THE NEUROGENETIC ANALYSIS OF SYNAPTOGENESIS AND SYNAPTIC PLASTICITY IN *DROSOPHILA MELANOGASTER*

By

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A dissertation to the Graduate Faculty in Biology in partial fulfillment of the requirements for the Doctoral of Philosophy,

The City University of New York

2012

This manuscript has been read and accepted for the
Graduate Faculty in Biology in satisfaction of the
dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

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Adviser: Dr. Tadmiri Venkatesh

Synaptogenesis is the process by which nascent axons from developing neurons target and form mature synapses with other neurons or cells. Specifically, synaptogenesis at the neuromuscular junction (NMJ) involves the motorneuron axon targeting its specific innervating muscle. The NMJ of the Drosophila larva is an excellent model system to study synaptogenesis due to genetic tools developed in the Drosophila melanogaster. The NMJ in Drosophila are glutamatergic, resembling mammalian central nervous system (CNS) excitatory synapse, and can provide insight into the molecular mechanisms that control synapse development and transmission. In addition, studying the physiology of the synapse can also provide for greater understanding of mechanisms that underlie synapse maintenance and plasticity.

In this dissertation, I have focused on the role of two functionally dynamic pathways, ubiquitination and cAMP-PKA (protein kinase A or A kinase) during development, and their role in NMJ synaptic structure and function. *rugose* (*rg*), which encodes for the *Drosophila* A kinase anchoring protein 550 (DAKAP550), belongs to a family of cAMP-PKA organizing centers that provide the scaffolding to permit PKA to bind to specific subcellular organelles, allowing for cAMP to activate PKA more effectively. There were changes observed in hypomorphic *rg* mutants in synaptic

transmission and plasticity as well as in basic forms of learning, specifically habituation, which involves the neuron, in this case, to cease responding to presented stimuli. Taken together, *rg* is necessary for the development of the synapse and synaptic transmission.

Our lab has previously shown that *Retina aberrant in pattern/Fizzy related* (*Rap/Fzr*), the activating subunit of the, E3 ubiquitin ligase, Anaphase Promoting Complex (APC/C) regulates cell fate determination in the developing Drosophila brain. Our cell biological, ultrastructural, electrophysiological, and behavioral data show that *rap/fzr* loss-of-function mutations lead to changes in synaptic structure and function, as well as locomotion defects. Specifically these changes were observed pre- and postsynaptically, represented by size and morphology of synaptic boutons, and number of neurotransmitter vesicles. Electrophysiologically, these were correlated with decreased transmission failure rates as well as increase in the size of synaptic potentials. In addition, larval locomotion and peristaltic movement are also impaired as. These findings suggest a novel role for Drosophila-Cdh1-mediated ubiquitination during development of functional synapses in the peripheral nervous system.

The use of genetic modifier screens in our laboratory has identified several neuronal substrates that physically interact with APC/C$^{Cdh1/Rap/Fzr}$ in Drosophila. As a part of my thesis research, I focused on three proteins: Locomotion Defects (Loco), Nonstop (Not) and Twins. Their involvement in signaling pathways makes these proteins candidates together with Rap/Fzr/Cdh1 to regulate synaptogenesis at the pre- and postsynapse of the Drosophila NMJ. This data suggest Not, Loco and Twins are localized at the NMJ, and involved in regulating synaptic development by genetically

interacting with APC/$C^{Cdh1/Rap/Fzr}$.

Both of these pathways play an important role in neuronal development, transmission, learning and memory, and dysfunction. However, their role in modifying synaptic activity remains unclear. Using *Drosophila Melanogaster* as a model, this thesis will elucidate the role of the APC/C and Rugose in synaptic development and plasticity at the neuromuscular junction.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Tadmiri Venkatesh, wholeheartedly for his constant support and guidance throughout this challenging process. Dr. Venkatesh has been instrumental in helping me achieve this milestone in professional and personal growth. I would also like to thank Drs. Chun-Fang Wu, Atsushi Ueda and Shao-Ying Hu for their academic support and mentoring.

I would also like to thank my parents Janet Wise-Thomas and Larry Thomas for their support and steadfast love in this process. I like to thank my family Malva Wise-White and Robert White, Norma Martin and Keith for their love and support.

To the past and present members of the Venkatesh Lab, especially Julian Flores and Emma Schatoff, I would like to express my gratitude. Special appreciation also to Drs. Margarita Kaplow, Richard Able, Chiyedza Small and Adam Korayem, for their friendship and guidance. Also I would like to thank the greater CCNY and CUNY community without their support none of this would be possible. I would like to thank my committee members for their guidance.

Lastly, I would like to thank my funding sources, NIH-MBRS RISE program, NYC Louis Stokes Alliance for Minority Participation and Alliance for Graduate Education and the Professoriate as well as Dr. Claude Braitwaite for his continued support and mentorship.

DEDICATION

I dedicate this thesis to the memory of Dr. Josh Wallman, who will be greatly missed. Dr. Wallman was my neuroscience professor and committee member at my thesis proposal. He influenced the way I thought about neuroscience and helped me gain insight and perspective on my own research.

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OVERVIEW

The concept of synaptogenesis encompasses specific processes that govern neuron differentiation, specific cues from various growth factors that guide nascent axons to target cells as well as generating a stable synapse and functional active zone between that neuron and the adjacent cell. The event of forming and maintaining functional synapses at the neuromuscular junction (NMJ) requires the precise temporal and spatial regulation of the assembly of protein complexes during synaptogenesis. Two cellular pathways that act as major regulatory components of synaptogenesis and synaptic transmission are the ubiquitination proteasome system (UPS) and the cAMP pathway. The UPS promotes protein modification and degradation through a pathway of multiple ligases and has emerged as an important mechanism in protein regulation within the neuron. Similarly, cAMP pathway activation allows for amplification of ligand binding to g-protein coupled receptor causing a cascade that induces kinase activation and regulation of gene transcription, thus leading to both dynamic and permanent structural changes in neurons.

Synaptogenesis in Drosophila

In the Drosophila larval NMJ, each muscle cell receives input from each outgrowing motorneuron and forms a specific stable synapse that doesn't change significantly throughout development while the number of boutons can change dynamically (Keshishian et al., 1996). Boutons are small button-like swellings at the end of an axon that package, store and release neurotransmitters into the synaptic cleft of the junction between neuron and muscle, overlapping the muscle site where

the receptors are located. Target recognition of the axon to the correct muscle it innervates, begins as filopodia extend from the axon terminal at the end of a motor neuron (Fiala et al. 1998 and Jones et al. 2000). The filopodium is a unique structure that forms only during axonal growth and elongation, and contains specific receptors to chemoattractive molecules, which guide the axon to its proper position. High release of glutamate is the main component in postsynaptic receptor clustering (Marrus and DiAntonio, 2004). However, a prolonged period of high glutamate extracellular concentration can lead to suppression of clustering (Augustin et al., 2007).

At each NMJ there are on average 20-30 boutons that innervate each muscle and each of these boutons contain presynaptic release zones or active zones. Each active zone contains specialized machinery to release the neurotransmitter, glutamate, in the synaptic cleft. Once glutamate is released it binds to postsynaptic receptors on muscle tissue that are in direct apposition across the cleft from the active zones, which opens NMDA and AMPA receptors allowing for the influx of a hyperpolarizing current of ions, Ca^{+2} in particular, causing muscle contraction. Drosophila glutamate receptors are comprised of two subunits, typically DGluRIIA or DGluRIIB (Broadie et al., 1993). Other subunits differ in single channel properties, synaptic responses, regulation by second messengers and localization (Marrus et al. 2004; Qin et al. 2005; Featherstone et al. 2005). These active zones can undergo modification that is stimuli-dependent (Qin et al. 2005). Modification of the synapse involves specific protein-protein interaction to cause the expression of receptors on membrane surface pre- and postsynaptically and receptor stabilization through the

postsynaptic density. Protein degradation is necessary during development because UPS targets proteins that are no longer functional by polyubiquitinating, allowing for synaptic growth (DiAntonio et al., 2001). This process is mediated by a series of ligases and targets polyubiquitinated substrate protein to the 26S proteasome for degradation.

Development of the filopodia and subsequent presynaptic terminal structures involves various proteins including wingless (wg, Drosophila homologue of Wnt), which is a key regulatory molecule (Packard et al., 2002). Wg is secreated presynaptically by the growing filopodia which binds to *Drosophila* Frizzled (Dfz2) receptor on muscle cells to signal the release of TGF-β/BMP ligand, Glassboat bottom (Gbb). Gbb binds to Txv (Thickvein) receptor located on the presynaptic bouton that is coupled to preseynaptic receptors, wishful thinking (Wit) and LIM (LIM). LIM dissociates from the receptor and interacts with MAD (Mothers against decapentaplegic) homolog Smad and MEDEA to turn on transcription of presynaptic proteins. Another powerful regulator of synaptic growth is an E3 Ligase, highwire (HIW), which targets synaptic protein for degradation. Suppression of HIW leads to dramatic increase in boutons with functioning postsynaptic densities and also disrupts the function of a MAPKK identified as Wallenda (Wan et al 2000). HIW has also been shown to play a role in the Gbb-Wnt pathway (Wu et al 2005).

Postsynaptically, the Drosophila MAGUKs (membrane associated guaylate kinases) also serve as the key organizing proteins at the synapse (Budnik 1996). MAGUK family are proteins that contain three or more post-synaptic density (PDZ) domains, which are involved in scaffolding organization and ion channel organization,

namely Discs-Large (dlg), bear homology to the mammalian scaffold protein, PSD95 (Woods et al. 1993; Tejedor et al. 1997). The process ultimately forms a stable synapse and septate junction. They function in stabilizing the growing filopodia interaction with myopodia that extend from the muscle cell, forming prevaricosities. Through selective elimination and stabilization, these prevaricosities mature into boutons (Goda and Davis 2003).

retina aberrant in pattern/ fizzy related (rap/fzr) and synaptic regulation of development

A section of my research has focused on *retina aberrant in pattern/ fizzy related* (*rap/fzr*), an activator of the APC/C. Rap/Fzr is the Drosophila homolog of the mammalian Cdh1. The ubiquitin ligase, Anaphase promoting complex/cyclosome (APC/C) has been well characterized for its role in the timely ubiquitination and degradation of mitotic cell cycle kinases, specifically cyclin B (Speese et al., 2003). A functional role for APC/C in neuronal development has been recently uncovered. Anaphase Promoting Complex/Cyclosome, has been shown to regulate axonal growth developing neurons through its activating subunit, Cdh1/Rap/Fzr (Konishi et al 2004). Conversely, Emi, a protein that acts as a dominant-interfering form of the core APC subunit APC11, increases axon growth in granule neurons (Konishi et al 2004). In *Drosophila*, Rap/Fzr/Cdh1 is expressed neuronal cells both in the central as well as the PNS. However, its action in the PNS, specifically at the NMJ, remains unclear. We present data which support that *rap/fzr* loss-of-function leads to changes in the architecture of the NMJ as well as synapse physiology and behavior. More specifically

these changes are observable pre- and postsynaptically at the NMJ, represented by changes in bouton morphology, size, and number of neurotransmitter vesicles. Physiologically, there are observable changes that show a decrease in excitatory junction current (ejc), impaired plasticity and habituation. In addition, larval locomotion and peristaltic movement is impaired as well. These findings lead us to conclude Rap/Fzr/Cdh1 is critical for synaptogenesis, synaptic transmission and locomotion.

cAMP-PKA pathway role in synaptogenesis and transmission

The cAMP-PKA pathway is a well-known second messenger pathway that is involved with many aspects of cell function. It involves the transduction of an extracellular signal or ligand that binds to a transmembrane protein receptor (G-protein coupled receptor) and activates a cascade, which results in the activation of adenylate cyclase and the conversion of ATP to cAMP. In the cAMP pathway, the G-protein coupled receptor (GCPR) family is comprised of many different types linked to G-proteins. Heterotrimeric G-proteins are guanine nucleotide binding proteins that act as signal transducers that can mediate change in effector proteins like adenylate cyclase and alter levels of cAMP. Dissociation of the Gα from the $\beta\gamma$ subunits lead to excite or inhibit effector protein pathways, such as phospholipase C (PLC), adenylate cyclase (AC), and forskolin (Seamon et al., 1981; Cabrera-Vera et al., 2003; Cumbay and Watts, 2004). Activation of AC and the addition of ATP, results in the conversion of 5'AMP to cAMP. cAMP can bind to the regulatory subunits of PKA, releasing it from the catalytic subunits. PKA phosphorylates a variety of cellular substrates, namely activating CREB transcription factor, which has been shown to facilitate changes in

synaptic transmission (Tojima et al., 2003). cAMP can also bind directly to cyclic nucleotide gated channels acting a transmitter (Matulef and Zagotta 2003).

Kim and Wu (1996) have shown that disruption in the cAMP pathway can greatly impact the ability of the motorneuron axon filopodia to target the specific muscle that it will innervate and create presynaptic boutons at its terminal. The activation of the cAMP pathway in adult rat hippocampal slices by rolipram, an inhibitor of cAMP breakdown, increased cell proliferation, which induced mature granule cells that expressed neuron-specific markers (Nakagawa et al., 2002). They found that this proliferation was mediated by CREB activation. Another study found that adding dibutyryl cAMP (DBcAMP), a membrane permeable cAMP analog, increased the number of neurites and varicosities, while the addition of a inhibitor of PKA, accelerated neurogenesis and neurite outgrowth. They suggested that it was PKA activation of CREB that was responsible for this change (Tojima et al., 2003).

A Kinase Anchoring Protein role in synaptic transmission

More recently, studies have pointed to dysfunction of cAMP organizing structures called A Kinase Anchoring Protein (AKAPs), which bind multiple proteins that interact in the same pathway, to be the cause of changes in synaptic transmission and memory formation. AKAPs sequester various signaling enzymes to a specific subcellular environment to ensure that it is near its relevant targets and to prevent indiscriminate phosphorylation of other substrates. The majority of known AKAPS bind to RII holoenzyme, though a small few have the ability to bind to both PKA subtypes (Huang et al., 1997). Ht31, a molecule with affinity to bind to PKA RII, has been shown

to uncouple PKA from –amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptors which reduce the postsynaptic AMPA receptor currents and synaptic potential (Rosenmund et al., 1994). In addition uncoupled PKA dissociates cAMP-dependent regulation of the L-type Ca^{+2} channels, which can reduce neuronal excitability (Johnson et al., 1997; Earl and Tietz 2011).

rugose (*rg*) which encodes a Drosophila AKAP (DAKAP), shares BEACH binding-domain homology with mammalian Neurobeachin (Wang et al., 2000; Shamloula et al., 2002). Mutations in Drosophila have shown rough eye phenotype caused by disorganized cone cells and aberrant cone cell differentiation (Shamloula et al., 2002). It was identified that *rg* interacts with components of the EGFR and Notch signaling pathways to regulate cell fate determination. However, *rg's* role in synaptic development and transmission has not been characterized. Here we present data that *rg* mutants show changes in synaptic development and physiology as well as changes basic forms of learning, habituation. This is considered to be a very simple demonstration of learning because habituation involves the process by which the neuron ceases responding to stimuli. Taken together, both Rap/Fzr and Rg have great potential in regulating synaptogenesis and plasticity at the neuromuscular junction both directly and indirectly. Their roles many provide more information in the dynamic puzzle of protein interaction, which provides the synapse with targeted growth and modification in signaling.

Altered habituation and synaptic properties in Drosophila AKAP (*rugose*) mutants.

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ABSTRACT

Habituation is a form of non-associative conditioning in which there is a reduction in response to a specific stimulus presented repetitively over time. Components of the cAMP mediated signaling pathway have been previously shown to be important for normal habituation in most neurons. A kinase anchoring proteins (AKAPs) are a large family of proteins which modulate the specificity of protein kinase A (PKA) function by targeting and compartmentalizing PKA to various sub-cellular structures. This allows integration of the cAMP-PKA pathway with other pathways. *rugose* (*rg*) encodes a Drosophila A kinase anchor protein (DAKAP550), which has been previously shown to be required for normal pattern formation in the developing eye (Shamloula et al. 2002). We present data which show mutations in *rugose* (*rg*) alter habituation and synaptic properties. Data from behavioral, electrophysiological and cell biological studies on the adult, as well as the larval neuromuscular junction are presented here. Taken together, we suggest that the AKAP,Rugose is necessary for normal synaptic transmission. This is consistent with the importance of the cAMP pathway in plasticity and memory formation.

INTRODUCTION

The process of basic learning involves many cellular pathways that regulate dynamic changes at the synapse, which occur at a molecular as well as at a cellular level. Among the various forms of learning, habituation has been considered as a form of non-associative learning whereby it is defined as the moment in which responding to repetitive stimuli diminishes and ceases, and is not attributed to sensory adaptation, motor fatigue or desensitization (Thomas and Spencer 1966; Rankin et al 2009; Engel and Wu, 2009). Although habituation is often cited as the "simplest form of learning," the underlying mechanisms are not fully understood (Rankin et al., 2009). A detailed understanding of the basic mechanisms of habituation will likely provide insights into higher forms of learning. Habituation has been studied in a wide variety of organisms and the behavioral and electrophysiological characteristics of habituation have been extensively characterized in Drosophila (reviewed in Wu and Engel, 2009). Neurogenetic analyses in Drosophila have implicated several signaling pathways and thus provide insights into the molecular underpinnings of habituation.

Genetic studies in Drosophila, mice and worms have shown that the cAMP-PKA (protein kinase A or A kinase) signaling pathway mediates many processes in cells such as oogenesis, tissue polarity, synaptic plasticity, learning, memory and synaptic organization (Edelman et al., 1987; Taylor et al., 1992; Engel and Wu 1996; Engel at al., 1998; McKnight et al., 1998; Song and Huganir, 2002; Bauman et al., 2004; Abel and Nguyen 2008; Szaszak et al., 2008). Our previous studies have shown that changes in the levels of cAMP, as examined in the *rutabaga* and *dunce* mutants,

lead to changes in habituation behavior (Wu. et al 1997). Alteration in cAMP levels and PKA signaling are known to modulate the synaptic terminal growth at Drosophila larval NMJs, synaptic plasticity and memory formation. Well-documented examples include studies on dunce (dnc) and rutabaga (rut), encoding phosphodiesterase and adenylyl cyclase, respectively (Budnik et al., 1990; Davis et al., 1996; Schuster et al., 1996; Zhong & Wu, 2004).

Activation of the cAMP pathway results in the elevation of cAMP levels, which in turn activates PKA. PKA phosphorylates a diverse variety of cellular substrates. The cellular and subcellular specificity of PKA is mediated by A kinase anchoring proteins (AKAPs). AKAPs are a large family of proteins originally identified in mammals, which modulate the specificity of protein kinase A (PKA) function by targeting and compartmentalizing PKA to various sub-cellular structures. AKAPs function as major cellular signal transduction organizing centers for the cAMP pathway (Krebs 1985; Lu et al., 2007, 2008; Chen et al., 2007; Hoshi et al., 2005; Smith et al., 2006). AKAPs contain two conserved domains, a domain that binds to the regulatory (R) subunit of PKA and a second domain that anchors to subcellular structure allowing for greater specificity of targeting PKA to the local substrate (Taylor et al., 1990; Li & Rubin 1995; Hausken et al., 1994,1996; Travalin et al. 2003; Dell' Acqua et. al., 2002, 2003). AKAPs localize PKA holoenzymes near membranes and organelles such as the plasma membrane, microtubules, and mitochondria (Rubin 1994; Edwards and Scott 2000; Dodge and Scott 2000). Failure in recruit PKA to appropriate sites might decrease the synaptic growth. On the other hand, the increase in neurotransmitter release may enhance cAMP signaling and facilitate the same process. Therefore, it is

interesting to examine how AKAP modulate motor terminal projection patterns in this preparation.

In addition AKAPs also contain additional sequences that allow for binding sites for protein kinase C (PKC), protein phosphatase 1 (PP1) and protein phosphatase 2 (PP2), (Coghlan, 1995). AKAPs have also been implicated in short-term memory consolidation and long-term potentiation (Song and Huganir, 2002; Malenka 2003; Malenka and Bear, 2004; Lu et al., 2007; Liu 2004; Dell' Acqua et al., 2006). In hippocampal neurons, AKAPs have been shown to anchor Ca^{2+} channels and NMDA receptors, facilitating efficient phosphorylation of Ca^{2+} or K^{+} channels and thus regulating changes in membrane potential at the synapse in the hippocampus (Hoshi et al., 2005). Previous work from our lab has shown that the Drosophila *rugose* (*rg*) locus encodes an AKAP (DAKAP550) required for neural pattern formation in the developing eye and interacts with multiple cellular signaling pathways (Shamoula et al. 2002; Han et al.1997).

METHODS

Drosophila Culture

Flies were cultured on standard cornmeal–agar medium and kept at 23° C. The isolation and characterization of the rg mutant alleles used in this study has been described previously (Shamloula et al., 2002). They include *w,rg$^{\gamma 1}$, w, rg$^{\gamma 5}$, w, rg$^{\gamma 7,}$ w,rg$^{\gamma 8}$, w,rg$^{\gamma 9}$, w,rg$^{\gamma 11}$, rg^{p2}, rg^{p4}, rg^{p5},* and *rg^{p6}.*These mutants are described as hypomorphs (Shamloula et al., 2002).

Immunocytochemistry

Third-instar larvae males of the same age were selected and dissected in calcium-free media modified minimal hemolymph-like solution (HL3.1) with Ca^{+2} and fixed for 25 min in 4% paraformaldehyde. The preparations were washed three times for 5 minutes with 0.2 M Phosphate Buffer Solution containing 0.2% Triton-X 100 (PBST). The whole mount preparations of dissected larvae were incubated overnight at 4oC in mouse anti-Discs Large supernatant at 1:5 (4F3) from Hybridoma Bank (University of Iowa Developmental Studies Hybridoma Bank). The preparations were then washed 3 times (5 minutes each time) in 0.2 % PBST. Secondary antibodies were applied for 2 hours: Alexa Fluor 635 conjugated anti-Phalloidin at 1:1000 (Invitrogen), goat anti-mouse FITC (Jackson Laboratories) at 1:100 and TRITC conjugated goat anti-Horseradish Perioxidase (Jackson Laboratories) at 1:100. Preparations then were washed twice for 5 min and mounted using Vectashield (Vector Laboratories). Confocal microscope images were taken with LSM 510 Confocal Laser Scanning System (Carl Zeiss Inc.) at 40x and were processed with Image J 1.24 and Adobe Photoshop 5.5. Boutons were counted using muscle 6 & 7 from segment A2 and A3 and analyzed using Prism.

Electrophysiology

We dissected post-feeding third instar larvae and recorded excitatory junctional potentials (ejps) from muscles #6 and 7 of abdominal segments 3 – 6, as described previously (Jan et al., 1977; Wu et al., 1978). Briefly, larvae were dissected in Ca^{2+} free HL3 saline (Stewart et al., 1994) containing (in mM) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 5 Trehalose, 115 Sucrose, and 5 HEPES, at pH 7.2. Ejps were recorded in low Ca^{2+} HL3.1 (Feng et al., 2004), which has the same ionic composition except for a

reduced Mg^{2+} concentration (4mM). The final Ca^{2+} concentration in recording saline is specified for each experiment. To evoke ejps, the segmental nerves were severed from the ventral ganglion and stimulated with a suction electrode (10μm inner diameter) through the cut end. Stimulation amplitude was adjusted to 2.0 times the threshold voltage to ensure a uniform stimulation condition among experiments. Stimulus duration was 0.1 ms. Intracellular glass microelectrodes were filled with 3 M KCl and had a series resistance of about 60 MΩ. Ejps were recorded with a direct current pre-amplifier (model M701 micro-probe system, WPI, Conn., USA, and an additional custom-built amplifier). Severed nerve bundles were stimulated using a suction electrode connected to a linear stimulus isolate. Data was acquired through an Axoclamp 2-A amplifier (Axon Instruments). EJPs were recorded on pclamp 1.0 then further analyzed using Axograph X. Miniature EJPs were analyzed using Fetchex 1.0. For EJP recordings, stimulus was presented from 0.8 to 3 Hz. mEJP for 2mins. For each mutant, recordings were made at each of the $[Ca^{2+}]$. For short-term synaptic plasticity, paired-pulses were presented at 3 intervals of 100ms and 50ms respectively (described in Zhong and Wu 1991) at $[Ca^{2+}]$ of 0.5 mM. All recordings in which the resting potential was higher than -60mV and/or all those resting potential and/or input resistance changed by 20% during the duration of data acquisition were excluded from analyses.

Adult Habituation

Preparation of flies, stimulation, recording, and analysis of muscle responses were performed as described previously (Engel and Wu, 1992, 1994b) with some

modifications. The Faraday cage was covered with black plastic to reduce ambient light because strong illumination was found to inhibit the long-latency response. Stimulation (0.1 msec pulse, Grass S8, Quincy MA) was passed between insulated tungsten electrodes inserted in the eyes. Signals were recorded from the right tergotrochanteral (TTM) jump muscle and left dorsal longitudinal a (DLMa) flight muscle, which are innervated by the same side of the giant fiber pathway (Levine, 1974; Wyman et al., 1984) (Fig. IA). Precise latency values were measured as described previously (Engel and Wu, 1992, 1994b).

RESULTS

rg mutants show changes in synaptic bouton morphology

All of the rugose mutant alleles tested (*w,rg*$^{\gamma 1, 4, 5, 6, 7, 8, 9, 11,}$ *and rg*$^{P2, P3, P3, P4}$ *and* ^{P6}) showed abnormal bouton morphology. Generally, we observed a decrease in the number of boutons at the synapse of the NMJFigure 1). We specifically observed only Type IB boutons that contained both pre- and postsynaptic staining structures at the NMJ. Moreover, their appearance and shape differed significantly from wild type in that the boutons were less oval shaped and more oblong/rectangular. In addition, the boutons also appeared to have grown continuously, connecting with each bouton, forming into one large bouton. By comparison, wildtype boutons are discrete oval-shaped. *rg* mutant boutons also appeared to be enlarged and some boutons contained spaces that had no staining, appearing as clusters of bouton that formed by

over-laying each other and vacuolated, specifically in mutant alleles w,rgγ8 and 9 (Figure 1). Compared to wild type all the *rg* alleles examined showed a significant decrease in bouton number (Figure 2). The size of boutons appeared to be slightly increased although we did not record changes in size. Only rg^{P2} show little to no change in the number of boutons. Anti-Horseradish peroxidase (HRP) positive bouton counts were performed on muscles 6 and 7, segments A2. Counts were analyzed by ACT-1 software (Nikon Digital Eclipse DXM 1200) and p values were derived by Student's two-tailed t-test (Prism). Significant decreases in Type-IB bouton number were observed in w, $rg^{\gamma\ 1,\ 5,\ 7,\ 8,\ 9}$ and ^{11} ($w,rg^{\gamma 9}$, p<0.0001, n=10; $w,rg^{\gamma 1}$, p<0.0001, n=10; $w,rg^{\gamma 6}$, p<0.001, n=11; $w,rg^{\gamma 5}$, p<0.01, n=8; $w,rg^{\gamma 8}$,p<0.01, n=11; $w,rg^{\gamma 11}$, p<0.01, n=7; $w,rg^{\gamma 7}$,p<0.05, n=15). Type-II boutons positive for anti-HRP also had significant decrease in bouton number (Figure 2). Significant changes in Type-II bouton number for segment A3 were also observed (data not shown). Our rationale was that these changes seen in bouton morphology may be indicative of changes in synaptic plasticity.

***rg* mutants show altered habituation**

In adult flies, changes in synaptic properties appear to be evident in *rg* mutants. Flight muscles are driven by a central pattern generator but also associated with the adult giant fiber escape response (Engel and Wu 1996) and allows for habituation studies to be conducted. *rugose* mutant alleles $w,rg^{\gamma 5}$, $w,rg^{\gamma 6}$, and $w,rg^{\gamma 7}$ showed strong increase in the rate of habituation, that is, it takes a significantly shorter time interval for the GF circuit to habituate to a set criterion of 20 consecutive failures than wild type; $w,rg^{\gamma 7}$ being the most rapid to reach the criterion, followed by $w,rg^{\gamma 6}$ and $w,rg^{\gamma 5}$

(Figure 3). The number of stimuli to different failure criteria (1, 2, 5, 20) is significantly decreased in *w,rg*^{v5,6,7}, i.e. the number of stimuli to attain a number of consecutive failures was decreased in *rugose* mutants compared to wild type. Plotting the mean number of stimuli to attain criteria of one to twenty consecutive failures (Figure 4) shows that attenuation occurs consistently in *rg* mutants, regardless of the frequency number. These finding further implicate the cAMP pathways in changes of the habituation properties of the giant fiber synapse.

***rugose* mutants exhibit changes in evoked responses and short-term plasticity**

In order to examine the role of *rugose* in synaptic function at the cellular level, we examined the properties of neuromuscular transmission in 3rd instar larvae. This preparation has been extensively used for studies on synaptic function (Jan et al., 1977; Wu et al., 1978), development (Broadie and Bate 1993; Kidokoro and Nishikawa 1994; Keshishian and Kim 2004), plasticity (Budnik et al., 1990; Zhong and Wu 1991; Zhong et al., 1992; Sigrist et al., 2003; Ueda & Wu, 2009), and transmitter vesicle mechanisms (Ramaswami et al., 1994; Song et al., 2002; Kuromi et al., 2010). It is therefore ideal for characterizing physiological and morphological defects in *rg* mutants.

We found that nerve-evoked evoked junctional potential (ejps) in *rg* mutants were abnormally large compared to those in WT control (Figure 5A). This reflects increased transmitter release, because the sizes of spontaneous miniature ejps were similar between *rg* and WT larvae (for w,rg^{vg}; 1.9 + 0.68 mV, n = 4; for WT, 1.5 + 0.48 mV, n = 7; $p < 0.05$). In addition, the short-term plasticity was altered as evidenced by

abnormal response to paired-pulse stimulation. We observed a weakening of facilitation in *rg* mutants (Figure 5B), consistent with the stronger release from presynaptic terminals in *rg*. Because of the non-linear summation effects of overlapping ejps, facilitation at shorter intervals near the ejp peak time could not be accurately determined. Therefore, we applied the focal loose-patch recording technique to observe synaptic currents (Ueda, et. al. 2000). At shorter inter-pulse intervals, we found a significant increase in facilitation (see facilitation index in figure 5D). This is interesting in that a short-lived facilitation process was also documented for the cyclic-AMP pathway mutants such as *dunce* (Ueda et. al. 2000). These data support the idea that AKAPs play an important role in regulating synaptic strength & plasticity.

DISCUSSION

Habituation has been studied in a wide variety of organisms, and is considered as a form of non-associative learning and is thought to provide a model for the simplest form of learning (Rankin et al 2009; Engel and Wu, 2009). Although the molecular basis of habituation is not fully understood key cellular signaling pathways such as the one mediated by cAMP-PKA have been implicated (Brandon et al., 1995; Kojima et al., 1997; Antonov et al., 2003; Huang et al., 2005). In this paper we have presented data that show that loss-of-function of an AKAP leads to disruption of normal habituation properties. Our studies show that in *rg* mutants there is an increase in the rate of habituation, that is, the behavior of ceasing to repetitive stimuli occurred in fewer number of stimuli trials than in wild type. In addition, our data also show that

morphological features and physiological properties of the larval NMJ synapses are significantly altered. We have shown that *rg* mutants show slight enlargement in bouton size and less oval-shaped boutons, with significant changes in the number of boutons at the NMJ. Overall, there was a decrease in the number of boutons in more severe alleles of *w,rg^{v1,} w,rg^{v5}, w,rg^{v7}, w,rg^{v8,} w,rg^{v9}* and *w,rg^{v11}*. These results support our conclusion that disruption of AKAP function can lead to significant changes at the synapse. Taken together we have presented data that suggests AKAPs specifically Rugose (DAKAP550) facilitates key intermediate steps that govern proper synaptic growth, transmission and plasticity. Our results are consistent with earlier studies on the effects of altered cAMP metabolism on synaptic plasticity and habituation in adults (Engel and Wu 1996), and larval NMJ morphology and neurotransmission, (Zhong and Wu 1991, Zhong et. al 1992). In addition, other studies in vertebrate preparations have implicated AKAPs in LTP and LTD (Hoshi et at., 2005; Rosenmund et al.,1994; Snyder et al., 2005; Tavalin et al., 2002). One model suggests that AKAPs regulate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) phosphorylation trafficking and indirectly regulate NMDA-dependent plasticity (Hoshi et al., 2005).

AKAPs are key mediators of cAMP as well as other a signal transduction pathways. Presynaptically AKAPs have been shown to regulate ion channel conduction, particularly Ca^{2+} channels, which are required for vesicle fusion (Rizo and Südhof, 2002). PKA and AKAPs together can increase the efficiency of the this channel by 3- to 10 fold, allowing for greater current to move intracellular (Pelzer et al., 1990). However, the molecular mechanism that links Rugose function to evoked synaptic

transmission remains unclear. In addition, some AKAPS have been shown to maintain postsynaptic scaffolds by simultaneously associating with other kinases and phosphatases. For example, AKAP79/150 has been shown to be targeted to dendritic spines by a binding motif in the N-terminus which complexes with phosphatidylinositol-4,5-bisphosphate (PIP2), F-actin, and actin-linked cadherin adhesions molecules (Michel and Scott 2002; Matsuzaki et al., 2004;). Moreover, this association allows AKAPs to also organize NMDARs and AMPARs to the postsynaptic membrane. This organization presents AKAP as dynamic protein that is motile within the cell. This implicates AKAP in LTP and LTD because it can change the movement and incorporation of NMDARs and AMPARs in the postsynaptic membrane. AKAPs have also been shown to directly interact with adenylate cyclase in neurons, and regulating the amount of cAMP that is produced in the cell (Dell'Acqua et al., 2006). Other studies on AKAPs in Drosophila have also suggested for a role in synaptic plasticity and long-term memory formation. Schwaerzel (2007) have reported the involvement of Drosophila AKAPs in anesthesia sensitive memory using the olfactory associative paradigm. In the Drosophila Yu mutants long term memory is disrupted (Lu et al 2007). Yu encodes another Drosophila AKAP and is expressed in the mushroom body (Lu et al 2007). This may provide a mechanism by which changes in presynaptic vesicular release occurs leading to observed changes in transmission and plasticity. Studies on neurobeachin (nbea) gene, the mammalian homolog of rugose, lend further support for a role for AKAPs at the synapse. Loss-of -function of nbea completely blocks evoked synaptic transmission at neuromuscular junctions while nerve conduction, synaptic structure and spontaneous neurotransmitter release are

completely normal (Su et. Al 2004). Nbea has also been implicated in vesicular traffic at the synapse and has been shown to be required for normal development of the synapses (Medrihan et al., 2009). Recent studies have shown that nbea gene is disrupted in individuals with Autism spectrum Disorder and the nbea gene spans the common Fragile site FRA 13A in human (Savelyeva et al., 2006; Medrihan, 2009). In addition, nbea gene is also disrupted in the human Chediak-Higashi syndrome, which presents as mental retardation and can cause fatal fetal developmental complications (Wang et al., 2000; Medrihan, 2009). Studies have shown that in individuals with Fragile X Syndrome and autism, have reduced levels of cAMP (Berry-Kravis, 1990; Berry-Kravis and Ciurlionis,1998). This has been shown to lead to a decrease in evoked synaptic potential, dendritic architecture and actin clumping in areas near the postsynapse (Kelley et al., 2007; Medrihan et al., 2009; Niesmann et al., 2011).
Our findings suggest a functional role for Rugose at the synapse on several aspects of synaptic properties, from formation and development of synaptic structures, to synaptic release, and postsynaptic response amplitude, and is consistent with the working hypothesis of Rugose is important for targeting and/or sequestering various proteins of cAMP-PKA signaling pathways to specific areas in the neuron. The high degree of structural and functional similarity between Rg and Nbea, suggests an evolutionarily conserved functional role essential for synapse formation and transmission, making *rg* a good candidate gene for studies on autism.

Acknowledgements:

We thank Confocal Microscopy center of the City University of New York for preparation of TEM samples and Dr. Chun-Fang Wu for the use of his laboratory, expertise and equipment. Supported by grants from the NIH (T.V and C-F. W).
A.W. was supported by NIH-RISE and LSAMP grants at the City College. The work at City College was supported by NIH-RCMI infrastructure grant.

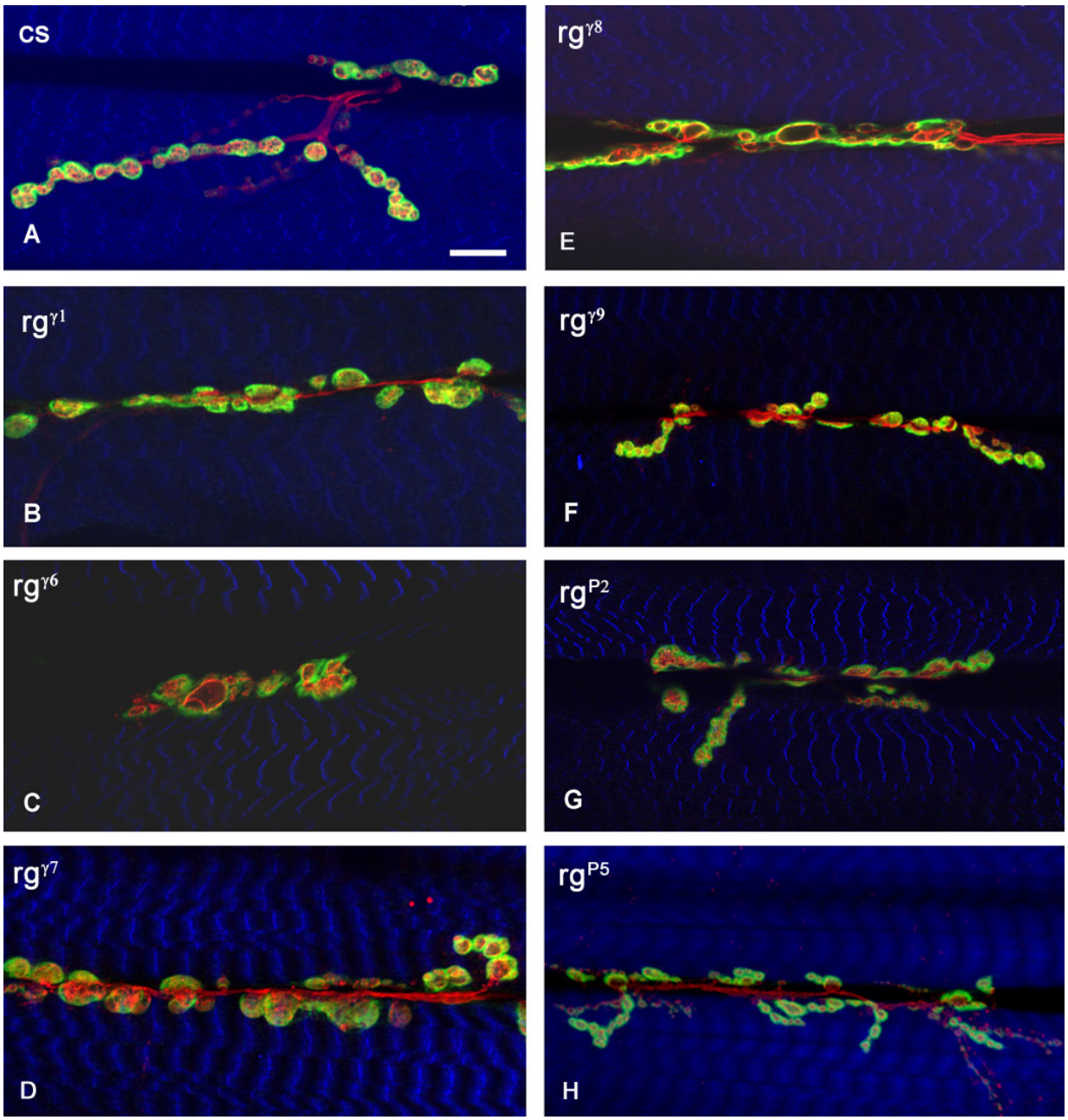

Figure1. rugose mutants exhibit altered synaptic structure. Confocal images of the Neuromuscular junction from third-instar larvae stained with antibodies, anti-Dlg (green) anti-HRP (red) and Phalloidin (blue). Canton-S (A). In *rugose* mutant alleles, boutons number appears decreased and boutons are slightly enlarged (B-H) with limited axonal branching and vacuous spacing in the boutons are seen (E) *rugose* mutant allele rg^{p2} (G) and rg^{p5} (H) exhibit less severe phenotype than the gamma allelic series. Note all images (A-H) are from Muscles 6 and 7-segment 3.

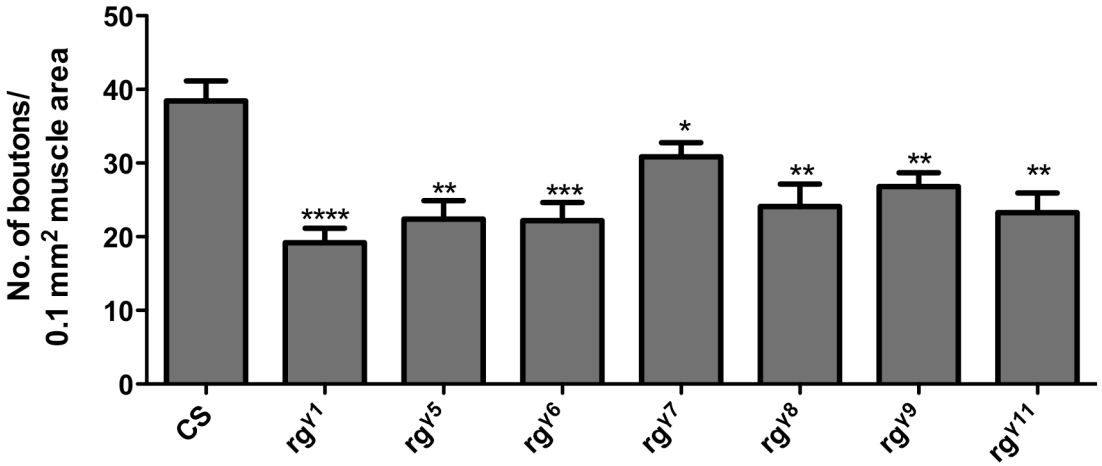

Figure 2. rugose mutants show aberrant synaptic bouton number. Anti-Discs large (dlg) and anti-Horseradish peroxidase (HRP) positive bouton counts were performed on muscles 6 and 7, segments A2 and A3. Counts were analyzed by ACT-1 software (Nikon Digital Eclipse DXM 1200) and p values were derived by Student's two-tailed t-test (Prism). Significant decrease in Type-IB bouton number was observed in alleles *w, rg$^{v\ 1,\ 5,6,\ 7,\ 8,\ 9}$ and ^{11}* (*w,rg^{v9}*, p<0.0001, n=10; *w,rg^{v1}*, p<0.0001, n=10; *w,rg^{v6}*, p<0.001, n=11; *w,rg^{v5},* p<0.01, n=8; *w,rg^{v8}*,p<0.01, n=11*; w,rg^{v11}*, p<0.01, n=7; *w,rg^{v7}*,p<0.05, n=15).

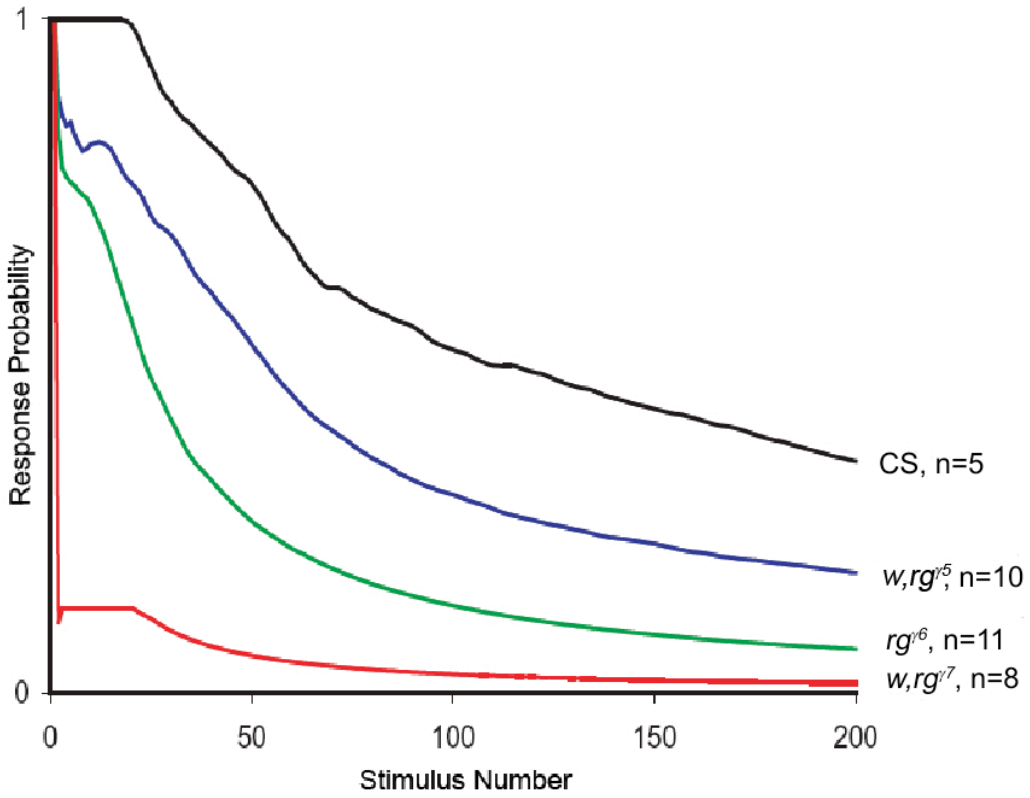

Figure 3. Kinetics of habituation probability response. A, Frequency-dependent decrement of response probability in *w,rg*$^{\gamma 5, 6}$ and ^{7} mutants are shown here. *rg* mutants require fewer number of stimuli to habituate, reducing their overall response probability.

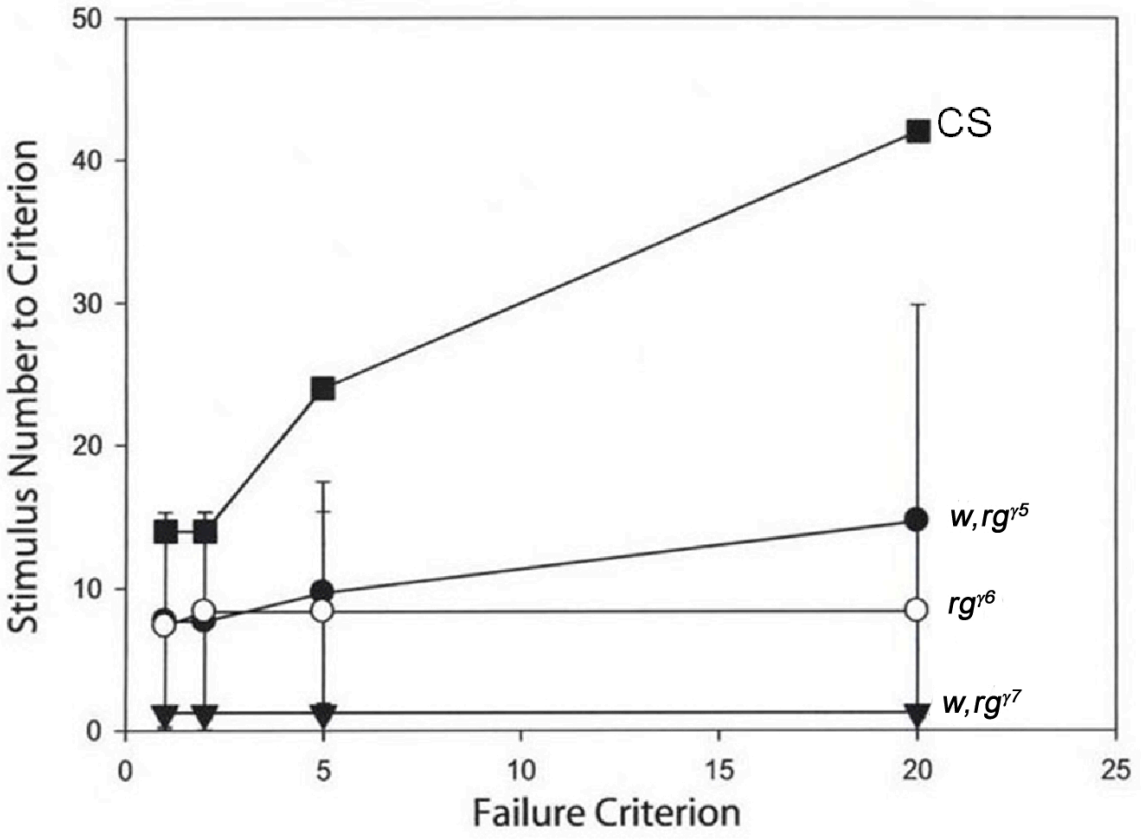

Figure 4. Numbers of stimuli to attain criteria of one to twenty consecutive failures. Comparing the difference between one and twenty consecutive failures shows that failure was not only earlier but also more abrupt for *rg* mutants. From these plots, it is clear that habituation requires fewer number of stimuli for *rg* mutants.

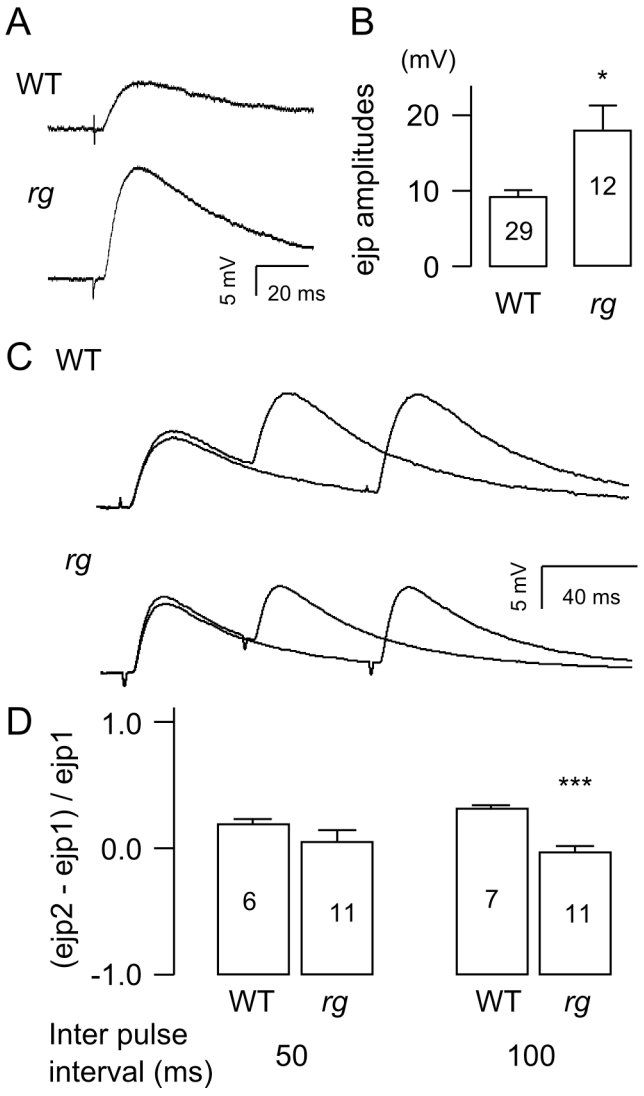

Figure 5. Abnormal synaptic transmission in rugose larval NMJs. (A,B) show increased ejps size in rg mutant larvae. (C) Sample trace displaying decreased paired pulse facilitation in rugose. Ejp traces of 50 and 100 ms inter-pulse intervals were overlaid for both WT and rg. Each trace is from the average of 7-8 trials. (D) Decreased paired pulse facilitation in w,rg. Data from w,rgr1 and w,rgr7 are pooled. * and *** indicates $p < 0.05$ and $p < 0.001$, respectively. (t-test with sequential Bonferroni correction). For B and D, error bars indicate SEM. Numbers of NMJs are indicated. Saline contained 0.2 mM of Ca2+.

Drosophila-Cdh1 (Rap/Fzr) a regulatory subunit of APC/C, is required for synaptogenesis, synaptic transmission and locomotion.

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ABSTRACT

The assembly of functional synapses requires the orchestration of the synthesis and degradation of a multitude of proteins. Protein degradation and modification by the conserved ubiquitination pathway has emerged as a key cellular regulatory mechanism. The anaphase promoting complex/cyclosome (APC/C) is a multi-subunit ubiquitination complex primarily characterized for its role in the regulation of mitosis. In recent years, APC/C has been implicated in various aspects of mammalian central nervous system development (Konishi et al. 2004). However, its role in the peripheral nervous system is not clear. The ubiquitin ligase subunit of the Drosophila APC/C was shown to be required for proper synaptic growth (van Roessel et al. 2004). Here, we examined the role of Rap/Fzr, a homolog of the mammalian Cdh1, an activating subunit of the APC/C, during the development of the larval neuromuscular junction in Drosophila. Our cell biological, ultrastructural, electrophysiological, and behavioral data show that *rap/fzr* loss-of-function mutations lead to changes in synaptic structure and function, as well as locomotion defects. Specifically these changes were observed pre- and postsynaptically, represented by size and morphology of synaptic boutons, and number of neurotransmitter vesicles. Electrophysiologically, these were correlated with decreased transmission failure rates as well as increase in the size of synaptic potentials. In addition, larval locomotion and peristaltic movement are also impaired as. These findings suggest a novel role for Drosophila-Cdh1-mediated ubiquitination during development of functional synapses in the peripheral nervous system.

INTRODUCTION

The assembly of functional synapses requires the balance between synthesis, sequestration and timely degradation of a multitude of proteins. Components of the ubiquitin proteosome system (UPS) are located both pre- and post-synaptically and play a key role during synaptogenesis and synaptic function (Speese et al 2003). For instance, components of the UPS at the synapse provides attractive molecular mechanism to regulate proteins locally by degrading activator as well as repressor proteins, ultimately acting as an “on/off switch” (Haas and Broadie 2008). Specifically, the UPS plays a crucial role in modifying intracellular trafficking of proteins or targeting proteins for degradation by the 26s proteasome (Hick 2001; Glickman and Ciechanover 2002) by recognizing a specific regulatory sequence and adding a 76 amino acid ubiquitin typically to the N-terminus (Glickman and Ciechanover 2002). Polyubiquination results in protein degradation, while mono-ubiquitination results in protein modification (Schnell and Hick 2003).

Within the ubiquitin pathway several ligase proteins function to bind the ubiquitin to the substrate protein. Briefly, the E1 ligase sequesters ubiquitin and activates it by adding an ATP before transferring it to an E2 conjugating ligase. Next, the E2 ligase-ubiquitin complex binds directly to the substrate protein or to an E3 Ligase that is already attached to the substrate protein. Finally, the E3 ligase protein removes the ubiquitin from E2 and adds it to the substrate protein. Four domain classes exist for E3 ligases: HECT (Homologus to E6-AP carboxyl terminus) and RING/U-Box (really interesting new gene)-finger domain, Skp, Cullin, F-box containing complex (SCF), and APC (Ohi

et al. 2003). These differ in organization and structure but all function as a multi-subunit protein complex that facilitates ubiquitination of proteins.

Several elegant genetic and molecular studies the E3 ubiquitin ligase, *highwire* (*hiw*) in *Drosophila* have implicated ubiquitination in synaptogenesis. First, loss-of-function *hiw* mutation leads to increased synaptic branching of terminal arbors, while at the same time decreases quantal content compared to wild-type (Wan et. al. 2000; Zhen et. al. 2000; DiAntonio et al. 2001). Hiw also negatively regulates the transforming growth factor-β/Bone morphogenetic protein (TGF-β/BMP) signaling pathway by binding to and promoting degradation of Medea, a SMAD transcription regulatory protein (Mccabe, et al. 2004). Wallenda, a MAPKK was also shown as a target for degradation by protein Hiw and FSN-1 to restrain synaptic terminal growth. Consistent with these data, loss-of-function mutation in *fat facets* (*faf*), which encodes a deubiquitinating enzyme, genetically suppresses the overgrowth phenotype found in *hiw* mutants (Wu et al., 2007; 2005; Collins, et al. 2006). Bendless (ben) is a ubiquitin conjugating enzyme that has been shown to regulate axon connectivity (Uthaman et al. 2008). This dynamic process of ubiquitination and deubuiquitination of substrates presents an efficient way of controlling levels and density of proteins at the synapse by creating an “on/off” switch.

As a regulator of synaptic transmission and plasticity, the UPS functions both pre- and postsynaptically to ultimately affect the rate of neurotransmission. Presynaptically, UPS functions to degrade proteins involved with vesicular release. In

the presence of a proteosome inhibitor, there was an increase in the amount of vesicle release (Speese et al., 2003). Specifically, UNC-13 which is found in the nematode *C. Elegans* is a component of the vesicle membrane and is essential for vesicle docking and fusion with the presynaptic membrane. UNC-13 can be ubiquitinated and accumulates after proteosome inhibition as the strength of the synapse increases (Aravamudan et al 1999). Suggesting that there's more UNC-13 available at the synapse to allow for increase in release of neurotransmitters. The questions remain, however, identifying which proteins are specifically involved with these ligases.

Postsynaptically, UPS also functions in regulating glutamate receptors through regulating endocytosis of postsynaptic density (PSD) scaffolding proteins. In *C. elegans*, the APC/C functions to regulate the number of GLR-1 receptor in the postsynaptic cell by directly targeting proteins involved in clathrin-mediated endocytosis (Juo and Kaplan 2004). In *Drosophila*, Frizzed-2 (Dfz2) at the NMJ was transported to the nucleus from the postysynaptic density by the protein d-GRIP. Presumably, Dfz2 is involved in regulating transcription associated with synaptic growth (Ataman et al 2006).

The Anaphase Promoting Complex/cyclosome APC/C is an evolutionarily conserved multi-subunit ubiquitin ligase complex that has been characterized primarily for its regulatory role during cell cycle progression. APC/C consists of at least 11 core subunits and facilitates the ubiquitination and timely degradation of key mitotic regulators such as cyclins, cyclin dependent kinases and securin. APC/C also mediates the ubiquitination and destruction of the inhibitors of chromosome segregation, regulators of DNA replication, and centrosome duplication, thus

regulating cell cycle progression (Peters 2002). Targeting of protein substrates and the subsequent activation of the APC/C complex for ubiquitination is dependent on two proteins with WD (Trp-Asp) repeat domain, Cdh1 and Fzy/Cdc20 (Kashevsky *et al.* 2002). Rap/Fzr (Retina aberrant in pattern/Fizzy related) is the Drosophila homolog of the mammalian Cdh1 (Sigist and Leher 1997; Jacobs et al 2002; Pimentel and Venkatesh, 2005.)

In addition to its role in cell cycle progression APC/C is also involved in synaptic development and plasticity. SiRNA knockdown of Cdh1 has been shown to promote axon growth in mammalian cerebellar neurons, suggesting that Cdh1 is an inhibitor of axon growth (Konishi et al. 2004). In Drosophila, van Roessel and colleagues (2004) showed that in loss-of-function morula (APC2) mutants, which have defects in an E3 ligase subunit of APC/C, showed synaptic overgrowth, an increase in the number of synaptic boutons, an increase in glutamate receptors expression levels, and abnormally strong synaptic transmission at the larval NMJ. APC2 was localized to the pre- and post-synaptic regions. Drosophila α-Liprin contains a D-box, localizes to the synapse, and is a scaffolding protein involved with glutamate receptor stabilization. α-Liprin is a substrate of APC/C and mutants show changes in bouton size (van Roessel et al., 2004). Reduction of active APC/C at the post-synaptic region leads to a decrease in the abundance of glutamate receptors in *C. elegans* (Juo et al. 2004) by clatherin-mediated endocytosis (Burbea et. al 2002) and is likely that APC/C is involved in regulating this pathway. The A PC/C was identified as a regulator of axonal growth by degrading Id2, a transcriptional repressor protein (Lasorella et al. 2006).

One of the substrates of Id2 is the Nogo receptor, an inhibitor of axonal growth (Lasorella et al 2006).

Recent studies have suggested a role for Cdh1 in synaptic plasticity learning and memory formation. Li and colleagues (2008) found in KO mice no gross morphological changes in hippocampal slices, however, they did show changes in long-term potentiation (L-LTP) at the Schaffer collateral-CA1 synapses of Cdh1 knockout mutants. LTP evoked by multiple spaced trains of stimuli was impaired in Cdh1 heterozygous knockout mice slices. These results suggest that APC/C ^{Cdh1} may mediate the timely protein modification and/or degradation of proteins at the synapse of hippocampal cells facilitating potentiation. In addition, heterozygote mice of *cdh1* knockouts (*cdh1*/-) showed poor performance in contextual fear-conditioning, a hippocampus-dependent process. However, performance was not significantly altered in auditory (cue)-dependent conditioning.

To further understand the role of Cdh1 at the synapse, in the present study, we have examined the effects of loss-of-function Drosophila-Cdh1 at the larval neuromuscular junction. Our finding suggests the activating Drosophila subunit *rap/fzr* modulates NMJ morphology, synaptic plasticity and behavior. Using loss-of-function alleles we were able to determine changes in bouton morphology, electrophysiology and larval locomotion ability. Loss-of-function mutants exhibit larger than wild-type sized boutons as well as a decrease in the number of boutons. Moreover, EM revealed that these larger boutons are mostly devoid of neurotransmitter vesicles. This change in the number of vesicles available for release at the presynapse significantly increased the size of the evoked response postsynaptically. It appears that

postsynaptically, the postsynaptic density is greatly disorganized. Behaviorally, mutants exhibit significant decrease in their forward locomotion and angular trajectory. Short-term potentiation is also disrupted in mutants where facilitation occurs at a greatly reduced rate in comparison to wild-type. This paper suggests novel role of Drosophila *rap/fzr* in synaptic development and synapse plasticity at the NMJ.

METHODS

Genetics and Transgenetic Lines

The following mutant *rap/fzr* flies were used: rap^{E2}, rap^{E4}, rap^{E6}, rap^{X-2}, rap^{X-3}, w,rap^{3} (Jacobs et al., 2002; Karpilow et al., 1989). Null alleles of *rap/fzr* are embryonic lethal, so mutants that were used here are all hypomorphic (partial loss-of-function).

Immunohistochemistry

Wandering larvae were dissected in calcium-free media HL3.1 solution (Fergestad and Broadie 2001) and fixed with 4% Paraformaldehyde for 25 minutes (Paraformaldehyde in Phosphate Buffer Solution). Washes and antibody incubations were performed in Phosphate Buffer Solution with Triton-X (0.1%). Dlg (disc-large, presynaptic NMJ marker from Iowa Hybridoma bank), GluRIIa (glutamate receptor IIA, postsynaptic NMJ marker from Iowa Hybridoma bank) and BRP (bruchpilot, presynaptic NMJ marker from Iowa Hybridoma bank), were used at 1:5. Secondary antibodies conjugated to FITC (Jackson Immunochemicals), Rhodamine (Jackson Immunochemicals), or Cy5 (Invitrogen) were used at 1:100. Cy5-conjugated phalloidin (25 nM, Molecular Probes) was used to stain muscles at 1:1000 and anti-HRP-Rhodamine Red-X (Jackson Immunochemicals) was used to stain neuronal

membrane at 1:100. All imaging was performed using a LSM 510 Confocal Laser Scanning System (Carl Zeiss). Dlg and HRP positively stained boutons counts were performed on muscle 6 and 7, abdominal segment A3. Students' t-test was used to derived p-value and significance. All of the strains were subjected to immunohistochemistry, however, rap^{X-3} and w,rap^{3} were looked at more extensively.

Quantitative Immunofluorescence

To identify changes in immunofluorescence intensity, control and mutant larvae were dissected and stained together. Immunohistochemistry was performed was described above, using anti-BRP and anti-GluRIII antibodies. Segment A3 was observed for each mutant and control. Laser intensity levels were kept low and used at a magnification of 40X. Plug-in software from Image-J was used to define level of saturation for each image using an arbitrary intensity threshold. Mean intensity values of wild-type and mutants were identified.

Method of TEM

To prepare ultrathin sections for transmission electron microscopy, the dissected larva muscle samples were first chemically fixed at 4 °C for 24 hours. The fixative contained 2.5 % glutaraldehyde, 2 % paraformaldehyde, 1 % low molecular weight tannic acid in 0.1 M Na Cacodylate, pH 7.4. The samples were washed with 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide in cacodylate buffer with 1.5% $K_4[Fe(CN)_6]$, 90 min on ice in darkness. The samples were then washed with cacodylate buffer and incubated with 1% tannic acid in cacodylate buffer for 30 min at room temperature. The samples were washed with distilled water and further incubated with 1% osmium tetroxide water solution for 30

min on ice and in darkness. After the osmium treatment and wash with distilled water, the samples were dehydrated with alcohol with a gradual increase in concentration from 30 % to 100%. The samples were washed with 100% propylene oxide for 5 min before embedded in resin (made of 20 ml of EMbed 812, 9 ml of DDSA and 12 ml of NMA). In the process of embedding, the samples were first immersed for a few hours in each of 30% and 70% of resin in propylene oxide at room temperature, further incubated in pure resin and then transferred into activated resin (10 ml of resin activated with 0.18 ml of DMP-30) in embedding capsules. The resin with samples was polymerized at 67 °C for 72 hours. After polymerization, cross sections of ~ 70 nm were cut at the center of muscles 6 and 7, segments A2 through A6, using LKB ultrotome (LKB-Produkter AB, Stockholm, Sweden). The sections were set on copper grids and stained with 0.5% lead citrate basic solution for 20 min. The stained sections were examined using Philips CM12 transmission electron microscope) operated at 80 KV. Images were taken with a Gatan 4K X 27K digital camera (Pleasanton, CA). Synaptic vesicles located within 50 nm from the presynaptic membrane were considered as potentially docked at release sites (Tartaglia et al., 2001).

The following chemicals were purchased from Electron Microscopy Sciences (Hatfield, PA): Low molecular weight tannic acid ($(C_{14}H_{10}0_9)_n$), paraformaldehyde solution, glutaraldehyde aqueous solution, osmium tetroxide aqueous solution, sodium cacodylate butter, propylene oxide, EMbed 812, DDSA, NMA and DMP-30.

Electrophysiology

Wandering Third- instar larvae were used to record from the neuromuscular junction. Dissections will be performed in HL3.1 solutions buffered at pH 7.2 (as described in Robinson et al. 2002) at [Ca^{2+}] varying 0.15mM, 0.2mM, 0.3mM and 0.5mM. All of the wild-type and mutants were subjected to each Ca^{2+} were solutions were washed three times between changing to a solution of greater [Ca^{2+}]; in order of increasing [Ca^{2+}] only. Muscle segments A2, A3, A4, and A5 of muscles 6 and 7 were used using sharp intracellular electrodes (20-50 MΩ), farbricated from borosilicate glass capillaries and filled with 3 M KCl solution. For evoked EPSPs, severed nerve bundles were stimulated using a suction electrode connected to a linear stimulus isolate. Data was acquired through an Axoclamp 2-A amplifier (Axon Instruments). EJPs were recorded on pclamp 1.0 then further analyzed using Axograph X. Miniature EJPs were analyzed using Fetchex 1.0. For EJP recordings, stimulus was presented from 0.8 to 3 Hz. mEJP for 2mins. For each mutant, 5 recordings were made at each of the [Ca^{2+}]. For short-term synaptic plasticity, paired-pulses were presented at 3 intervals of 100ms, 50ms and 20ms, respectively (described in Zhong and Wu 1991) at [Ca^{2+}] of 0.5 mM. All recordings in which the resting potential was higher than -60mV and/or all those resting potential and/or input resistance changed by 20% during the duration of data acquisition were excluded from analyses.

Muscle Degeneration

Immunocytochemistry was performed on third-instar larva in the methods described in the immunocytochemistry methods section. Antibodies to phalloidin and DAPI were used to stain muscle and muscle nuclei of CS and *w,rap*^{3}. Images were taken with

Zeiss Confocal Microscope and statistical analysis of muscles were performed using Prism 5 software.

Behavior assay

Larval Behavioral Crawling Assays

Larval behavioral crawling assays were performed according to Min and Condron (2005). Adult flies were allowed to lay eggs in standard fly food vials, after which the adults were removed. The larvae were grown until they were foraging third instars (72-78 hours pos-hatching). 20-25 larvae of each strain were collected using a moistened paintbrush and washed three times in 1ml of water in order to free them of any yeast residues, with each wash lasting seven minutes. After removing the dirty water with a micropipette, the larvae were allowed to recover for ten minutes on a clean, dry 35 x 10-mm Petri dish with the lid on.

Prior to testing the larvae, a 5 mm hole was dug out in the center of a 100 x 15-mm agar plate. Cold baker's yeast-water paste was applied to the center of the plate until it filled to the level of the agar. After placing the plate underneath a videorecorder (Apple iSight) the larvae were carefully laid out 5mm from the edge of it using a spatula and paintbrush. Approximately 110-150 of each control and mutant strains were tested in groups of 20-25. The plates were each recorded for thirty minutes at room temperature in a darkroom, in order to prevent their negative larval phototaxis from interfering with their migration towards the yeast. Ratios comparing the number of larvae that made it to the yeast paste to those did not were used as a better method of comparison.

Peristaltic Wave

Larval Peristaltic Contraction Counting Assay Protocol

As described in Nagai *et al.* (2010) all fly stocks were maintained at 22 ˚C on standard food. Agar plates were made using 12.5% Ultra-pure Agarose and stored at 4˚C. An agar plate was incubated at 30˚C for 15 min. A 12 well plate was used for washing the larvae, 6 wells filled with 500 μL d.i. water, and 6 wells with 500 uL 15% sucrose solution. Late third instar larvae in the wandering stage were identified as crawling on the side of the bottle (not submerged in the food). They were removed from the bottle using a paintbrush, and about 4 were placed in each of the sucrose wells. The well plate was put on a shaker for 1 wash (7 minutes). Using the paintbrush, the larvae were then transferred from the sucrose wells to the d.i. water wells, and returned to the shaker for 2 washes (14 minutes). An individual larva was removed from a well, placed on the agar plate, and allowed to acclimate for 30 seconds. The number of peristaltic contractions was counted for 2 minutes. If the larva was still alive but no contractions were observed, it was recorded as ∞ time, and the trial was redone with a different larva. 100 larvae were observed for each mutant strain.

Worm Locomotion Analysis

Further locomotory studies were performed on Third-instar “wandering” larvae using Multi-Worm Tracker (Janelia Farms) software. MWT is a set of software that tracks, in real time, small moving high-contrast against a static background. Larvae were first washed in dH_2O and then placed on thinly coated 1.5% agar gel plates, while Nikon camera was used to image the plates. Up to five larvae at a time were allowed to remain on the plate for 62 seconds while the software captured their images. After 62 seconds, the larvae were discarded and five new larvae were plated on the same agar

plate. Each trial contained approximately five larvae and total of 15 trials were used for each *rap/fzr* mutant. Data were compiled in Excel and analyzed in Prism 5. Data produced looked at speed, length, angular speed and kink. Speed refers to the speed of the objects in mm/second. Angular speed was measured in radians/second of objects; this is calculated over the same interval as speed, but reports the greatest difference in angle between primary axes over that time. Length refers to the distance spanned by objects along their major axis (defined to be the axis of a least squares fit), in mm. Finally, kink, which was defined by the angle in radians between the line from the first to third point of the skeleton and the fourth through last points.

RESULTS

Abnormal bouton morphology in *rap/fzr* neuromuscular junctions

To study the role of Drosophila-Cdh1 (Rap/Fzr) in the regulation of synaptogenesis at the NMJ, we first examined the bouton morphology of loss-of-function *rap/fzr* mutants. We examined loss-of-function mutant alleles $rap^{E2, E4, E6, x\text{-}2, x\text{-}3}$ and w,rap^{3} previously generated in our laboratory (Jacobs et al., 2002; Karpilow et al., 1989) using EMS, X-Ray, and P-element. CS appears oval in shape and the spacing between each bouton are the roughly the same, which forms normal branching patterning axons that innervate the muscles. The general morphology of the boutons changed, becoming more circular in shape and larger in size (Figure 1). While rap^{E2} appears the most similar to wild type, the boutons of the remaining *rap/fzr* mutants in the allelic series appear to have decreased in number: (bouton /0.1mm2 muscle surface) CS 32.95 $\pm$ 7.51, n=20; rap^{E2}, 15.7 $\pm$ 3.79, n=20; p<0.0001; rap^{E4} 14.6 $\pm$ 4.68, n=23, p<0.0001;

rap^{E6} 13.6 ± 5.48, n=18, p<0.0001; *rap* $^{X-2}$ 17.2 ± 7.71, n=21, p<0.0001; *rap* $^{X-3}$ 17.9 ± 3.55, n=12, p<0.0001; *w,rap*^{3} 30 ± 8.03, n= 15, not significant (Figure 2A). Our data show an increase in average bouton area significantly for *rap*^{E4} (19.4 ± 7.24, n=29, p<0.0005), *rap*$^{X-2}$ (15.9 ± 6.67, n=29, p<0.05) (data not shown); and *rap*$^{X-3}$ (Figure 2*B*); CS 12.39 ± 6.08, n=29. These changes are further evidenced by transmission electron microscope data, which shows increase in the size of the boutons as well (data not shown). While *w,rap*^{3} shows no difference in size compared to wildtype, images taken of *w,rap*^{3} show boutons grossly misshapen, their postsynaptic density convoluted, making the process of counting and defining the area difficult to accurately quantify. This demonstrates that Rap/Fzr also works to regulate the pathways involved in bouton morphology and synaptogenesis. We report no changes in branch number in *rap/fzr* mutants. The number of branch networks generated during synaptogenesis is not significantly affected in the mutants (data not shown).

To test whether there were changes in synaptic markers, we used two synaptic markers, one presynaptic Bruchpilot (BRP) and one postsynaptic marker, glutamate receptors type-IIA (GluRIIIA). We quantified the average level of intensity of clusters (Figures 2C -D). *rap*$^{X-3}$ mutant showed significant changes in the intensity of BRP (CS 112.6 ± 2.98 n=11, *rap*$^{X-3}$ 122.8 ± 2.88, n=11, p<0.05) and GluRIII staining (CS 118.6 ± 2.452, n=12; *rap*$^{X-3}$ 147.8 ± 3.347, n=12, p<0.0001). In addition, BRP puncta were also increased in *rap*$^{X-3}$ and *w,rap*^{3} (Figure 2E; n=30, p<0.001). While *w,rap*^{3} saw only a slight change in BRP and GluRIII intensity. Together, these results indicate changes in vesicle release machinery as well as changes to postsynaptic receptor density. It is not yet clear whether *rap/fzr* affects both of these mechanisms or if one is a

consequence of the other. Our results demonstrate that *rap/fzr* regulates size and number. This further indicates that *rap/fzr* it is important for synaptogenesis at the larval NMJ synapses.

TEM reveals empty boutons at the NMJ **in most severe mutant alleles**

Ultrasucture analysis by TEM showed evidence consistent with changes in neurotransmitter vesicle release (Figure 3A). In some of the alleles, the boutons appear virtually empty, containing little to no vesicle reserve pools for release, specifically *w,rap*^{3}. These data suggest these observed changes maybe related to an increase in vesicle trafficking and release at the presynapse, which may indicate changes in synaptic transmission. The vesicle size does not seem to be different in mutants and CS. However, it is possible because some terminals with no vesicles were missed due to the lack of vesicles, and therefore we were unable to quantify the vesicle size. *rap*^{E2} and *rap*^{E6} do not appear to be dramatically different than CS (data not shown). *w,rap*^{3} samples also have a greater reduction in the number of terminals than CS samples. As a precaution, additional dissections were fixed in EGTA-BAPTA buffer was used to chelate free Ca^{2+} to prevent release of vesicles during the fixation process yet *w,rap*^{3} showed virtually empty boutons. *rap*^{E4} also have larger terminals than CS but the same density of vesicles (data not shown). In addition, we counted vesicles that were fused to the presynaptic membrane (Atwood et al.,1989). We found a significant decrease in the number of fused vesicles in the *w,rap*^{3} mutant and slightly decreased in *rap*$^{X-3}$ (Figure 3B). We also see changes in SSR and postsynaptic density

Muscle Degeneration

We investigated changes located in muscle in loss-of-function *rap/fzr* mutants that specifically involved muscle fiber organization. *w,rap*^{3} presented dramatic phenotypic changes in muscle fiber organization (Figure 4). CS contained clearly visable, repeating sarcomere units. The darker staining regions are associated with A band that contain overlapping thin and thick filaments. I bands are the segments in between each A band. All were found normal in CS. *w,rap*^{3} at 20X magnification, contained patches of similar organization found in CS, however in the majority of the muscle, these structures were not present and had the disappearance of I bands and blending of A bands; an appearance that looked 'rubbed-out'. At closer inspection at a magnification at 100X, the muscle in *w,rap*^{3} provides further evidence in the changes of these specialized structures associated with sarcomeres. We observed that the majority of the muscles appeared this way in *w,rap*^{3} mutants (WT total muscles n=329, normal muscles were 108 $\pm$ 7; WT abnormal muscles 1.67$\pm$ 2.89; *w,rap*^{3} total muscles n=260, normal muscles were 9 $\pm$ 5.57, abnormal muscle 77.67$\pm$13.58). The total number of abnormal muscles was significantly increased in *w,rap*^{3} (Figure 5). Also, the total numbers of normal muscles were significantly decreased. Indicating that *rap/fzr* is necessary in normal development of muscle fiber structure and maintenance. Alternatively, because the ejp size of *rap/fzr* mutants was increased, it is likely that this additional synaptic activity would cause heightened activity in the muscles resulting in stress and eventually degeneration. In addition, Rap/Fzr dysfunction in muscles might also inhibit

Enhanced synaptic transmission at the *rap/fzr* neuromuscular junction

To understand the role of APC/$C^{Rap/Fzr/Cdh1}$ in synaptic transmission, we carried out electrophysiological experiments with *rap/fzr* mutants (Figure 6A-D). The area under the trace recording also was increase significantly for rap^{X-3} and w,rap^{3} (Figure 6A). This might be due to a longer time constant and distance at which Ca^{2+} channels are located from the vesicle release sites. Figure 6A shows representative traces, which demonstrate the differences in evoked potential responses. Compared to CS, mutant EJP characteristically differ in that their decay time is slower and smaller spontaneous EJP can also be seen (Figure 6A). These 'mini' responses are not related to the stimulus presented and were a consequence of independent release.

Recordings of evoked response recorded from *rap/fzr* mutants found that mutants rap^{X-3} and w,rap^{3} were significantly larger than wild-type (CS at 0.15, 0.2, and 0.5mM Ca^{2+}, were 0.123 mV$\pm$ 0.04, n=7; 0.208V + 0.08, n=2; 0.770mV $\pm$ 0.131, n=5; 0.956 mV $\pm$ 0.210, n= 3; rap^{X-3} 0.145 mV $\pm$ 0.63, n=1, p< 0.0001; 0.624 mV $\pm$ 0.149, n=2, p<0.0001; 0.838 mV + 0.047, n=1; 1.3V $\pm$ 0.513, n=3, p<0.0001; w,rap3 0.194 mV $\pm$ 0.068, n= 5, p<0.0001; 0.354 mV $\pm$ 0.097, n= 3, p<0.0001; 0.934 mV $\pm$ 0.14, n=7, p<0.0001; 1.66 mV $\pm$ 0.31, n=3, p<0.0001). We determined that the neurotransmitter release is directly proportionate to [Ca^{2+}] by recording EJP in a gradient series using 4 different physiological Ca^{2+} concentrations, 0.15, 0.2, and 0.5 mM extracellular bath solutions (Figure 6B). This demonstrated that EJP are Ca^{2+} dependent and that neurotransmitter release was not due to additional action potentials. This data was further corroborated by the addition of Tetrodotoxin (TTX), which inhibits Na^{+} gated channels, EJP were no longer still present (Figure not shown). The size of the

amplitude of the EJP was also significantly larger with increasing Ca^{2+} concentration in mutants rap^{X-3} and w,rap^{3} than in wildtype (Figure 6A). At 0.15mM [Ca^{2+}], only w,rap^{3} largely different from wild-type. At that [Ca^{2+}], the remaining mutants were not significantly different than wild-type (data not shown). What remains to be determined are some of the effector proteins that APC/$C^{rap/fzr/Cdh1}$ interacts with that causes changes in neurotransmitter release.

Due to the increase in EJP amplitudes, we tested whether quantal release was similar to wild type in low Ca^{2+} extracellular solution, thus failure rates were recorded for *rap/fzr* mutants. Based on Liao et. al (1995) silent synapse assay, synaptic failure occurs when in the release probability is low due to the [Ca^{2+}] which is also kept low. As a result, the evoked response from the postsynaptic membrane is unlikely. 100 stimuli were presented to each synapse and the number of evoked responses was recorded. At low Ca^{2+} (0.1mM), failure events were recorded and showed significant decrease in w,rap^{3} ($n= 3$, $p< 0.01$) than in wildtype (Figure 6C).

Note: More recently, when we reassessed CS ejp, we found that our CS stock had greater amounts of variability in ejp size. As a result, we found no difference between CS and *rap/fzr* mutants. We are currently backcrossing our rap mutant stocks into w^{1118} CS stock to generate heterozygous and then use it as a control.

Synaptic plasticity is significantly diminished in *rap/fzr* mutants

In addition to changes in EJPs, we also report changes in short-term synaptic plasticity. Figure 6D, shows that at 0.5mM [Ca^{2+}] allele w,rap^{3} shows significant paired-pulse depression, when normalized to wild-type. Based on the work by Fatt &

Katz (1952) and del Castillo & Katz (1954a), synapse release is proportional to the number of Ca^{2+} that are capable of binding to the vesicle docking and release proteins. In the case of the *rap/fzr* mutants, the first pulse causes virtually all the vesicles to be released causing a decrease in vesicle release in the second pulse, hence facilitated depression. It is possible that in *rap/fzr* mutants the first pulse triggers vesicles to be released in larger than wild type quantity and therefore it is unable to release vesicles in the second pulse. This indicates that the ability for the synapse to release more quanta than it should in response to a pulse stimulus causing rapid vesicle pool depletion. These results indicate Rap/Fzr plays a necessary role in synaptic plasticity.

Rap/Fzr present locomotion impairment

Crawling

To test whether *rap/fzr* mutants show impairments to locomotion due to previously described changes in synapse physiology, we conducted locomotion assays. Locomotion behavior also appears to be greatly affected in *rap/fzr*. We observed significant locomotory deficiencies in the more severe mutants. Interestingly, these are the same mutants that exhibited severe changes in physiology as described above, further implicating Rap/Fzr in synaptic development and neural transmission. In one assay, the locomotion of wandering third-instar larvae toward a chemoattractant (yeast) was observed. We report that not only the more severe mutants exhibited longer crawling times but that they also stopped moving. The actual distribution of the number of larvae that made it to the yeast throughout thirty minutes was also

calculated for each strain, in intervals of six minutes (Figure 7A, $P<0.05$). The majority of CS larvae arrived to the yeast in the first five minutes, with very few arriving in the later minutes. All of the *rap/fzr* alleles tested had significantly fewer number of larvae arriving within the first ten minutes, with *w,rap3* having the fewest larvae arriving at any of the time intervals. The most severe mutants who never reached the yeast center were noted and ratio to those that did. In comparison to CS, these strains exhibited significantly smaller rates of larvae that reached the yeast. This implies the decrease in mobility in severe mutants. CS had greatest number of larvae that made it to the yeast paste, with 3.65:1, or 78.5%, of the total larvae arriving to the food within thirty minutes. rap^{E4} larvae presented a 3.16:1 ratio, with 76.0% of them arriving. rap^{x-3} larvae presented a .40:1 ratio, with only 29% of them arriving to the yeast within thirty minutes. w,rap^{3} mutants were least able to arrive to the yeast, with only .23:1, or 21% of them arriving within thirty minutes.

Peristaltic contractions

To further investigate larvae locomotion, we observed the number of peristaltic contractions of third instar larvae in the wandering stage of all the *rap/fzr* mutants and wildtype. Peristaltic contractions alternate between “shortening” and “lengthening” phases in a rhythmic motion that allow the larva to move, which can be easily observed by the naked eye. Although the larvae generally wandered in random directions, wild-type larvae on average contracted more frequently than any of the mutant strains. Wildtype tended to begin moving almost immediately after being placed on the plate, while the mutants seemed to require more time to acclimate to the plate before contracting. The mutants used were rap^{x-2}, rap^{x-3}, w,rap^{3}. In comparison

to wild-type, mutants ranged in severity with *rap*$^{x-2}$ being the most impaired in locomotion (n= 100, p<0.0001), followed by *rap*$^{x-3}$ (n= 100, p<0.0001), and *w,rap*^{3} (n=100, p<0.0001) (Figure 7B). These peristaltic waves are generated by central pattern (Choi et al., 2004). This data further reinforces APC/C$^{rap/fzr/Cdh1}$ underlying role in proper synaptic development and function.

Locomotion behavior further analyzed

The average speed was significantly decreased in *rap*$^{X-3}$ (n= 87, p<0.0001), and *w,rap*^{3} (n= 91, p<0.0001), mutants than in wild type (Figure 7C). This data supports the changes seen in the other behavioral assays in that motility greatly affected in rap/fzr loss-of-function mutants.

DISCUSSION

Here we presented evidence that *rap/fzr* facilitates synaptic transmission. As result of loss-of-function mutations in the *rap/fzr* gene, bouton morphology has increased in size but has decreased in number at the synapse. Most interestingly, *rap*$^{X-3}$ and *w,rap*^{3} showed an increase in glutamate receptors, suggesting that Rap/Fzr may have some role in organizing postsynaptic receptors and scaffolding proteins. This is a plausible role given the function of APC/C in *C. elegans*, whereby APC/C functions to regulate the number of GLR-1 at the postsynapse by directly targeting proteins involved in clathrin-mediated endosytosis (Juo and Kaplan 2004). This change is further characterized muscle degeneration as well as changes in construction of the PDZ post-synaptically as reveled by TEM. This also suggests that changes associates with mutation in *rap/fzr* are not just localized to the presynaptic motorneuron. Changes in

the postsynaptic density (PSD) may reflect other proteins that are involved in regulating glutamate receptors and other PSD-associated proteins.

Alterations in synaptic transmission reflect these changes found in the postsynaptic muscle but may also involve presynaptic vesicle releasing mechanisms. This is consistent with the immunocytochemical data that we have presented which suggests that the presynaptic Ca^{2+} protein, Bruchpilot, maybe over expressed in *rap/fzr* mutants (Figures 1 and 2B, C). Bruchpilot is an important protein at the synapse, which functions in a similar manner to mammalian vesicle docking proteins ELKS/CAST/ERC in its homology in N-terminal binding domain sequence (Wagh et. al 2006). The study found that adult Drosophila lacking this gene were unable to fly properly and named "*crash pilot*." Hence, changes to Bruchpilot levels at the synapse can greatly influence the rate of synaptic transmission (Fouget et al. 2009).

The aberrant locomotion behavior in *rap/fzr* further supports deficiencies at the synapse and ultimately results in uncoordinated muscle contraction, prohibiting normal locomotion to occur. Peristaltic waves are generated by central pattern generators in the central nervous system, which direct motor neurons (Choi et al., 2004). Sensory neurons also contribute to controlling locomotion, thought to influence the relaxation of a muscle segment after a contraction (Hughes and Thomas, 2007). In addition, glia cells have been found to modulate synaptic transmission and also play important roles in synapse formation and maintenance (Zuo and Bishop, 2008). In wildtype larvae, synaptic transmissions functions through maintenance of signal pattern generators (cpg), this allows the larva to move in a coordinated manner (Choi et al., 2004). Studies of *rap* mutants have shown alterations in glia number and structure (Kaplow,

2008), suggesting possible changes at the neuromuscular junction. This assay tested if such mutations would translate to the behavioral level and disrupt locomotion. The statistically significant decrease in the number of peristaltic contractions of mutant strains (compared to wild-type) implies some defect in mutant larval movement. This may be attributed to any number of changes observed. Clearly, the muscles that are degenerated in rap/fzr mutants are numerous and effect locomotion significantly, whether it is due to the changes in synaptic release or APC/C$^{rap/fzr/cdh1}$ dysfunction within muscle, has yet to be determined. This leads us to conclude that *rap/fzr* is necessary for synaptic transmission and plasticity.

These results beg to ask the question, what are the proteins that interact with APC/C$^{rap/fzr/Cdh1}$ that could lead to this dysfunction? Our lab employed a genetic modifier screen to identify genes that interact with Rap/Fzr (Kaplow et. al 2007). These interacting genes encode proteins involved in ubiquitin-mediated proteolysis, signal transducers, transcription factors and several genes of unknown function (Kaplow et. al 2007). Three genes that stood out among the thirty-three were *locomotion defects* (*loco*), *non-stop* (*not*), and *twins*. All have significant involvement in nervous system development.

Loco is involved in the regulation of thehetero-trimeric G protein signaling pathway (Han et al. 2006) and *loco* encodes a protein of the RGS (regulators of G-protein signaling) family (Han et al. 2006). In the genetic modifier screen, *loco* was identified as a dominant suppressor of rough eye phenotype of *rap/fzr*. In loco mutants, glia cells fail to properly ensheath longitudinal axon tracts (Schwabe et al.,

2005). Rap/Fzr targets Loco for ubiquitination, thereby regulating glial differentiation in the developing nervous system (Kaplow et al 2008).

Notstop (Not) is a ubiquitin-specific protease. *not* mutants have glial and axonal targeting defects and glial cells, which do not migrate properly in the developing eye (Poeck et al 2001). In nonstop loss-of-function mutants, epithelial, marginal and medulla glia fail to migrate from glial precursor areas (GPC) located at dorsal and ventral edges of the R cell projection field. As a result, defective glia migration causes R1-R6 axons to mistarget (Poeck et al 2001). Nonstop may regulate Rap/Fzr by either deubiquitination its substrate/s such as Loco or removing a ubiquitin tag from Rap/Fzr itself, thereby keeping the APC active for a longer time period. Studies from our laboratory have shown that Rap/Fzr targets Not for ubiquitination and Not deubiquitinates Loco and promotes glia differentiation (Kaplow et al 2008). We propose that deubiquitination of Loco by Not may be critical for synaptogenesis. It is thought that Rap/Fzr is also regulated by both autoubiquitination and is activated by Fizzy (Cdc20). Rap/Fzr may also be activated following deubiquitination by Not and may also act as a local regulator of Rap/Fzr.

Twins (PP2A) acts a dominant enhancer of the *rap/fzr* rough eye phenotype. PP2A was shown to be involved in a number of different and unrelated pathways. In the peripheral nervous system, *twins* mutation caused similar defects to *numb* and *musahi*, which cause increase number of support cells at the expense of neurons in the mechanosensory organ (Shiomi et al., 1994). Twins also functions in wing morphogenesis as loss-of functions show pattern duplication in imaginal discs (Uemura et. al., 1993). In addition Twin interacts with Ras pathway during eye

development. Loss-of-function compounds *ras* mutants and enhance the function of unregulated Raf defects (Wassarman et al., 1995). Rap/Fzr is inhibited by phosphorylation and activated by dephosphorylation (Listovsky et al. 2000). Genetic interactions between Twins and Rap/fzr suggest the twins may activate Rap/Fzr by dephosphorylation at the neuromuscular junction, allowing for temporal control of APC activation. However, these proteins and their involvement in synaptic development and transmission require further study.

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Acknowledgements:

We thank Confocal Microscopy center of the City College of New York, City University of New York for preparation of TEM samples and Dr. Chun-Fang Wu for the use of his laboratory, expertise and equipment. Supported by grants from the NIH (T.V and C-F. W). A.W. was supported by NIH-RISE and LSAMP grants at the City College. The work at City College was supported by NIH-RCMI infrastructure grant.

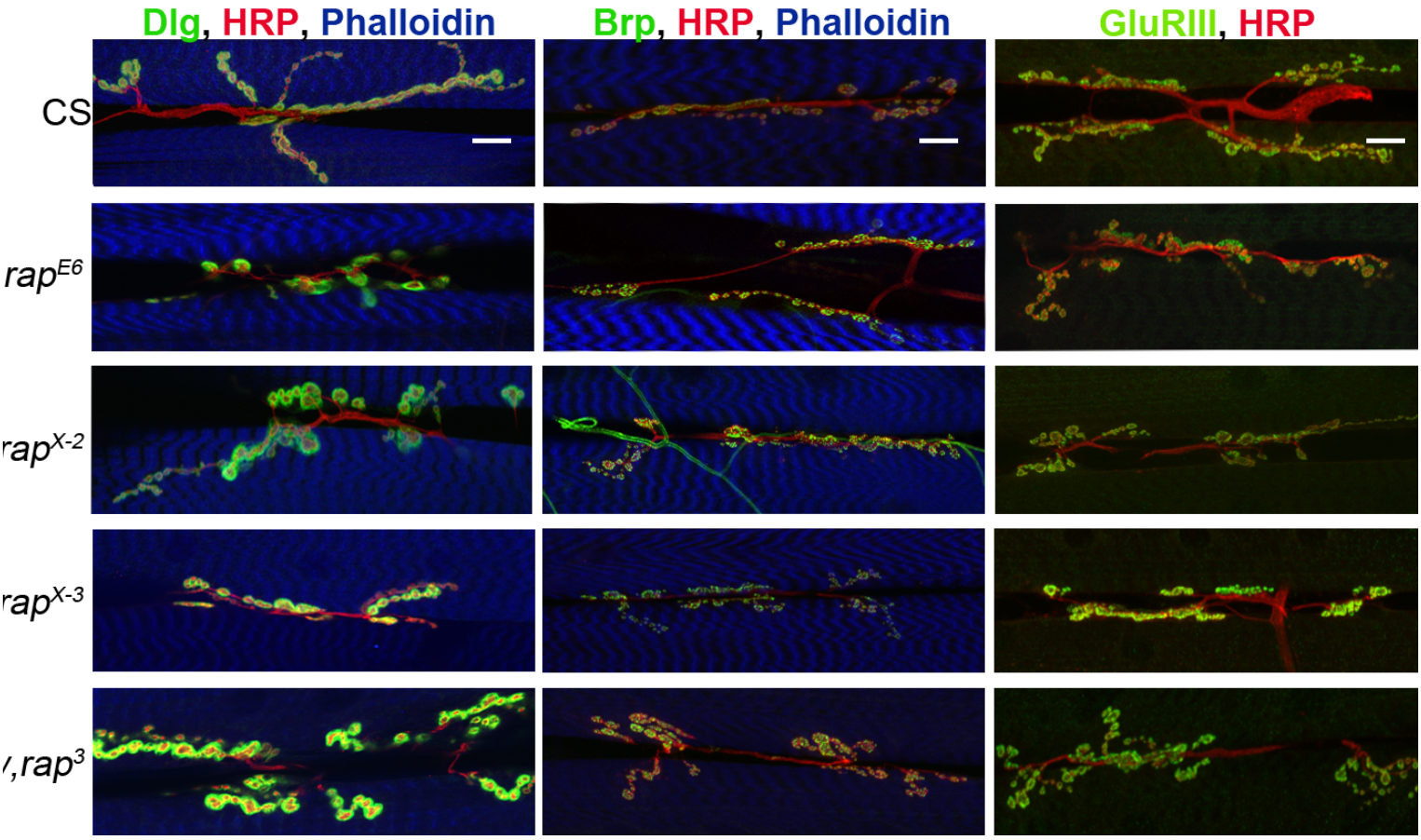

Dlg, HRP, Phalloidin
Brp, HRP, Phalloidin
GluRIII, HRP
CS
rap^E6
rap^X-2
rap^X-3
,rap^3

Figure 1. *rap/fzr* mutants show significant changes in bouton morphology. Loss-of-function *rap/fzr* mutants exhibit changes in bouton morphology at the NMJ as seen in this composite of *rap/fzr* mutant alleles with various antibodies. Sample confocal images of larval NMJs from muscle segment 3 (muscles 7 and 6) stained with HRP (Red), phalloidin (Blue), and either Dlg, GluRIIIA or BRP (green). When compared to CS, *rap/fzr* mutants rap^{E6}, rap^{X-2}, rap^{X-3} and w,rap^{3} alleles exhibit fewer numbers of boutons. In addition, boutons tend to be more oblong and misshapen with less smooth postsynaptic density staining, especially with w,rap^{3}. w,rap^{3} also seen here having enlarged Dlg staining. In the GluRIIIA staining, puncta appear to have greater intensity particularly in rap^{E6} and w,rap^{3}. Similarly, BRP staining not only showed increase in staining intensity but also increase in the number of puncta in rap^{X-3} and w,rap^{3}.

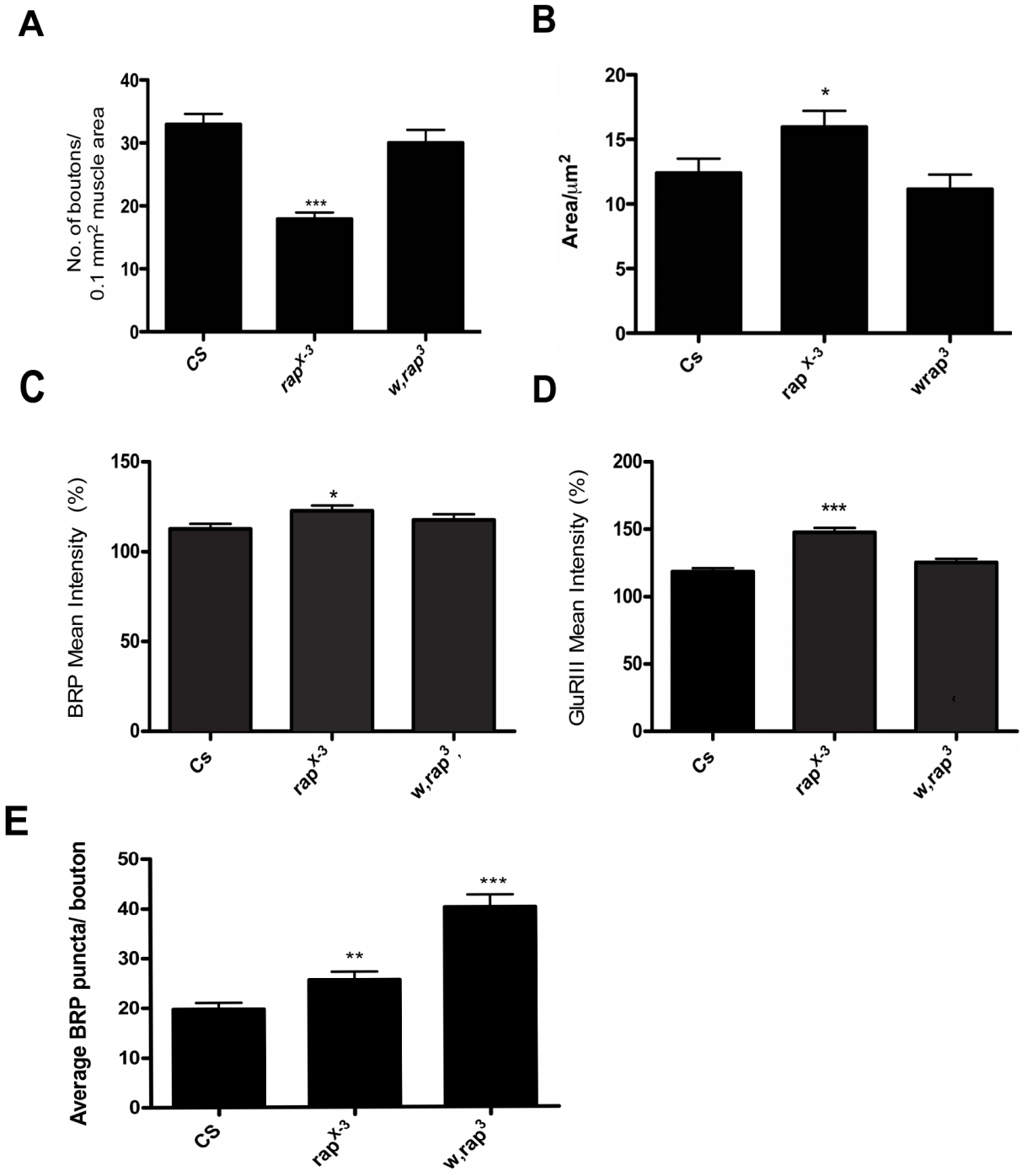
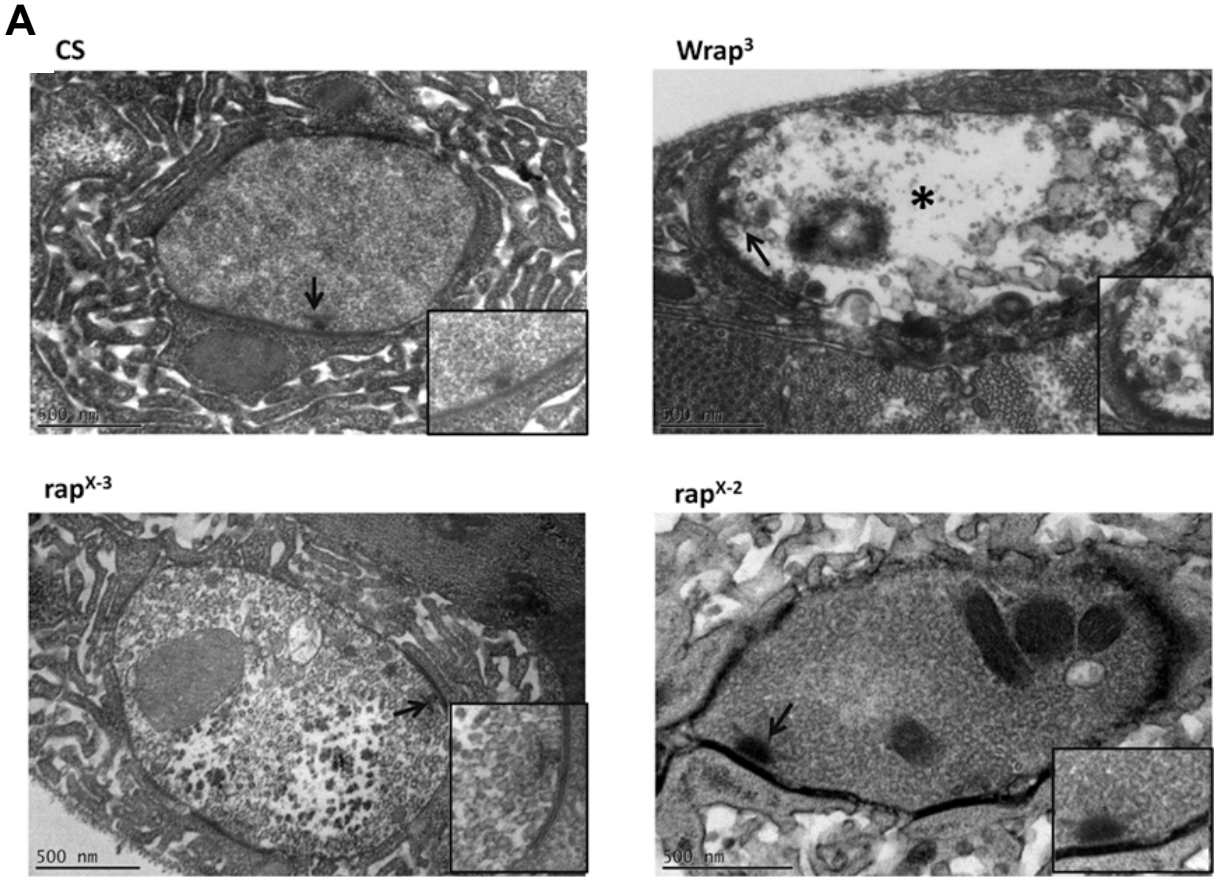

A
No. of boutons/ 0.1 mm2 muscle area
40
30
20
10
0
***
CS
rapX-3
w,rap3
B
Area/μm2
20
15
10
5
0
*
Cs
rap X-3
wrap3
C
BRP Mean Intensity (%)
150
100
50
0
*
Cs
rapX-3
w,rap3
D
GluRIII Mean Intensity (%)
200
150
100
50
0
***
Cs
rapX-3
w,rap3
E
Average BRP puncta/ bouton
50
40
30
20
10
0
**
***
CS
rapX-3
w,rap3

Figure 2. *rap/fzr* mutants have decreased number of boutons but show an increase in bouton size and fluorescent intensity of BRP and GluRIIIA.
(A) Comparison of NMJ boutons number at the NMJ show decrease in *rap/fzr* mutants, rap^{E2}, rap^{E4}, rap^{E6}, rap^{X-2} and rap^{X-3} n=29, p<0.0001. w,rap^{3} did not show decrease in boutons. (**B**) There is a significant increase in size in rap^{E4} n=29, p<0.0005, rap^{X-2} n=29, p<0.05 and rap^{X-3} n=29, p<0.05. The other alleles also show an increase in size but, however, are not significant. Only w,rap^{3} shows no difference in size compared to wildtype. (**C-D*)*** Fluorescent intensity of BRP significantly increased in rap^{X-3} n=11, p<0.05 and not significant in w,rap^{3} p<0.186, n=11. GluRII staining is also significantly increased in rap^{X-3} p<0.0001, n=11 and not significantly increased in w,rap^{3} p<0.25, n=11.(E) Average BRP punta count per bouton are significantly increased, rap^{X-3} , n=30, p<0.001 and w,rap^{3} n=30, p<0.001.


A
CS
Wrap3
rapX-3
rapX-2
500 nm
500 nm
500 nm
500 nm

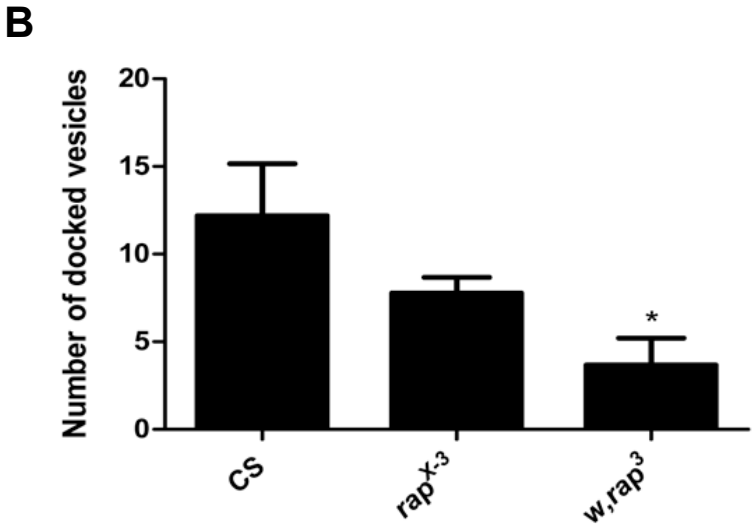

B
Number of docked vesicles
20
15
10
5
0
*
CS
rapX-3
w,rap3

Figure 3. *rap/fzr* mutants nerve terminals are shown to have either terminals 'empty' of vesicles or have terminals with relatively fewer vesicles than CS. *rap/fzr* mutants also show significant decrease in the number of docked vesicles at the presynaptic terminal. (**A**) Images of synaptic boutons on muscle fibers from wild type larvae (CS) and two mutants (*w,rap*^{3} and *rap*$^{X\text{-}3}$). Large space without synaptic vesicles (indicated with *) was often observed in the *w,rap*^{3} mutant. **(*B*)**, Histogram of docked vesicles on the presynaptic membrane. *w,rap*^{3} has significantly decrease in number of docked vesicles *w,rap*^{3} n=6 $p<0.05$.

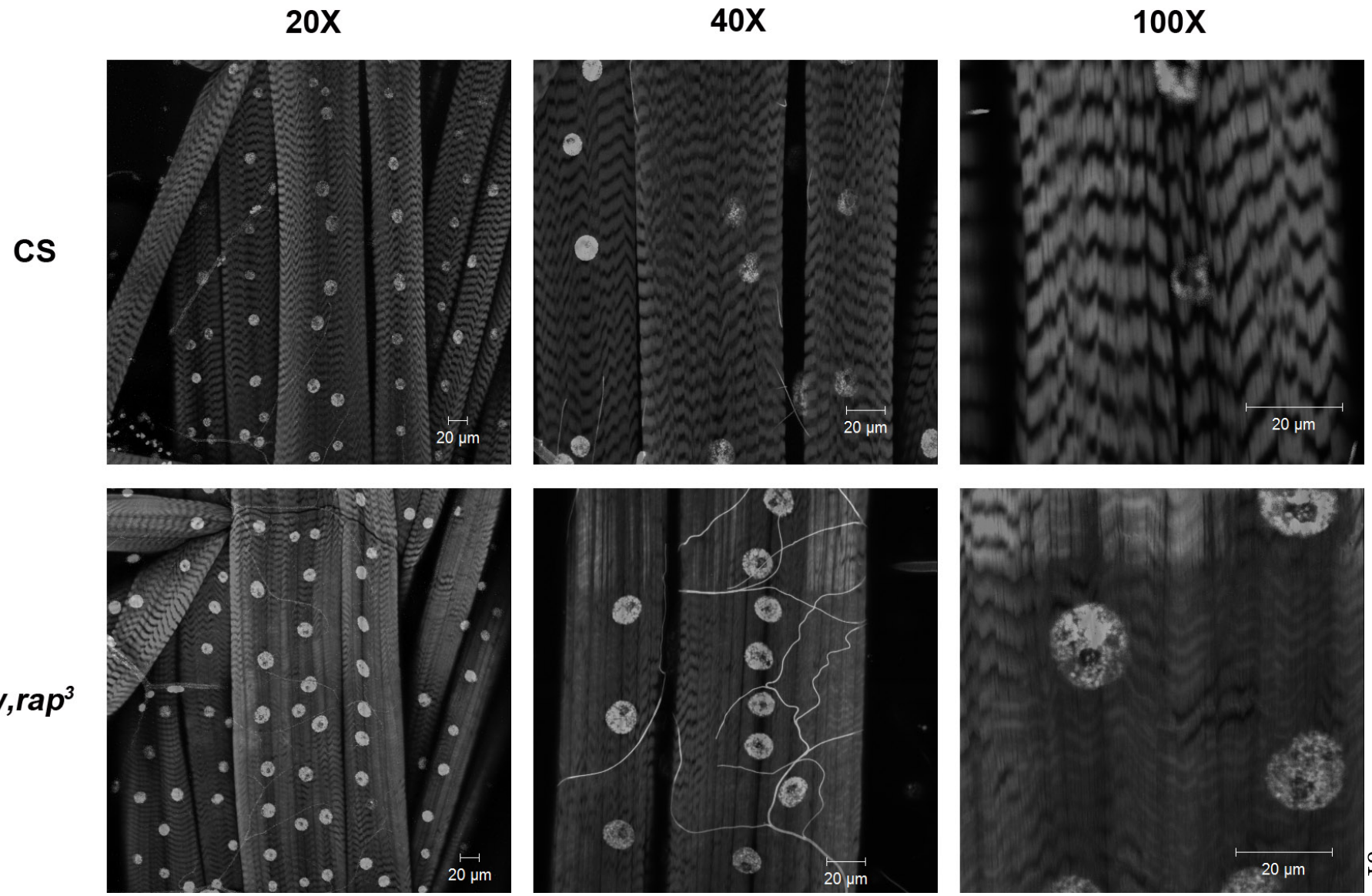


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Figure 4. Muscle degeneration in *w,rap*^{3} mutants at 20, 40 and 100 magnification levels. Confocal images contain stain using DAPI and phalliodin. These images show changes in sarcomere patterning in *w,rap*^{3} when compared to wt (CS). There is significant disruption in the structure of the I and A bands in that their boundaries are not present. We also see increase in the number of nuclei staining

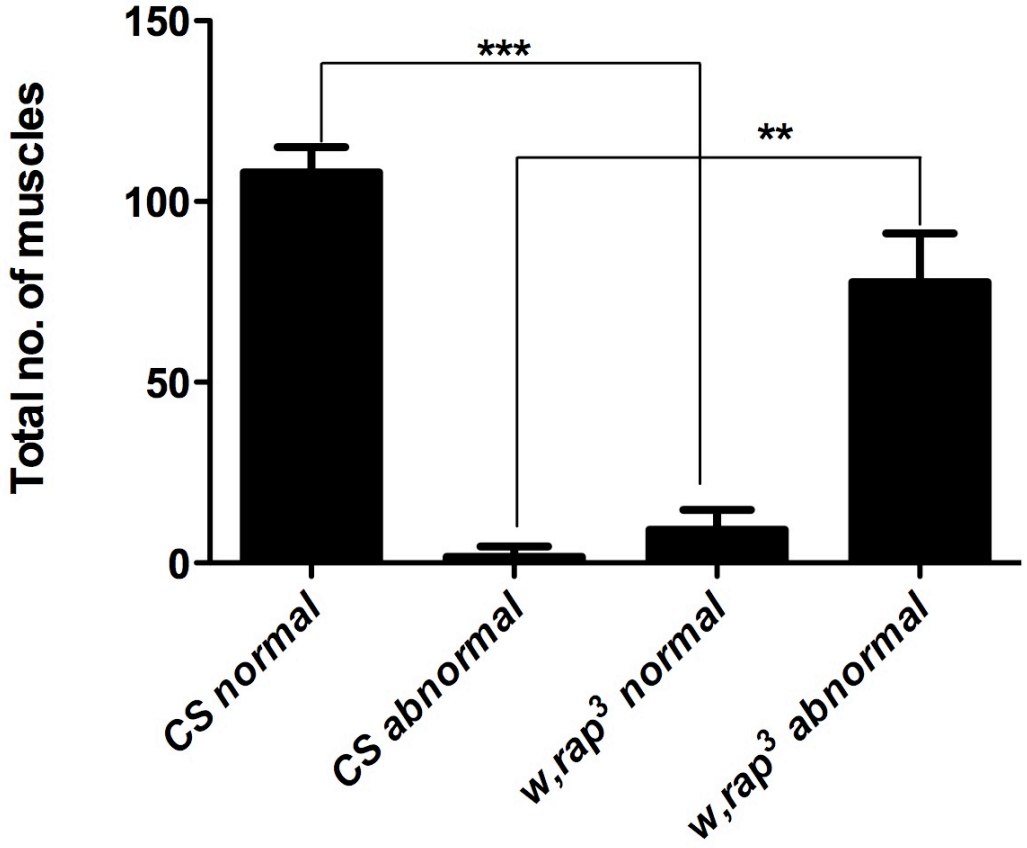

Total no. of muscles
150
100
50
0
***
**
CS normal
CS abnormal
w,rap3 normal
w,rap3 abnormal

Figure 5. Quantification of the number of normal and abnormal muscle fiber in CS and *w,rap*^{3}. This figure demonstrates the increase of number of muscles that exhibited degeneration in sarcomere structure in rap/fzr mutants. Comparison of CS normal (n=3) and *w,rap*^{3} normal (n=3), $p<0.0001$. The number of abnormal muscles in *w,rap*^{3} was also significantly increased in comparison to CS ($p<0.0089$).

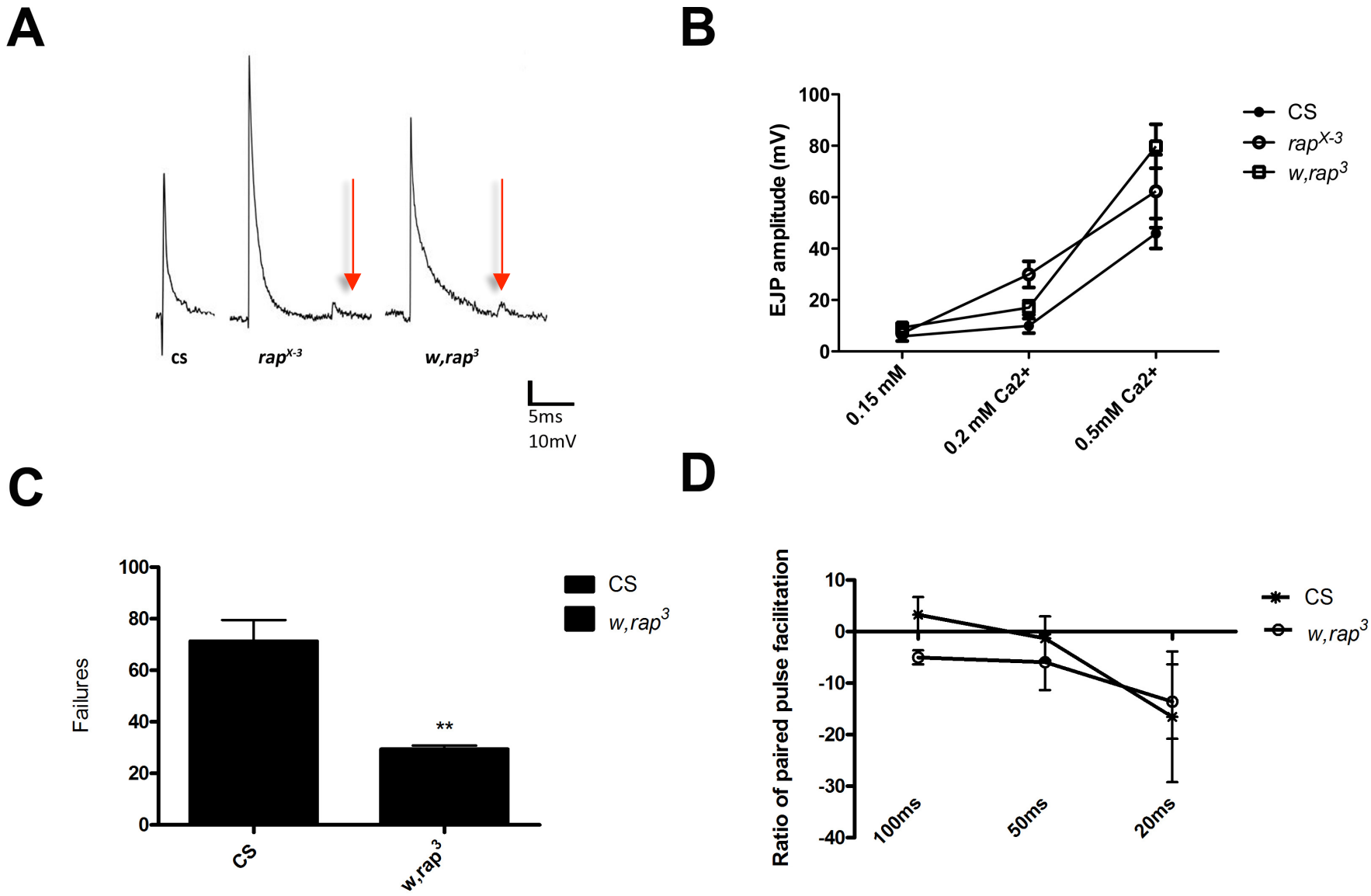
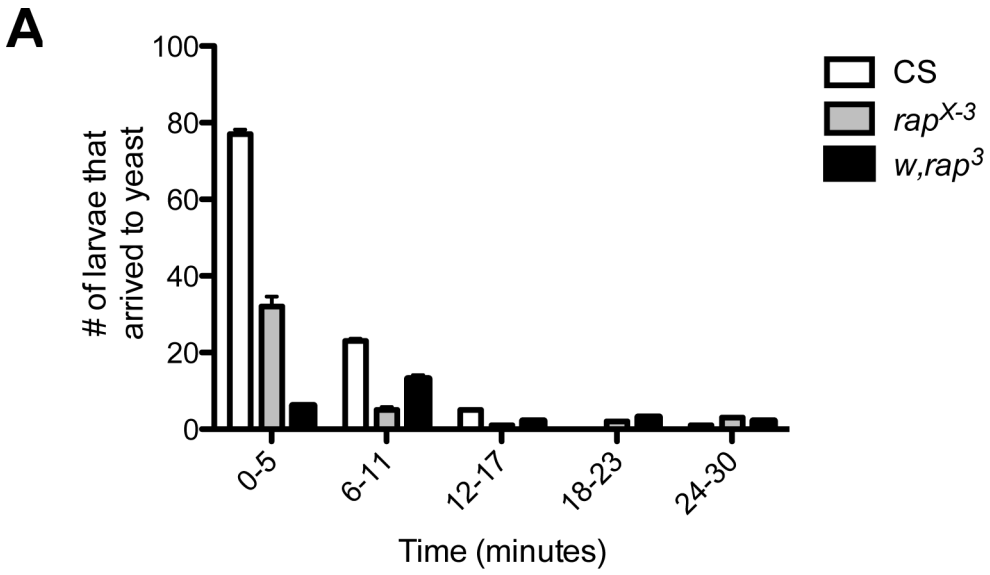
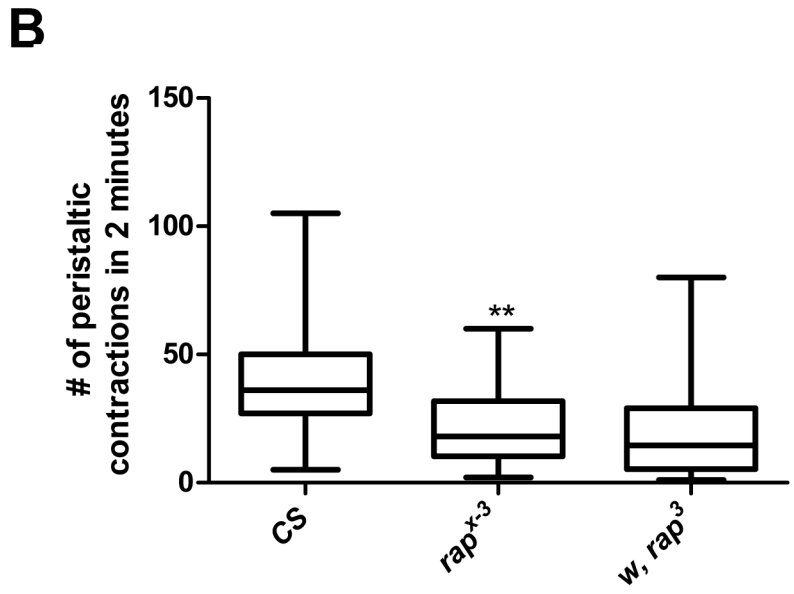

A
cs
rapX-3
w,rap3
5ms
10mV
B
EJP amplitude (mV)
100
80
60
40
20
0
0.15 mM
0.2 mM Ca2+
0.5mM Ca2+
CS
rapX-3
w,rap3
C
Failures
100
80
60
40
20
0
**
CS
w,rap3
CS
w,rap3
D
Ratio of paired pulse facilitation
10
0
-10
-20
-30
-40
100ms
50ms
20ms
CS
w,rap3

Figure 6. *rap/fzr* mutants exhibit strong increase in synaptic activity. (A) Example traces show slightly larger amplitude in mutants. In addition, latent “miniature” evoked potentials can be seen in rap^{X-3} and *w,rap*^{3} as indicated by (*). **(B)** Mean EJP show increase evoked response at four different extracellular Ca^{2+} concentrations**. (C)** Synaptic failures are decreased in rap^{X-3} and w,rap^{3} when 0.1mM Ca^{2+} bath is applied. (**D*)*** PPR at extracellular Ca^{2+} concentration of 0.5 mM were given at intervals of 20ms, 50ms and 100ms. w,rap3 exhibited severe depression throughout each of intervals.


A
100
80
60
40
20
0
# of larvae that arrived to yeast
0-5
6-11
12-17
18-23
24-30
Time (minutes)
CS
rap^X-3
w,rap^3


B
150
100
50
0
# of peristaltic contractions in 2 minutes
**
CS
rap^X-3
w, rap^3

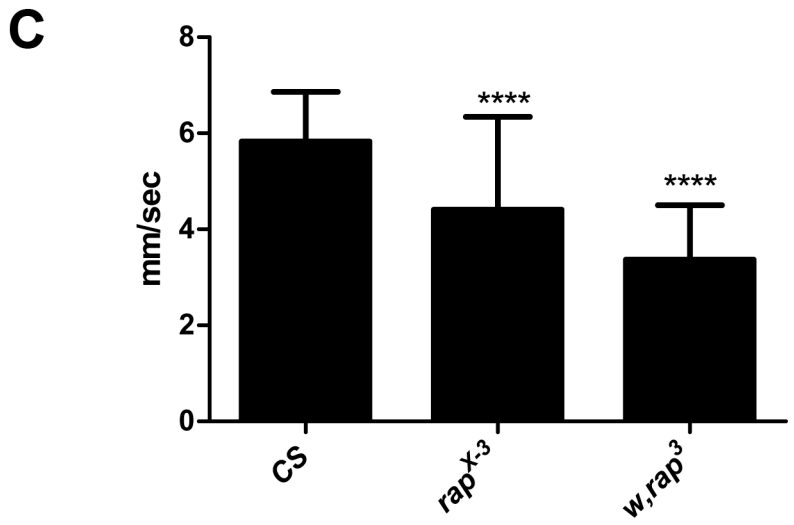

C
8
6
4
2
0
mm/sec
****
****
CS
rap^X-3
w,rap^3

Figure 7. *rap/fzr* mutants show decrease in locomotion and peristaltic contractions. (A)Shows the locomotion distribution of larvae that reached the chemoattractant center of an agar filled petri dish larvae after a 30-minute period. The majority of CS larvae reached the yeast center within 0-5 minutes, while the other mutants range from 11-30 minutes. All mutants were significantly slower, $p<0.05$, rap^{E4} (n=154), rap^{X-3} (n=148), w,rap^{3} (n=114). (**B)** Number of peristaltic contractions by different mutant strains but relative to CS, contracted far less. In comparison to wild-type, CS n=100, mutants ranged in severity with rap^{x-2} being the most impaired in locomotion (n= 100, $p<0.0001$), followed by rap^{x-3} n= 100, $p<0.0001$ and w,rap^{3} n=100, $p<0.0001$. The number of peristaltic contractions is significantly decreased in the most severe *rap/fzr* mutants. **(C)** "Worm Tracker" revealed that *rap/fzr* were significantly impaired in locomotion behavior by having a slower velocity than CS, rap^{X-3} n= 87, $p<0.0001$ and w,rap^{3} n= 91, $p<0.0001$.

Strain	# that made it	# that did not	Ratio
Cs	106	29	3.65:1
rap^{E4}	117	37	3.16:1
rap^{X3}	43	105	0.40:1
w*rap*^{3}	24	90	0.23:1

Table 1. Ratio of genotypes tested in yeast-plate assay. This table describes the genotype ratio individual larvae that reached the center of the yeast plate.

Studies on the interactions of Rap/Fzr with Loco, Nonstop and Twins at the larval neuromuscular junction

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ABSTRACT

The mechanisms that regulate synaptogenesis through the ubiquination pathway at the neuromuscular junction in *Drosophila Melanogaster* remains poorly understood. Our previous study has shown physical interactions of Rap/Fzr with a variety of other proteins, however, we have focused on a few specific ones, Locomotion defects (Loco), nonstop (Not) and Twins. These proteins have been show to interact with Rap/Fzr in the central nervous system. Here, we show localization and physical interaction of these proteins at the neuromuscular junction. Loco is a regulator of G-protein signaling, and may play an important role in G-coupled protein signaling. Not is a deubiquitinase that can deubiquinate other proteins including Rap/Fzr. Twins is the α-subunit protein phosphatases subunit which acts to dephosphorylate substrate proteins. We present a possible mechanism by which Rap/Fzr interacts with different pathways downstream of APC/C, allowing for the regulation of synaptogenesis. The components of the UPS at the synapse provides an attractive molecular mechanism to regulate proteins locally by degrading activator as well as repressor proteins, ultimately acting as an “on/off switch.”

INTRODUCTION

The ubiquitin proteasome system (UPS) plays a crucial role in modifying intracellular trafficking of proteins or targeting proteins for degradation by the 26s proteasome (Hick L. 2001; Glickman M.H. and A. Ciechanover 2002) by recognizing a specific regulatory sequence and adding a 76 amino acid ubiquitin typically to the N-terminus (Glickman M.H. and A. Ciechanover 2002). Polyubiquination results in protein degradation, while monoubiquitination results in protein modification (Schnell and Hick 2003). Within the ubiquitin pathway several ligase proteins function to bind the ubiquitin to the substrate protein. Briefly, the E1 ligase sequesters ubiquitin and activates it by adding an ATP before transferring it to an E2 conjugating ligase. Next, the E2 ligase-ubiquitin complex binds directly to the substrate protein or to an E3 Ligase that is already attached to the substrate protein. Finally, the E3 ligase protein removes the ubiquitin from E2 and adds it to the substrate protein. Four domain classes exist for E3 ligases: HECT (Homologus to E6-AP carboxyl terminus) and RING/U-Box (really interesting new gene)-finger domain, SCF, and APC (Ohi, MD. et al. 2003). These differ in organization and structure but all function as a multi-subunit protein complex that facilitates ubiquitination of proteins.

Perviously our laboratory employed a genetic modifier screen to identify genes that interact with Rap/Fzr, the activating subunit of the E3 ubiquitin ligase, Anaphase Promoting Complex/Cyclosome (APC/C), These interacting genes encode proteins involved in ubiquitin-mediated proteolysis, signal transducers, transcription factors and several genes of unknown function (Kaplow et. al 2007). Three genes that stood out

among the thirty-three were *locomotion defects* (*loco*), *non-stop* (*not*), and *twins*. All have significant involvement in nervous system development.

loco encodes a protein of the RGS (regulators of G-protein signaling) family (Han et al. 2006) and is involved in the regulation of hetero-trimeric G protein signaling. In the genetic modifier screen, *loco* was identified as a dominant suppressor of the rough eye phenotype of *rap/fzr*. In loco mutants, glia cells fail to properly ensheathe the longitudinal axon tracts (Schwabe et al., 2005). Rap/Fzr targets Loco for ubiquitination, thereby regulating glial differentiation in the developing nervous system (Kaplow et al 2008).

Not is a ubiquitin-specific protease. Not mutants have glial and axonal targeting defects and glial cells which do not migrate properly in the developing eye (Poeck, B. et al 2001). In *not* loss-of-function mutants, epithelial, marginal and medullary glia fail to migrate from glial precursor areas (GPC) located at dorsal and ventral edges of the R cell projection field. As a result, defective glia migration causes R1-R6 axons to mistarget (Poeck, B. et al 2001). Not may regulate Rap/Fzr by either deubiquitination its substrate/s such as Loco or removing a ubiquitin tag from Rap/Fzr itself, thereby keeping the APC active for a longer time period. Studies from our laboratory have shown that Rap/Fzr targets Not for ubiquitination and Not deubiquitinates Loco and promotes glia differentiation (Kaplow et al 2008). We propose that deubiquitination Loco by NOT may be critical for synaptogenesis. It is thought that Rap/Fzr is also regulated both autoubiquitination and by the action of Fizzy (Cdc20). Rap/Fzr may also be activated following deubiquitination by Not and may also act as a local regulator of Rap/Fzr.

twins acts a dominant enhancer of the *rap/fzr* rough eye phenotype. PP2A was shown to be involved in a number of different and unrelated pathways. In the peripheral nervous system, *twins* mutations caused similar defects to *numb* and *musahi*, which caused an increase in the number of support cells at the expense of neurons in the mechanosensory organ (Shiomi et al., 1994). Twins also functions in wing morphogenesis as loss-of functions show pattern duplication in imaginal discs (Uemura et. al., 1993). In addition Twin interacts with Ras pathway during eye development. Loss-of-function compounds *ras* mutants and enhance the function of unregulated Raf defects (Wassarman et al., 1996). *rap/fzr* is inhibited by phosphorylation and activated by dephosphorylation (Listovsky et al. 2000). Genetic interactions between Twins and Rap/fzr suggest the twins may activate Rap/Fzr by dephosphorylation at the neuromuscular junction, allowing for temporal control of APC/C$^{rap/fzr/cdh1}$ activation. Here we show the presence of Nonstop, Loco and Twins at the synapse and loss-of-function mutants have changes in bouton morphology. In addition, we show *rap/fzr/cdh1* dominantly interacts with *nonstop*, *loco* and *twins* at the synapse, revealing possible regulatory role at the NMJ through these proteins. This may contribute further to understanding the mechanisms in which APC/C$^{rap/fzr/cdh1}$ regulates synaptogenesis and synaptic transmission.

Materials and Methods

Drosophila Culture

The following mutant flies were used: loco^{p452} (Yu et al., 2005) and not^{1}/TM6;Tb (Martin et al., 1995; Poeck et al., 2001) have been previously described. *rap*^{E2}, *rap*^{E4},

rap^{E6}, *rap*$^{X-2}$, *rap*$^{X-3}$, *w,rap*^{3} were used as previously described (Jacobs et al., 2002; Karpilow et al., 1989). Null alleles of *rap/fzr* are lethal, so mutants that were generated are partial loss-of-function. Several loco stock obtained from the Bloomington stock center include *P{lacZ-un1}loco*^{rC56}*, y1 w67c23; P{EPgy2}locoEY04589, w[*]; P{w[+mC]=EP}loco[GE24954], and y[1]; ry[506] P{y[+mDint2] w[BR.E.BR]=SUPor-P}loco[KG02176]/TM3, Sb[1] Ser[1].* Flies were cultured on standard cornmeal–agar medium and kept at 23° C. The isolation and characterization of the mutant alleles used in this study have described previously (Kaplow et al., 2007). CS were used as the control genotype.

Immunocytochemistry

Third-instar larvae males were selected, dissected in calcium-free media modified minimal hemolymph-like solution (HL3.1) with Ca^{+2} and fixed for 25 min in 4% paraformaldehyde. The preparations were washed three times for 5 minutes with 0.2 M Phosphate Buffer Solution containing 0.2% Triton-X 100 (PBST). The larvae sections were incubated overnight at 4^{o}C in mouse anti-Discs Large supernatant at 1:5 (4F3) from Hybridoma Bank (University of Iowa Developmental Studies Hybridoma Bank). The sections were then washed 3 times (5 minutes each time) in 0.2 % PBST. Secondary antibodies were applied for 2 hours: Alexa Fluor 635 conjugated anti-Phalloidin at 1:1000 (Invitrogen), goat anti-mouse FITC (Jackson Laboratories) at 1:100 and TRITC conjugated goat anti-Horseradish Perioxidase (Jackson Laboratories) at 1:100. Sections then were washed twice for 5 min and mounted using Vectashield (Vector Laboratories). Confocal microscope images were taken with LSM 510 Confocal Laser Scanning System (Carl Zeiss Inc.) at 40x and were processed

with Image J 1.24 Adobe Photoshop 5.5. Boutons were counted using muscle 6 & 7 from segment A2 and A3 and analyzed using Prism. Significance and p values were derived by Student's t-test.

RESULTS

Localization of loco, nonstop and twins to the synapse

Our laboratory has shown Rap/Fzr physically interacts with Loco, Nonstop and Twins in the central nervous system (Kaplow et al., 2008). To test whether these proteins were present at the neuromuscular junction in third instar larva, we performed immunolocalization experiments using specific antibodies of Nonstop, Loco and Twins. We have shown that all three proteins localize to the neuromuscular junction (Figures. 1-3). By using an anti-loco antibody developed in *Drosophila* (courtesy of Dr. Bill Chia) show that Loco is present at both the pre- and postsynaptic regions (Figure 1). However, its involvement in synaptic development and transmission still remains unclear. Nonstop appears to localize specifically to the presynaptic regions (Figure 2). This may indicate that the role of this dequbiquitination protein is only in the presynapse. Lastly, Twins appears both pre- and postsynaptically with discrete puncta in motorneuron and muscle cells (Figure 3). Using these specific antibodies, we were able to conclude that Nonstop, Loco and Twins localize to the synapse. Their pre- and postsynaptic function at the synapse has yet to be determined.

Loco and Not loss-of-function mutants show abnormal synaptic morphology

Homozygous *loco* and *nonstop* mutations are larval/pupal lethal. However, we were able to analyze 'escaper' mutants, which are loss-of-function mutants with a

small percentage of a residual functional copy of *loco* or *nonstop.* We analyzed homozygous *loco* and *nonstop* mutant 'escapers' for changes in the NMJ synapse morphology. Both *loco and not* mutants showed abnormal bouton morphology and a decrease in bouton number (Figure 4). Their bouton appears much larger in morphology, while type II boutons remain intact and wildtype in appearance. In addition, the individual bouton size was decreased. Quantification of boutons in escaper mutants from both *nonstop* and *loco* loss-of-function mutants was done (Figure 5). Both *loco* (n=12) and *nonstop* (n=12) mutants had decrease number of bouton, $p<0.001$ for both mutants. Bouton size is altered in nonstop and loco mutants (Figure 6). There is a significant change in the size of the boutons. In nonstop mutants, the boutons are significantly increased and in loco the boutons are significantly decreased in comparison to wild-type.

Loco, Nonstop and Rap/Fzr interact at the synapse

To test whether Loco and/or Non-stop interact with Rap/Fzr at the synapse, we generated *rap/fzr*, *loco* double mutants. We crossed heterozygous *loco* mutants and *non-stop* with the various *rap/fzr* loss-of-function mutants. The F1 hemizygous *rap/fzr* males were heterozygous for *rap/fzr* and either *loco or not.* In larvae, rap^{E2}*; not/+* and $rap^{E6,}$*;not/+* mutants showed an increase in number of boutons when compared to their *rap* mutant loss-of-function counterpart. We found that rap^{E4}*; loco/+* and w,rap^{3}*; loco/+* also showed increase in bouton numbers. Lastly, in rap^{X-2} and rap^{X-3} mutants that were crossed to both *not* and *loco* showed an increase in bouton number and terminal arborization (Figure 7) in both *loco* and *not*. We looked at the remaining *rap/fzr* and *loco* or *rap/fzr* and *not* double mutant alleles, which showed a gain of

function phenotype in terms of the number of boutons. This demonstrated dominant interactions between Rap/Fzr and Loco as well as Rap/Fzr and Not at the NMJ (Figure 8). The boutons in the *nonstop* mutants appeared to have increased in number. This demonstrates Rap/Fzr, Loco and Nonstop importance in synaptogenesis and development.

DISCUSSION

The APC/C is a multi-subunit E3 ligase, that functions in polyubiquitination of a substrate and leads to protein degradation by the 26s proteasome. Rap/Fzr/Cdh1 is the activating subunit of the APC/C and its regulatory role involves activating the $APC/C^{rap/fzr/cdh1}$ during specific stages of mitosis. It has also been shown to regulate glia cell differentiation (Kaplow et al., 2008). $APC/C^{ap/fzr/cdh1}$ has been shown to be a potent regulator of synaptic plasticity and synaptic development at the neuromuscular junction (van Rossel et al. 2004). More recently, we have shown Rap/Fzr/Cdh1 necessary for synaptic development and transmission. We report that loss-of- function mutants present pleiotrophic anomalies at the NMJ (Wise et al., in press). Morphological changes at the synapse were seen, as bouton number and individual bouton size was significantly decreased. Ultrastructurally, boutons appeared to have significantly decreased number of synaptic vesicles and docked vesicles. Boutons essentially looked empty. Our data suggests that $APC/C^{ap/fzr/cdh1}$ plays an important role in synaptic transmission, as ejp average potentials are significantly larger than wild-type and paired-pulse facilitation is significantly decreased. Normal peristaltic locomotion was significantly disrupted and *rap/fzr/cdh1* mutants moved more slowly

than wild-type, indicating lack of coordination. Lastly, we observed muscle degeneration within these mutants.

Kaplow et al., 2007 has shown that Rap/Fzr/Cdh1 physically interacts with several different proteins. Here we have presented data that support Loco, Nonstop and Twins are localized at the NMJ and loss-of-function mutants of show changes in synaptic bouton morphology. We have also presented data that show that Loco and Nonstop act as dominant suppressors of Rap/Fzr/Cdh1 at the neuromuscular junction.

Loco is targeted for ubiquitination by Rap/Fzr through its D-box and/or KEN box, two signature ubiquitination-targeting motifs recognized by the APC/C (Pfleger and Kirschner 2000; Burton and Solomon 2001; Hilioti et al. 2001; Kaplow et al., 2008). From the data we can assume that Rap/Fzr also works similarly at the NMJ. Loco functions as an RGS protein, inactivating the inhibitory α subunit of G-coupled protein receptor. This interaction may lead to changes in synaptic transmission that was previously observed (Wise et al., in press). $G\alpha_i$ regulates cAMP levels through inhibiting adenylate cyclase, ultimately regulating levels of cAMP.

nonstop encodes for a ubiquitin specific protease and is required for glial cell development (Poeck et al., 2001). Nonstop has also been shown to be necessary for axon guidance. However, its role in synaptic development and transmission remain unclear. Kaplow et al., has shown Rap/Fzr/Cdh1 and Not physically interact at in glia cells in the central nervous system. We have presented that Not expression is also localized at the NMJ and that its interaction with Rap/Fzr dominantly suppresses the *rap/fzr* mutant phenotype. It is likely that Not specifically targets Rap/Fzr at the synapse, aiding to specific temporal regulation of Rap/Fzr.

Taken together, Rap/Fzr can interact with proteins at the synapse, which can have major effects on synaptic transmission and development. The interaction of Not and Rap/fzr could have specific temporal control of Rap/Fzr by regulating when it is deubiquitinated. However, the role of Rap/Fzr in targeting Loco for deubiquitination has potential in regulating the cAMP pathway resulting in changes within the synapse that are long-term and structurally permanent.

Acknowledgements:

We thank the Confocal Microscopy center of the City College of New York (City University of New York) for preparation of TEM samples and Dr. Bill Chia for Loco antibodies.

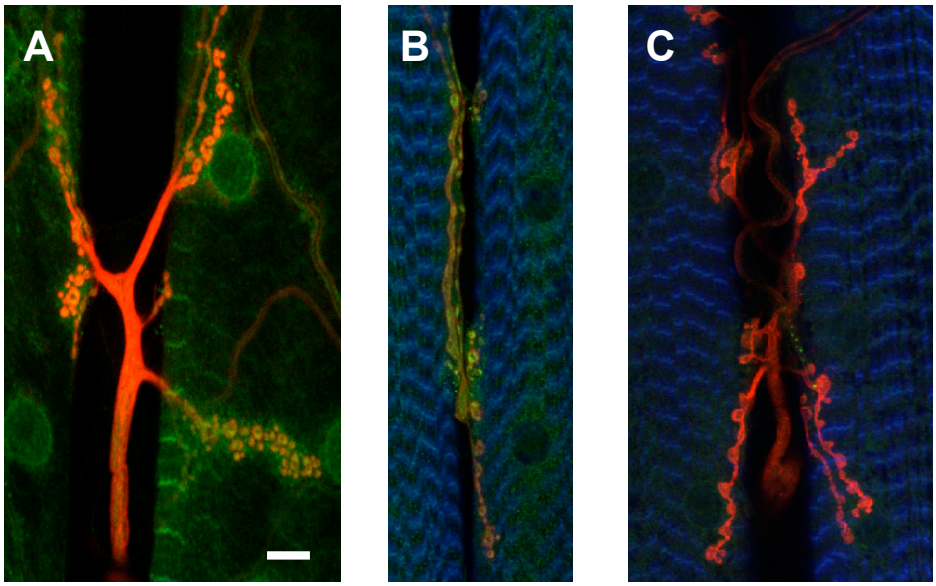

Figure 1. Immunolocalization of Loco to the NMJ in CS.
(A-B). Confocal image showing anti-loco (green), motorneuron is labeled with anti-Horseradish perioxidase (red) and muscle is labeled with anti-phalloidin (blue). Loco is present both pre- and post-synaptically. **(C)** Stained with anti-Loco in homozygous loss-of-function mutants, no staining or puncta are represented. This suggests that Loco does appear both pre- and postsynaptically.

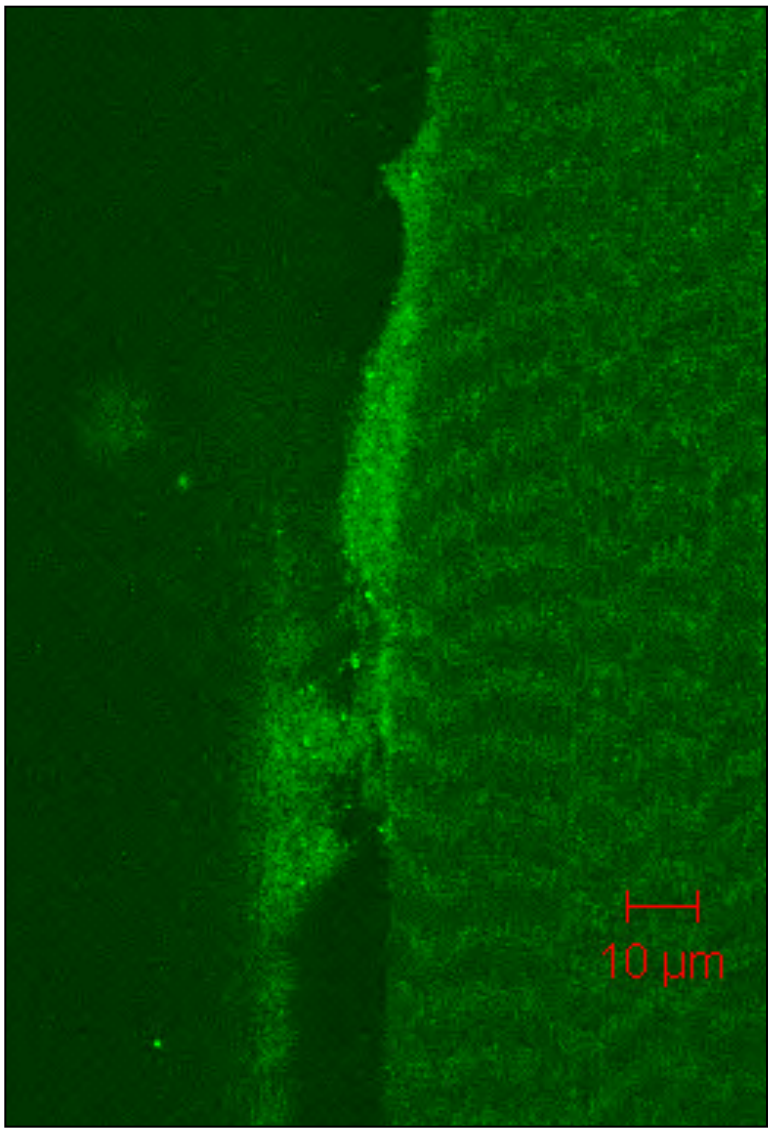

Figure 2. Immunolocalization of Nonstop at the NMJ. Not puncta appear to be localized to the presynaptic terminals using an antibody against Not. The staining also shows that Not might be located in the muscle as well.

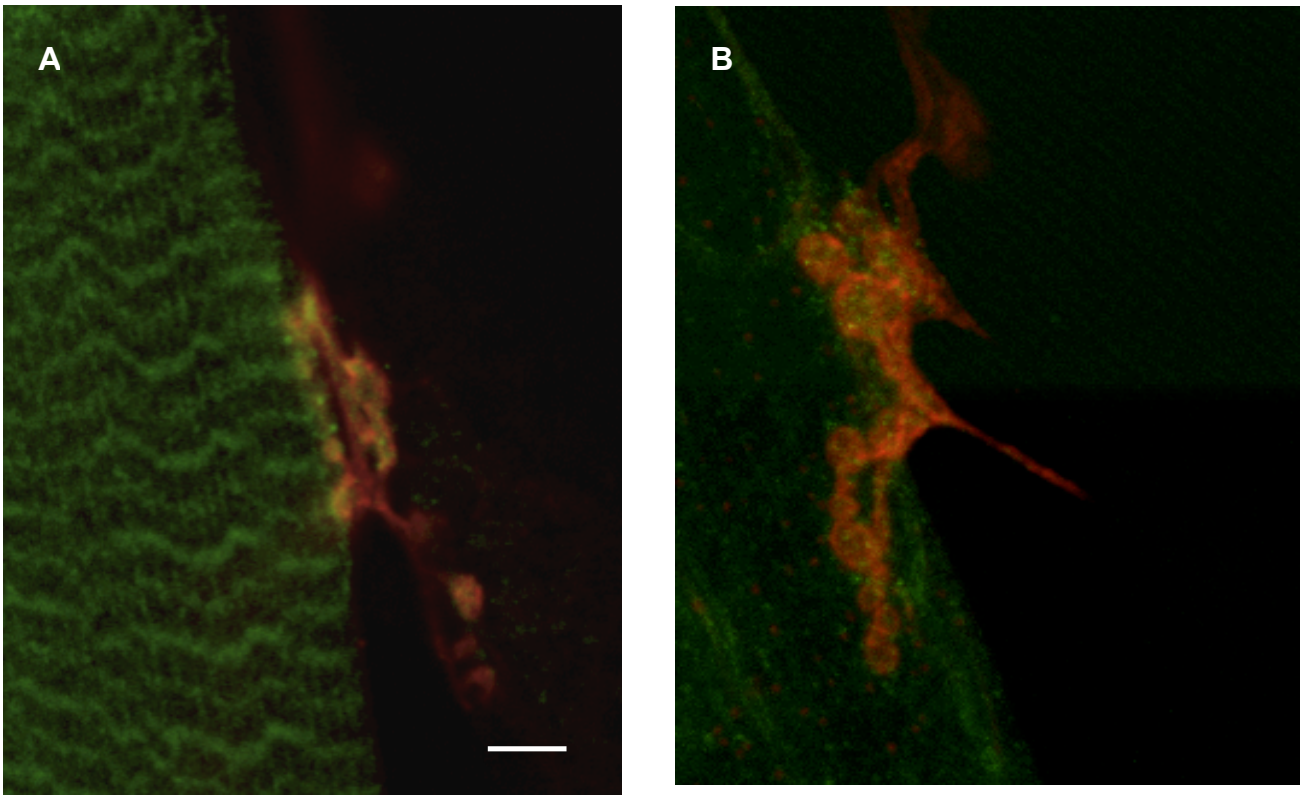

Figure 3. Localization of Twins pre- and postsynaptically. (A-B) These images reveal Twins (green) subunit localization to the pre- and postsynapse of the NMJ. Note the puncta arrangement around the postsynapse, which may indicate Twins involvement at the postsynaptic density. The motorneuron is labeled with anti-Horseradish perioxidase (red).

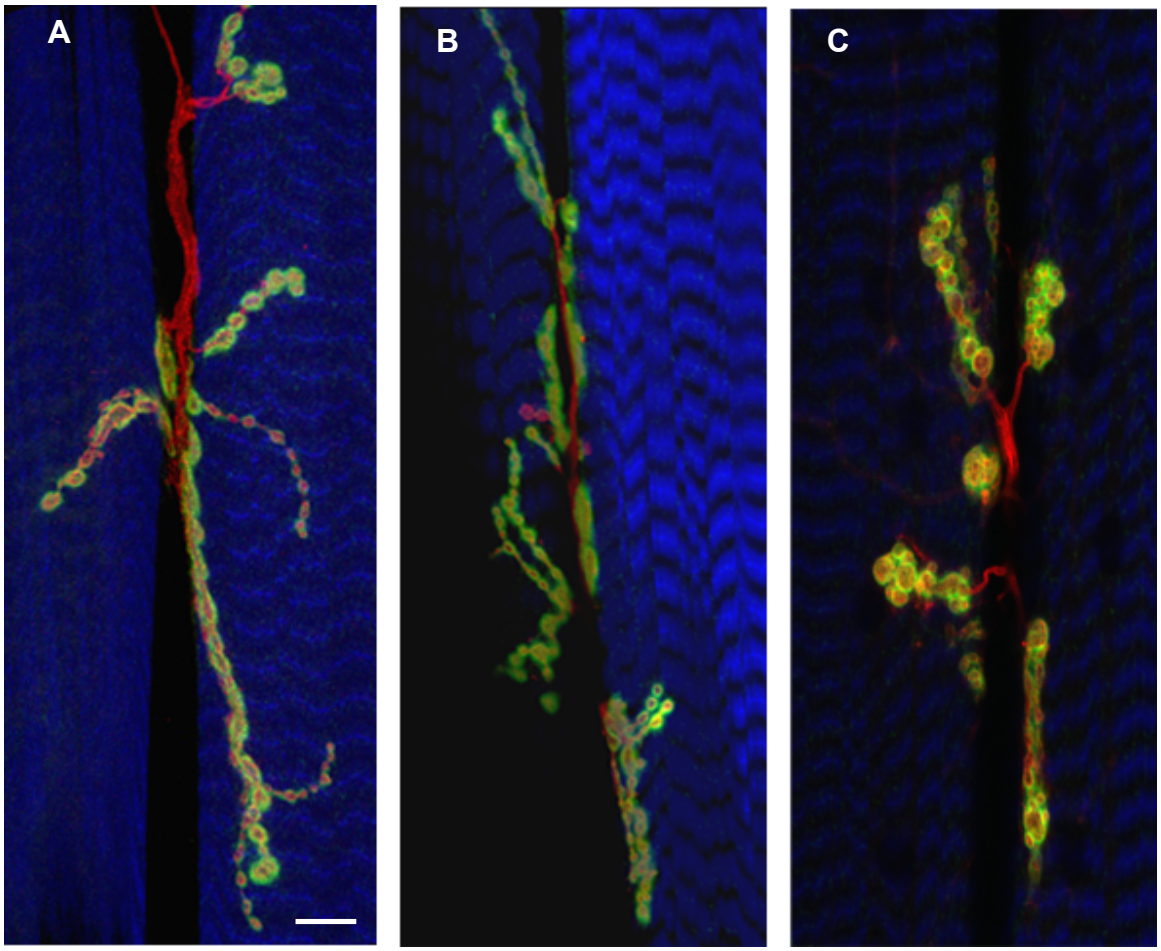

Figure 4. *loco* and *nonstop* mutants show altered synaptic structure and morphology. The motorneuron is labeled with anti-Horseradish perioxidase (red), postsynaptic density is labeled with Disc Large (Dlg) in green, and muscle is labeled with anti- phalliodin (blue). (**A**) Show CS normal synaptic NMJ bouton architecture. (**B**) loco^{P452} homozygous mutants. (**C**) nonstop homozygous mutant. (**B-C)** The changes associated with the synapse of loss-of function loco^{P452} and not showed decrease in bouton number, however, the size of the bouton vary. In the loco mutants, bouton size has decreased while nonstop show an enlargement in size.

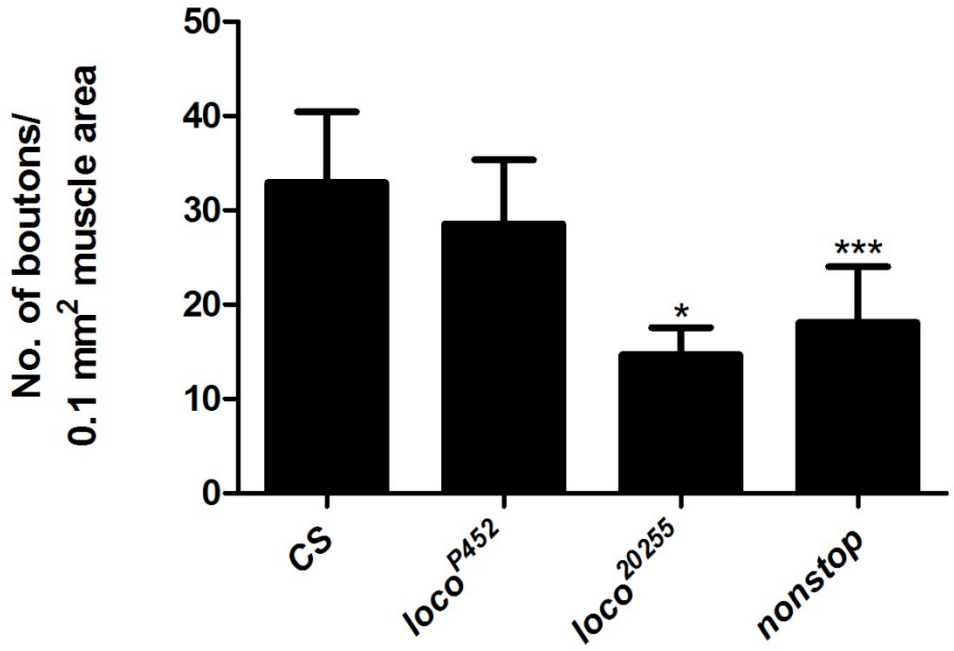

Figure 5. Bouton counts of *loco* and *not* mutants. Quantification of boutons from both *nonstop* and *loco* loss-of-function mutants. *locoP*^{452}, n=7, p<0.05; *loco*^{20255}, n=6, p<0.05; and *nonstop*, n=12, p<0.0005. Mutants showed a decrease in the number of bouton.

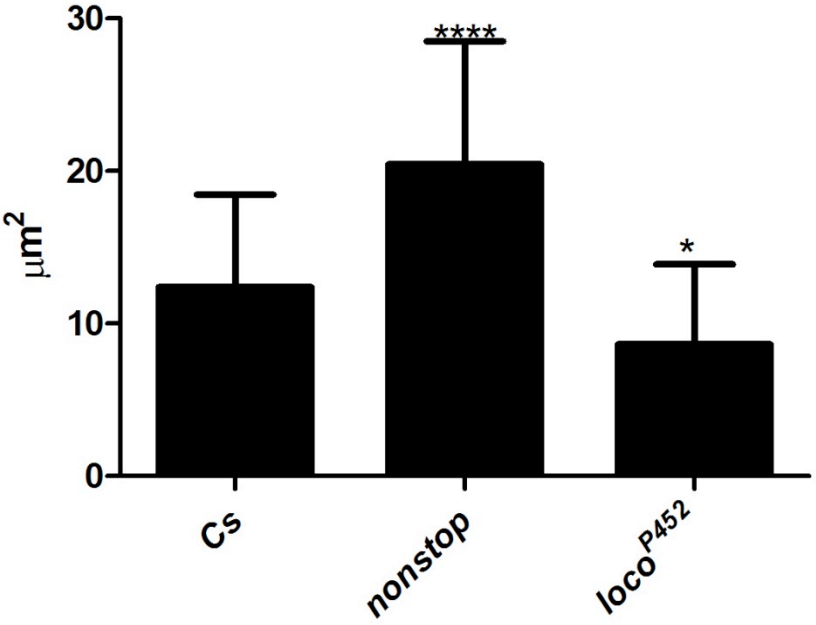

Figure 6. Bouton area is altered in *nonstop* and *loco* mutants. There is a significant change in the size of the boutons. In *nonstop*, the boutons are significantly increased ($p<0.0001$) and in *loco* the boutons are significantly decrease ($p<0.05$) in comparison to wild-type.

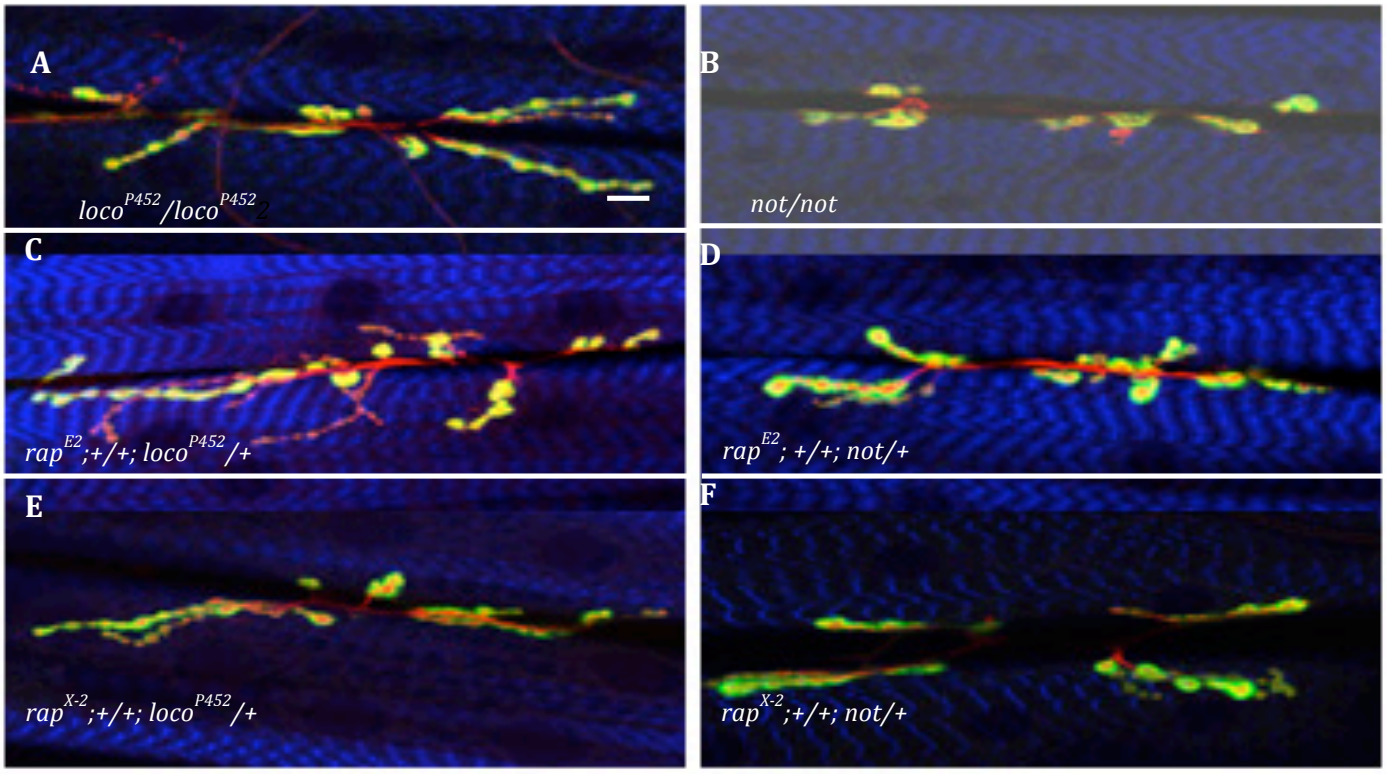

Figure 7. Double mutants of *loco* and *nonstop* with rap^{E2} and rap^{X-2}, respectively. (A) Show loss-of- function mutant $loco^{P452}$ (**B)** and *not*. (**C-F)** show rap^{E2} and rap^{X-2} mutants crossed with both $loco^{P452}$ and nonstop. Only male larvae were imaged. **(C and E)** In the rap^{E2} *and* rap^{X-2}, $loco^{P452}$ mutants there is an increase in type II boutons and observed increase in bouton size. (**D and F**) Similarly, there is a smaller increase in the number of bouton in heterozygous rap^{E2} *and* rap^{X-2}; *nonstop* mutants but a large increase in size. Postsynapse is labeled with Dlg (FITC), presynapse is labeled with TRITC-conjugated anti-horseradish perioxidase and the muscles are labeled with phalliodin-conjugated Cy5.

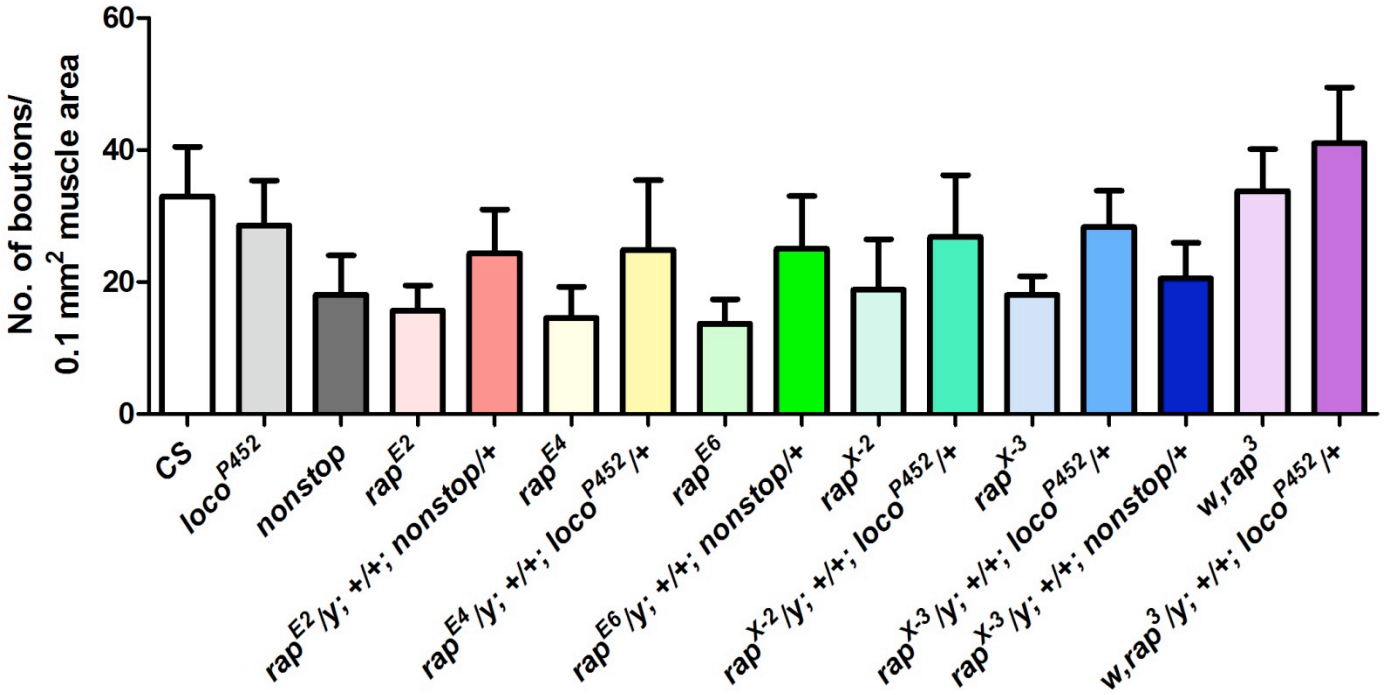

Figure 8. Double mutants counts of loco and nonstop with rap E2 and rap X-2, respectively. The following table is a composite of the wild-type, *loco, not, rap/fzr* and double mutants bouton counts. Compared to wild type, *loco, not, rap/fzr* mutants (rap^{E2}, rap^{E4}, rap^{E6}, rap^{X-2}, rap^{X-3} and w,rap^{3}) exhibit significant decrease in the number of boutons. However, one copy of loss of function *rap/fzr* in the background of loco or not mutant shows suppression of the *rap/fzr* phenotype, indicating that *rap/fzr* interacts with these proteins at the synapse. This also confirms previous data which showed Rap/Fzr upstream of Not and Loco.

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