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AXONAL TRAFFICKING OF BMP SIGNALS IN *DROSOPHILA* MOTONEURONS

by

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A DISSERTATION

Submitted to the graduate faculty of the University of Alabama at Birmingham
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2009

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2009

AXONAL TRAFFICKING OF BMP SIGNALS IN *DROSOPHILA* MOTONEURONS

REBECCA BURT SMITH

GENETICS GRADUATE PROGRAM

ABSTRACT

The *Drosophila* Bone Morphogenetic Protein (BMP) Glass bottom boat (Gbb) is a ligand for the BMP type II receptor, Wishful thinking (Wit). Mutations in either of these proteins impair synaptic growth at the neuromuscular junction (NMJ). Gbb is a retrograde signal (from target to innervating neuron) that is essential for proper NMJ development and function. Tissue-specific rescue of pathway mutants and immunolocalization experiments indicate that the receptor-ligand interaction occurs at the NMJ, and results in the nuclear accumulation of the phosphorylated form of the transcription factor Mad, indicating that retrograde transport along the axon is critical for pathway activation. We have investigated the mechanism of retrograde axonal transport of the BMP using a signaling endosome model of signal propagation. In this model, the receptor complex undergoes internalization at the synaptic terminal and is transported along axon microtubules to the cell body where it then activates down-stream molecules. Tools that we have utilized to characterize our pathway include fluorescent fusion proteins of our BMP receptors and Mad transcription factor as well as high resolution live imaging techniques. From our studies, we find that the receptors Wit and Tkv are being transported in a bi-directional manner along the motoneuron axon. Mad along the axon, however, appears diffuse and does not travel with the receptors. We see p-Mad in the nucleus and at the synaptic terminal, however we do not see p-Mad along the axon. A known inhibitor of the pathway, DN-Glued, dramatically decreases the amount of

moving receptor vesicles, linking receptor transport to pathway activity. Additionally, we have found that Tkv-YFP and Wit-CFP colocalize along the axon in predominately retrograde moving particles and lose this directionality in *gbb* ligand mutants, consistent with our hypothesis of a signaling endosome. In further support of the signaling endosome model, manipulation of the endocytic pathway results in alterations in p-Mad levels and alterations in receptor vesicle transport. Our data suggests that, to propagate the BMP pathway along the motoneuron axon, the receptors are traveling in a signaling endosome that phosphorylates Mad in separate events at the synaptic terminal and proximal cell body.

DEDICATION

I dedicate this dissertation to my family.

To my beautiful three-month-old son, Austin, who is the best thing that has ever happened to me. I hope to spend much more time with you after I complete graduate school. You are a blessing and a miracle and I feel so lucky to have you.

To my husband of seven years, Lee, who has stood by me through all of graduate school and amazingly is still with me. I feel eternally lucky to have found such a great husband and look forward to spending the rest of my life with you.

To my parents and sister, who have always believed in me and made me believe that I could do anything that I set my mind to. Thank you for all of your endless support and for always going the extra mile to help me meet my goals.

Again to my parents and my parents in law, who baby sat for me during the completion of this dissertation and without whose help, I could not have finished. Thank you for your huge support and sacrifice while I finished my dissertation.

ACKNOWLEDGEMENTS

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Next I would like to acknowledge my co-workers, Jim Machamer and Nam Chul Kim as well as past lab member Rina Kara. In my thesis, I have included experiments that Jim (Chapter 2, Figure 1c) and Nam Chul (Chapter 1, Supplementary figure 2) have completed. Jim and Nam Chul have consistently helped me when I had a problem with an experiment, or needed to help to understand a concept. Rina helped with much of my training when I first started the lab. You have been great to work with and I thank you for your friendship.

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LIST OF ABBREVIATIONS

ATF	activating transcription factor
BDNF	brain-derived neurotrophic factor
BMP	Bone Morphogenic Protein
CREB	cAMP response element binding
Co-Smad	Common Partner Smad
Dlg	Discs large
DN-Glued	Dominant Negative p150/Glued
Dpp	Decapentaplegic
Erk	extracellular signal-regulated kinases
Gbb	Glass bottom boat
JNK	c-Jun N-terminal kinase
LTF	Long-term facilitation
LTP	Long-term Potentiation
Mad	Mothers against decapentaplegic
MAP	Mitogen-activated protein
MIS	Mullerian Inhibiting Substance
NGF	nerve growth factor
NMJ	neuromuscular junction

Nwk	Nervous wreck
p-Mad	phosphorylated Mad
PKA	protein kinase A
R-Smad	Receptor-phosphorylated Smad
SARA	Smad anchor for receptor activation
Sax	Saxophone
SE	signaling endosome
SMA	Spinal Muscular Atrophy
Spict	Spichthyin
Syt	Synaptotagmin
TGF-β	Transforming Growth Factor-β
Tkv	Thick veins
Trk	tropomyosin-receptor-kinase
Wit	Wishful thinking

CHAPTER 1

INTRODUCTION: RETROGRADE SIGNALING IN NERVOUS SYSTEM DEVELOPMENT AND FUNCTION

Disorders of neural function such as intellectual disability and Alzheimer 's disease affect approximately 8.8 million US citizens (Larson et al., 2000). Understanding the basic biology of development and maintenance of learning and memory functions is of major importance in determining the basis of these diseases and will allow development of methods to detect, prevent and treat neuronal defects. Retrograde axonal transport is critical for neuronal viability and differentiation, demonstrating the importance of target-derived signals (Goldstein, 2003). Defects in axonal transport contribute to the pathology of some inherited disorders such as Charcot-Marie Tooth disease (type 2A) and some neurodegenerative diseases, such as Alzheimer's and Huntington's (Hirokawa and Takemura, 2004; Holzbaur, 2004). Retrograde axonal transport is also crucial for target-derived pathways that initiate a nuclear response such as regulating synaptic plasticity by transcription of new genes (Fitzsimonds and Poo, 1998). Determining the mechanism of target-derived signaling is critical to understanding how it regulates synaptic plasticity and contributes towards the development and survival of the neuron. This dissertation provides new information about the role of axonal transport in retrograde signaling, which is crucial for synaptic

plasticity, learning and memory, as well as nervous system development and overall health.

Learning, Memory, and Synaptic Plasticity

The human brain is composed of trillions of neurons interconnected via synapses. Despite this complexity, however, the cellular mechanisms of learning and memory appear to be conserved in basic form in all species from invertebrates to mammals (Koh et al., 2000). Synaptic plasticity, the process by which connections between a neuron and its target are modified, is required for nervous system development and is believed to be the basic mechanism underlying memory and learning (Waites et al., 2005). Transcription and translation have each shown to be important contributors to structural and functional changes during synaptic plasticity (Alberini, 2009; Costa-Mattioli et al., 2009). Of significant importance are signaling pathways that originate at the synapse and regulate transcription, and as a result require retrograde transport for delivery of the signal to the neuron cell body and nucleus (Fitzsimonds and Poo, 1998). Defining the molecular mechanisms of retrograde signaling is therefore necessary for understanding the neurobiology of learning and memory.

Retrograde signaling

Retrograde signaling often requires axonal transport to provide proper communication between the synaptic terminal and cell body of the motoneuron. This is critical when target-derived factors initiate a nuclear response such as gene transcription (Fitzsimonds and Poo, 1998). Because of the long distance between the distal and

proximal ends of the neuron (Figure 1), target-derived pathways that drive gene transcription depend on active transport (Goldstein, 2003). Neurons are specifically

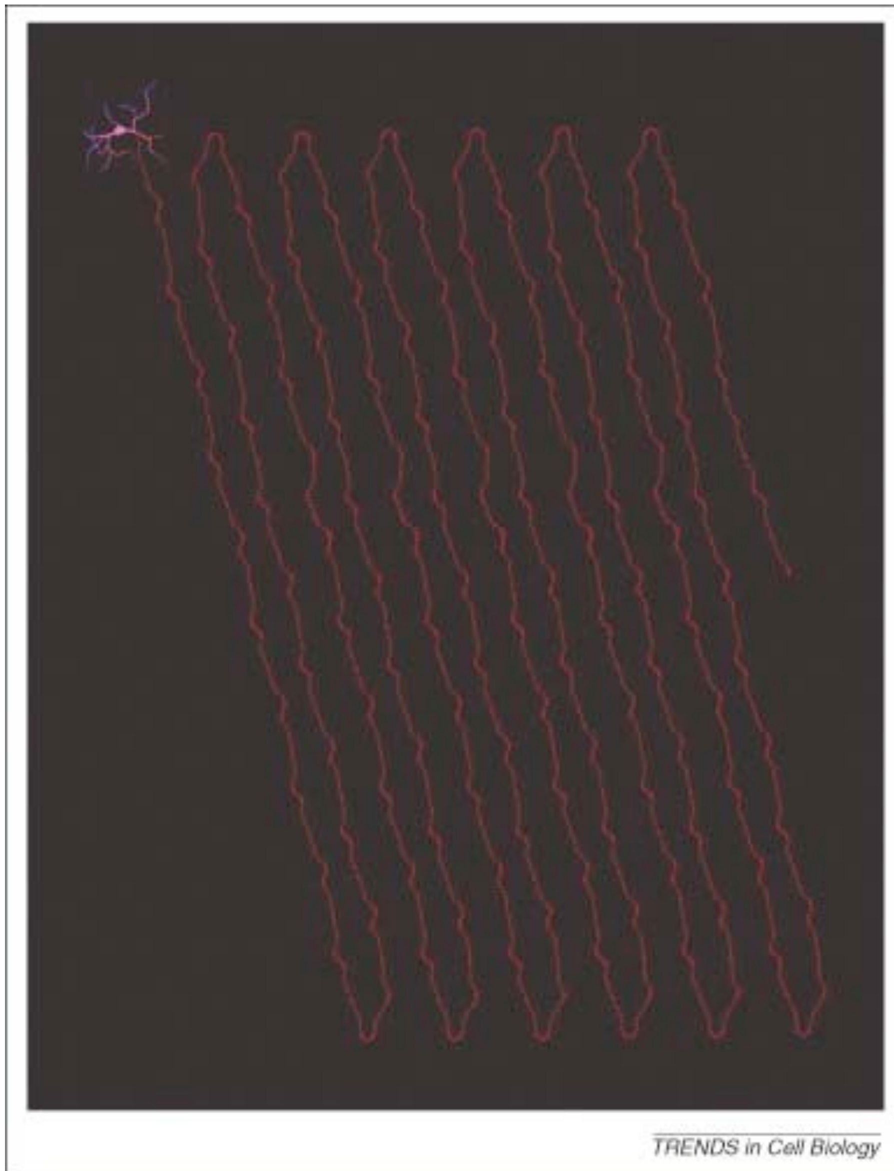

Figure 1: The daunting task of retrograde axonal signaling.

Photomontage of a rat hippocampal neuron with dendritic tree (purple) and an axon 1 cm in length (red) shown at scale. Human lumbar motoneurons can have axons exceeding 1 m in length.

Note: From "Message in a bottle: long-range retrograde signaling in the nervous system" by Carlos F. Ibáñez, 2007, *Trends in Cell Biology*, 17/11. p. 519-528. Copyright 2007 by Elsevier Ltd. Reprinted with permission.

susceptible to errors in active transport and defective axonal transport is a hallmark phenotype of some neurodegenerative diseases (Goldstein, 2003). Alzheimer's disease, Huntington's disease, and polyglutamine diseases all have axonal blockages that inhibit transport (Fitzsimonds and Poo, 1998; Holzbaur, 2004). Transport occurring from the synapse to cell body, usually mediated by the molecular motor dynein, is termed retrograde transport. Anterograde transport is transport occurring in the opposite direction from the cell body to the synapse, usually mediated by kinesin molecular motors (Figure 2). Errors in retrograde transport have been shown to cause neuron degeneration in diseases such as Motor Neuron disease, Amytrophic Lateral Sclerosis, and Spinal Muscle Atrophy (Hafezparast et al., 2003). In these diseases, critical retrograde factors that are essential for synapse development and maintenance as well as neuron survival cannot be transported effectively, leading to the death of the neuron (Goldstein, 2003). In conclusion, retrograde transport of critical signaling molecules is important for synaptic plasticity in development, and the maintenance of established synapses to prevent neuron death.

TGF-β Superfamily and Synaptic Plasticity

Growth factors of the Transforming Growth Factor-Beta (TGF-β) superfamily are necessary for synaptic plasticity and maintenance. In *Aplysia*, the ligand TGF-β1 has been found to promote long-term facilitation of the sensitization of defensive withdrawal reflexes (Zhang et al., 1997). Long-term facilitation (LTF) is one cellular mechanism that underlies non-associative learning and memory, and is similar to vertebrate Long-term Potentiation (LTP) (Packard et al., 2003). This long-term change suggests

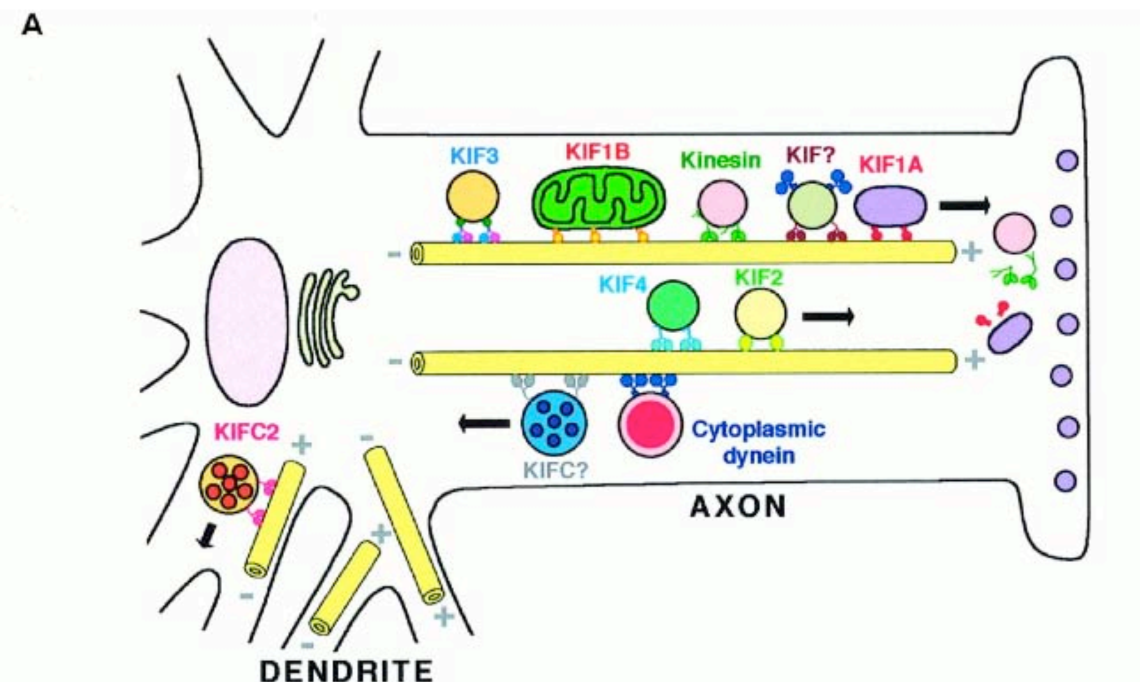

Figure 2: Motor proteins are involved in directional axonal transport of organelles and cargo

Motor proteins including cytoplasmic dynein and members from the kinesin family move cargo in motoneuron axons. Retrograde transport is from the synapse to cell body (plus to minus) and anterograde transport is from the cell body to the synapse (minus to plus).

Note: Adapted from "Kinesin and Dynein Superfamily Proteins and the Mechanism of Organelle Transport" by Nobutaka Hirokawa, 1998, *Science*, 279, p.519. Copyright 1998 by AAAS. Adapted with permission.

mediation through transcriptional and translational events (Zhang et al., 1997). TGF-β1 appears to be both necessary and sufficient for the long-term induction of synaptic plasticity in *Aplysia* (Chin et al., 1999).

In mammalian cells, another member of the TGF-β superfamily, Bone Morphogenic Protein (BMP) has been linked to synaptic plasticity. Studying mutants of the BMP antagonist Chordin, Sun and colleagues demonstrated that the BMP pathway contributes to synaptic plasticity in mice (Sun et al., 2007). Chordin mutants displayed

greater LTP in hippocampal neurons and performed better in behavioral memory tests (Sun et al., 2007). Accordingly, BMP-7 enhances dendritic outgrowth in the hippocampus. Treating neurons with BMP-7 for three days led to dendritic branching similar to that of 2 week old control neurons (Withers et al., 2000). Additionally, in this study BMP-7 accelerated the rate at which neurons became receptive to innervation, increasing the rate of synapse formation (Withers et al., 2000). Likewise, BMP-5 induced dendritic growth in sympathetic neurons, which was inhibited by antagonists of BMP function (Beck et al., 2001). BMPs contribute to mammalian synaptic plasticity and are potential research targets to find therapeutic agents for diseases of learning and memory.

Conservation of TGF-β Superfamily Signaling

As well as being crucial for proper synaptic development and neuron maintenance, members of the TGF-β superfamily regulate other developmental and physiological processes, including apoptosis, proliferation, differentiation, motility, and adhesion (Schiller et al., 2004). This superfamily is also widely conserved in the animal kingdom (Herpin et al., 2004), and these growth factors are so well conserved that there is cross reactivity between mammalian ligand and *Drosophila* receptors and vice versa. For example, the *Drosophila* ligand, Decapentaplegic (Dpp) induces bone formation in mammals (Sampath et al., 1993) and human BMP4 ligand sequences can substitute for the *dpp* gene rescuing *dpp* mutant phenotypes (Padgett et al., 1993). All TGF-β superfamily members signal through a family of single transmembrane protein serine/threonine kinase receptors (Herpin et al., 2004; Massague, 1998).

Based on structural and functional properties, the receptors are divided into two subfamilies: type I and type II (Massague, 1998). The TGF-β superfamily receptors have a high degree of sequence conservation in all species (Herpin et al., 2004) and the standard mechanism for pathway activation is similar for each member of the superfamily (Massague, 1998). In the standard mechanism for pathway activation (Figure 3), the ligand brings together the type II receptor and the type I receptor to form a complex. The type II receptor is a constitutively active kinase that phosphorylates the type I receptor, allowing the type I receptors in turn to activate Receptor-phosphorylated Smad (R-Smad) proteins. In the nuclei, phosphorylated R-Smad together with the Common Partner (Co-Smad) Smad4 modulates the expression of TGF-β responsive genes (Herpin et al., 2004). In Drosophila there exists seven TGF-β family ligands (Dpp, Gbb, Screw, dActivin, Myoglianin, Maverick, and dwadle), three type I receptors (Baboon, Sax, and Tkv) and two type II receptors (Punt and Wit) (Newfeld et al., 1999; Raftery and Sutherland, 1999).

The TGF-β ligand superfamily can be divided into TGF-β, BMPs and activins, and all of these subgroups share Smads as common downstream signaling proteins (Herpin et al., 2004). Mammals have several isoforms of R-Smads that are pathway-specific. Smads 1, 5, and 8 mediate BMP signaling while Smads 2 and 3 mediate TGF-β and activin signaling. The universal mammalian Co-Smad is Smad 4 (Chacko et al., 2004) . *Drosophila* Smad proteins include one BMP R-Smad (Mad) and one TGF-β/activin R-Smad (dSmad2). As in mammals, there exists only one *Drosophila* Common Smad, Medea (Herpin et al., 2004). Mad and Medea are each more similar in sequence and

function to the cognate vertebrate Smad than they are to each other (Raftery and Sutherland, 1999).

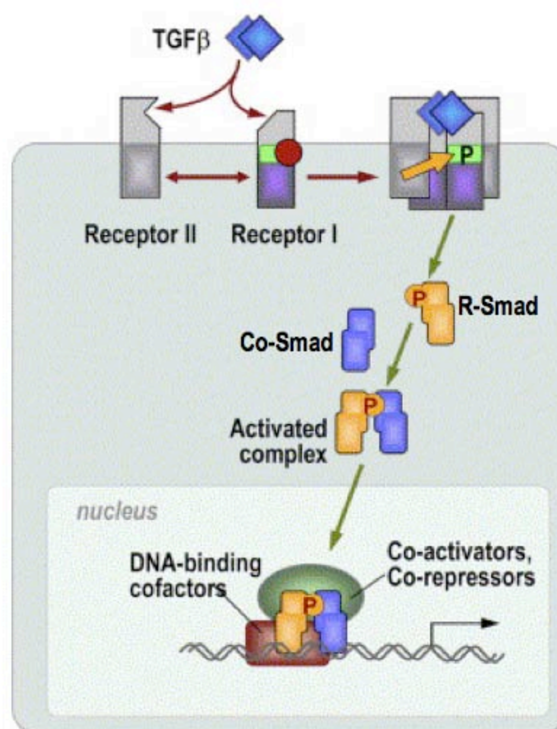

Figure 3: The standard activation pathway for TGF-β family members

TGF-β signaling via Smads: Converging in and branching out of a simple signaling engine. (Top) The basic signaling engine: The ligand assembles a receptor complex that phosphorylates Smads, and the Smads assemble a transcriptional complex that regulates target genes. The type II receptors are activators of the type I receptor. R-Smads are direct substrates of type I receptors. The assembly of receptor-phosphorylated Smads with co-Smads is essential for many transcriptional responses. Smads gain access to target genes by synergistically binding to DNA with cell-specific cofactors, many of which remain unknown. The Smad complex can recruit coactivators or corepressors that determine the outcome.

Note: Adapted from “The logic of TGFβ signaling” by Joan Massagué and Roger R. Gomis, 2006, *FEBS Letters*, p. 2811-2820. Copyright 2006 by Federation of European Biochemical Societies. Adapted with permission.

Drosophila Model for Studying the Synapse

The *Drosophila* larva neuromuscular junction (NMJ) is an excellent model for the study of synaptic plasticity (Koh et al., 2000). *Drosophila* is a powerful genetic model that can be easily manipulated to study the function of genes or proteins and transgenes *in-vivo*. Its ease of anatomical and electrophysiological accessibility and well-defined synaptic pattern also make *Drosophila* a useful biological tool (Koh et al., 2000). In addition, many key synaptic molecules found in *Drosophila* can also be found in mammals (Koh et al., 2000). Like most CNS excitatory synapses in mammals, the *Drosophila* NMJ is glutamatergic and demonstrates activity-dependent remodeling (Koh et al., 2000). Many *Drosophila* neurons are defined and can be individually identified because their innervation patterns are stereotyped and repetitive. It is therefore possible to study identified synaptic terminals, such as those between motoneurons and the body-wall muscle fibers that they innervate (Keshishian and Kim, 2004). The existence of evolutionarily conserved synapse components along with the ease of genetic and physical manipulation make the *Drosophila* model ideal for investigating the mechanisms underlying synaptic plasticity and learning in higher organisms (Koh et al., 2000).

Drosophila BMP Pathway

In Drosophila, a BMP pathway provides a target-derived signal that is critical for proper synaptic terminal development in motoneurons (Figure 4). The type II receptor in this pathway, Wishful thinking (Wit), is the *Drosophila* homolog of the vertebrate BMP type II receptor (Aberle et al., 2002; Marques et al., 2002). Mutations in *wit* cause a

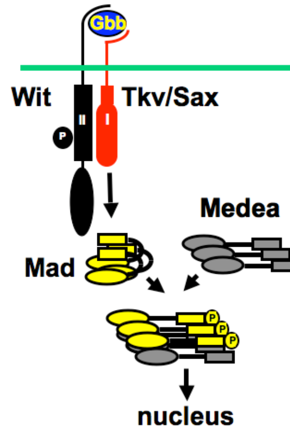

Figure 4: Wit/Gbb pathway

The type II receptor, Wit, phosphorylates the type I receptor, Tkv or Sax upon Gbb ligand binding. Tkv then phosphorylates Mad and recruits Medea to form an active complex. This active Smad complex regulates gene transcription in the nucleus. This is a simplified version because the receptor complex is a heterotetramer, with two type II receptors and two type I receptors and the ligand is a dimer formation.

Note: Image kindly provided by G. Marques

dramatic decrease of presynaptic neurotransmitter release in motoneurons and a reduction in synaptic terminal size and synaptic bouton number (Aberle et al., 2002; Marques et al., 2002) (Figure 5). Neuron-specific expression of a *wit* transgene rescued the mutant phenotype while the muscle expressed Wit did not (Marques et al., 2002). In addition, *wit* mutants appeared to affect p-Mad accumulation only in the central nervous system (Marques et al., 2002) (Figure 5). This shows the importance of Wit for normal motoneuron development, while another type II receptor, presumably Punt, is responsible for pathway activation in all other tissues (Marques et al., 2002). *wit* mutants also showed altered synaptic active zone morphology, demonstrated by the detachment of the presynaptic membrane from the muscle's postsynaptic membrane (Aberle et al., 2002; Marques et al., 2002). Wit does not control axonal guidance but rather plays a role in

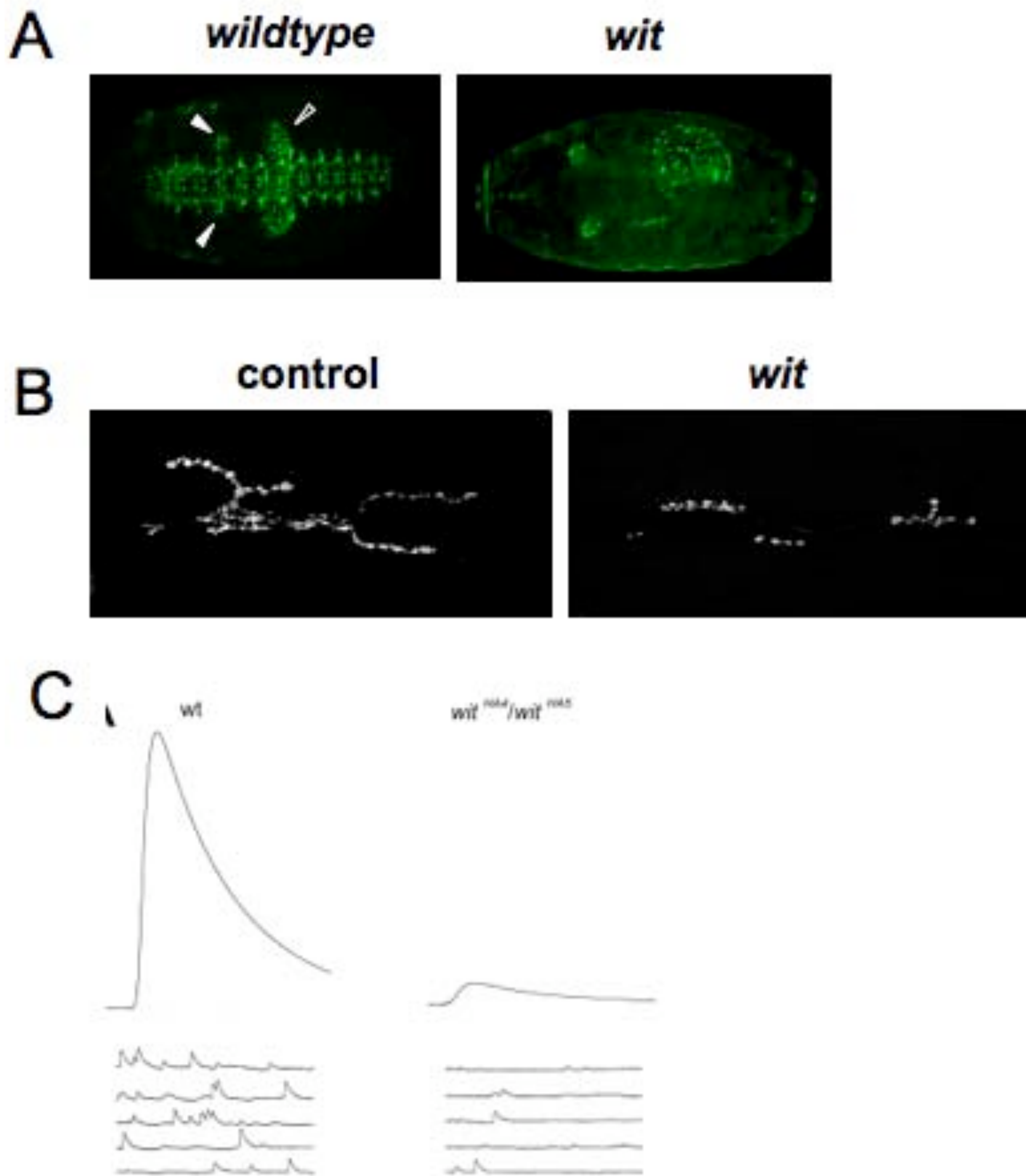

Figure 5: *wit* mutant phenotype

(A) *wit* mutants have eliminated p-Mad in the CNS. Gut p-mad staining remains in *wit* mutants. (B) *wit* mutants have small synapses when compared with control. (C) *wit* mutants have a reduction in electrophysiological potential. Representative traces of evoked and spontaneous potentials from wild-type (CD8-GFP-Sh), and in *wit* mutants wit^{HA4}/wit^{HA5}.

Figure 5: *wit* mutant phenotype (cont.)

Note: “A” panel adapted from “The Drosophila BMP Type II Receptor Wishful Thinking Regulates Neuromuscular Synapse Morphology and Function” by Guillermo Marqués, Hong Bao, Theodor E. Haerry, Mary Jane Shimell, Peter Duchek, Bing Zhang, and Michael B. O'Connor, 2002. Neuron, 33, p.529. Copyright 2002 Cell Press. Adapted with permission.

Note: “B” panel unpublished G.Marques.

Note: “C” panel adapted from “Wishful thinking Encodes a BMP Type II Receptor that Regulates Synaptic Growth in Drosophila” by Hermann Aberle, A. Pejmun Haghighi, Richard D. Fetter, Brian D. McCabe, Tiago R. Magalhães and Corey S. Goodman, 2002, Neuron, 33, p. 545. Copyright 2002 Cell Press. Adapted with permission.

regulating the growth of the synaptic terminal and synaptic transmission (Marques et al., 2002), as well as synaptic stability (Eaton & Davis 2005). In the motoneuron, Wit has been shown to signal through the type I receptors Thick veins (Tkv) and Saxophone (Sax) and through the R-Smad Mad and Co-Smad Medea. When these other components of the pathway are down-regulated, NMJ phenotypes similar to the *wit* mutant are observed (McCabe et al., 2004; Rawson et al., 2003; Sweeney and Davis, 2002). These results demonstrate the importance of Wit and the BMP signaling pathway to motoneuron synapse development.

Wit’s ligand, Glass bottom boat (Gbb), is also required for proper synapse formation. Similar to the *wit* mutant, *gbb* mutant larvae have reduced NMJ synapse size, decreased neurotransmitter release, and aberrant presynaptic ultrastructure (McCabe et al., 2003). When expressed in muscle, Gbb ligand restored normal synaptic size and synaptic bouton number to *gbb* mutants, while Gbb expressed only in the neuron did not (McCabe et al., 2003). There does appear to be an additional role for Gbb in the neuron, however, because Gbb expressed in all neurons rescued the electrophysiological

phenotype of *gbb* mutants while muscle or motoneuron expressed Gbb did not (McCabe et al., 2003). McCabe and colleagues hypothesize that this result may indicate that Gbb is used as a retrograde signal between neurons and that a full rescue of the *gbb* mutant phenotype may require Gbb produced in the muscle and neuron. Nevertheless, the *gbb* mutant phenotype of small synapses and nuclear p-Mad accumulation is rescued by signaling through BMP receptors at the motoneuron synaptic terminal, indicating that Gbb acts as a retrograde signal (McCabe et al., 2003).

Because this pathway is activated at the synaptic terminal with the ligand binding the receptors, but has its effect in the nucleus by regulating transcription with activated Smad transcription factors, this pathway must depend on active transport to move the signal along the axon. The Dynactin protein complex is implicated as an essential determinant of synapse stability and is thought to mediate all Dynein function in the cell (Karki and Holzbaur, 1999). Using a dominant negative form of one of its critical subunits, p150/Glued (DN-Glued), McCabe *et al.* found that p-Mad accumulation in the nuclei of motoneurons was blocked (Figure 6). In addition, these animals have small synapses similar to the *gbb* and *wit* mutants (Figure 6) (McCabe et al., 2003) and have reduced synaptic transmission (Eaton et al., 2002). These results, summarized in Table 1, demonstrate that retrograde transport of the BMP signal is required for the proper formation of the NMJ in *Drosophila*.

Although it has been demonstrated that this signaling pathway depends on retrograde transport, it is not known which component transports the signal from the synaptic terminal to the nucleus along the axon. The possible candidates are the

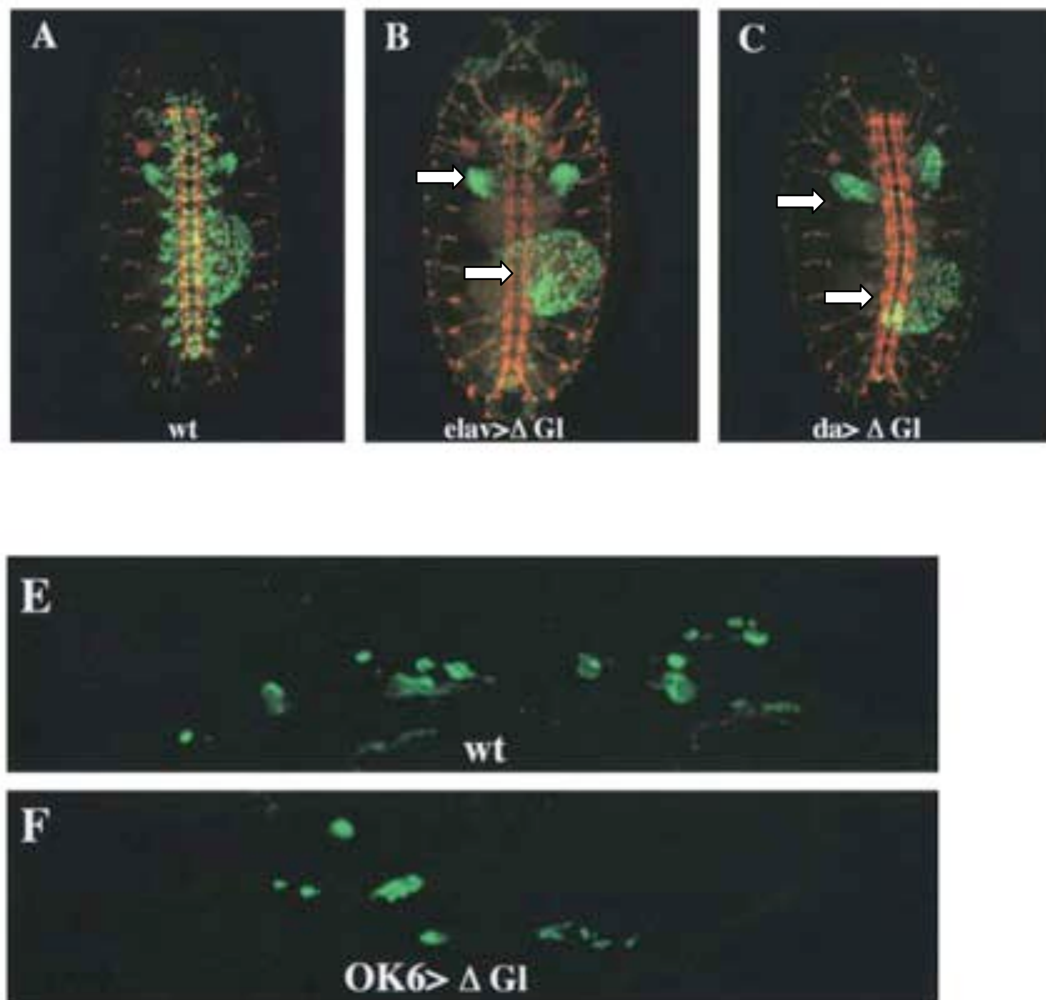

Figure 6: Expression of Dominant-Negative Glued eliminates p-Mad accumulation in motor neurons and results in small synapses

(A) Wild-type stage 17 embryo stained for p-Mad (green) and FasII expression (red). (B) Expression of ΔGl using the pan-neuronal elav driver. Note the loss of p-Mad staining in the CNS. (C) Expression of ΔGl using the ubiquitos daughterless driver. Note specific loss of p-Mad in the CNS versus the gastric ceca or gut (arrows). (E and F) Boutons at muscle 6 and 7 stained with anti-csp. (E) Wild-type and (F) OK6 motoneuron driver expressing ΔGl.

Note: From “The BMP Homolog Gbb Provides a Retrograde Signal that Regulates Synaptic Growth and the Drosophila Neuromuscular Junction” by Brian D. McCabe, Guillermo Marqués, A. Pejmun Haghighi, Richard D. Fetter, M. Lisa Crotty, Theodore E. Haerry, Corey S. Goodman, Michael B. O'Connor, 2003, Neuron, 39, p.241. Copyright 2003 by Cell Press. Reprinted with permission.

receptors or the Smad protein Mad, but neither has been shown to be transported along the axon. The goal of this dissertation is to determine if the receptors, Mad protein, or a combination of Mad with the receptors are transporting the BMP pathway signal along the axon.

Table 1: Summary of key background data

	P-Mad in MTN nuclei	**NMJ size**	**Synaptic Transmission**
Wild-type	Yes	Normal	Normal
gbb	No	Small	Reduced
wit	No	Small	Reduced
DN Glued	No	Small	Reduced

Neurotrophic Factors and the Signaling Endosome Model

Neurotrophins promote the survival of specific populations of developing neurons and support the maintenance of mature neurons (Wang and Poo, 1997). LTP is also impaired when neurotrophin function is abolished, demonstrating the importance of neurotrophins in synaptic plasticity (Schinder et al., 2000). Accordingly, neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are implicated in both developmental and adult synaptic plasticity (Kang et al., 1997). NGF is the prototypic neurotrophin and has been well established as a target-derived factor in the regulation of the survival and differentiation of neurons (Lessmann et al., 2003). When released, NGF binds to tropomyosin-receptor-kinase A (TrkA) receptors located on axon terminals of the innervating neurons, and thus activates the signaling pathway

sending the signal retrogradely along the axon to the neuronal cell bodies (Ye et al., 2003).

Studies with NGF have confirmed that neurotrophins are produced/released from target tissues and internalized and transported to the cell body of the innervating neuron (Chao, 2003). Evidence shows that the ligand, NGF, as well as the phosphorylated receptor, TrkA, are retrogradely transported in neurons (Delcroix et al., 2003; Ehlers et al., 1995; Ye et al., 2003). Accordingly, the pathway is dependent on endocytic proteins and Dynein function for proper propagation and transport of the active signal (Heerssen et al., 2004; McPherson et al., 2001). Addition of the NGF ligand to the neuron induced TrkA receptor internalization and transport (Grimes et al., 1996; Heerssen et al., 2004), signifying activity-dependent internalization of the receptors. Together, these findings provide evidence for a signaling endosome model (Figure 7) in which the ligand/receptor complex undergoes internalization at the synapse and then is transported along the axon microtubule network to the cell body where it propagates the signal activation pathway.

Rab Proteins and Roles in Endocytic Sorting

Rab proteins are GTPases that coordinate steps in membrane traffic or endocytic sorting, implicated principally in the control of vesicle docking and fusion (Grosshans et al., 2006). Rab-GTPases function as switches cycling between GTP-bound and GDP-bound states (Grosshans et al., 2006). Initially, Rab proteins are recruited to the target membrane and participate in vesicle budding (Somsel Rodman and Wandinger-Ness, 2000). They also have been implicated in facilitating endosome transport along the cytoskeleton and they have roles in vesicle docking and fusion of their endosome cargo

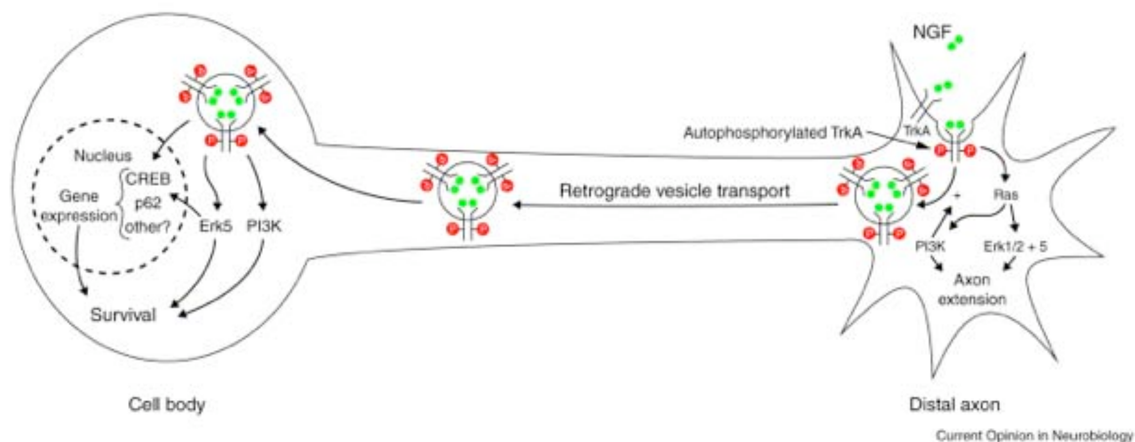

Figure 7: The signaling endosome model.

Trk receptors on distal axons are activated upon binding to neurotrophin. The ligand–receptor complex is internalized through clathrin-mediated endocytosis. Some of the vesicles become specialized endosomes that serve as platforms for continued Trk signaling and are transported retrogradely to the cell body using a Dynein-dependent and microtubule-dependent transport mechanism. The vesicle-associated Trk receptor remains autophosphorylated and capable of promoting a unique set of signals upon arrival at the cell bodies. These include phosphorylation of PI3K and Erk5.

Note: From "Retrograde neurotrophin signaling:Trk-ing along the axon" by David D Ginty and Rosalind A Segal, 2002, Current Opinion in Neurobiology, 12, p.268. Copyright 2002 by Elsevier Science Ltd. Reprinted with permission.

(Jordens et al., 2005; Somsel Rodman and Wandinger-Ness, 2000; Zerial and McBride, 2001). From sequences in the human genome, at least 60 different Rab family members have been identified (Bock et al., 2001) and each is likely associated with a particular organelle or pathway (Somsel Rodman and Wandinger-Ness, 2000) (Figure 8). Some well-characterized Rabs are the early endosomes Rab5 and Rab4 as well as the late endosome Rab7. Rab5 and Rab4 likely act together to control the influx into and out of early endosomes, respectively (Sonnichsen et al., 2000). In accordance, Rab4 is an early endosome protein that is responsible for recycling cargo back to the membrane (Li and Stahl, 1993) and Rab5 has been shown to play a role in endocytosis and early endosome

Figure 8: Intracellular vesicle transport pathways and localization of selected Rabs.

Of special interest to discussions within this dissertation are:
Rab5- early endosome protein
Rab4- early endosome and recycling endosome protein
Rab7- late endosome protein

Note: From "The Rab GTPase family" by Harald Stenmark and Vesa M Olkkonen, 2001, *Genome Biology*, 2 (5). Copyright 2001 by BioMed Central. Reprinted with permission through open access policy.

fusion in endocytic traffic (Bucci et al., 1992). Rab5 might also facilitate cargo selection because it was shown to be required for ligand sequestering to clathrin coated pits (McLauchlan et al., 1998), which would provide an avenue for targeted endosomal transport of signaling pathways. Rab7 is a late endosome protein that transfers its cargo to the lysosome (Meresse et al., 1995). Rab family proteins are essential for targeted transport of endosomes.

Rab5 and Rab7 are both implicated in axonal transport of neurotrophins and appear to act subsequently in endosome transport. In a recent report, Rab5 was shown to regulate an early endosome sorting step preceding axonal transport, while Rab7 regulates long-range retrograde axonal transport in motoneurons (Deinhardt et al., 2006). Over time in cell culture, Rab5 positive endosomes convert to Rab7 endosomes, displaying a converting mechanism (Rink et al., 2005). As Rab5 positive endosomes move toward the interior of the cell, Rab5 begins to be lost correlating with the gain of Rab7 (Rink et al., 2005). Rab5 and Rab7 work sequentially to transport endosome along the early to late endocytic route in cells.

Mammalian TGF-β Similarities to Signaling Endosome Model

Some members of the TGF-β super family act similarly to neurotrophins in mechanism and function. Mullerian Inhibiting Substance (MIS) and TGF-β2 are target-derived retrograde signals that function as motor neuron survival factors (Jiang et al., 2000b; Wang et al., 2005). MIS supports the survival of mouse embryonic motor neurons and is utilized as a communication signal between mature neurons (Wang et al., 2005). Another TGF-β ligand, TGF-β2, reduced cell death when applied to injured

motoneurons (Jiang et al., 2000b). Additionally, TGF-β2 is produced by the subsynaptic regions of muscle fibers and potentially is presented to the neuron in a paracrine fashion (Jiang et al., 2000a). This ligand and its receptors were transported in nerves (Jiang et al., 2000a) mirroring the transport of NGF and its receptors along the axon. Members of the TGF-β family appear to be critical retrograde survival signals in mammalian neurons.

Mechanistically, there is also evidence that mammalian TGF-β receptors are endocytosed, which is a necessity of the signaling endosome pathway. TGF-β receptors interact with a subunit of AP2, the clathrin-associated adaptor complex (Yao et al., 2002). Additionally, pathway activity has been linked to endocytosis because ligand exposure caused the cell to internalize heteromeric TGF-β receptor complexes (Anders et al., 1997). Consistently, the kinase of the type II TGF-β receptor is necessary for optimal endocytosis of the complex and this effect was dependent on the type II receptor's transphosphorylation ability (Anders et al., 1998). Endocytosis of TGF-β family receptors has also been found to be necessary for transcriptional activation. Smad activation and downstream signaling only occurred after endocytic vesicle formation (Penheiter et al., 2002). Additionally, BMP receptor endocytosis by clathrin lead to continued Smad signaling and pathway transcriptional response, while receptors remaining at the plasma membrane did not (Hartung et al., 2006). In summary, endocytosis and endocytic traffic of mammalian TGF-β /BMP family receptors is required to elicit the proper transcriptional response from Smad effector proteins, which is reminiscent of the NGF signaling endosome model and its activation of effector molecules that enter the nucleus to regulate transcription.

Drosophila BMP Pathway is Regulated by Endocytosis

Meeting one condition of the signaling endosome model, the *Drosophila* BMP pathway also is known to interact with endocytic proteins. The BMP pathway has been shown to be regulated by two recently discovered endocytic proteins, Nervous wreck and Spichthyin (O'Connor-Giles et al., 2008; Wang et al., 2007). These proteins showed early and recycling endosome localization and loss of these proteins led to BMP-dependent synaptic terminal overgrowth (O'Connor-Giles et al., 2008; Wang et al., 2007). At the late endosome/lysosome stage, Spinster is another protein that serves to down-regulate the BMP pathway (Sweeney and Davis, 2002). Mutations in Spinster cause enhanced/misregulated BMP signaling and have altered late endosome/lysosome function (Sweeney and Davis, 2002). Additionally, loss of Vps35, an endocytic sorting protein, leads to TGF-β /BMP dependent upregulation of synaptic size (Korolchuk et al., 2007). These data demonstrate that BMP receptors are endocytosed and that the endocytic pathway regulates BMP signaling during motoneuron synaptic growth.

Neurotrophin Effector Protein Activation- Clues for Mad Activation?

To fully characterize the BMP pathway mechanism, we need to determine the subcellular localization where Mad is activated. p-Mad is localized at the nucleus (Marques et al., 2002; Marques et al., 2003) and synaptic terminals in wild type animals (O'Connor-Giles et al., 2008; Wang et al., 2007). The question is if the activated p-Mad is transported from the synaptic terminal to the nucleus, and whether its hypothetical transport is dependent on active retrograde transport. In the neurotrophin signaling endosome model, some of the proteins that the receptor complex phosphorylates is

transported with the receptor complex along the axon. Phosphorylated Mitogen-activated protein (MAP) Kinases and phosphorylated extracellular signal-regulated kinases (Erk) 1/2 and p-p38, have been observed to be transported with activated TrkA receptors, and this transport depends on NGF activity (Delcroix et al., 2003). Additionally, NGF pathway activation leads to cAMP response element binding (CREB) translation along the axon and the CREB protein subsequently is transported with the activated receptor complex to the cell body of the neuron (Cox et al., 2008). Effector proteins that are activated by the neurotrophin signaling endosome complex are transported with the activated receptor/ligand complex. In our study, we will determine if Mad is being transported along the axon with the receptor complex.

Based on thesignaling endosome model, there are indications that subcellular location of neurotrophin activation determines specificity in responses. Neurotrophin stimulation at the cell body results in activation of both the Erk1/2 and Erk5 (Cosker et al., 2008; Watson et al., 2001). Activation of neurotrophins at the distal axon; however, leads to activation of Erk5 but not Erk1/2 because the latter doesn't transmit a retrograde signal (Cosker et al., 2008; Watson et al., 2001). It appears that the activation of neurotrophin pathways has differing effects whether it is at the synaptic terminal or cell body, and hence endosome traffic helps compartmentalize signaling.

If the BMP receptors are being transported in a signaling endosome, it is possible that it could serve as a platform for Mad activation, and pMad could be transported with the signaling endosome to maintain its activation on the way to the nucleus. Smad anchor for receptor activation (SARA) is an early endosome protein that has been shown to be necessary for proper TGF-β and BMP signaling (Fumiko Itoh, 2002) by recruiting

the R-Smad to be phosphorylated by the type I receptor. If the active receptors move along the axon in an endosome, SARA and Mad could be transported with the vesicle in contact with the type I receptor. In lieu of the signaling endosome model, p-Mad could be transported along the axon taking the active signal to the nucleus itself. In either situation, we would expect Mad to be transported by Dynein, given the need for Dynein activity for pathway activation (Marques et al., 2003; McCabe et al., 2003). Active transport is necessary for long range signaling between the synaptic terminal and cell body a few millimeters away because the time required for cytoplasmic diffusion is likely longer than the lifetime of the activated molecule (Fitzsimonds and Poo, 1998). Our study will address these possibilities for Mad activation and dynamics.

Presented Dissertation Study and Results

As previously described, the Gbb/Wit BMP pathway is a retrograde pathway in motoneurons that must depend on axonal transport to activate Smad transcription factors. The evidence for this finding is that Gbb expression is required largely in the post-synaptic muscles, as opposed to the neuron (McCabe et al., 2003). Muscle expression, of the Gbb ligand rescued nuclear p-Mad in the ventral ganglion of *gbb* mutants better than neuronal expression, indicating that the nuclear activation of p-Mad must depend on axonal transport (McCabe et al., 2003). By expressing DN-Glued, a subunit of the Dynactin complex required for Dynein activity, the BMP pathway is inhibited to a similar extent as pathway mutants (Marques et al., 2003; McCabe et al., 2003). This evidence suggests that motor proteins might be involved in pathway activity. Although there is evidence that this BMP pathway activation requires axonal transport, the identity of the

transported signaling has not been deciphered. The possible candidates for axonal transport are the Mad transcription factor or the receptors, however neither has been shown to be transported along the axon. We intend to determine if there is evidence for the receptors to be transported along the axon in a signaling endosome structure or if the Mad transcription factor can be effectively transported along the axon independently of the receptors.

To address the question of whether Mad or the receptors are carrying the signal from the synapse to the nucleus, we have created fluorescent fusion proteins of the receptors and Mad and examined their subcellular location and dynamic movement along the axon. We have altered pathway activity and examined the effect on receptor transport and Mad localization. Dynein-mediated transport was inhibited and we examined changes in receptor movement along the axon. Additionally, we have perturbed the Rab endocytic pathway, presumably affecting receptor endocytic sorting, and tested for changes in pathway activity read-outs such as p-Mad and synaptic terminal size.

From our studies we have found that the receptors are transported along the axon in a bidirectional manner. This traffic of the type I receptor is affected by levels of the type II receptor, suggesting an endosome packaging mechanism. The direction of traffic of vesicles containing the co-localized type I and type II receptors is overwhelmingly retrograde, consistent with these vesicles being signaling endosomes. This directionally disappears when the Gbb ligand is absent from the system, strongly supporting a signaling endosome model of BMP signaling. Additionally, a known inhibitor of the BMP pathway, DN-Glued, was shown to inhibit receptor transport, linking receptor transport and pathway activity. Further support for the signaling endosome model is seen

when Rab activity levels are manipulated. When Rab endocytic sorting is perturbed we saw that p-Mad levels are altered in the nucleus and synaptic terminal of motoneurons, signifying that receptor endocytic sorting regulates Mad activation. Finally, we saw that Mad is not transported with the receptor endosomes and that Mad's movement along the axon appears to be diffuse. These data supports the model of a signaling endosome of the BMP pathway, phosphorylating Mad during separate events at the distal and proximal ends of the motoneuron.

BMP RECEPTORS TRAFFIC IN A SIGNALING ENDOSOME THAT DISPLAYS RETROGRADE MOVEMENT IN RESPONSE TO PATHWAY ACTIVITY

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CHAPTER 2

BMP RECEPTORS TRAFFIC IN A SIGNALING ENDOSOME THAT DISPLAYS RETROGRADE MOVEMENT IN RESPONSE TO PATHWAY ACTIVITY

Abstract

Neurodegeneration can be caused by the faulty transport of growth and survival signals from the synaptic terminal, where the neuron interacts with its targets, to the nucleus where new proteins are synthesized in response to these critical signals. Activation of the Bone Morphogenetic Protein (BMP) pathway at the *Drosophila* larval neuromuscular junction (NMJ) by muscle-derived signals results in synaptic growth. Pathway activation at the NMJ results in nuclear accumulation of the phosphorylated form of the transcription factor Mad in the motoneuron nucleus. We are interested in how the signaling event at the synaptic terminal is relayed to the nucleus. Other target-derived factors, such as neurotrophins, utilize retrograde axonal transport of a signaling endosome for signal propagation. We have investigated if this signaling endosome model applies to BMP pathway activation in motoneurons. We find that the receptors Wishful thinking (Wit) and Thick veins (Tkv) are transported in a bi-directional manner along the motoneuron axon, and that disruptions of axonal transport that are known to inhibit the BMP pathway dramatically decrease the amount of moving receptor vesicles. Additionally, the amount of Wit receptor influences the vesicular distribution of Tkv-YFP. When Tkv and Wit are coexpressed, they colocalize along the axon in retrograde

moving vesicles, consistent with our hypothesis of a signaling endosome. In the absence of the ligand Glass bottom boat (Gbb), the pathway is silent and colocalized traffic of Tkv and Wit lacks retrograde directionality. Our data support a signaling endosome model to propagate the BMP pathway in Drosophila motoneurons, and we propose that the colocalized axonal retrograde traffic of Wit and Tkv represents an activated receptor complex that relays the BMP signal from the synaptic terminal to the neuron soma.

Introduction

The nervous system contains neurons with long processes that are especially sensitive to errors in axonal transport. Because of the large distance between areas of the neuron that are responsible for biosynthesis and those where cell signaling occurs, the cell must rely on long-distance transport systems for the proper distribution of important cargo (Goldstein, 2003). Blockages in transport result in the death of the neuron and, if widespread, disease pathology. Alzheimer's disease, Huntington's disease, and polyglutamine diseases all have axonal blockages that inhibit transport (Goldstein, 2003). Axonal transport can occur in two directions: retrograde transport moves cargo from the distal synaptic terminal to the proximal cell body and anterograde transport, which moves in the opposite direction, from the cell body to the synaptic terminal. Errors in retrograde transport have been shown to cause neuron degeneration in diseases such as Motor Neuron Disease, Amyotrophic Lateral Sclerosis (ALS), and Spinal Muscle Atrophy (Hafezparast et al., 2003). Retrograde transport is critical for the health of the neuron because it allows for proper intracellular communication to ensure that the appropriate targets have been contacted or that the physical integrity of the axon is still intact

(Goldstein, 2003). Because of the large size of motoneurons, it is unlikely that simple diffusion would be sufficient to distribute factors that are important in gene regulation (Goldstein, 2003). Without active retrograde transport from the synapse to the cell body, biochemical responses to target-derived signals that require gene transcription would not occur.

To coordinate their growth during development, retrograde signaling is critical for bidirectional communication between the neuron and target-cell. Retrograde signaling is cued by a target-derived factor, being synthesized in the postsynaptic cell, and then presented to the innervating neuron (Fitzsimonds and Poo, 1998). Nerve Growth Factor (NGF) is a target derived peptide that is critical for maturation and survival of sympathetic neurons (Fitzsimonds and Poo, 1998). Other members of the neurotrophin family, such as BDNF and neurotrophin-3 and 4/5, have been identified as retrograde factors and provide critical growth and survival signals (Cosker et al., 2008; Ginty and Segal, 2002). Trafficking of the neurotrophin signals from synaptic terminal to the cell body has been shown to be a critical step in pathway activation, and inhibition of trafficking of the NGF signal causes neurodegeneration (Bronfman et al., 2007; Cosker et al., 2008).

Retrograde signals such as NGF and BDNF use a signaling endosome (SE) to activate down-stream effectors at the cell body (Delcroix et al., 2003; Ibanez, 2007; Ye et al., 2003). In this model, the ligand binds its receptors at the synaptic terminal and then the complex is endocytosed. The ligand/ receptor complex is then retrogradely transported along the axon to activate down-stream proteins that confer neuron survival. Retrograde transport of this complex is necessary for neuron survival, as neutralization of

the kinase of the NGF receptor within the cell body and axon results in cell death (Heerssen et al., 2004; Ye et al., 2003). Endocytosis of the ligand/receptor complex is important to induce survival by NGF (Heerssen et al., 2004; Ye et al., 2003).

In addition to the neurotrophin family, some members of the Transforming Growth Factor-ß family, such as TGF-ß2 (Jiang et al., 2000b) and Mullerian Inhibiting Substance (Wang et al., 2005) also are classified as motoneuron survival factors. A Bone Morphogenic Protein (BMP), another TGF-ß superfamily member, is a retrograde signal that facilitates coordination between the motoneuron and the muscle in *Drosophila* (Aberle et al., 2002; Marques et al., 2002; McCabe et al., 2003). The ligand in this pathway, Glass bottom boat (Gbb) has been found to signal through the BMP type II receptor Wishful thinking (Wit) (Marques et al., 2003; McCabe et al., 2003) and the type I receptors, Saxophone (Sax) and Thick veins (Tkv) (McCabe et al., 2004; Rawson et al., 2003). Both endogenous (McCabe et al., 2004) and transgenic receptors expressed with a motoneuron driver were found to localize to the synaptic terminal (McCabe et al., 2004; Wang et al., 2007) and muscle expression of Gbb is required to establish proper synaptic terminal size and bouton number (McCabe et al., 2003). These data suggest that Gbb activates the BMP receptor complex at the NMJ to act as a retrograde signal to induce growth of the synaptic terminal (McCabe et al., 2003). An important question is how the active BMP signal gets from the synaptic terminal to the nucleus.

There is evidence that the Wit/Gbb pathway activation depends on axonal transport. Similar to neurotrophin signaling (Heerssen et al., 2004), Dynein has been linked to BMP pathway activation. A truncated form of p150/Glued (DN-Glued) has been shown to block assembly of Dynein retrograde motors (Martin et al., 1999). When

this protein is overexpressed in neurons, it leads to inhibition of the BMP pathway (Marques et al., 2003; McCabe et al., 2003). In normal circumstances, when the ligand binds to the type I and type II receptors, a Smad transcription factor, Mad, is phosphorylated (p-Mad) and enters the nucleus to regulate transcription. The phenotype of neuronal expression of DN-Glued expression is elimination of p-Mad in motoneuron nuclei and small synapses similar to the phenotype of BMP pathway mutants (Eaton et al., 2002; McCabe et al., 2003). The involvement of Dynein in pathway activation suggests that active retrograde transport of a BMP pathway component is involved in signal transfer from the synaptic terminal to the nucleus of the motoneuron.

Yet to be identified is the BMP pathway component that transfers the active pathway signal from the synaptic terminal, where Gbb secreted from muscle can activate receptors, to the neuron soma where Mad can enter the nucleus and regulate transcription of genes required for synaptic terminal growth. The probable candidates for long-range transport of BMP signaling are either Mad or the receptors, but neither has been shown to be transported along the axon. We set out to investigate the molecular mechanisms of BMP signal relay from NMJ to cell body.

Other reports show that BMP and TGF-ß receptors interact with transport machinery, indicating that transport of these receptors could be important in pathway activation. In mammalian motoneuron cell culture, TGF-ß2 was found to be transported both in anterograde and retrograde directions along axons (Jiang et al., 2000a). Additionally a dynein light chain adapter protein, Tctex-1, has been shown to interact with and be phosphorylated by BMPR-II (Machado et al., 2003). Because mammalian BMP and TGF-ß receptors interact with transport machinery, the receptors are a likely

candidate for transporting the pathway signal and this mechanism could be utilized in multiple cell types.

Endocytosis of receptors, which is a requirement for the receptors to transport the active signal, also has been shown to play an important role in BMP and TGF-ß pathway signaling in mammalian cells. Upon ligand binding the active kinase of TGF-ß receptors was shown to elicit endocytosis of the receptor complex (Anders et al., 1997; Anders et al., 1998). In cell culture, the TGF-ß receptors interact with clathrin-associated adaptor protein, AP2 (Yao et al., 2002) and BMP receptors are endocytosed by clathrin coated pits and caveloae (Di Guglielmo et al., 2003; Hartung et al., 2006). Another study describes that internalization of the TGF-ß receptors is required to activate the downstream Smads to propagate pathway signaling (Penheiter et al., 2002). Consistently, an early endosomal protein, Smad Anchor for Receptor Activation (SARA) has been shown to be necessary for efficient TGF-ß as well as BMP signaling (Itoh et al., 2002) by recruiting the R-Smad for phosphorylation by the type I receptor. Receptor endocytosis appears to have an important role for BMP or TGF-ß signaling in various mammalian cells.

In *Drosophila*, there have been several studies showing that endocytic pathway regulates BMP signaling in motoneurons. Recently two endocytic proteins, Spichthyin (Spict) and Nervous wreck (Nwk), have been identified that down-regulate the BMP signal at the synaptic terminal and, when mutated, cause BMP dependent synaptic terminal overgrowth (O'Connor-Giles et al., 2008; Wang et al., 2007). Additionally, a late endosomal/lysosomal protein named Spinster, when mutated, causes enhanced/ misregulated BMP pathway signaling resulting in synaptic terminal expansion (Sweeney

and Davis, 2002). Finally, loss of Vps35, an endocytic sorting protein, led to TGF-β/ BMP dependent upregulation of synaptic size (Korolchuk et al., 2007). Taken together, these reports show that endocytic proteins regulate BMP signaling, indicating that a signaling endosome is a plausible mechanism of signal sorting and attenuation for this pathway.

In this study we have used fluorescent fusion proteins of the *Drosophila* BMP receptors to investigate the existence of a BMP signaling endosome. We have found that the receptors form endosomes at the NMJ and are transported along the axon. Blockade of axonal retrograde motors eliminates BMP signaling and transport of the receptors, linking pathway activation to receptor traffic. We also show data demonstrating that receptor transport is dependent on pathway activity, which is consistent with neurotrophin models of signaling endosomes (Heerssen et al., 2004). Additionally, using two-color time lapse imaging, we find that Wit and Tkv co-localize in vesicles that are predominately moving in the retrograde direction, differing significantly from single receptor transport. When the receptors are expressed in a *gbb* mutant this colocalization between Wit and Tkv is still present, but there is a large portion of the colocalized particles moving in an anterograde direction, indicating that the presence of the Gbb ligand is important to confer directionality to the co-localized receptor vesicle. We propose that the retrograde moving vesicle containing both BMP receptors constitutes a signal endosome for BMP signal propagation from the synaptic terminal to the motoneuron nucleus. This mechanism of signal relay is likely to function in TGF-ß signaling in other cells and developmental contexts.

Results

Type I and Type II BMP receptors tagged with fluorescent proteins are functional

To investigate the localization and dynamics of the BMP receptors we tagged the type I receptor Thick veins (Tkv) with YFP and the type II receptor Wishful thinking (Wit) with EGFP (Marques 2003) and CFP, in all cases at the C-terminal end of the receptor (Figure 1A). To verify that these fusion proteins were functional we conducted experiments to rescue the phenotype of the cognate mutants by expression of the fluorescent fusion proteins. We found that when expressed with a pan-neuronal (elav) Gal4 driver, Wit tagged with either EGFP or CFP fully rescued the lethality of *wit* mutants (not shown), much as the wild type untagged Wit receptor (Marques et al., 2002; Marques et al., 2003). Additionally, when driven by elav, Wit-EGFP rescued the synaptic bouton number of *wit* mutant larvae (Figure 1C). When driven by the ubiquitous driver armadillo>Gal4, Tkv-YFP rescued the embryonic lethality phenotype of *tkv* to pupal stage (not shown). The transgene also rescued the larval synaptic size phenotype of *tkv* mutants (Fig 1B). These experiments indicate that the fluorescent fusion proteins of the receptors are functional and can be used confidently for experimental purposes as true reporters of endogenous proteins subcellular localization.

Wit and Tkv are localizing to early endosomes

To examine the subcellular localization of the receptors, we used motoneuron driver OK6 to express the fluorescent fusion receptors. We see that the receptors localize to the plasma membrane of the synaptic boutons and form punctate structures within the synaptic terminal, along the axon and cell body of the motoneuron. Vesicles of punctate

Tkv can be seen in Figure 2 A1, B1, C1 and vesicles of punctate Wit can be seen in Figure 2 D2, H2, I2.

To determine if these punctate receptors could have been endocytosed we looked at co-localization of the receptors with early endosome proteins Rab5 and Rab4. Rab5 specifically is involved in early endosome functions and regulates early endosome fusion and motility (Clague and Urbe, 2001). Rab4 is also an early endosome protein but additionally controls the recycling of early endosome cargo (Somsel Rodman and Wandinger-Ness, 2000).

When expressing Tkv-YFP and Rab5-GFP in motoneurons, we see co-localization at the synaptic terminal, along the axon, and at the cell body (Figure 2A-2C). However, when we co-express Rab4-RFP and Tkv-YFP or Wit-GFP we see co-localization only at the synaptic terminal and not in the axons or cell bodies (Figure 2D-I). This is in agreement with the more restricted localization of Rab4 to early and recycling endosomes, and argues that receptor internalization and recycling occurs mainly at the NMJ. From the partial co-localization of BMP receptors and Rabs we conclude that Wit and Tkv are endocytosed at the synaptic terminal, in accordance with the genetic and immunolocalization data.

To investigate the receptor localization within the synapse in more detail, we expressed the fluorescently tagged receptors with OK6 while simultaneously staining for active zones utilizing the active zone marker NC82 (Bao et al., 2005) (Supplementary Fig. 1). Tkv-YFP and Wit-CFP was localized to punctate structures in the synaptic terminal that partially colocalized with NC82, indicating that the BMP receptors localize to active zones in the synaptic bouton. The co-localization between Tkv-YFP and NC82

persisted in *wit* mutants, indicating that targeting of Tkv to active zones occurs independently of the type II receptor Wit.

Other groups have shown p-Mad at the synaptic terminal (O'Connor-Giles et al., 2008; Wang et al., 2007), something we have confirmed (unpublished). Newly described endocytic proteins, Spict and Nwk, regulate p-Mad amounts at the synaptic terminal by down-regulating the receptors (O'Connor-Giles et al., 2008; Wang et al., 2007). Mutants in these proteins display elevated p-Mad staining at the NMJ (O'Connor-Giles et al., 2008; Wang et al., 2007). These data along with our evidence demonstrate that the receptors, regulated by endocytic proteins, are likely phosphorylating Mad at the synaptic terminal.

Wit and Tkv traffic along the axon

After observing that the receptor vesicles colocalize with Rab endosome markers, suggesting that the receptors are internalized in endosomes at the synaptic terminal, we wondered if we could visualize the dynamic movement of these receptor vesicles within the motoneuron axon. In the signaling endosome model, the receptors are transported along the axon, thus we explored receptor axonal transport.

Within the neuron axon, there is a certain orientation of microtubules and thus axonal traffic. In axons, microtubules are unipolar, and the plus-end points distally to the synaptic terminal (Hirokawa and Takemura, 2004). Axonal retrograde transport, or minus-end directed transport, is transport from the neuromuscular junction to the cell body (Ibanez, 2007), and is driven by Dynein and Dynactin (Holzbaur, 2004). Powered by Kinesin and proteins in the Kinesin superfamily, anterograde transport is plus-end

directed and travels from the cell body to the neuromuscular junction, or synaptic terminal (Holzbaur, 2004).

When the fluorescently tagged receptors are expressed by motoneuron driver OK6, we observed their dynamic movement in a bidirectional manner along the motoneuron axon, in addition to receptor vesicles that did not move within the time frame of our experiments (30-60 s runs) (Fig. 3 and 4). We quantified the fraction of motile vesicles for each receptor, the proportion of vesicles moving anterogradely and retrogradely, and the velocity distribution of those vesicles. We found that approximately 1/3 of moving particles were being transported in an anterograde direction while 2/3 were moving in a retrograde direction for both Tkv-YFP and Wit-GFP receptors (Fig. 3E, 4E).

The skewed directionality of receptor traffic is difficult to interpret with our current knowledge of the signaling mechanism. If the purpose of anterograde transport was to replenish the receptors at the synaptic terminal after they are activated, one might expect a 1:1 ratio of receptors traveling toward the synaptic terminal and back, but that would assume an equilibrium or steady state situation, something unlikely in a developmental process. One possible explanation for the larger number of retrogradely moving vesicles is that the size of the vesicles is different coming from the synaptic terminal or from the cell body. The anterograde vesicles could be smaller or the receptors might be less concentrated and not detected as readily by the resolution of the imaging equipment. Alternatively, the anterograde moving vesicles could contain more receptors than the retrograde vesicles, thus less vesicles would be required to be shipped to the synaptic terminal. Another possibility is that translation of the receptors could be occurring near the synapse adding to the receptor pool at the synaptic terminal. Axonal

translation of synaptic proteins has been proposed to occur in other pathways (Giuditta et al., 2008) and could be occurring in the BMP pathway as well. Further study is necessary to clarify this issue.

Similar to other pathways, retrograde axonal transport of the BMP receptors could have important implications on pathway activity, thus we quantified the retrograde trafficking of the receptors in more detail (Fig 3C, 4C). Wit and Tkv were both traveling in a retrograde direction (towards the cell body and away from the synaptic terminal) with velocities between 0.27-2.8 µm/s. The retrograde speeds have a uni-modal distribution. Wit (average 1.55 µm/s, standard deviation 0.49 µm/s) and Tkv (average 1.36 µm/s, standard deviation 0.55µm/s) were traveling at similar retrograde speeds, which are compatible with Dynein-mediated fast axonal transport (Hirokawa, 1998).

Contrary to the similar average velocity and population distribution of retrograde transport, there are differences in anterograde transport of the BMP receptors (Fig 3D, 4D). Both receptors had a similar velocity range of 0.19-3.36 µm/s that appeared to be bi-modal or tri-modal, indicating that there are multiple populations of moving particles at different velocities. The velocities of these populations are similar for both the type II receptor Wit and the type I receptor Tkv, however the distribution of receptors within each population varies. Tkv-YFP has more vesicles moving at slower speeds; with the largest fraction being contained in the slowest population. The majority of Wit-GFP anterograde vesicles are in the fastest population. Reflecting this different distribution, the mean velocity was 1.50 µm/s for anterograde moving Tkv-YFP (standard deviation 0.78 µm/s) and 1.87 µm/s for Wit-GFP (standard deviation 0.64 µm/s) ($p<0.0001$). A

possible interpretation of this result is that Wit and Tkv are transported independently anterogradely towards the NMJ.

Wit and Tkv colocalize at the synaptic terminal in moving vesicles

To directly assess the co-localized retrograde traffic of Wit and Tkv in a signaling endosome we utilized two-color live imaging of Wit-CFP and Tkv-YFP. When we expressed the tagged receptors with motoneuron drivers BG380 and OK6, we found that they colocalize in punctate structures that dynamically move within the synaptic terminal (Figure 5). Analysis of these vesicles shows a mixture of vesicles containing one or both receptors. While the convoluted traffic pattern makes it difficult to describe and quantify the movement of these vesicles, there are clear examples of retrograde traffic of vesicles containing both Tkv and Wit within the synaptic terminal, consistent with our SE model.

Wit and Tkv colocalize in retrograde moving vesicles along the axon

After observing colocalization of the receptors at the synaptic terminal and cell body (not shown), we analyzed the traffic of Tkv-YFP and Wit-CFP in the motoneuron axons. When driven by OK6 and BG380, Wit-CFP and Tkv-YFP colocalize along the axon in dynamic vesicles that are predominately moving in a retrograde direction (Figure 6A-C). Of the vesicles that contain both receptors, 95% were traveling in a retrograde direction while only 5% were traveling in an anterograde direction (n=120 particles) (Fig 6C). There were retrograde moving particles that changed directions briefly to travel anterograde for a few time frames, and then returned to moving retrograde. These particles were not included in either population. The velocity range of retrograde

movement was 0.09-2.20 μm/s. The velocity was normally distributed and the mean speed was 0.78 μm/s (standard deviation 0.39 μm/s) (Figure 6D). This was significantly slower than the average retrograde velocity of vesicles carrying individual receptors. The vast majority of colocalized receptors are traveling in a retrograde direction, supporting the signaling endosome model for BMP signaling.

Tkv-YFP vesicular distribution and retrograde speed is decreased in *wit* mutants

Because we saw that the receptors co-localize in predominantly retrograde moving particles along the axon, we wondered if the type I receptor transport was altered when the type II receptor was absent. If the endocytosis and transport of the vesicles is activity dependent and both receptors are packaged together for retrograde shipment in an endosome, we would expect to see differences in amounts of Tkv receptor vesicles and retrograde transport when Wit is not present and the pathway is inactive. To address this question, we expressed Tkv-YFP in the motoneurons of *wit* mutants and imaged the cell body (Fig. 7C) and synaptic terminal (Fig 7A). We found that there was a significant decrease in Tkv-YFP vesicular distribution in both of these locations (Figure 7A-C). As an important control, we verified that expression of the driver, OK6, is not affected in *wit* mutants. (Supplementary figure 2).

After we saw this effect on Tkv vesicular distribution at the synaptic terminal and cell body in fixed tissue, we analyzed velocity of Tkv-YFP axonal transport in *wit* mutants. If Wit is not present, we hypothesize that there would be significant impact on Tkv transport, linking receptor transport to pathway activity. Recently it has been reported that general axonal transport was impaired in *tkv* mutants and when a novel

inhibitor to the BMP pathway, Spict, was overexpressed (Wang et al., 2007). In *tkv* mutants, anterograde and retrograde fast axonal transport of Synaptotagmin (Syt)-GFP was reduced and large non-motile Syt-EGFP accumulations were seen (Wang et al., 2007). We wondered if this was a general response when the pathway is inhibited, as when Wit is not present, or one that was selective to *tkv* mutants and the Spict protein. When we examined Tkv-YFP transport in *wit* mutants (Supplementary Fig 3), there was not an increase in the amount of Tkv-YFP stationary vesicles and Tkv-YFP was transported in both directions (Supplementary Fig 3). In our data, there was a slight decrease in velocity in the *wit* mutants, but the Tkv-YFP vesicles retained movement (Supplementary Fig 3A). The reported impairment of transport when the BMP pathway is inhibited must be a specialized interaction between the BMP pathway and the Syt protein or the transport defect is specific to *tkv* mutants and not shared by *wit* mutants.

Wit levels modulate Tkv-YFP vesicle trafficking along the axon

After observing the Wit dependent decrease in Tkv vesicular accumulation but not a significant reduction in velocity, we wondered what the effects were on the amount of Tkv vesicular transport. If Wit and Tkv travel in an endosome to relay the pathway signal, as in the signaling endosome model, we would expect to see alterations in Tkv vesicle transport when Wit is absent or overexpressed.

To better visualize the flow of moving vesicles, we bleached a center portion of the axon and then quantified the particles that were transported into the bleached area imaging the area a total of 60 seconds (Fig 8A). In different images the axon sizes would vary so we normalized the particle data by dividing by the width of the axons quantified.

Additionally, to eliminate viewer bias, we assigned a fluorescence intensity threshold to the movies counting only those vesicles that reached or surpassed the fluorescent threshold.

Compared with control, we observed differences in the number of Tkv-YFP vesicles that were being transported in both the *wit* mutant and when Wit is overexpressed. For retrograde transport, in *wit* mutants there was a 57% decrease in retrograde moving Tkv-YFP particles. When Wit is overexpressed, there was a 409% increase in moving retrograde Tkv-YFP particles (Fig 8B). This decrease of vesicular Tkv-YFP in a *wit* mutant and increase of Tkv-YFP when Wit is overexpressed indicates that the amount or activity of the type II receptor regulates the type I receptor vesicle transport and further supports that the transport of these receptors is activity dependent.

We also examined the amount of anterograde moving Tkv-YFP particles while varying Wit expression levels. In the *wit* mutant, there was not a significant difference of the amount of anterograde moving Tkv-YFP vesicles when compared with control. When Wit is overexpressed however, we do see a 220% increase in the amount of Tkv-YFP vesicles traveling in an anterograde direction (Fig 8C). In the signaling endosome model, packaging and active signaling transport could only explain an increase in receptor transport amounts in the retrograde direction. Seeing an increase in overall amounts of Tkv vesicle transport suggests that there is another method of regulation taking place, such as a reduction in degradation. A possible explanation for this data is that there is decreased degradation or up-regulation of the Tkv receptor when there is more signaling taking place.

DN-Glued reduces transport of the receptors

DN-Glued has been shown to inhibit the BMP pathway in motoneurons (Marques et al., 2003; McCabe et al., 2003). Glued is a subunit of the Dynein/Dynactin complex, and its truncated form DN-Glued acts as a dominant negative to inhibit Dynein activity. When DN-Glued is expressed, it inhibits the Wit pathway as assessed by accumulation of p-Mad in the motoneuron nuclei (Marques et al., 2003; McCabe et al., 2003) and results in small synapses (Eaton et al., 2002; McCabe et al., 2003). DN-Glued has been shown to inhibit the Wit pathway, suggesting that this pathway depends on retrograde trafficking. It is not known, however, the pathway member that DN-Glued inhibits. We wondered how receptor transport was affected when DN-Glued is expressed. When DN-Glued is co-expressed with Tkv-YFP (Figure 9) or Wit-GFP (not shown), we observe a large increase in stationary receptor vesicles (vertical particle tracks in kymograph, Figure 9A-B). Quantification yields a 75% decrease in the fraction of moving vesicles upon DN-Glued expression (Figure 9C). Additionally, there is a significant reduction in retrograde velocity of Tkv-YFP without a reduction in anterograde velocity (Supplementary figure 4A-B). Finally, of the remaining moving Tkv-YFP particles, a larger fraction of them is moving in an anterograde direction compared to control when DN-Glued is expressed (Supplementary figure 4C). All these observations are consistent with preferential blocking of retrograde motor Dynein by DN-Glued and a consequent decrease in retrograde transport of Tkv. Differing from Tkv-YFP, when Wit-GFP is expressed simultaneously with DN-Glued, we observe a significant decrease in the velocity of both retrograde and anterograde traffic (Supplementary figure 5A and B). We additionally do not see an increase of the percentage of the amount of anterograde

moving Wit-GFP when compared to the control (Supplementary figure 5C). It has been proposed that Dynein and retrograde motors can be associated with the same cargo, and that there must be coordination between the two opposing motor proteins to elicit cargo transport (Ma and Chisholm, 2002). A possible explanation for the effect of DN-Glued on Wit anterograde transport is that the retrograde and anterograde motors associated with Wit are both affected by DN-Glued and therefore Wit transport in both directions is perturbed. Wit and Tkv being transported anterogradely by different motors is consistent with the different velocity distribution of anterograde moving Wit and Tkv particles (Fig 3, 4).

The retrograde transport of colocalized receptors is dependent on pathway activity

Under normal conditions the receptors colocalize in vesicles that move nearly exclusively in retrograde direction (Fig. 6). This is consistent with these vesicles carrying the BMP signal from NMJ to cell soma in the form of an activated receptor complex of Tkv and Wit. If this were the case, this retrograde traffic should be a signaling-dependent process, as is the situation with the neurotrophin receptors TrkA and TrkB (Heerssen et al., 2004). We decided to look at how the colocalized retrograde traffic would be affected by lack of the Gbb ligand. Behaving as genetic nulls, gbb^{1} and gbb^{2} mutants do not have any Gbb protein (Wharton et al., 1999), but despite extensive embryonic and larval lethality a small fraction of the animals make it to third instar larva. When we expressed Wit-CFP and Tkv-YFP in motoneurons of *gbb* mutants, we found that the receptors were still co-localizing, both at the NMJ and in axons. However, quantification of axonal traffic shows a decrease of retrograde transport and an increase in anterograde traffic of

the vesicles containing both Wit-CFP and Tkv-YFP, whose distribution now resembles that of vesicles carrying just one type of receptor (Fig 10). In the control, 95% of colocalized vesicles are moving in the retrograde direction. In the *gbb* mutant retrograde traffic of the colocalized particles was reduced to 60% and anterograde transport of particles was increased to 40% (Fig 10C). This loss of retrograde directionality indicates BMP signaling drives vesicles containing both BMP receptors towards the cell body. These data suggest that under control conditions Wit-CFP and Tkv-YFP are forming an active receptor complex. This active complex would signal directly or through uncharacterized adaptors to molecular motors attached to the endocytic vesicle to effect retrograde traffic, and thus relay the BMP signal from synaptic terminal to cell body in the form of a BMP signaling endosome. In *gbb* mutants the receptors still co-localize, but we propose that they are not interacting in an active complex. In the absence of the ligand, the receptors lose directionality, implying that when the pathway is active the colocalized receptors traffic in a BMP signaling endosome.

Discussion

Because neurons are such long cells they are specifically susceptible to errors in transport. There are numerous devastating neurodegenerative diseases that have errors in axonal transport (Goldstein, 2003; Hafezparast et al., 2003; Holzbaur, 2004). Specifically, mutations in the motor protein Dyenin that is responsible for retrograde axonal trafficking, has been linked to motor neuron degeneration (Hafezparast et al., 2003). When Dynein is mutated, critical neuron survival signals cannot be properly transported from the synaptic terminal to the cell body (Heerssen et al., 2004). The

health of neurons is dependent upon the proper communication between the distal and proximal ends of the cell, and when axonal transport is inhibited it leads to neuronal death (Goldstein, 2003; Hafezparast et al., 2003; Holzbaur, 2004).

Neurotrophins are critical target-derived retrograde signals that ensure the survival and growth of selected neurons. Neurotrophins have been shown to travel in a signaling endosome to propagate pathway signaling (Delcroix et al., 2003; Ye et al., 2003). In this model of signaling, the ligand in the pathway binds the receptors and then the complex is endocytosed and retrogradely transported in a Dynein dependent manner along the axon to activate downstream effector molecules. Because of the neuron's dependence on active transport for communication between its distal and proximal ends, it is probable that many critical retrograde factors travel in a signaling endosome to propagate signaling.

The BMP pathway is a retrograde signal that is necessary for proper synaptic terminal formation in Drosophila motoneurons. The ligand binds the receptors at the synaptic terminal membrane, which allows for the type II receptor to phosphorylate the type I receptor. The next step in the pathway is activation of the R-Smad transcription factor, which will allow it to enter the nucleus and regulate transcription. Yet to be identified is the pathway component that transports the active signal from the synaptic terminal to the cell body. The possible candidates are the receptors or the Smad transcription factor Mad, but neither has been shown to be transported along the axon. By studying the traffic of BMP receptors in motoneurons, we present the following critical evidence supporting the existence of a BMP signaling endosome.

First, we show that both type I and type II BMP receptors are present in early and recycling endosomes at the synaptic terminal. Receptor endocytosis is a critical requisite for generation of a signaling endosome, and similar to the endocytosis of neurotrophin receptors (Delcroix et al., 2003; Ye et al., 2003) we see colocalization of the BMP receptors with early endosome proteins Rab4 and Rab5.

Second, we show that the BMP receptors shuttle along the motoneuron axons. Just as with neurotrophins (Deinhardt et al., 2006; Delcroix et al., 2003; Heerssen et al., 2004), the BMP pathway is activated at the synaptic terminal but has its effect regulating transcription, thus it must depend on active retrograde transport to have efficient signaling at the nucleus. From our study, we can visualize transport of the BMP receptors in a bidirectional manner along the axon providing more evidence of a signaling endosome.

Third, we show that when the BMP pathway is inactive, as in *wit* mutants, there is a decrease of Tkv vesicles at the NMJ and of Tkv vesicle transport along the axon. We see a decrease in the amount of retrograde moving vesicles in *wit* mutants and an increase in the amount of Tkv vesicles when Wit is overexpressed. Our interpretation of this result is that when the Wit receptor is present, it is more readily able to recruit Tkv into vesicles that then are shipped retrogradely along the axon. In the neurotrophin signaling endosome model, the transport of Trk receptors is regulated by pathway activity (Delcroix et al., 2003; Ehlers et al., 1995). Our data demonstrate that the same is true in the BMP pathway. Analysis of the relationship between anterograde transport and signaling is more complicated. In the *wit* mutant there is no change of the amount of anterograde traffic, as expected if this traffic represents the steady state synthesis of

receptors. However, when Wit is overexpressed we see an increase in anterograde traffic indicating a positive feedback mechanism or decrease in Tkv degradation, resulting in more anterograde transported Tkv-YFP in response to extra retrograde signal. In summary, the trafficking of the type I receptor Tkv is affected by alterations in pathway activity, thus linking pathway activity and receptor transport.

Fourth, we show that DN-Glued inhibits BMP receptor retrograde transport, linking receptor transport and pathway signaling, and thus supporting the signaling endosome model for BMP signaling in motoneurons. Neurotrophin pathway activity and transport has been shown to be dependent on Dynein function (Heerssen et al., 2004). When transport by Dynein is interrupted, neurotrophins do not support neuron survival (Heerssen et al., 2004). DN-Glued is also an inhibitor of the BMP pathway, demonstrating that Dynein is necessary for pathway activation (McCabe et al., 2003). As of yet, it has been unclear which critical pathway component is affected by DN-Glued expression. The dramatic decrease in moving BMP receptors by DN-Glued expression strongly suggests that retrograde transport of the receptors plays a key role in pathway signaling.

Fifth, we show that the type I receptor Tkv and the type II receptor Wit co-localize in vesicles that move overwhelmingly in the retrograde direction. The vesicles that contain just one of the receptors have a moderate preference for the retrograde direction, with about 30-40% of the receptors traveling in an anterograde direction while 60-70% travel in a retrograde direction. We interpret the preference for retrograde traffic of co-localized receptors as evidence for a BMP signaling endosome that containins the activated receptors and relays the signaling event at the NMJ terminal to the cell body for

Mad phosphorylation. When we tested this hypothesis by looking at receptor traffic in the absence of ligand we still detected co-localized Tkv and Wit. However, in *gbb* mutants the strong preference for retrograde trafficking disappears and the directional distribution of colocalized receptor traffic is similar to that of single receptors. These receptors would be inactive, and then unable to drive retrograde transport of the vesicle. In the control situation the ligand activates the receptors, which then form an active complex able to confer a signal directing the vesicle toward the cell body. The directionality of the colocalized receptors disappears in *gbb* mutants, providing strong evidence that the BMP receptors travel retrogradely in a signaling endosome. The presence of co-localized receptors in the absence of ligand may reflect co-internalization at the plasma membrane independent of signaling or the fusion of endosomes containing the individually internalized receptors. Whether this also occurs in the control situation at a low level or reflects abnormal membrane traffic in *gbb* mutants, perhaps as a result of defects in the microtubule cytoskeleton (Wang et al., 2007), awaits further studies.

Taken together these results show that Gbb signaling at the synaptic terminal results in endocytosis of the type I receptor Tkv and the type II receptor Wit. These two receptors are transported retrogradely in a BMP signaling endosome to the cell body by Dynein-mediated axonal transport, and this transport is critical for BMP signaling in larval motoneurons. We propose that Wit and Tkv form a complex of active receptors in this endosome, and that this activity is required to confer retrograde direction to the signaling endosome movement and to phosphorylate Mad in the soma.

The existence of a BMP signaling endosome in *Drosophila* motoneurons raises a number of questions about BMP signaling mechanisms. One first aspect is how the BMP

receptors attach to the Dyenin motor. It is possible that this is mediated by direct interaction between the receptors and Dynein accessory proteins. The dynein light chain Tctex-1 interacts with, and is phosphorylated by BMPR-II indicating that it is a possible candidate as a Dyenin adaptor protein (Machado et al., 2003). An alternative hypothesis is that receptor phosphorylation upon activation allows interaction with an unknown scaffolding molecule. The receptors could then act on this or other molecules in the complex to drive traffic preferentially towards the cell body. An example of a scaffolding protein that links vesicles and motor proteins for targeted movement is the JNK-interacting protein 1 (JIP1) and its *Drosophila* ortholog, APLIP1 (Sandhya, 2008). Both of these proteins interact directly with Kinesin light chains (Sandhya, 2008). Mutations of *Aplip1* resulted in effects similar to that of kinesin inhibition, such as paralysis, axonal swellings, and reduced transport in both directions (Horiuchi et al., 2005). Although this data demonstrates that JIP is an adaptor protein for Kinesin, it also appears to have a role in retrograde transport with Dynein (Horiuchi et al., 2005). *Aplip1* mutant had a decrease of transport of mitochondria only in the retrograde direction (Horiuchi et al., 2005). Consistently, heterozygous mutation in *Aplip1* and *Dyenin heavy chain 64C* lead to axonal transport defects suggesting a genetic interaction (Horiuchi et al., 2005). It appears that there is also a control mechanism for JIP release of motor-cargo interaction. When Aplip1 interacts with proteins in the JNK pathway (Wallenda, Hemiperterous, or Basket) then Aplip1 disassociates from Kinesin (Horiuchi et al., 2007). This JNK dependent dissociation of kinesin and JIP scaffolding protein represents a control mechanism that can be used to regulate targeted transport of selected cargo, and

much like the BMP signaling endosome, allows control of traffic directionality in response to signaling (Horiuchi et al., 2007).

Another important question is where Mad is phosphorylated by the signaling endosome. p-Mad is present at the synaptic terminal (O'Connor-Giles et al., 2008; Wang et al., 2007) and nucleus (Marques et al., 2002; Marques et al., 2003), so it is possible that Mad could be phosphorylated at the NMJ and then transported to the cell body and eventually nucleus, independently or bound to the receptor endosome. A similar phenomenon occurs in neurotrophin signaling, where the transcription factor CREB has been shown to be transported along the axon with the receptor complex (Cox et al., 2008). Alternatively, it is possible that Mad is subject to two different phosphorylation events, at the NMJ and at the soma, much like ERK isoforms are phosphorylated by neurotrophin receptors both at the synaptic terminal and the cell soma (Cosker et al., 2008; Watson et al., 2001). It is possible that synaptic p-Mad is a different population with a different role than p-Mad in the nucleus, perhaps local signaling through cross talk with other pathways (Guo and Wang, 2009).

A final mechanistic question that a BMP signaling endosome poses is the fate of the ligand, and it would be interesting to determine if the ligand is part of the BMP signaling endosome, holding together the activated receptor complex. This would further parallel the situation in the neurotrophin signaling endosome.

The existence of a BMP signaling endosome in Drosophila larval motoneurons also raises questions as to how general this mechanism is for sorting and regulating the TGF–ß signal. In *Drosophila* wing imaginal discs, there is an example of a BMP signal that is reliant on an endosomal protein to properly distribute a gradient of the morphogen

Decapentaplegic (Dpp) (Bokel et al., 2006). Smad Anchor for Receptor Activation (SARA) is an endocytic protein that regulates the subcellular distribution of Smad proteins and presents these R-Smads for phosphorylation to the activated TGF-ß receptor complex (Fumiko Itoh, 2002). SARA, the Dpp ligand, and the type I receptor Tkv were found in a population of endosomes that associated with the spindle machinery in wing imaginal discs (Bokel et al., 2006). During mitosis this association allowed for equal distribution of the Dpp morphogen into the two daughter cells (Bokel et al., 2006). In *sara* mutants, the receptors were not targeted to the spindle and resulting p-Mad distribution was severely altered (Bokel et al., 2006). SARA activity demonstrates that receptor endosome targeting on microtubules has a major significance in the distribution of BMP pathway activity, possibly representing a signaling endosome in the wing imaginal disc. Whether the type II receptor Punt is also present in that endosome is unknown.

The role of TGF-ßs as neuroprotective signals for mammalian neurons suggest that disregulation of BMP signal endosome transport could have implications for neurodegenerative diseases as well as other diseases caused by faulty BMP or TGF-ß pathways. A TGF–ß pathway has been shown to contribute to Alzheimer's pathology (Tesseur et al., 2006) and syndromes attributed to TGF–ß receptor mutations involve developmental delay (Ades et al., 2006; van Steensel et al., 2008). Additionally, in other cell types BMPs and TGF-ß mutations have been the cause of diseases such as primary pulmonary hypertension (Consortium et al., 2000; Thomson et al., 2000) and cancer (Biswas et al., 2008). To thoroughly search for the causes of neurodegenerative diseases, researchers should examine components that assist in pathway signaling as well as errors

in the pathway components themselves. In pathways that depend on signaling endosomes, it would be important to examine accessory proteins such as endocytic machinery or motor proteins that could contribute to errors in signaling. We have taken the first steps to identify the signaling mechanism for this BMP pathway critical for motoneuron synaptic development, which will help us understand the role of related pathways in human disease.

Materials and Methods

Constructs

Using NotI sites in the c-terminal of the Wit receptor, CFP and GFP was inserted into pUAST-Wit. Using an Acc651/Age1 cleavage sites, *tkv* ORF was inserted into pUAST-EYFP vector (GM, unpublished). All subcloned transgenes were prepared for germ line transformation (Model Systems Genomics, Duke University, Eric Spana, Director), and inserts in all three major chromosomes mapped and balanced.

Fly stocks and genetics

We used Gal4 drivers OK6 (2nd chromosome) (Aberle et al., 2002), OK371 (2nd chromosome) (Mahr and Aberle, 2006) and BG380 (X chromosome) (Budnik et al., 1996) to express BMP receptors in larval motoneurons. In most cases second chromosome inserts of the receptor transgenes were recombined with the drivers. We received UAS-DN-Glued from Thomas Hays.

To identify that the receptors are colocalizing with Rab proteins, we used UAS>Rab5-GFP (Entchev et al., 2000) and UAS>Rab4-RFP (Bloomington #8505). The

genotypes were as follows: OK6>Gal4, UAS>Tkv-YFP/+; UAS>Rab5-GFP/+ and OK6, UAS>Tkv-YFP/ UAS>Rab4-RFP.

See Flybase (http://flybase.org/) for the description of the mutant alleles used: wit^{A12}, wit^{B11}, gbb^{1} and gbb^{2}, tkv^{5}, tkv^{8}. The *gbb* mutants that were analyzed for receptor colocalization derived from the cross of OK6, UAS>Wit-CFP, gbb^{2}/CyO-eYFP2 and BG380>Gal4, Tkv>YFP/FM7i-GFP; gbb^{1}/Cyo-eYFP2, and had the following genotypes: BG380>Gal4, UAS-Tkv-YFP/+; OK6>Gal4, UAS-Wit-CFP, gbb^{2}/ gbb^{1} (mutant) and BG380>Gal4, UAS-Tkv-YFP/+; OK6>Gal4, UAS-Wit-CFP, gbb^{2}/ + (control). The analysis of colocalized speed was performed using the following genotype: OK6, UAS>Tkv-YFP; UAS>Wit-CFP.

Immunofluorescence

For analysis of UAS-Tkv-YFP fluorescence intensity, third instar larvae with the appropriate genotype were selected by the presence of fluorescence in salivary glands before dissection. These larvae were dissected and fixed with 4% PFA in PBS for 5 minutes so not to quench the YFP fluorophore. All larvae that compared to one another were stained simultaneously in the same dissecting dish. After immunostaining the larvae pelts were cleared in 80% Glyoh PBS and mounted in Permafluor.

When examining Tkv-YFP fluorescence intensity at the synaptic terminal, anti-HRP was used to identify the presynaptic membrane. After fixation the larvae was blocked in PBS containing 0.1% Triton-X100 and 2.5% BSA. The anti-HRP Cy3 conjugate was from Jackson Immuno Research and used in a dilution of 1:200 in block and incubated overnight at 4ºC. DAPI was also incubated with the anti-HRP Cy3 at a

dilution of 1:200. After incubation, the larvae were washed in PBS containing 0.2% Triton-X100 six times for 6-10 minutes each.

When testing for fluorescence intensity at the ventral ganglion, the larvae were fixed as above, but stained only with Dapi. Dapi was used at a dilution of 1:200 and incubated with the larvae after dissection.

Microscope confocal imaging

Timelapse movies were obtained in a Nikon Eclipse TE2000-U Lambda LS Stutter microsope equipped with a Perkin Elmer Ultraview confocal head and an Ultraview ERS Rapid Confocal Imager using Ultraview or Volocity software. The animals were dissected live in HL3 medium (Stewart BA, 1994), pinned to Sylgard in a specially designed imaging chamber, eviscerated keeping CNS, nerves and muscle intact and covered with a coverslip. The axonal transport was imaged on a 1.49 and 1.30 oil 100x objective. When imaging one receptor, exposure times of 100-200 msec were used (488 laser, Bin 2, emission filter 527nm) for acquisition speeds of 9-5 frames per second (fps). For dual color imaging, spectral separation setting was used, with exposure times of 30-300msec for Tkv-YFP (514 laser, Bin2, emission filter 587nm) and 200-300 msec for Wit-CFP (440 laser, Bin2, emission filter 485nm, 705nm), for a combined acquisition speed of 0.5-1 fps.

Confocal imaging of fixed tissues for synaptic and ventral ganglion comparisons was completed on a Leica SP2. All of compared images were taken at the same settings.

Spectral separation between GFP and YFP

We imaged Rab5-GFP and Tkv-YFP on the Leica SP2 microscope to spectrally separate YFP and GFP. The Leica settings were as follows: YFP channel: Excitation laser 514nm 100% power, emission band pass 565-612, pinhole 370, dichroic mirror- DD 458/514. GFP channel: Excitation laser: 488-100%, Emission Band pass- 496-515, Pinhole 370, dichroic mirror- RSP500. Controls containing only YFP or only GFP were used to ensure lack of cross talk between the channels.

Quantifying fixed Tkv-YFP fluorescence

Vesicular distribution of Tkv was assessed measuring the YFP fluorescence intensity of Tkv-YFP endosomes per synaptic terminal area defined by the presynaptic membrane marker anti-HRP. Tkv-YFP driven by the OK6 driver was imaged on a Leica SP2 upright microscope with an SP2 confocal head system on an 100x objective with the following settings: YFP channel- laser line 514-100%, Emission Band Pass 519-571, dichroic mirror DD 458/514, Pinhole 253.47, No binning. Alexa 568 channel: Excitation Laser line 561-100%, Emission Band Pass 606-728, dichroic Mirror 488/568, Pinhole 419.81, No binning. All comparison images were imaged with sequential scan and were imaged in two channels, presynaptic HRP (Red) and Tkv-YFP (Green). An example of threshold analysis is in shown in the supplementary data (Supplementary Fig 5). Tkv-YFP fluorescence intensity was analyzed using IPlab.

Quantifying vesicle amounts

To examine the effect of Wit receptor levels on Tkv vesicle amounts, we dissected third instar larvae live in HL3 medium (Stewart et al., 1994). The larvae had

the following genotypes: OK6>Gal4, UAS>Tkv-YFP/+ (control), OK6>Gal4, UAS>Tkv-YFP/+; wit[A12]/wit[B11] (*wit* mutant), OK6>Gal4, UAS>Tkv-YFP/ UAS>Wit (Wit overexpressed). Utilizing the Perkin Elmer, Ultraview software, we imaged along the axon and bleached a center portion for a clear view of moving vesicles. The microscope settings were: laser line 514-20%, Bin2, emission filter 587nm, exposure 100ms. Settings for photokinesis: bleaching laser-100%, Pre-bleach 30 frames, post-bleach 600 frames, bleach cycles 30. The time-lapse movies were then uploaded into ImageJ and a threshold was assigned to the image. All particles that met or surpassed the threshold that crossed the initial bleaching line on the corresponding side of the ROI within 60 seconds were counted.

Statistical Analysis and Graphs

Statistical analysis was performed and bar graphs were created using Prism4 statistical software (GraphPad Software Inc.) Comparing the experimental data set to the control yielded all p-values. A student's unpaired t-test with one-tailed p-value was completed for all data sets. Chi-Squared tests were applied to population distributions to get the p-values for the comparison pie charts. The pie charts were made in Excel (Microsoft).

Generating Kymographs

We used ImageJ (Version 1.38x, NIH) and the plug-in "Kymograph" (EMBL website http://www.embl-heidelberg.de/eamnet/html/body_kymograph.html) to create kymograph plots. On an image stack a line was drawn tracing the axon and ImageJ

generated a Kymograph. The program does this by taking the area of the drawn line following it through time, stacking each frame under the previous one, making a two-dimensional view of particle movement though time. In this way, time is descending in the Y plane and distance is in the X plane. Because the Y plane is time, the smaller the slope of a trajectory equals the faster the particle is moving. Slower particles had very steep slopes while faster particles had smaller slopes, representing closeness to horizontal lines. Stationary particles (vertical lines) were not measured. The ventral ganglion was always to the right of axon movies. Particles with descending slopes are moving retrograde, towards the ventral ganglion, while particles with asending slopes are moving anterograde toward the synaptic terminal. A more detailed description is available upon request.

Measuring velocities

Using the straight-line tool in Image J, a line was drawn over the longest pathway section of movement of each trajectory. Then the Macros plug-in "tsp050706" was used. If the particle changed speeds, the longest continuous section of one speed was measured. For most Kymographs, all sloping pathway lengths were measured and it was recorded which direction each particle was traveling. Kymographs were excluded from the population distribution pie graph if all of the sloping trajectories within the kymograph were not measured, which is the reason the number of moving particles differs in the velocity analysis and the population distribution pie graph. A more detailed description is available upon request.

Kymograph analysis of co-localized Tkv-YFP and Wit-CFP

Using Image J, the two channels of each movie were opened as different stacks. Using the "Colocalization Analysis" plug-in, an 8-bit stack was created where the colocalized particles in each frame were highlighted with white. After this stack was created we generated a kymograph and analyzed the velocity of colocalized particles using the methods described above.

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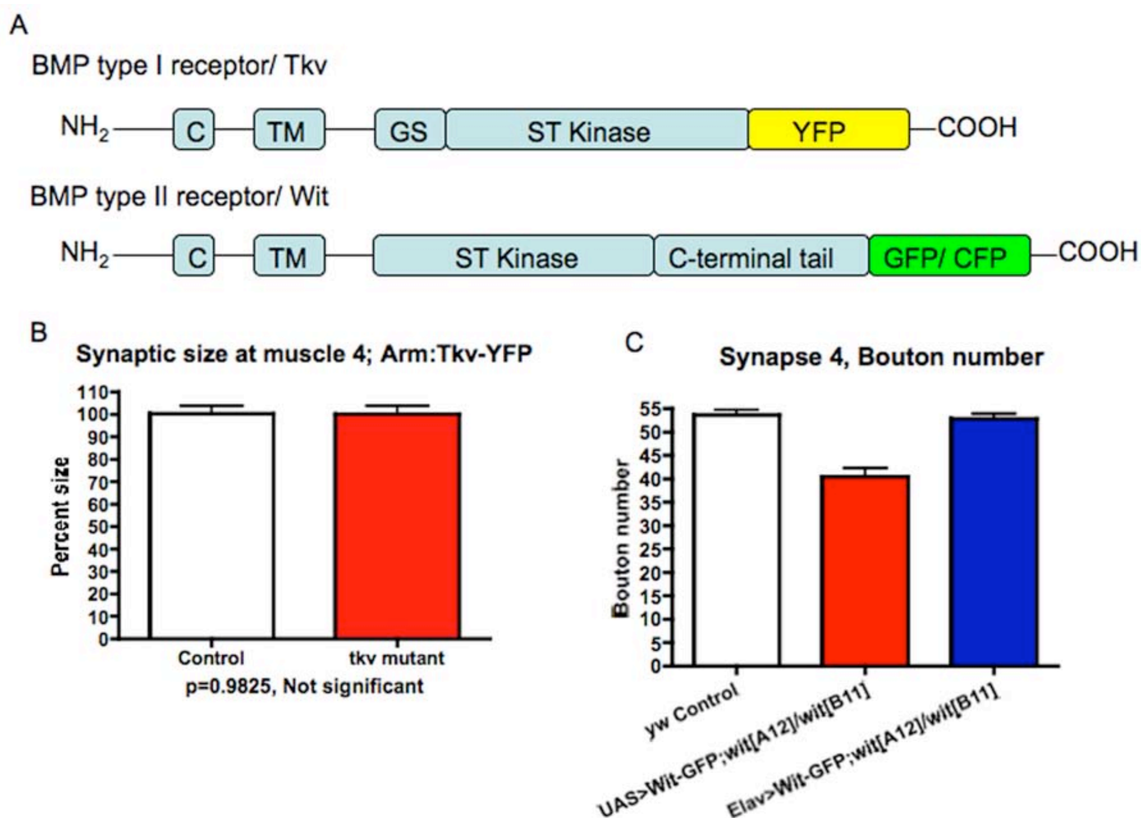


Figure 1: Fluorescent fusion proteins of the receptors are functional

Transgenes of the Type II and Type I receptor were engineered to have the XFP protein attached to the C-terminal end of the receptor (A). Arm:Tkv-YFP rescued synaptic terminal size in at muscle 4 in *tkv* mutants (tkv^{5}/tkv^{8}) (B). Elav:Wit-GFP rescued bouton number in *wit* mutants (wit^{A12}/wit^{B11}) (C). *wit* mutants are significantly different from control and rescued groups ($p<0.0001$) while the control and rescue groups are not significantly different from each other (C).

Note: Panel "C" experiment and figure completed by James B. Machamer

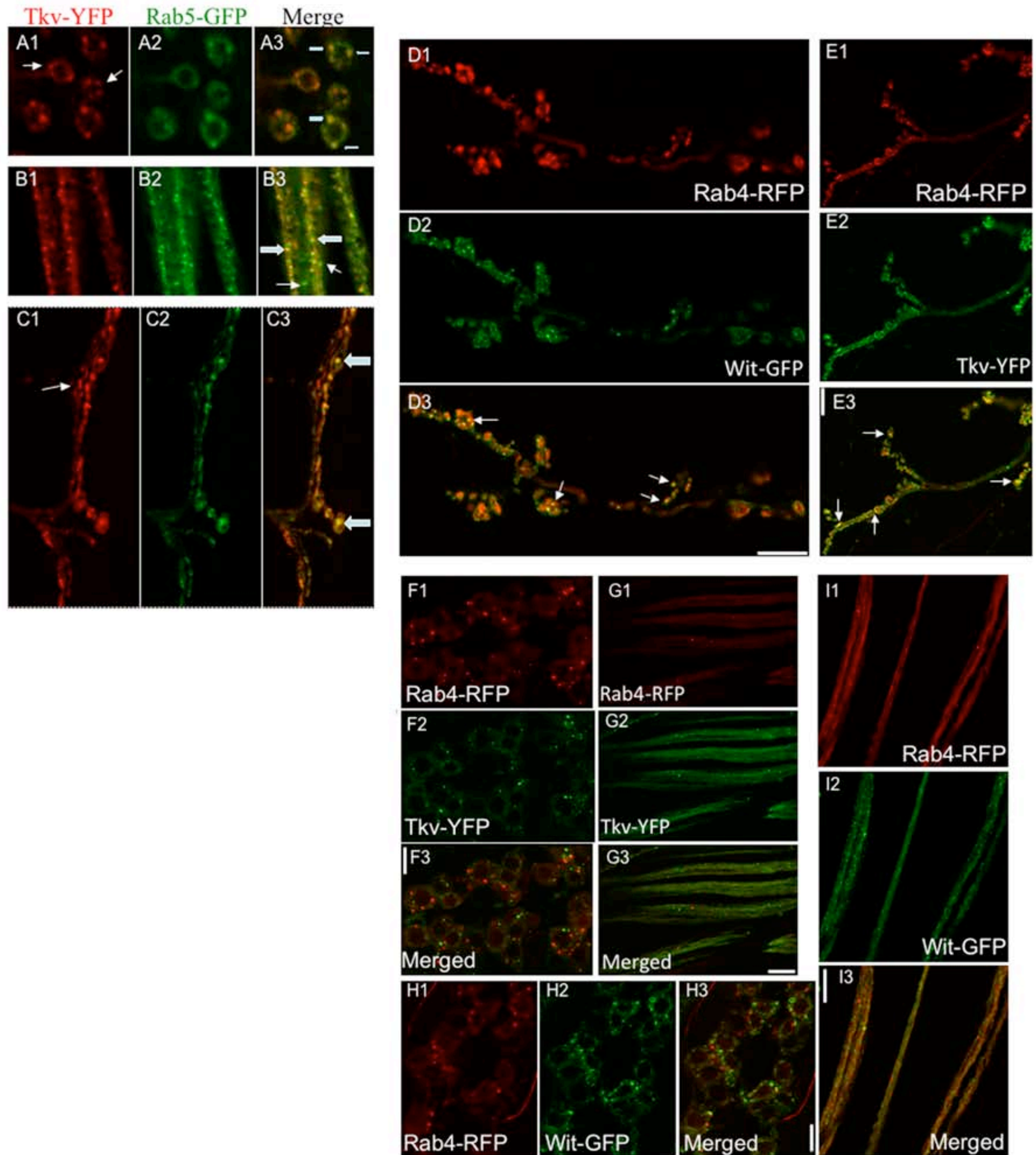


Figure 2: Receptors colocalize with Rabs labeling early endosomes

Rab5-GFP colocalizes with Tkv-YFP at the cell body (A1-A3), along the axon (B1-B3), and at the synaptic terminal (C1-C3). Wit (D1-D3) and Tkv (E1-E3) colocalize with Rab4 at the synaptic terminal. Rab4 does not display colocalization with Tkv along the axon (G1-G3) or at the cell bodies (F1-F3). There is also no colocalization between Wit and Rab4 along the axon (I1-I3) or at the cell bodies (H1-H3). All transgenes are expressed with OK6 driver. All scale bars are 10 microns in length.

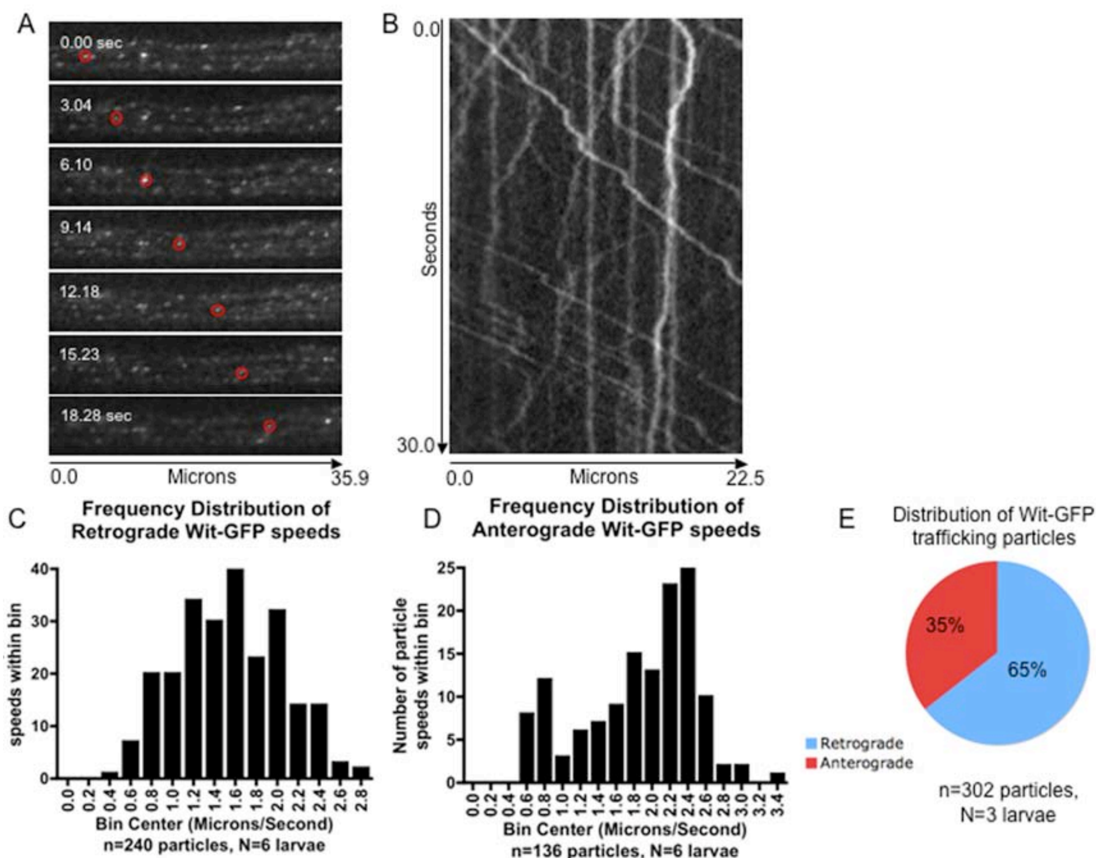


Figure 3: The Wit receptor is transported along the axon

Wit is transported along the axon in vesicles (A). A kymograph was constructed tracing vesicle movement on the x-axis, through time on the y-axis (B). This kymograph demonstrates retrograde Wit vesicle movement (descending slope projections), anterograde Wit vesicle transport (ascending slope projections) and stationary Wit vesicles (vertical lines). When we analyzed the retrograde movements of Wit-GFP we measured 240 vesicles in 6 larvae (C). The range of punctae speed was 0.47-2.8 μm/s. The median of the speeds was 1.52 μm/s while the mean of the group was 1.55 μm/s. The standard deviation was 0.49 μm/s and the standard error was 0.032 μm/s. For anterograde Wit-GFP transport we measured 136 vesicles in 6 larvae (D). The range of speeds was from 0.53-3.33 μm/s. The median speed was 2.03 microns per second while the mean was 1.87 μm/s. The standard deviation was 0.64 μm/s and the standard error was 0.055 μm/s. When we plotted the distribution of all moving Wit vesicles within the kymographs, we found that 65% of the vesicles were moving in a retrograde direction while 35% moved in an anterograde direction (E).

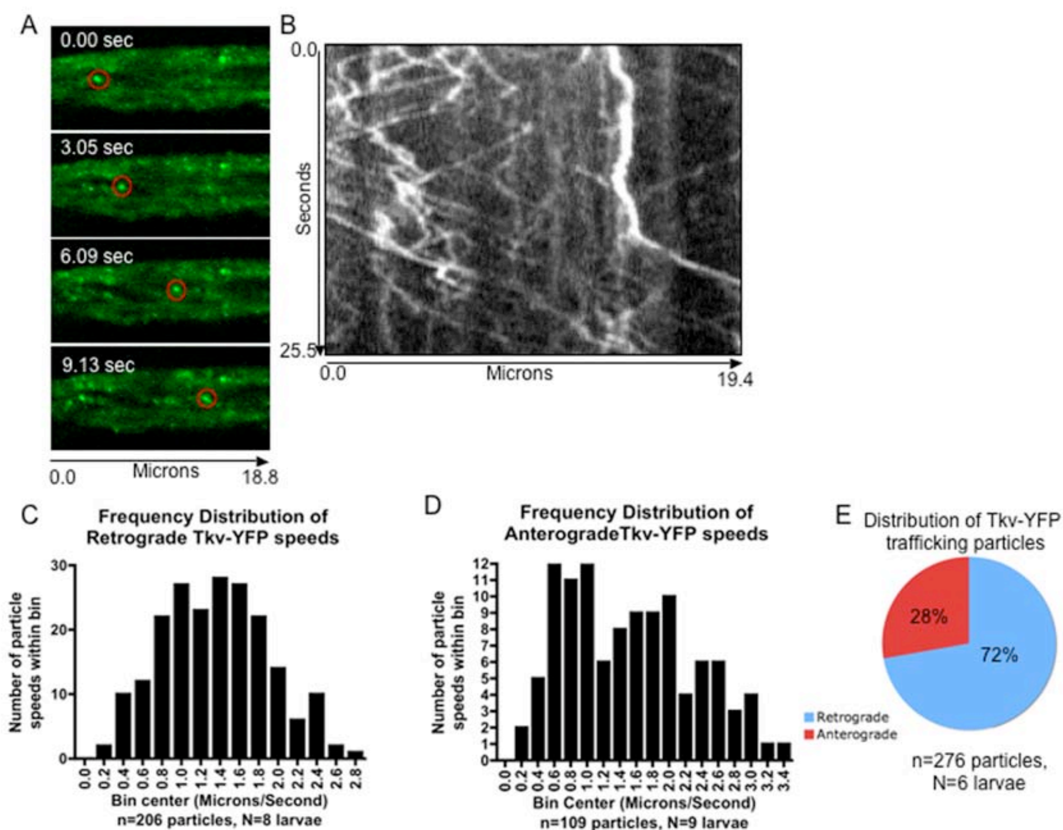


Figure 4: The Tkv receptor is transported along the axon

Tkv receptor is transported along the axon in vesicles (A). A kymograph was constructed tracing vesicle movement on the x-axis, through time on the y-axis (B). This kymograph demonstrates retrograde Tkv vesicle movement (descending slope projections), anterograde Tkv vesicle transport (ascending slope projections) and stationary Tkv vesicles (vertical lines). When we measured retrograde Tkv-YFP analysis, the total number of particles tracked was 206 in 8 larvae (C). The range of vesicle speeds was 0.27-2.7 µm/s. The median speed was 1.33 µm/s while the average was 1.36 µm/s. The standard deviation was 0.55 µm/s and the Standard Error was 0.038 µm/s. When we measured the anterograde speeds for Tkv-YFP, we measured a total of 109 punctae in 9 larvae (D). The range of speeds was from 0.19-3.36 µm/s. The median speed was 1.46 µm/s and the mean was 1.50 µm/s. The standard deviation was 0.78 µm/s and the standard error was 0.075 µm/s. When we plotted the distribution of all Tkv moving vesicles within the kymographs, we found that 72% of the vesicles were moving in a retrograde direction while 28% moved in an anterograde direction (E).

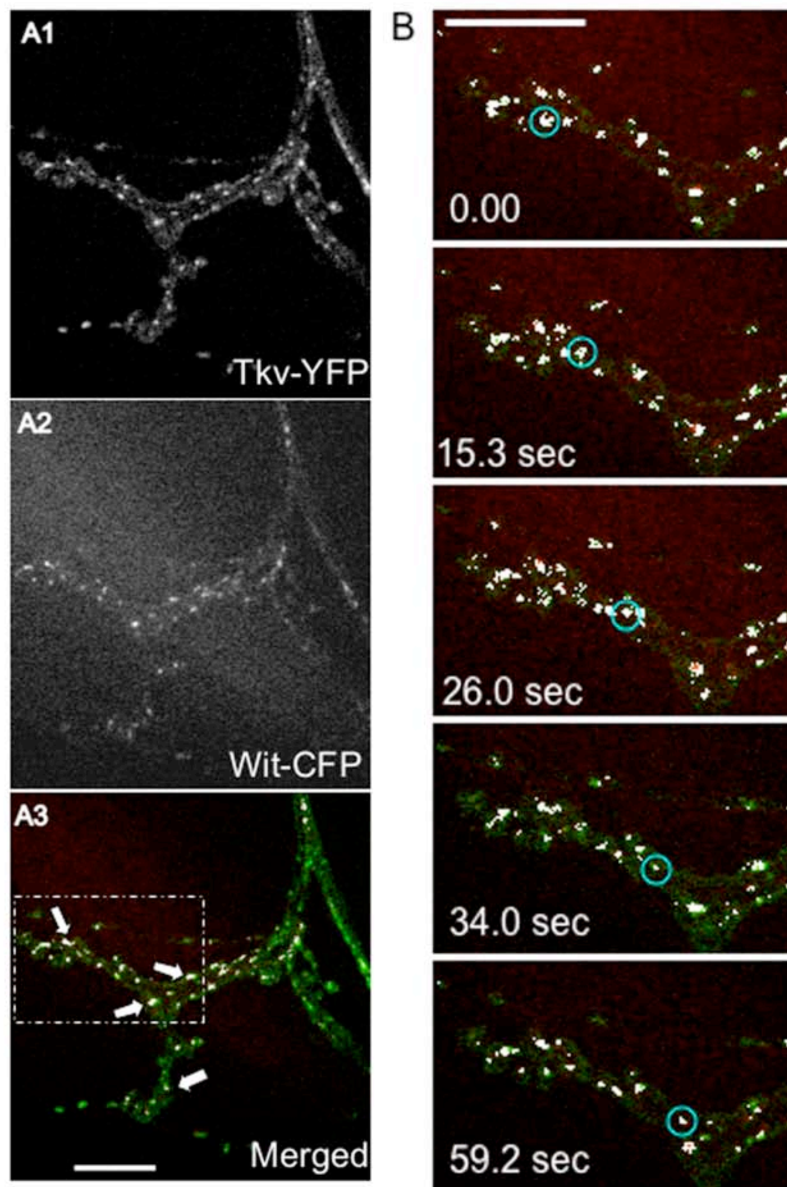


Figure 5: Wit and Tkv colocalize at the synaptic terminal in dynamic vesicles.

Tkv-YFP (A1) and Wit-CFP (A2) colocalize at the synaptic terminal in still frames of two-color time-lapse. Colocalized vesicles are labeled in white (A3, arrows). An enlargement of this area through time shows that vesicles dynamically move at the syanaptic terminal (B). The highlighted vesicle is moving in a retrograde direction, away from the distal end of the synaptic terminal (B-blue circle). Tkv-YFP and Wit-CFP were expressed with motoneuron drivers BG380 and OK6. The scale bar is 10 microns.

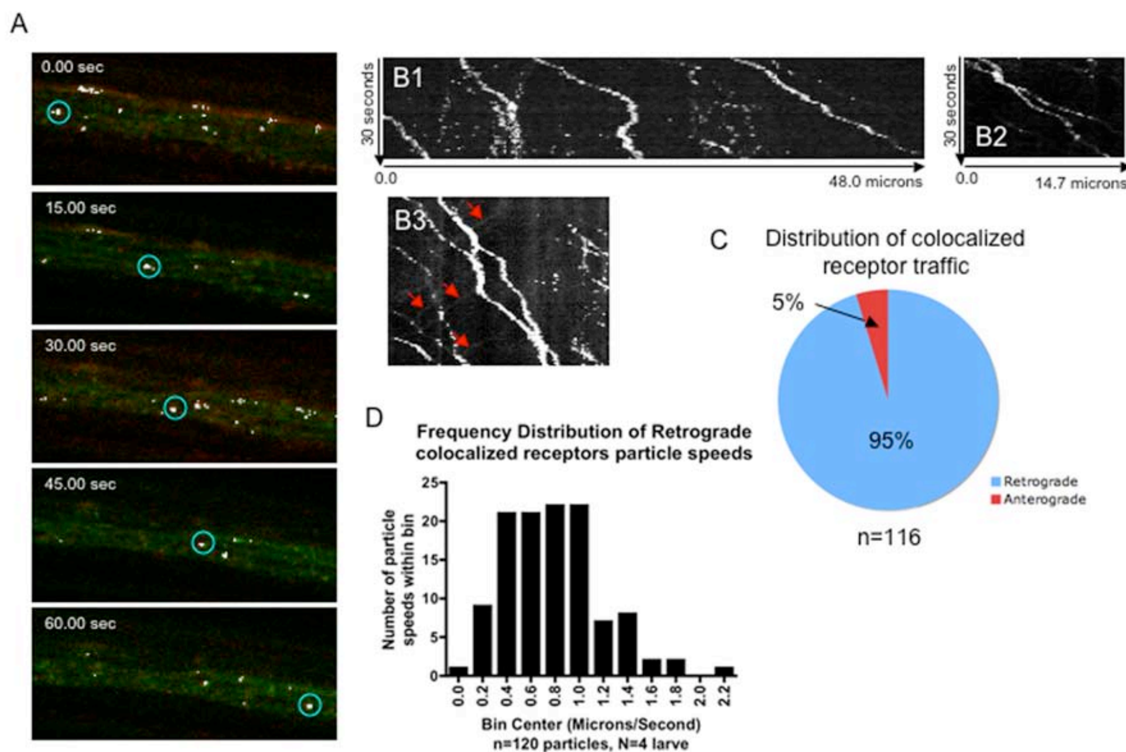


Figure 6: Wit and Tkv colocalize along the axon in predominately retrograde moving vesicles

Wit-CFP and Tkv-YFP were found to colocalize in dynamic vesicles along the axon (A- white punctate structures). Kymographs of the colocalized particles projection paths were created (B1 and B2). In the Kymographs, note that colocalized particles are predominantly moving in a retrograde direction (descending slope projections) (B1-B3). Anterograde (ascending slope projections) vesicle movement is also observed (B3- red arrows) but represents only single receptor vesicle movement (gray projections) and not colocalized receptor vesicles (white projections). For velocity analysis of retrograde colocalized receptor vesicles, we measured a total number of 120 particles in 4 larvae (D). The range of speed was 0.09-2.20 µm/s. The median speed was 0.75 µm/s while the mean was 0.78 µm/s. The standard deviation was 0.39 µm/s and the standard error of the mean was 0.037 µm/s. When we plotted the direction of moving particle in the kymographs (C), we found that 95% of colocalized vesicles are moving in a retrograde direction. 5% of colocalized vesicles were found to move in an anterograde direction during the duration of the time-lapse movie.

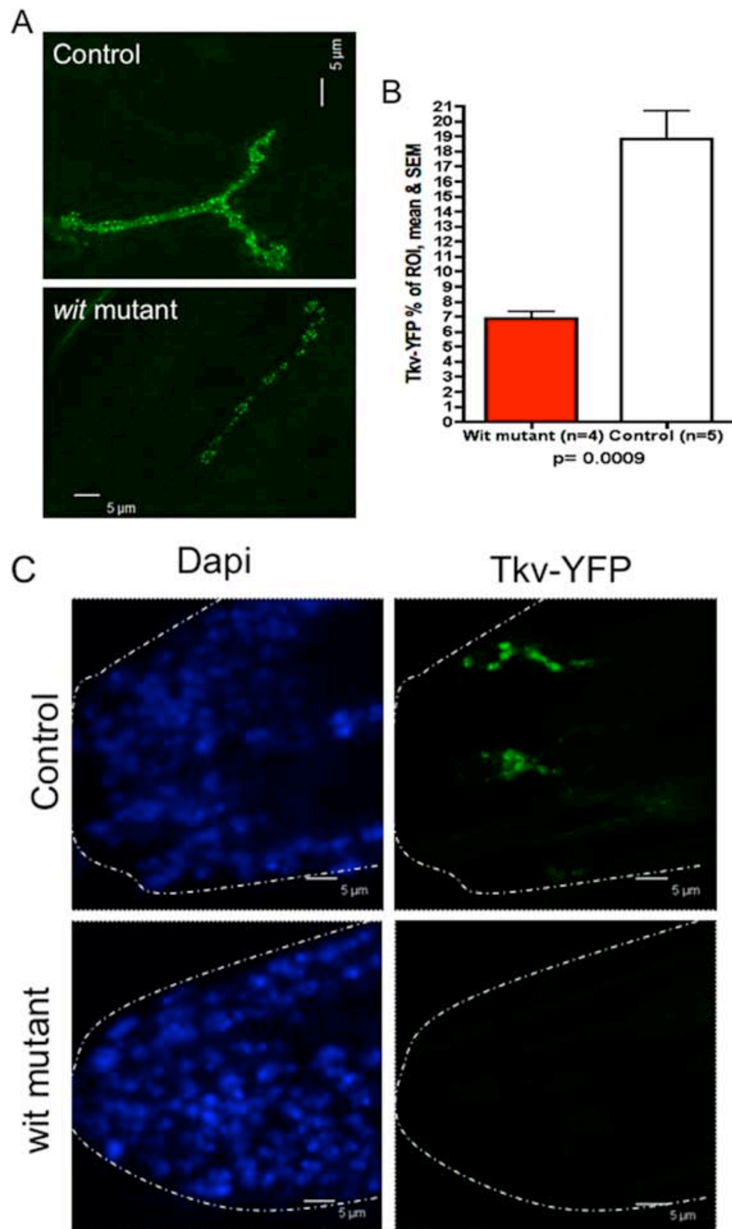


Figure 7: Tkv vesicle distribution is decreased at the cell body and synaptic terminal.

Tkv-YFP was expressed with OK6 motoneuron driver and larvae were dissected, fixed and stained. Images were taken of muscle 4 synaptic terminal (A) in control and *wit* mutant animals. Synaptic terminal muscle four images were quantified for fluorescence and measured for comparisons between control and *wit* mutants (B). Compared to control (n=5), *wit* mutants (n=4) had approximately 63% decrease in Tkv-YFP intensity at the synaptic terminal. Additionally, there was a decrease in Tkv-YFP vesicles at the ventral ganglion in *wit* mutants (C).

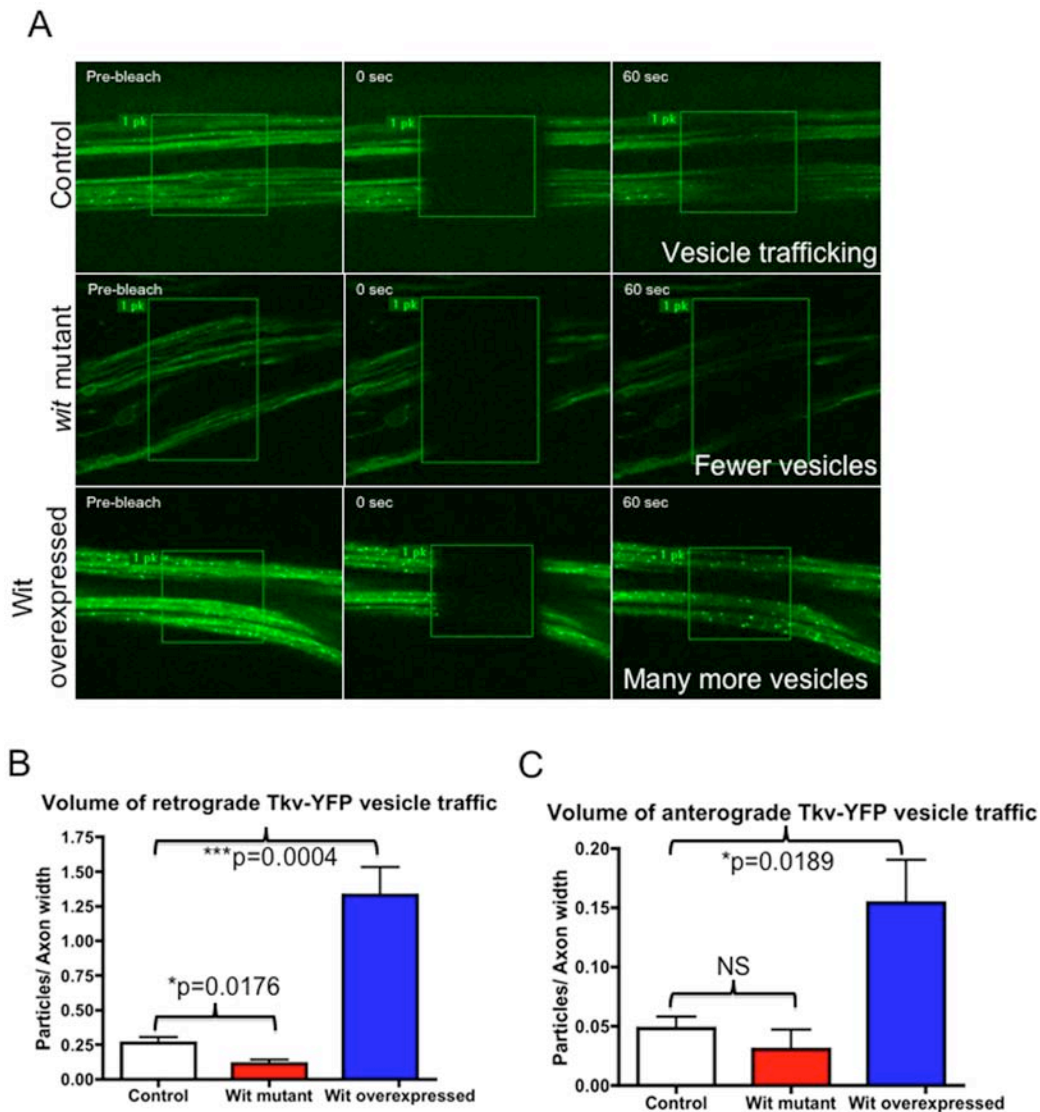


Figure 8: The amount of Tkv vesicle transport is dependent on Wit receptor levels

Tkv-YFP was expressed in motoneurons with varying levels of the Wit receptor: In control with endogenous levels, in *wit* mutants without any Wit receptor, or when Wit is overexpressed (A). To more clearly view the Tkv vesicle movement, we bleached the center area of the axons and then counted the vesicles moving into the bleached area within 60 seconds. We observed that there is a clear effect on the amount of Tkv receptor vesicles being trafficked. We saw a 57% decrease in retrograde moving Tkv vesicles in *wit* mutants and a 409% increase when Wit is overexpressed (B). When examining anterograde traffic of Tkv vesicles, there was no significant change between the control and *wit* mutant vesicle volume but there was a 220% increase when Wit is overexpressed (C). Control (N=3 larvae, 6 movies), *wit* mutant (N=3 larvae, 6 movies), Wit overexpression (N= 2 larvae, 6 movies).

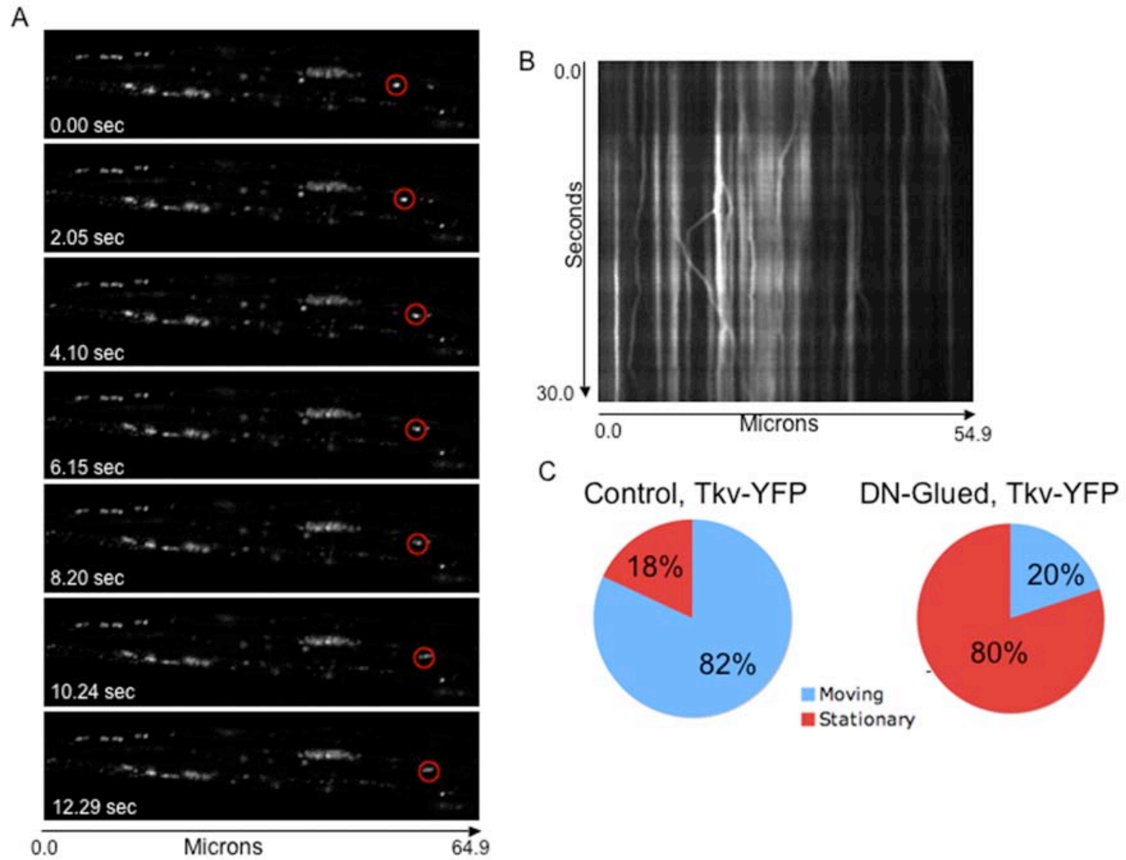


Figure 9: DN-Glued expression reduces the transport of Tkv-YFP

Tkv-YFP and DN-Glued were expressed in motoneurons. Tkv-YFP vesicle movement along the axon was severely impaired (A). The kymograph plotting Tkv-YFP vesicle movement shows an increase in vertical lines, demonstrating the increase in stationary vesicles (B). The population distribution of moving receptors was decreased 75% when DN-Glued is expressed (C).

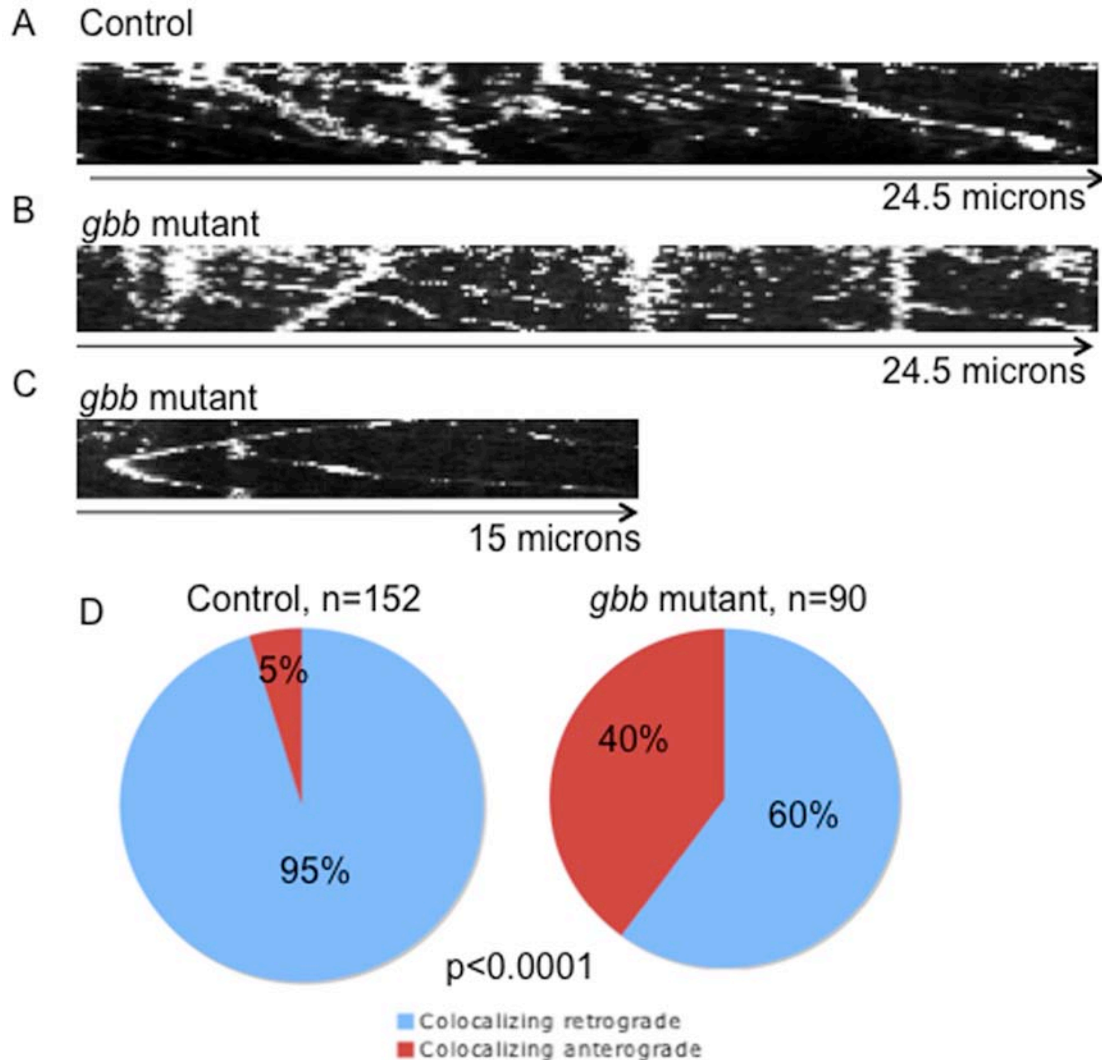
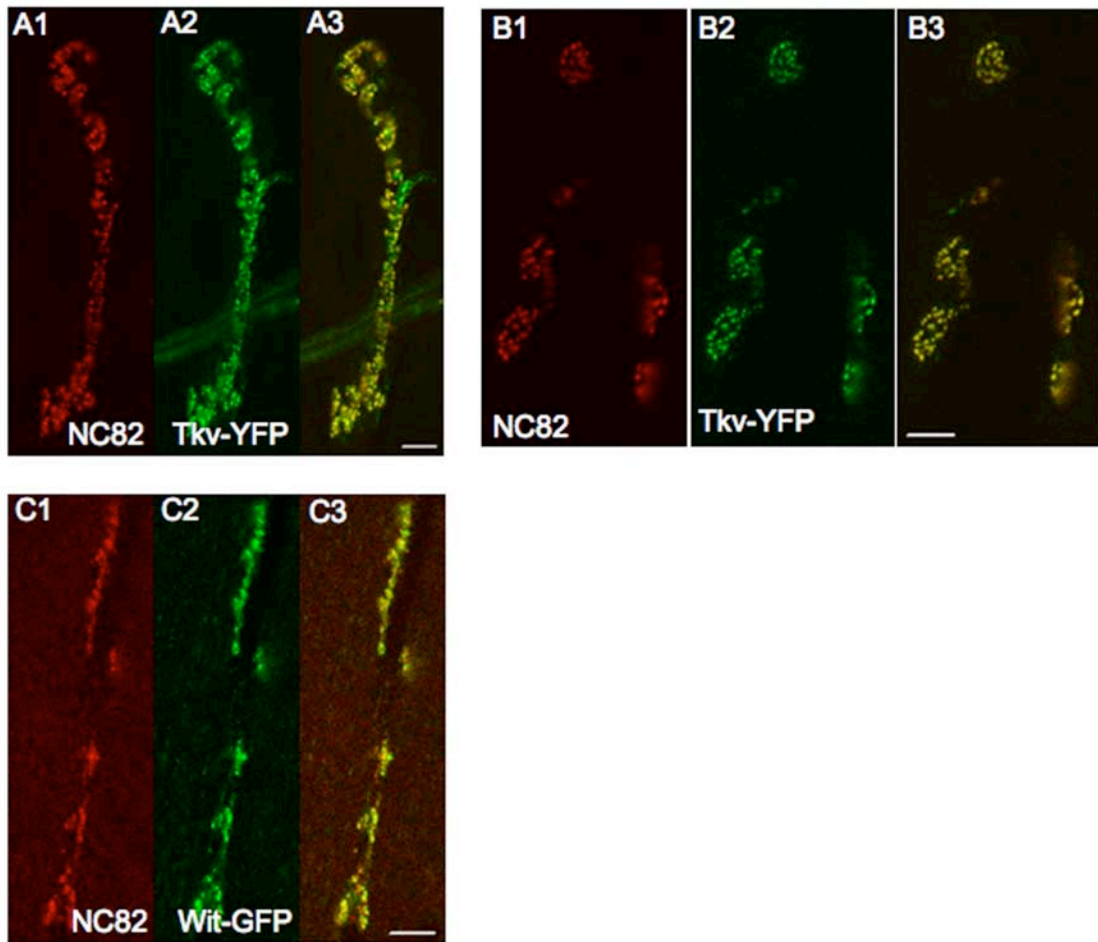


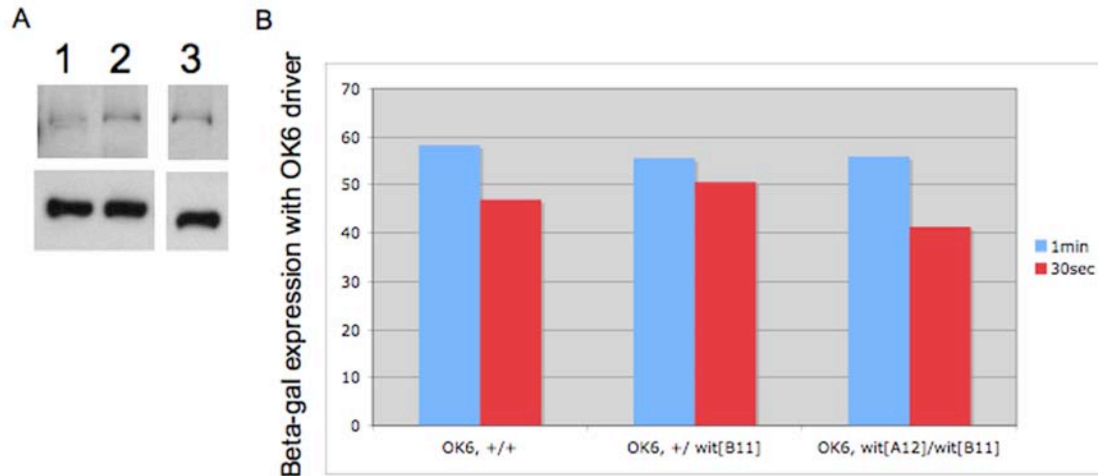
Figure 10: Directionality of colocalized receptor vesicles is eliminated in *gbb* mutants

In the kymographs, retrograde moving colocalized particles projections are white diagonal lines with descending slope. Anterograde particle projections are white diagonal lines with ascending slope. In control animals (5 movies, 3 larave), transport of colocalized receptor vesicles traveled in predominantly in a retrograde direction (A and D). In the *gbb* mutant (6 movies, 3 larave) there was an increase percentage of anterograde moving particles. (B and D). This directionality shift could reflect a lack of active signal and result in vesicles that change direction frequently (C). We found that *gbb* mutants had decreased directionality and had similar directional behavior as vesicles containing just one receptor (D).



Supplementary figure 1: Tkv colocalizes with NC82 in control and *wit* mutants

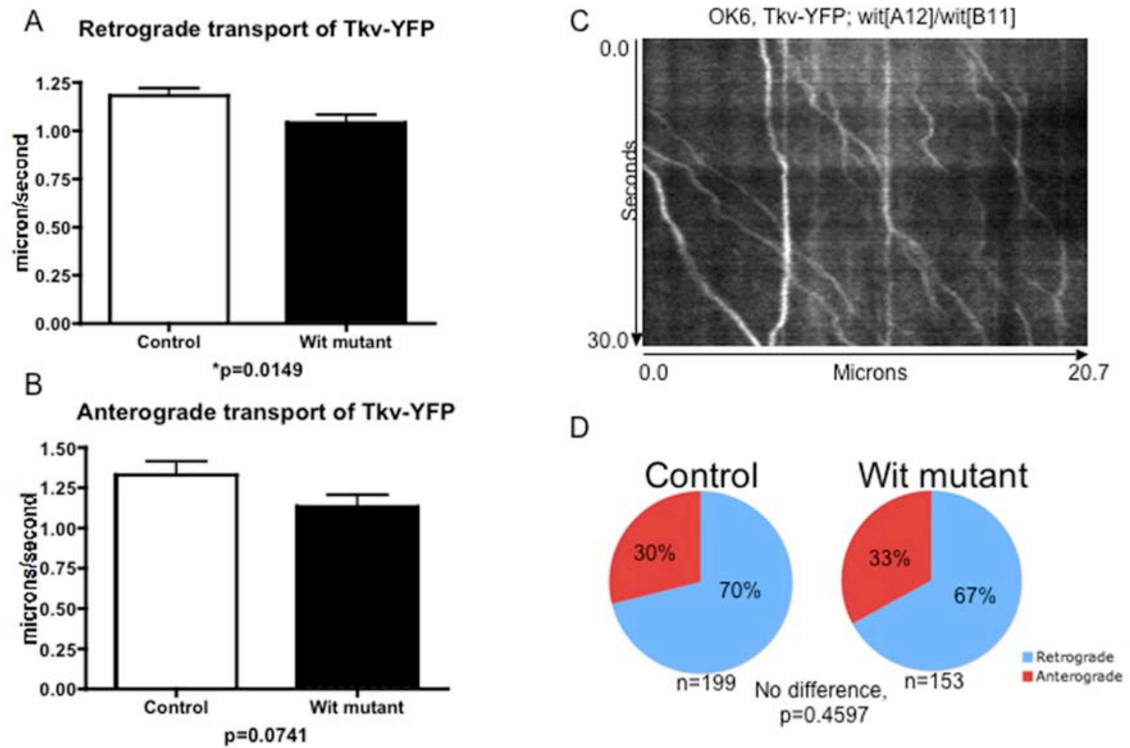
Tkv-YFP (A1-A3- muscle 4) and Wit-GFP (B1-B3- muscle 13) colocalizes with NC82 immunostaining in motoneurons. In *wit* mutants, this colocalization persists between Tkv-YFP and NC82 staining (B1-B3- muscle 6/7). Scale bar is 5 microns.



Supplementary figure 2: OK6 driver expression is not down-stream of Wit

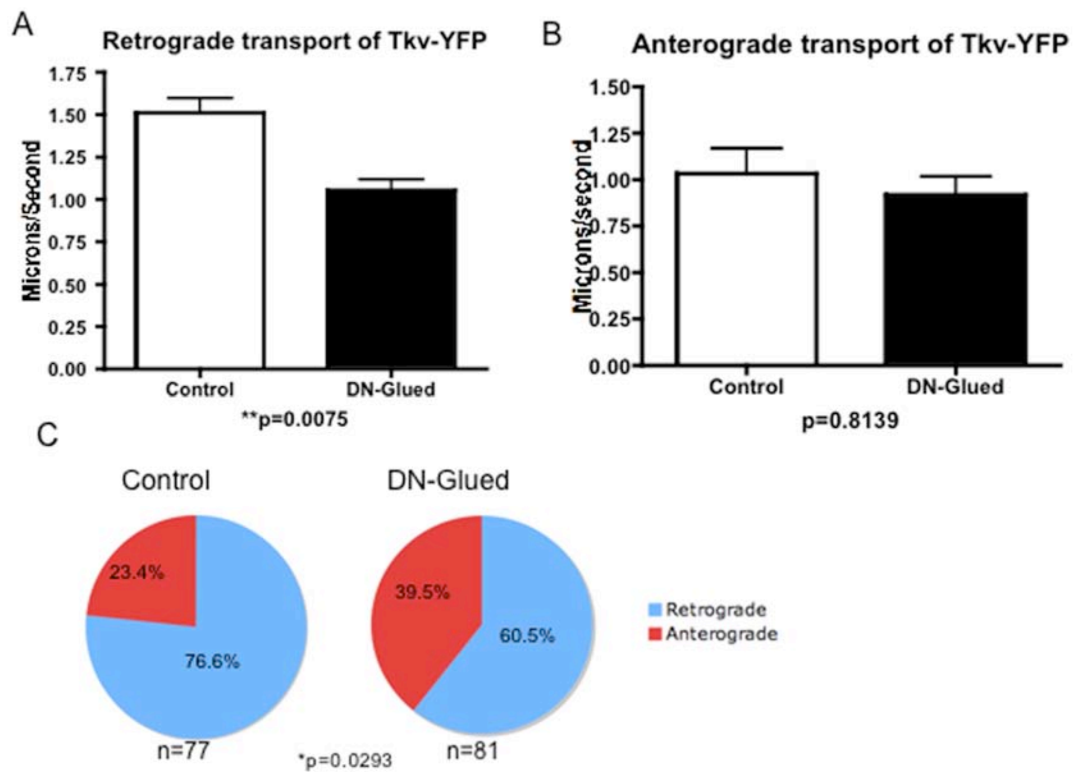
Western of UAS>Beta-Gal expression under driver OK6 (A). Third instar larval brains were run on a Western with the following genotypes: 1. Control- OK6>gal4; +/+ 2. *wit* heterozygote- OK6>Gal4; +/wit^{B11} 3. *wit* mutant- OK6>Gal4; wit^{A12}/wit^{B11}. Tubulin is the lower band control. Each lane contains the equivalent of 2 larval brains. Quantification of the Western bands with exposure times of 1min (blue) or 30 sec (red) shows that Beta-Gal expression by OK6 driver is not altered in *wit* mutants (B).

Note: Experiment and figure completed by Nam Chul Kim.



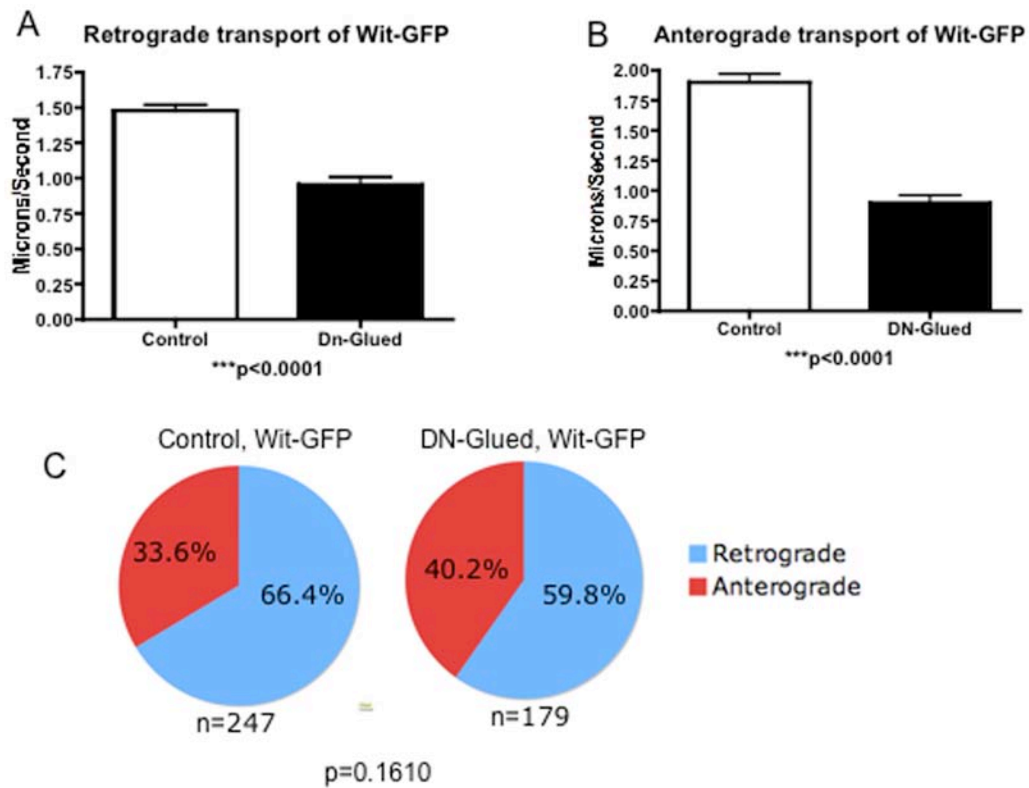
Supplementary Figure 3: Tkv-YFP vesicles are transported in *wit* mutants

The velocity of Tkv-YFP vesicles was measured in control and *wit* mutants. The retrograde speed of Tkv vesicles was slightly reduced in *wit* mutants (p=0.0149) (A). The anterograde speeds were also slightly reduced but the data was not significantly different from control (p=0.0741) (B). The kymograph demonstrates that retrograde moving Tkv vesicles (descending slope projections) are still present in a *wit* mutant (C). When we look at the population distribution between retrograde and anterograde vesicles, we find that there is not a significant difference (0.4597) between the two groups (D).



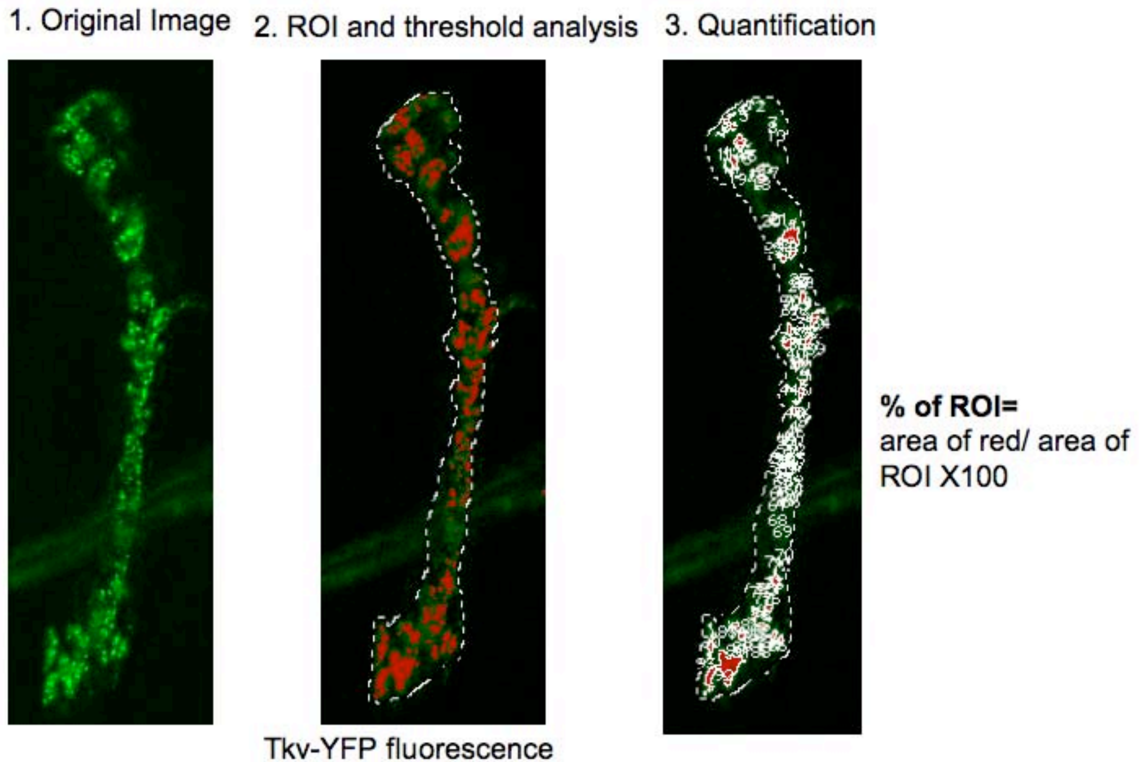
Supplementary Figure 4: DN-Glued decreases Tkv-YFP retrograde velocity

DN-Glued and Tkv-YFP were expressed in motoneurons. DN-Glued severely reduced the amount of moving receptors (See fig 9). For the remaining moving vesicles, we found that there was a significant (p=0.0075) decrease in the velocity of retrograde Tkv-YFP vesicles. We however did not observe a significant decrease in the velocity of anterograde moving vesicles (B). When examining the directionality of moving Tkv vesicles, we see that there is a significant difference between the control and animals expressing DN-Glued, with a decrease in the percent of retrogradely moving vesicles (C).



Supplementary Figure 5: DN-Glued decreases Wit-GFP retrograde and anterograde velocity

DN-Glued and Wit-GFP were expressed in motoneurons. DN-Glued severely reduced the amount of moving receptors (See fig 9). For the remaining moving Wit vesicles, we found that there was a significant ($p<0.0001$) decrease in the velocity of retrograde Wit-GFP transport. There was also a significant decrease in the velocity of anterograde moving vesicles ($p<0.0001$) (B). When examining the population distribution of Wit vesicles moving in a retrograde or anterograde direction, we see that there is no significant change between the control and when DN-Glued is expressed (C).



Supplementary Figure 6: Quantifying Fixed Tissue Tkv-YFP Fluorescence

To quantify Tkv-YFP fluorescence at the synaptic terminal the following steps were taken: An ROI was drawn around the synaptic terminal in the HRP channel and then transferred to the Tkv-YFP channel. Threshold analysis was conducted identifying the regions within the ROI that had signal intensity above a given threshold. The combined area of the regions divided by the ROI area is the function of Tkv-YFP in the synaptic terminal.

MAD PHOSPHORYLATION AND SYNAPTIC TERMINAL SIZE IN MOTONEURONS IS REGULATED BY THE RAB ENDOCYTIC PATHWAY

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CHAPTER 3

MAD PHOSPHORYLATION AND SYNAPTIC TERMINAL SIZE IN MOTONEURONS IS REGULATED BY THE RAB ENDOCYTIC PATHWAY

Abstract

Deciphering the signaling pathways that control synaptic plasticity would provide more insight into the processes of memory and learning. Synaptic growth at the *Drosophila* neuromuscular junction (NMJ) requires retrograde Bone Morphogenetic Protein (BMP) signaling mediated by the muscle derived Glass bottom boat (Gbb) ligand and the neuronal Wishful thinking (Wit) and Thick veins receptors. Gbb is a target-derived factor that acts on its receptors at the synaptic terminal, and consequently, this pathway must depend on retrograde transport to carry the active signal to the nucleus where phosphorylated Mad (p-Mad) regulates transcription. Our studies have focused on identifying the pathway component that carries the activated signal from the NMJ to the nucleus. As a possible signal carrier candidate, we have investigated the subcellular localization of YFP-Mad, it's activated form p-Mad, as well as its transport along the axon. We see diffuse YFP-Mad at the synaptic terminal, along the axon, and at the cell body and nucleus. Coexpressing fluorescently tagged Tkv does not result in colocalized transport of Mad and Tkv, arguing against pMad piggybacking retrogradely on a signaling endosome. Using an antibody to the activated form of Mad, we see Wit-dependent p-Mad at the synaptic terminal and neuron nucleus, but we do not detect p-

Mad along the axon. When we examine transport along the axon, Mad is not punctate, doesn't travel with receptor endosomes and appears to be diffuse. Manipulation of endocytic traffic effectors Rab5 and Rab7 results in changes in the BMP pathway readout of synaptic terminal size, as well as significant alterations in BMP receptor transport. Additionally there are changes in p-Mad accumulation at the synaptic terminal and nucleus. Taken together these data suggest that Mad does not carry the active pathway signal retrogradely along the axon, and hence pMad accumulation at the NMJ terminal and the nucleus must be the result of two different phosphorylation events. Our findings support a model in which Mad is phosphorylated at the motoneuron synaptic terminal and nucleus separately by endocytosed BMP receptors, analogous to the well-characterized independent phosphorylation of ERKs in the periphery and the cell body by signaling endosomes carrying neurotrophin receptors.

Introduction

The mammalian nervous system is composed of trillions of neurons all of which must establish critical connections, or synapses, with their target cell and maintain and refine these connections throughout life (Atwood and Wojtowicz, 1999). Synaptic plasticity, or the ability of neurons to undergo activity-dependent synaptic modification, is the basis of learning and memory (McKay et al., 1999). Long-term synaptic plasticity and memory formation relies on transcription (Alberini, 2009) and translation (Costa-Mattioli et al., 2009) of new proteins for the establishment or structural remodeling of the synaptic terminal. There are a few established signaling pathways that are known to be essential for synaptic plasticity and memory formation and operate through

transcriptional regulation (Alberini, 2009). Some of these are triggered by retrograde factors, target-derived proteins that are secreted in the post-synaptic cell and signal to the innervating neuron (Fitzsimonds and Poo, 1998). Determining how critical transcription factors are activated is essential to determine how problems in these pathways can lead to diseases of learning and memory.

Retrograde signaling allows the post-synaptic target cell to communicate with the pre-synaptic neuron for growth coordination, maintenance of the synapse and activity dependent modulation (Fitzsimonds and Poo, 1998). The presynaptic propagation of most signals theoretically could occur in two different ways, by diffusion or active transport (Fitzsimonds and Poo, 1998). The spread of cytosolic signals by diffusion is limited by the rate of transport and the lifetime of the signal (Fitzsimonds and Poo, 1998), and the time required for a signal to travel from the neuron terminal to the cell body is likely to become longer than the lifetime of the activated molecule (Fitzsimonds and Poo, 1998). Because of the extraordinary length of many neurons, a target-derived signal that has its ultimate effect regulating gene transcription in the nucleus must depend on active axonal retrograde transport (Goldstein, 2003).

The best-characterized retrograde signals are neurotrophins, critical factors for the differentiation, maintenance and survival of select neurons (Lo, 1995; Schinder and Poo, 2000). Neurotrophins promote the survival of specific population of developing neurons and maintain characteristic functions of mature neurons (Wang and Poo, 1997). Long term potentiation (LTP) is also impaired when neurotrophin function is abolished, demonstrating the importance of neurotrophins in synaptic plasticity (Schinder et al., 2000). Accordingly, neurotrophins such as nerve growth factor (NGF) and brain-derived

neurotrophic factor (BDNF) are implicated in both developmental and adult synaptic plasticity (Kang et al., 1997). NGF is the prototypic neurotrophin and has been well established as a target-derived factor in the regulation of the survival and differentiation of neurons (Lessmann et al., 2003). When released by the postsynaptic cell, NGF binds to TrkA receptors located within axon terminals of the innervating neuron, and thus activates the signaling pathway sending the signal retrogradely along the axon to the neuronal cell bodies (Ye et al., 2003).

Studies show that NGF utilizes a signaling endosome for signaling (Delcroix et al., 2003; Ibanez, 2007; Ye et al., 2003). In this model, NGF is produced and released from the target tissues and binds its receptors in the membrane of the innervating neuron. Then the ligand/receptor complex is endocytosed and transported retrogradely to the cell body in a Dyenin-dependent manner along the axon (Heerssen et al., 2004). Transport of the NGF receptor complex is important for activation of transcription factors that can enter the nucleus and activate transcriptional programs essential for neuron survival, synaptic plasticity, and growth signals (Cox et al., 2008; Schinder and Poo, 2000; Ye et al., 2003).

Endocytic proteins that have been identified to influence the transport of neurotrophin signaling components include Rab proteins (Deinhardt et al., 2006). Rab proteins are GTPases that coordinate steps in membrane traffic or endocytic sorting, implicated principally in the control of vesicle docking and fusion (Grosshans et al., 2006; Somsel Rodman and Wandinger-Ness, 2000; Zerial and McBride, 2001). Rab-GTPases function as switches cycling between GTP-bound and GDP-bound states (Grosshans et al., 2006). Initially Rab proteins are recruited to the target membrane and

participate in vesicle budding (Somsel Rodman and Wandinger-Ness, 2000). They also have been implicated in facilitating endosome transport along the cytoskeleton (Jordens et al., 2005). Rab5, early endosome protein, and Rab7, late endosome protein, have been shown to regulate axonal transport of neurotrophin pathway components, regulating the transport of potential signaling endosomes (Deinhardt et al., 2006).

Based on this signaling endosome model, there are indications that subcellular location of neurotrophin activation determines specificity in responses. Neurotrophin stimulation at the cell body results in activation of both Erk1/2 and Erk5 (Cosker et al., 2008; Watson et al., 2001). Activation of neurotrophins at the distal axon; however, leads to activation of Erk5 but not Erk1/2 because the latter doesn't transmit a retrograde signal (Cosker et al., 2008; Watson et al., 2001). It appears that the activation of neurotrophin pathways has differing effects whether it is at the synaptic terminal or cell body, and hence endosome traffic helps compartmentalize signaling.

Additionally, an endocytic vesicle with activated receptors could serve as a signaling platform that would support down-stream effector activation (McPherson et al., 2001). In some pathways, activated transcription factors have been also shown to be transported along the axon independently or with the kinase by which they are phosphorylated. In the neurotrophin pathway, the transcription factor CREB has been shown to be translated in the axon and the protein retrogradely transported together with the signaling endosome (Cox et al., 2008). Phosphorylated MAP Kinases, p-Erk1/2 and p-p38, have been observed to be transported with activated TrkA receptors, and this transport depends on NGF activity (Delcroix et al., 2003). In the JNK signaling pathway, axonal transport of c-Jun N-terminal kinase (JNK) and pathway transcription factors,

activating transcription factor (ATF)3 as well as ATF2/p-AFT2 has been shown to take place (Lindwall and Kanje, 2005). NFkB transcription factor is also retrogradely transported along the axon (Meffert et al., 2003) and this transport has been shown to be dependant on Dynein/Dynactin (Mikenberg et al., 2007). Transcription factors can then be transported along the axon with or without their activating signaling components, and this transport is required for retrograde signals such as injured axonal response (Lindwall and Kanje, 2005).

Importins have been implicated as a transport mechanism for transcription factors from distal synaptic sites to the nucleus. Importins, which are soluble transport factors that mediate the translocation of substrates through the nuclear pore complex, are seen in the neuron axoplasm, indicating a role for these proteins other than at the nucleus (Hanz et al., 2003). Long-lasting facilitation triggers importin translocation from the synapse to the nucleus (Thompson et al., 2004). Additionally cyclic AMP response element binding protein (CREB)2/ ATF4, a transcriptional repressor that modulates long-term synaptic plasticity and memory, was shown to bind Importin-α isoforms (Lai et al., 2008). This binding is essential for CREB2 translocation from distal dendrites to the nucleus (Lai et al., 2008). Nerve lesions trigger the local translation of Importin-ß, that binds to Importin-α, and this nuclear location signal-binding complex traffics retrogradely to the soma in a Dynein-dependent manner (Hanz et al., 2003). The interaction of importins with Dynein and critical transcription factors suggests a model where retrograde signaling results in the secondary activation of transcription factors that use importins as molecular linkers to Dynein, and thus the transcription factors relay the signaling event

from the synaptic terminal to the nucleus. This traffic is essential for the establishment of synaptic plasticity (Lai et al., 2008).

In *Drosophila* motoneurons, a target-derived BMP pathway that activates Smad transcription factors is responsible for proper synaptic terminal development (Aberle et al., 2002; Marques et al., 2002). Gbb is secreted from the muscle as a retrograde signal to coordinate growth of the muscle and innervating neuron (Haghighi et al., 2003; McCabe et al., 2003). In the motoneuron, Gbb brings together the Wit receptor and the type I receptor Thick veins (Tkv) or Saxophone (Sax) at the synaptic terminal membrane, resulting in phosphorylation of the type I receptor (McCabe et al., 2004; Rawson et al., 2003; Sweeney and Davis, 2002). As a result of this activation an R-Smad transcription factor named Mad is phosphorylated by the type I receptor and binds the co-SMAD Medea (McCabe et al., 2004; Rawson et al., 2003). Then these activated transcription factors enter the nucleus to regulate genes important for synaptic terminal development and function.

Because Gbb is a retrograde signal that binds the Wit receptor at the synaptic terminal (McCabe et al., 2003), the BMP pathway signal must travel from the motoneuron synaptic terminal to the cell body. There is evidence that the pathway depends on Dynein for pathway activation, and hence retrograde axonal transport. When DN-Glued, an inhibitor of Dynein, is expressed it causes small synaptic terminals (Eaton 2002) and loss of p-Mad in the motoneuron nuclei (Marques et al., 2003; McCabe et al., 2003), thus phenocopying *wit* mutations. Yet to be identified is the pathway component that carrys the active BMP signal along the axon.

An obvious candidate is a BMP signaling endosome carrying the activated receptors from neuromuscular junction (NMJ) to soma. However, phosphorylation of Mad is detected at the synaptic terminal (O'Connor-Giles et al., 2008; Wang et al., 2007), reflecting the local receptor activation and opening the possibility that activation of the receptors at the NMJ results in generation of pMad that is then transported retrogradely to the cell body. It is also possible that pMad is transported to the cell body by attaching to a signaling endosome containing the receptors. In the present study, we investigate if the Mad protein could be transporting the active signal from the synaptic terminal to the nucleus of the motoneuron in a complex with or independently of the BMP receptors.

Given the presence of pMad at the synaptic terminal, we wondered if the activated transcription factor was transported along the axon. We have found that while Mad can be seen at the synaptic terminal, axon, and cell body, there is no evidence of p-Mad along the axon. YFP-Mad can be seen diffusely along the axon, and moves bidirectionally. No Mad punctae are seen to be transported with the type I receptor vesicles. These data argue against a role for Mad's axonal transport in pathway activity. When we examined the expression of Rab mutant transgenes that alter receptor endocytic sorting, we found that pathway readouts of synaptic size and p-Mad accumulation were changed, revealing a level of BMP pathway regulation by these proteins. p-Mad was altered in animals expressing the Rab mutant transgenes but the effects on the synaptic terminal and nuclear accumulation were not consistent with each other. In larvae expressing DN-Rab5 or CA-Rab7 the synaptic terminal size was increased. Additionally, BMP receptor transport was significantly altered when the Rab mutant transgenes were expressed. The combined results argue for two independent Mad activation steps. First, Mad would be locally

activated at the synaptic terminal, and this pMad would be involved in some kind of local signaling. Second, the activated receptors would be transported to the cell body for Mad phosphorylation and translocated to the nucleus to regulate transcription.

Results

Mad-YFP localizes to the synaptic terminal, axon and cell body

To establish a reporter for Mad localization, we constructed a Mad protein tagged with YFP. This protein is fully functional by rescuing the viability of Mad mutants and can be phosphorylated, identified with the p-Mad antibody. We can then use this fluorescent fusion protein to study the transcription factor's movement and phosphorylation.

We wanted to see where Mad-YFP localizes within the motoneuron so we expressed the transgene with an OK6 motoneuron driver while using an antibody to stain YFP to amplify the signal. We saw that Mad-YFP localized the nucleus, synaptic terminal and axon of motoneurons (Figure 1).

Phosphorylated Mad at the synaptic terminal but not along the axon

While Mad appears throughout the motoneuron, we do not see the same pattern for activated Mad, p-Mad. Previous studies have shown that p-Mad is found in motoneuron nuclei and disappears in *wit* mutants (Marques et al., 2002; Marques et al., 2003). Additional recent studies have described p-Mad at the synaptic terminal post (Dudu et al., 2006) and presynaptically (O'Connor-Giles et al., 2008; Wang et al., 2007). However, these authors did not establish the dependence of this synaptic p-Mad on BMP signaling. Control (*yw*) and *wit* mutant third instar larvae were dissected and stained with

antibodies recognizing p-Mad and a post-synaptic membrane marker, Discs large. All larvae of compared genotypes were stained in the same dissection dish and imaged at the same settings. Using threshold analysis by IPlab, we measured p-Mad staining intensity at the NMJ at muscle 4. We compared synaptic staining of third instar larvae with genotypes of *yw* (n=11), (Fig. 2A1-3). *wit* mutant (n=16) (Fig. 2A4-6), and *yw* background staining (no primary antibody, n=11). We found that the p-Mad staining is eliminated in *wit* mutants (p<0.0001), much as the p-Mad nuclear signal (Marques et al., 2002) (Fig.5). The background bar in this figure represents *yw* synaptic staining without a primary antibody recognizing p-Mad, but with secondary antibody staining, illustrating that *wit* mutant synaptic terminals contain little or no p-Mad (Figure 2C). Given that *wit* is expressed and required only presynaptically, this result strongly argues that p-Mad is presynaptic. This is further supported by careful analysis of the synaptic p-Mad signal. Enlarged portions of two control synaptic terminals show that post-synaptic Dlg staining (green) largely surrounds the p-Mad staining (red) (Fig. 2B1, 2B2), suggesting that this p-Mad staining is presynaptic. Our own novel data is that p-Mad at the synaptic terminal disappears in *wit* mutants demonstrating that synaptic terminal p-Mad is Wit dependent, and then largely if not exclusively presynaptic. This data shows that p-Mad is at the synaptic terminal, suggesting that Mad can be phosphorylated at the distal end of the neuron, as expected from the neuronal localization of the BMP receptors and the source of ligand in the muscle.

Now that we know that p-Mad localizes both to the motoneuron nucleus and at the synaptic terminal in a Wit dependent manner, we looked next at p-Mad staining along the axon. When we examine the axons, we do not see any specific p-Mad staining (Fig.

2D). The p-Mad signal in the wild type axon (Fig. 2D1) is the same as in the *wit* mutant negative control (Fig. 2D2), and is then background. Because we do not see p-Mad along the axon, this argues against a role for Mad in transporting the active pathway signal from the synaptic terminal to the nucleus.

Mad-YFP appears diffuse along the axon and does not travel with receptor vesicles

Because we observed Mad-YFP in the axon when expressed with a motoneuron driver, we wondered if there was movement of Mad-YFP along the axon. To examine Mad-YFP movement along the axon, we expressed Mad-YFP in motoneurons and examined the samples live (Fig 3A). We could not detect any particulate structure in cell soma, axons or NMJ. Within the motoneuron nucleus several structured appeared enriched in Mad-YFP, but we have not made attempts to characterize them. Given the absence of Mad-YFP containing particles, we studied axonal transport of Mad-YFP by bleaching the center area along the axon and visually examining recovery (Fig. 3A). We found that after a section of the axon was bleached, recovery occurred slowly from both sides (Fig 3A). It is possible that Mad could be associated with punctate, discrete structures that are being transported by motor proteins and are below the sensitivity of our camera. One possibility is that activated Mad could be traveling retrogradely with the receptors, much as synaptically translated CREB is transported to the cell body on a Trk signaling endosome (Cox et al., 2008). We have observed directional transport of the receptors along the axon in vesicles (Smith et al, submitted) causing us to wonder if Mad could be traveling together with the receptors, but just below the level of detection. We reasoned that if we overexpressed both proteins to amplify the signal, Mad traveling with

a receptor endosome would appear as a punctate structure and look characteristically similar to the receptor transport. We overexpressed both Mad-YFP and Tkv-CFP, but did not observe any Mad-YFP punctate structures traveling with the Tkv punctate receptor vesicles (Fig. 3B). Even when the area was bleached in the YFP field and vesicles of Tkv-CFP receptors moved into the bleached area, no Mad-YFP fluorescence was observed to be traveling with the receptor vesicle, leading us to the conclusion that Mad does not travel associated with the retrogradely transported receptors along the axon.

p-Mad accumulation is altered when endocytic sorting is perturbed

Taken together, the studies of Mad-YFP traffic and subcellular localization as well as p-Mad distribution support a model in which Mad is phosphorylated independently at the synaptic terminal and the cell body. If this is true, what is the mechanism and function of Mad's proximal (cell body for nuclear p-Mad accumulation and transcriptional regulation) and distal (at the synaptic terminal for local signaling) phosphorylation? The ligand of the pathway, Gbb, is a retrograde signal and its activation of the BMP receptors occurs at the neuromuscular junction (McCabe et al., 2003). From other groups work, we know that endocytic proteins influence the BMP pathway activity by downregulating the BMP receptors at the synaptic terminal. Nervous wreck and Spichthyin are two recently discovered endocytic proteins that regulate the BMP pathway. Their proposed role is either as an early endosome, as with Spict, or recycling endosome, as with Nwk. (O'Connor-Giles et al., 2008; Wang et al., 2007). Additionally, a late endosome protein, Spinster, has been implicated in regulation of the BMP pathway. (Sweeney and Davis, 2002). Loss of Vps35, another endocytic sorting

protein, led to TGF-β /BMP dependent upregulation of synaptic size (Korolchuk et al., 2007). We wondered if perturbing endocytic sorting would provide insight into the mechanism of Mad activation at one or both subcellular locations containing p-Mad.

To address this question we altered receptor endocytic trafficking to see if it would affect phosphorylated Mad levels (a direct readout of BMP signaling). To perturb the early endosomal sorting we utilized mutant transgenes of Rab5, and to affect late endosomal sorting we utilized mutant transgenes of Rab7. Rabs are GTPases that are responsible for membrane traffic and endocytic sorting. By regulating SNARE complex assembly, the Rab GTPase are able to target the endosome to the correct membrane (Clague and Urbe, 2001; Li and Liang, 2001; Zerial and McBride, 2001). Dissecting the workings of the Rab endocytic pathway may aid in describing the mechanism of Mad phosphorylation on the distal and proximal ends of the motoneuron.

Rab5 is an early endosome protein that regulates membrane docking and fusion of the early endosomes (Nielsen et al., 1999). There is an established role for Rab5 in early endosome fusion (Gorvel et al., 1991; Li and Liang, 2001; Stenmark et al., 1994). Rab5 has also been described to be necessary for ligand sequestration into clathrin coated pits (McLauchlan et al., 1998; Somsel Rodman and Wandinger-Ness, 2000) as well as for regulating the motility of early endosomes along microtubulues (Nielsen et al., 1999) and being responsible for sorting of cargo for axonal transport (Deinhardt et al., 2006). To determine the role that early endocytic sorting has on BMP pathway signaling, we utilized Rab5 constitutively active (CA) as well as dominant negative (DN) mutant transgenes. Rab5S43N, referred to as DN-Rab5 in the extent of this paper, is a dominant negative mutant of Rab5 that is trapped at the inactive GDP-bound state (Entchev et al.,

2000) completely abolishing GTP binding (Li et al 1994). The constitutively active Q79L mutant of Rab5 (in this paper referred to as CA-Rab5) is fixed in the GTP bound form of the protein (Stenmark et al., 1994). Use of this constitutively active form of Rab5 caused the internalization of the transferrin receptor to be accelerated resulting in accumulation of the receptor in large endosomes while inhibiting recycling (Stenmark et al., 1994). Rab5 transgene mutants will be utilized to perturb early endocytic sorting.

To alter late endocytic sorting we manipulated the activity levels of the late endosomal/lysosomal effector Rab7. DRab7Q67L, or CA-Rab7, is a dominant gain-of-function mutant that is blocked in the active GTP-bound state (Meresse et al., 1995; Vitelli et al., 1997). Over expression of CA-Rab7 in *Drosophila* wing imaginal discs was found to increase degradation of internalized Dpp, a BMP ligand that like Gbb binds to the type I receptor Tkv (Entchev et al., 2000). When a dominant negative version of Rab7, Rab7N125I, was expressed it caused an internalized G protein to accumulate specifically in early endosomes (Feng et al., 1995). When expressed in *Drosophila* photoreceptors, Rab7N125I tagged with a YFP protein, referred to as DN-Rab-YFP exhibits a more dispersed localization pattern, as opposed to large aggregates (Zhang et al., 2007). A related DN-Rab7 blocked the retrograde transport of neurotrophins receptors, p75 and TrkB (Deinhardt et al., 2006). Manipulating Rab7 should provide information about the late endosome potential to regulate Mad phosphorylation.

p-Mad accumulation at the synaptic terminal is affected by manipulation of the early endocytic pathway

First we wanted to observe the effect of manipulating the endocytic pathway on p-Mad accumulation at the synaptic terminal. Presence of p-Mad at the synaptic terminal has been shown previously by other groups (O'Connor-Giles et al., 2008; Wang et al., 2007) and confirmed by our data (Fig 2). Newly described endocytic proteins, Spict and Nwk, regulate p-Mad amounts at the synaptic terminal by down-regulating the receptors and mutants of these endocytic proteins display elevated p-Mad staining (O'Connor-Giles et al., 2008; Wang et al., 2007). This data along with our evidence of synaptic p-Mad depending on Wit demonstrate that the receptors are likely phosphorylating Mad at the synaptic terminal.

We expressed DN-Rab5, CA-Rab5 and CA-Rab7 in motoneurons with the OK371 driver and then dissected and stained the larvae for p-Mad (Fig 4–red) and the post-synaptic marker Discs large (Fig 4-green). To have a negative and positive control, *yw* and *wit* mutants were simultaneously dissected and stained, and all genotypes were processed in the same dish to eliminate experimental variations.

Manipulation of Rab activity altered p-Mad levels at the synaptic terminal (Fig 4F). In larvae expressing DN-Rab5 (Fig 4C) and CA-Rab5 (Fig 4D), p-Mad levels were significantly decreased at the synaptic terminal. CA-Rab7 (Fig 4E) on the other hand had normal p-Mad levels. These data demonstrate that alterations in early endosome sorting inhibit the accumulation of p-Mad at the synaptic terminal, while changes in late endosomal traffic has no effect on synaptic Mad phosphorylation.

We also looked at p-Mad at the synaptic terminal in animals expressing DN-Glued (Supplementary Fig. 1). In other studies, when DN-Glued is expressed p-Mad is lost in the nuclei of motoneuron, but p-Mad level at the synaptic terminal has not previously been examined. When DN-Glued is expressed in motoneurons we saw loss of p-Mad at the synaptic terminal similar to that of *wit* mutants (Supplementary Fig1). We have previously shown that Wit anterograde transport is perturbed when DN-Glued is expressed. A possible explanation of this data is that DN-Glued expression affects the normal localization of the receptors. It is possible that DN-Glued inhibits the receptors from being properly inserted into the membrane, which would affect the activity of the pathway in this location. Further studies should be completed to investigate this possibility.

p-Mad accumulation at the nucleus is affected by manipulation of the early and late endocytic pathway

After observing that synaptic p-Mad accumulation is affected at the synaptic terminal, we wanted to see if the same effects occurred in the nucleus. As in the previous experiment, DN-Rab5, CA-Rab5, and CA-Rab7 were expressed with a motoneuron driver, OK371, and then dissected and stained with p-Mad (Figure 5- red). We co-expressed Mad-YFP (Figure 5-green) in these motoneurons to increase our sensitivity of detection, although similar results were obtained by staining for the endogenous p-Mad, without expression of Mad-YFP. Control larvae containing only the driver and Mad-YFP transgene were used as a positive control and *wit* mutants with the driver and Mad-YFP transgene were used as a negative control, both dissected and stained simultaneously. To

ensure that we were examining the same set of nuclei in the ventral ganglion, DAPI (Figure 5-blue) was used as a counter-stain to aid in finding the area of interest. In each sample we imaged the Mad-YFP transgene and p-Mad in the strip of motoneurons along the dorsal side of the ventral ganglion.

As expected we saw that p-Mad staining was present in the control nuclei (Fig 5A) while in *wit* mutants (Fig 5B) it disappeared. Surprisingly, in animals expressing DN-Rab5 we saw a slight increase in p-Mad staining in the nuclei. Furthermore, in animals expressing CA-Rab5 (Fig 5D) or CA-Rab7 (Fig 5E) we see that nuclear p-Mad is eliminated similar to a *wit* mutant (Fig 5F). This result was unexpected because it differs from the synaptic terminal p-Mad results dramatically.

In addition to the effect on p-Mad accumulation in the nucleus, we also see alterations on Mad-YFP nuclear localization (Fig 5, column 1). In muscles, Mad has been described previously to shuttle in and out of the nucleus in an inactive basal state (Dudu et al 2006). When Mad is phosphorylated, the nuclear export rate is decreased resulting in an increase of Mad protein within the nucleus (Dudu et al. 2006). Similar dynamics have been described for mammalian Smads (Hill, 2009; Pierreux et al., 2000). Our data suggest that a similar motion could also be occurring in motoneurons, because when the pathway is inactive as in *wit* mutants there is a decrease in nuclear Mad protein (Fig 5B1 and Supplementary Fig2). Although not quantitatively measured, when we manipulate the Rab endocytic pathway, the Mad levels visually correlate with p-Mad levels. The presence of nuclear Mad-YFP in the absence of p-Mad (*wit* mutant) confirms the previously published data of signal-independent nuclear translocation of Smads (Hill, 2009; Pierreux et al., 2000).

Presence of synaptic p-Mad correlates with synaptic terminal growth

The BMP pathway is the major synaptic growth signal for the NMJ synaptic terminal, and synaptic size is a read-out of BMP pathway activation. When the pathway is inactive such as in the *wit* receptor mutant, the result is small synaptic terminals. Current thinking is that the ligand will bind the receptors and then Mad transcription factor will be phosphorylated allowing it to regulate transcription of genes allowing for synaptic terminal growth. We hypothesized that the changes in nuclear p-Mad levels upon manipulation of Rab activity would correlate with changes in synaptic size. Surprisingly, this was not the case. Normalized to muscle size, expression of DN-Rab5 caused an overall enlargement of the synaptic terminal by 66% (Fig 6A and 6E) while animals expressing CA-Rab5 led to no significant change in synaptic size (Fig 6B and 6E). Expression of CA-Rab7 (Fig 7A and 7E) led to a 75% enlargement of the synaptic terminal while animals expressing DN-Rab7-YFP had no significant change compared to control (Supplementary Fig 3). This data was unexpected because when CA-Rab7 or CA-Rab5 are expressed there is no p-Mad in the nucleus, which conflicts with the current thinking that nuclear p-Mad is required for synaptic terminal expansion.

Given that the BMP pathway is the best-characterized synaptogenic signal at the larval NMJ, we hypothesized that the increased synaptic terminal size caused by DN-Rab5 and CA-Rab7 over expression was due to enhanced BMP signaling. To test this hypothesis we over expressed DN-Rab5 in the absence of Wit (Fig 8). When one *wit* allele was eliminated, the DN-Rab5 overgrowth phenotype was suppressed by 40% (Fig 8A) percent and CA-Rab7 was suppressed by 43% (Fig 8B). When both *wit* alleles were mutant, the synaptic size overgrowth caused by DN-Rab5 (Fig 8A) and CA-Rab7 (Fig

8B) was fully suppressed to *wit* mutant levels. This suppression of the overgrowth phenotype by *wit* alleles demonstrates that excess BMP signaling is responsible for synaptic terminal overgrowth induced by DN-Rab5 and CA-Rab7 over expression.

Although p-Mad in the nucleus does not correlate with synaptic expansion, p-Mad presence (if not levels) at the synaptic terminal does. When there is at least some p-Mad at the synaptic terminal, as in DN-Rab5, CA-Rab5 and CA-Rab7 (Fig 5F), we see either normal synaptic terminals or synaptic terminal expansion. When there is no p-Mad at the synaptic terminal, as in *wit* mutants (Fig 5F) or DN-Glued, we see small synaptic terminals. However, the levels of p-Mad do not correlate with the degree of synaptic terminal expansion. This is in disagreement with data reported by O'Connor-Giles and colleagues, where the number of satellite boutons (indicative of synaptic terminal growth) were increased as p-Mad levels at the synaptic terminal increased (O'Connor-Giles et al., 2008). p-Mad at the synaptic terminal could play a role in synaptic terminal expansion.

Rab transgenic mutants affect receptor transport

We have recently found that axonal transport of receptors is linked to pathway activation (Smith et al, submitted). In order to substantiate that the effect of Rab activity manipulations on synaptic terminal size and Mad phosphorylation is due to aberrant endosomal traffic of BMP receptors, as suggested by the suppression of the phenotypes by *wit* mutations, we imaged Tkv-YFP (Fig 9) and Wit-GFP (not shown) traffic and localization in the presence of Rab mutant isoforms. We have found that receptor endosomal localization throughout the motoneuron is affected by manipulation of the Rab's function. When CA-Rab5 is expressed we see large endosomes within the synaptic

terminal, along the axon, and at the cell body (Fig 9B, F, L). When DN-Rab5 is expressed, we see elevated levels of Tkv-YFP in the membrane of the synaptic terminal, and along the axon, however Tkv-YFP endosomes at the cell body appear unaffected (Fig 9C, I, K). When CA-Rab7 is expressed, although displaying an elevated bouton phenotype, Tkv-YFP at the synaptic terminal looks relatively similar to the control (Fig 9G). In animals expressing CA-Rab7, the axon (Fig 9D) and cell bodies (Supplementary Fig 4) contain large stationary vesicles.

To interpret these data, we will examine alterations in Mad phosphorylation that correlate to receptor endosome alterations. It appears that where the receptor endosomes are normal, we see normal or elevated p-Mad. CA-Rab5 expression alters receptor endosomes throughout the motoneuron, has decreased p-Mad at the synaptic terminal and no p-Mad in the nucleus. DN-Rab5 has normal endosomes at the cell body and this correlates with p-Mad in the nucleus. CA-Rab7 has normal endosomes at the synapse, which correlates with normal p-Mad at the synaptic terminal. This data suggests that normal receptor endocytic sorting is important for Mad phosphorylation.

In summary, expression of Rab mutant transgenes alters receptor transport, synaptic terminal size and the distribution of p-Mad, and it is likely that there is a causal connection between these observations. The key role of Rabs as regulators of membrane vesicular traffic for signal routing and attenuation is evidenced by the altered transport of the receptors, defective accumulation of p-Mad in the nucleus of motoneurons and the aberrant morphology of the synaptic terminals due to excess receptor signaling.

Discussion

Causative agents of neural disease, such as learning and memory dysfunction, include faulty signaling of neuronal survival signals (Goldstein, 2003; Hafezparast et al., 2003; Hirokawa and Takemura, 2004). Faulty signaling could result from mutations in trophic pathway components or, less directly, from accessory proteins that are required for proper distribution or transport of active survival signals. Because of the extensive distance between the synaptic terminal and cell body in some neurons, it is unlikely for critical signaling factors to be traveling by simple diffusion (Goldstein, 2003). Transport of growth and survival signals is necessary to achieve viable neurons and, in order to treat diseases of learning and memory; it is essential that we understand signaling mechanisms of critical pathways.

BMP signaling is critical for proper synaptic terminal development in *Drosophila* motoneurons. This pathway provides an essential retrograde signal that coordinates muscle and synaptic terminal development. BMP pathway genetic analysis provides evidence that the Gbb ligand is produced in muscles and is signaling through BMP receptors at the motoneuron synaptic terminal (McCabe et al., 2003). Gbb is a target-derived factor that acts on its receptors at the synaptic terminal, and consequently, this pathway must depend on retrograde transport to carry the active signal to the nucleus where phosphorylated Mad (p-Mad) regulates transcription. The goal of this study is to determine if Mad is transporting the activated pathway signal along the axon.

We present two independent lines of evidence that argue against a role for Mad as the signal relay from synaptic terminal to nucleus. First, a Mad-YFP fluorescent fusion protein expressed with a motoneuron driver localizes to the synaptic terminal, the nucleus

and along the axon. However Mad movement along the axon does not display directionality and appears diffuse. The related molecule Smad2 interacts with kinesin, and this interaction and an intact microtubule cytoskeleton is required for presenting Smad2 to the receptors and pathway activation in Xenopus embryos (Batut et al., 2007). The role of retrograde transport of pSmad2 in that same system has not been investigated, and although in our study Mad-YFP appears diffuse along the axon, further studies are needed to rule out the possibility that Mad is being transported by molecular motors in our system.

Second, when we look at the subcellular distribution of the activated form of Mad, p-Mad, we detect it at the synaptic terminal and nucleus, but not along the axon. Critically, p-Mad disappears from both NMJ and nucleus in *wit* mutants, as expected from Wit being the type II receptor responsible for all BMP signaling in neurons (Aberle et al., 2002; Marques et al., 2002; Marques et al., 2003). It is unlikely that p-Mad could be moving along the axon from the synaptic terminal to the nucleus by diffusion. First, transport to the nucleus by diffusion would require large levels of phosphorylated Mad and we are not able to see any p-Mad along the axon. Second, it is unlikely that p-Mad would stay phosphorylated over the long distance from the synapse to the nucleus unless it was traveling with an activated receptor endosome (Fitzsimonds and Poo, 1998) and we do not detect Mad moving with receptor vesicles.

Taken together, these data argue against Mad being phosphorylated at the NMJ and p-Mad transported retrogradely towards the nucleus, and in favor of Mad being subject to two independent phosphorylation events, one at the NMJ, perhaps related to local signaling; and another one at the cell soma, resulting in nuclear accumulation of p-

Mad. Further support for this model is seen when receptor endocytic sorting is altered, because of the independent effects on p-Mad at the synaptic terminal and nucleus.

A possible model that fits the presented data is that the receptors in a BMP signaling endosome phosphorylate Mad at the NMJ, shortly after endocytosis, and then the signaling endosome proceeds to the cell body to phosphorylate Mad for its accumulation in the nucleus, while non-activated Mad is diffuse in the axoplasm throughout the motoneuron (Fig 10A). When CA-Rab5 is expressed, the receptors could be sequestered in a large early endosome that prevents efficient signaling at the synaptic terminal and prevents the complex from being ac tive more down-stream at the cell body, preventing Mad phosphorylation in the cell body (Fig 10B). When DN-Rab5 is expressed, the endosomes could be immature early endosomes that cannot phosphorylate Mad efficiently at the synaptic terminal (Fig 10C). Later on in transport, the endosome might mature, independently of Rab5's function, and is able to phosphorylate Mad at the cell body (Fig 10C). When CA-Rab7 is expressed, synaptic terminal phosphorylation of Mad is unaffected, but the transport of the signaling endosome is blocked by its transfer to the lysosome (Fig 10D). These models provide an explanation of the presented data combined with other evidence about BMP receptor transport (Smith, et al. submitted).

In mammalian cells, the endocytosis of TGF-ß receptors has been shown to be a requirement for pathway propagation. A study describes that internalization of the TGF-ß receptors is required to activate the downstream Smads to propagate pathway signaling (Penheiter et al., 2002). Upon ligand binding the active kinase of TGF-ß receptors was shown to elicit endocytosis of the receptor complex (Anders et al., 1997; Anders et al., 1998). Additionally there is evidence that TGF-ß receptors interact with endocytic

machinery. In cell culture, the TGF-ß receptors interact with clathrin-associated adaptor protein, AP2 (Yao et al., 2002) and BMP receptors are endocytosed by clathrin coated pits and caveloae (Hartung et al., 2006). Consistently, an early endosomal protein, Smad Anchor for Receptor Activation (SARA) has been shown to be necessary for efficient TGF-ß as well as BMP signaling (Itoh et al., 2002) by recruiting the R-Smad for phosphorylation by the type I receptor. Our results revealing that the endocytic pathway regulates Mad phosphorylation could be of general relevance for related pathways in mammals.

A surprising result was that synaptic terminal size increased in a *wit*-dependent manner when CA-Rab7 was over expressed, despite the lack of p-Mad in the nucleus. A correlation between p-Mad at the NMJ and synaptic growth has been described previously (O'Connor-Giles et al., 2008), and although we do not see a quantitative correlation, p-Mad being present at the synaptic terminal correlates with synapses of normal or enlarged size. This finding that synaptic p-Mad may dictate synaptic size, while nuclear p-Mad does not, is controversial and will require further study to substantiate. Our current hypothesis is that p-Mad at the NMJ serves a local signaling role, perhaps through cross talk to other pathways (Moustakas and Heldin, 2005; Vardouli et al., 2008). While it is difficult to envision synaptic growth in the absence of Mad transcriptional role, it is possible that a parallel pathway involving local signaling could compensate for the absence of p-Mad in the nucleus. There is indeed a precedent for this, as over expression of *Drosophila* LIM domain kinase 1 (dLIMK1) can rescue the synaptic terminal size phenotype of *wit* and *Mad* mutants (Eaton and Davis, 2005).

We have shown previously evidence of a BMP signaling endosome along the axon (Smith, et al., submitted). The data from this study further supports this model, as the data shows that endocytic traffic regulates BMP receptor traffic and synaptic growth. In addition to their traditional roles as early and late endosomal proteins, Rab5 and Rab7 have been found to have critical roles in axonal transport of neurotrophin endosomes in mammalian motoneurons (Deinhardt et al., 2006). Specifically, Rab7 was required for the axonal transport of possible signaling endosomes- vesicles that contain neurotrophins and their receptors (Deinhardt et al., 2006). In the same study, Rab5 was found to regulate an early endosome step preceding axonal transport at the synaptic terminal (Deinhardt et al., 2006). Rab5 and Rab7 play critical roles in signaling endosome transport of neurotrophins, and could have similar effects on the BMP signaling endosome.

In summary, this study supports a model in which Mad is phosphorylated separately at the synaptic terminal and cell body by BMP receptors that are regulated by the Rab endocytic pathway, and suggests that Mad is not the carrier of the BMP signal from periphery to cell body. This is a significant step forward toward the dissection of the BMP signaling pathway, which is critical for proper synaptic terminal development in *Drosophila*. In addition, our proposed model has implications for mammalian pathways that are critical for synaptic plasticity. BMPs are also essential regulators of synaptic plasticity in the mammalian brain (Sun et al., 2007) and other related mammalian growth and survival pathways could signal in similar manner to the BMP pathway. Analysis of the causative pathology underlying neurological disorders should consider the role of

endosomal traffic of growth factor receptors other than neurotrophins and the differential consequences of localized pathway activation.

Materials and Methods

Constructs

Utilizing PCR, oligos were used to add NdeI and XbaI sites onto the C-terminal domain of the *Mad* ORF. Then the amplified *Mad* was placed in TOPO (Invitrogen) vector and subcloned using NdeI and HindIII sites into pUAST-EYFP vector (GM, unpublished). To create the Tkv-YFP and Tkv-CFP constructs, Acc65I/AgeI sites were used to insert *tkv* ORF into pUAST-EYFP or pUAST-ECFP-N1 vector (GM, unpublished). All subcloned transgenes were prepared for germ-line transformation (Model Systems Genomics, Duke University, Eric Spana, Director), and inserts in all three major chromosomes mapped and balanced.

Fly stocks and genetics

We used Gal4 drivers OK6 (2nd chromosome) (Aberle et al., 2002), and OK371 (2nd chromosome) (Mahr and Aberle, 2006) to express BMP receptors in larval motoneurons. In most cases second chromosome inserts of the receptor transgenes were recombined with the drivers.

We received the stocks containing Rab transgenes from Hugo Bellen. The genotypes of overexpression of these constructs were as follows: OK371>Gal4/ UAS>Rab5[SN] (DN-Rab5) (Entchev et al., 2000), OK371>Gal4/+;UAS>Rab5[QL]/+ (CA-Rab5) (Stenmark et al., 1994), and OK371>Gal4/UAS>Rab7[QL] (CA-Rab7)

(Feng et al., 1995) and OK371>Gal4/+; UASp-YFP-Rab7-T22N/+ (DN-Rab7-YFP) (Bloomington stock 9778) (Zhang et al., 2007). The control for bouton counting of these larvae, was the above genotypes but without OK371 driver.

Genotypes used for the suppression of synaptic overgrowth when DN-Rab5 was expressed are as follows: OK371>Gal4/+; UAS>Rab5[SN], wit[A12]/ wit[B11] (transgene expression plus wit mutant), OK371>Gal4/+; UAS>Rab5[SN], wit[A12]/ TM3 (one wit mutant allele). The controls were from the same vial but did not contain UAS>Rab5[SN], wit[A12].

Genotypes used for the suppression of synaptic overgrowth when CA-Rab7 was expressed are as follows: OK371>Gal4/ UAS>Rab7 [QL]; wit[A12]/ wit[B11] (transgene expression plus wit mutant), OK371>Gal4/ UAS>Rab7 [QL]; wit[A12]/ TM3 (one wit allele), UAS>Rab7 [QL]/ CyO-YFP; wit[A12]/ wit[B11] (wit mutant), UAS>Rab7 [QL]/ CyO-YFP; wit[A12]/ TM3 (control).

Genoypes for the Mad-YFP rescue of *Mad* mutants were Mad[12]/Mad[1]; UAS>YFP-Mad/ daughterless>Gal4.

See Flybase (http://flybase.org/) for the description of the mutant alleles used: wit^{A12}, wit^{B11}, Mad^{12}, Mad^{1}

Phosphorylated Mad Immunofluorescence

Third instar larvae were dissected live in HL3 medium. They were then fixed at room temperature for 10 minutes with 4% PFA containing 0.1% Triton-X in PBS. They were washed 3-5 times with PBS. After dissection and fixing, the remaining larvae tissue was blocked at room temperature for 45 minutes-1 hour with PBS containing 0.1%

Triton-X and 2.5% BSA. After blocking, the larvae tissue was incubated overnight at 4ºC with the primary diluted 1:500 rabbit anti-p-Mad (PS1 from Carl-Henrik Heldin) (Urban et al., 1998). To maximize the signal's intensity, we incubated serially with two different secondary antibodies that are imaged in the same channel. The next antibody incubation was Goat anti-Rabbit Alexa 568 at 1:200 followed by Donkey anti-Goat-Cy3 at 1:200, both of which occurred at room temperature for 4 hours or more. Both of these secondary antibodies had been pre-absorbed with control larvae tissue overnight at 4ºC, before incubating with the experimental larvae pelts. After triple staining, the larvae were incubated with a Mouse anti Discs large (DSHB, Iowa) 1:50 overnight at 4ºC to label the post-synaptic membrane. Then incubated with Goat anti-Mouse Alexa-488 1:200 and DAPI 1:200 at room temperature for at least 4 hours. All antibody dilutions were in block. After each incubation, the larvae were washed with PBS-T containing 0.2% Triton-X 6 times for 6-10 minutes each. All larvae in statistical comparisons were stained simultaneously in the same dissecting dish. After staining the larvae pelts were mounted in Permafluor.

Synaptic Phosphorylated Mad Imaging and Threshold analysis

Phospho-Mad staining in the red channel was imaged with a Leica Confocal SP2 system. All comparison images were imaged with sequential scan and were imaged in two channels, postsynaptic DLG (green) and p-Mad (red).

For synaptic p-Mad quantification, the synaptic area identified by Dlg staining was outlined in IPLab and designated as an ROI. The ROI was then transferred to the presynaptic p-Mad staining. A threshold was established, designating all of the signal

intensity that was above the threshold. This designated staining above threshold was summed and quantified as percent of the ROI area. Because this measurement is a percent of area, it takes into consideration different size synaptic terminals that occur in various genotypes, such as *wit* mutants and control larvae. The percent of staining above threshold within the ROI was analyzed for several synaptic terminals in each group for every bar graph.

The same technique was used for nuclear p-Mad. The nuclei of the ventral ganglion were encircled in the Mad-YFP channel with one ROI and the transferred to the p-Mad channel. Quantification proceeded as described above.

Statistical Analysis

Statistical analysis was preformed and bar graphs were created using Prism statistical software. Comparing the experimental data set to the control yielded all p-values unless noted. A student's unpaired t-test with one-tailed p-value was completed for all data sets.

Immunofluorescence for Bouton Counting

Third instar larvae were heat killed and dissected in PBS containing, 1mM EGTA, 1mM MgCl. They were dissected and fixed with 4% PFA in PBS containing 1mM EGTA, 1mM MgCl, and 0.2% Triton-X. After fixation, the larvae were washed with PBS 3-5 times. After dissection, the larvae tissue was blocked at room temperature for approximately 1 hour with PBS containing 0.1% Triton-X and 2.5% BSA. Primary antibodies targeting the pre and post-synaptic membranes were incubated with the larvae

pelts overnight at 4 degrees. The primary antibodies are Goat anti-HRP-Cy3 conjugate (Jackson Laboratories, 1:200) and Mouse anti-Dlg (1:50). The secondary antibody Goat anti-mouse Alexa 488 (1:200) and with DAPI were incubated with the larvae pelts for at least 4 hours at room temperature. All antibody dilutions were in block. After each antibody incubation, the larvae pets were washed at least 6 times for 6-10 minutes in PBS containing 0.1-0.2% Triton-X. After staining the larvae pelts were mounted in Permafluor.

Bouton Counting

Synaptic terminal boutons of third instar larvae were counted for muscle 4 segments A2-A5. Boutons were identified as a circular delineation of the surrounding synaptic area. Only boutons that were seen in both the pre-synaptic red channel and post-synaptic green channel were counted. The boutons were counted on a Zeiss microscope 63X lens. To obtain the area of the muscles, pictures were taken with a Nikon Olympus 20X objective. With IPLab the length and width of the muscles were measured using the drawing tool. The rectangular area of the muscle was then obtained. To obtain a normalized synaptic terminal size, the number of boutons was divided by the area (length times width). To establish percent size, each value was divided by the average of the normalized control synaptic terminal size.

Bouton Size Analysis

To image all the boutons for each synaptic terminal, z-stacks of each post-synaptic membrane were taken by imaging Dlg staining with a Leica SP2 microscope.

The synaptic area imaged by the z-stacks range in thickness between 3-10 microns. The maximum projection of the z-stack is captured and the bouton size of each synaptic terminal is analyzed. The segmental circular drawing tool in IPLab was used to outline each bouton, measuring 10 boutons per synaptic terminal. The Measure segments command gave an average area of each ROI size. This average area was taken for several synaptic terminals per genotype.

Mad-YFP Immunofluorescence

For analysis of UAS-Mad-YFP fluorescence intensity, larvae with the genotype OK6>Gal4, Mad-YFP/ OK6>Gal4, Mad-YFP were analyzed. These larvae were dissected and fixed with 4% PFA in PBS for 5 minutes so not to quench the YFP fluorophore. After immunostaining the larvae pelts were cleared in 80% glycerol in PBS and mounted in Permafluor.

When examining Mad-YFP fluorescence intensity at the synaptic terminal, anti-HRP was used to identify the presynaptic membrane. After fixation the larvae was blocked in PBS containing 0.1% Triton-X100 and 2.5% BSA. The anti-HRP Cy3 conjugate was from Jackson Immuno Research and used in a dilution of 1:200 in block and incubated for 6 hours at room temperature. Next, secondary antibody Goat anti Mouse- 488 alexa (1:200) and DAPI (1:200) were incubated with the samples overnight at 4ºC. After incubation, the larvae were washed in PBS containing 0.2% Triton-X100 six times for 6-10 minutes each.

Mad axonal movement analysis

Larvae with the genotype OK6>Gal4, UAS-Mad-YFP/+ were imaged (microscope settings were: Exposure 100ms, no binning, laser excitation 514-20%, emission filter- 587nm.) and OK6>Gal4, UAS-Mad-YFP/ UAS-Tkv-CFP (microscope settings were YFP: Exposure 50ms, Bin 2, laser excitation 514-20%, emission filter- 587nm; CFP: Exposure 100ms, Bin 2, laser excitation 440-30%, emission filter 485nm/ 705 nm). Utilizing the Perkin Elmer, Ultraview software, we imaged along the axon and bleached a center portion for a clear view of moving particles. Movies lasted 30-60 seconds.

Microscope confocal imaging

Timelapse movies were obtained in a Nikon Eclipse TE2000-U Lambda LS Stutter microsope equipped with a Perkin Elmer Ultraview confocal head and an Ultraview ERS Rapid Confocal Imager using Ultraview or Volocity software. The animals were dissected live in HL3 medium (Stewart et al., 1994), pinned to Sylgard in a specially designed imaging chamber, eviscerated keeping CNS, nerves and muscle intact and covered with a coverslip. Confocal imaging of fixed tissues for synaptic and ventral ganglion comparisons was completed on a Leica SP2. All of compared images were taken at the same settings.

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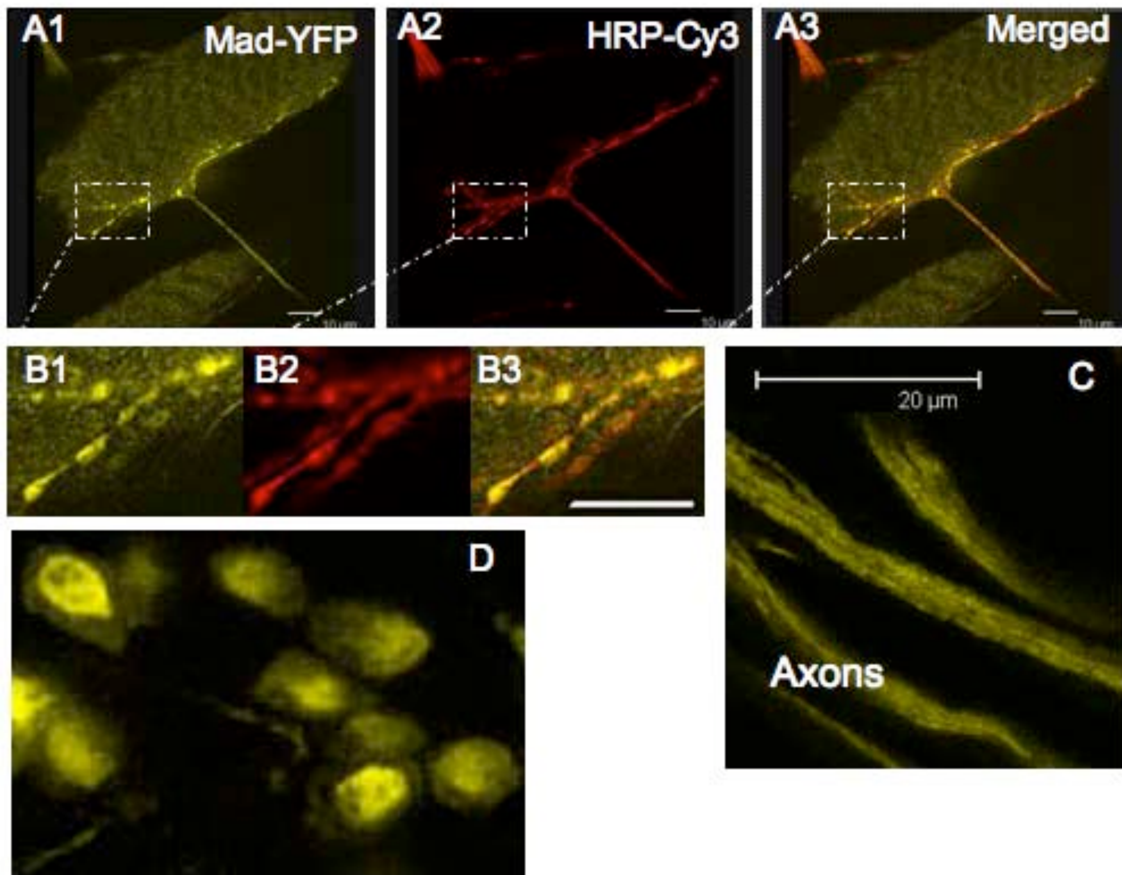


Figure 1: Mad-YFP localizes to the synaptic terminal, axon and cell body

Mad-YFP was expressed with OK6 motoneuron driver and third instar larvae were dissected, fixed and stained with an anti-YFP for signal amplification. The YFP signal from Mad was seen at the synaptic terminal (A&B panels), along the axon (C), and in the nucleus (D) of motoneurons. An enlarged area of the synaptic terminal is shown (B1-B3). Scale Bar is 10 microns.

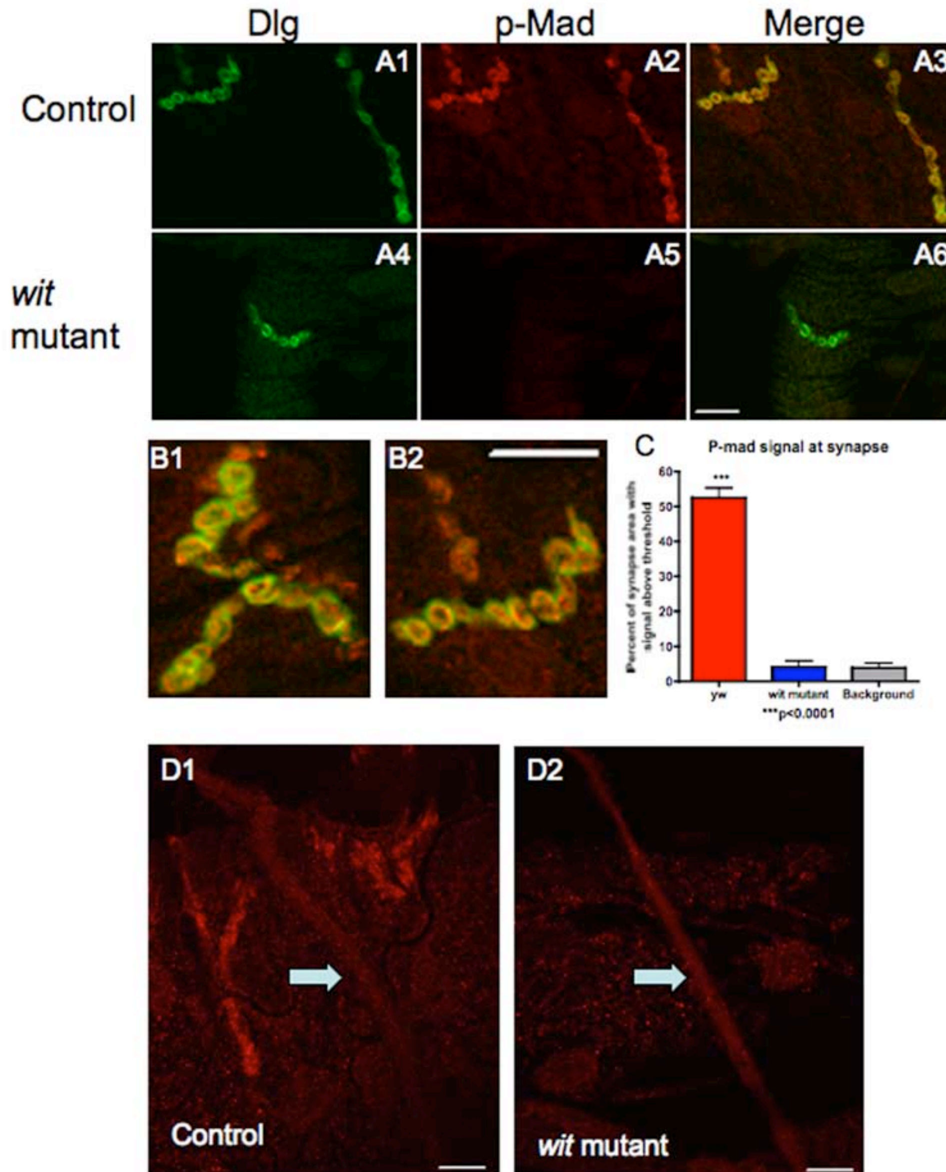


Figure 2: Phosphorylated Mad is at the synaptic terminal but not along the axon

Synaptic terminal 4 in control (A1-A3) and *wit* mutant (A4-A6) are immunostained for post-synaptic protein Discs large (Dlg) (A1, A4) and p-Mad (A2, A5). Dlg and p-Mad are merged (A3, A6). Merged panels are enlarged (B1, B2) to demonstrate that post-synaptic Dlg (green) surrounds the p-Mad (red) indicating that p-Mad is pre-synaptic. When quantifying the p-Mad staining (C), we compared synaptic staining of third instar larvae with genotypes of *yw* (n=11), *wit* mutant (n=16), and *yw* background staining (n=11, no primary antibody). We found that the p-Mad staining at the synapse is eliminated in *wit* mutants ($p<0.0001$) because the *wit* mutant level of p-Mad is equivalent to background staining done with no primary antibody and only secondary. There is no p-Mad present in the axons of control larvae despite the positive synaptic staining (D1). The axon in control (D1) is compared to a *wit* mutant (D2) to demonstrate the lack of specific p-Mad staining. Scale bar equals 10 microns.

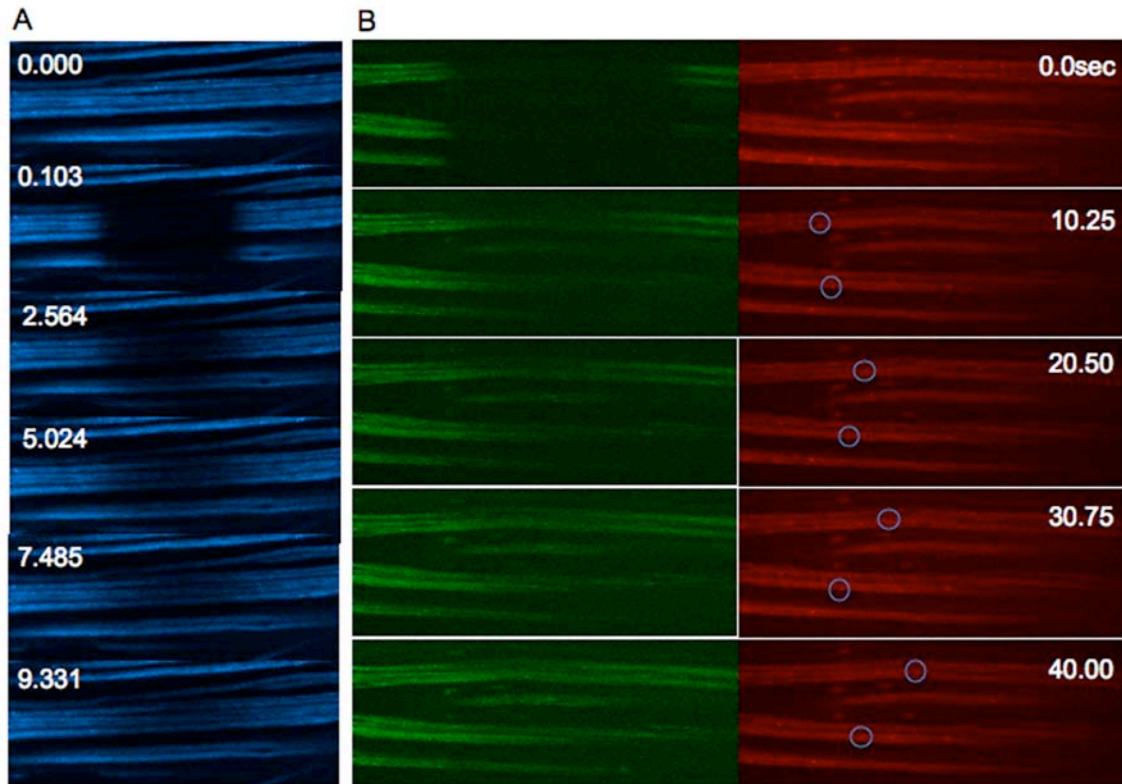


Figure 3: Mad-YFP appears diffuse along the axon and does not travel with receptor vesicles

Mad-YFP appears diffuse along the axon and demonstrates no preferential directional movement along the axon (A). For better resolution the center portion of the axon is photo-bleached and then imaged for 60 seconds to observe Mad movement. Mad-YFP recovers from both sides of the bleached area at the same rate demonstrating no directionality to Mad's movement (A). Mad-YFP (green channel) and Tkv-CFP (red channel) were co-expressed to observe if Mad-YFP's movement would correspond to Tkv vesicles transport (B). After bleaching the middle area of the axon in the YFP channel, we did not see any Mad-YFP movement corresponding with moving receptor vesicles (blue circles) (B).

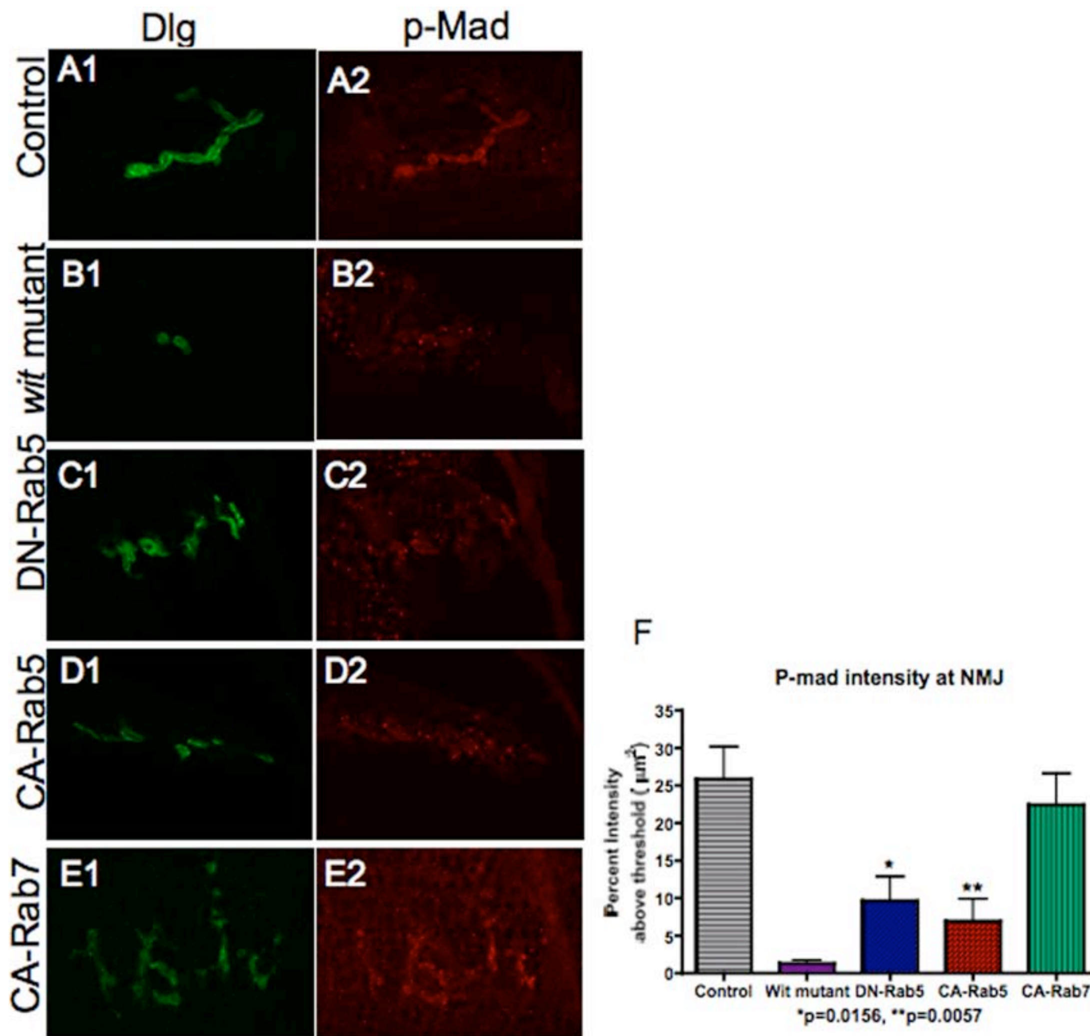


Figure 4: p-Mad levels at the synaptic terminal are affected by the perturbation of the early endocytic pathway

Larvae were stained with antibodies recognizing p-Mad in the red channel and Dlg in the green channel. We stained 5 larvae of each of the following genotypes: Control (Ok371>Gal4/+, n=11) (A), Wit mutant (OK371>Gal4/+, wit^{A12}/wit^{B11}, n=9) (B), DN-Rab5 (OK371>Gal4/+; UAS>DN-Rab5/+, n=7) (C) CA-Rab5 (OK371>Gal4/+; UAS>CA-Rab5/+, n=7) (D), CA-Rab7 (OK371>Gal4/+; UAS>CA-Rab7/+, n=7) (E). The quantification of the synaptic staining intensity shows that both CA-Rab5 and DN-Rab5 have reduced levels of p-Mad staining (F). The negative control *wit* mutant p-Mad staining has a 95% reduction compared to control staining. Compared to control, DN-Rab5 has a significant 63% reduction in synaptic staining (p=0.0156). Additionally, CA-Rab5 has a 73% reduction in synaptic p-mad staining (p=0.0057). However, CA-Rab7 did not have a significant change in synaptic p-Mad staining. These results indicate that Rab5, but not Rab7, regulates p-Mad at the synapse.

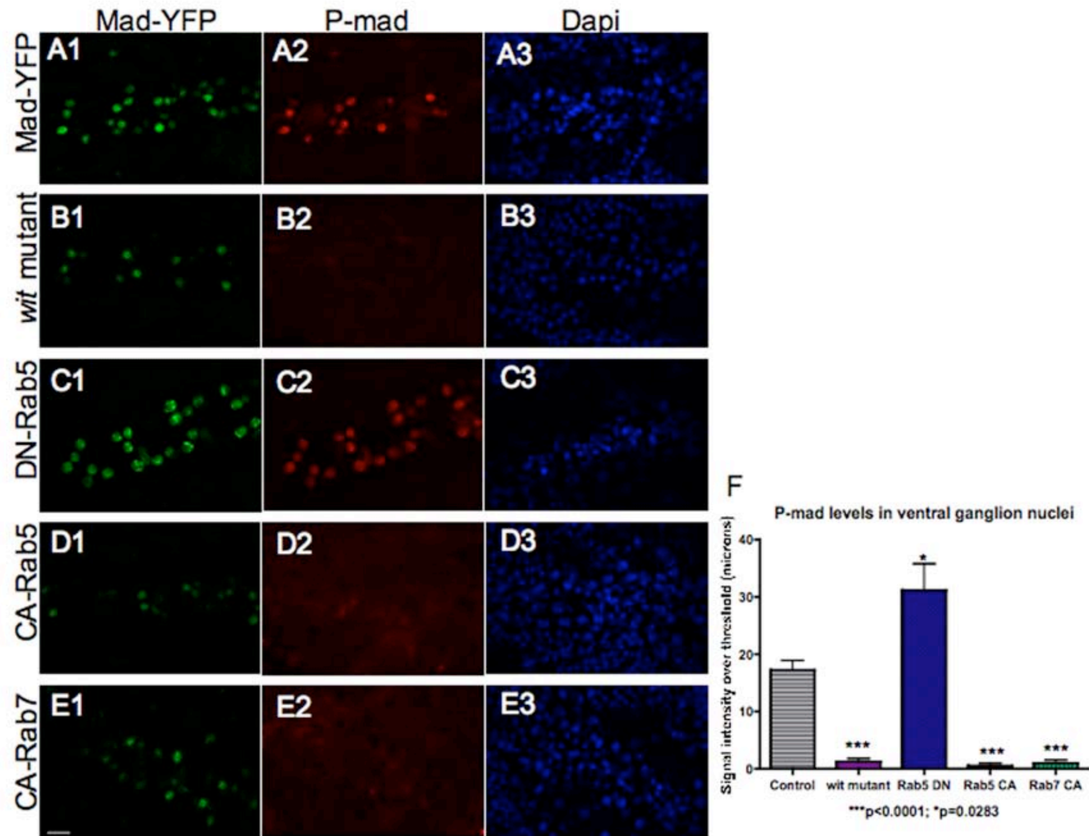


Figure 5: p-Mad levels at the nucleus are affected by the perturbation of the late endocytic pathway

Third instar larvae expressing Mad-YFP were dissected and stained for p-Mad (red channel) and DAPI (blue channel). All animals expressed Mad-YFP driven by the OK371 driver. The control (A) animals displayed nuclear p-Mad while *wit* mutants (B) did not. In addition to Mad-YFP the other larvae expressed: DN-Rab5 (C) CA-Rab5 (D), CA-Rab7 (see fig. 4 legend for genotypes) (E). The quantification of the nuclear staining intensity shows that both CA-Rab5 and CA-Rab7 have *wit* mutant levels of p-Mad staining (F), indicating that there is no p-Mad in the nucleus. Expression of DN-Rab5 resulted in a significant increase in p-Mad in the nuclei of motoneurons when compared to control.

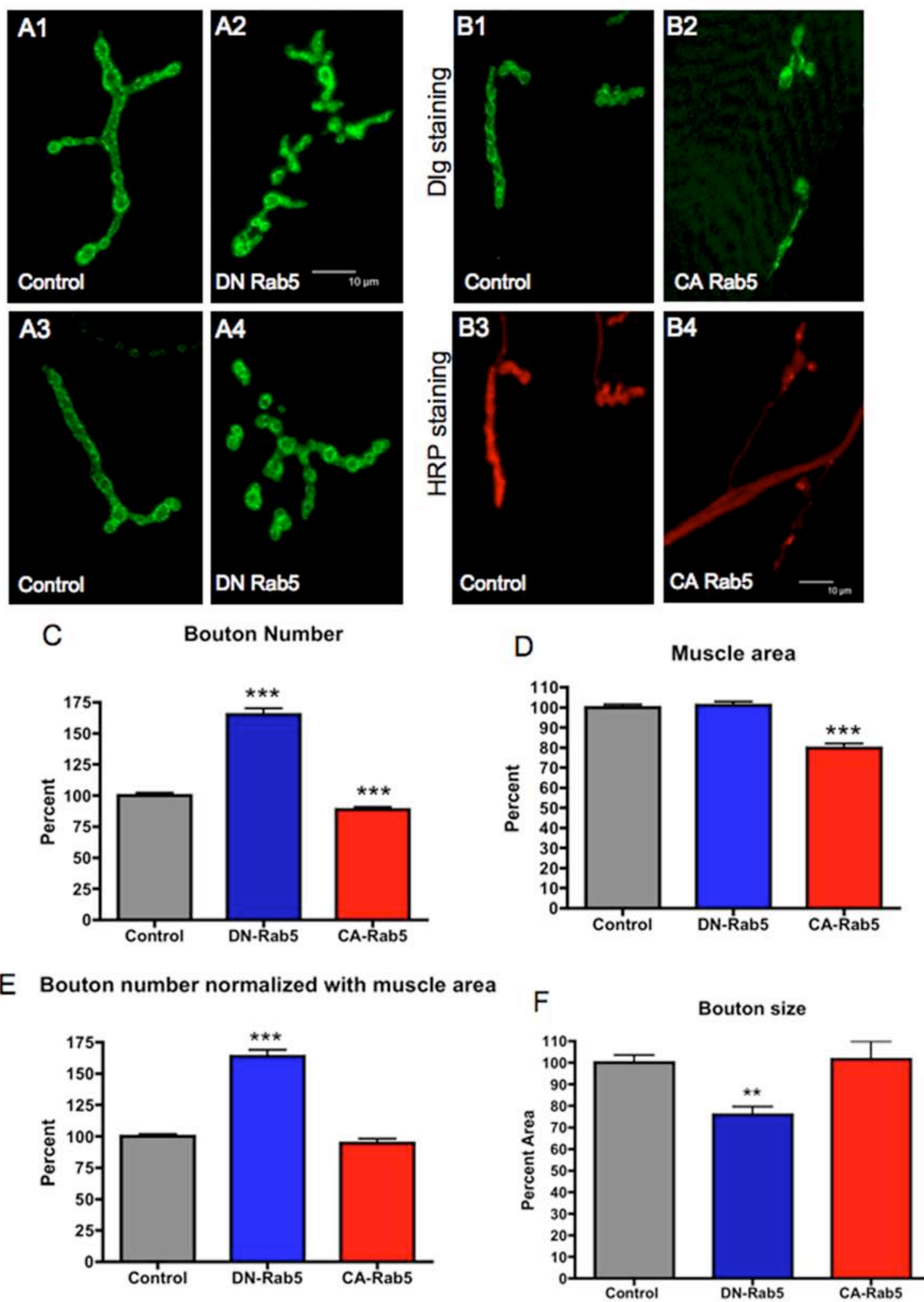


Figure 6: Expression of Dominant negative Rab5 affects synaptic size

Figure 6: Expression of Dominant negative Rab5 affects synaptic size (cont.)

Expressing DN-Rab5 in motoneurons, we looked at synaptic size as a readout of BMP signaling. We found that the synaptic terminals had a phenotype of increased bouton number (A1-A4). Conversely, when CA-Rab5 was expressed in motoneurons a reduction in bouton number was observed (B1-B4). Larvae with both control and experimental genotypes were raised in the same vial to eliminate environmental differences. Large HRP-containing vesicles and decreased HRP membrane staining in larvae expressing CA-Rab5 demonstrate an increase in membrane endocytosis (B4), in agreement with other studies indicating that CA-Rab5 functions as a constitutively active molecule. DN-Rab5 synaptic terminals (n=99) had an average of 65% more boutons than control (total synapses n=106) ($p<0.0001$) (C). CA-Rab5 synaptic terminals (total synapses n=100) had an average of 23% less boutons than control (n=112) ($p<0.0001$) (C). The synaptic bouton number was normalized to muscle area to compensate for differences in muscle size. DN-Rab5 animals' muscle 4 areas (n=100) were not significantly different from the control (n=105) (D). However, CA-Rab5 muscles (n=99) were 21% smaller than their control counterparts (n=112) ($p<0001$) (D). When the muscle area normalizes the bouton data, DN-Rab5 synaptic terminals are 64% larger than control ($p<0.0001$) while CA-Rab5 synaptic terminals are not significantly different than control synaptic terminals (E). Individual boutons areas were also measured. DN-Rab5 had boutons with 24% smaller area than control ($p=0.0020$) while CA-Rab5 animals had similar size boutons (F) (n=60 boutons, 6 synaptic terminals for all groups). In summary, motoneurons expressing DN-Rab5 demonstrate decreased synaptic bouton size and increased synaptic terminal size when normalized with muscle. Expression of CA-Rab5 has no significant effect on bouton or synaptic terminal size. Images and quantifications are from synaptic terminal at muscle 4. Scale bar equals 10 microns.

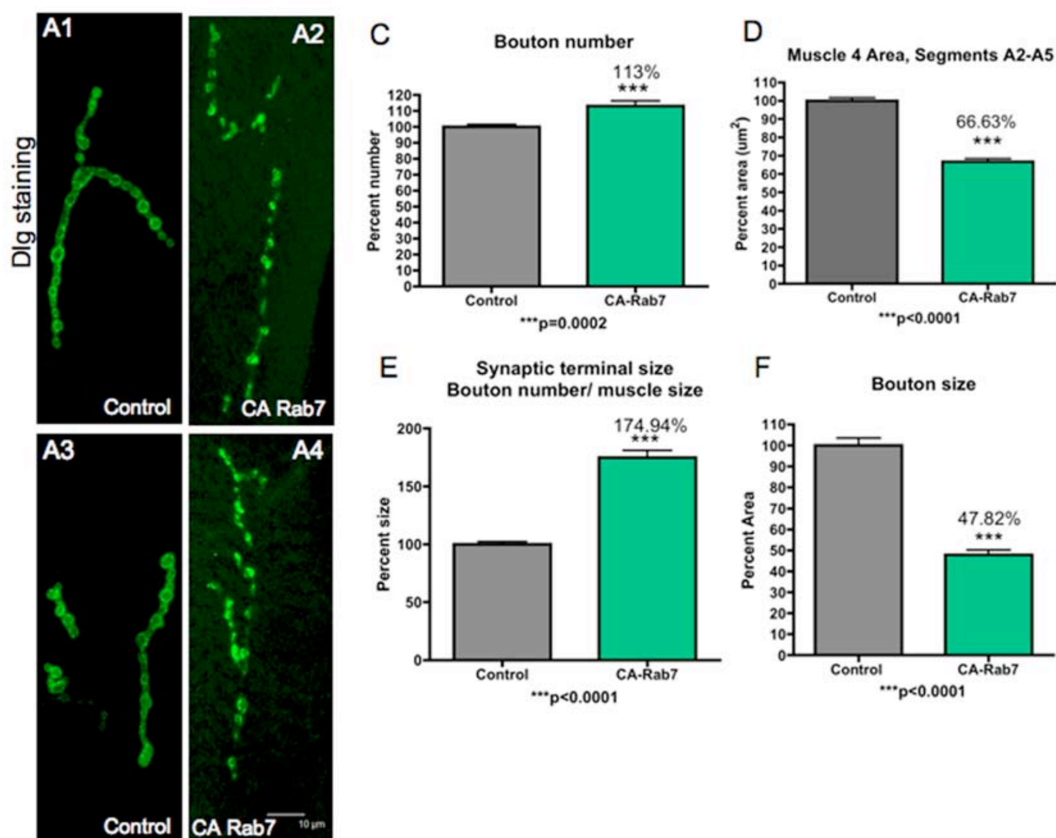


Figure 7: Expression of Constitutively active Rab7 affects synaptic size

Utilizing UAS-CA-Rab7 expression, we examined synaptic morphology of third instar larvae. Muscle 4 segments A2-A5 right and left side were quantified in graphs Fig 4D-E (CA-Rab7: n=96 synapses, and Control group n=115 synapses). Images A1-A4, demonstrate the phenotype of Rab7CA synapses. These images are compacted z-stacks and are representative of the average bouton number in each group. Expression of CA-Rab7 by OK371 motoneuron driver resulted in an increase in bouton number by 13% (p=0.0002) (C). Although they had slightly more boutons, CA-Rab7 animals were much smaller than control animals, with a 67% reduction in muscle area (p<0.0001) (D). After normalizing the bouton number with the muscle area we found the CA-Rab7 had a 75% increase in synaptic size compared with the control group (p<0.0001) (E). Measuring bouton area, we found that CA-Rab7 (n=69 boutons, 7 synapses) individual boutons size were reduced 52.18% when compared with control (n=80 boutons, 8 synapses) (p<0.0001) (F).

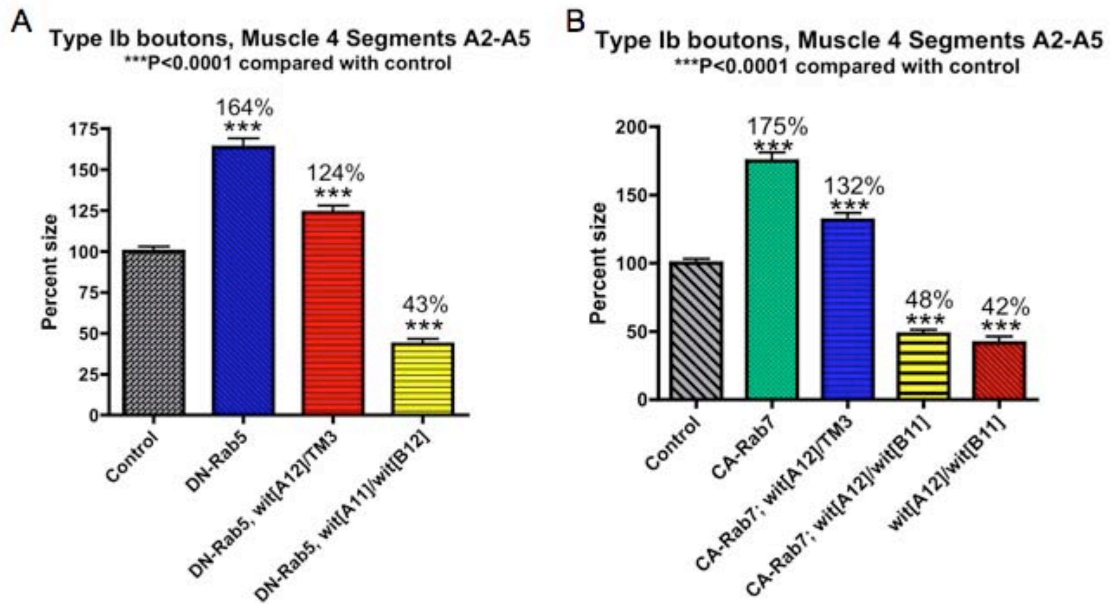


Figure 8: DN-Rab5 and CA-Rab7 expression expands synaptic terminal by way of Wit

In the case of DN-Rab5 and CA-Rab7 expression, we wanted to verify that synapse expansion was a result of BMP pathway disregulation. When one *wit* allele was eliminated, the DN-Rab5 overgrowth phenotype was suppressed by 40% percent (A). When both *wit* alleles were mutant, the synaptic size overgrowth caused by DN-Rab5 was fully suppressed to *wit* mutant levels around 45% of the control (A). In animals expressing CA-Rab7, one *wit* mutant allele caused partial suppression (43%) of the overgrowth phenotype (B). Animals carrying two *wit* mutant alleles showed a full suppression of the CA-Rab7 overgrowth phenotype, with the synaptic terminal size diminishing to *wit* mutant levels (42%) (B). *wit* mutant synaptic size and CA-Rab7 synaptic size with two *wit* alleles are not significantly different from each other (B). This suppression of the overgrowth phenotype by *wit* alleles demonstrates that excess BMP signaling is responsible for synaptic terminal overgrowth induced by CA-Rab7 over expression

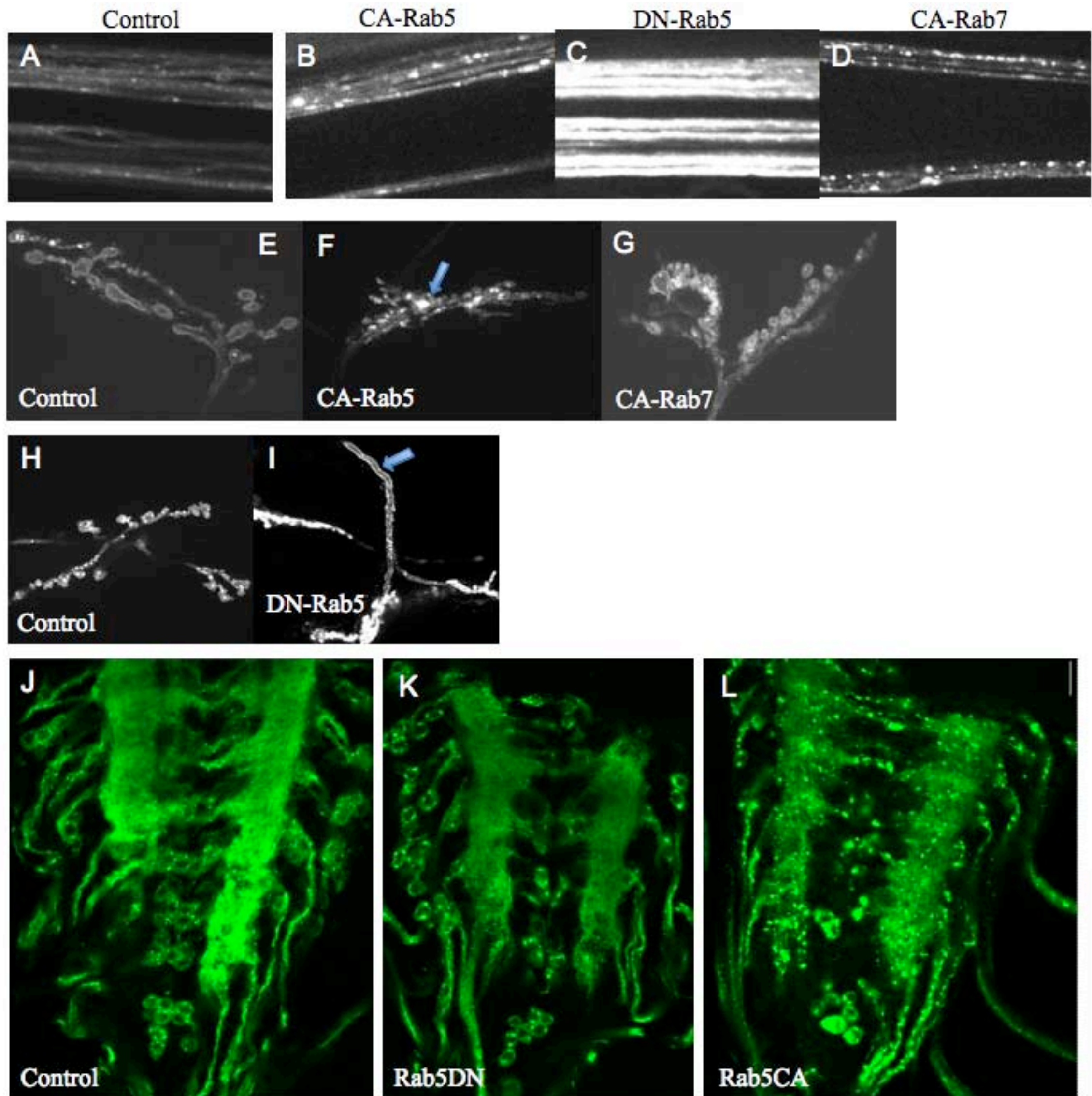


Figure 9: Endocytic sorting alters receptor localization

Tkv-YFP transport was altered in animals expressing Rab mutant transgene with OK371, a motoneuron driver. In the control there are vesicles along the axon (A) and these vesicles are dynamically moving. This is in contrast to when CA-Rab5 (B) or CA-Rab7 (D) are expressed, because there is an increase in large stationary vesicles along the axon. Additionally, transport of Tkv-YFP is altered when DN-Rab5 is expressed showing a decrease of vesicular Tkv, which could be an increase in plasma membrane bound receptor levels or an increase in small vesicles below the resolution of the microscope (C).

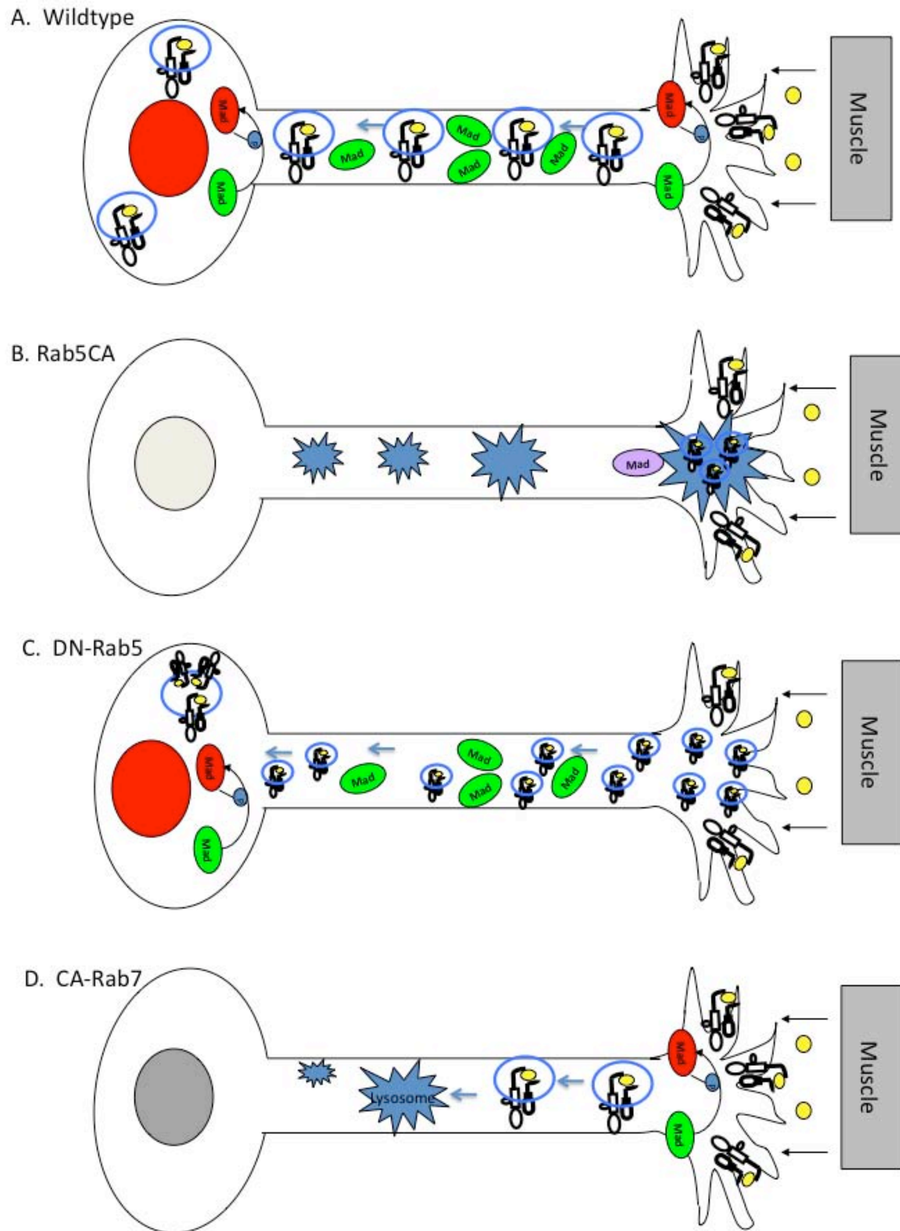
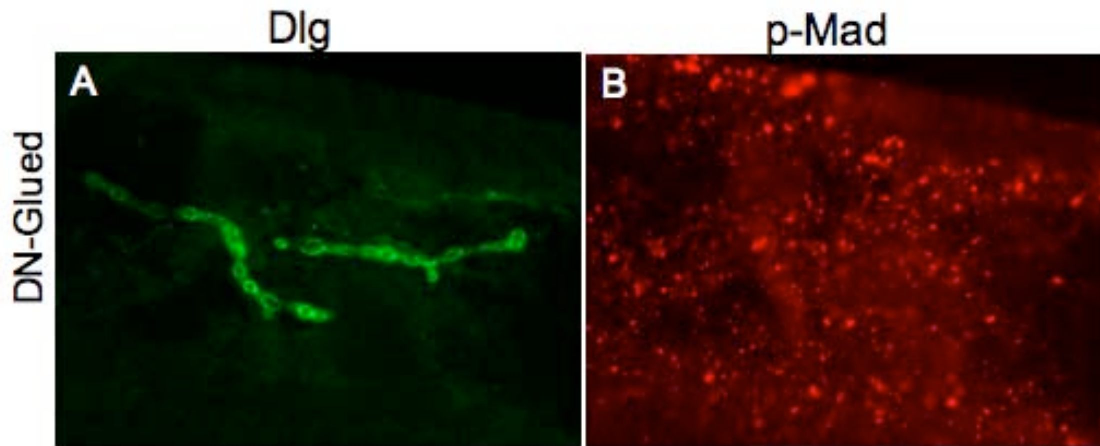


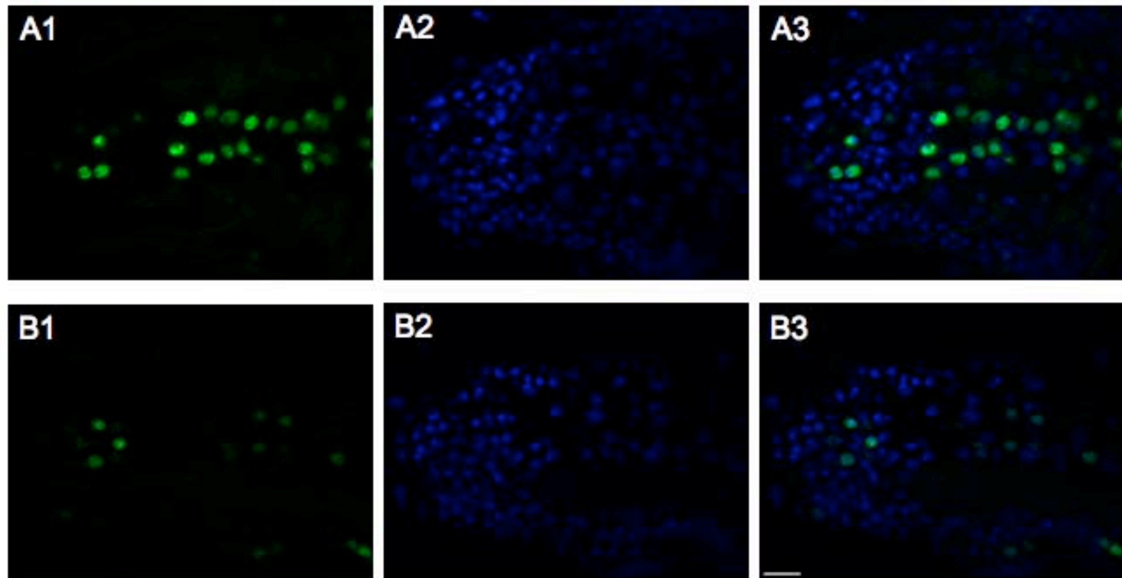
Figure 10. Possible models of results

Models of the wild type BMP signaling (A). Possible model of CA-Rab5 expression- Receptors are endocytosed in large fused endosomes and unable to phosphorylate Mad (B). Possible model of DN-Rab5 expression- There could be less receptor inactivated by endocytosis so there are more receptors traveling to the nucleus (or in the plasma membrane). Endosomes at synaptic terminal may exist as small immature early endosomes unable to phosphorylate Mad efficiently. Endosomes may mature at the cell body where they can phosphorylate Mad for entry into the nucleus (C). Possible model of CA-Rab7 expression- Receptors could successfully signal at the synapse but then get transported to the lysosome, inhibiting more proximal Mad phosphorylation at the cell body (D).



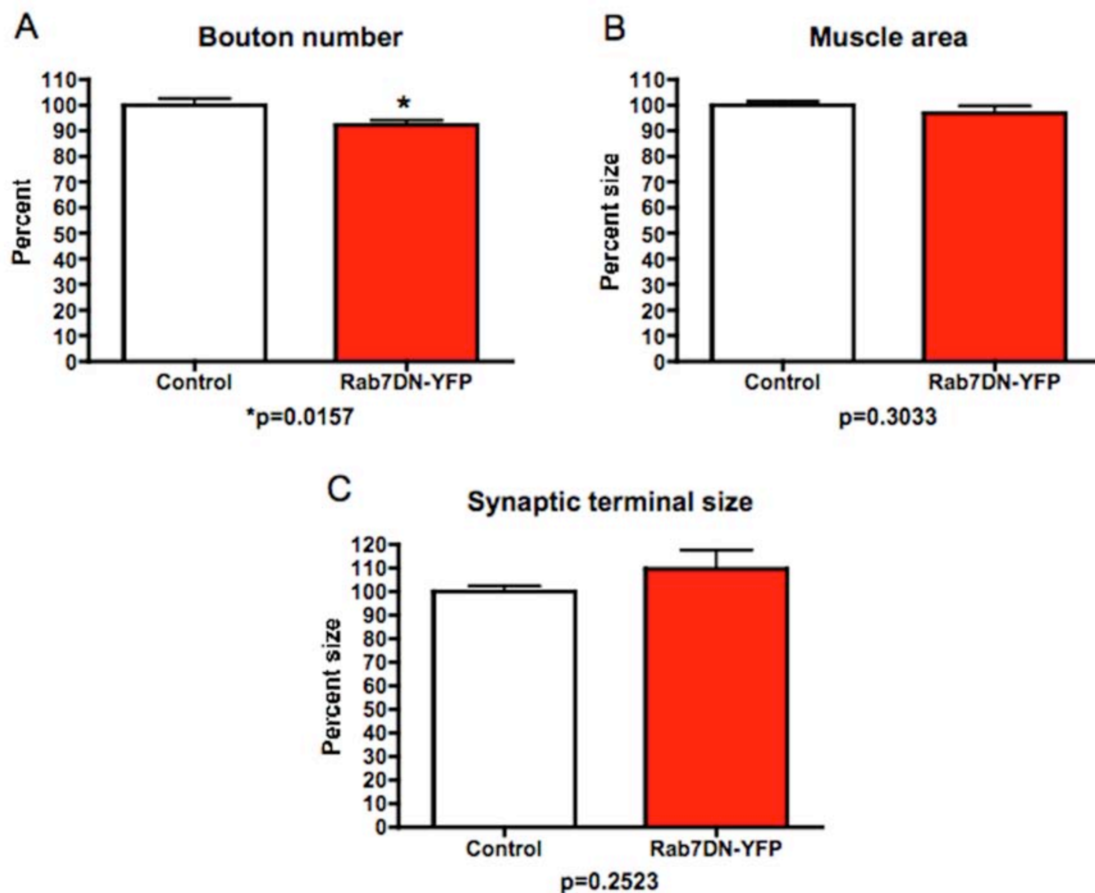
Supplementary figure 1: DN-Glued expression causes a loss of p-Mad at the synaptic terminal

DN-Glued expression demonstrates lack of p-Mad staining when compared with control (not shown) Muscle 4 was immunostained with post-synaptic protein marker Dlg (A) and p-Mad (B).



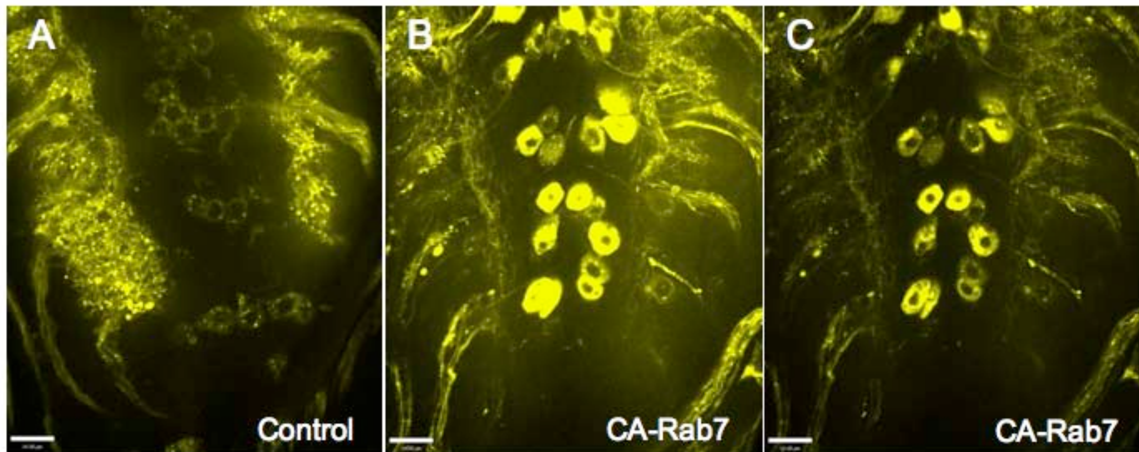
Supplementary figure 2: *wit* mutants have decreased nuclear Mad-YFP

Mad-YFP expressed by motoneuron driver OK6. There is a decrease in nuclear Mad-YFP in *wit* mutants (B1-B3) when compared with control (A1-A3).



Supplementary figure 3: DN-Rab7-YFP has no effect on synaptic size

Quantification of boutons and synaptic size of muscle 4, segments A2-A5. When DN-Rab7-YFP is expressed in motoneurons, we observe a 4% (p=0.0157) decrease in bouton number (A). These animals were similar in size with control (B). When the bouton numbers are normalized with the muscle area, we find that there is no difference in synaptic terminal size between these two groups (N= 12 larvae each, 92 synaptic terminals each).



Supplementary figure 4: CA-Rab7 expression causes receptor aggregates in the cell body of motoneurons

When CA-Rab7 is expressed, Tkv-YFP receptor vesicles are mis-localized to aggregates in the cell body. The control (A) contains normal appearing vesicles but when CA-Rab7 is expressed (B) there are large aggregates of receptor. Compared with control CA-Rab7 was imaged at the same exposure (B) and then at a lower exposure (C) in order to capture more detail of the cell body. Transgenes are expressed by the OK371 driver. Scale bar is 14 microns.

CHAPTER 4

DISSCUSSION: MODEL OF BMP SIGNALING IN *DROSOPHILA* MOTONEURONS

Diseases associated with impairments in learning and memory are devastating illnesses that affect millions of people yearly (Braddock et al., 2001; Herbert et al., 2003; Larson et al., 2000). The basic mechanism of learning and memory is synaptic plasticity, the process by which connections between a neuron and its target are modified (Koh et al., 2000). Bidirectional communication between the pre and post-synaptic cell is critical for the development, maintenance, and activity-dependent modulation of synaptic connections (Fitzsimonds and Poo, 1998). Proteins that are responsible for these communications are released from the target cell but have an effect in the presynaptic cell and are termed retrograde factors (Fitzsimonds and Poo, 1998). When these factors initiate nuclear responses such as gene transcription, these pathways must depend on axonal transport to transport active pathway signals from the proximal and distal ends of the neuron (McKay et al., 1999). Studying how critical signaling pathways utilize axonal transport is essential for understanding diseases of learning and memory.

The BMP pathway in *Drosophila* motoneurons includes a retrograde factor that is critical for synaptic plasticity (Aberle et al., 2002; Marques et al., 2002). The ligand in this pathway, Gbb, is a target-derived factor that acts on its receptors at the synapse, and

consequently, this pathway depends on retrograde transport to carry the active signal to the nucleus where phosphorylated Mad (p-Mad) regulates transcription (McCabe et al., 2003). Dynein has been shown to be necessary in pathway activation, because when Dynein is inhibited by DN-Glued expression, pathway activity is inhibited (McCabe et al., 2003). Critical evidence that reflects the dependency of nuclear p-Mad on long-distance signaling is the result that muscle expressed Gbb rescued the nuclear p-Mad phenotype in motoneurons of *gbb* mutants (McCabe et al., 2003). The goal of this thesis was to determine which component carries the active signal from the synapse to the cell body.

In our study, we detect individual BMP receptors being transported along the axon in bidirectional manner, providing the first clue that they could be carrying the active signal. There are multiple possibilities for receptor trafficking along the axon: The receptors, Wit and Tkv, could be transported individually by themselves, they could be in the same endosome without being in a complex, or the receptors could be in an active complex capable of phosphorylating Mad. We have observed examples of receptor trafficking that are consistent with all of these possibilties. When we look at individual transport, we observe bidirectional transport of the receptors. Although over half of these particles display movement towards the cell body, single receptor endosomes do not exhibit a dramatic preference for transport directionality. Retrograde transport of receptor vesicles could reflect signaling endosomes or individual receptors that are being transported to the cell body for degradation. Anterograde movement could reflect newly translated receptors that are being shipped from the cell body so that they can function at the synaptic terminal, or they might be receptors whose transport was initiated at the

synaptic terminal but change directions randomly because their vesicles lack an activation signal. Additionally, we have seen colocalizing receptors in the *gbb* mutant that have not been activated by the ligand that do not demonstrate a preference for directionality, and behave similarly to single receptor traffic. We interpret this observation as receptors that happen to be transported together, hence the colocalization between them, but are not in a complex because they do not demonstrate directionality. Finally, when the receptors are expressed in the presence of the ligand, we see 95% of colocalized traffic moving in a retrograde direction. When the ligand is eliminated, we observe loss of directionality, which is strong evidence for a signaling endosome. We conclude that the preference for retrograde transport of colocalized receptors in the presence of the Gbb ligand supports the signaling endosome model.

Additional evidence supports the signaling endosome model, inconsistent with Mad transcription factor carrying the active signal to the cell body (Fig1). The type I receptor vesicle transport depends on the presence of the type II receptor, reflecting a possible packaging dependency on both receptors as a complex. Additionally, a known inhibitor of the pathway, DN-Glued inhibits receptor transport, linking receptor transport to pathway activity. Mad is diffuse along the axon and doesn't appear to travel with the receptor vesicles arguing against Mad's role in axonal transport of the active pathway signal. Additionally, Mad's phosphorylation at the synapse and cell body depend on the Rab endocytic pathway. Regulation of the pathway could reflect a signaling endosome mechanism for pathway activation, because the overexpression of Rab mutant transgenes interfere with this endosomal traffic and block Mad from being phosphorylated. We have

presented evidence that the receptors are traveling in a signaling endosome and the receptors, not Mad, transport the active signal from the synapse to the cell body.

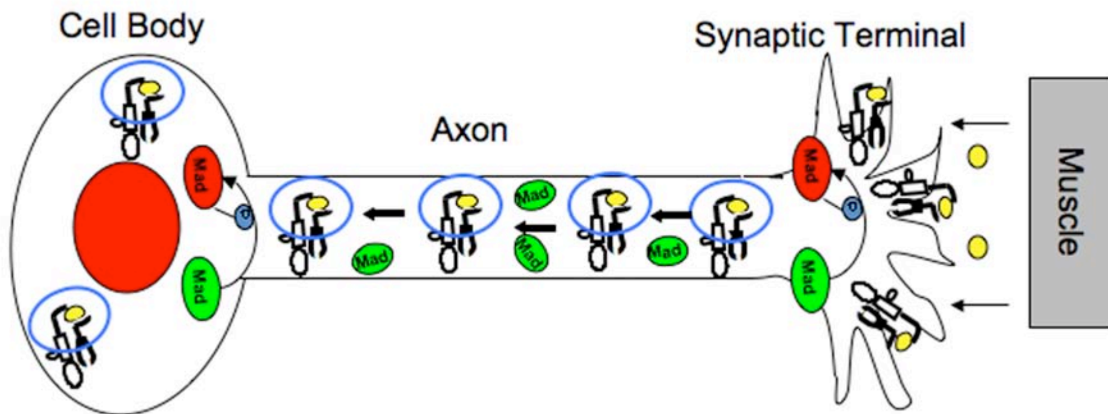


Figure 1: Model of BMP signaling in motoneurons.

The receptors travel from the synaptic terminal to the cell body in a signaling endosome to phosphorylate Mad for nuclear entry. Wit and Tkv colocalize in predominately retrograde moving structures. Mad is phosphorylated in separate events at the nucleus and synaptic terminal. The Rab endocytic pathway regulates receptor endosomes resulting in p-Mad alterations at both distal and proximal locations of the motoneuron.

Resulting from our conclusion that p-Mad is not present along that axon and that Mad is not transported, we also conclude that Mad must be phosphorylated in separate events at the synapse and cell body. This study and others, have shown p-Mad at the synapse and cell body, and we showed that p-Mad is not present along the axon. Additionally, we did not observe Mad transported with receptor vesicles, which are potentially signaling endosomes. When early and late Rab mutant transgenes were expressed, we saw differences in Mad phosphorylation in different subcellular locations. These combined data lead us to conclude that Mad can be phosphorylated in independent events at the synaptic terminal and nucleus.

Evidence from other studies suggests that the endocytic proteins Spict and Nwk regulate p-Mad levels at the synaptic terminal. The Spict protein was found to localize on early endosomes by colocalizing with Rab5 but not any late endosome markers (Wang et al., 2007). Additionally, Nwk colocalized with Rab11, a recycling endosome marker (O'Connor-Giles et al., 2008). Both of these proteins appeared to be both at the early and recycling endosome stage and to function at the synaptic terminal. We also observed that altering endosome traffic modifies p-Mad levels at the synaptic terminal. It was not reported whether Nwk or Spict affected nuclear p-Mad levels, but based on our hypothesis that synaptic Mad and nuclear Mad are phosphorylated at separate events, it would be interesting to test what effect Nwk and Spict have on nuclear p-Mad levels.

What are the implications of Mad being phosphorylated separately at the synaptic terminal and soma of the motoneuron? We can model our observations on the retrograde neurotrophin pathway that preferentially activates different proteins depending on the subcellular location of the activated receptors, thus determining specificity of response. Neurotrophin stimulation at the cell body resulted in activation of both Erk 1/2 and Erk 5; however activation of neurotrophins at the distal axon leads to preferential activation of Erk5 (Cosker et al., 2008; Watson et al., 2001). Locations of subcellular activation of neurotrophin pathways determine the specificity of the response, revealing a high level of detail in neurotrophin pathway regulation. Is it possible that Mad could have differing roles at the synapse and cell body?

A possible role for synaptic terminal p-Mad is regulating synaptic terminal expansion. When CA-Rab5 and CA-Rab7 are both overexpressed, there is elimination of p-Mad in the nucleus, while these flies have normal and expanded synaptic boutons

respectively, casting doubts on the current model that nuclear p-Mad is necessary for synaptic terminal growth. Indeed, O'Conner-Giles et al. have revealed a correlation of p-Mad levels at the synaptic terminal with synaptic expansion (O'Connor-Giles et al., 2008). Our data differs from their findings, because we do not see a correlation of the levels of p-Mad with the amount the synaptic terminal expansion. When we express DN-Rab5, p-Mad levels are reduced, but still present at the synaptic terminal, and there is an expansion of the synaptic terminal. Small synapses are observed only when p-Mad is eliminated from the synaptic terminal in *wit* mutants and when DN-Glued is expressed. Perhaps synaptic terminal p-Mad is interacting with cytoskeletal proteins, interacting with another non-canonical pathway, or providing a permissive signaling that allows for either normal or enlarged synapses.

There is evidence that R-Smads can interact with other pathways that could theoretically contribute to the synaptic terminal overgrowth. Smad3 was shown to form a complex with the pro-survival signal Akt to control a TGF-β pro-apoptotic signal (Remy et al., 2004). Additionally through direct physical interaction, Smad3 has been shown to activate the protein kinase A (PKA) protein (Zhang et al., 2004). In this study, TGF-β activation of CREB was dependent on both Smads and PKA (Zhang et al., 2004). Another protein that could be regulating the structure of the synaptic terminal in response to Smad pathway activation is LIM kinase (LIMK). LIMK is a key regulator of actin dynamics because it phosphorylates and inactivates cofilin, an actin depolymerizing factor (Bernard, 2007). TGF- β1 induced the regulation of actin assembly through the function of the kinase LIMK2 (Vardouli et al., 2005). Additional results showed that an R-Smad inhibitor prevented TGF-β induced actin reorganization, suggesting that R-

Smads are involved in regulating actin through LIMK (Vardouli et al., 2005). p-Mad could be activating these non-canonical pathways at the synaptic terminal to bring about synaptic terminal expansion.

Another possibility is that Wit and/or Tkv actually interact with cytoskeletal proteins at the synaptic terminal and p-Mad is a by-product without function at the synaptic terminal. The receptors could be required to be in an active complex at the synaptic terminal for the effective regulation of the cytoskeleton. In this situation, Mad might be phosphorylated as a by-product, simply identifying where the receptors are in an active conformation, but has no function at the synaptic terminal. Non-canonically, and without the involvement of Smads, the Wit receptor has been shown to interact with the cytoskeleton regulator LIM kinase (LIMK) to stabilize the *Drosophila* synaptic terminal (Eaton and Davis, 2005). Presynaptic expression of LIMK in *wit* mutants restored growth and function to the synapse (Eaton and Davis, 2005). It appears that LIMK has a potential role at the synaptic terminal and can be regulated by Wit. Could the Wit receptor at the synaptic terminal be affecting the synapse architecture, while more proximal BMP endosomal signaling would lead to nuclear p-Mad activation and to a different outcome? Our results suggest that nuclear p-Mad is not required for synaptic terminal expansion and that other non-canonical BMP signaling mechanisms, perhaps local signaling at the synaptic terminal, are responsible for this role. Further studies are needed to address this controversial finding. It is possible that other aspects of synaptic development and function that depend on BMP signaling, such as synaptic transmission and proper apposition of pre and postsynaptic membrane, are mediated by nuclear p-Mad.

Other groups have proposed that the BMP receptors are being endocytosed by novel endocytic machinery for down regulation of receptor signaling, however it is feasible that these recently discovered proteins are involved in receptor recycling, a separate pathway from endosome signaling. In work by O'Conner-Giles and colleagues, Nervous wreck (Nwk) was shown to interact with Tkv to down-regulate signaling. Because Nwk was shown to colocalize with Rab11, and Tkv levels are not altered when Nwk is overexpressed or in mutant form, the group proposes that Nwk could attenuate levels of BMP signaling by regulating the levels of vacant Tkv at the plasma membrane surface (O'Connor-Giles et al., 2008). Another recently described protein, Spichthyin (Spict), has been described to down-regulate BMP signaling (Wang et al., 2007). Wang and colleagues propose that Spict inhibits BMP signaling at the early endosome stage, and levels of Tkv at the synaptic terminal were increased by *spict* knockdown (Wang et al., 2007). Spict, like Nwk, could be involved in recycling vacant receptors and, in that manner, down-regulate the BMP signal. We too show data suggesting that the receptors are recycled because we see Rab4 colocalization with Tkv and Wit only at the synapse and not in other locations in the cell. It appears that some endocytic or transport proteins may be responsible for recycling of the receptors while other accessory proteins would be preferentially involved with the signaling endosome endocytosis and transport.

Adaptor proteins that function to attach cargo to motor proteins might be a specialized way to preferentially select and transport active signaling endosomes. In the mammalian cells, BMPR type II interacts functionally with TcTex-1, a light chain of Dynein (Machado et al., 2003). Another adaptor protein, JIP (JNK interacting protein) is known to connect cargo to Kinesin, but is also implicated in retrograde signaling with

Dynein (Horiuchi et al., 2005). Loss of JIK gene in mammalian cells led to a reduction in retrograde moving cargo, indicating that JIK might also serve as an adaptor protein for Dynein (Sandhya, 2008). Consistently, heterozygous mutation in *Aplip1,* the *Drosophila* ortholog of JIP, and *Dyenin heavy chain 64C* caused axonal transport defects, suggesting a genetic interaction (Horiuchi et al., 2005). Additionally, there is also a control mechanism for JIP release of motor protein interaction. When Aplip1 interacts with proteins in a JNK pathway (Wallenda, Hemiperterous, or Basket), Aplip1 dissociates from Kinesin (Horiuchi et al., 2007). This JNK dependent dissociation of Kinesin and JIP scaffolding protein represents a control mechanism that can be used to regulate targeted transport of selected cargo (Horiuchi et al., 2007). TcTex-1 or a JIP could be a specialized adapter protein that links the BMP signaling endosome to the Dynein motor protein for retrograde axonal transport.

Another endosomal protein, APPL, has been shown to label signaling endosomes in other pathways and is a good candidate for regulating BMP signaling endosomes. EGF was shown to be internalized to APPL positive endosomes and then these APPL positive endosomes translocate from the cell surface to the nucleus (Miaczynska et al., 2004). APPL also appears to have a role in neurotrophin signaling. APPL1 was found to associate with TrkA receptor in endosomal fractions (Lin et al., 2006). APPL1 was also required for NGF induced MEK, Erk1.2 and Akt activation (Lin et al., 2006). For APPL to be released from the membrane it requires GTP hydrolysis from Rab5 (Miaczynska et al., 2004). The signaling endosome route for EGF and neurotrophins appears to be regulated by specific endosomal proteins. APPL could regulate the BMP pathway

signaling endosomes similarly to the EGF and NGF pathways and future studies should explore this possibility.

From our studies, we have found evidence that BMPs receptors are the likely carriers of the active BMP signal along the axon to the motoneuron nucleus. Our evidence suggests that the receptors are transported from the synaptic terminal to the cell body in a signaling endosome. Additionally, we find no evidence in support of the Mad transcription factor actively transporting the active signal along the axon. Finally, early and late endosome signaling regulates Mad phosphorylation. Our model consists of two signaling events that take place by an active BMP receptor complex in motoneurons. Based on our model, one event takes place at the synaptic terminal and the other event takes place at the cell body and is regulated by a long distance BMP signaling endosome (Fig 1). Further study is needed to determine Mad's role in synaptic terminal expansion.

Identifying the mechanism by which BMPs are transported along the axon is a first step towards understanding the regulation of synaptic plasticity. Intriguingly, a recent study has linked Spinal Muscular Atrophy (SMA) and Wit pathway signaling (Chang et al., 2008). Spinal Muscle Atrophy is a recessive hereditary neurodegenerative disease in humans that results in early onset lethality, motor neuron loss, and skeletal muscle atrophy. Using a *Drosophila* ortholog to model this disease, the Wit receptor and Mad transcription factor were identified to modify the disease phenotype (Chang et al., 2008). Loss of function of Dad, a Mad inhibitor rescued the *Smn* disease phenotype at the synaptic terminal (Chang et al., 2008). The authors propose that increasing BMP signaling could be a possible therapeutic approach for SMA patients. Our results describing the mechanism of BMP signaling in *Drosophila* motoneurons can only

increase the success of further disease studies by contributing to a deeper understanding of synaptic plasticity.

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