggest that BMP signaling promotes synaptic growth by repressing *dFMR*, allowing higher levels of Futsch to promote MT stabilization and synaptic expansion.

BMP signaling also exerts control on synaptic growth through regulation of the presynaptic actin cytoskeleton. Overexpression of Rac1, but not RhoA or Cdc42, during larval stages caused synaptic overgrowth at *Drosophila* NMJs. Rac-induced overgrowth at NMJs was suppressed in either a *mad* or *wit* mutant background. However, growth was not suppressed when a constitutively active Rac was overexpressed, suggesting BMP signaling regulates Rac activation (Ball et al., 2010). Many Rho-type guanine nucleotide exchange factors (GEFs) are

enriched in the *Drosophila* nervous system and a subset of these have been linked to neuronal growth (Awasaki et al., 2000; Sanchez-Soriano et al., 2007). Among these, the RhoGEF *trio*, which has Rac1-specific GEF activity, is required for normal synaptic growth at NMJs, however, *trio* mutant synapses are not as severely undergrown as *wit* mutant synapses (Newsome et al., 2000; Ball et al., 2010). Trio expression requires normal BMP signaling and overexpression of Trio in a BMP pathway mutant background partially restores synaptic growth defects (Ball et al., 2010).

Non-canonical BMP signaling in *Drosophila* motorneurons

BMP signaling at synapses has also been demonstrated to signal through molecular pathways that are distinct from Smad transcriptional regulation. Although there are no direct signaling roles attributed to synaptic P-Mad, a number of observations strongly suggest that BMP signaling modifies synaptic function by regulating the phosphorylation state of a synaptic pool of Mad. While BMP receptors are present in synaptic terminals at the plasma membrane and on early endosomes, BMP receptors are also bidirectionally transported along axons and retrograde transport of colocalized Tkv and Wit occurs in a Gbb-dependent manner. Mad also traffics bidirectionally within axons, however, Mad in axons is not phosphorylated (Smith et al., 2012). Cell body/nuclear P-Mad is believed to be phosphorylated by ligand-receptor complexes that are retrogradely transported from synaptic terminals to cell bodies. These data suggest that distinct synaptic and cell body/nuclear P-Mad pools are generated by local

phosphorylation of Mad in synaptic terminals or in cell bodies. Furthermore, mutations that diminish only synaptic P-Mad or only cell body/nuclear P-Mad have distinct effects on synaptic function (Merino et al., 2009; Higashi-Kovtun et al., 2010). Synaptic but not cell body/nuclear P-Mad levels are sensitive to activity through postsynaptic glutamate receptors (Sulkowski et al., 2014). Synaptic P-Mad is therefore distinct from cell body/nuclear P-Mad and is alternatively regulated. This strongly suggests that synaptic P-Mad has a unique function and therefore participates in non-canonical BMP signaling. These observations have increasingly gained attention in the field within the last few years and the search for functional roles for synaptic P-Mad are ongoing.

BMP-LIM Kinase signaling

BMP signaling also diverges from Smad-dependent signaling at the type II receptor Wit. A study of mutations that increase the incidence of synaptic footprints, molecular markers for partial synaptic degeneration that very rarely occur at NMJs, revealed a critical role for *wit* and *LIM domain kinase* (*limk*). BMP pathway mutations cause synaptic undergrowth and also increase the observed frequency of synaptic footprints at larval NMJs. While all BMP pathway components examined increased the frequency of footprints, mutations in *wit* had a significantly greater frequency of footprints than mutations in components further downstream including *tkv*, *mad* and *med* (Eaton and Davis, 2005). This suggests that Wit has additional synapse stabilization properties that do not require canonical BMP signaling components. The additional stabilization

properties of Wit mapped to the intracellular C-terminal region of the protein, which is not required for BMP type I receptor phosphorylation and therefore not required for Smad signaling (Eaton and Davis, 2005). The C-terminal region of Wit contains a conserved Limk binding domain that has been shown to increase Limk activity in mammals in a BMP ligand- and Pak-dependent fashion (Foletta et al., 2003; Lee-Hoeflich et al., 2004; Eaton and Davis, 2005; Podkawa et al., 2013). A C-terminal truncated *wit* transgene lacking the Limk-binding domain (wit^{dCT}) was able to rescue synaptic growth but not the frequency of synaptic footprints when neuronally expressed in a *wit* mutant background, thereby demonstrating that Wit-Limk binding enhances synapse stabilization. Furthermore, overexpression of Limk in a *wit* mutant background was sufficient to partially rescue the synaptic footprint phenotype (Eaton and Davis, 2005).

The researchers also examined how synapse stability contributed to overall synaptic growth. Neuronal overexpression of Limk in *wit* mutants was able to fully rescue synaptic growth at NMJs while neuronal overexpression of Limk in *mad* mutants only partially rescued overall synaptic growth (Eaton and Davis, 2005). These data argue that some of the deficit in synaptic growth in BMP pathway mutants is accounted for by a lack of Wit-Limk-mediated synaptic stabilization, presumably regulated by Gbb-Wit binding. Complete loss of Limk has been reported to cause slight synaptic overgrowth whereas overexpression of a constitutively active Limk causes undergrowth (Ang et al., 2006). In contrast, partial loss of Limk or overexpression of wild type Limk has no effect on synaptic growth (Eaton and Davis, 2005; Ang et al., 2006). It is possible that in a larva

with normal BMP signaling, only the most extreme changes in Limk activity cause synaptic growth phenotypes. These observations have led to the hypothesis that in additional to stabilizing synapses in a BMP-dependent mechanism, Limk activity restrains synaptic growth.

Summary of BMP signaling

BMP signaling through the muscle released ligand Gbb, presynaptic receptors Tkv, Sax and Wit, and divergent Smad-mediated and Limk-mediated presynaptic pathways controls synaptic growth, ultrastructure and transmission at *Drosophila* NMJs. BMP signaling is regulated at numerous stages and importantly, by changes in neuronal activity. While BMP signaling is a major regulator of synaptic function at fly NMJs, many synapses in the animal kingdom do not utilize retrograde BMP signaling as a plasticity mechanism. However, the study of BMP signaling at this model synapse is informative by shedding light on the timing and parameters of synaptic plasticity mechanisms generally, which are likely to be utilized by many types of neurons and synapses.

Important parameters of synaptic plasticity revealed by the study of BMP signaling include MT and actin cytoskeleton regulation. Synaptic growth at *Drosophila* larval NMJs is controlled in large part by regulating stability of the MT cytoskeleton via the MAP1B homolog Futsch, which is achieved in part by BMP-regulated dFMR repression (Roos et al., 2000; Nahm et al., 2013). Actin cytoskeleton regulation, which also controls synaptic growth, is achieved by regulating transcription of the RhoGEF *trio*, which regulates Rac1 activity (Ball et al., 2010). We also demonstrate here that BMP signaling controls the presynaptic

actin cytoskeleton via Limk and its target Cofilin, an actin-severing protein (Ohashi et al., 2000; Ang et al., 2006). The details of regulation of synaptic plasticity by cytoskeleton are broadly applicable to the study of synaptic plasticity and these pathways may serve as important therapeutic targets in the treatment of neuronal dysfunction.

Additional growth and plasticity pathways at *Drosophila* NMJs

Wnt/Wingless signaling, MAP kinase cascades, cAMP signaling, non-Futsch regulators of MT stability, and additional regulators of the actin and Spectin cytoskeleton also regulate growth and plasticity of NMJs (Menon, 2013).

Wingless (Wg) is a member of the Wnt family of growth factors and signals through canonical and non-canonical pathways at *Drosophila* NMJs to regulate growth and ultrastructure of synapses (Marqués, 2005; Speese and Budnik, 2007). Like BMP signaling, Wg signaling is required during development for embryonic patterning, and also functions at motorneuron synapses later in life to control synaptic growth and neuronal transmission and proper development of the postsynaptic membrane specialization, the SSR. (Rijsewijk et al., 1987; Packard et al., 2002). Wg signaling at NMJs involves Wg ligand release from motorneurons that is received by muscle and by motorneurons in an autorcrine fashion through the receptor Frizzled and co-receptor Arrow (Packard et al., 2002; Bejsovec, 2013). In the canonical pathway, receptor-ligand binding causes inhibition of GSK-3β and subsequent stabilization of β-catenin, which acts as a transcription factor (Bejsovec, 2013). GSK-3β is localized presynaptically at

NMJs and also regulates the MAP1B homolog Futsch. Autocrine Wg signaling has been shown to regulated NMJ size via GSK-3β and Futsch (Franco et al., 2004). In the non-canonical pathway, a portion of Frizzled is cleaved as a result of Wg binding and the cleaved fragment enters muscle nuclei to act as a transcription factor to regulate postsynaptic development (Mathew et al., 2005; Ataman et al., 2006, 2008; Korkut et al., 2009).

The role of MAPK signaling in regulating growth of NMJs was revealed through a screen for synaptic growth mutants that found a strong negative regulator of synaptic growth. Mutants in the E3 ubiquitin ligase *highwire* (*hiw*) display one of the most extreme synaptic overgrowth phenotypes observed to date. One function of Hiw is to target the Co-Smad Med for degradation. Loss of Hiw causes excessive BMP signaling mediated by Mad/Med (McCabe et al., 2004). However, synaptic overgrowth still occurred in *mad; hiw* double mutants, indicating that *hiw* has additional function in suppressed synaptic growth not mediated by BMP signaling (Wu et al., 2005). A screen for suppressors of synaptic overgrowth in hiw mutants found that a mutation in a *Drosophila MAPKKK* completely suppressed *hiw* overgrowth. The MAPKKK, Wallenda, signals through JNK/Fos to regulate NMJ growth. Wallenda does not appear to interact with any BMP pathway components (Collins et al., 2006).

Synaptic growth and plasticity at Drosophila NMJs is regulated through multiple pathways, however, synapse size and function is tightly and consistently regulated to allow appropriate function of larval musculature. The BMP, Wnt/Wg and JNK/Fos coordinate synaptic growth through crosstalk and feedback

mechanisms. Canonical BMP signaling and canonical Wg signaling regulate presynaptic MT stability via the MAP1B homolog Futsch and evidence exists that these pathways regulate gene transcription in tandem (Nishita et al., 2000; Attisano and Labbe, 2004). The E3 ubiquitin ligase Hiw restrains synaptic growth by regulating presynaptic levels of the Co-Smad Med. These examples are unlikely to be the full extent of communication among these pathways considering that coordinated locomotion by the neuromuscular system is an evolutionarily important task for fruit flies.

Figures

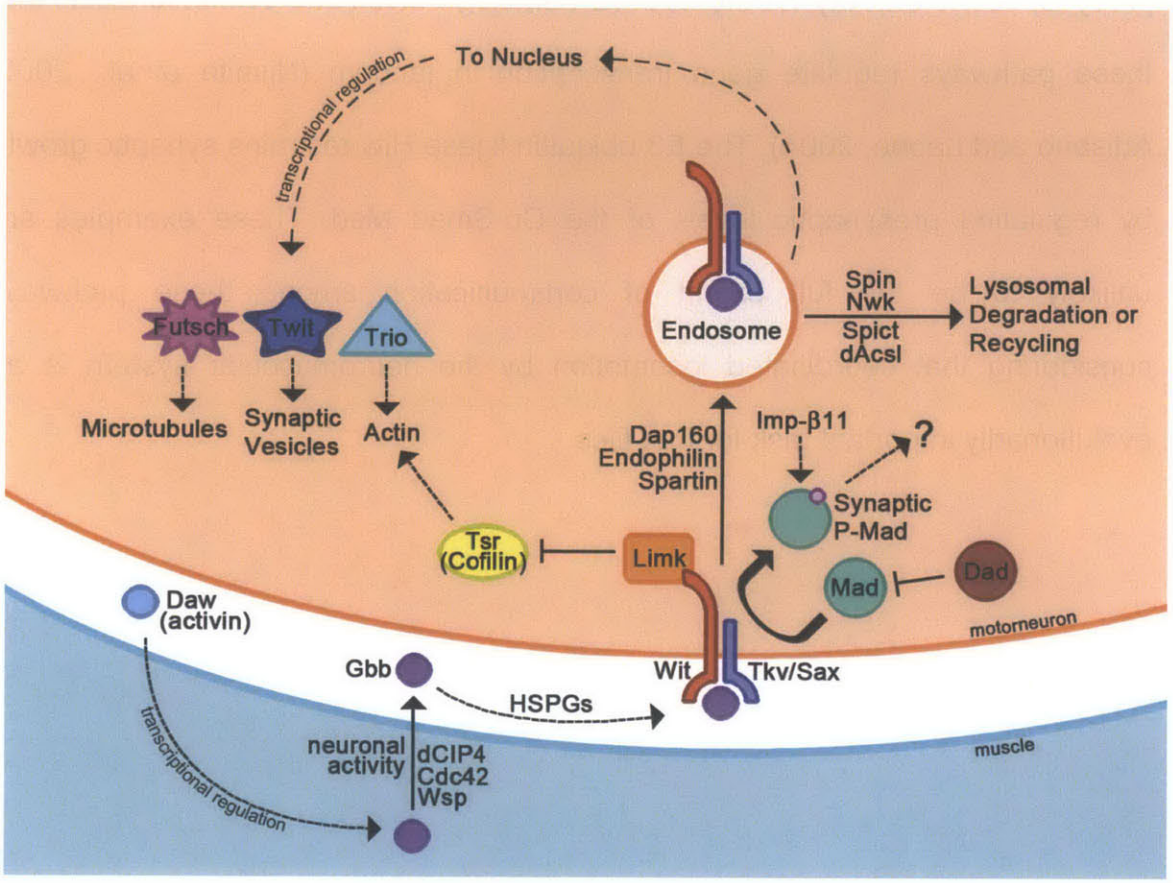
transcriptional regulation
To Nucleus
Futsch
Twit
Trio
Microtubules
Synaptic Vesicles
Actin
Endosome
Spin
Nwk
Spict
dAcsl
Lysosomal Degradation or Recycling
Dap160
Endophilin
Spartin
Imp-β11
?
Synaptic P-Mad
Tsr (Cofilin)
Limk
Mad
Dad
Daw (activin)
motorneuron
Gbb
Wit
Tkv/Sax
HSPGs
neuronal activity
dCIP4
Cdc42
Wsp
muscle
transcriptional regulation

Figure 1. BMP signaling controls synaptic growth and plasticity at *Drosophila* NMJs and is regulated at many steps. The schematic diagrams the components of BMP signaling including the ligand Gbb, receptors Tkv/Sax and Wit, and cytoplasmic R-Smad Mad. Also diagramed are genes involved in regulating BMP signaling, often identified by mutants for those genes that recapitulate BMP mutant phenotypes or create phenotypes that can be rescued by modifying BMP signaling. Canonical BMP signaling regulates transcription of downstream targets including Trio, Twit, and indirectly the MAP1B homolog Futsch via dFMR. The effects of these downstream proteins on synaptic growth and function account for many of the observed phenotypes in BMP pathway mutants.

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Chapter 2

Rapid Activity-Dependent Synaptic Growth Requires Synaptic Transmission and Retrograde BMP Signaling

Zachary Piccioli performed the majority of the work described in this chapter.

Abstract

Drosophila neuromuscular junctions expand drastically during larval life to accommodate growth of the animal. Synaptic growth is regulated to keep pace with muscle growth and is modulated by chronic and short-term changes in neuronal activity. We observed rapid growth events at NMJs that consisted of budding of new boutons from existing synaptic boutons in a 1 to 2 minute timeframe. These growth events are distinct from previously described slow bouton addition mechanisms and result in morphologically distinct bouton products. Here we show that these rapid growth events are triggered by high neuronal activity and produce ghost boutons, immature boutons that do not participate in synaptic signaling. Ghost bouton budding requires local signaling and Ca^{2+}, as well as synaptic transmission machinery and Synaptotagmin 4 (Syt4) -mediated retrograde signaling. We also demonstrate that BMP signaling throughout larval development is required to potentiate NMJs to undergo ghost bouton budding in response to high activity. Developmental BMP signaling induces transcription of the Rho GEF *trio*, and we find that Trio protein levels correlate with rapid activity-dependent synaptic growth and that Trio is enriched in newly formed ghost boutons.

Introduction

In additional to developmental synapse formation during the larval stages (Zito et al., 1999), the NMJ displays acute structural plasticity in the form of rapid presynaptic bouton budding in response to elevated levels of neuronal activity

(Ataman et al., 2008). These rapidly generated presynaptic varicosities, referred to as ghost boutons, lack pre- and post-synaptic transmission machinery when initially formed. Ghost boutons were first described by the Budnik group, who observed a high incidence of morphologically distinct boutons in mutants for *dgrip*, a gene involved in postsynaptic Frizzled nuclear import downstream of Wingless/Wnt signaling. Ghost boutons are highly spherical structures as opposed to the normally ellipsoid shape of typical synaptic boutons. They are identified based on staining for neuronal membrane using anti-HRP, but lack staining for a variety of postsynaptic proteins including the PSD-95 homolog Discs Large (DLG), glutamate receptors, and Spectrin. Ghost boutons do contain synaptic vesicles labeled by Cysteine String Protein and Synapsin and do occasionally contain active zones (Ataman et al., 2006; Mosca and Schwarz, 2010). Mutants in the Wingless signaling pathway exhibit defects in synaptic growth and postsynaptic development including deficits in subsynaptic reticulum (SSR) formation resulting in thin sections of SSR or bouton unopposed by SSR (Packard et al., 2002). One possible explanation for the increased frequency of ghost boutons in *dgrip* mutants is that reduced SSR size lowers physical barriers to new bouton budding. Alternatively, the observed frequency of ghost boutons could be increased because of a failure of proper postsynaptic maturation of ghost boutons while the frequency of ghost bouton budding remains unchanged (Ataman et al., 2006; Fuentes-Medel et al., 2009; Mosca and Schwarz, 2010).

The budding of ghost boutons requires retrograde signaling mediated by the postsynaptic Ca^{2+}-sensitive vesicle trafficking regulator Synaptotagmin 4

(Syt4) (Korkut et al., 2013). Syt4 also participates in developmental synaptic growth and controls retrograde signaling that mediates enhanced spontaneous release at the NMJ (Barber et al., 2009; Yoshihara et al., 2005). The contents of postsynaptic Syt4 vesicles are currently unknown, but based on the ability of postsynaptic Syt4 to drive changes in presynaptic growth and function; it is reasonable to predict that Syt vesicles contain retrograde growth factors that can be received by motorneurons. Synaptic P-Mad levels are sensitive to activity through postsynaptic glutamate receptors that flux Ca^{2+} into muscle and a potential Ca^{2+}-sensitive release mechanism for the BMP ligand Gbb is through Syt4-dependent vesicle fusion (Sulkowski et al., 2014).

Beyond the role of Syt4 in ghost bouton budding, little is known about the signaling pathways that underlie this rapid form of structural synaptic plasticity. In particular, it is unclear if pathways that regulate synaptic growth over the longer time scales of larval developmental also trigger acute structural plasticity. To address these issues, we identified synaptic pathways that are required for rapid structural plasticity at *Drosophila* NMJs. We find that ghost bouton budding can be locally regulated at the synapse level, occurring in axons that have been severed from the neuronal cell body. In addition, activity-induced ghost bouton formation requires Synaptotagmin 1-mediated neurotransmitter release and postsynaptic glutamate receptor function. Like developmental growth, retrograde BMP signaling is required for ghost bouton budding. BMP signaling functions through a permissive role mediated by developmental Smad and Trio signaling.

Results

Rapid synaptic growth at *Drosophila* NMJs requires local activity-dependent signaling

Prior studies at the *Drosophila* NMJ identified patterns of developmental synaptic growth by imaging through the cuticle of intact larvae genetically expressing fluorescent synaptic markers at multiple time points separated by several days (Zito et al., 1999). To more acutely analyze patterns of synaptic growth over a shorter time interval, we performed live imaging of NMJs of dissected larvae genetically expressing fluorescently tagged synaptic proteins. For our initial analysis, transgenic animals were generated that expressed *UAS-mRFP-Syntaxin 13*. Syntaxin 13 is a t-SNARE protein that decorates endosomal compartments and the presynaptic membrane when expressed in motor neurons with *elav*-GAL4 (Fig. 1A), and proved to be an effective marker for visualizing synaptic growth dynamics. mRFP-Syntaxin 13 expressing 3rd instar larvae were dissected, and selected NMJs were imaged at 1 Hz over 30 minutes in live preparations with the brain intact. Using this approach, rapid presynaptic budding events were observed that triggered formation of a new bouton in less than one minute during normal central pattern generated muscle contraction (Fig. 1A, Movie 1). These budding events occurred very rarely and generated extremely round boutons with thin axonal connections to the parent bouton. These newly formed presynaptic varicosities morphologically resembled ghost boutons that have been previously described (Ataman et al., 2006). Ghost boutons lack postsynaptic specializations, including the PSD-95 homolog Discs Large (Dlg)

and glutamate receptor clusters (Fig. 1B). Undifferentiated boutons have been previously observed developmentally in the absence of external stimulation (Ataman et al., 2006; Fuentes-Medel et al., 2009; Korkut et al., 2009; Mosca and Schwarz, 2010;), indicating they may contribute to normal synaptic development. Muscle and surrounding glia have also been shown to engulf a subset of these immature boutons (Fuentes-Medel et al., 2009), indicating some ghost boutons are normally eliminated during development. In unstimulated preparations, we observed that ghost boutons represented ~1% of all synaptic boutons at muscle 6/7 NMJs, with their frequency positively correlated with overall NMJ size ($n = 68$, $r^2 = 0.3002$, $p < 0.0001$; Fig. 1C). In several cases, we observed ghost bouton-like presynaptic structures that were surrounded by trace amounts of DLG (Fig. 1D), suggesting a subset of these varicosities may be maturing into functional connections.

During live imaging of normal larval synaptic dynamics, we observed two general patterns by which new synaptic boutons appeared. In the first, an existing presynaptic bouton would bud off new membrane in a relatively rapid fashion over 10-30 seconds, appearing to split following the emergence of the new bouton (Movie 1, Fig. 1A). These events were often associated with muscle contraction. Given the presynaptic arbor extends into the muscle at the *Drosophila* NMJ, we hypothesize that adhesive interactions between the muscle and the presynaptic bouton at particular attachment points may participate in "pulling" out a new bouton from an existing varicosity, with force for the process generated during muscle contraction. In a second pathway, often observed in

preparations with less muscle contraction, presynaptic membrane would slowly flow from an existing bouton into a small bud over several minutes to form a bigger structure that would take on the shape of a new bouton and eventually separate from the main arbor (Movie 2). This budding mechanism appeared to rely more on presynaptic force generation than the postsynaptic "pulling" events shown in Movie 1. Syntaxin 13-positive compartments were often observed near these budding sites, suggesting local endosomal dynamics may contribute to membrane addition or to recycling of cell adhesion proteins at budding sites. In both cases, newly formed varicosities were highly dynamic, often moving tens of microns within the muscle while pulling on small axons that connected the bouton to the main arbor. Since we were only able to image over 30 minutes in dissected preparations before signs of tissue damage, it is unclear how many newly formed boutons mature into functional connections or are disassembled by neighboring glia or muscle.

Due to the rarity in capturing synaptic budding events with live imaging in unstimulated larvae, we proceeded to examine the molecular mechanisms that underlie rapid synaptic growth using a modified high K^+ stimulation protocol in dissected larvae that has been previously shown to rapidly induce presynaptic bouton budding (Ataman et al., 2008). We dissected 3rd instar wandering larvae such that the resulting fillets were relaxed enough to allow for muscle contraction. Dissected preps were stimulated by washing on high K^+ solution three times for two minutes each spaced by 10 minutes in HL3 dissecting solution. After the third incubation with high K^+ solution, larvae were allowed to rest in HL3 for two

minutes before being stretched and fixed. Ghost boutons were identified either by live imaging of NMJs in animals presynaptically expressing *UAS-CD8-GFP*, or by staining with DLG and HRP antibodies to identify ghost boutons that were not surrounded by a postsynaptic specialization in fixed animals. This short protocol improved upon prior efforts to promote synaptic growth and robustly induced budding of new presynaptic varicosities in live animals at approximately 12% of existing boutons within 30 minutes, providing an easily quantifiable assay for rapid activity-induced synaptic growth.

Using live imaging before and after stimulation, we observed that new ghost boutons can be generated within a single 2-minute incubation with 90 mM K^+ solution (Fig. 2B: 3'). NMJs continue to bud new boutons throughout the synaptic terminal with repeated exposures to high K^+ solution (Fig. 2B: 15'). We did not observe budding by live imaging in mock treated animals that were incubated with HL3 solution in place of high K^+ solution (Fig. 2E; mock treated, n = 26). In all instances in which synapses were continually monitored throughout the duration of the K^+ stimulation protocol, ghost boutons emerged during periods of high K^+ incubation (Fig. 2A). Following three rounds of K^+ stimulation, the number of ghost boutons at an NMJ was no longer correlated with the baseline bouton number, indicating that this form of rapid-activity dependent growth is not dependent on prior size of the synaptic field (Fig. 2C; n = 123, r^2 = 0.0031, p = 0.54). Ghost bouton budding was observed from both type 1b and 1s boutons. However, boutons were less likely to bud from terminal boutons compared to all other boutons (p = 1.17e-7, binomial test), suggesting the terminal bouton is not

a favored site for new bouton addition. In addition, we never observed budding of new varicosities from an axon segment that lacked a preexisting bouton, suggesting budding events always initiated from previously formed synaptic varicosities. A similar conclusion was made based on prior *in vivo* imaging of developmental synaptic growth (Zito et al., 1999), suggesting preexisting boutons likely contain important molecular components for bouton addition that are not concentrated in the axon.

Further analysis of the conditions permitting ghost bouton budding in response to elevated activity revealed that budding is a local signaling event that requires Ca^{2+}. When Ca^{2+} was removed from the HL3 and 90 mM K^+ solution, the number of ghost bouton budding events fell significantly (Fig. 2D, E; mean ± SD: control = 6.605 ± 5.998, n = 38; 0 mM Ca^{2+} = 0.2 ± 0.5, n = 25, $p < 0.0001$, ANOVA), but were not completely eliminated. Addition of 0.5 mM EGTA to the 0.0 mM Ca^{2+} solution completely eliminated ghost bouton budding events following stimulation (n = 13). Formation of ghost boutons also did not require an axonal connection to the cell body (Fig. 2D, E; axon cut = 6.667 ± 5.073, n = 21), indicating that the signaling events that initiate bouton budding, as well as the machinery that physically drives new bouton addition, are unlikely to acutely require transcription or translation. Additionally, some boutons were observed to bud within seconds of exposure to high K^+, indicating that a subset of synaptic terminal sites are likely to be pre-potentiated for budding in response to elevated neuronal activity. We conclude that ghost bouton budding in response to

elevated activity is a local signaling event that does not acutely require function of the neuronal cell body.

Ghost bouton budding requires synaptic transmission and retrograde BMP signaling

It has been demonstrated previously that ghost bouton budding in response to K^+ stimulation requires muscle depolarization (Korkut et al., 2013). To further characterize the requirements for synaptic transmission in ghost bouton budding, we examined mutants in the presynaptic Ca^{2+} sensor *synaptotagmin 1* (*syt1*). Mutations in *syt1* decrease neurotransmitter release at *Drosophila* NMJs by specifically disrupting the synchronous component of evoked fusion (Yoshihara and Littleton, 2002; Jorquera et al., 2012). We found that ghost bouton budding was substantially reduced in $syt1^{AD4/N13}$ mutants (Fig. 3A, B; wild type = 8.361 ± 7.403, n = 57; $syt1^{AD4/N13}$ = 0.9778 ± 1.5, n = 45, $p < 0.0001$, ANOVA). We also tested the postsynaptic requirement for glutamate receptor subunits in ghost bouton budding. Expression of RNAi directed against the glutamate receptor subunits *DGluRIIA* or *DGluRIIB* in muscle using the 24B-Gal4 driver caused a reduction in ghost bouton budding frequency (Fig. 3A, B; *24B,* $gluRIIA^{RNAi}$ = 0.8065 ± 1.276, n = 31, $p < 0.0001$, ANOVA; *24B,* $gluRIIB^{RNAi}$ = 2.586 ± 3.978, n = 29, $p < 0.0001$, ANOVA). Knockdown of DGluRIIA reduced budding frequency significantly more than knockdown of DGluRIIB ($p = 0.0214$, Student's t-test). Given the prominent role of DGluRIIA-containing glutamate receptor complexes in mediating postsynaptic Ca^{2+} entry, these findings suggest

a potential Ca^{2+}-dependent postsynaptic process may initiate rapid structural plasticity. A prime candidate for such a role would be the release of growth promoting factors following activity-triggered fusion of postsynaptic vesicles containing the Ca^{2+} sensor Synaptotagmin 4 (Syt4) (Yoshihara et al., 2005; Barber et al., 2009). Indeed, the mammalian Syt4 homolog has been shown to regulate BDNF release (Dean et al., 2012), a key modulator of structural plasticity at mammalian synapses. We therefore tested the role of Syt4 in regulating rapid activity-dependent structural plasticity at *Drosophila* NMJs using null mutations in the locus we previously generated. Loss of Syt4 substantially reduced ghost bouton budding in response to K^+ stimulation (Fig. 3A, B; $syt4^{BA1}$ = 4.585 ± 5.08, n = 65, p = 0.0037, ANOVA), similar to observations made by Korkut and colleagues (2013). Postsynaptic knockdown of Syt4 phenocopied $syt4^{BA1}$ mutants, indicating that a postsynaptic source of Syt4 contributes to this effect (Fig. 3A, B; *24B,* $syt4^{RNAi}$ = 3.548 ± 2.694, n = 31, p = 0.0016, ANOVA). These data indicate ghost bouton budding is sensitive to the levels of both presynaptic neurotransmitter release and postsynaptic glutamate receptor and Syt4 function, rather than being triggered only by changes in presynaptic membrane depolarization induced by high K^+.

Retrograde BMP signaling from the muscle to presynaptic terminal has been well characterized for its role in normal developmental synaptic growth. To determine if BMP signaling is also required for ghost bouton budding at larval NMJs, we manipulated components of the BMP signaling pathway and assayed their potential for rapid activity-induced presynaptic growth. Postsynaptic

reduction of the BMP ligand Gbb in muscle by RNAi significantly reduced ghost bouton budding (Fig. 3A, B; *24B*, gbb^{RNAi} = 1.8 ± 1.795, n = 20, $p < 0.0001$, ANOVA), although baseline bouton number remained unchanged (in contrast to *gbb* mutants) (Fig. 3C). These results suggest partial knockdown of Gbb is sufficient to disrupt activity-induced ghost bouton budding, but does not alter normal developmental synaptic growth. This observation indicates developmental versus acutely triggered synaptic growth is likely to have different sensitivity or distinct molecular components for driving new synapse formation. To examine if ghost bouton budding is locally regulated at individual synapses by BMPs, or is instead controlled mainly by a BMP-dependent developmental transcriptional signal that would affect all synapses of a given motor neuron, we drove RNAi against Gbb using H94-Gal4, which expresses predominantly in muscle 6, but not muscle 7 (Davis et al., 1997). Muscle 6 and 7 are innervated by two motor neurons that branch onto both muscle fibers. If BMP signaling solely functioned in a developmental role to allow NMJs to express the potential to undergo structural plasticity, we would expect Gbb expression from either muscle fiber to be sufficient to promote normal ghost bouton budding. However, if BMP signaling plays a more acute instructive role in structural plasticity, we would expect to see preferential defects in new synaptic budding events at muscle 6, which would have reduced local Gbb output due to preferential RNAi expression in this muscle driven by the H94-Gal4 promoter. In wild type animals there is a slight bias for ghost bouton budding onto muscle 6, as this is the larger of the two muscles. This bias is eliminated when Gbb levels are reduced in muscle 6 and

not muscle 7 (Fig. 3A, D; p = 0.0058, Fisher's exact test), indicating bouton budding is likely to require in part a local postsynaptic source of Gbb given the comparatively enhanced ability of muscle 7 to support budding. However, total bouton budding events were also decreased at muscle 7 compared to controls, indicating a developmental role for Gbb also contributes to this form of structural plasticity.

Canonical BMP signaling potentiates budding via the RhoGEF *trio*

To further analyze the requirement for BMP signaling in rapid structural plasticity, we examined how disruptions of additional components of the signaling pathway would alter developmental versus acute synaptic growth. Null mutants for the BMP type II receptor *wit* ($wit^{A12/B11}$) displayed a reduction in K^+-stimulated bouton budding (Fig. 3A, B; $wit^{A12/B11}$ = 1.162 ± 2.089, n = 37, p < 0.0001, ANOVA). *Wit* mutant animals also strongly reduced developmental synaptic growth. However, bouton budding as a fraction of baseline bouton number was still significantly reduced (wild type = 0.1369 ± 0.1327 ghost boutons/baseline boutons, n = 66; $wit^{A12/B11}$ = 0.0353 ± 0.0662, n = 37, p < 0.0001, Student's t-test). Given that Wit signals through multiple pathways, we sought to determine how *wit* contributes to ghost bouton budding. Canonical BMP receptor signaling leads to Smad phosphorylation and, together with cofactors, translocation to the nucleus to act as a transcription factor (Bayat et al., 2011). To assay Smad signaling in ghost bouton formation, we overexpressed the inhibitory Smad, *daughters against dpp (dad)* (Tsuneizumi et al., 1997; Kamiya et al., 2008).

Overexpression of *UAS-dad* in motor neurons strongly reduced levels of phosphorylated Mad and caused slight but not significant synaptic undergrowth (Fig. 4B; Eaton and Davis, 2005; Dudu et al., 2006). Overexpression of *UAS-dad* in motor neurons inhibited ghost bouton formation (Fig. 4A, E; *c164, dad* = 2.633 ± 3.944, n = 60, $p < 0.0001$, ANOVA), indicating that Wit signals through Mad to developmentally regulate ghost bouton formation.

One well-characterized transcriptional target of Mad that regulates synaptic growth in *Drosophila* motor neurons is the Rho GEF *trio* (Ball et al., 2010). We assayed for a requirement for Trio in rapid activity-dependent growth by performing K^+ stimulation in $trio^{S137203}$ mutant animals. We observed a quantitatively similar reduction in ghost bouton budding in $trio^{S137203}$ animals compared to *wit* mutants (Fig. 4A, E; $trio^{S137203}$ = 1.486 ± 1.995, n = 37, $p < 0.0001$, ANOVA). Conversely, overexpression of *trio* caused an increase in ghost bouton budding well above wild type levels (Fig. 4A, E; *c164, trio* = 15.41 ± 11.39, n = 27, $p < 0.0001$, ANOVA). These data indicate that Trio may play a key role in the execution of activity-induced synaptic growth given this bidirectional modulation. We therefore sought to determine if Trio protein preferentially targeted to sites of new synaptic growth. Antisera raised against Trio poorly detect the protein at NMJs in wild type animals, but robustly detected overexpressed protein. As such, we performed immunostaining for Trio in larvae presynaptically expressing *UAS-trio*. Strikingly, Trio immunoreactivity was enriched in ghost boutons following high K^+ stimulation (Fig. 4C). Average fluorescence intensity detected in ghost boutons was significantly greater than

that in normal boutons throughout the terminal (Fig. 4C, D; normalized ghost bouton fluorescence intensity = 1.419 ± 0.363, n (ghost boutons) = 77, n (NMJs) = 9, $p < 0.0001$, paired t-test).

We hypothesize that Trio may be enriched at sites primed for bouton budding, generating a higher concentration of the protein that later becomes trapped in newly formed boutons following K^+ stimulation. To test this hypothesis we observed the localization pattern of overexpressed Trio at NMJs in the absence of external stimulation. We found that in a significant subset of boutons on branches running parallel to the muscle 6/7 boundary, Trio protein was enriched in the outer portion of the bouton (Fig. 5 A,B). This localization pattern is consistent with sites of ghost bouton budding, which typically bud from boutons on branches running parallel to the muscle cleft. Furthermore, sites of budding nearly always exist on the sides of these boutons that are distal to muscle 6/7 boundaries. We also observed that Trio is concentrated in ghost boutons generated by endogenous activity (Fig. 5 A,C). These observations are consistent with a model in which localized enrichment of Trio protein primes sites for ghost bouton budding in response to activity and more or less Trio protein changes the number of primed sites.

Taken together, these data indicate that Wit signaling through a canonical Smad transcriptional pathway is likely to mediate the developmental role for BMP signaling in ghost bouton budding. In terms of Smad-dependent transcriptional targets, rapid structural plasticity at the NMJ is bi-directionally correlated with levels of the Rho GEF Trio.

Discussion

BMP signaling in ghost bouton budding

Multiple genetic perturbations of BMP signaling were identified that altered the frequency of activity-dependent bouton budding at the NMJ. Although several of these mechanisms are shared with those previously characterized to control BMP-mediated developmental synaptic growth, several manipulations separated rapid activity-dependent BMP-mediated bouton budding from the slower forms of developmental growth. In the case of *wit* mutants or motor neuron overexpression of *dad,* a reduction in baseline bouton number was observed that showed varying degrees of severity. *Wit* mutants displayed strongly undergrown synapses, while *dad* overexpression animals had only modest synaptic undergrowth. In contrast, both these manipulations strongly suppressed ghost bouton budding. Additionally, synaptic undergrowth with partial knockdown of Gbb using postsynaptic RNAi was not observed, while this manipulation caused a strong reduction in ghost bouton budding. These observations indicate that rapid ghost bouton budding is more sensitive to modest perturbations in BMP signaling compared to developmental synaptic growth. One explanation for this differential sensitivity is that BMP signaling potentiates NMJs for activity-dependent bouton budding via transcriptional regulation of molecular components that are not required for normal synaptic growth. Alternatively, similar molecular pathways are required, but at different levels of output. In particular, *trio* mutants display a less severe synaptic undergrowth phenotype than *wit* mutants, but show similar severe defects in ghost bouton budding.

Because *trio* expression is strongly dependent on BMP signaling (Ball et al., 2010), a modest reduction in BMP output could reduce Trio levels such that ghost bouton budding is significantly reduced, while normal synaptic growth is less affected. It will be interesting to determine in future studies if the developmental role for BMP signaling for acute structural plasticity shares a critical period as has recently been found for BMP function during developmental synaptic growth (Berke et al., 2013).

Given the requirement of the postsynaptic Ca^{2+} sensor, Syt4, for normal levels of ghost bouton budding, an attractive model is that BMP is released acutely in response to elevated activity though the fusion of Syt4 positive postsynaptic vesicles. However, our analysis indicates that retrograde BMP signaling through *trio* transcriptional upregulation is unlikely to be an instructive cue for bouton budding, as severing of axons and inhibition of retrograde trafficking of P-Mad before stimulation does not reduce budding in response to elevated activity. It is possible that synaptic P-Mad may play an instructional role in ghost bouton budding, as a local decrease in budding frequency was observed when Gbb expression is specifically reduced in muscle 6. Neuronal overexpression of *dad* also reduced synaptic P-Mad (Fig. 4B). Therefore, *dad* overexpression could inhibit ghost bouton budding by decreasing synaptic P-Mad, in addition to decreasing nuclear Smad signaling. However, we did not observe dosage-dependent genetic interactions between *syt4* and *wit* (Chapter 3, Fig. 1D), suggesting that Syt4 may participate in a separate pathway to regulate ghost bouton budding. Activity-dependent fusion of Syt4 postsynaptic vesicles

(Yoshihara et al., 2005) could release a separate unidentified retrograde signal that provides an instructive cue for budding that would function in parallel to a developmental requirement for retrograde BMP signaling.

Synaptic actin cytoskeleton regulation via Trio

In addition to instructive cues from the postsynaptic compartment that trigger ghost bouton budding, the presynaptic nerve terminal must have molecular machines in place to read out these signals and execute the budding event. Regulation of Rho GTPases via Rho GEFs and GAPs downstream of extracellular cues is an attractive mechanism, as these proteins play critical roles in regulation of neuronal morphology and axonal guidance (Luo, 2000; Dickson, 2001). Several studies have shown that retrograde synaptic signaling regulates Rho GTPase activity to alter synaptic function and growth in *Drosophila* (Tolias et al., 2011). Ghost bouton budding mediated by developmental BMP signaling also shares some similarities with mechanisms underlying homeostatic plasticity at *Drosophila* NMJs. The Eph receptor is required for synaptic homeostasis at the NMJ, and it interfaces with developmental BMP signaling via Wit (Goold and Davis, 2007; Frank et al., 2009). While Eph receptor-mediated homeostatic plasticity predominantly requires the downstream RhoA GEF Ephexin, the Eph receptor may also signal through Rac1 (Frank et al., 2009). *Drosophila* VAP-33A may also act as a ligand for synaptic Eph receptors, as it has been shown to regulate NMJ morphology and growth, while preferentially localizing to sites of bouton budding (Pennetta et al., 2002; Tsuda et al., 2008). Our analysis indicates

that the levels of Trio, which functions as a Rho GEF, are bi-directionally correlated with ghost bouton budding activity and that overexpressed Trio is localized to ghost boutons after budding. We also observed that overexpressed Trio had a subsynaptic localization consistent with sites of ghost bouton budding. As such, acute Trio regulation represents another attractive pathway for rapidly modifying bouton budding activity.

Methods

Drosophila genetics and molecular biology

Flies were cultured on standard medium at 25°C. All stocks were obtained from the Bloomington Stock Center unless otherwise specified. Wild type flies used in the analysis were Canton S. cDNA encoding the endosomal t-SNARE Syntaxin 13 was modified to add an mRFP tag to the N-terminus of the encoded protein. The sequence was subcloned into PUAST and transgenic flies were generated. The following RNA hairpin lines from the Harvard TRiP collection were used: *UAS-syt1*^{RNAi} (JF01234), *UAS-gbb*^{RNAi} (HMS01243), *UAS-gluRIIA*^{RNAi} (JF02647), *UAS-gluRIIB*^{RNAi} (JF03145). *Wishful thinking* mutants were analyzed as the heterozygote allelic combination *wit*^{A12}/*wit*^{B11}. *Trio*^{S137203} mutants (Ball et al., 2010) were analyzed as homozygotes. Homozygous *Syt4*^{BA1} (Yoshihara et al., 2005) mutants were used for Syt4 null animals. The following Gal4 lines were used: *elav-Gal4* (C155), *c164-Gal4* (Torroja et al., 1999) and *24B-Gal4* (Brand and Perrimon, 1993). *UAS-mRFP-Syx13*, *UAS-syt1*^{RNAi}, *UAS-gluRIIA*^{RNAi}, *UAS-gluRIIB*^{RNAi}, *UAS-gbb*^{RNAi}, *UAS-dad,* and *UAS-trio* were analyzed as

transheterozygotes with the indicated Gal4 driver. Live imaging was performed using the following lines: (1) *elav-Gal4, UAS-mRFP-syx13;* (2) *c164-Gal4*, *UAS-CD8-GFP/+*.

High K^+ stimulation of larval NMJs

The activity-dependent ghost bouton growth protocol was adapted from Ataman et al. (2008). Wandering 3rd instar larvae were dissected in HL3 saline solution (in mM: 70 NaCl, 5 KCl, 0.2 $CaCl_2$, 20 $MgCl_2$, 10 $NaHCO_3$, 5 Trehalose, 115 sucrose, 5 HEPES-NaOH, pH 7.2). Larvae were dissected according to a guide with consistent dissecting pin locations varying in size by 5% increments. Dissecting pins were then moved inward to the same guide shape at 60% of the original size for each larva. Relaxed fillets were subjected to three 2-minute incubations in 90 mM K^+ solution (in mM: 40 NaCl, 90 KCl, 1.5 $CaCl_2$, 20 $MgCl_2$, 10 $NaHCO_3$, 5 Trehalose, 5 sucrose, 5 HEPES-NaOH, pH 7.2) spaced by 10 minutes in HL3 solution. After the third 90 mM K^+ incubation, larvae were returned to HL3 solution for 2 minutes and then stretched to the original position by moving the dissecting pins outward according to the original guide. Ghost boutons were identified by appearance of a bouton that was not previously observed in live imaging, or by the presence of a presynaptic bouton (HRP labeled) that lacked DLG staining in fixed preparations. Muscle 6/7 NMJs from abdominal segments 2 through 5 were included in the analysis. Histograms show mean ± SEM and the imbedded text indicates number of replicates (n). Statistical significance in two-way comparisons was determined by a Student's t-test, while

ANOVA analysis was used when comparing more than two data sets. P values associated with ANOVA tests were obtained from a Tukey's post-test.

Live imaging of NMJ growth

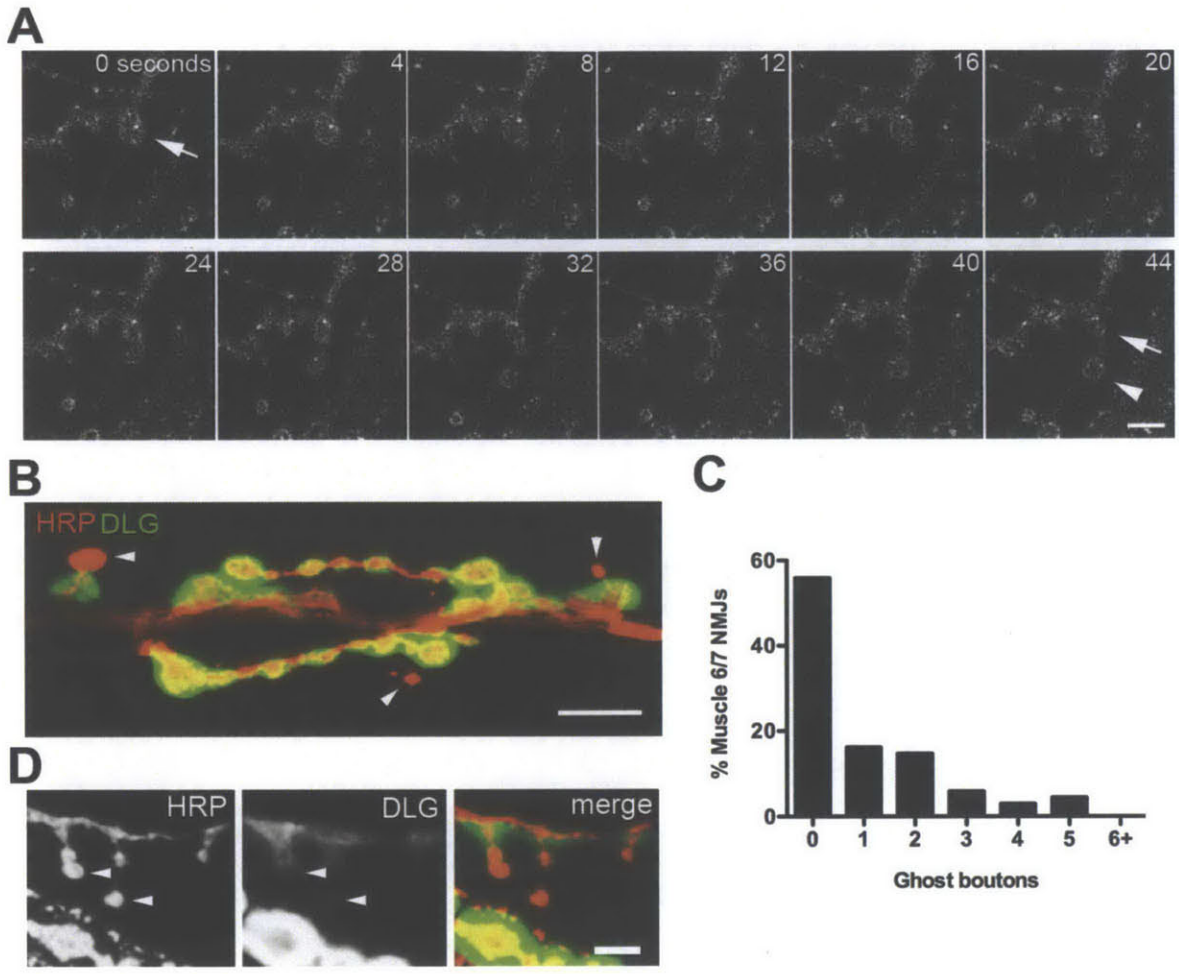
Wandering 3rd instar larvae expressing *UAS-mRFP-syx13*, *UAS-CD8-GFP* or *UAS-GMA* were dissected in HL3 saline solution. For experiments involving high K^+ stimulation, after initial imaging, larvae were subjected to the high K^+ protocol as described above and imaged again after one, two or three 2-minute 90 mM K^+ solution incubations Images were acquired with a PerkinElmer Ultraview Vox spinning disc confocal microscope equipped with a Hamamatsu C9100-13 ImagEM EM CCD at 8-35 Hz with a 40X 0.8 numerical aperture (NA) water-immersion objective (Carl Zeiss).

Immunostaining

Larvae were fixed for 40 min in HL3.1 containing 4% formaldehyde. Following washes in PBS and PBST (1% Triton-X-100), larvae were incubated overnight with primary antibody at 4°C, incubated with secondary antibodies for 4 hrs at room temperature the following day, and mounted in 70% glycerol in PBS for imaging. Antibodies were diluted as follows: mouse anti-DLG (1:500) (Developmental Studies Hybridoma Bank) mouse anti-Trio (1:250) (Developmental Studies Hybridoma Bank) TRITC-conjugated anti-HRP (1:500) (Jackson ImmunoResearch Laboratory), Alexa Fluor 488 goat anti-mouse (1:1000) (Invitrogen). Images were acquired with a 40X 1.3 NA oil-immersion

objective (Carl Zeiss) and analyzed with Velocity Software.

Figures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DLG
merge

Figure 1. Rapid synaptic growth occurs spontaneously and in response to elevated activity. **A.** Live confocal imaging of a dissected larval NMJ presynaptically expressing mRFP-Syx13. The arrow indicates the site of a new bouton spontaneously budding and stabilizing (arrow) over the course of a minute. A second bouton can also be seen to emerge from the same budding site, but later collapses. Scale bar = 5 μm. **B.** Ghost boutons can be detected in fixed tissue by staining for the presynaptic neuronal membrane (anti-HRP, red) and the postsynaptic scaffold protein DLG (green), appearing as presynaptic varicosities that lack DLG staining. Scale bar = 11 μm; arrowheads indicate ghost boutons. **C.** Histogram of ghost bouton frequency observed at unstimulated NMJs. N = 68 NMJs, 12 animals. **D.** Putative ghost boutons identified by morphology in a fixed preparation can display faint DLG accumulation (arrowheads), suggesting some newly formed varicosities are likely to be undergoing synaptic maturation. Scale bar = 5 μm.

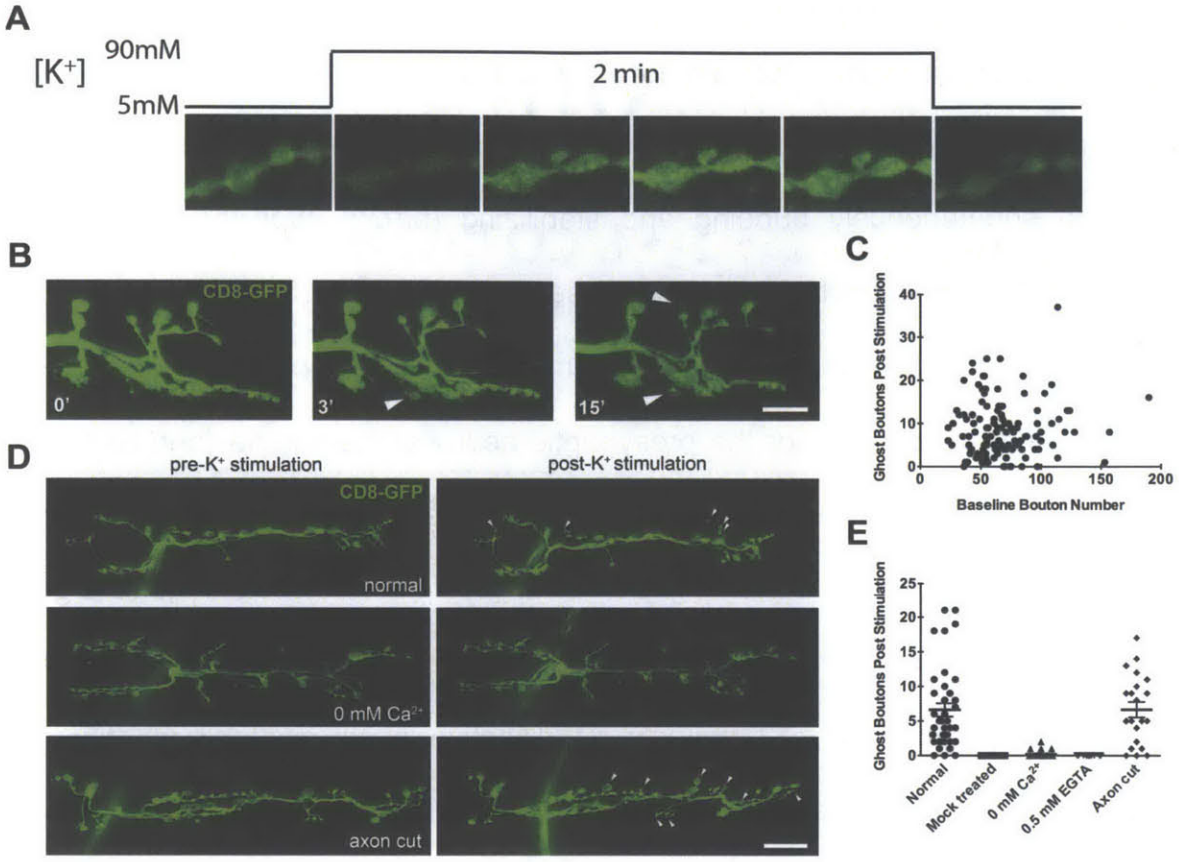

A
[K+]
90mM
5mM
2 min
B
CD8-GFP
0'
3'
15'
C
Ghost Boutons Post Stimulation
Baseline Bouton Number
D
pre-K+ stimulation
post-K+ stimulation
CD8-GFP
normal
0 mM Ca2+
axon cut
E
Ghost Boutons Post Stimulation
Normal
Mock treated
0 mM Ca2+
0.5 mM EGTA
Axon cut

Figure 2. Ghost bouton budding induced by high K^+ stimulation is a rapid local signaling event. **A.** Ghost boutons are generated during periods of high K^+ stimulation and the process of budding closely resembles budding events generated by endogenous activity. New varicosity formation was visualized by presynaptic expression of membrane tethered CD8-GFP. **B.** Live imaging of bouton budding (arrowheads) in response to 2-minute incubations in high K^+ spaced 10 minutes apart. Scale bar = 7 μm. **C.** Quantification of ghost boutons in relation to existing bouton number at the NMJ following high K^+ stimulation. N = 123 NMJs, 18 animals. **D.** New bouton budding (arrowheads) in response to high K^+ stimulation is strongly dependent upon external Ca^{2+}, but is not changed when axons are severed from motor neuron cell bodies. Scale bar = 14 μm. **E.** Quantification of ghost bouton budding detected by live imaging of animals presynaptically expressing membrane tethered CD8-GFP at the indicated conditions. N (NMJs, animals): control = 38, 7; mock treated = 26, 4; 0 mM Ca^{2+} = 25, 4; 0.5 mM EGTA = 13, 4; axon cut = 21, 4. Error bars indicate SEM.

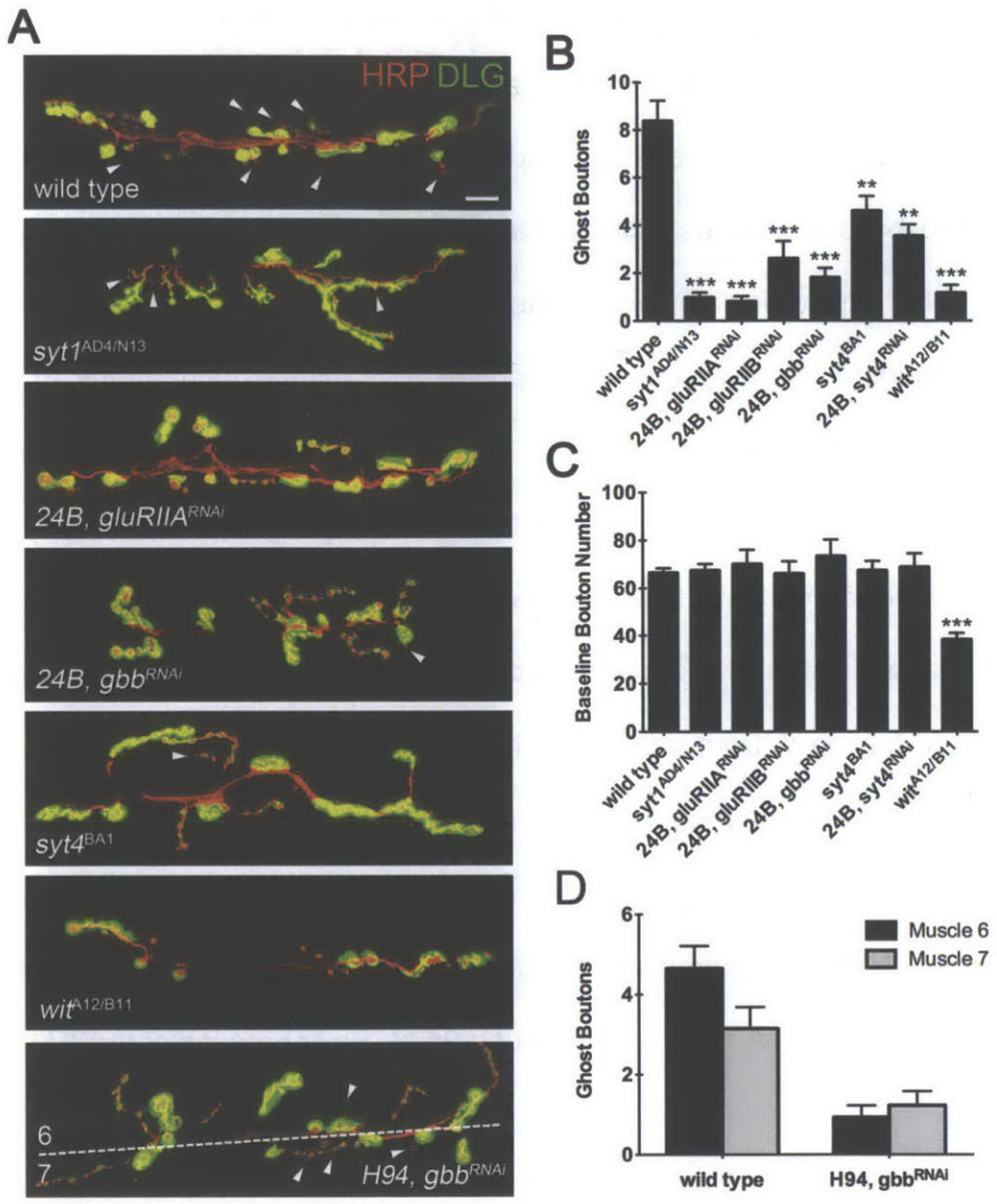

A
HRP DLG
wild type
syt1AD4/N13
24B, gluRIIARNAi
24B, gbbRNAi
syt4BA1
witA12/B11
6
7
H94, gbbRNAi
B
Ghost Boutons
10
8
6
4
2
0
***
***
***
***
**
**
***
wild type
syt1AD4/N13
24B, gluRIIARNAi
24B, gluRIIBRNAi
24B, gbbRNAi
syt4BA1
24B, syt4RNAi
witA12/B11
C
Baseline Bouton Number
100
80
60
40
20
0
***
wild type
syt1AD4/N13
24B, gluRIIARNAi
24B, gluRIIBRNAi
24B, gbbRNAi
syt4BA1
24B, syt4RNAi
witA12/B11
D
Ghost Boutons
6
4
2
0
Muscle 6
Muscle 7
wild type
H94, gbbRNAi

Figure 3. Ghost bouton budding requires normal synaptic transmission and local retrograde BMP signaling. **A.** Wandering 3rd instar animals were fixed in formaldehyde after high K^+ stimulation and stained with anti-HRP and anti-DLG to identify ghost boutons. Presynaptic knockdown of Syt1 and postsynaptic knockdown of GluRIIA and GluRIIB reduce activity-dependent budding. Likewise, loss of the postsynaptic Ca^{2+} sensor Syt4, or postsynaptic knockdown of Gbb with the muscle driver 24B-Gal4, reduces ghost bouton budding. Knockdown of Gbb with the muscle 6 specific H94-Gal4 preferentially reduces budding at muscle 6. Scale bar = 12 μm; arrowheads indicate ghost boutons. **B.** Quantification of ghost boutons per NMJ in the indicated genetic backgrounds. N (NMJs, animals): wild type = 57, 11; $syt1^{AD4/N13}$ = 45, 6; *24B,* $gluRIIA^{RNAi}$ = 31, 4; *24B,* $gluRIIB^{RNAi}$ = 29, 4; *24B,* gbb^{RNAi} = 20, 3; $syt4^{BA1}$ = 65, 11; *24B,* $syt4^{RNAi}$ = 3.548 ± 2.694, n = 31, 3; $wit^{A12/B11}$ = 37, 6. **C.** Quantification of baseline bouton number in the indicated genetic backgrounds. N: same as in (B). **D.** The average number of ghost boutons that bud onto muscle 6 or muscle 7 is quantified for Gbb knockdown by the muscle 6 specific driver H94-Gal4. N (NMJs, animals): wild type = 52, 7; *H94,* gbb^{RNAi} = 40, 6. **: $p < 0.01$; ***: $p < 0.001$; ANOVA. Error bars indicate SEM.

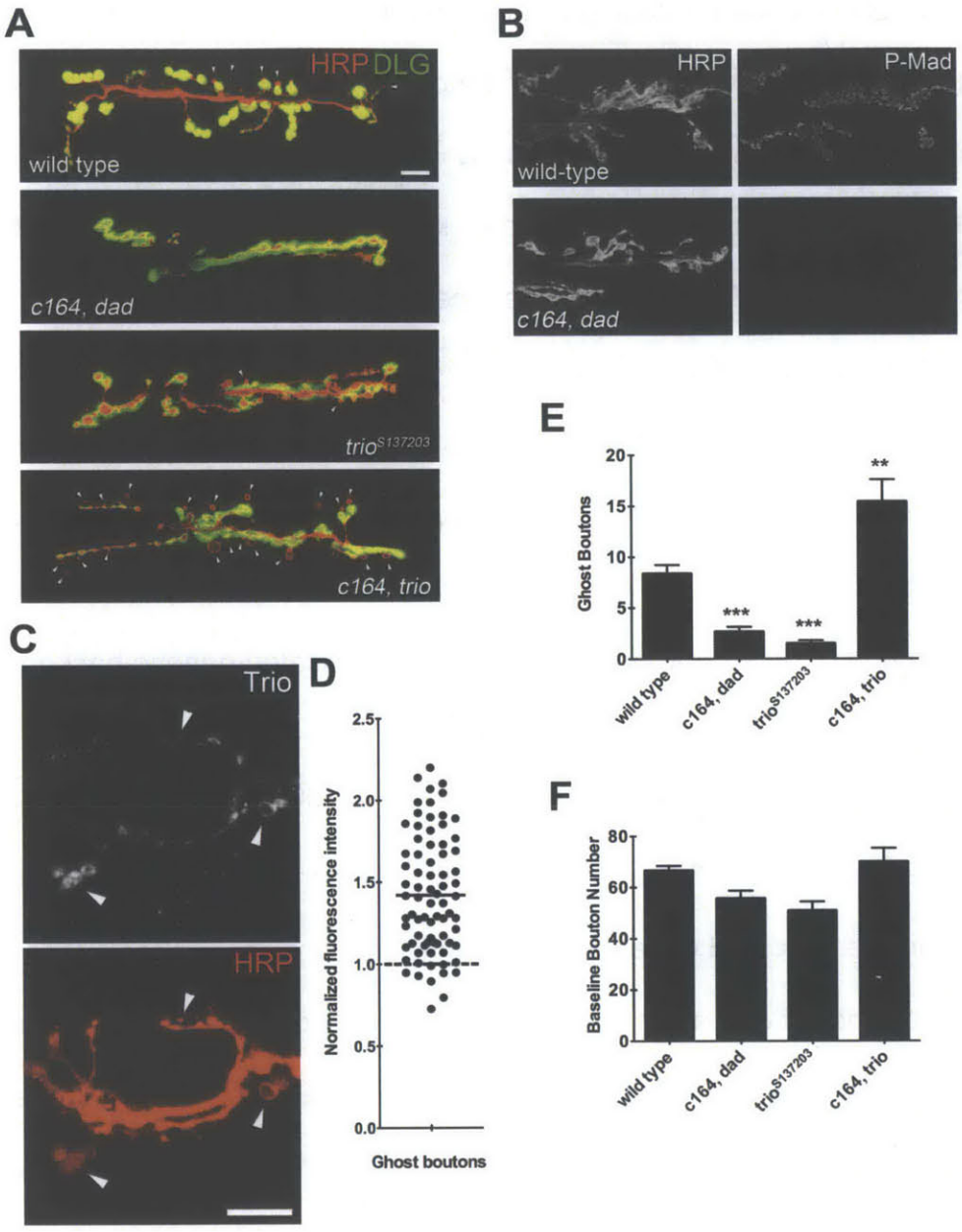

A
HRP DLG
wild type
c164, dad
trio^S137203
c164, trio
B
HRP
P-Mad
wild-type
c164, dad
C
Trio
HRP
D
Normalized fluorescence intensity
2.5
2.0
1.5
1.0
0.5
0.0
Ghost boutons
E
Ghost Boutons
20
15
10
5
0
**
***
***
wild type
c164, dad
trio^S137203
c164, trio
F
Baseline Bouton Number
80
60
40
20
0
wild type
c164, dad
trio^S137203
c164, trio

Figure 4. Ghost bouton budding requires Smad signaling and correlates with Trio levels. **A.** Wandering 3rd instar larvae were fixed in formaldehyde after high K^+ stimulation and stained with anti-HRP and anti-DLG to identify ghost boutons. Overexpression of the inhibitory Smad *dad*, causes synaptic undergrowth and a reduction in ghost bouton budding frequency. Trio protein levels correlate with ghost bouton budding frequency. Scale bar = 12 μm; arrowheads indicate ghost boutons. **C.** *c164, UAS-trio* animals were stimulated and fixed and stained with anti-Trio antibody. Scale bar = 12 μm; arrowheads indicate ghost boutons identified by morphology. **D.** Normalized fluorescence intensity of Trio antisera staining within ghost boutons was normalized to average fluorescence intensity of all other normal boutons at the same NMJ. N (ghost boutons, NMJs) = 77, 9. Solid line indicates mean; dashed line indicates average normal bouton fluorescence intensity. **E.** Quantification of ghost boutons per NMJ in the indicated genetic background. N (NMJs, animals): wild type = 57, 11; *c164, dad* = 70, 8; *trio*^{S137203} = 37, 5; *c164, trio* = 27, 4. **F.** Quantification of baseline bouton number. N: same as in (E).

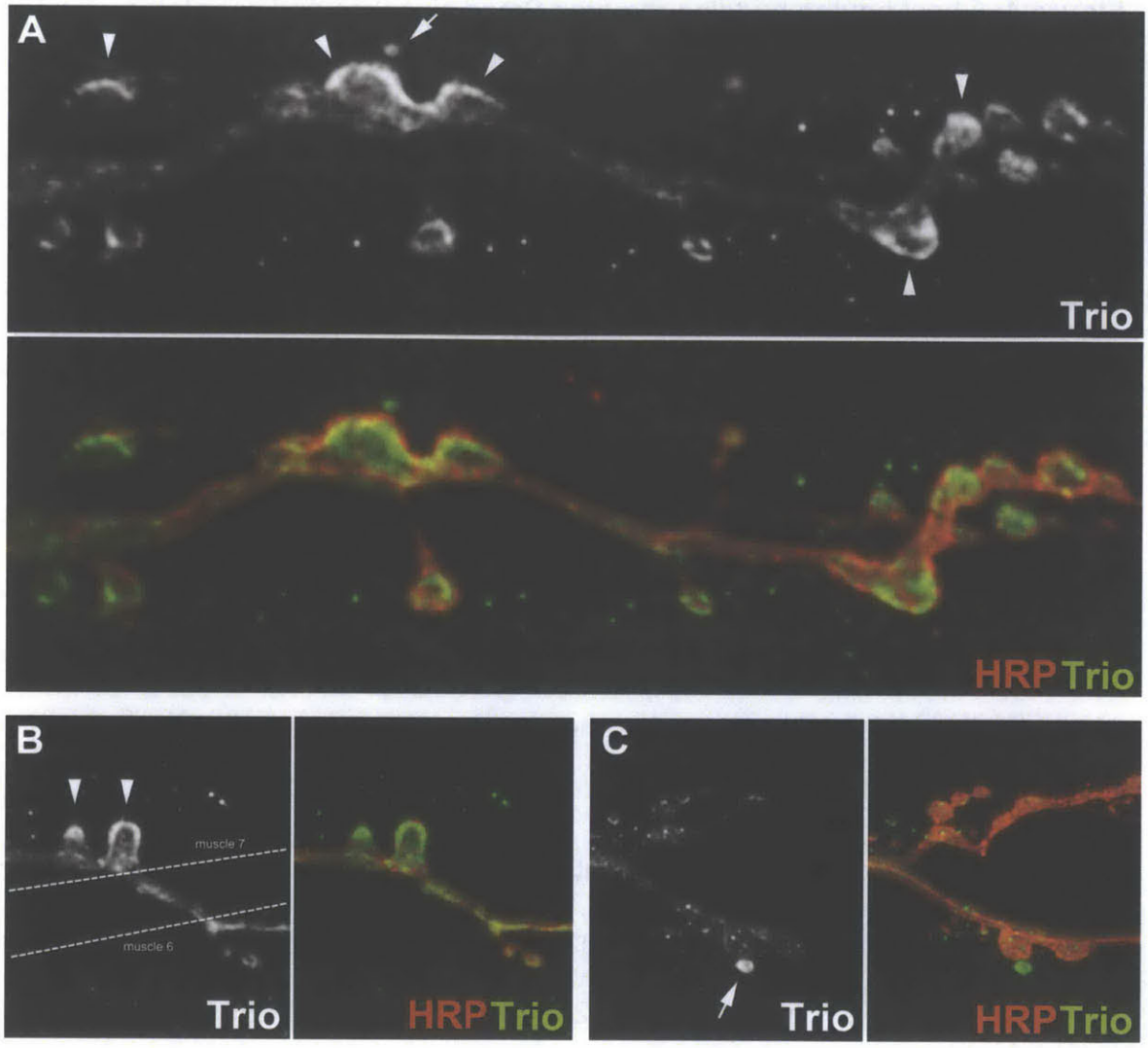
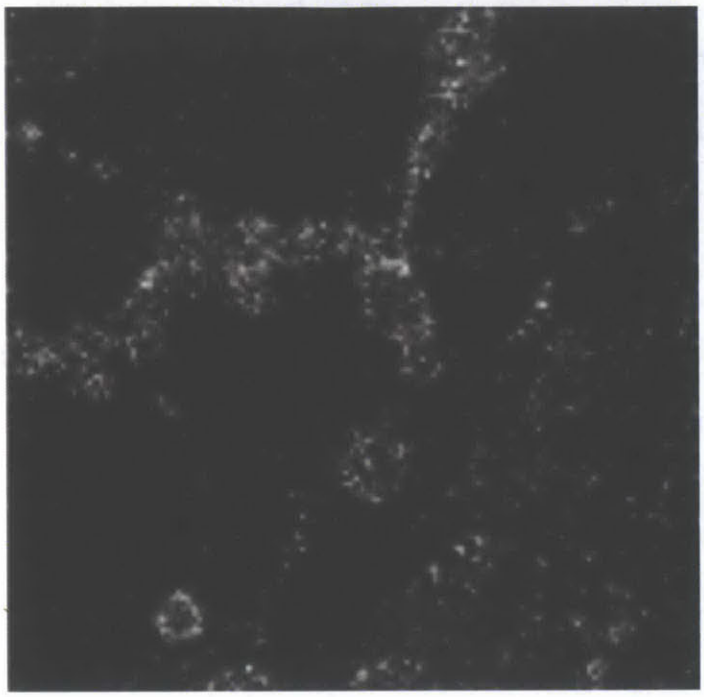

A
Trio
HRP Trio
B
muscle 7
muscle 6
Trio
HRP Trio
C
Trio
HRP Trio

Figure 5. Trio localizes to the outer leaflets of bouton membranes in the absence of external stimulation. *c164, UAS-trio* animals were fixed and stained with anti-Trio antibody in the absence of external stimulation. **A,B.** A subset of synaptic boutons at muscle 6/7 displayed a characteristic localization of overexpressed Trio. Trio protein was enriched at the outer leaflet of the bouton membrane, distal to the muscle 6/7 boundary. These sites are representative of ghost bouton budding sites. Arrowheads indicate sites of Trio enrichment; arrow indicates ghost bouton. C. Ghost boutons generated by endogenous activity are enriched for Trio. Arrow indicates ghost bouton.

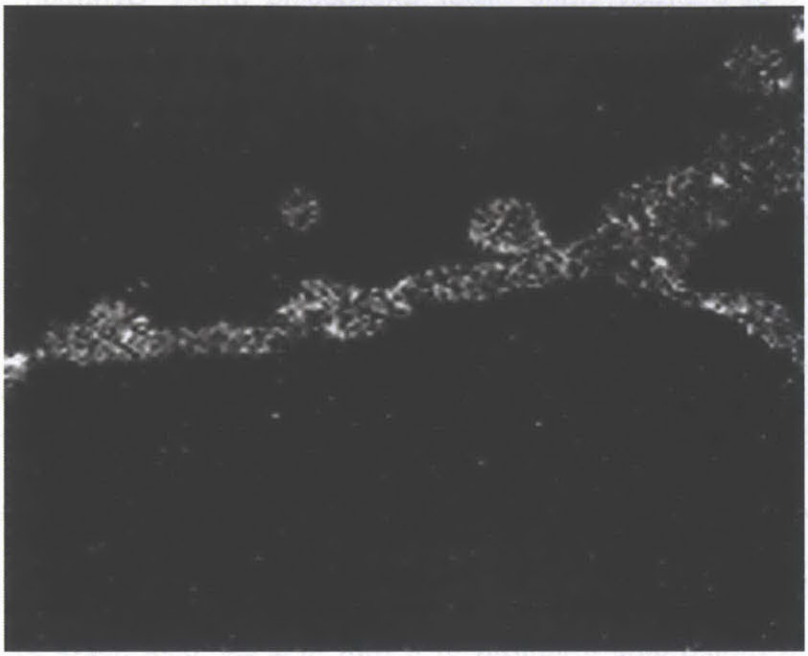
Movie Legends

Movie 1. http://www.jneurosci.org/content/34/12/4371.long



Movie 1. Rapid presynaptic bouton budding imaged at Muscle 12/13. Visualization of a presynaptic arbor expressing mRFP-Syntaxin 13 at muscle 12/13 in a dissected 3rd instar larva with the nervous system intact. The parent bouton swells at a restricted site (approximately 2 μm across), producing two discernable boutons within 20 seconds. The new bouton (labeled A) then separates from the parent bouton at the budding site, but remains connected by a thin axon. A second, smaller bouton (labeled B) buds from the same initial site, but collapses rapidly and is not maintained. The first newly budded bouton is stabilized within 1 minute at ~4 mm from the parent bouton and has a strikingly round morphology. The parent bouton appears incrementally smaller after budding. The movie was acquired at a rate of 1 Hz, with video speed set at 10 frames per second, 10X real time.

Movie 2: www.mit.edu/flybrain/littletonlab/Piccioli



Movie 2. Multiple budding events and subsequent bouton relocalization at muscle 4. The presynaptic terminal at muscle 4 in a dissected 3rd instar larva with the nervous system intact is visualized by neuronal expression of mRFP Syntaxin 13. **A.** A large bouton shows signs of swelling at a restricted site similar to Movie 1, but over a longer period. A small bouton buds from the parent bouton and adopts the characteristic morphology of rapidly budded boutons: highly rounded and connected to the parent bouton by a thin axon. This new bouton moves away from the parent bouton by more than 10 μm and retains its axonal connection. **B.** Membrane swelling then occurs on the same synaptic branch at the bouton most proximal to the synaptic branch point. Growth of this new bouton also occurs more slowly than is observed in Movie 1. The growth of this new bouton occurs steadily and at the time of separation from the parent, this bouton is considerably larger than the parent bouton. The second bouton initially maintains its axonal connection to its parent bouton as it migrates away. **C.** The axonal connection is then lost while the large bouton continues to migrate away from the synaptic terminal, catching the smaller, initially budded bouton in its wake. Both boutons appear disconnected from the synaptic terminal and eventually reverse migration direction, moving together in the muscle tissue towards the synaptic branch point. The movie was acquired at a rate of 1 Hz. The video speed is 10 fps; 10x real time.

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Chapter 3

Presynaptic Actin Cytoskeleton Regulation Controls Activity-Dependent Synaptic Growth

Zachary Piccioli performed all of the work described in this chapter.

Abstract

BMP signaling via Gbb-Wit binding diverges into multiple signaling pathways downstream of Wit that contribute to synaptic growth and plasticity through differing mechanisms. In additional to participating in Smad signaling, Wit has been shown to signal locally through direct binding to LIM domain Kinase (Limk). Previous work has demonstrated that synaptic stabilization mediated by the Wit-Limk interaction is required for normal synaptic stabilization. Here we show that the Wit-Limk interaction also regulates rapid activity-dependent synaptic growth. The Limk-binding domain of Wit inhibits ghost bouton budding, as does overexpression of Limk. This is consistent with a model in which Wit binding Limk increases Limk activity. We also observe that Cofilin activity controls ghost bouton budding in a manner that is consistent with our expectations based on Limk activity. Limk functions to inhibit Cofilin via phosphorylation at Ser3 and overexpression of a phosphomimetic or phosphoincompetent Cofilin decreases or increases ghost bouton budding frequency, respectively. That is, decreased Cofilin activity inhibits budding while increased Cofilin activity increases budding frequency. Overexpression of mutant Cofilin perturbs normal presynaptic F-actin organization, suggesting that mutant Cofilin affects rapid activity-dependent plasticity via changes to actin dynamics. In support of this idea, we observe that bouton budding is accompanied by local changes in the F-actin cytoskeleton and that pharmacological disruption of normal F-actin turnover inhibits bouton budding.

Introduction

Neurons have highly specialized morphologies that are critical to proper cellular function. The actin cytoskeleton contributes greatly to the formation and maintenance of these morphologies: actin regulatory molecules are involved in neurite outgrowth, axonal pathfinding and synapse formation (Luo, 2000; Cingolani and Goda, 2008). Actin is the most abundant cytoskeletal protein at mature synapses and greatly contributes to structural synaptic plasticity (Carlisle and Kennedy, 2005). Additional roles for actin presynaptically include Synapsin-dependent synaptic vesicle sequestration (Hilfiker et al., 1999; Jordan et al., 2005; Evergren et al., 2007), synaptic vesicle recruitment to active zones (Kuromi and Kidokoro, 1998; Nunes et al., 2006), and synaptic vesicle docking at active zones (Siksou et al., 2007). Actin is involved in the regulation of both synaptic size and synaptic vesicle trafficking and is therefore an important regulator of synaptic plasticity. The importance of actin-based regulation of synaptic plasticity is evident in the number of Rho family GTPases and regulators of Rho GTPases that are linked to intellectual disability (Tolias et al., 2011; Ba et al., 2013).

Rho GEFs and GAPs and Rho GTPases exert their effects on the actin cytoskeleton through networks of effector proteins, the downstream components of which directly bind actin to affect its polymerization state in a variety of ways. The Rho GTPase effector Limk is activated downstream of Rac1 via Pak and downstream of RhoA via Rock (Edwards et al., 1999; Maekawa et al., 1999; Dan et al., 2001). Limk phosphorylates and inhibits the actin-depolymerizing factor Cofilin (Arber et al., 1998; Ohashi et al., 2000; Ang et al., 2006). The *Drosophila*

genome encodes a single Cofilin gene called *twinstar* (*tsr*). Knockout mice for *limk1* have abnormal dendritic spine morphologies and deficits in spatial learning, indicating that Limk has important neuronal functions and is involved in synapse biology (Meng et al., 2002). In *Drosophila*, Limk is localized presynaptically at NMJs where it regulates synapse stabilization and growth (Eaton and Davis, 2005, Ang et al., 2006) and has been shown to inhibit axonal outgrowth in mushroom body neurons (Ng and Luo, 2004).

Here we report a novel role for Limk in synaptic plasticity. We find that the Limk-binding domain of Wit has a slight inhibitory effect on ghost bouton budding and that overexpression of Limk strongly inhibits budding. Consistent with these observations, we find that Cofilin activity promotes budding and that budding requires local actin turnover.

Results

Wishful Thinking controls ghost bouton budding through multiple signaling pathways

In addition to Smad-dependent transcriptional changes, Wit can signal locally through Limk to promote synapse stabilization (Eaton and Davis, 2005). Limk phosphorylates and inactivates Cofilin (*Drosophila Twinstar; Tsr*) at serine 3 and can induce changes in actin cytoskeleton structure to restrain normal developmental synaptic growth (Ohashi et al., 2000; Ang et al., 2006). Limk has been shown to interact directly with BMP type II receptors, which increases its kinase activity (Foletta et al., 2003; Lee-Hoeflich et al., 2004; Podkowa et al.,

2013). To test if the Wit-Limk interaction also regulates ghost bouton budding, we rescued $wit^{A12/B11}$ animals with motor neuron expression of full length *UAS-wit* or a truncated copy of the gene, *UAS-*wit^{dCT}, that lacks the Limk binding domain (Eaton and Davis, 2005). Expression of either full length or truncated *wit* rescued developmental synaptic growth (Fig. 1C), indicating that the Limk-binding domain of Wit is not likely to significantly contribute to normal developmental synaptic growth. In contrast, we observed only a partial rescue of ghost bouton budding frequency in full-length *wit* rescue animals (Fig. 1A, B; wit rescue = 3.733 ± 3.875, n = 45, p = 0.0004, ANOVA), though budding frequency was significantly higher than in $wit^{A12/B11}$ mutant animals (p = 0.0414, ANOVA). Wit^{dCT} rescued animals showed significantly greater bouton budding frequencies than full-length *wit* rescue animals (Fig. 1A, B; wit^{dCT} rescue = 7.821 ± 5.769, p = 0.0054, ANOVA). To further examine the Wit-Limk link, we overexpressed full-length *wit* or truncated wit^{dCT} in wild type motor neurons. Overexpression of full-length *UAS-wit* reduced ghost bouton budding (Fig. 1B; *c164, wit* = 3.891 ± 3.629, n = 55, p = 0.0003, ANOVA), while overexpression of *UAS-*wit^{dCT} did not alter budding (*c164,* wit^{dCT} = 6.364 ± 4.507, n = 55, p > 0.999, ANOVA). These observations suggest the Limk binding domain of Wit normally functions to inhibit activity-dependent ghost bouton budding events.

Given the link between Wit signaling and Limk, we sought to determine if Cofilin might function in the same pathway for ghost bouton budding by assaying if *wit* and *tsr* showed genetic interactions. Heterozygote wit^{B11}/+ and tsr^{1}/+ animals both show similar deficits in ghost bouton budding (Fig. 1D; tsr^{1}/+ = 4.29

± 3.95, n = 24; wit^{B11}/+ = 3.82 ± 3.30, n = 45, p > 0.999, ANOVA), suggesting this form of rapid structural plasticity is highly sensitive to incremental disruptions in BMP signaling and partial loss of Tsr function. Analysis of double heterozygous tsr^{1}/+;wit^{B11}/+ animals revealed a significantly greater reduction in activity-dependent plasticity than either single heterozygote (Fig. 1D; tsr^{1}/+;wit^{B11}/+ = 1.25 ± 1.65, n = 37, tsr^{1}/+ vs tsr^{1}/+;wit^{B11}/+: p = 0.0242, wit^{B11}/+ vs tsr^{1}/+;wit^{B11}/+: p = 0.0206, ANOVA). This dosage-dependent interaction is consistent with a model in which Wit and Tsr participate in a similar pathway to regulate ghost bouton budding.

Based on the function of Syt4 as a Ca^{2+} sensor that regulates postsynaptic vesicle fusion, we hypothesized that Syt4 may participate in retrograde BMP signaling. In order to address this possibility we created *syt4, wit* double heterozygous animals. We found that ghost bouton budding frequency was not significantly different in these animals compared to $syt4^{BA1}$ mutants or wit^{B11}/+ single heterozygotes (Fig. 1D; $syt4^{BA1}$/+; wit^{B11}/+ = 4.750 ± 3.36). This observation does not exclude the possibility that Syt4 participates in BMP signaling although it does suggest that Syt4 does not participate. We have characterized BMP signaling as being required developmentally to supply the synaptic terminal with growth factors including Trio, and based on a lack of observed genetic interaction, Syt4 is unlikely to participate in this pathway. However, we have not yet addressed if BMP signaling occurring acutely in response to activity, perhaps to contribute to a synaptic pool of P-Mad, is

involved in ghost bouton budding. Syt4 could potentially have a role in this process and not interact genetically with Smad signaling downstream of Wit.

Interestingly, we did observe an interaction between *syt4* and *tsr*. While ghost bouton budding was not further reduced in *syt4, tsr* double heterzygotes compared to $syt4^{BA1}$ mutants or tsr^{1}/+ heterozygotes, overall synaptic size was significantly reduced (Fig. 1E; baseline bouton number: wild type = 66.47 ± 23.62; tsr^{1}/+; $syt4^{BA1}$/+ = 42.16 ± 18.11; $p < 0.0001$, ANOVA). This interaction suggests that *syt4* may participate in a common signaling pathway with presynaptic *tsr*. This observation suggests that if *syt4* and *tsr* participate in a common pathway, that pathway does not regulate ghost bouton budding or does not occur in the appropriate temporal or spatial dimensions.

Limk regulation of presynaptic Cofilin activity controls rapid activity-dependent synaptic growth

To determine how Limk activity modulated Cofilin and ghost bouton budding, we assayed acute synaptic growth in strains with altered Limk function or disrupted Cofilin regulation by Limk. Motor neuron overexpression of *UAS-limk* strongly inhibited activity-dependent bouton budding (Fig. 2A, B; *c164, limk* = 1.675 ± 2.702, n = 40, $p < 0.0001$, ANOVA), indicating Limk suppresses ghost bouton formation, potentially through phosphorylation-mediated inhibition of Cofilin (Tsr). To examine if Cofilin activity regulates ghost bouton budding, we overexpressed either a constitutively inactive ($UAS\text{-}tsr^{S3E}$) or a constitutively active ($UAS\text{-}tsr^{S3A}$) *twinstar* transgene bearing a phosphomimetic or

phosphoincompetent Limk phosphorylation site, respectively. A decrease in bouton budding frequency was observed when the inactive transgene was overexpressed (Fig. 2A, B; *c164, tsr*^{S3E} = 3.107 ± 3.059, n = 28, p = 0.0006, ANOVA), while a strong increase in bouton budding frequency was found upon overexpression of the active transgene (Fig. 2A, B; *c164, tsr*^{S3A} = 13.39 ± 7.958, n = 36, p = 0.0003, ANOVA). *c164, tsr*^{S3E} and *c164, tsr*^{S3A} animals showed no changes in number of ghost boutons in the absence of stimulation compared to wild type (wild type = 0.9706 ± 1.393, n = 68; *c164, tsr*^{S3E} = 1.032 ± 1.703, n = 31; *c164, tsr*^{S3A} = 1.423 ± 1.46, n = 52; p = 0.233, ANOVA).

We presynaptically expressed the F-actin marker GMA to visualize actin within synaptic terminals. GMA contains the actin-binding domain of Moesin fused to GFP (Dutta et al., 2002). Live imaging of GMA expressing larvae revealed that synaptic boutons contain dynamic F-actin puncta (Fig. 3A). These puncta typically persist for 1 to 2 minutes, although a subset of puncta are stable over a longer period of time. GMA-labeled F-actin puncta behave similarly to F-actin visualized by presynaptic expression of GFP-tagged actin (Nunes et al., 2006).

As predicted based on the ability of Cofilin to disassemble actin filaments, expression of either phosphoincompetent or phosphomimetic transgenes altered the presynaptic actin cytoskeleton when visualized by GMA. In addition to labeling dynamic actin puncta within synaptic boutons, GMA normally labeled more stable actin structures in axons and interbouton regions (Fig. 3A,B). In contrast to controls, GMA formed puncta in axons and extended interbouton

regions of animals expressing *UAS-tsr*^{S3E} or *UAS-tsr*^{S3A} (Fig. 3B). We occasionally observed boutons that lacked discernable F-actin puncta in wild type animals, whereas boutons lacking F-actin puncta occurred more frequently in *tsr*^{S3E} expressing animals (Fig. 3B). We also observed large and bright GMA positive clusters in *tsr*^{S3E} and *tsr*^{S3A} animals that were not observed at wild type NMJs (Fig. 3B). These findings suggest that BMP signaling through Limk is likely to alter Cofilin activity, with subsequent effects on the presynaptic actin cytoskeleton and its ability to support activity-induced bouton formation.

Local actin turnover is required for bouton budding

Manipulations to the actin cytoskeleton regulators *limk* and *tsr* resulted in changes to ghost bouton budding frequency, and in the case of *tsr*, observable changes to the presynaptic actin cytoskeleton. To directly examine the role of presynaptic actin in bouton budding, we performed live imaging in animals expressing GMA and the membrane marker CD8-RFP expressed in motor neurons. 3rd instar larvae were dissected and imaged, and then subjected to a single 2-minute incubation in high K^+, and imaged again. We then identified sites of ghost bouton budding to observe how K^+ stimulation affected the local actin cytoskeleton at sites of new bouton formation by comparing before and after images. We consistently observed the emergence of new F-actin puncta localized at the sites of ghost bouton budding from previously existing boutons (Fig. 4A), suggesting local actin rearrangements occur at regions where new

boutons form. To test if local actin rearrangements are required for ghost bouton budding, we directly interfered with actin turnover through bath application of the F-actin depolymerizing drug latrunculin A or of the F-actin stabilizing drug jasplakinolide (Spector et al., 1999). Application of 10 mM latrunculin A caused dispersal of F-actin puncta with minutes, as well as a reduction in the number of puncta (Fig. 4B). Application of 10 mM of jasplakinolide stabilized existing F-actin puncta and caused the formation of new puncta within minutes (Fig. 4B). The effects of the drugs were still observed 15 minutes after wash out. To examine how these manipulations regulate bouton budding, we incubated dissected preps in HL3 containing 10 mM of drug for 15 minutes and then proceeded with the K+ stimulation protocol with solutions containing 10 mM of drug. Disruption of normal actin turnover with either drug resulted in a decrease in bouton budding compared to controls (Fig. 4C: No Drug = 8.767 ± 7.035, n = 30; Latrunculin A = 4.829 ± 6.046, n = 35, p = 0.0155, ANOVA; Jasplakinolide = 1.933 ± 2.586, n = 30, $p < 0.0001$, ANOVA). Some F-actin puncta were still observed in animals treated with 10 mM latrunculin A, indicating that this treatment may allow formation of a limited number of new puncta. In contrast, the actin cytoskeleton appeared highly stable in jasplakinolide treated animals, and caused a much greater reduction in ghost bouton budding. These data indicate that local actin rearrangement occurs during ghost bouton budding, and that actin turnover contributes to the rapid formation of new synaptic varicosities in an activity-dependent manner.

Discussion

Limk regulation of Cofilin controls ghost bouton budding

Rho GTPase signaling can produce distinct effects in differing systems and cell types depending on the presence or absence of downstream effectors, although most of these pathways ultimately impinge on regulation of the actin cytoskeleton (Luo, 2000). Indeed, we have found a key role for Limk regulation of Cofilin activity in the control of ghost bouton budding. The current findings indicate that Limk activity normally functions to inhibit the formation of ghost boutons, as neuronal overexpression of Limk strongly suppressed activity-dependent bouton budding. Consistent with an inhibitory role for Limk, Cofilin activity promotes budding, while overexpression of an inactive Cofilin inhibited budding. Expression of mutant Cofilin transgenes resulted in visible changes to the presynaptic actin cytoskeleton at NMJs, indicating these manipulations likely alter rapid budding events by changing local actin dynamics as sites of potential growth. Using live imaging of F-actin dynamics before and after bouton budding, formation of new F-actin puncta was observed at sites of bouton budding. Elevated Cofilin activity is sufficient to increase ghost bouton budding frequency, and is predicted to increase actin turnover and formation of F-actin structures (Michelot and Drubin, 2011). Pharmacological disruption of actin polymerization dynamics also disrupts rapid bouton addition in response to elevated activity.

These findings support a model whereby Wit has opposing signaling roles with respect to bouton budding (Fig. 5). Providing a permissive role via Smad signaling and an inhibitory role via Limk activation may provide for a system in

which increased potential for rapid synaptic expansion is directly coupled to enhanced synaptic stability. This coupling could set a threshold for ghost bouton budding downstream of synaptic activity. In the background of moderate or low synaptic activity, Limk prevents ghost bouton budding. When synaptic activity is elevated, additional signaling events promote new synaptic growth by either reducing or outcompeting Limk activity, with a concurrent activation of Cofilin. Decreased Limk activity downstream of extracellular cues has been shown to regulate cell morphology in other systems as well (Sparrow et al., 2012), providing an attractive mechanism for rapid activity-dependent regulation of synaptic structure at *Drosophila* NMJs.

Methods

Drosophila genetics and molecular biology

Flies were cultured on standard medium at 25°C. All stocks were obtained from the Bloomington Stock Center unless otherwise specified. Wild type flies used in the analysis were Canton S. cDNA encoding the endosomal t-SNARE Syntaxin 13 was modified to add an mRFP tag to the N-terminus of the encoded protein. The sequence was subcloned into PUAST and transgenic flies were generated. *Wishful thinking* mutants were analyzed as the heterozygote allelic combination wit^{A12}/wit^{B11}. $Limk^{P1}$ (*P[EY08757]*; Eaton and Davis, 2005) animals were analyzed as male hemizygotes. The following Gal4 lines were used: *24B-Gal4* (Brand and Perrimon, 1993). *UAS-wit,* $UAS\text{-}wit^{dCT}$, *UAS-limk,* $UAS\text{-}tsr^{S3E}$, $UAS\text{-}tsr^{S3A}$, and *UAS-ssh* were analyzed as transheterozygotes with the

indicated Gal4 driver. Live imaging was performed using the following lines: (1) *c164-Gal4, UAS-CD8-GFP/+*; (2) *c164-Gal4, UAS-mCD8-RFP/+; UAS-GMA/+*; (3) *UAS-tsrS3E/+; c164-Gal4/+; UAS-GMA/+*; (4) *UAS-tsrS3A+; c164-Gal4/+; UAS-GMA/+*. Rescue lines consisted of the following genotypes: (1) wit rescue: *c164-Gal4 / UAS-wit; wit^{A12}/wit^{B11}*; (2) wit^{dCT} rescue: *c164-Gal4 / UAS-wit^{dCT}; wit^{A12}/wit^{B11}*.

High K^+ stimulation of larval NMJs

The activity-dependent ghost bouton growth protocol was adapted from Ataman et al. (2008). Wandering 3rd instar larvae were dissected in HL3 saline solution (in mM: 70 NaCl, 5 KCl, 0.2 $CaCl_2$, 20 $MgCl_2$, 10 $NaHCO_3$, 5 Trehalose, 115 sucrose, 5 HEPES-NaOH, pH 7.2). Larvae were dissected according to a guide with consistent dissecting pin locations varying in size by 5% increments (schematic representation of guide with pins placed at line termini: -|--|-). Dissecting pins were then moved inward to the same guide shape at 60% of the original size for each larva. Relaxed fillets were subjected to three 2-minute incubations in 90 mM K^+ solution (in mM: 40 NaCl, 90 KCl, 1.5 $CaCl_2$, 20 $MgCl_2$, 10 $NaHCO_3$, 5 Trehalose, 5 sucrose, 5 HEPES-NaOH, pH 7.2) spaced by 10 minutes in HL3 solution. After the third 90 mM K^+ incubation, larvae were returned to HL3 solution for 2 minutes and then stretched to the original position by moving the dissecting pins outward according to the original guide. Ghost boutons were identified by appearance of a bouton that was not previously observed in live imaging, or by the presence of a presynaptic bouton (HRP

labeled) that lacked DLG staining in fixed preparations. Muscle 6/7 NMJs from abdominal segments 2 through 5 were included in the analysis. Histograms show mean ± SEM and the imbedded text indicates number of replicates (n). Statistical significance in two-way comparisons was determined by a Student's t-test, while ANOVA analysis was used when comparing more than two data sets. P values associated with ANOVA tests were obtained from a Tukey's post-test.

Live imaging of NMJ growth

Wandering 3rd instar larvae expressing *UAS-mRFP-syx13*, *UAS-CD8-GFP* or *UAS-GMA* were dissected in HL3 saline solution. For experiments involving high K^+ stimulation, after initial imaging, larvae were subjected to the high K^+ protocol as described above and imaged again after one, two or three 2-minute 90 mM K^+ solution incubations. Latrunculin A (Sigma) and jasplakinolide (Invitrogen) were prepared as 1 mM stocks in DMSO and diluted in HL3 and 90 mM K+ solutions. Drug treatments were performed by pre-treating dissected larvae in HL3 solution containing 10 mM latrunculin A or 10 mM jasplakinolide for 15 minutes. Stimulation using HL3 and 90 mM K^+ solutions containing either 10 mM latrunculin A or 10 mM jasplakinolide was then employed as described above. Images were acquired with a PerkinElmer Ultraview Vox spinning disc confocal microscope equipped with a Hamamatsu C9100-13 ImagEM EM CCD at 8-35 Hz with a 40X 0.8 numerical aperture (NA) water-immersion objective (Carl Zeiss).

Immunostaining

Larvae were fixed for 40 min in HL3.1 containing 4% formaldehyde. Following washes in PBS and PBST (1% Triton-X-100), larvae were incubated overnight with primary antibody at 4°C, incubated with secondary antibodies for 4 hrs at room temperature the following day, and mounted in 70% glycerol in PBS for imaging. Antibodies were diluted as follows: mouse anti-DLG (1:500) (Developmental Studies Hybridoma Bank), TRITC-conjugated anti-HRP (1:500) (Jackson ImmunoResearch Laboratory), Alexa Fluor 488 goat anti-mouse (1:1000) (Invitrogen). Images were acquired with a 40X 1.3 NA oil-immersion objective (Carl Zeiss) and analyzed with Velocity Software.

Figures

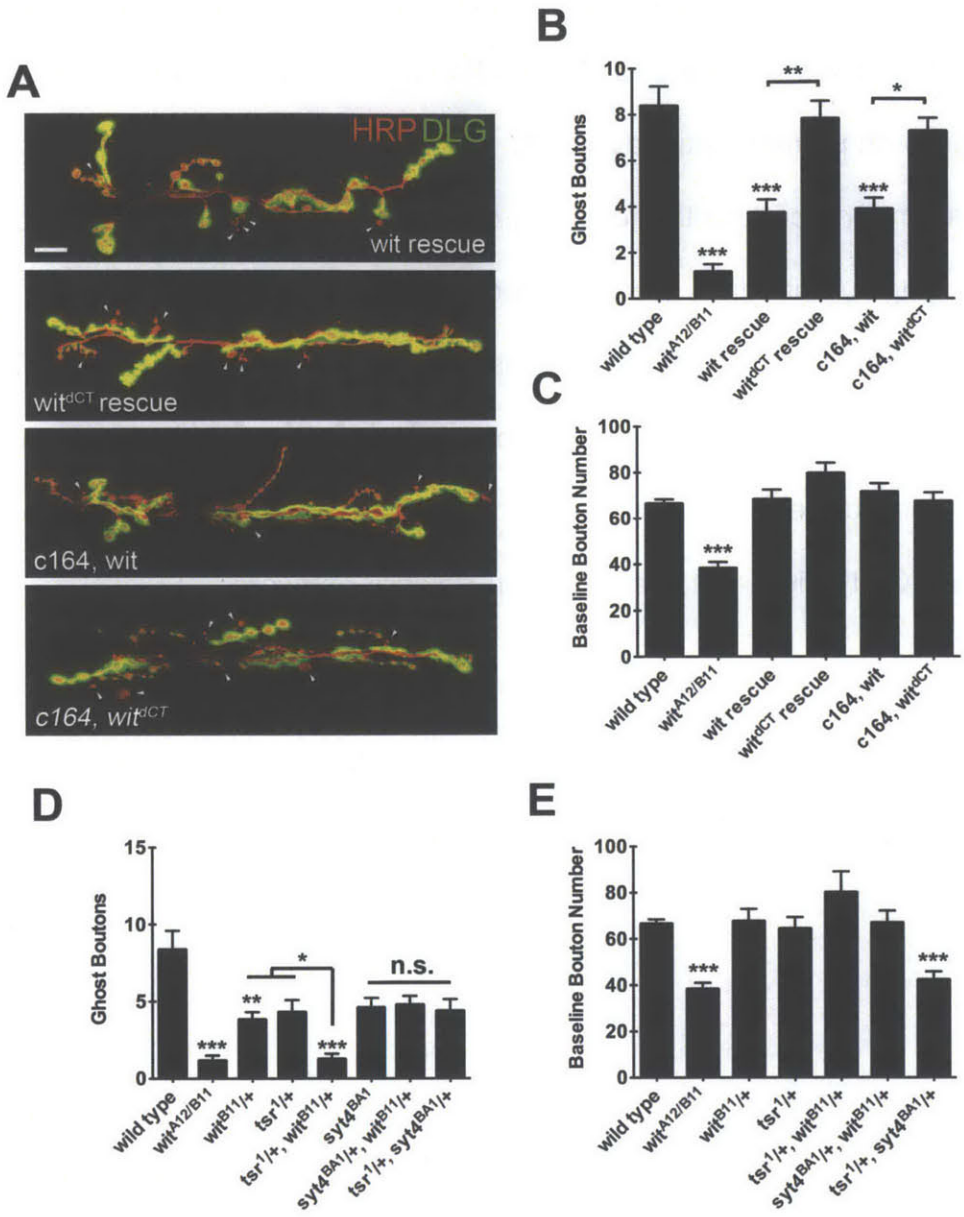
A
HRP DLG
wit rescue
wit^{dCT} rescue
c164, wit
c164, wit^{dCT}
B
Ghost Boutons
wild type
wit$^{A12/B11}$
wit rescue
wit^{dCT} rescue
c164, wit
c164, wit^{dCT}
C
Baseline Bouton Number
D
Ghost Boutons
n.s.
tsr^{1}/+
tsr^{1}/+, wit^{B11}/+
syt4^{BA1}
syt4^{BA1}/+, wit^{B11}/+
tsr^{1}/+, syt4^{BA1}/+
E
Baseline Bouton Number

Figure 1. Ghost bouton budding is modulated by Wit-Limk binding **A.** Wandering 3rd instar larvae were fixed in formaldehyde after high K^+ stimulation and stained with anti-HRP and anti-DLG to identify ghost boutons. Motor neuron rescue with full-length *UAS-wit* did not completely rescue ghost bouton budding frequency, while rescue with *UAS-wit^{dCT}* rescued ghost bouton budding to a significantly greater extent. Scale bar = 12 μm; arrowheads indicate ghost boutons. **B.** Quantification of ghost boutons per NMJ in the indicated genetic background. N (NMJs, animals): wild type = 57, 11; *wit$^{A12/B11}$* = 37, 6; wit rescue = 45, 7; wit^{dCT} rescue = 56, 7; *c164, wit* = 55, 8; *c164 wit^{dCT}* = 55, 8. **C.** Quantification of baseline bouton number. N: same as in (D). **D,E.** Wit and Tsr show genetic interactions for defective ghost bouton budding. Syt4 does not show a genetic interaction with Wit but does interact with Tsr not in terms of ghost bouton budding frequency but rather for overall synaptic growth. Quantification of ghost boutons per NMJ in the indicated genetic background is shown. N (NMJs, animals): wild type = 57, 11; *tsr^{1}*/+ = 24, 3; *wit^{B11}*/+ = 45, 6; *tsr^{1}*/+; *wit^{B11}*/+ = 37, 5; *syt4^{BA1}* = 65, 11; *syt4^{BA1}*/+; *wit^{B11}*/+ = 32, 4; *tsr^{1}*/+; *syt4^{BA1}*/+ = 25, 4; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ANOVA. Error bars indicate SEM.

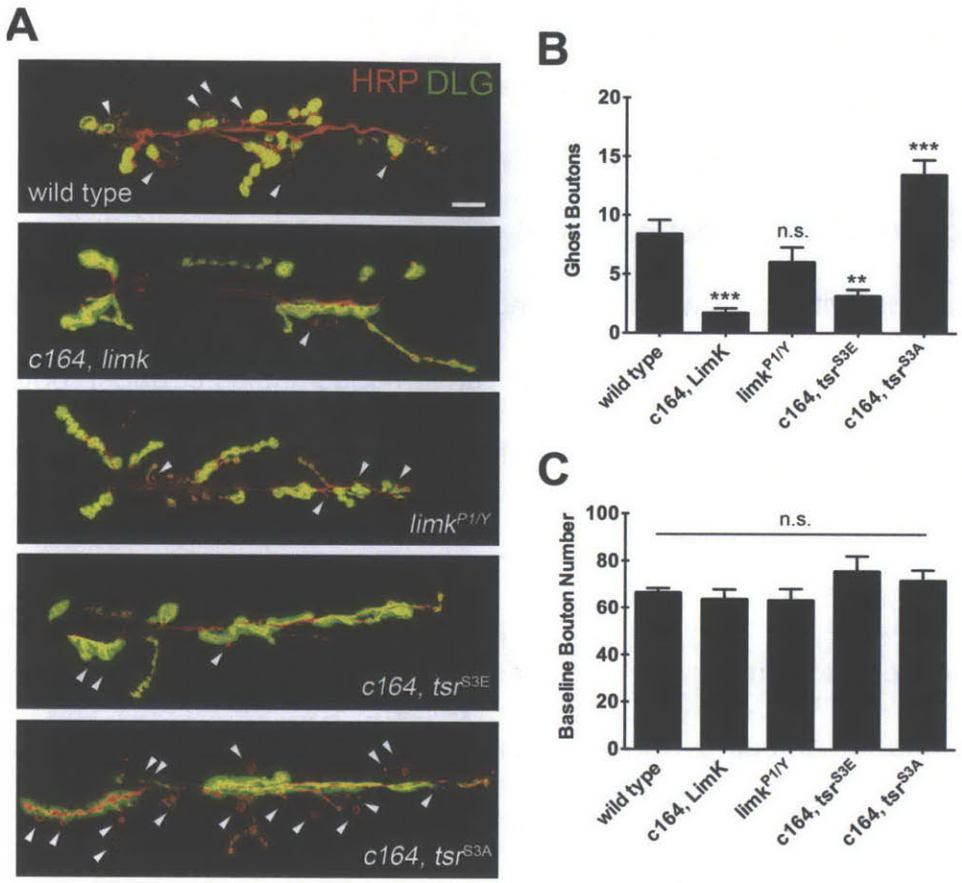
A
HRP DLG
wild type
c164, limk
limkP1/Y
c164, tsrS3E
c164, tsrS3A
B
Ghost Boutons
20
15
10
5
0
***
n.s.
**
***
wild type
c164, LimK
limkP1/Y
c164, tsrS3E
c164, tsrS3A
C
Baseline Bouton Number
100
80
60
40
20
0
n.s.
wild type
c164, LimK
limkP1/Y
c164, tsrS3E
c164, tsrS3A

Figure 2. Ghost bouton budding is regulated by Limk and Cofilin activity. **A.** Presynaptic overexpression of *limk* strongly reduces activity-dependent bouton budding. Presynaptic overexpression of constitutively inactive tsr^{S3E} reduces ghost bouton budding, while presynaptic overexpression of constitutively active tsr^{S3A} increases ghost bouton budding above wild type levels. Scale bar = 12 μm; arrowheads indicate ghost boutons. **B.** Quantification of ghost bouton budding frequency in the indicated genetic background. N (NMJs, animals): wild type = 57, 11; *c164, limk* = 40, 5; $limk^{P1/Y}$ = 36, 5; *c164,* tsr^{S3E} = 28, 4; *c164,* tsr^{S3A} = 36, 6. **C.** Quantification of baseline bouton number in the indicated genetic background.

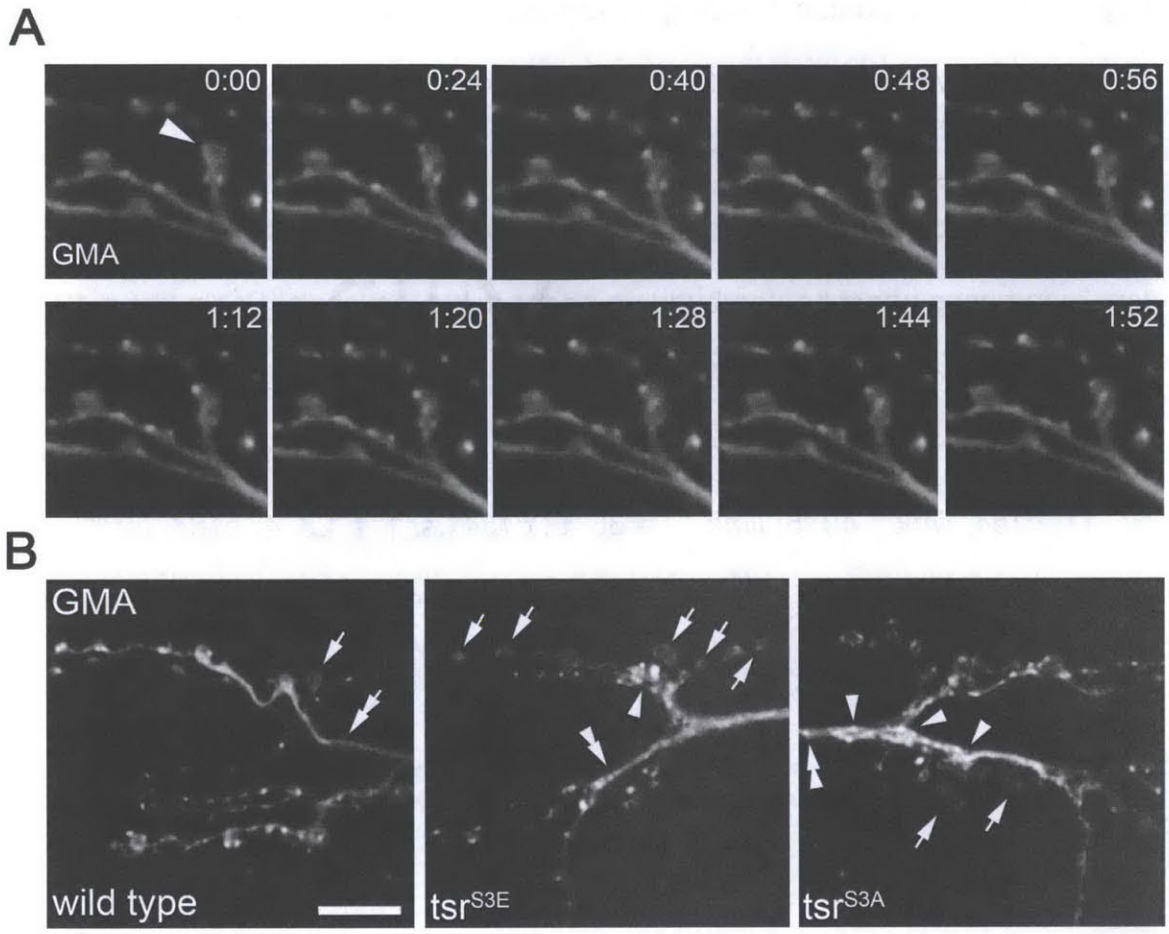
A
0:00
0:24
0:40
0:48
0:56
GMA
1:12
1:20
1:28
1:44
1:52
B
GMA
wild type
tsrS3E
tsrS3A

Figure 3. The presynaptic F-actin cytoskeleton as visualized by GMA is highly dynamic. **A.** The NMJ of a wild type animal presynaptically expressing GMA to label the F-actin cytoskeleton in motorneurons. Discrete F-actin puncta typically form and disappear inside boutons within minutes. GMA labels a more stable population of F-actin in interbouton regions. Arrowhead indicates an F-actin puncta that appears and disappears within the 2-minute imaging period. **B.** Live confocal imaging of GMA-GFP at NMJs driven by c164-Gal4. F-actin labeled by GMA appears as dynamic puncta with relatively even size and spacing in wild type animals. GMA labeling in axons and extended interbouton regions is stable and uniform at wild type NMJs (double arrow), but is interrupted by puncta and appears less uniform in tsr^{S3E} and tsr^{S3E} NMJs (double arrowheads). Boutons lacking discernable F-actin puncta occurred rarely at wild type NMJs and occur more frequently at tsr^{S3E} NMJs (arrows). Large and bright GMA labeling was observed in some boutons in tsr^{S3E} and tsr^{S3A} NMJs that was not observed in wild type (arrowheads). Scale bar = 12 μm. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ANOVA. Error bars indicate SEM.

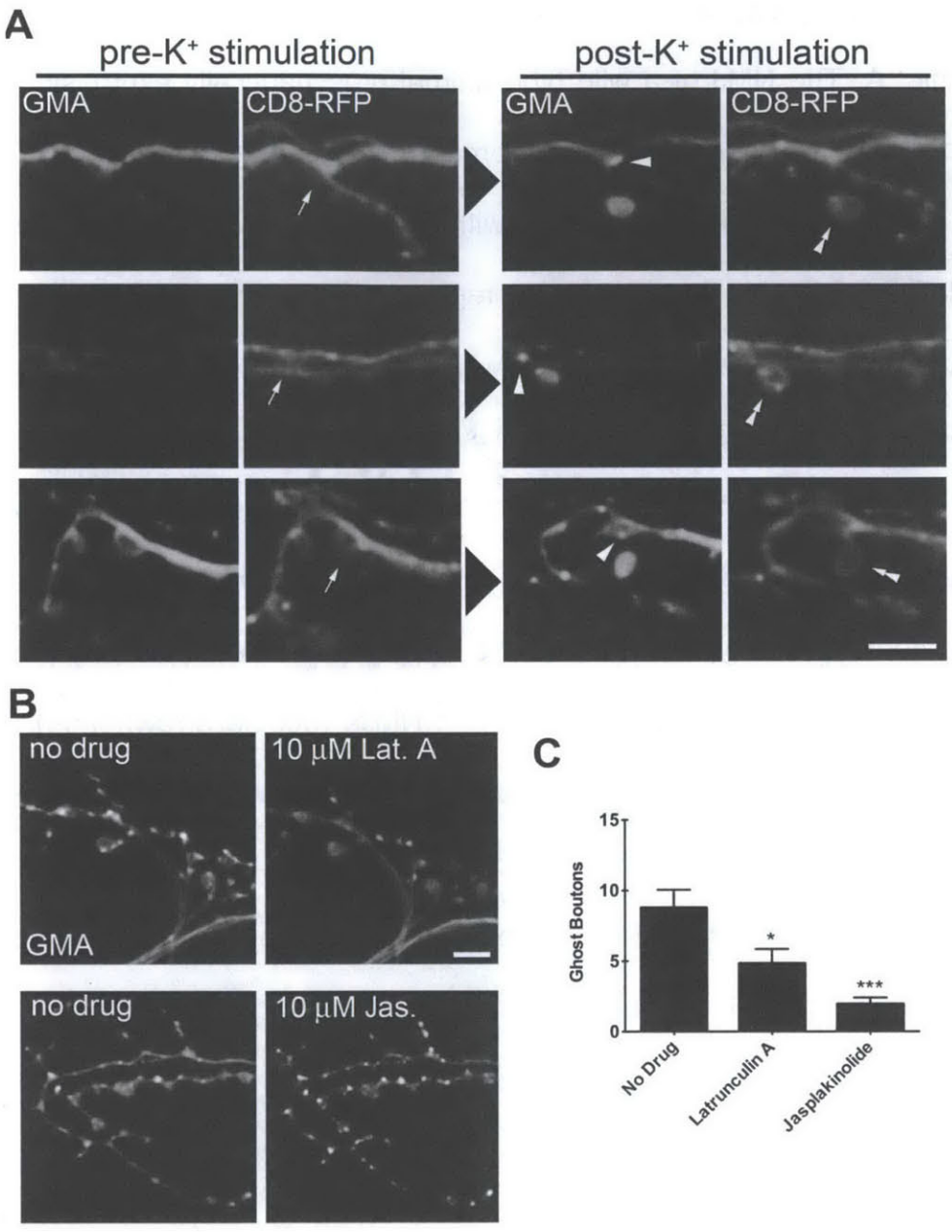
A
pre-K+ stimulation
post-K+ stimulation
GMA
CD8-RFP
GMA
CD8-RFP
B
no drug
10 μM Lat. A
GMA
no drug
10 μM Jas.
C
Ghost Boutons
15
10
5
0
*
***
No Drug
Latrunculin A
Jasplakinolide

Figure 4. Ghost bouton budding is accompanied by local rearrangements of the presynaptic actin cytoskeleton. **A.** Animals presynaptically expressing the membrane marker CD8-RFP and the F-actin marker GMA-GFP were imaged before and after high K^+ stimulation. New F-actin puncta (arrowheads) are observed at sites of budding (arrows) where newly formed ghost boutons (double arrowheads) attach to the main axonal arbor. Scale bar = 6 μm. **B.** Application of 10 mM latrunculin A to the bath solution rapidly disperses F-actin puncta, while application of 10 mM jasplakinolide causes formation and stabilization of F-actin puncta. Scale bar = 6 μm. **C.** Wild type animals pretreated with latrunculin A or jasplakinolide for 15 min before high K^+ stimulation display a reduction in ghost bouton budding frequency. N (NMJs, animals): no drug = 30, 4; Latrunculin A = 35, 5; Jasplakinolide = 30, 4. *: $p < 0.05$; ***: $p < 0.001$; ANOVA. Error bars indicate SEM.

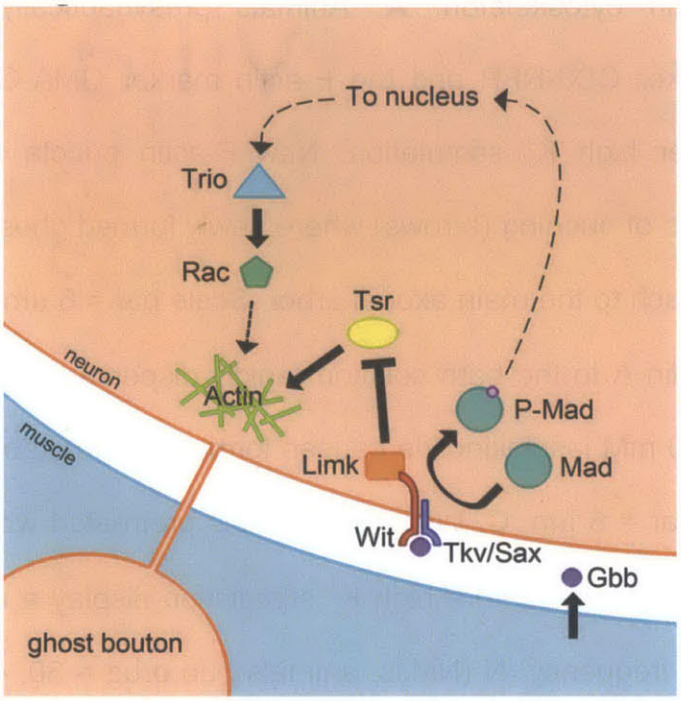
To nucleus
Trio
Rac
Tsr
neuron
Actin
P-Mad
muscle
Limk
Mad
Wit
Tkv/Sax
Gbb
ghost bouton

Figure 5. Model for ghost bouton formation through parallel signaling pathways involving Gbb and Wit. BMP signaling through Wit is predicted to both promote and inhibit changes to the actin cytoskeleton that regulate ghost bouton formation. Gbb signaling developmentally through the phosphorylation and nuclear translocation of Mad potentiates synaptic terminals for activity-induced bouton budding by promoting transcription of the Rho GEF *trio*. Trio activity may also be regulated locally and acutely by synaptic activity. Wit also signals locally through Limk to inhibit Cofilin (Tsr), thereby suppressing ghost bouton formation.

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Chapter 4

Conclusions and Future Directions

Summary

Our experimental analysis of ghost bouton budding at the *Drosophila* NMJ indicates that rapid activity-dependent synaptic growth requires retrograde BMP signaling at this synapse. The current data support a model in which BMP signaling through the type II receptor Wit is required developmentally to potentiate synapses for budding in response to elevated synaptic activity. This pathway requires Smad-dependent expression of the Rho GEF *trio*, and parallels a requirement for BMP signaling and Trio in developmental synaptic growth that occurs during the larval stages. In a parallel pathway, Wit interaction with Limk inhibits bouton budding through regulation of Cofilin activity. Both pathways regulate the synaptic actin cytoskeleton and may converge on similar actin regulatory molecules such as Limk and Cofilin via Rac1 or RhoA. Manipulating Cofilin activity levels by overexpression of Limk or expression of constitutively active/inactive Cofilin demonstrates that high Cofilin activity favors bouton budding, while low Cofilin activity inhibits budding. Local changes in the actin cytoskeleton that accompany activity-dependent bouton budding were also observed at sites of new synaptic growth. In addition, pharmacological disruption of normal actin turnover inhibits budding, suggesting increased actin turnover mediated by Cofilin potentiates rapid activity-dependent synaptic plasticity.

Future Directions

Instructive retrograde cue for ghost bouton budding

This work identifies the underlying molecular mechanisms that potentiate synapses for rapid activity-dependent growth as well as factors that are able to bidirectionally influence the frequency of these rapid alterations in synaptic structure. Based on the requirement for synaptic transmission through postsynaptic glutamate receptors, we propose that budding is triggered by a retrograde message that is activated based on the activity level of the postsynaptic cell. We initially hypothesized that the BMP ligand Gbb could act as a retrograde instructive cue for budding. However, we found that retrograde BMP signaling was required throughout development and halting canonical BMP signaling by cutting axons immediately before stimulation did not reduce budding frequency. When we knocked down Gbb in only one of two muscle targets of muscle 6/7 motorneurons, we observed a developmental effect, in that budding was reduced globally. We also observed a local effect: the ratio of ghost bouton budding that favored muscle 6 over muscle 7 was abolished. This result indicates that the muscle source of Gbb has some role in determining bouton budding locations. Information about the source of Gbb could be relayed to the presynaptic neuron through local synaptic phosphorylation of Mad. Synaptic P-Mad levels are sensitive to activity through postsynaptically opposed glutamate receptor clusters, although the time course of this sensitivity is not yet clear (Sulkowski et al., 2014). When we assayed synaptic P-Mad levels before and after the high K^+ stimulation protocol, we did not observe changes in

fluorescence intensity (data not shown), indicating that levels of P-Mad do not robustly change in response to elevated synaptic activity in a 30-minute window. We therefore predict that any effects of local synaptic P-Mad on ghost bouton budding are integrated over longer time periods and that Gbb is unlikely to act as an instructive cue for bouton budding.

We found that Syt4 is partially required for ghost bouton budding. Syt4 has previously been characterized as acting in a retrograde signaling pathway to regulate acute activity-dependent changes in synaptic signaling and developmental synaptic growth (Yoshihara et al., 2005, Barber et al., 2009). We did not observe a genetic interaction between *syt4* and *wit*, suggesting that Sy4 acts in an independent retrograde signaling pathway. A major question left unanswered by this work is, "what is the identity of the retrograde instructive cue for ghost bouton budding?" Given that Gbb is unlikely to be the instructive cue and that Syt4 is likely to be involved in an independent pathway that regulates ghost bouton budding, further experimentation involving *syt4* is a promising avenue for answering this question. Purification of Syt4-containing vesicles using biochemical approaches would be an attractive mechanism to identify candidate retrograde factors that directly instruct bouton budding. Current work in the lab is taking advantage of genetic approaches to identify regulators of fusion of Syt4 vesicles using a tagged Syt4-pHlourin fusion protein that can follow postsynaptic exocytosis. This work has identified the t-SNARE Syntaxin 4 as the key postsynaptic SNARE for Syt4-dependent fusion. Syntaxin 4 represents another genetic and biochemical entry point into identifying the instructive cue that is

released in an activity-dependent fashion from the postsynaptic compartment. Although we originally assumed this would be a diffusible molecule like BMP ligand, one cannot rule out the possibility that exocytosis of a transmembrane factor could mediate the transsynaptic signaling required for ghost bouton budding.

Our data is also consistent with a model in which Trio activity is regulated by an extracellular cue to control ghost bouton budding. Budding is strongly reduced in *trio* mutants. In contrast, overexpression of Trio results in more ghost boutons after stimulation. We did not observe an increase in the number of ghost boutons at *c164, UAS-trio* NMJs in the absence of stimulation (data not shown), indicating that high levels of trio protein are not sufficient to increase ghost bouton budding, but rather that elevated neuronal activity is also critical. *Drosophila trio* genetically interacts with the receptor phosphatase *dlar*, which has been shown to regulate synaptic growth (Kaufmann et al., 2002; Johnson et al., 2006; Pawson et al., 2008). Interestingly, dLar is predicted to act in a pathway either downstream or in parallel to canonical BMP signaling, as overexpression of dLar rescues canonical BMP pathway inhibition (Berke et al., 2013). As such, signaling through presynaptic dLar may be involved in ghost bouton budding and therefore indicate the nature of the retrograde instructive cue.

Actin regulatory proteins including Rho GEFs and GAPs that are regulated by extracellular cues are also candidates for instructive cues for budding (Luo, 2000; Tolias et al., 2011). We have demonstrated that regulated actin turnover

controls rapid activity-dependent plasticity and we propose that ghost bouton budding is in part triggered by changes to the F-actin cytoskeleton. While we have implicated Limk and Cofilin, it is plausible that the necessary modification to the actin cytoskeleton could be mediated through other actin effector proteins. Additionally, signaling through Limk occurs via multiple upstream pathways that integrate the activity of many Rho GEFs and GAPs, including Trio (Ng and Luo, 2004). Limk and Cofilin activity could therefore be regulated by any of their upstream signaling components to control bouton budding. One such candidate pathway occurs through the Eph receptor and the downstream Rho GEF Ephexin and Rho GTPase Rac1 (Frank et al., 2009). Eph-Ephexin signaling is required for synaptic homeostasis at *Drosophila* NMJs, a process that also requires developmental BMP signaling (Goold and Davis, 2007). Homeostatic plasticity is acutely sensitive to neuronal activity through postsynaptic glutamate receptors on the minute time scale, indicating that Eph-Ephexin signaling can be regulated within the same duration (Frank et al., 2009). Two ligands for Eph receptors, Ephrin and Vap33A, are expressed in muscle and could therefore signal in a retrograde manner (Pennetta et al., 2002; Tsuda et al., 2008).

Contribution of ghost bouton budding to synaptic growth

While this work indicates that ghost bouton budding is an activity-dependent synaptic growth mechanism utilized at *Drosophila* NMJs, the contribution developmental to synaptic growth of this process remains unclear. Ghost boutons are defined by the mechanisms of budding and their

morphological characteristics, specifically lack of postsynaptic markers. Work by the Budnik and Freeman groups has demonstrated that ghost boutons will either mature into functional boutons or be cleared by muscle and glial engulfment (Ataman et al., 2008; Fuentes-Medel et al., 2009). Ghost bouton maturation consists of accumulation of pre- and postsynaptic markers and subtle changes in shape such that ghost boutons become indistinguishable from all other boutons (Ataman et al., 2008). Therefore, a snapshot of the NMJ at the third instar larval stage provides no information about the number of boutons that were initially ghost boutons. Clearing of ghost boutons can occur within hours while maturation is complete within 18 hours (data for shorter time intervals is not available). Ghost boutons that contribute to synaptic growth may only be detectable within the first few hours after they are generated. These studies do not indicate the fraction of ghost boutons that will undergo maturation as opposed to degradation. Without this data, we cannot use potential for ghost bouton budding to estimate the total number of ghost boutons that contribute to synaptic growth. Additionally, we have not yet determined the total number of ghost boutons that are generated throughout larval life.

Ghost bouton budding events occurring the absence of stimulation were very rare, but we can determine the approximate total number of ghost boutons generated at muscle 6/7 NMJs by observing the frequency of not yet matured or degraded ghost boutons through different stages of larval life. We restricted our analysis to late third instar larvae (L3), however larval NMJs experience the greatest periods of growth during the L2 and early L3 stages (Keshishian et al.,

1993; Broadie and Bate, 1993; Zito et al., 1999). Quantifying ghost boutons in fixed animals at these stages may indicate that ghost bouton budding is more or less utilized during phases of rapid synaptic growth and provide a clearer picture of the total number of ghost boutons generated during larval life.

Determining how ghost bouton budding contributes to overall synaptic growth in a quantitative manner requires in part knowing the frequency of ghost boutons that will mature and appear indistinguishable from normal boutons. An accurate measurement of this parameter requires identifying ghost boutons within a few hours from their generation while they are morphologically distinct from other boutons. Our approach of using high K^+ stimulation robustly generated ghost boutons, which was a critical feature in allowing us to quantitatively assay the frequency of budding in differing genetic backgrounds. The major drawback to this approach was the requirement to dissect larvae before stimulation, which limits the lifespan of the animal to approximately one hour in ideal conditions. During this hour-long window, we did not observe changes in ghost bouton morphology. While this procedure provides an ample number of ghost boutons to analyze, it is not suitable for following ghost bouton fate due to the restricted window of observation in living animals. An alternative possibility is to analyze naturally occurring ghost boutons that can be identified through the larval cuticle in intact animals. A significant challenge in quantifying ghost bouton fate in intact animals is the low incidence of ghost boutons at unstimulated L3 NMJs, ~1% of boutons. Identifying a suitable number of ghost boutons in anesthetized animals for repeated imaging sessions would be prohibitively laborious. Existing

observations concerning ghost bouton fate were obtained by stimulating intact, anesthetized larval via genetically expressed Channelrhodopsin, which generates ghost boutons at a lower frequency than high K^+ stimulation (Ataman et al., 2008). Although this method still relies on exogenously generated neuronal activity, it is possible to utilize stimulation paradigms that closely resemble endogenous activity patterns. In this way, ghost boutons can be generated at a known time point and in a location amendable to imaging through the larval cuticle. This technique has proven successful for qualitatively determining ghost bouton fate and could be performed at a scale that would render quantitatively accurate results.

We can also attempt to quantify ghost bouton budding contribution to synaptic growth through genetic means. Overexpression of Tsr^{S3E} or Tsr^{S3A} caused changes in high K^+-stimulated ghost bouton budding frequency, however, *c164, tsr*^{S3E} and *c164, tsr*^{S3A} animals showed no changes in ghost bouton number in the absence of external stimulation compared to controls. Overexpression of these transgenes also did not change overall synaptic growth. This observation suggests that normal laboratory conditions for animal rearing do not manifest genetic differences in activity-dependent ghost bouton budding that significantly affect total synaptic growth. Wild type animals have the capacity for increased activity-dependent synaptic growth when raised at elevated temperatures (Sigrist et al., 2003). Increased synaptic activity mediated by increased ambient temperature may increase ghost bouton budding frequency such that it significantly contributes to synaptic growth. If this is the case, we

would predict that *c164, tsr*^{S3E} animals would show deficits in synaptic growth at high temperatures compared to controls. In contrast, we would expect *c164, tsr*^{S3A} animals to show increased synaptic growth. Differences in synaptic growth between these groups could then be attributed in part to ghost bouton budding and provide quantitative data for the contribution of ghost bouton budding to overall synaptic growth in the context of elevated neuronal activity.

Mechanisms of rapid synaptic growth

The molecular processes that work to rapidly construct new synaptic structures are of general interest to the study of synaptic plasticity as these mechanisms could be conserved across many species and different synaptic structures within organisms. Work in this area has demonstrated a key role for regulated actin turnover in rapid bouton formation. We have identified general roles for the Rho GEF Trio and effectors Limk and Cofilin. Further molecular characterization of the presynaptic actin regulatory pathway at play in ghost bouton budding is complicated by the fact that many of the genes implicated in this process (Rac1, Pak, RhoA, and Rock) are essential for life, with mutations in these genes resulting in embryonic lethality. Furthermore, the effects of removing any of these gene products on the actin cytoskeleton is not trivial to determine, nor is the role of presynaptic actin (Cingolani and Goda, 2008; Michelot and Drubin, 2011). There are, however, other elements to rapid bouton addition that we have not yet explored. In particular, the source of membrane that constitutes ghost boutons is not yet known.

Membrane addition is required for growth of dendritic spines in mammalian neurons (Park et al., 2006; Holtmaat and Svoboda, 2009). The exocyst is a multiprotein complex that regulates membrane delivery to cell surfaces (He and Guo, 2009). The exocyst has been shown to contribute to activity-dependent plasticity at *Drosophila* NMJs by contributing to growth of the postsynaptic SSR (Teodoro et al., 2013). Studies in yeast have demonstrated that the exocyst complex is regulated by Rho-family GTPases, suggesting the possibility that membrane deposition may be regulated by proteins that participate in ghost bouton budding (Wu et al., 2008). In the future it will be straightforward to test for a requirement for exocyst component genes in high K^+-induced ghost bouton budding.

Ghost bouton budding morphologically resembles membrane blebbing that occurs during apoptosis and some forms of cellular motility (Elmore, 2007; Charras and Paluch, 2008). A cleaved peptide of Limk1 has been implicated in apoptotic membrane blebbing (Tomiyoshi et al., 2004). Although cleaved Limk1 promotes rather than inhibits apoptotic blebbing, this observation nevertheless illustrates that actin regulation contributes to membrane blebbing. Additional evidence for actin regulation of membrane blebbing comes from studies of chemotaxis in *Dictyostelium*. The parallels between bleb-mediated motility in *Dictyostelium* and ghost bouton budding at *Drosophila* NMJs are striking. Zatulovskiy et al. (2014) found that Dictyostelium utilize directed blebbing as a form of motility in response to increased mechanical resistance. Blebs leave behind short-lived F-actin scars at sites where membrane appears to detach from

the cortical cytoskeleton. Zatulovskiy and colleagues also observed that mutations in cytoskeleton regulatory proteins including *arp2/3* and *profilin* alter blebbing. This led the authors to hypothesize that hyper-stabilization of F-actin inhibits blebbing. They also found that blebbing required PI3-kinase (Zatulovskiy et al., 2014).

Overexpression of PI3K in motorneurons causes synaptic overgrowth at *Drosophila* NMJs in an Akt-dependent manner. Furthermore, PI3K activity was able to induce formation of new functional synapses in the central brains of adult flies (Martín-Peña et al., 2006). Akt has been implicated in numerous cellular functions including cell growth and membrane addition (Manning and Cantley, 2007). Postsynaptic Akt1 at NMJs has been shown to regulate glutamate receptor abundance and also growth of the SSR (Lee et al., 2013). It will be interesting to determine if presynaptic Akt1 and PI3K are involved in ghost bouton budding.

Conclusion

We find that ghost bouton budding requires developmental BMP signaling that potentiates rapid activity-dependent growth through the Rho GEF Trio. Our observations are consistent with a requirement for a retrograde instructive cue that triggers bouton budding in response to elevated activity. A number of lines of evidence indicate that the BMP ligand Gbb is unlikely to be an instructive cue for budding. We observed a requirement for Syt4 in a signaling pathway that is likely to be independent of BMP signaling. Retrograde signaling pathways mediated by

Syt4 are therefore good candidates for instructive cues for budding. Ongoing work in our lab and others seeks to determine the identity of Syt4-mediated retrograde signaling pathways. Presynaptic regulation of the actin cytoskeleton via Rho GAPs and GEFs is a potential instructive signaling pathway for budding and genetic experiments with actin regulatory network proteins is another viable route for determining the retrograde instructive cue for budding.

We have characterized a form of rapid activity-dependent synaptic growth at *Drosophila* NMJs. This form of plasticity can dramatically alter the morphology of synaptic terminals within short time periods. How rapid bouton budding contributes to overall synaptic growth, which is a proxy measurement for synaptic plasticity, is still unclear. Careful observations of ghost bouton numbers at different stages of larval life will lend insight to the total number of ghost boutons generated throughout development. This figure combined with the knowledge of the fraction of ghost boutons that mature will allow us to approximate the total contribution of ghost bouton budding to synaptic growth. We can determine the fraction of ghost boutons that mature in a quantitative manner by generating ghost boutons via genetically expressed Channelrhodopsin in intact animals. Raising larvae at elevated temperatures is another potential method for assaying ghost bouton contribution to synaptic growth using genetic tools.

We have shown that regulation of the presynaptic actin cytoskeleton affects ghost bouton budding frequency. We do not yet have a detailed understanding of how actin regulation leads to rapid formation of morphologically distinct boutons but comparison of ghost bouton budding to morphologically

similar structural rearrangements in other cell types may identify additional molecular mechanisms. Ghost bouton budding presumably requires membrane addition to the motorneuron plasma membrane, as parent bouton size is not dramatically reduced. The exocyst complex is responsible for postsynaptic activity-dependent membrane addition and it may play a similar role presynaptically. Ghost bouton budding strikingly resembles chemotactic membrane blebbing in *Dictyostelium*. Molecular characterization of chemotaxis by blebbing revealed a requirement for PI3K, which also functions to control synaptic growth at *Drosophila* NMJs. Experiments involving PI3K manipulation may shed additional light on the molecular process of rapid bouton budding. Investigation along these lines will expand the understanding of the molecular processes of rapid activity-dependent synaptic growth and how it contributes to synaptic plasticity.

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