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In vivo and in real-time mechanisms of synaptic bouton formation

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Dissertação orientada por:
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2017

Abstract

A functional nervous system relies on the communication between neurons via highly specialized structures, called synapses. During development, neuronal morphology and wiring establishment are genetically determined but neuronal structural and function can be altered by changing levels of activity, a process known as synaptic plasticity. Adult neuronal circuits remain plastic and this feature is what allows us to store information and adapt to the environment. Defects in synaptic connectivity and activity-dependent plasticity are characteristic of neurodevelopmental and neurodegenerative disorders. Notoriously, recent studies highlight a link between functional defects in the presynaptic elements of neurons, called synaptic boutons, and the origin of several of these diseases. Presynaptic boutons, round varicosities conserved from invertebrates to man, are highly dynamic structures where synapses are located and where neurotransmission occurs. Despite being one of the main synaptic compartments, very little is known about the mechanism and dynamics of their genesis. Several studies contributed to the understanding of this question, but detailed mechanistic information regarding bouton outgrowth is still lacking.

The purpose of this work is to acquire fundamental knowledge regarding how presynaptic boutons are formed and integrated into wired neurons. The powerful model synapse of *Drosophila* neuromuscular junction (NMJ) was adopted to dissect the details of bouton outgrowth *in vivo* and in real-time since it exhibits robust structural plasticity and the synapses formed on particular muscles have characteristic shapes and discernible boutons. The analysis was performed on the 3rd instar larvae because developmental bouton addition is nearly complete, and at this stage acute structural plasticity can be induced by patterned stimulation of motor neurons (MNs) using a variety of stimuli (high K^+ solution, electrical activity and optogenetics). Well-established protocols using spaced high K^+ paradigms were adopted to induce the rapid addition of synaptic boutons at this synapse.

Neuronal migration and growth are critical events for the correct development and wiring of the nervous system. To date, the mechanisms described to give rise to presynaptic boutons involve the formation of filopodia or lamellipodia structures. However, performing high temporal resolution time-lapse imaging of unanesthetized *Drosophila* larval NMJs revealed a new, unreported, mechanism of presynaptic bouton addition into mature neurons. It was found that addition of synaptic boutons in response to acute activity does not occur like in the embryonic stage, where a growth cone differentiates into round boutons. Instead, new boutons rapidly emerge in a manner strongly resembling a mechanism known-to-be used by some cells in migration and tissue invasion. Considering that the NMJ is deeply inserted into the muscle, for the MN to include new boutons it must further invade the muscle, which mechanistically is not very different from migration across other tissues. It is suggested that MNs have possibly adopted a strategy that combines this form of migration with activity-dependent signaling pathways to modulate the formation of synaptic boutons during intense muscular activity. Additionally, manipulating the pathways that regulate this mechanism exposed an intricate interplay between MNs and the muscle in the regulation of the number of activity-dependent boutons. Interestingly, the movies also showed a strong correlation between muscle contraction and bouton formation. This finding implied that the muscle mechanics probably has an active role on the MN, that can be either by setting up de formation of new boutons in primed sites or by increasing the dynamics of this process in situations of increased stress. It is proposed that a balance of mechanical forces and biochemical signaling are probably coordinated during structural plasticity upon intense muscle activity.

This research further expands our understanding of the mechanisms that control presynaptic growth and assembly in mature neurons. Further dissection of this phenomenon will contribute to uncover general principles that link normal development and function to dysfunction and disease, providing new insights into neuronal disease etiology and opening new avenues for the development of strategies to promote neuronal complexity and possibly delay symptoms associated with neuronal diseases.

Keywords: presynaptic bouton; activity-dependent; structural plasticity; *Drosophila*, neuromuscular junction.

Resumo

O funcionamento do sistema nervoso depende do estabelecimento da associação e comunicação entre neurónios, que ocorre através de estruturas altamente especializadas, designadas de sinapses. Durante o desenvolvimento, a morfologia dos neurónios e a estrutura das redes neuronais são geneticamente programados, mas a estrutura e a função dos neurónios pode ser remodelada em resposta a alterações nos níveis de atividade, um processo conhecido como plasticidade sináptica. No adulto, os circuitos neuronais retêm características plásticas e é esta maleabilidade do tecido nervoso que nos permite adquirir e armazenar informação e adaptar a alterações no ambiente. Defeitos na conectividade sináptica e na plasticidade dependente da atividade são característicos de doenças neurodegenerativas, como a Doença de Alzheimer e a Esclerose Lateral Amiotrófica, e, usualmente precedem a morte neuronal, que é a última causa dos sintomas molestos que acompanham este tipo de doenças. Adicionalmente, alterações na plasticidade estrutural também são um mecanismo comum a contribuir para os defeitos cognitivos e funcionais observados em doenças do desenvolvimento neuronal, como o Autismo e a Deficiência intelectual. Notoriamente, estudos recentes salientam uma ligação entre defeitos funcionais observados nos elementos pré-sinápticos dos neurónios e a origem de várias doenças destes tipos.

As sinapses consistem em junções intercelulares assimétricas nas quais é possível distinguir, morfológica e funcionalmente, dois compartimentos, um pré-sináptico e um pós-sináptico. Os terminais pré-sinápticos dos neurónios são constituídos por varicosidades redondas conhecidas como botões sinápticos, que são estruturas altamente conservadas desde os invertebrados até ao homem. Os botões sinápticos são o local onde as sinapses são organizadas e onde a transmissão nervosa ocorre. Estas estruturas são muito dinâmicas e estruturalmente plásticas, alterando o tamanho e o número em resposta a modificações na atividade. Apesar de constituírem um dos principais compartimentos sinápticos, pouco se sabe acerca dos mecanismos que regulam a sua formação. Durante o desenvolvimento, os axónios dos neurónios, que ainda não estabeleceram contactos sinápticos, têm estruturas altamente dinâmicas e móveis na sua extremidade distal designadas de cones de crescimento. Morfologicamente, os cones de crescimento têm uma forma cónica e formam dois tipos de protrusões celulares: filopódios e, entre estes, lamelipódios. Os cones de crescimento guiam os axónios até ao seu alvo-sináptico, que pode ser outro neurónio ou outro tipo de célula, e após chegada ao seu destino final os cones de crescimento diferenciam-se em botões redondos. Mas, durante o crescimento, os axónios pré-conectados devem continuar a alongar para acompanhar a mudança de tamanho do animal. Além disso, no adulto a formação de circuitos neuronais funcionais e a ocorrência de plasticidade sináptica são processos que dependem criticamente da montagem e desmontagem de sinapses. Assume-se que durante o crescimento dos organismos e durante a plasticidade sináptica a formação de novos botões segue os princípios do crescimento neuronal. Contudo, a visualização detalhada e informação sobre o mecanismo e a dinâmica do crescimento dos botões no adulto é quase inexistente.

Este trabalho tem como propósito a aquisição de conhecimento básico, mas fundamental, acerca do mecanismo de formação e integração de novos botões pré-sinápticos em neurónios maduros e pré-conectados. Um dos exemplos mais estudados de uma sinapse química, a forma predominante de comunicação entre os neurónios, é a sinapse formada entre um neurónio motor e o músculo, a qual é designada de junção neuromuscular. As junções neuromusculares são estruturas especializadas para proporcionar excitação transiente e à prova de falhas da célula muscular pós-sináptica assegurando contração muscular sempre que o neurónio motor estiver ativo para suportar a geração de movimento, uma qualidade importante dos animais. Adotou-se a junção neuromuscular da *Drosophila* para estudar o mecanismo de formação dos botões sinápticos dado que esta sinapse exibe plasticidade estrutural robusta e as sinapses formadas em músculos particulares apresentam formas bem características e botões claramente discerníveis. A análise foi efetuada no terceiro estadio larvar uma vez que neste estadio a adição de botões que acompanha o desenvolvimento da junção neuromuscular está quase completa e é possível induzir plasticidade estrutural de forma aguda.

A rápida adição de botões pré-sinápticos pode ser induzida em resposta a determinados padrões de atividade utilizando uma variedade de estímulos (solução com K^+ elevado, atividade elétrica e optogenética). Neste estudo, para induzir plasticidade estrutural na junção neuromuscular adotou-se protocolos baseados em paradigmas de despolarização espaçada usando uma solução com K^+ elevado. A observação dos novos botões (designados botões dependentes-da-atividade) foi efetuada em amostras vivas e em amostras fixadas de forma a extrair não só a dinâmica como também informação quantitativa a partir de larvas sujeitas a plasticidade estrutural.

A migração e o crescimento neuronal são eventos críticos para o desenvolvimento correto e estabelecimento dos circuitos neuronais. Até à data, os mecanismos descritos que participam na formação dos botões pré-sinápticos, envolvem a formação de filopódios ou lamelipódios. No entanto, a realização de microscopia confocal com lapso de tempo em junções neuromusculares de larvas de *Drosophila* submetidas a plasticidade estrutural revelou um novo mecanismo de adição de botões pré-sinápticos em neurónios maduros. Alterando dois parâmetros em relação a outros estudos, nomeadamente a taxa de aquisição em segundos e não bloquear a contração muscular, foi possível observar a formação de botões sinápticos *in vivo* e em tempo-real. Descobriu-se que a adição de botões pré-sinápticos em resposta a atividade aguda não ocorre como no período embrionário e não requer uma estrutura de crescimento especializada. Em vez disso, observou-se que os botões dependentes-da-atividade emergem de uma forma muito semelhante a um tipo de migração usado por algumas células na invasão de tecidos. Considerando que a junção neuromuscular não assenta apenas superficialmente no músculo, mas encaixa neste tecido, significa que os botões sinápticos estão embebidos no músculo. Assim, para que o neurónio motor forme botões funcionais é necessário que "invada" o músculo, o que não é muito diferente de mecanismos de migração através de outros tecidos. É possível que os neurónios motores tenham adotado uma estratégia em que combinam este mecanismo de migração com vias de sinalização dependentes-de-atividade para modular a adição de novos botões sinápticos durante a atividade muscular. Adicionalmente, observou-se a existência de uma elevada correlação entre a emergência de novos botões e a contração muscular, o que aponta para um possível papel mecânico do músculo durante este processo. Os vídeos analisados mostram pelo menos dois tipos de formação de botões: eventos rápidos associados com contração muscular intensa ("botão explosivo") e fenómenos lentos que acompanham movimentos mais subtis ("botão gradual"). Notavelmente, ao manipular a via que regula este tipo de migração, possivelmente usado também pelos neurónios motores, expôs uma interação complexa entre os neurónios motores e o músculo na regulação do número de botões dependentes-da-atividade. Considerando a proximidade entre os dois tipos de células bem como as propriedades contractivas do músculo é plausível propor que a ação mecânica do músculo possa ter um papel ativo no neurónio motor para a formação de novos botões a partir de locais determinados ou mesmo acelerar o processo em situações de maior tensão. Sugere-se que a atividade do músculo pode ter um papel importante na modelação da frequência e dinâmica dos botões que emergem dos neurónios motores em resposta à atividade.

Este estudo descreve um novo modo de crescimento neuronal, nomeadamente de formação de botões pré-sinápticos, que ainda não foi reportado em neurónios, mas que é bastante usado em migração em outros tipos celulares. De mencionar que as vias que levam à iniciação deste mecanismo são diversas sendo pouco provável que todas participem neste tipo de plasticidade, especialmente se o neurónio motor adaptar partes do mecanismo ou usar diferentes moléculas para atingir um resultado equivalente. Assim, o conhecimento dos genes ligados à formação de botões dependentes-da-atividade é necessário para compreender plenamente e de forma integrada o mecanismo de formação de botões num neurónio maduro. Por outro lado, este trabalho sugere que um balanço entre forças mecânicas e sinalização bioquímica são provavelmente coordenados durante a plasticidade estrutural em resposta a atividade muscular e expande a nossa compreensão dos mecanismos que regulam o crescimento e organização do terminal pré-sináptico. Uma análise mais aprofundada deste fenómeno, bem como das vias dependentes-da-atividade com papel no crescimento neuronal, irá contribuir para desvendar os princípios gerais que ligam o desenvolvimento e funcionamento normal à disfunção e à doença. Este conhecimento permitirá uma nova perceção e discernimento da etiologia da doença neuronal e o desenvolvimento de novas estratégias para promover a complexidade neuronal e possivelmente retardar os sintomas associados com doenças neuronais.

Palavras-chave: botão pré-sináptico: atividade: plasticidade estrutural; *Drosophila*, junção neuromuscular.

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Acknowledgments

First and fore mostly, I would like to thank Rita Teodoro for giving me the opportunity to study neuroscience and for encouraging me throughout my studies. This would not be possible without your enthusiasm for research, everlasting support, and positive attitude, which motivated me to continue. I would like to thank Lara Carvalho who was always willing to help with the live imaging and for her kindness. I would like to thank my family and friends that have shown excitement for my research and for their endless support. Lastly, I would speciallly like to thank Daniel for being my strength and pushing me to achieve my goals. I would not have done this without you.

List of Abbreviations

NMJ……..…..... Neuromuscular junction
MN……Motor neuron
SV……...…. Synaptic vesicle
NT……Neurotransmitter
AZ……Active zone
Dlg……...Discs Large
HRP……...Horseradish peroxidase
CNS……...Central nervous system
SSR……...…… Subsynaptic reticulum
GluR……...…. Glutamate receptor
Wg……..……..... Wingless
PKA……..…Protein kinase A
CaMKII……. Ca^{2+}/Calmodulin-dependent Kinase II
BMP…… Bone morphogenetic protein
Wit……...Whisful thinking
LimK……...Lim domain Kinase
Gbb……Glass bottom boat
Syt1……. Synaptotagmin 1
Syt4……. Synaptotagmin 4
Syx4……...Syntaxin 4
Nlg1……Neuroligin 1
CAM…… Cell adhesion molecule
ECM……...Extracellular matrix
Shv……...Shirveled
NMII……...Non-muscle myosin II
F-actin……..…Filamentous actin
Wild-type……. WT
Knockdown ……....……K/D
MHC……...Myosin heavy chain
RLC……...…. Regulatory light chain
ELC……..... Essential light chain
MLCK……...Myosin light chain kinase
ROCK……...Rho kinase
PP1…….....…Protein phosphatase 1
IR …….....…. RNAi
Sqh……..... gene encoding NMII RLC in *Drosophila*
Zip……gene encoding NMII MHC in *Drosophila*
SqhCA……...…. Constitutively active NMII
SqhDN……. Dominante negative NMII
Rok…….....gene encoding ROCK in *Drosophila*
Strn-Mlck……..... gene encoding MLCK in *Drosophila*
Tsr ……gene encoding cofilin in *Drosophila*
Cher…….....gene encoding filamin in *Drosophila*
ADF……. Actin depolymerizing factor
NASPM……. Reversible GluR antagonist

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I. INTRODUCTION

1 How are presynaptic boutons inserted into a wired neuron?

1.1 Introduction

The correct wiring of the nervous system is required for functions such as learning and memory, locomotion, or perception. During development, neuronal morphology and wiring establishment are genetically determined, but neuronal structure and function can be altered by changing levels of activity, a process known as synaptic plasticity. In the adult, neuronal circuits remain plastic and this feature is what allows us to acquire and store information and adapt to the environment[1–4]. Defects in synaptic connectivity and activity-dependent plasticity are a hallmark of neurodegenerative disorders[5], such as Alzheimer's disease or Amyotrophic lateral sclerosis. Moreover, alterations in structural plasticity have also been proposed as a common mechanism contributing for cognitive and functional defects observed in developmental neuropathologies, such as Intellectual Disability or Autism Spectrum Disorders[6].

Neurons connect and communicate with other cells via highly conserved specialized structures, called synapses. A synapse formed between a motor neuron (MN) and the muscle is called neuromuscular junction (NMJ) and is responsible for muscle contraction. The presynaptic terminals of neurons, called synaptic boutons, are a nearly universal feature of synapses from invertebrates to man. Boutons are round varicosities and it is inside boutons where synapses assemble and where neurotransmission occurs. Like its postsynaptic counterparts, boutons are equally plastic and increase in size and number upon increased levels of activity. Despite being one of the main synaptic compartments, very little is known about the mechanisms that regulate their formation. During embryonic development, axons, that are not yet synaptically connected, have highly dynamic and motile structures, called growth cones, at their leading edges. Morphologically, growth cones have a conical shape and two types of protrusions: thin fingerlike filopodia and, between them, flat lamellipodia. Growth cones guide the axon's migration to their synaptic target, which can be another neuron or a muscle cell in the case of NMJs, and upon arrival to their destination growth cones differentiate into round boutons. However, as the animal grows, networked axons must continue to increase in length and, in the adult, synapse assembly and disassembly is crucial for the maintenance of functional neuronal circuits. It is assumed that during organismal development and during synaptic plasticity new bouton formation follows the principles of neurite outgrowth and initial synapse formation. Though, the mechanism by which new boutons are added to a mature neuron or the cytoskeletal remodeling required for these morphological changes remain unclear. The dissection of this question will allow us to have an increased understanding on how neurons grow and remodel, and this has recently become even more significant because a link has been made between the functional defects in presynaptic elements of neurons and the origin of several neuronal diseases[7,8].

This master thesis is focused on a long-standing question in basic neurobiology, which concerns how presynaptic boutons are formed and integrated into wired neurons. The main objectives of this dissertation were: 1) to uncover the mechanism/s of synaptic bouton addition *in vivo* and in real-time; and 2) to dissect the pathway/s or molecular players regulating bouton formation. This research is necessary to understand how structural changes affect synaptic function and may inform the discovery of the mechanism by which synaptic boutons form in the adult.

1.2 The *Drosophila* larval NMJ as model to study presynaptic assembly

Many forms of synaptic plasticity are required for neuronal circuits to adapt their function to environmental challenges. Among these, the capacity of neurons to alter their morphology in response to activity, known as structural plasticity, is one of the most durable forms of plasticity. Structural plasticity can involve the addition, remodeling or removal of pre- and postsynaptic elements and has been shown to be a fundamental process during development, for both formation and refinement of synaptic connectivity, and, in the adult, to modulate the organization and functional properties of neuronal circuits[9].

NMJs are specialized structures that function to provide reliable yet transient excitation of muscle cells to support movement. At this synapse signal transmission is mediated by small molecules called neurotransmitters (NTs), which are packed in synaptic vesicles (SVs) at the presynaptic terminal of MNs. Upon synaptic activation, in response to a calcium influx, SVs rapidly fuse with the plasma membrane and release their content into the synaptic cleft. Once released NTs diffuse across the cleft and bind to specific postsynaptic receptors leading to muscle activation[10,11]. To assure high temporal and spatial control, SV release occurs at highly specialized areas of the presynaptic membrane called active zones (AZs), that are closely and precisely aligned with the postsynaptic receptors. At most NMJs each MN activates target muscle cells and does so in response to changing patterns of nerve activity, which mirror fluctuations in the organism functional demands. Since neuromuscular transmission is highly modifiable, the architecture of this synapse must also be very dynamic. Using vertebrate NMJ models much has been learned about the postsynaptic morphogenesis[12], however considerably less is known about the mechanisms underlying presynaptic structural plasticity.

The *Drosophila* larval NMJ (Fig 1.1) provides an excellent model for such studies because, in addition to the vast genetic toolkit available, it exhibits robust structural plasticity and the synapses formed on particular muscles have very characteristic shapes and clearly discernible boutons[13,14]. Moreover, fluorescent imaging of living dissections of intact larvae is possible, providing an increasingly dynamic view of presynaptic morphogenesis. Unlike the mammalian NMJ, *Drosophila* NMJ is a glutamatergic synapse, rather than cholinergic synapse. Despite this, the cytoskeleton regulation and the principles that guide synaptogenesis and morphology are similar[15]. Therefore, since boutons are conserved in humans, this model synapse was adopted to dissect the details of synaptic bouton outgrowth. These boutons form during embryogenesis and increase in size and number during larval life to accommodate the growth of the animal[16]. NMJ patterning is genetically determined but, after initial synapse formation, synaptic activity also influences and refines the final arbor morphology. In addition to developmental synapse formation during the larval stages, the NMJ also displays acute structural plasticity in response to elevated levels of neuronal activity[17]. The method routinely used by several laboratories to induce rapid formation of new synaptic boutons at *Drosophila* larval NMJ is based on spaced or patterned depolarization of MNs using a variety of stimuli (high K^+ solution, electrical activity and optogenetics)[17,18]. When new varicosities (called ghost boutons) from, they lack postsynaptic transmission machinery, despite possessing highly differentiated presynaptic compartments including SVs and occasionally AZs[18]. Another difference is that ghost boutons are highly spherical as opposed to the more oblong mature boutons[19].

In this study, the 3rd instar larval NMJ was chosen as a model system to investigate the mechanisms underlying presynaptic bouton formation in wired neurons because at this stage developmental bouton addition is nearly complete and acute structural plasticity can be induced. All experiments were conducted on the NMJ formed on muscles 6/7, the most characterized synapse in *Drosophila*, because it is relatively simple and accessible for study. Well-established protocols using spaced high K^+ depolarization paradigms were adopted to induce rapid addition of synaptic boutons at

this synapse[17,18]. Post-stimulation, new activity-dependent boutons (or ghost boutons) were identified in two ways: by direct observation of live events in animals expressing fluorescent membrane markers in MNs; or they were inferred from fixed larvae post stimulation by staining with Horseradish Peroxidase (HRP) (neuronal membrane marker) and Discs Large (Dlg) (postsynaptic marker) antibodies to identify ghost boutons that were not surrounded by a postsynaptic specialization. The analysis of activity-dependent bouton formation in both live-samples and fixed-samples was a powerful approach to extract both the dynamics and quantitative information from larvae subjected to activity-dependent structural plasticity.

The *Drosophila* larval NMJ

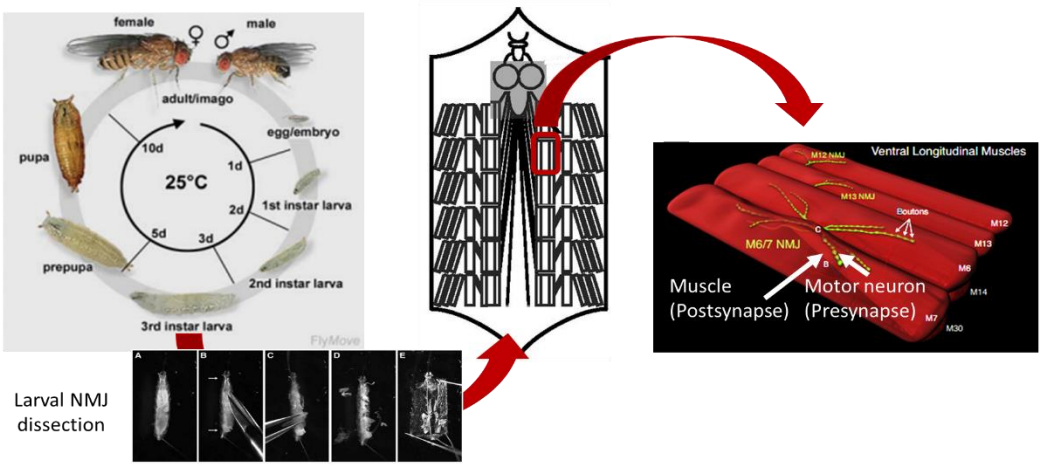


Figure 1.1 – Model synapse: *Drosophila* Neuromuscular junction (NMJ). Schematic of Drosophila life cycle, with emphasis on the 3rd instar larvae, which is the stage used throughout this thesis. On the right side is represented the larval motor system and the most studied NMJ, which innervates muscles 6/7.

1.3 Synaptic bouton formation and structural plasticity at the *Drosophila* NMJ

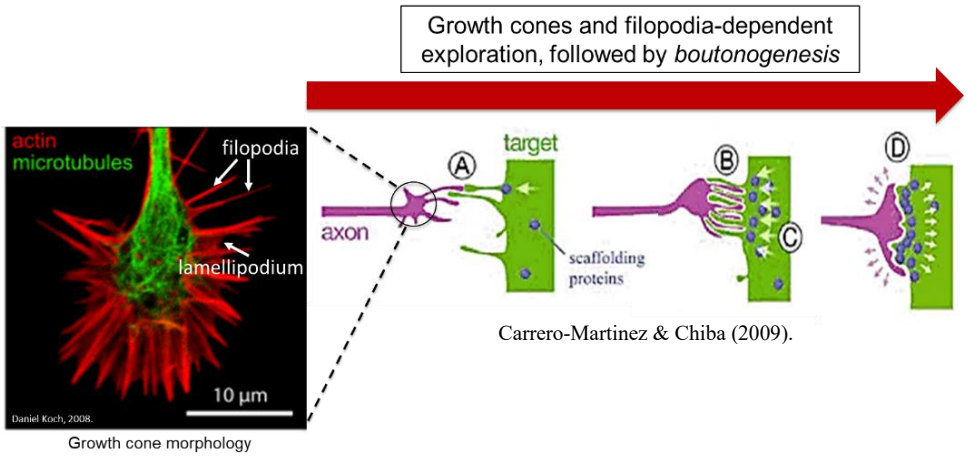
The developmental events intrinsic to the formation of the *Drosophila* NMJ have been well characterized, where embryogenesis lasts 20-22h at 25ºC, from the time the egg is laid until a 1st instar larvae emerges from the egg case. During the first 10h of embryonic development, neuroblasts segregate to form the neuroectoderm, divide and differentiate both glia and neurons. During the next 3h, the postmitotic MNs axons leave the central nervous system (CNS) and extend, via fasciculations along the body wall, to the developing musculature, to make synaptic connections with specific target muscle(s). Embryonic axonal navigation is accomplished by a motile structure at the distal tip of the elongating axon, called the growth cone (Fig 1.2A). Growth cones search for and detect molecular cues (attractants and repellents) that are displayed throughout the developing muscle field, and respond to these signs by advancing, pausing, and turning, until reaching their proper destination. Upon arrival to their synaptic muscle-target, actin-base motile processes in the growth cone (filopodia) and in the muscle (myopodia) closely interact to facilitate target recognition and site selection (perhaps by presenting specific recognition molecules)[20]. In *Drosophila*, as in vertebrates, mature muscles are formed from the fusion of myoblasts to form a single mature multinucleated fiber. At the time of initial growth cone contact, fusion is complete, but at this stage, muscles, which are interconnected to neighbors via cytoplasmic bridges, are still developing membrane electrical properties towards maturity. Once a target is identified as "appropriate" the growth cone differentiates into round boutons over the course of several hours. Because myopodia are transient structures only observed at the onset of embryonic synaptogenesis this model is thought to account for the generation of the NMJ network pattern, which remains largely intact through the larval stages.

Following initial NMJ formation, a second phase of synaptic development occurs. Due to the rapid growth during the larval stages, the NMJ must continue to expand, adding new boutons and synaptic branches, as part of its normal developmental program (Fig 1.2B), but the mechanisms underlying these modifications are not well understood. Prior studies attempted to identify patterns of developmental synaptic growth at the *Drosophila* NMJ by performing live-imaging through the cuticle of intact larvae genetically expressing a fluorescent postsynaptic marker[16]. As synapses grow, from 1st to 3rd instar, new boutons often arose by division of existing boutons (asymmetric budding or symmetric division), but in some cases boutons emerged *"de novo"* from the axonal membrane. In either case, growth cone-like specializations weren't observed prior to bouton emergence. However this study had intrinsic limitations that could have prevented the visualization of such structures: the images were acquired at 24h intervals and only the outline of boutons was observable, and this outline was a postsynaptic label based on Dlg localization[16]. In fact, initial bouton formation cannot be inferred from this classical study, because the marker used only allowed visualization of boutons after they had matured and were surrounded by Dlg.

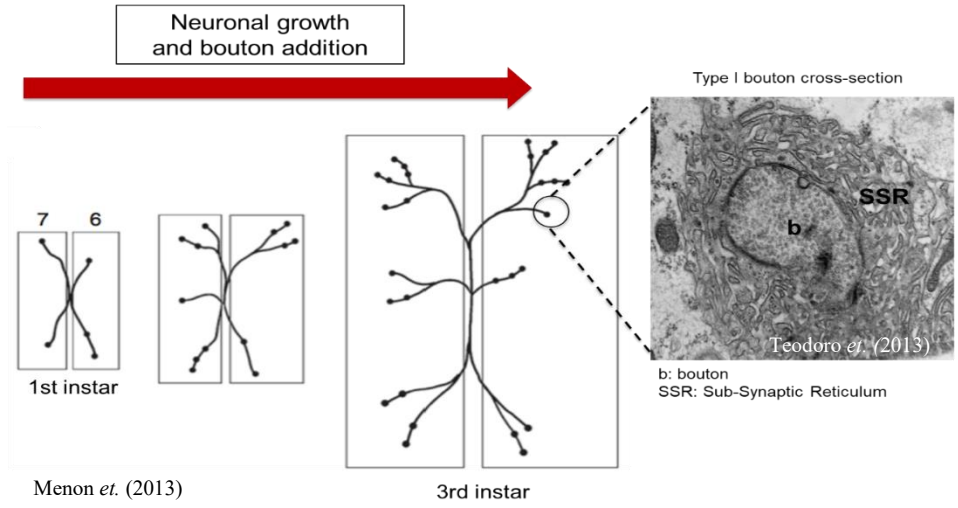
One class of mutations that affect synaptic growth are those that change MN's excitability[21], indicating that synaptic growth is regulated by neuronal activity (Fig 1.2C). It is thought that during developmental growth, NMJs adapt to patterns of neuronal activity that refine their final morphology. In the 3rd instar stage, after a period of morphogenetic growth, mature motor terminals branch across their target muscles and form many presynaptic boutons. The largest class of boutons (called type I) are glutamatergic and are surrounded by a complex system of membrane infoldings from the muscle called subsynaptic reticulum (SSR)[9] (Fig 1.2B). Each bouton contains spatially segregated active AZs that reside opposite a distinct cluster of glutamate receptors (GluRs) on the postsynaptic muscle surface. Although, some subsets of muscles are additionally innervated by type II or type III boutons, which contain octopamine or peptide neurotransmitters (and glutamate), muscles 6 and 7, which have been a particular focus of NMJ research, are innervated exclusively by type I boutons.

Prior studies used live-imaging at intact *Drosophila* larval NMJs expressing a membrane tethered GFP in MNs and demonstrated that the outgrowth of synaptic boutons can occur rapidly in response to patterned depolarization using a high K^+ solution[17,18]. New boutons (ghost boutons) normally form at the SSR perimeter and fill with SVs within minutes of high K^+ stimulation. However bouton maturation requires subsequent assembly of presynaptic AZs and postsynaptic specializations (containing GluRs clusters) before boutons can become functional, stable and reach full size[9]. On the other hand, boutons that fail to stabilize can be retracted or degraded by muscle and glia-mediated mechanisms[22]. Interestingly, acute spaced stimulation of MNs also induced the formation of dynamic filopodia-like processes (called synaptopods), that emerged from the stretch of neurite between boutons and from boutons themselves. Although the nature of synaptopods is still unresolved, it was suggested that they might act as precursors of boutons, like the filopodia observed at dendritic spines. However, since no transition from filopodia to bouton was ever seen, it was suggested that synaptopods most likely represent exploratory structures, a function also suggested for dendritic filopodia and for growth cone, prior to target innervation[17]. Given that time-lapse images were taken at the beginning and then at specific time-points during and after stimulation, the study by Ataman *et.* (2008) did not allow the visualization of bouton outgrowth in real-time. Nevertheless, this work was important because it showed that activity-dependent modifications in synapse structure at the larval NMJs are not simply the result of chronic changes in activity levels throughout development. Rather, rapid changes leading to the formation of new undifferentiated synaptic structures can be elicited by acute stimulation[17,22].

A) NMJ formation during embryogenesis



B) Larval development is accompained by NMJ expansion



C) Developmental NMJ growth adapts to patterns of neuronal activity

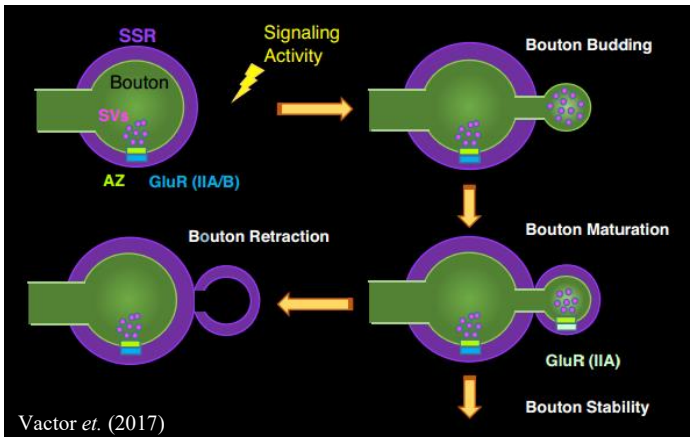


Figure 1.2 – *Drosophila* NMJ formation and development. A) Representation of NMJ formation during embryogenesis to show a phase of synaptogenesis followed by bouton differentiation: notice that this developmental phase is mediated by a specialized portion of the axon called growth cone. On the left, there is a confocal microscopy picture (Daniel Koch, 2008) of a growth cone to highlight its morphology. Growth cones have a conic shape and two types of motile protrusions: filopodia and, between them, lamellipodia. B) Schematic of NMJ development to show arbor growth and bouton addition during the three larval stages. On the right, an electron microscopy image of a synaptic bouton (Teodoro *et al.*, 2013)) to show the elaboration of the postsynaptic specialization called SSR. C) Developmental signaling or synaptic activity can induce the formation of new boutons that can persiste and mature or be eliminated by retraction.

1.4 Activity-dependent pathways involved in bouton formation at *Drosophila* NMJ

The capacity of neurons to rapidly modify their synaptic structure in response to activity is crucial for neuronal development and complex nervous system's functions. However, the molecular mechanisms that translate patterns of activity into specific structural, and functional, changes of synapses are largely unknown. Although activity-dependent control of *Drosophila* NMJ morphogenesis has been known for a long time, the finding of a simple acute stimulation paradigm made possible to study the molecular players required to initiate bouton formation in response to activity. Additionally, the *Drosophila* powerful genetics and well-conserved signaling pathways makes this system well-suited to study the mechanisms behind structural plasticity.

Drosophila NMJs undergo rapid changes in synaptic structure and function in response to patterned stimulation. These changes, which include the formation of motile presynaptic filopodia, formation of undifferentiated varicosities (ghost boutons) and potentiation of spontaneous release frequency, are dependent on transcription and translation. Several pathways have been shown to be required for this process, including MN derived Wnt family member Wingless (Wg) to act on both sides of the synapse to promote synaptic growth and maturation, with a major effector output being changes in the microtubule cytoskeleton[17]. Additionally, glia-derived Wg seems to play a selective role for bouton maturation[23]. Even though Wg signaling was shown to be involved in activity-dependent plasticity at the *Drosophila* NMJ, it is still not well understood how this signal mediates the cellular changes that accompany this process. Interestingly, it has recently been shown that the actin regulator, Cortactin, which acts as a major organizer of protrusion, membrane mobility and invasiveness, is present pre- and postsynaptically at the *Drosophila* NMJ and acts as a regulator of activity-dependent synaptic plasticity controlled by Wg[24]. Previous studies have also revealed that structural growth at the *Drosophila* NMJ is tighly coupled to synaptic transmission. Protein kinase A (PKA) has been shown to induce rapid bouton formation by promoting Synapsin mediated transport of SVs into nascent boutons, before the assembly of AZs[18]. In parallel, PKA also phosphorylates SV fusion clamp Complexin to selectively and transiently enhance spontaneous NT release[25]. In addition to PKA, Calmodulin-dependent kinase II (CaMKII), which has also been reported to participate in regulation of synaptic transmission, functions in an activity-dependent manner to modulate rapid bouton formation[26]. More recently, Bone morphogenetic protein (BMP) retrograde signaling events have been found to play an essential role in activity-dependent bouton formation at the *Drosophila* NMJ, acting largely through the actin cytoskeleton. BMP signaling through its receptor Wishfull thinking (Wit), has opposing signaling roles with respect to bouton formation, providing a permissive role via developmental Smad and Trio (canonical pathway), and an inhibitory role via Lim domain kinase (LIMK) (noncanonical pathway). It is suggested that in the background of moderated or low synaptic activity LIMK prevents bouton formation, but when activity is elevated, additional release of BMP family ligand Glass Bottom boat (Gbb) from muscles promotes new synaptic growth, by either reducing or outcompeting LIMK activity, with concurrent activation of cofilin, an actin depolymerizing protein[19]. While Gbb is also released via dense core vesicles by nerve terminals, the Gbb co-factor Crimpy has been shown to keep presynaptic information distinct from the postsynaptic signal[27]. Calcium influx into synaptic compartments during activity is a key mediator of neuronal plasticity. The role of presynaptic calcium in triggering SV fusion through the Ca^{2+} sensor Synaptotagmin 1 (Syt1) is well established. To tightly couple presynaptic remodeling with synaptic transmission, retrograde signals also need to be regulated by activity-dependent events. It has been proposed that Synaptotagmin 4 (Syt4) may act as a postsynaptic sensor to trigger Ca^{2+}-dependent release of retrograde signals. While the precise relationship of Syt4-triggered retrograde vesicular release and Gbb delivery is not yet known, it is though that Syt4 may participate in a separate pathway to regulate ghost bouton formation[19] by releasing an unidentified retrograde signal[28]. Interestingly, Syt4 has been shown to collaborate with Syntaxin 4(Syx4) to achieve calcium-dependent

release of the synaptic adhesion molecule neuroligin 1 (Nlg1) and, like Syt4, Syx4-dependent transport of Nlg1 is necessary for ghost bouton formation[28]. These studies showed that several synaptic molecules play a role in activity-induced synaptic plasticity, probably by coordinating efficient synaptic transmission between pre- and postsynaptic compartments. Likewise, at the synapse, the extracellular matrix (ECM) environment and cell adhesion molecules (CAMs) are thought to coordinate communication across the synaptic cleft to regulate synapse growth[29]. Integrins are one of the major CAM families and integrin receptors are important for coordinating activity-dependent synaptic modifications[30]. At the *Drosophila* NMJ, in response to activity, muscles downregulate laminin to reduce integrin activation and allow synaptic growth and expansion[31]. Interestingly, laminin levels at NMJs, which are inversely correlated with NMJ size, are regulated by larval crawling activity, synapse excitability, postsynaptic response and anterograde Wg signaling[31]. More recently, it was also found that an activator of integrin signalling, Shriveled (Shv) is released by MNs in an activity-dependent manner and regulates proper synaptic growth at the *Drosophila* NMJ. Shv is acutely induced during intense neuronal activity, but not during mild activity, and through integrin binding promotes bouton enlargement and an increase in glutamate receptor abundance[32].

Drosophila NMJ has been widely used as model to access the mechanisms required to initiate bouton formation in response to activity. Several pathways and molecular players have been discovered to participate in the regulation of activity-dependent bouton formation revealing that structural plasticity at this NMJ is most likely a complex sequence of factors and communication between MNs and its cellular partners. Despite this, the mechanisms that regulate presynaptic morphology are barely beginning to be understood and detailed visualization of bouton outgrowth and cytoskeletal dynamics during bouton formation is still lacking.

1.5 Migration mechanisms and structural plasticity

Cell migration is a fundamental process, being used from simple unicellular organisms, such as amoeba, for mating and search of food, to the more complex and specialized multicellular organisms, such as mammals, for tissue organization, organogenesis and homeostasis[33]. During multicellular organism development, morphogenesis can result in cell locomotion, from large-scale migration of epithelial sheets during gastrulation to the movement of individual cells of the nervous system. In a adult organism, cell migration is also essential for several functions, such proper immune response, wound repair, and tissue homeostasis, while aberrant cell migration is seen in pathological situations, such as cancer metastasis[34]. Most of these motility events share common cellular features: when observed in the process of movement, cells exhibit dynamic extension and retraction of two kinds of plasma membrane protrusions, lamellipodia (veil-like protrusions) and filopodia (finger-like protrusions).

Both types of protrusions are observed at the leading edge in migrating cells and in axonal growth cones[34] (Fig 1.3B), a semi-autonomous portion of the developing neuron that is highly specialized for motile activity[35] and that can be used as a model for the study of directional cell movements[36]. The formation of lamellipodia and filopodia results from a complex interaction of multiple cellular components and involves the coordinated activity of the plasma membrane and the actin cytoskeleton[34]. In the lamellipodium, actin filaments undergo constant polymerization and push against the plasma membrane. To achieve this, during polymerization, actin filaments are oriented with the growing ends (the plus end) to the periphery of the migrating cell's leading edges and of growth cones. Simultaneously, actin filaments are constantly pulled away from the periphery via a mechanism that involves myosin motor proteins, called retrograde flow[37]. However, peripheral actin filaments attach to their substrate through adhesion complexes and then the membrane and cytoplasm move toward the periphery over the anchored actin filaments, resulting in forward translocation. Whereas lamellipodia are composed of a meshwork of actin filaments oriented to the periphery, filopodia are formed by

bundles of long actin filaments that protrude away from the periphery, often originating in the lamellipodium. Thereby, a key condition for filopodial formation is that actin-filaments continue to grow at their plus ends. The protrusion of filopodia greatly enhances the extracellular area that a cell can sample in order to detect relevant signals during its movement. Filopodia mediate the ability of migrating cells and growth cones to navigate their environments and sense cues to guide their direction of migration and destination. Lamellipodia can also serve as sensors of extracellular signals but they usually contribute by generating the force required for movement, which is provided by a combination of plus-end actin polymerization, retrograde flow and cell adhesion. Both lamellipodia and filopodia exhibit similar types of behavior, characterized by cycles of extension and retraction.

Growth cone mediated axon guidance and synaptogenesis are critical processes for the formation of complex neuronal networks through the body, allowing neurons to connect into precise arrangements. However, the formation and maintenance of this neuronal organization and interconnectivity depends on additional communication between neurons, and associated glia, and can be further remodeled through adulthood. Synaptic contacts are dynamically formed, lost, and modified in size, and strength, in response to neuronal activity and this synaptic plasticity is what determines many functional aspects of neuronal circuitries. During synaptic plasticity, the protrusion of lamellipodia and filopodia from the surface of neurons is supposed to be fundamental for the formation of axon branches and assembly of synaptic structures. Dendritic spines, the postsynaptic compartment at most excitatory synapses in the CNS, can dynamically form, change their shape and disappear in response to activity indicating that spine morphology and density are important for structuring synaptic interactions and plasticity. There is clear evidence for a role of dendritic filopodia in synaptogenesis and in dendritic spine formation and these processes have been shown to be important for learning and memory[38]. Interestingly, because of their unique morphology and motility, it has also been suggested that filopodia, in addition to spinogenesis, can participate in environmental sampling of potential axonal partners, possibly lowering the threshold and reducing the time to form new dendritic spines and synapses, providing a substrate for fast learning[39]. Like dendritic spines, presynaptic boutons are dynamic and appear to be continuously added and eliminated throughout the lifetime of a neuron[40,41], and also seem to have an active role in activity-dependent synaptogenesis[40,42,43]. In spite of the emerging evidence that activity-dependent synapse formation can be initiated either through post or presynaptic structural modifications, the mechanisms that regulate the morphological alterations underlying the genesis of synaptic boutons in mature neurons are far from being understood.

1.6 Bleb-driven migration and neurons

Neuronal migration and growth are critical events for the correct development and wiring of the nervous system. To date, the mechanisms described to give rise to presynaptic boutons involve the formation of filopodia or lamellipodia structures. Once they form, most synapses are not static but rather continue to expand and retract throughout life. Understanding how experience and activity can influence structural changes that alter connectivity between a neuron and its target in the adult is key to understanding how the nervous system works and may help to better understand the origin of neurodegenerative diseases. Using the *Drosophila* NMJ as a model synapse we watched neuronal remodeling in mature neurons. Observation of bouton formation *in vivo* and in real-time revealed that activity-dependent bouton formation at the *Drosophila* NMJ does not occur like in the embryonic stage, where a growth-cone differentiates into round boutons, but instead strongly resembles blebbing (this work), another mechanism used by migrating cells. Is blebbing a novel mechanism of bouton addition into wired neurons?

In eukaryotic cells the plasma membrane is closely bound to the cell cortex, a layer of actin, myosin, and associated proteins. Normally myosin motor proteins maintain the cortex under tension

thereby exerting pressure on the cytoplasm. But, occasionally, as a result from local cortical contractions, the plasma membrane detaches focally from the underlying actin-cortex and the cell internal hydrostatic pressure and cytoplasmic flow leads to herniations of the membrane that grow into spherical protrusions called blebs[44,45]. Although in the past blebs have been associated with apoptotic cells, it is also clear that blebs are observed in healthy cells, during cell migration, cell spreading and cytokinesis[46]. The survival of blebbing cells over several days, the lack of apoptotic markers and nuclear fragmentation, as well as the reversible nature of this process generally confirmed the nonapoptotic nature of such blebs and proved a physiological role for blebbing[47–49]. In fact, in recent years it became well established in the field that migration by blebbing is an important form of migration used in 3D environments in combination with, or in alternative to, lamellipodia-driven migration. The bleb mode of migration is used by several different cell types, such as fish and *Drosophila* primordial germ cells movement, motility of Amoeba and *Dictyostelium*, and in mammalian tumor cell invasion[44].

Blebs can be triggered in different ways, but it usually involves the activation of non-muscle myosin II (NMII). In neurons, NMII has been correlated with growth cone dynamics and synapse formation and function[50,51], but nothing regarding blebs and NMJs or neurons has been reported. If activity-dependent boutons are forming by blebbing it would be the first time this mechanism is implicated in neurons, and would represent a paradigm shift in the field, where presynaptic elements have mostly been associated with lamellipodia or filopodia processes. Interestingly, leading edge extension through blebbing and lamellipodia is not mutually exclusive as some cells are capable of moving by switching between these two modes of migration[52,53]. However, an increase in blebbing leads to a decrease in lamellipodia and vice-versa[54], reflecting that these are very different mechanical processes[55]. A big difference between blebs and other cellular protrusions, such as lamellipodia or filopodia, is that their growth is pressure-driven, rather than regulated by polymerizing actin filaments against the membrane (Fig 1.3A). Usually, lamellipodia migration predominantes in 2D-adhesive substrates, whereas blebbing is especially prominent in 3D-environments, and has been correlated with tissue invasion in cancer cells. Considering that the NMJ arbor is deeply inserted into the muscle, as opposed to a growing axon without a synaptic target, for the MN to include new boutons it must further invade the muscle, which mechanistically is not very different from migration across other tissues. Can we introduce blebbing as a novel mechanism by which neurons regulate neuronal morphology and can we put forward the idea of neurons being capable of using growth cones and blebbing for the regulation of growth and plasticity in different environments? This thesis will explore this ideia.

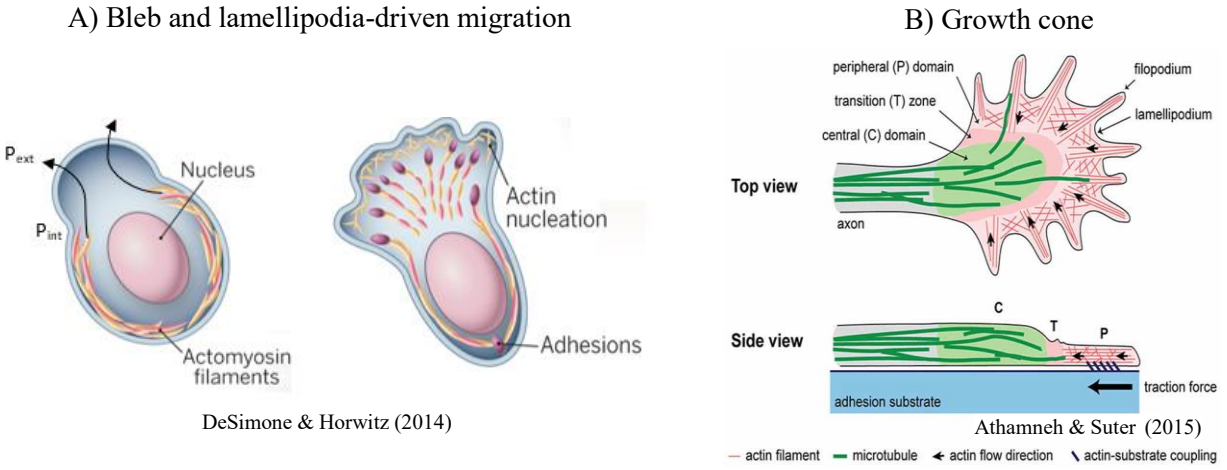


Figure 1.3 - Cellular migration modes. A) Bleb and lamellipodia-driven migration. In the bleb mode (left), contractile actomyosin filaments increase intracellular hydrostatic pressure to push the membrane out through areas of a weakened cortex. The lamellipodial mode (right) results of actin polymerization and adhesion at the front and by actomyosin contraction and de-adhesion at the rear. B) A schematic of a growth cone's cytoskeletal strutures to show the similarities to lamellipodial migration. The force for growth cone movement is driven as a result of coupling of F-actin flow in the peripheral domain to an extracellular adhesion substrate.

II. RESULTS AND DISCUSSION

2 Activity-dependent bouton formation occurs by a bleb-like mechanism

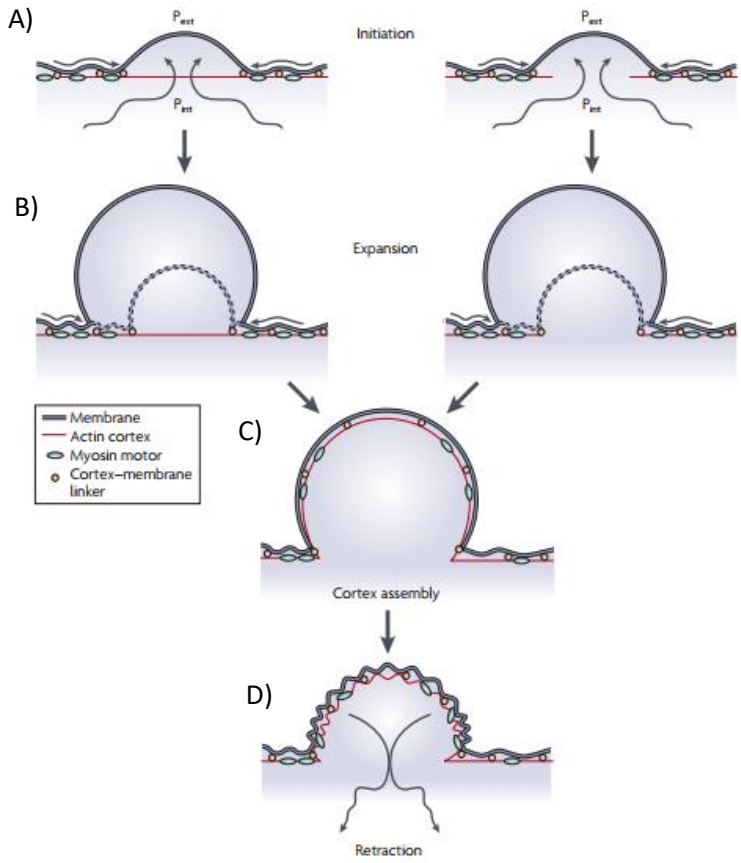
2.1 Abstract

Presynaptic boutons are conserved structures from invertebrates to man. However, the mechanisms that regulate their formation in wired neurons, or the cytoskeletal changes required for structural changes, remain unclear. By performing high temporal resolution time-lapse imaging of unanesthetized *Drosophila* larval NMJs subjected to acute structural plasticity, bouton formation was observed *in vivo* and in real-time. The dynamics and morphological changes observed during activity-dependent bouton formation were clearly distinct from embryonic growth-cone mediated bouton formation and, instead, strongly resembled a mechanism used by migrating cells, known as blebbing. This migration strategy could have been adapted by MNs to rapidly modulate the addition of new presynaptic boutons in response to intense activity. It was also noted that muscle contraction is highly correlated with bouton formation, suggesting that it may play a role during this process.

2.2 Introduction

2.2.1 Bleb formation and life cycle

Blebs are hydrostatic pressure and cytoplasmic-flow propelled cellular protrusions that appear as spherical expansions of the membrane and that have been shown to be instrumental for cell migration in 3D environments (Fig 2.1). Most of the knowledge of bleb formation comes from non-motile cells, where blebs follow a highly dynamic life cycle that lasts a few minutes and is characterized by 3 phases: initiation (or nucleation), rapid expansion and slow retraction. In migrating cells, the mechanisms appear conserved, but retraction is rarely seen. Blebs can be initiated by a variety of experimental and physiological triggers that either disrupt membrane-actin interactions or promote cortex rupture[44,56]. Experimentally, it is very difficult to distinguish between these two mechanisms because: cortex tears may be small, making them difficult to image; and the two mechanisms can act in combination, with a small cortex rupture favoring membrane delamination from the cortex (by breaking molecular links connecting the cortex to the membrane)[57]. Once nucleated, membrane detachment and bleb expansion are thought-to-being driven by intracellular pressure transients generated by myosin II contraction of the actin cortex. This pressure-driven growth is what distinguishes blebs form other cellular protrusions, such as lamellipodia or filopodia, whose growth is regulated by polymerizing actin filaments against the membrane. In fact, a hallmark of blebs is that when they are initially formed their membrane is not sustained by an actin cortex. Because cells support only small increases in area before rupture, a source of membrane is thought-to-be necessary for bleb growth[45]. Since endosomes are only rarely found in blebs, membrane unfolding and flow are possible candidates to provide membrane into the growing bleb, being mechanically able to resist expansion and effectively slow down the bleb growth[57]. Although flow of lipids through the bleb neck has been observed in some cells[44], little is known about the source of membrane and regulation of membrane supply into the growing bleb. Once expansion slows, unless the bleb is stabilized (for example by substrate adhesion), an actin cortex is reconstituted, and myosin contractility mediates the bleb retraction, which is slower than expansion. Blebs normally use signaling through Rho-ROCK-myosin as common machinery to provide actomyosin contractility for bleb retraction. The mechanisms that regulate actin polymerization in blebs are unclear, as the two best-characterized actin nucleators, the ARP 2/3 complex and the mammalian formin diaphanhous are not detected under the membrane of blebs of filamin-deficient cells (which bleb profusely and are commonly used in studies of blebbing)[44]. However several other formins have been linked to membrane blebbing suggesting that actin polymerization during bleb retraction can be formin-mediated[55]. In motile cells retraction doesn't always occur. Instead cells push the body forward as result of contraction at the rear and reversible disruption of the actin cortex at the leading edge, traveling by extending new blebs out of existing blebs (sequential blebbing)[44,55].



Charras & Paluch (2008)

Figure 2.1 - The bleb life cycle. In non-motile cells blebs exhibit a highly dynamic life cycle that includes three phases: A) Initiation; B) rapid expansion; C) and D) slow retraction. In migrating cells, the mechanisms appear conserved, but retraction is rarely seen.

2.3 Results and Discussion

To characterize how new boutons are formed in response to activity, we performed high-temporal resolution live-imaging of unanesthetized *Drosophila* larval NMJs subjected to acute structural plasticity (see methods – Appendix I), which allowed us to watch neuronal remodeling *in vivo* and in *real-time*. For our initial analyzes, a neuronal Gal4 driver (nSybg-Gal4) was used to promote the expression of CD4-GFP or CD4-Tomato in MNs allowing visualization of their membrane as readout for synaptic growth. Imaging of MN's membrane using a spinning disk microscope, which permitted an acquisition rate of seconds, showed that new boutons can form at different rates (from 60 to 1255s; 323s in mean, total of 24 boutons from 5 animals), and at different arbor locations (often from existing boutons and, in some cases, directly from the axonal membrane). From our movies it was also noted that muscle contraction was highly correlated with bouton formation. In agreement with previous studies[19], we saw two main types of bouton formation from existing boutons: 1) gradual flow of presynaptic membrane and slow elongation, often to reshape its size (by increasing it) (Fig 2.2A; Supplementary movie 1); and 2) rapidly budding of presynaptic membrane (Fig 2.2B; Supplementary movie 2). The first type was seen in preparations with subtle muscle movements, while the second type was frequently associated with visible muscle contraction. Additionally, we also saw that in some cases, upon intense muscle contraction, threads of new boutons emerged in an explosive manner from a single new event, which could be a way to rapidly add new branches. We do not exclude that more modes of bouton formation occur.

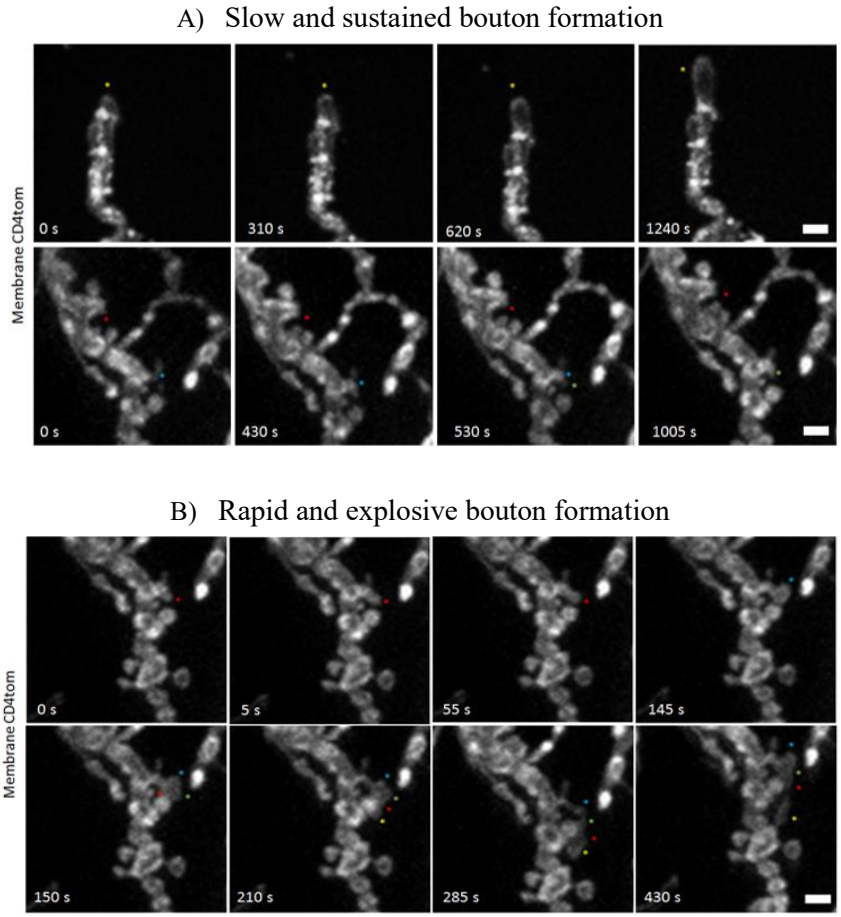
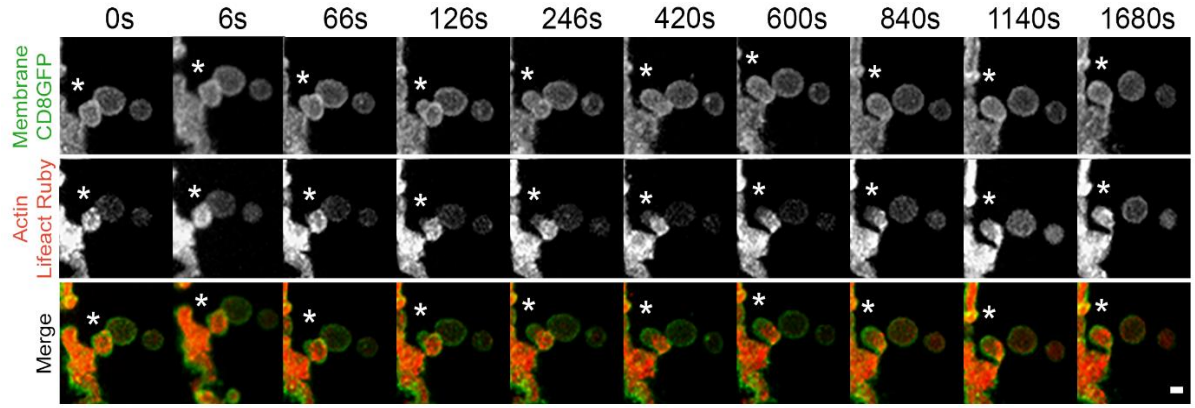


Figure 2.2 - High temporal resolution time-lapse imaging of *Drosophila* larval NMJs, after acute induction of structural plasticity. We observed that boutons form by extending round protrusions of the plasma membrane and not by filopodia or lamellipodia mediated growth. This process resembles blebbing. We observed two main modes of bouton formation in WT: A) a slower sustained growth and B) a fast and explosive mode. Usually muscle contraction precedes bouton formation, especially in the fast mode. Time in seconds is indicated in the bottom left. Neuronal membrane is labelled with CD4-tomato. Scale bar is 2 μm.

Likewise, from our movies, we did not see any growth-cone like structure and boutons do not form from visible lamellipodia/filopodia precursors. However, it was clear that new boutons often emerged rapidly and with a bulky, rounded morphology, which reminded us of a very dynamic migration mode called blebbing. Blebs differ from other cellular protrusions, such as lamellipodia/filopodia in that their growth is pressure-driven rather than relying on actin polymerization. In other words, when blebs form, they lack actin. By doing dual imaging of membrane and actin, we observed that new boutons show the hallmark of blebs: membrane growth, without actin (Fig 2.3). We observed absence of large amounts of filamentous actin (F-actin) in growing boutons and persistence of an actin cortex at the cell body beneath the growing bouton. Furthermore, we observed cases of bouton formation with and without retraction, similarly to the types of blebs that have been previously reported. In Fig 2.3A (Supplementary movie 3) we show an example of a bouton that forms and shows all the phases of the bleb life cycle. Notice the dynamic nature of actin, clearly the bouton formed with no (or very little actin) which later refilled the bouton, and this was followed by bouton retraction. Interestingly, the base of the bouton seems to increase over time, a possible indication of detachment of the membrane from the actin cortex, and actin continued at the cell membrane bellow the growing bouton. Also represented (Fig 2.3B; Supplementary movie 4) is an example of a bouton (in white) that forms and expands and then, rather than retracting, a second bouton emerged (in red) in a manner that resembles sequential blebbing. This type of bouton formation was more frequently observed than the retracting type. Notice that boutons formed with no actin, but at the base, a bright F-actin punctum, which in some studies is

used to identify blebs[58]. Interestingly, the emergence of new F-actin puncta localized at the sites of ghost bouton formation has been used by Piccioli & Littleton (2014) to identify new boutons. Additionally, they showed that pharmacological disruption of actin polymerization dynamics (with depolymerizing drug Latrunculin A or F-actin stabilizing drug Jasplakinolide) also disrupts rapid bouton addition in response to activity compared with a control situation, suggesting that local actin rearrangements occurs during ghost bouton formation[19]. Likewise a continuous F-actin turnover is supposed to be necessary to support blebbing in migrating cells.

A) Example of a bouton that shows al the phases of the bleb life cycle



B) Example of a bouton without retraction

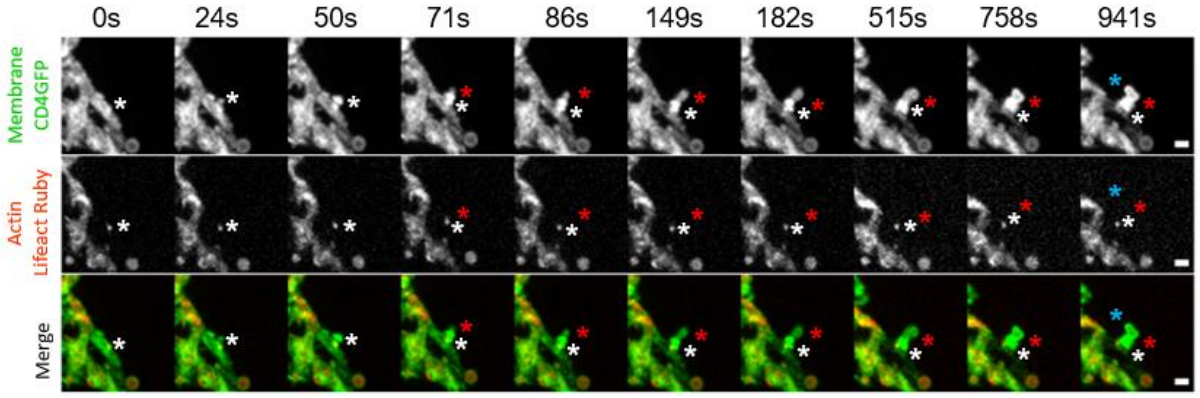
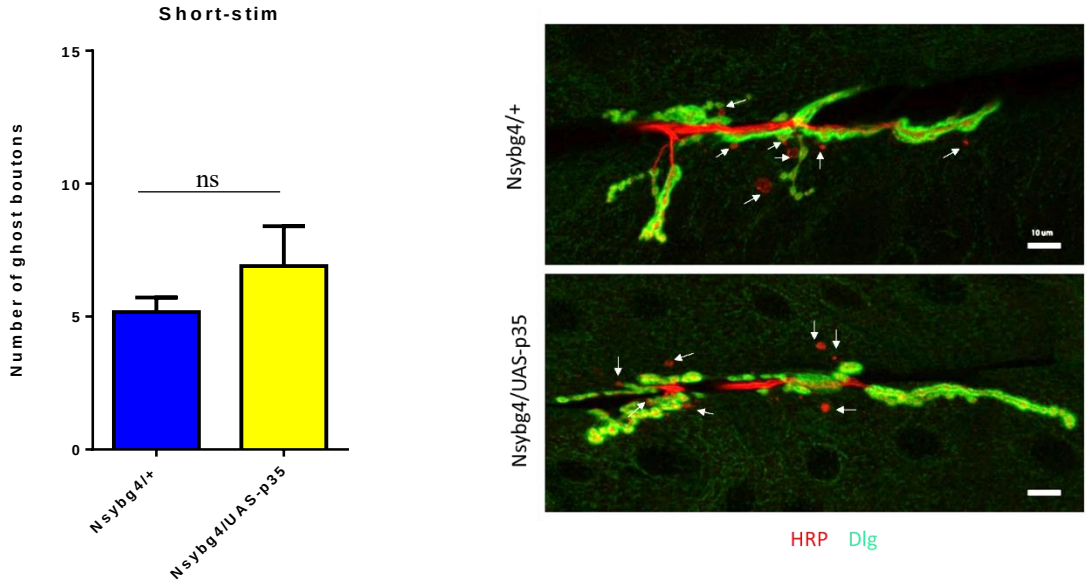


Figure 2.3 – Dual live imaging of MN's membrane and actin dynamics after High K^+ stimulation. Activity-dependent boutons show the hallmarks of blebs: plasma membrane without actin. We show an example of A) a bouton (white*) that shows all the phases of the bleb life cycle: initiation (0-6s), expansion (6-246s) and retraction (246-1680s) and an example of B) sequential bouton formation (white, red, and blue *s) without retraction. Neuronal membrane is labeled with CD8GFP or CD4GFP and actin is labeled with Lifeact-ruby. Time in seconds is represented in the top of the panels. Scale bar is 2µm.

Therefore, our data supports the hypothesis that activity-dependent boutons form by blebbing. Since blebs have also been associated with cell death, we blocked apoptosis in MNs and tested whether new boutons are the result from an apoptotic process. To investigate this, we induced acute structural plasticity (see methods) in MNs expressing the anti-apoptotic protein p35 and in wild-type (WT) MNs. In our hands, at 25ºC control NMJs exposed to the stimulation protocol showed an average of 5±0.55 ghost boutons, while unstimulated synapses showed an average of 2±0.38 ghost boutons. Expression of p35 in motor neurons did not alter the frequency of ghost boutons after the stimulation compared to control (Fig 2.4A), suggesting a non-apoptotic nature for new boutons formed with activity. This re-enforced the notion that these bleb-like structures are physiologically relevant[17]. Moreover, ghost bouton frequency was significantly enhanced during development (Fig 2.4B) and this was an interesting

result since p35 can also protect *C. elegans* from developmental cell death[59], suggesting that it may play a similar role in other organisms.

A) Expression of p35 in MNs did affect their capacity to form new boutons after acute stimulation



B) Expression of p35 in MNs increased ghost bouton frequency during development

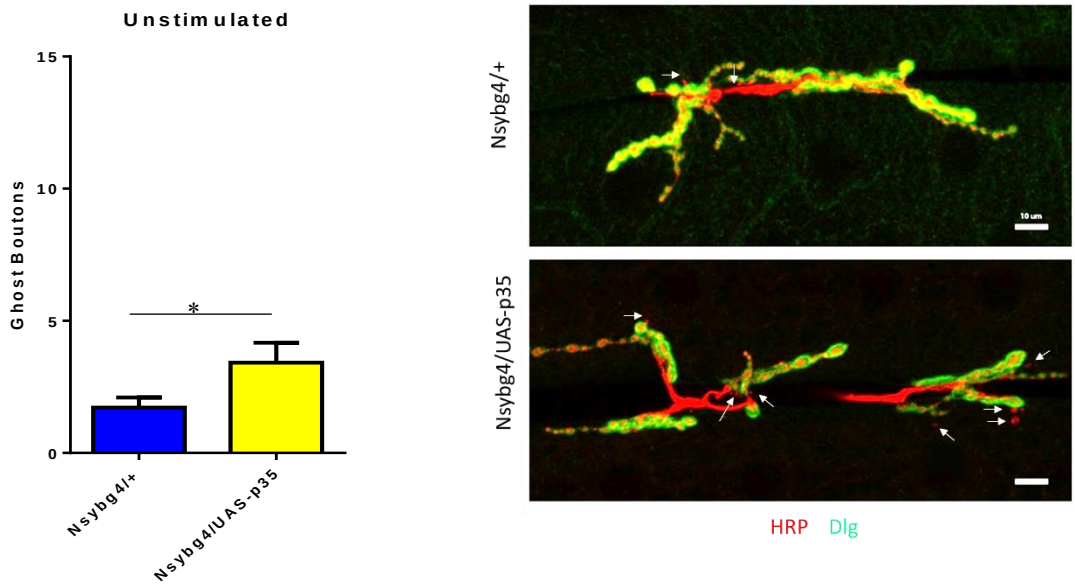


Figure 2.4 - Effect of neuronal expression of anti-apoptotic p35 in activity-dependent bouton formation. Neuronal expression of p35 A) did not affect the capacity of MNs to form new boutons after acute stimulation but B) increased ghost bouton frequency during development. White arrows indicate ghost boutons. Scale bar is 10 µm. *$p<0.05$. Error bars represent SEM. High K^+: data collected from 99 (Nsybg4/+) and 21 (Nsybg4/UAS-p35) NMJs. At rest: data collected from 35 (Nsybg4/+) and 12 (Nsybg4/UAS-p35) NMJs. At least 5 larvae were used per line and per condition.

2.4 Conclusions

From our data, it seems that activity-dependent bouton formation does not occur like in the embryonic stage and does not require any specialized growth structure. Instead new boutons form by blebbing or use an equivalent strategy to this mode of migration. Interestingly, we saw a strong correlation between muscle contraction and bouton formation, suggesting that the muscle plays a role in this process that could either be by setting up or increasing its dynamics. It is possible that MNs have adopted this migration mode and combined it with activity signaling pathways to modulate bouton formation during muscle activity.

3 The blebbing pathway is used for activity-dependent bouton formation

3.1 Abstract

Live imaging of WT larvae revealed a novel mode of bouton formation, which resembles and has the hallmarks of blebbing (Chapter 2). There are several pathways that have been reported to play a role in bleb initiation, some of which converge on NMII activation. Despite detected at low levels at NMJs of WT animals, NMII immunoreactivity was enriched in many ghost boutons following acute stimulation. We showed that interfering with NMII changes the capacity of MNs to respond to activity (by increasing it). This result lead us to hypothesize that muscle contraction may be sufficient to promote bouton blebbing, possibly by "squeezing" the neuron that has a weaken cortex due to NMII Knockdown (K/D). Our results suggest a dynamic interplay between the MNs and the muscle in the regulation of bouton formation.

3.2 Introduction

3.2.1 Role of non-muscle myosin II (NMII) for blebbing

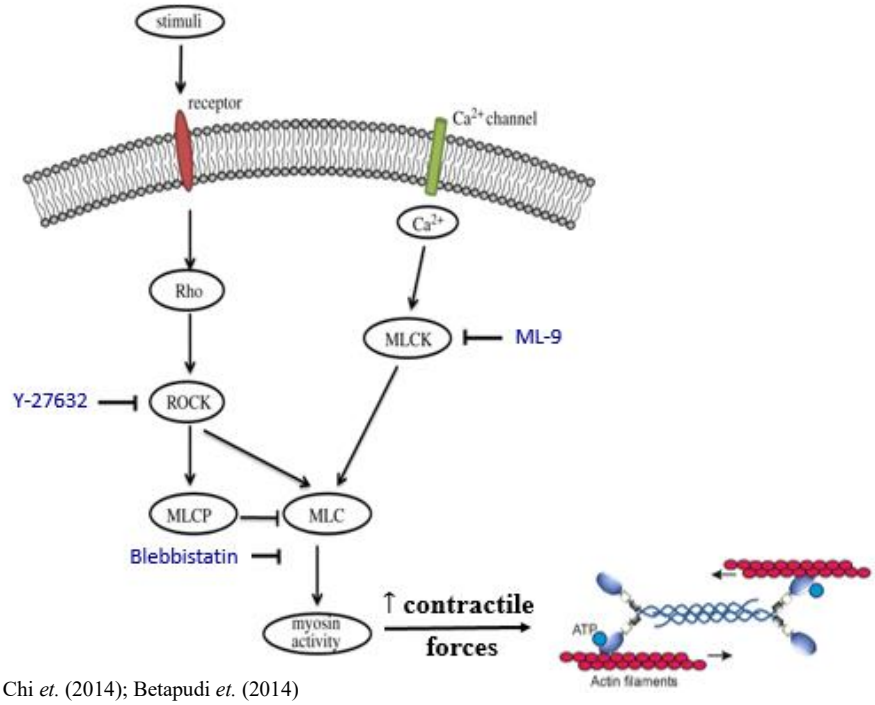
Myosins constitute a superfamily of actin-based cytoskeletal motors that use energy derived from ATP hydrolysis to generate movement and force. The catalytic sites are found in the head of myosins and are often activated when myosin binds to actin filaments. The tail of some myosins moves cargo in the cell, whereas in other myosins the tails self-associate into bipolar filaments, allowing the heads to tether actin filaments and exert tension on them. Additionally, myosins can act indirectly through actin to bring adhesion molecules, such as integrins or caderins, into close proximity[60]. There are 35 known classes of myosins, and humans have 40 myosin genes that fall into 13 classes[51]. Most myosins belong to the classe II and together with actin make-up the major contractile proteins of cardiac, skeletal and smooth muscle. Related myosins, that resemble their muscle counterparts, are also present in all non-muscle eukaryotic cells. NMIIs are proteins that have actin cross-linking and contractile properties and that function to control processes that require cellular reshaping and movement, such as cell adhesion, cell migration and cell division[60].

In neurons, NMII localizes to dynamic actin-enriched structures including growth cones and synapses. Whereas all three mammalian isoforms of NMII localize to growth cones, where they regulate process extension, NMIIb is the predominant isoform present in the synapse, being present in both pre- and postsynaptic terminals. At presynaptic terminals NMII regulates SV recycling and neurotransmission, whereas postsynaptically NMII controls the morphology and dynamics of dendritic spines, and has been shown to be required for synaptic plasticity and LTP induction[51]. Despite having prominent synaptic roles, there are no reported roles for NMII in the formation of blebs in neurons or NMJs. However, in the context of cell migration, NMII has extensively been shown to contribute for both mesenchymal-migration (lamellipodia-driven) and amoeboid-migration (bleb driven). During mesenchymal-migration cells polymerize actin at the cell front while integrin-based adhesions, mediate attachment to the ECM. NMIIa generates forces that alter actin organization at the cell front and initiate adhesion maturation, while NMIIb is involved in the formation of stress fibers, nucleus translocation and in the detachment of adhesions at the cell rear. For amoeboid-migration, increased NMII-mediated tension affects the cortical actin network generating the intracellular pressure that drives the bleb expansion. Indeed, NMII-null cells or treatment with Blebbistatin, a common inhibitor of NMII, prevents bleb formation and thereby NMII is considered an important regulator of blebbing initiation[54].

3.2.2 Pathways that regulate NMII in blebbing cells

Although blebs can be triggered in different ways it usually involves NMII activation and increased contractility of the cortex. Like muscle myosins, NMII molecules are comprised of three pairs of peptides: two heavy chains (MHCs) which bind and produce tension on actin, two regulatory light

chains (RLCs) that regulate its activity, and two essential light chains (ELCs) that stabilize the heavy chain structure[60]. In contrast to skeletal and cardiac myosins, which are regulated by a separate set of proteins, normally bound to the actin filaments, NMII motor activity, activation, and assembly state are determined by the reversible phosphorylation of the light and heavy chains[60]. In flies and mammals the activation of NMII by phosphorylation of the associated RLC controls assembly and activation of the NMII to produce force on F-actin. A multitude of pathways have been identified that control NMII activation, but the actomyosin contraction system is regulated by, at least, two independent pathways (Fig 3.1): a Ca^{2+}-dependent pathway and a Rho-dependent pathway[61]. Activation of both pathways has been observed in blebbing cells[62–66]. Ca^{2+}/Calmodulin leads to Myosin Light chain Kinase (MLCK) activation, which seems to be specific for NMII RLCs, whereas the small GTPase Rho activates both Rho Kinase (ROCK) and Citron Kinase[60], that usually phosphorylate several substrates in addition to the RLCs of NMII. Although ROCK can phosphorylate RLCs directly, it primarily acts to inhibit the major myosin phosphatase, Protein Phosphatase 1 (PP1), which is responsible for the dephosphorylation and inactivation of NMII, prolonging NMII activation in cells. Although other small GTPases, such as Rac and Cdc42, are able to phosphorylate NMII, they generally promote actin polymerization, whereas Rho activates actomyosin-mediated contractility.



Chi *et.* (2014); Betapudi *et.* (2014)

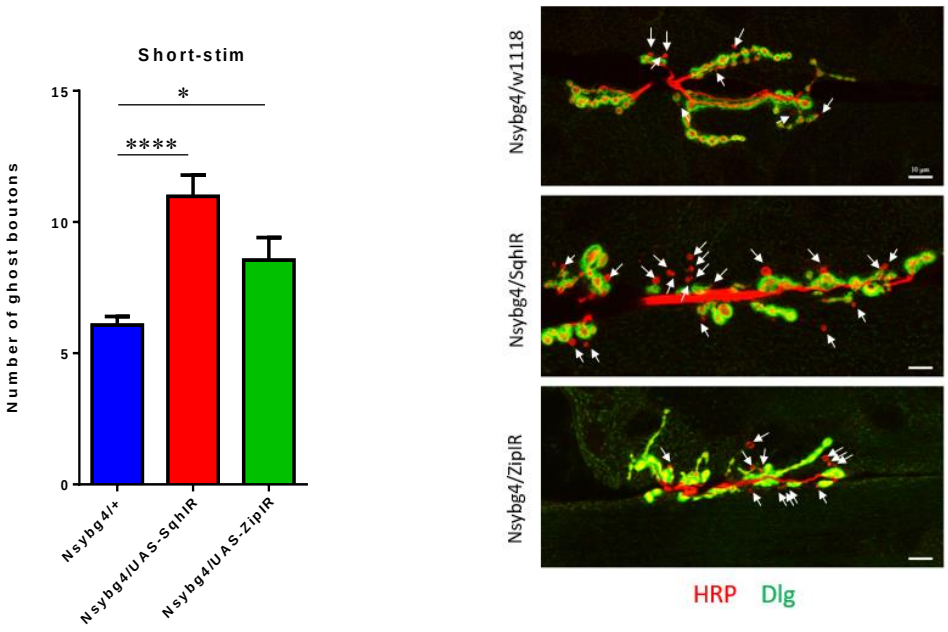
Figure 3.1 - Pathways that regulate NMII activation in blebbing cells. The Rho/ROCK and Ca^{2+}-Calmodulin/MLCK pathways are the major regulators of NMII. Both pathways are active in blebbing cells resulting in increased NMII-driven contractibility of the cell cortex.

3.3 Results and Discussion

Although NMII is not described as having a role in activity-dependent bouton formation, it is possible that it could regulate the structural modifications that occur during activity-dependent plasticity, since ghost boutons emerge in a bleb-like fashion and NMII is critical for bleb initiation. To investigate this, we induced acute structural plasticity in MNs that had reduced NMII. For this we used the neuronal Gal4 driver nSyb to express RNAi (IR) constructs against the RLCs (SqhIR) and the MHCs (ZipIR) of NMII, specifically in neurons. Disruption of the RLCs (Sqh) and the MHCs (Zip) of NMII in MNs rendered the synapse more sensitive to this treatment (Fig 3.2A). The average number of ghost boutons after stimulation was 11±0.81 in SqhIR and 9±0.87 in ZipIR compared to 6±0.33 in the control situation

at 30ºC. These changes were not due to developmental increased formation of ghost boutons since there is no difference between NMII K/D and the control in unstimulated preparations (Fig 3.2B).

A) NMII K/D in MNs increased their capacity to form new boutons after acute stimulation



B) NMII K/D in MNs did not alter ghost bouton frequency during development

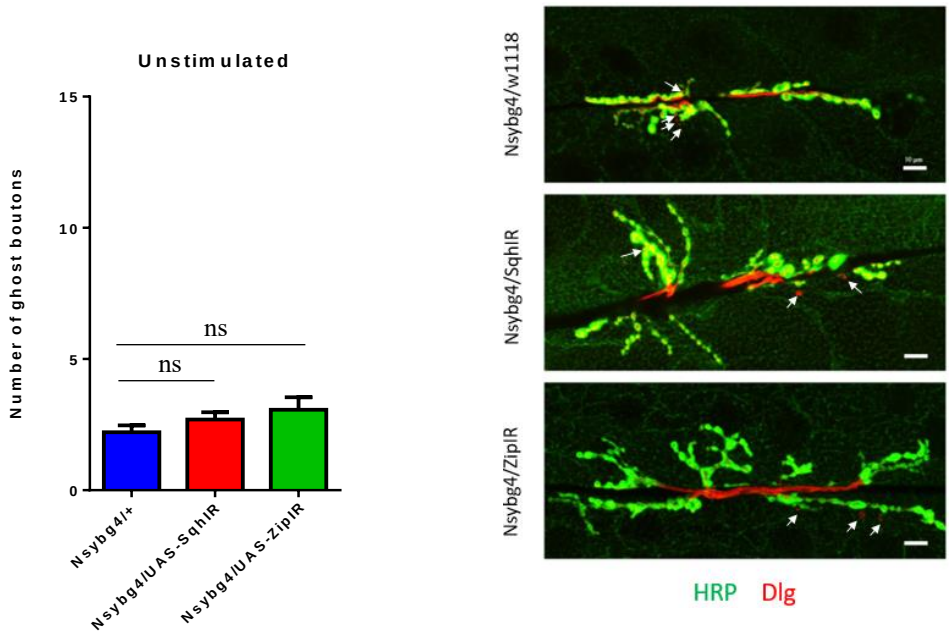
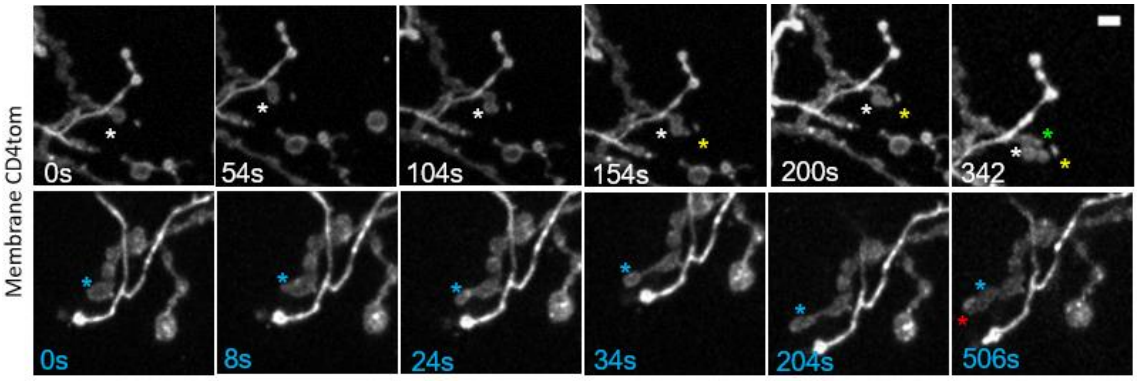


Figure 3.2 - Effects of altering the levels of NMII during ativity-dependent plasticity. Neuronal IR against two subunits of NMII (Sqh and Zip) A) altered the capacity (by increasing it) of MNs to form new boutons after acute stimulation B) but not during development. Scale bar is 10 μm. White arrows indicate ghost boutons. *p<0.01, ****p<0.0001. Error bars represent SEM. High K^+: data collected from 254 (Nsybg4/+), 105 (Nsybg4/UAS-SqhIR) and 69 (Nsybg4/UAS-Zip) NMJs. At rest: data collected from 85 (Nsybg4/+), 56 (Nsybg4/UAS-SqhIR) and 47 (Nsybg4/UAS-ZipIR) NMJs. ≥ 12 larvae per line and per condition.

To study the dynamics of bouton formation when NMII is reduced, we analyzed the speed at which new boutons were forming in the WT and NMII IR. We found a significant decrease in the time per bouton formation when NMII was K/D in MNs (Fig 3.3; Supplementary movie 5). However, this data is still preliminary because the n is still very low, and we are combining all categories of events, which results in a high variance.

A) Live-imaging of bouton formation in NMII K/D



B) Bouton formation in NMII IR is faster than in the NMII

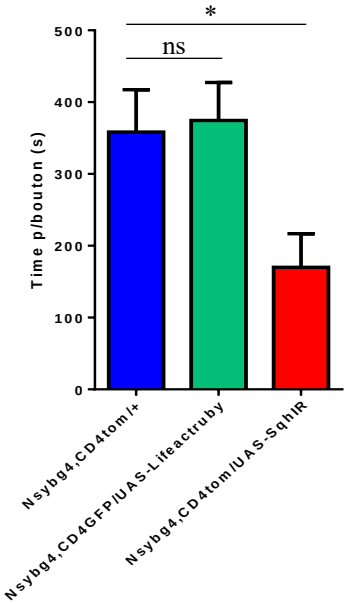


Figure 3.3 - Live-imaging of bouton formation in NMII K/D after acute induction of activity-dependent boutons. A) Our initial movies on NMII IR suggested that boutons were forming faster compared to the WT situation (see Fig 2.2). MNs membrane was labeled with CD4-Tomato. Scale bar is 2 μm. B) Our preliminary analysis of bouton formation speed supports this observation. Additionally, we also compared bouton formation dynamics when we labeled the membrane vs when actin was also labeled. No diferences were found in the two scenarios, suggesting that the constructs used did not interfered with the dynamicity of this process. *p<0.05. Error bars represent SEM. Data collected from 10 (Nsybg4, CD4Tom), 30 (Nsybg4,CD4GFP/UAS-Lifeactruby) and 7 (Nsybg4,CD4Tom/UAS-SqhIR) NMJs stimulated with HighK^+. ≥2 larvae per line.

In sum, we observed that when NMII was reduced in MNs, the NMJ was more plastic and the boutons formed at a faster rate. It is known that NMII is a master regulator of blebbing that when pharmacologically inhibited or K/D in cells prevents blebbing, indicating the cortical tension powered by NMII is what generates the intracellular pressure transients that drive the bleb expansion. Interestingly, when we KD NMII in neurons, rather than abolishing activity dependent bouton formation, it increases it. We were puzzled with these results because NMII is required for bleb formation. However, even in cells, if NMII is K/D, and the cells are pressured between agar, this pressure is sufficient to not only restore blebbing, but also to make it more frequent[54]. Therefore, NMII K/D cells are bleb competent, they just lack cortical tension and cannot initiate the process which is pressure driven. Because our movies had shown that bouton formation is highly correlated with muscle contraction we hypothesized that muscle contraction may be the factor that triggers "bouton blebbing", perhaps by "squeezing" (or pressuring) the neuron that has a weaken cortex due to NMII K/D.

Additionally, we also tested if NMII activation was necessary for "bouton blebbing". For that we expressed in MNs both the constitutively active (CA) and the dominant negative (DN) form of Sqh

(Fig 3.4). Because the cortical tension is thought to depend on NMII activity, we also wanted to explore if increasing and decreasing the cortical tension, respectively, at the NMJ arbor affected activity-dependent bouton formation. We found that expression of SqhDN (non phosphorylatable Sqh) in MNs increased activity-dependent bouton frequency (Fig 3.4A), similar to what was observed when we K/D NMII and further supporting the idea that weakening MNs cortex does not prevent bouton formation, but instead facilitates it. However, when MNs expressed SqhCA (phosphomimetic Sqh) we saw no significant differences in ghost bouton production compared to control (Fig 3.4A), which showed that expressing NMII CA in MNs is not sufficient to increase bouton formation. This may be because NMII activation needs to be highly localized (which does not occur in NMII CA), like it is thought to happen in blebbing cells, or, alternatively NMII is not necessary to initiate this mechanism. Because blebs form in conditions of high cortical contractility, if NMII is not driving the increase in cortex tension, it is possible that other intrinsic or extrinsic factors do so. Interestingly, it was recently shown that the actin cortex architecture, alongside with NMII, also regulates cell surface tension[67]. For example, increasing actin turnover also leads to an increase in cortical tension providing another possible way to generate the internal hydrostatic force that drives bleb formation. Furthermore, previous studies have proposed that filament bending under compression could also account for cortical tension generation in actomyosin networks[68,69]. While these are all valid possibilities, in our system and based on our observations, we favor a model where the muscle can directly press on MNs to further increase bouton formation.

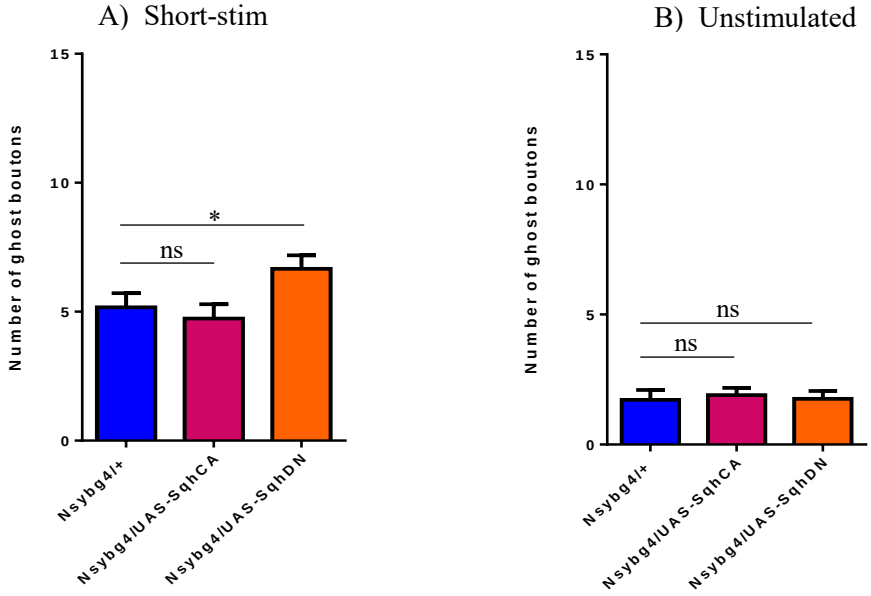


Figure 3.4 - Effects of altering activity of NMII in activity-dependent plasticity. Neuronal expression of NMII DN, but not of NMII CA, altered the capacity of MNs to form activity-dependent boutons after stimulation (A) but not during development (B). *p<0.05. Error bars represent SEM. High K^+: data collected from 99 (Nsybg4/+), 97 (Nsybg4/UAS-SqhCA) and 89 (Nsybg4/UAS-SqhDN) NMJs. At rest: data collected from 35 (Nsybg4/+), 41 (Nsybg4/UAS-SqhCA) and 42 (Nsybg4/UAS-SqhDN) NMJs. ≥7 larvae per line and per condition.

While our data suggests a role for NMII in acute bouton formation, the localization of Sqh or Zip at the NMJ, before and after stimulation, is unknown. Using an antibody against NMII, or a GFP trap in the Sqh *locus* (which recapitulates the endogenous localization), we detected low levels of the protein at the presynaptic terminals of NMJs in WT animals (most of NMII was detected at the nerve, trachea and in the muscle), and this was true even when we overexpressed the protein in neurons using nSybg-Gal4. Despite this, following high K^+ stimulation, NMII immunoreactivity was enriched in many ghost boutons both in WT and with overexpression, as it can be seen in the insets of Fig 3.5. This finding suggests that NMII may have a dynamic localization, being recruited to newly formed boutons during structural plasticity at the *Drosophila* NMJ. One hypothesis is that NMII may be enriched at sites

primed for bouton formation, generating a higher concentration of the protein that later becomes trapped in newly formed boutons. Interestingly, when we acquired live movies with fluorescently tagged versions of NMII we never observed any events (in several experiments), suggesting that it is possible that the tag is interfering with NMII function and bouton iniation, therefore precluding live observation of NMII dynamics during this process.

In cells, there are many cases where blebs extend from cortexes that had little NMII and, similar to actin, NMII was not found along the leading edges of blebs immediately after extension and the pre-existing myosin remained in the basal region. In these studies, when myosin appeared at the leading edge it was followed by bleb retraction[47,54]. These findings together with that we found NMII surrounding several ghost boutons, led us to hypothesize that NMII may participate in bouton retraction, of boutons that do not stabilize (for example by adhering to the muscle surface). However, since in our movies we did not see many boutons retracting, another possibility is that NMII may act indirectly through actin to recruit molecules that help to stabilize some of the newly formed boutons, playing a role in bouton maturation. Both of these hypothesis can explain the increased frequency in ghost boutons when we K/D NMII in MNs.

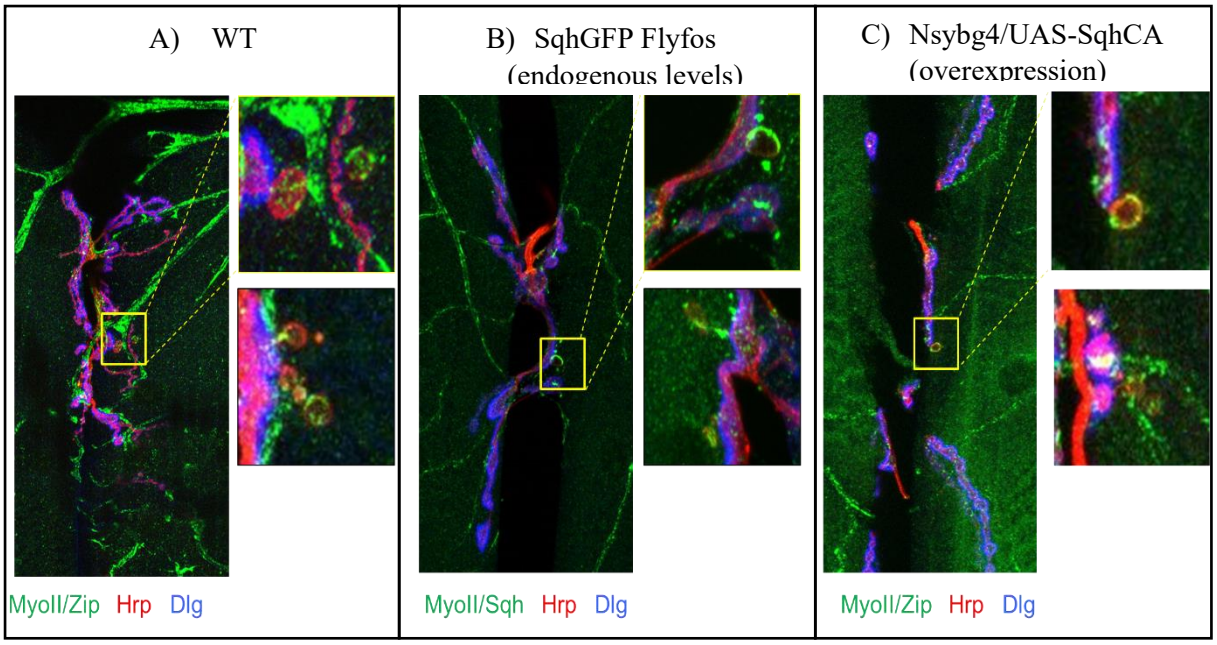


Figure 3.5 - NMII localization at the NMJ after High K^+ stimulation. NMMII is recruited to newly formed activity-dependent boutons. A) We show an antibody staining for NMII in the WT to see the endogenous distribution of NMII at the NMJ. B) We also used a protein trap (where GFP is inserted into the locus of Sqh and is expressed as a fusion protein that reflects the endogenous distribution of the protein) and GFP antibody in an attempt to amplify the signal of NMII (middle panel). C) We show antibody staining for NMII with overexpression of SqhCA. Despite, being detected at low levels at the presynaptic terminal in all cases, NMII was clearly present in the newly formed boutons.

To investigate if NMII local activation is implicated in bouton initiation and whether there is a preferred route for bouton formation within the bleb pathway, we interfered with NMII immediate regulators (Fig 3.6). However our preliminary data, shows that K/D of ROCK (RokIR) or MLCK (Strn-MlckIR) in MNs (Fig 3.6, left bars) does not alter the MN's capacity to form activity-dependent boutons. This may be due to low IR efficiency, because these pathways are redundant, or because compensatory pathways may be activated. Moreover, by interfering with these kinases a lot of cellular functions are affected. The ideal would be to interfere with the GEFs associated with these kinases that confer specificity for a given function, such as bleb initiation. However such experiments are not possible because the identity of these molecules is not known.

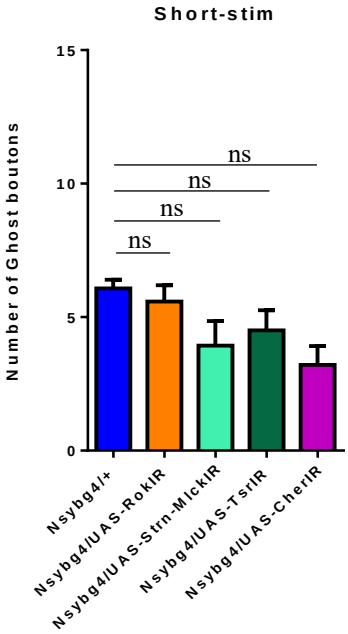


Figure 3.6 - Effect of interfering with molecular players associated with blebbing in activity-dependent bouton formation. Neuronal K/D of NMII immediate regulators – ROCK (RokIR) and MLCK (Strn-MlckIR) – showed no effect on activity-dependent plasticity at the NMJ. The same was seen with other two candidates to initiate bouton formation – Cofilin (TsrIR) and Filamin (CherIR). Error bars represent SEM. Data collected from 254 (Nsybg4/+), 60 (Nsybg4/UAS-RokIR), 15 (Nsybg4/UAS-Srn-MlckIR), 34 (Nsybg4/UAS-TsrIR) and 14 (Nsybg4/UAS-CherIR) NMJs stimulated with $HighK^{+}$. ≥4 larvae per line.

Additionally, we also wanted to do a candidate approach and test molecular players that could participate in bouton formation. Two distinct mechanisms of bleb initiation have been observed experimentally: local delamination of membrane from the cortex, or local rupture of the actin cortex. Although both types can occur because of localized myosin-driven contraction of the actin cortex, any local loss of membrane-cortex adhesion or intra/extracellularly-induced local cortex weakening, could achieve the same results when coupled to a uniform intracellular hydrostatic pressure. Therefore, we decided to test the possible role of filamin, a membrane-actin linker protein that also acts as actin-crosslinking protein, and of cofilin, an actin depolymerization protein that also induces F-actin rupture, by exercing a mechanical effect on actin filaments, just like NMII[70]. Both of these proteins have been linked to blebbing. Filamin-deficient cells bleb constitutively and are widely used for bleb studies and cofilin levels were extremely reduced in Calpain K/O cells, that have impaired blebbing[58]. Interestingly, cofilin-mediated severing accelerates the turnover and spatial reorganization of F-actin, that is though-to-be important to sustain blebbing dynamic behavior. However, our preliminary results indicate that neuronal K/D of either Cofilin (TsrIR) or filamin (CherIR) did not alter the ghost bouton frequency at the *Drosophila* NMJ (Fig 3.6, right bars). Even though TsrIR in MNs did not show any affect in bouton formation, previous studies at the *Drosophila* NMJ showed that expression of a inactive form of cofilin impaired bouton formation in response to activity, while expression of a constitutively active form of cofilin increases it[19]. Moreover, they also showed that cofilin activity downstream of the BMP pathway, which promotes local actin remodeling, is important for bouton formation in response to activity. Nonetheless, our results do not discard the possibility for a role of cofilin in bouton formation in response to activity. Therefore, and given that both cofilin and NMII can cause cortex rupture, we hypothesize that cofilin either acts in concert with NMII activity or may bypass the need of NMII during bouton formation when coupled to muscle contraction. Not surprisingly, Actin depolymerizing factor (ADF)/cofilin proteins have been shown to control actomyosin assembly (by competitive binding to F-actin) and intracellular contractile force generation, a function of equal physiological importance to their established roles in mediating F-actin turnover[71].

On the other hand, the antibody staining of a presynaptic isoform of filamin showed that filamin is present at MNs terminals and surrounding synaptic boutons in a punctuate pattern, like it was previously reported[72], but it did not localize in ghost boutons after stimulation (Fig 3.7B). Though our data did not show significant differences from the control, filamin presence at MNs terminals points to a presynaptic role for this molecule, which is still to discover. Because, filamin has been shown to govern the growth of postsynaptic membrane at the *Drosophila* larval NMJ[72] it is possible that it may also participate in membrane addition at the presynaptic compartment. Strikingly, in *Drosophila* filamin interacts with NMII (Zip)[73] and Rab11[74], a key regulator of membrane trafficking known to interact with the Sec15[75] component of the exocyst complex, that is involved in vesicle trafficiking to the plasma membrane.

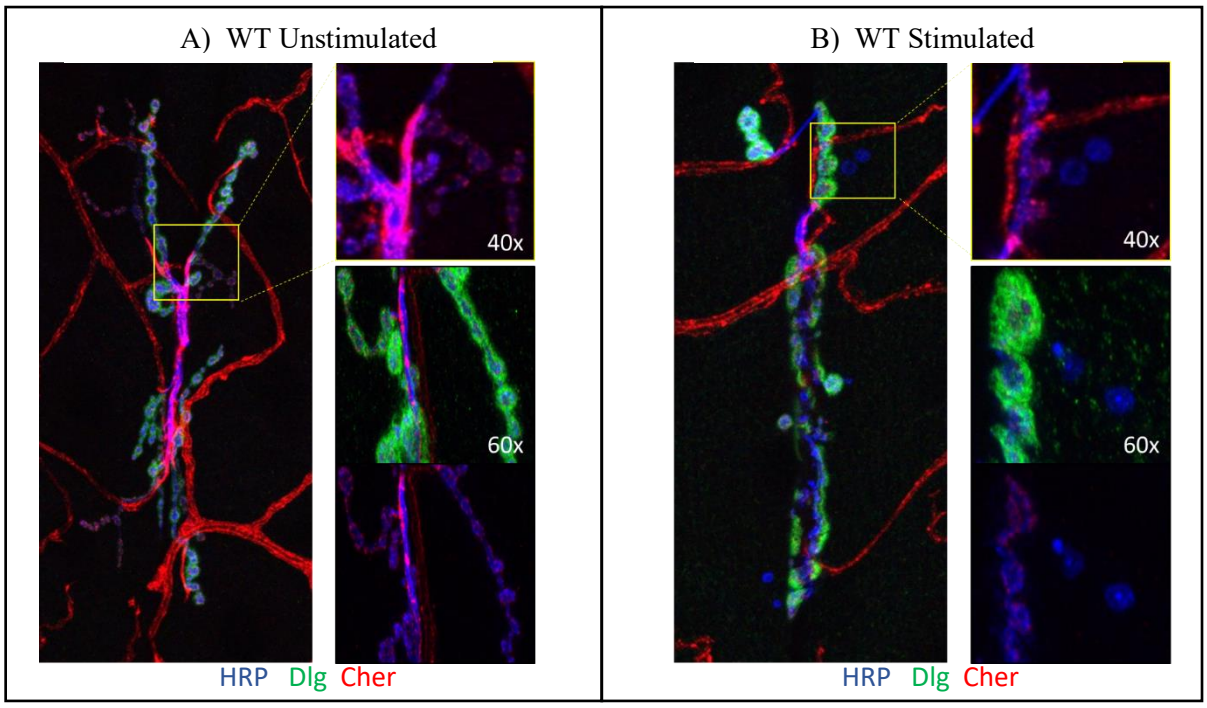


Figure 3.7 - Filamin localization at the NMJ before and after High K^+ stimulation. We used an antibody directed against the presynaptic isoform of filamin, which was gently provided by M. Uhlirova Lab. At the Drosophila NMJ the antibody immunoreactivity has been previously reported by GaYoung Lee & Tomas Schwartz (2016), with this isoform being present at the nerve, trachae, glia and puncta in presynaptic boutons. Our results reproduced this distribution (A) and, additionally, we saw that despite being present in MNs terminal boutons, filamin is not recruited to newly formed boutons (B), like NMII is (this work).

3.4 Conclusions

Our live-imaging of WT larvae revealed a novel mode of bouton formation, which resembles and has the hallmarks of blebbing. NMII is a master regulator of blebbing that when K/D in cells inhibits this process. However, if these cells are compressed between two layers of agar, blebbing is restored showing that these cells are still bleb competent, but in the absence of NMII the intracellular pressure is insufficient to drive bleb formation. Because blebbing also depends on increased cortical contractility NMII is hypothesized to be the main driver of the intracellular pressure that powers the bleb expansion. At the NMJ, NMII is recruited to new boutons, however when we used RNAi against two of NMII subunits (Zip and Sqh) we observed more boutons rather than fewer. This result raised the possibility of the existence of an interplay between MNs and muscle and exposed rather interesting relationships between these cells in the regulation of neuronal growth in response to synaptic activity. Despite having identified NMII as player in bouton formation, it remains to determine which blebbing pathways contribute for bouton formation and whether there is a preffered route used for this process.

4 Activity-dependent bouton formation is regulated by a complex interplay between MNs and the muscle

4.1 Abstract

Manipulation of NMII in neurons exposed an intricate interplay between MNs and the muscle (Chapter 3) in the regulation of the number of activity-dependent boutons. This MN-muscle interplay poses a series of interesting possibilities, given that the factors required for blebbing can be regulated by this interaction, namely muscle contraction can directly influence MNs confinement. By blocking muscle contraction using a GluR antagonist, NASPM, and a general myosin inhibitor, Blebbistatin, we decreased activity-dependent bouton formation in WT and NMMII K/D NMJs, suggesting that both muscle activity and muscle contraction can participate in the regulation of bouton formation. We propose that a balance of mechanical forces and biochemical signaling are probably coordinated during structural plasticity upon intense muscle activity. It is possible that MNs adapted this migration mechanism and combined it with activity-dependent signaling pathways to modulate the addition of presynaptic elements during intense muscle activity.

4.2 Introduction

4.2.1 Factors that promote bleb formation

Many migrating cells can switch between mesenchymal (lamellipodia-driven) and amoeboid-like (bleb-driven) migration modes and this plasticity provides an important advantage for cells that migrate through changing environments *in vivo*. Blebs occur in conditions of high cortical contractibility, and bleb formation is further favored in conditions of low adhesion and high confinement (Fig 4.1A), which allows rapid and polarized movement[76]. High cortical contractility is crucial to generate the propelling forces that drive bleb-migration, but cortical contractility also favors rounded cell shapes and may directly counteract adhesion. Indeed, transitions from mesenchymal-to-amoeboid motility favor non-adhesive modes of migration. On the other hand, decreasing cell adhesion, for instance by lowering the levels of integrins or promoting their inactivity, or placing cells in a non-adhesive substrate, for example between two non-adhesive gels, also favors rapid non-adhesive migration, provided that there's some physical confinement, which is essential for maintaining some level of cell-substrate interaction and providing force transmission between the two, which is required for locomotion.

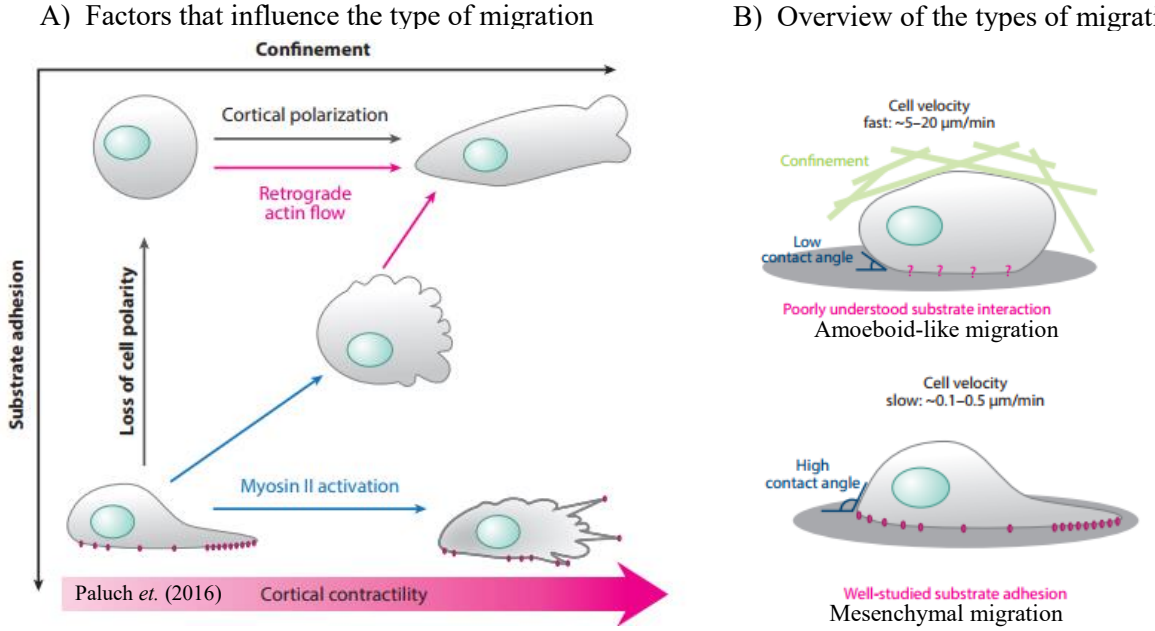


Figure 4.1 - Factors that promote bleb-driven migration. A) Graph representing how contractile forces, cell adhesion and confinement influence the type of cell migration. Blebs form preferentially in conditions of high

cortical contractility, low adhesion, and high confinement. B) Overview of mesenchymal and amoeboid migration to highlight typical cell shapes and migration velocities.

4.2.2 Mechanisms of bleb-based migration

While several physical mechanisms have been proposed for force transmission between cell and substrate during adhesion-free migration, most remain theoretical. We will focus on the mechanisms that account for migration in confinement. A commonly proposed mechanism is based on cell-substrate intercalation, namely on the interpolation of protrusions formed at the sides of the cell into gaps of the substrate, which may serve as footholds to drive cell locomotion. Despite conceptually attractive, the extent of force generation via such mechanism has not been experimentally investigated and remains unknown if this strategy is sufficient to propel a cell forward in a 3D matrix. An alternative mechanism, termed chimneying, has been proposed to explain migration in confined environments without requiring a discontinuous substrate. To achieve this kind of motility the cell must be sufficiently confined so that it can exert significant lateral pushing forces against the surrounding substrate, to wedge the cell body into place, allowing for high cortical contractility at the rear and protrusion at the cell front. In this model the pushing forces may result either from actin polymerization on the wall behind the leading edge, or from the intracellular pressure. At last, mechanisms based on nonspecific friction between the cell and the substrate have also been suggested. This model proposes that intracellular forces generated by the cytoskeleton, such as flows of actomyosin cortex, are transmitted to the substrate via nonspecific friction. These friction forces could result from transient, and weak, interactions between molecules at the cell surface and the substrate and, in principle, any molecule with an extracellular domain that is coupled to the actin cortex, such as cadherins, could contribute. Such non-specific friction would necessarily occur if the cells were close enough to the substrate and in strong confinement[76].

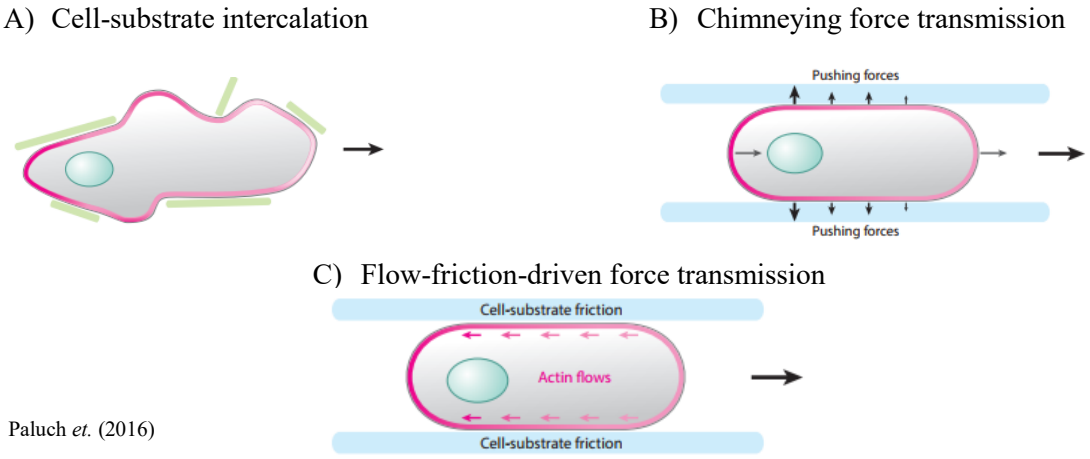


Figure 4.2 - Mechanisms of bleb-based migration with confinement. We show different proposed mechanisms of force generation and transmission during adhesion-free migration in confinement: A) Intercalation of lateral protrusions into substrate gaps into 3D matrices. B) Chimneying migration force transmission in confinement. C) Flow-friction-driven migration forces in strong confinement.

4.3 Results and Discussion

Because blebbing depends on increased cortical contractibility, NMII is hypothesized to be the source of intracellular pressure that powers bleb formation. However, when we K/D NMII in MNs it increased activity-dependent bouton formation, rather than preventing it. Because our movies showed that bouton formation is highly correlated with muscle contraction, we hypothesized that muscle contraction could be sufficient to induce “bouton blebbing” by pressuring the neuron that had a weaken cortex due to NMII IR. Additionally, to try to understand and explain our results, we also looked for the factors that determine bleb formation. Blebs are favored in conditions of increased cortical contractibility, low adhesion and high confinement (Fig 4.1). These variables posed very interesting

possibilities because: 1) MNs are set on a cell that has contractile properties – the muscle; and 2) muscle contraction itself, can influence MNs confinement.

To test our hypothesis that bouton addition can be a mechanical consequence from muscle contraction, we decided to block muscle contraction during the stimulation protocol to see if we could revert NMIIR results. We started by using a reversible GluR antagonist, NASPM, that should prevent muscle contraction without affecting neurotransmission, at least directly or at a short scale. During the stimulation we maintained the larva fully stretched to minimize residual contraction. We chose the lower NASPM concentration reported to be effective in GluR inhibition - 100 µM of NASPM. However, using this concentration most muscles were destroyed, suggesting that larvae were still able to contract resulting in muscles pulling away from the body wall, and consequent destruction of the preparation. Therefore, these experiments at 100 µM of NASPM could not be quantified. To find a condition where the use of this drug resulted in effective blockade of muscle contraction, we tested a range of concentrations (Fig 4.3). At these higher concentrations, even though, some contraction was observed during the protocol, muscles were not so damaged, indicating that muscle activity was indeed attenuated. In the WT, blocking muscle contraction with NASPM lead to a substantial decrease in activity-dependent bouton formation compared with the no NASPM control (Fig 4.3A). This result is in agreement with previous studies showing that ghost bouton formation in response to K^+ stimulation requires muscle depolarization. Likewise mutations in Syt1 that decrease presynaptic NT release or RNAi against the glutamate receptor subunits in muscle also lead to a reduction in ghost bouton frequency[19]. Altogether, these findings indicate that, in addition to presynaptic membrane depolarization by high K^+, activity-dependent bouton formation also requires muscle activation.

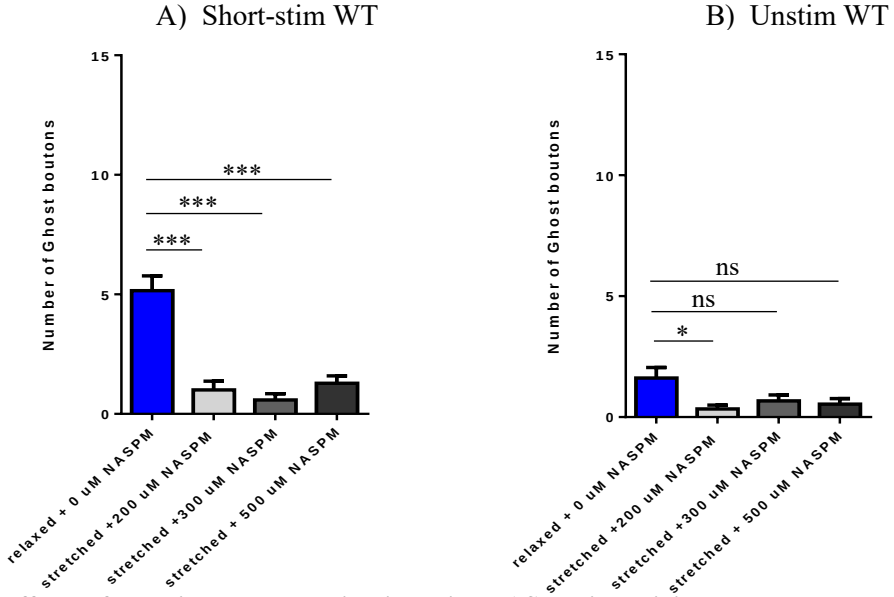


Figure 4.3 - Effects of blocking muscle activation with NASPM in activity-dependent bouton formation in the WT. Blocking muscle contraction with NASPM in the WT decreases bouton formation after stimulation (A) but not during development (B). *p<0.05, ***p<0.001. Error bars represent SEM. HighK^+: data collected from 99 (relaxed + 0µM NASPM), 12 (stretched + 200µM NASPM), 12 (stretched + 300µM NASPM), 14 (stretched + 300 µM NASPM) NMJs. At rest: data collected from 35 (relaxed + 0µM NASPM), 15 (stretched + 200µM NASPM), 15 (stretched + 300µM NASPM), 15 (stretched + 300 µM NASPM) NMJs. ≥3 larvae per line per condition.

We then used NASPM to block muscle activity both in WT and in NMII K/D to ask if elimination of muscle pressure on MNs could revert NMII K/D results. By blocking muscle contraction using 300 uM NASPM there was a decreased activity-dependent bouton formation in both the WT and in NMII K/D (Fig 4.4 C and D). This reduction was similar in both genotypes (Fig 4.4B) further reinforcing that muscle activity is one of the main drivers of structural plasticity at the *Drosophila* NMJ.

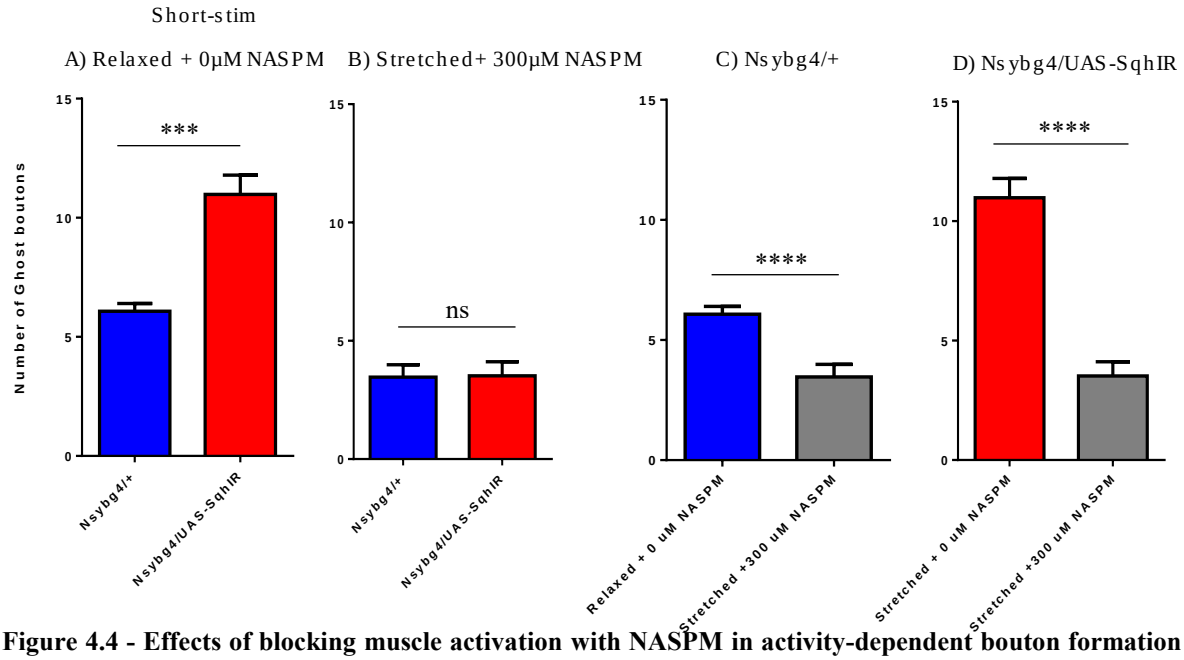


Figure 4.4 - Effects of blocking muscle activation with NASPM in activity-dependent bouton formation in NMII IR. Blocking muscle contraction with NASPM decreases activity-dependent bouton formation in NMIIR and in the control (C and D) to similar levels (B). ****p<0.0001. Error bars represent SEM. Relaxed + 0µM NASPM: data collected from 254 (Nsybg4/+) and 105 (Nsybg4/UAS-SqhIR) NMJs. Stretched + 300µM NASPM: data collected from 21 NMJs. ≥7 larvae per line per conditions.

We reasoned that if NMII can also induce bouton formation independently of muscle contraction, by increasing contractility, we would expect that when NMII CA is expressed in neurons, we would see a smaller effect of NASPM. To test this, we repeated this experiment in neuronally expressed NMII CA and DN. Our preliminary data shows that application of 300 µM NASPM reduced activity-dependent bouton formation in the WT and in SqhDN, but not in SqhCA (Fig 4.5 D and E). On the other hand, in the absence of muscle activity, SqhDN showed a pronounced decrease in bouton formation (Fig 4.5B). Together these findings indicate that, in addition to biochemical mechanisms, mechanical tension acting on MNs may also participate in the regulation of activity-dependent bouton formation.

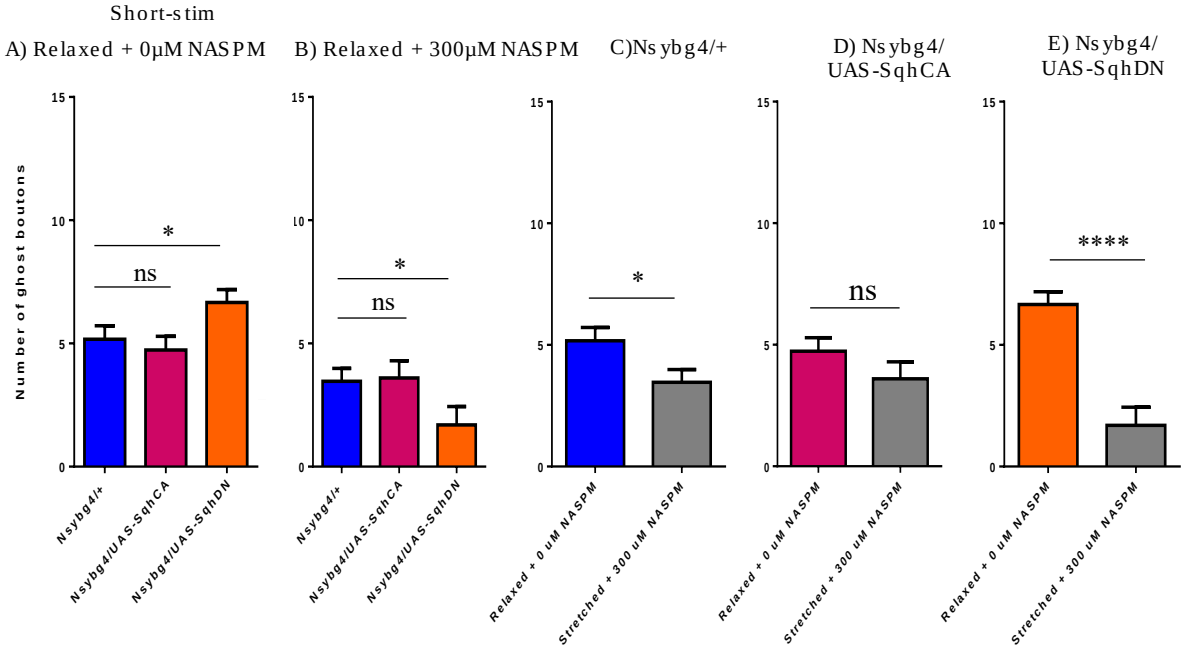


Figure 4.5 - Effects of blocking muscle activation with NASPM in NMII CA and NMII DN. Blocking muscle contraction with NASPM decreased activity-dependent formation in the WT and in NMII DN (C e E). *p<0.05, ****p<0.0001. Error bars represent SEM. Relaxed + 0µM NASPM: data collected from 99 (Nsybg4/+) and 97 (Nsybg4/UAS-SqhCA) and 89 (Nsybg4/UAS-SqhDN) NMJs. Stretched + 300µM NASPM: data collected from 7 (Nsybg4/+), 15 (Nsybg4/UAS-SqhCA) and 13 (Nsybg4/UAS-SqhDN) NMJs. ≥4 larvae per line per condition.

These experiments show that muscle activity is important for activity-dependent bouton formation, but they don't inequivocally show that muscle contraction itself is required for this process of bouton formation. When we block GluRs in the muscle, muscle contraction is prevented but we also interfer with postsynaptic signaling and muscle-neuron communication, which may participate in the regulation of this process. In fact, when the muscle is activated, GluRs mediate postsynaptic Ca^{2+} entry and this influx of Ca^{2+} may lead to the release of a retrograde signal to instruct MNs to initiate rapid structural plasticity. Though it was previously reported that loss of postsynaptic Ca^{2+} sensor Syt4 substantially reduces ghost bouton formation in response to K^+ stimulation, suggesting that Syt4 regulates the release of such signal, our results with Syt4 null ($Syt4^{BA1}$) show no differences in activity-dependent bouton formation compared to control (Fig 4.6). This could be explained if there is redundancy in the machinery necessary for postsynaptic secretion or if Syt4 is not absolutely required for this process, or at short-term, since there are parallel pathways, such as BMP signalling, that have been shown to also be important for acute plasticity.

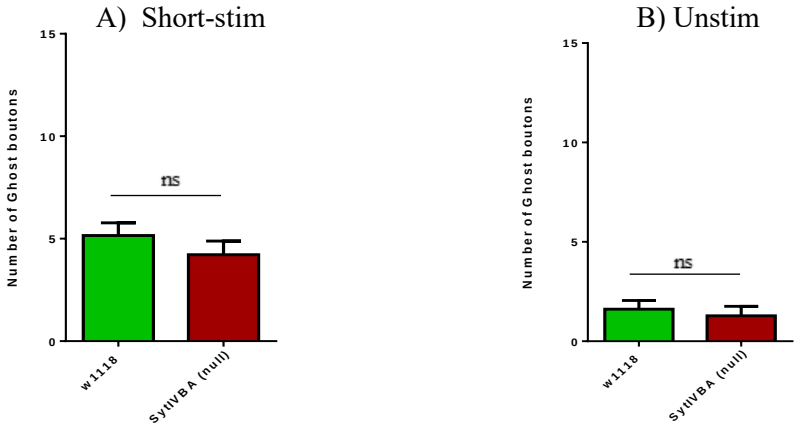


Figure 4.6 - Effects of blocking Syt4-dependent retrograde signaling in activity-dependent bouton formation. Syt null ($Syt4^{BA1}$) MNs do not show altered ghost bouton frequency after acute stimulation (A) and during development (B). Error bars represente SEM. High K^+: data collected from 46 (w1118) and 32 ($SytIV^{BA1}$) NMJs. At rest: data collected from 26 (w1118) and 14 ($SytIV^{BA}$) NMJs. ≥3 larvae per line per condition.

To test if muscle contraction plays an active role in bouton formation we tried to block muscle myosin II, the motor protein required for muscle contraction itself, because in this way we would not interfere with the postsynaptic signaling events. For that we used Blebbistatin, which is a small inhibitor of class II myosins. Normally Blebbistatin blocks muscle and nonmuscle myosin 2, however *Drosophila* NMII is uniquely insensitive to this drug[77]. Moreover, since Blebbistatin had been used in a previous study to block cardiac myosin in *Drosophila* we decided to test if this drug also worked on muscle myosin[78]. The larvae were stretched during the protocol to minimize contraction. We used 100 µM of Blebbistatin, the same concentration that had been previously used by Viswanathan *et*. (2014), and, at this concentration, contraction was visibly reduced during the stimulation and muscles did not show visible signs of tearing. Our preliminary data shows that blocking muscle contraction with Blebbistatin decreased ghost bouton formation in SqhIR and SqhCA, but not in the WT (Fig 4.7 D and E). This result indicates that muscle contraction can indeed promote bouton formation in NMII K/D neurons. However, because blocking muscle contraction does not seem to alter MNs capacity for bouton formation in the WT we suggest that MNs may have a presynaptic mechanism that also generates force that could drive bouton blebbing. Both actomyosin contractility mediated by NMII and/or the actin turnover mediated

by cofilin are possible candidates to drive bouton formation via a presynaptic force. Interestingly, expressing NMII CA in MNs gives the most pronounced reduction in ghost bouton formation, further suggesting that NMII is involved in retraction or maturation of ghost boutons. This also suggests that if NMII participates in bouton initiation it will be by strictly regulating the localization where it is activated and not by having widespread NMII activity, as seen in NMII CA.

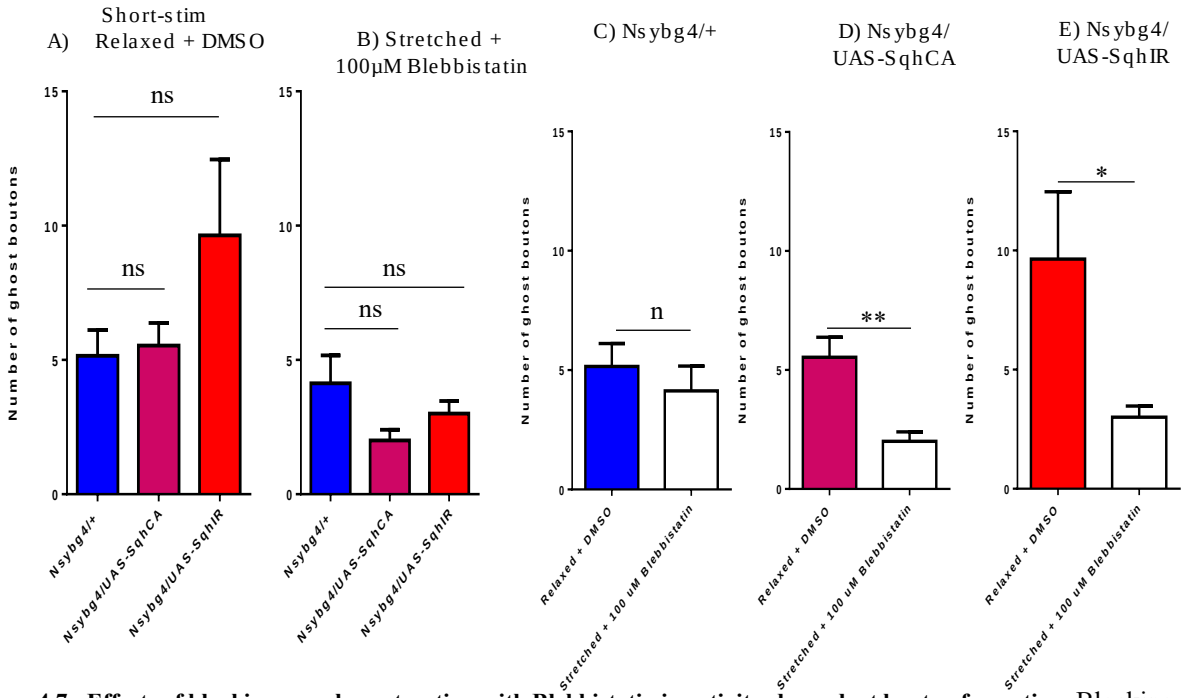


Figure 4.7 - Effects of blocking muscle contraction with Blebbistatin in activity-dependent bouton formation. Blocking muscle contraction with Blebbistatin decreased activity-dependent bouton formation in NMII IR and in NMII CA (D e E), but not in the WT (C). Error bars represent SEM. Relaxed + DMSO: data collected from 13 (Nsybg4/+), 13 (Nsybg4/UAS-SqhCA) and 11 (Nsybg4/UAS-SqhIR) NMJs. Stretched + 100µM Blebbistatin: data collected from 15 (Nsybg4/+), 17 (Nsybg4/UAS-SqhCA) and 16 (Nsybg4/UAS-SqhIR) NMJs. ≥3 larvae per line per condition.

Altogether, our results suggest a very interesting and intricate interplay between MNs and muscle that could possibly regulate activity-dependent bouton formation at *Drosophila* NMJ. We suggest that there are two main opposing forces at the NMJ, one exerting pressure from inside the neuron onto the muscle and the other being muscle contraction that exerts pressure onto the MN. Understanding this interplay between MNs and muscle will be key to predict how to manipulate NMJs to increase neuronal complexity.

4.4 Conclusions

We propose that a balance of mechanical forces and biochemical signaling are probably coordinated during structural plasticity. It is possible that neurons adapted blebbing, a well-known migration mechanism, and combined it with activity-dependent pathways to modulate the addition of presynaptic elements during intense muscle activity. In a way, for the MN to form new functional boutons, it is necessary for MNs to invade the muscle, which mechanistically is not very different from migration into other tissues. On the other hand, muscle contraction can modulate MNs confinement to facilitate or increase the dynamics of bouton formation in situations of increased stress. Additionally, the formation of new boutons in response to elevated levels of activity seems to be highly coordinated between MNs and the muscle.

III. CONCLUSIONS

5 General discussion

5.1 Contributions of my research to the field of synaptic remodeling

Despite increasing evidence that synaptic boutons are important modulators of neuronal output and that presynaptic morphological changes (growth of axonal filpodia and remodeling of boutons) also contribute to activity-dependent plasticity and synaptogenesis, the underlying mechanisms that regulate bouton formation itselft remain mostly unknown to date. In this thesis we wanted to uncover the mechanism by which mature wired neurons form new presynaptic structures. To dissect this mechanism, the *Drosophila* 3rd instar larval NMJ was adopted as a model synapse, since at this stage developmental bouton addition is nearly complete and structural plasticity can be induced acutely in response to elevated neuronal activity. The method employed to induce the rapid formation of new boutons relied on well-established paradigms of pattern depolarization using a solution rich in K^+. Throughout the project, standard methods of *Drosophila* genetics, immunohistochemistry, live-imaging and molecular biology were used to study how boutons form in response to these activity patterns. Analyzing activity-dependent bouton formation, in live and fixed samples, was a powerful way to extract both the dynamics and quantitative information from larvae subjected to acute structural plasticity.

The formation of new synaptic boutons is thought to be governed by highly dynamic processes. By labelling the plasma membrane and components of the cytoskeleton and by performing high-temporal resolution time-lapse imaging it was possible to characterize presynaptic growth in real-time. Additionally, because muscle depolarization has been shown to be required for activity-dependent bouton formation, we did not completely block muscle contraction during imaging. Our experimental analysis at the *Drosophila* NMJ revealed that boutons induced by activity do not form like in the embryonic stage, which uses a growth-cone mediated mechanism that relies on lamellipodia and filopodia. Instead, new boutons resembled morphologically and showed the hallmarks (emerged without actin) of blebbing, another mechanism used by migrating cells. From our movies we also saw a strong correlation between muscle contraction and bouton formation in response to activity, which suggested that the muscle may have a role in this process. Despite detected at low levels at NMJs of WT animals, NMII, which activation is though to be required for the bleb formation, was recruited to new boutons after acute stimulation. However, when we K/D NMII in MNs it increased activity-dependent bouton formation rather than abolishing it. This result was further supported by neuronal expression of NMII DN, which also increased MN's capacity to respond to acute stimulation, and by live-imaging on NMII IR, that indicated that boutons formed faster than in the WT. Given that blebs form in conditions of high contractility, and muscle contraction was often associated with bouton formation, we hypothesized that the muscle may act directly to promote "bouton blebbing" by pressuring the neuron that had a weaken cortex due to NMII IR. To test this hypothesis, we blocked muscle contraction with a GluR blocker, NASPM, and with a myosin II inhibitor, Blebbistatin. Our preliminary data showed that blocking muscle with NASPM or Blebbistatin decreased activity-dependent bouton formation in the WT, however the change was only significant for NASPM. This finding was interesting because, by using NASPM, we disrupted retrograde signaling from the muscle, which was not the case when using Blebbistatin, meaning that uncoupling MN-muscle communication, but not mechanical uncoupling, severily affected MN's capacity to form ghost boutons, further suggesting the existence of a presynaptic mechanism of force generation that act during activity-dependent bouton formation. However, when we used NASPM or Blebbistatin, NMII IR (just like NMII DN) was no longer able to form more boutons compared to control, indicating that muscle force could indeed promote bouton formation. Additionally, these results show that without muscle contraction, reducing NMII levels in MNs impaired bouton formation in response to activity. Because, NMII was dynamically recruited to new boutons, our data suggests a possible role for NMII in the regulation of bouton formation at the NMJ. However, expressing NMII

CA in MNs was not sufficient to increase bouton formation compared to WT suggesting localized activation of NMII may be required to induce bouton initiation, similarly to what has been proposed for blebbing cells. Additionally, because previous studies have shown that actin turnover mediated by cofilin potentiates rapid activity-dependent plasticity at the *Drosophila* NMJ, and, since, cofilin was also shown to control actomyosin assembly and intracellular contractile force generation, it is tempting to speculate that these proteins may act in concert for bouton formation and, that during elevated levels of muscle activity, cofilin may bypass the need of NMII during bouton formation when coupled to muscle contraction (and our results when blocking muscle contraction in NMII IR support this idea). Additionally, NMII can also participate in bouton retraction or stabilization, adding a new layer of complexity to the interpretation of these experiments. Further research will be necessary to elucidate the exact role of NMII, and cofilin, in the regulation of activity-dependent bouton formation.

Overall our data indicates that at the NMJ, besides considering the MN, we need to take into account the muscle, given its close association with the neuron and because it has contractile properties. Our results suggest that there are two main opposing forces at the NMJ, one exerting pressure from inside the neuron onto the muscle, and the other being muscle contraction that exerts pressure on the MN. This MN-muscle dynamic interplay poses interesting relationships between these cells in the regulation of neuronal growth in response to synaptic activity, given that the factors required for blebbing can be regulated by this interplay, namely muscle contraction can influence MNs confinement to promote rapid polarization and further increase bouton formation dynamics. Moreover, because muscle activation, and consequent retrograde signaling events, seem to instruct the formation of new activity-dependent boutons, we propose that a balance of mechanical forces and biochemical signaling are tighly coordinated during structural plasticity upon intense muscle activity. We suggest that MNs may have adopted this migration mechanism, and combined it with, activity-dependent signaling pathways to modulate bouton formation during muscle activity, an environment were MNs confinement is favored. While some mechanisms have been proposed to explain bleb-migration in confined environments, the chimneying model (Fig 4.2B) is particularly interesting because MNs may use a similar strategy to add new boutons during muscle activity. For this kind of movement, the cell must be sufficiently confined to exert significant pushing forces against the substrate and move the cell body into place and the pushing forces may result either from actin polymerization on the wall behind the leading edge or from the intracellular pressure.

5.2 General conclusions

This work has provided new insights into regulation of synaptic bouton formation using the *Drosophila* NMJ as a model system. We report a new mode of neuronal growth, namely of synaptic bouton formation, that relies on a mechanism used by migrating cells – called blebbing – but that has never been described in neurons. Our preliminary data also suggests that a balance of mechanical forces and biochemical signaling is probably coordinated during structural plasticity in response to muscle activity. We propose that MNs may possibly have adopted a strategy that combines this form of migration with activity-dependent signaling pathways to modulate the formation of synaptic boutons during muscle activity, and that muscle contraction may have an active role in triggering or increasing the dynamics of bouton formation in situations of elevated neuronal activity. Future research on this phenomenon, as well as of activity-dependent pathways with a role in neuronal growth, will address open questions and will strengthen our understanding of the regulatory mechanisms underlying presynaptic growth and assembly in mature neurons. This knowlegde will contribute to reveal general principles that link normal function to dysfunction providing a new perception of neuronal disease etiology. Additionally, we hope that the unveiling of this mechanism will open new ways to promote neuronal growth and rewiring in neurons. In the context of neurodegenerative disorders, this could serve as a rehabilitation strategy to recover neuronal function.

References

1. Personius, K. E. & Balice-gordon, R. J. Activity-Dependent Synaptic Plasticity : Insights from Neuromuscular Junctions. *Neurosci.* **8,** 414–422 (2015).
2. Power, J. D. & Schlaggar, B. L. Neural plasticity across the lifespan. *Dev. Biol.* **6,** 1–9 (2017).
3. May, A. Experience-dependent structural plasticity in the adult human brain. *Trends Cogn. Sci.* **15,** 475–482 (2011).
4. Lövdén, M., Wenger, E., Mårtensson, J., Lindenberger, U. & Bäckman, L. Structural brain plasticity in adult learning and development. *Neurosci. Biobehav. Rev.* **37,** 2296–2310 (2013).
5. Dugger, B. N. & Dickson, D. W. Pathology of Neurodegenerative Diseases. (2017).
6. Bernardinelli, Y., Nikonenko, I. & Muller, D. Structural plasticity : mechanisms and contribution to developmental psychiatric disorders. **8,** 1–9 (2014).
7. Waites, C. L. & Garner, G. C. Presynaptic function in health and disease. *Trends Neurosci.* **34,** (2011).
8. Monday, H. R. & Castillo, P. E. Closing the Gap: long-term presynaptic plasticity in brain function and disease. *Curr. Opin. Neurobiol.* **45,** 106–112 (2017).
9. Vactor, D. V & Sigrist, S. J. Presynaptic morphogenesis, active zone organization and structural plasticity in Drosophila. *Curr. Opin. Neurobiol.* (2017).
10. Augustine, J. Synaptic Structure and Function : Dynamic Organization Yields Architectural Precision synapses. **83,** 187–194 (1995).
11. Mcallister, A. K. Dynamic Aspects of CNS Synapse Formation. (2007). doi:10.1146/annurev.neuro.29.051605.112830
12. Wu, H., Xiong, W. C. & Mei, L. To build a synapse: signaling pathways in neuromuscular junction assembly. *Development* **137,** 1017–1033 (2010).
13. Collins, C. A. & Diantonio, A. Synaptic development : insights from Drosophila. doi:10.1016/j.conb.2007.01.001
14. Frank, C. A. *et al.* New approaches for studyng synaptic development, function and plasticity using Drosophila as a model system. *J Neurosci.* **33,** 17560–17568 (2013).
15. Broadie, K. & Bate, M. The Drosophila NMJ : a genetic model system for synapse formation and function. **6,** 221–231 (1995).
16. Zito, K. & Parnas, D. Watching a synapse grow: noninvasive confocal imaging of synaptic growth in Drosophila. *Neuron* **22,** 719–29 (1999).
17. Ataman, B. *et al.* Article Rapid Activity-Dependent Modifications in Synaptic Structure and Function Require Bidirectional Wnt Signaling. 705–718 (2008). doi:10.1016/j.neuron.2008.01.026
18. Vasin, A. *et al.* Synapsin Regulates Activity-Dependent Outgrowth of Synaptic Boutons at the Drosophila Neuromuscular Junction. **34,** 10554–10563 (2014).
19. Piccioli, Z. D. & Littleton, J. T. Retrograde BMP Signaling Modulates Rapid Activity-Dependent Synaptic Growth via Presynaptic LIM Kinase Regulation of Cofilin. **34,** 4371–4381 (2014).
20. Featherstone, D. E. & Broadie, K. Surprises from Drosophila : Genetic mechanisms of synaptic development and plasticity. **53,** 501–511 (2001).
21. Y, Z., V, B. & CF, W. Synaptic plasticity in Drosophila memory and hyperexcitable mutants: role of cAMP cascade. *J. Neurosci.* (1992).
22. Fuentes-medel, Y. *et al.* Glia and Muscle Sculpt Neuromuscular Arbors by Engulfing Destabilized Synaptic Boutons and Shed Presynaptic Debris. **7,** (2009).
23. Kerr, K. S. *et al.* Glial Wingless/Wnt regulates glutamate receptor clustering and synaptic physiology at the Drosophila Neuromuscular Junction. *J. Neurosci.* 2010–2920 (2014).
24. Alicea, D., Perez, M., Maldonado, C., Dominicci-cotto, C. & Marie, B. Cortactin is a regulator of activity-dependent synaptic plasticity controlled by Wingless. *J. Neurosci.* (2017).
25. Cho, R. W. *et al.* Phosphorylation of Complexin by PKA regulates activity-dependent spontaneous neurotransmitter release and structural synaptic plasticity. *Neuron* (2015).
26. Nesler, K. R., Starke, E. L., Boin, N. G., Ritz, M. & Barbee, S. A. Presynaptic CamKII regulates activity-dependent axon terminal growth. *Mol. Cell. Neurosci.* 33–41 (2016).

27. James, R. E. *et al.* Crimpy enables discrimination of pre and postsynaptic pools of a BMP at the Drosophila NMJ. *Dev. Cell* **31,** 586–598 (2014).
28. Harris, X. K. P., Zhang, Y. V, Piccioli, Z. D., Perrimon, N. & Littleton, J. T. The postsynaptic t-SNARE Syntaxin 4 controls traffic of Neuroligin 1 and Synaptotagmin 4 to regulate retrograde signalling. *Elife* (2016).
29. Menon, K. P., Carrilo, R. A. & Zinn, K. Development and plasticity of the Drosophila larval NMJ. *Wiley Interdiscip. Rev. Dev. Biol.* **2,** 647–670 (2013).
30. Park, Y. K. & Goda, Y. Integrins in synapse regulation. *Nat. Publ. Gr.* (2016). doi:10.1038/nrn.2016.138
31. Tsai, P. *et al.* Activity-dependent retrograde laminin A signaling regulates synapse growth at Drosophila neuromuscular Junctions. *PNAS* **109,** 17699–17704 (2012).
32. Lee, J. Y. *et al.* Activity-induced synaptic structural modifications by an activator of integrin signaling at the Drosophila neuromuscular junction. *J Neurosci.* (2017).
33. Vicente-Manzanares, M., Webb, D. J. & Horwitz, A. R. Cell migration at a glance. *Cell Sci. a Glance* (2005).
34. Lundquist, E. The finer points of filopodia. *PLoS Biol.* (2009).
35. Devoto, S. H. Neuronal growth cone migration. *Experientia* (1990).
36. Suter, D. M. Live cell imaging of neuronal growth cone motility and guidance in vitro. *Methods Mol Biol.* (2011).
37. Lin, C. H., Espreafico, E. M., Mooseker, M. S. & Forscher, P. Myosin drives retrograde F-actin flow in neuronal growth cones. *Neuron* (1996).
38. Ziv, N. E. & Smith, S. J. Evidence for a Role of Dendritic filopodia in Synaptogenesis and Spine formation. *Neuron* (1996).
39. Ozcan, A. S. Filopodia: A rapid structural plasticity substrate for fast learning. *Front. Synaptic Neurosci.* (2017).
40. V, D. P. *et al.* Cell type-specific structural plasticity of axonal branches and boutons in the adult neocortex. *Neuron* 861–75 (2006).
41. Stettler, D. D., Yamahachi, H., Li, W., Denk, W. & Gilbert, C. D. Axonal and synaptic boutons are highly dynamic in adult visual cortex. *Neuron* **49,** 877–887 (2006).
42. Colicos, M. A., Collins, B. E., Sailor, M. J. & Goda, Y. Remodeling of Synaptic Actin Induced by Photoconductive Stimulation. *Cell* **107,** 605–616 (2017).
43. Nikonenko, I., Jourdain, P. & Muller, D. Presynaptic remodeling contributes to activity-dependent synaptogenesis. *J. Neurosci.* 8498–8505 (2003).
44. Charras, G. & Paluch, E. Blebs lead the way : how to migrate without lamellipodia.
45. Waters, S. *et al.* Cellular blebs : Pressure-driven , axisymmetric , membrane protrusions protrusions. (2013). doi:10.1007/s10237-013-0509-9
46. Tinevez, J., Schulze, U., Salbreux, G., Roensch, J. & Joanny, J. Role of cortical tension in bleb growth. (2009).
47. Charras, G. T., Hu, C., Coughlin, M. & Mitchison, T. J. Reassembly of contractile actin cortex in cell blebs. **175,** 477–490 (2006).
48. Charras, G. T., Coughlin, M., Mitchison, T. J. & Mahadevan, L. Life and Times of a Cellular Bleb. **94,** 1836–1853 (2008).
49. Fackler, O. T. & Grosse, R. Cell motility through plasma membrane blebbing. 879–884 (2002). doi:10.1083/jcb.200802081
50. Newell-litwa, K. A., Horwitz, R. & Lamers, M. L. Non-muscle myosin II in disease : mechanisms and therapeutic opportunities. 1495–1515 (2015). doi:10.1242/dmm.022103
51. Kneussel, M. & Wagner, W. Myosin motors at neuronal synapses : drivers of membrane transport and actin dynamics. (2013). doi:10.1038/nrn3445
52. Amoeboid, F. *et al.* Cortical Contractility Triggers a Stochastic Switch to Article Cortical Contractility Triggers a Stochastic Switch to Fast Amoeboid Cell Motility. 673–685 (2015). doi:10.1016/j.cell.2015.01.008
53. Diz-muñoz, A., Romanczuk, P., Yu, W., Bergert, M. & Ivanovitch, K. Steering cell migration by alternating blebs and actin-rich protrusions. *BMC Biol.* 1–13 (2016). doi:10.1186/s12915-016-0294-x
54. Sugiyama, T., Pramanik, K. & Yumura, S. Microtubule-Mediated Inositol Lipid Signaling Plays

Critical Roles in Regulation of Blebbing. 1–20 (2015). doi:10.1371/journal.pone.0137032
55. Ridley, A. J. Review Life at the Leading Edge. *Cell* **145,** 1012–1022 (2011).
56. Charras, G. T. A short history of blebbing. **231,** 466–478 (2008).
57. Paluch, E. K. & Raz, E. The role and regulation of blebs in cell migration. *Curr. Opin. Cell Biol.* **25,** 582–590
58. Larsen, A. K. *et al.* Genetic disruption of calpain correlates with loss of membrane blebbing and differential expression of RhoGDI-1 , cofilin and tropomyosin. **666,** 657–666 (2008).
59. Sugimoto, A., Friesen, P. D. & Rothman, J. H. Baculovirus p35 prevents developmentally programmed cell death and rescues a ced-9 mutant in the nematode Caenorhabditis elegans. *EMBO J.* **13,** 2023–2028 (1994).
60. Vicente-Manzanares, M., Ma, X., Adelstein, R. S. & Horwitz, A. R. Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nat. Rev. Mol. Cell Biol.* (2009).
61. Chi, Q. *et al.* Rear actomyosin contractility-driven directional cell migration in three-dimensional matrices: a mechano-chemical coupling mechanism. *J. R. Soc. Interface* **11,** (2014).
62. Charras, G. T., Yarrow, J. C., Horton, M. A., Mahadevan, L. & Mitchison, T. J. Non-equilibration of hydrostatic pressure in blebbing cells. *Nature* **435,** 365–369 (2005).
63. Gadea, G., de Toledo, M., Anguille, C. & Roux, P. Loss of p53 promotes RhoA–ROCK-dependent cell migration and invasion in 3D matrices. *J. Cell Biol.* **178,** 23–30 (2007).
64. Sahai, E. & Marshall, C. J. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat Cell Biol* **5,** 711–719 (2003).
65. Godin, C. M. & Ferguson, S. S. G. The Angiotensin II Type 1 Receptor Induces Membrane Blebbing by Coupling to Rho A, Rho Kinase, and Myosin Light Chain Kinase. *Mol. Pharmacol.* **77,** 903 LP-911 (2010).
66. Meshki, J. *et al.* Neurokinin 1 Receptor Mediates Membrane Blebbing in HEK293 Cells through a Rho/Rho-associated Coiled-coil Kinase-dependent Mechanism. *J. Biol. Chem.* **284,** 9280–9289 (2009).
67. Chugh, P. *et al.* Actin cortex architecture regulates cell surface tension. *Nat Cell Biol* **19,** 689–697 (2017).
68. Silva, M. S. e *et al.* Active multistage coarsening of actin networks driven by myosin motors. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 9408–9413 (2011).
69. Lenz, M., Thoresen, T., Gardel, M. L. & Dinner, A. R. Contractile Units in Disordered Actomyosin Bundles Arise from F-Actin Buckling. *Phys. Rev. Lett.* **108,** 238107 (2012).
70. Blanchoin, L., Boujemaa-paterski, R., Sykes, C. & Plastino, J. Actin dynamics, architecture, and mechanics in cell motility. 235–263 (2014). doi:10.1152/physrev.00018.2013
71. Wiggan, O., Shaw, A. E., DeLuca, J. G. & Bamburg, J. R. ADF/Cofilin regulates actomyosin assembly through competitive inhibition of myosin II binding to F-actin. *Dev. Cell* **22,** 530–543 (2012).
72. Lee, G. & Schwarz, T. L. Filamin, a synaptic organizer in Drosophila, determines glutamate receptor composition and membrane growth. *Elife* **5,** e19991 (2016).
73. Külshammer, E. & Uhlirova, M. The actin cross-linker Filamin/Cheerio mediates tumor malignancy downstream of JNK signaling. *J. Cell Sci.* **126,** 927–938 (2013).
74. Ramel, D., Wang, X., Laflamme, C., Montell, D. J. & Emery, G. Rab11 regulates cell-cell communication during collective cell movements. *Nat. Cell Biol.* **15,** 317–324 (2013).
75. Langevin, J. *et al.* Drosophila exocyst components Sec5, Sec6, and Sec15 regulate. *Dev. Cell* **9,** 365–376 (2005).
76. Paluch, E. K., Aspalter, I. M. & Sixt, M. Focal Adhesion – Independent Cell Migration. 1–22 (2016). doi:10.1146/annurev-cellbio-111315-125341
77. Heissler, S., Chinthalapudi, K. & Sellers, J. Kinetic characterization of the sole nonmuscle myosin-2 from the model organism Drosophila melanogaster. *FASEB j.* (2015). doi:10.1096/fj.14-266742
78. Viswanathan, M. C., Kaushik, G., Engler, A. J., Lehman, W. & Cammarato, A. A Drosophila Melanogaster Model of Diastolic Dysfunction and Cardiomyopathy Based on Impaired Troponin-T Function. *Circ. Res.* **114,** e6-17 (2014).

IV. APPENDICES

I – Materials and Methods

i. *Drosophila* Stocks

Flies were cultured at 25ºC and maintained on standard media. Crosses were set with the minimum of 5-10 virgin females and 3-5 males of the appropriate stocks. Adults were removed 7-8 days after each cross to ensure segregated generations. For larval collection, eggs were laid and grown on apple juice plates and yeast paste at 25ºC. For RNAi experiments, collection of RNAi expressing strains and their controls were set up at 29-30ºC to maximize the efficiency of knockdown. The TM6b balancer chromosome was used to facilitate genotyping of larva (non-tubby larvae were selected). The w1118 line served as a control genotype, as the stocks used are in this genetic background. For tissue-specific transgene expression Nsyb-Gal4 (Pan-neuronal driver) was used to drive UAS-constructs' expression in neurons. Transgenic UAS-lines used, UAS-driven RNAi lines, mutant alleles and recombinant and other lines used are describe in tables bellow. As a control, we used nSyb-Gal4 crossed with w1118.

Table 1 -UAS-transgenic lines used

Name	Description	Reference
UAS-ZipIR	Expresses RNAi against Zipper (heavy chain of NMMII) under the control of UAS; on chromosome 3	BDSC #36727
UAS-SqhIR	Expresses RNAi against Spaghetti Squash (regulatory light chain of NMMII) under the control of UAS; on chromosome 3	BDSC #32439
UAS-SqhCA	Expresses a phosphomimetic sqh protein with T20E and S21E aa substitutions under the control of UAS; on chromosome 3	BDSC #64411
UAS-SqhDN	Expresses a nonphosphorylatable sqh protein with T20A and S21A aa substituitons under the control of UAS; on chromosome 3	BDSC #64114
UAS-p35	Expresses the anti-apoptotic protein p35 under the control of the UAS; on chromosome 3	BDSC
UAS-RokIR	Expresses RNAi against Rho kinase under the control of UAS; on chromosome 3	BDSC #34324
UAS-Strn-MlckIR	Expresses the RNAi against Stretchin (MLCK) under the control of UAS; on chromosome 2	BDSC #37523
UAS-CherIR	Expresses RNAi against Cheerio (filamin) under the control of UAS; on chromosome 3	BDSC #26037
UAS-TsrIR	Expresses RNAi against Twinstar (cofilin) under the control of UAS; on chromosome 3	BDSC #65055

Note: BDSC stands for Bloomington Stock Center.

Table 2 -Mutant lines used

Name	Description	Reference
$SytIV^{BA1}$	Functionally null allele	(Yoshihara et al.,2005) /Troy Littleton Lab

Table 3 - Recombinants and other lines used

Label	Genotype/Description	Reference
Nsyb-Gal4, CD4Tom	Expresses Tomato tagged CD4 in neurons; on chromosome 3 N	This study
Nsybg-Gal4, CD8GFP	Expresses GFP tagged CD8 in neurons; on chromosome 3	This study
Nsyb-Gal4, CD4GFP	Expresses GFP tagged CD4 in neurons; on chromosome 3	This study
Sqh-mCherry	Expresses Cherry tagged sqh protein under the control of the sqh native promoter; on chromosome 3	BDSC
Sqh-GFP (FlyFos)	Expresses GFP tagged sqh at endogenous levels; on chrosome 3	BDSC
Zip-GFP (FlyTrap)	Expresses GFP tagged zipper at endogenous levels; on chromosome 3	BDSC
UAS-LifeactRuby	Ruby tagged Lifeact (stains F-actin); on chromosome 3	BDSC
UAS-LifeactGFP	GFP tagged Lifeact (stains F-actin); on chromosome 3	BDSC

Note: BDSC stands for Bloomington Stock Center.

ii. Immunohistochemistry

In preparation for immunohistochemistry and imaging of the neuromuscular junction, 3rd instar larvae were dissected in PBS (phosphate-buffered saline) using a procedure similar to Brent *et.* (2009). Gut and fat body were removed, while the CNS was kept intact until after fixation. The resulting larval fillets were fixed either with Bouin's fixative (saturated picric acid + formaldehyde + glacial acetic acid) or with PFA (4% paraformaldehyde diluted in 1x PBS) at room temperature for 5 and 20 min, respectively, then extensively washed in PBT (1x PBS + 0,3% Triton) to permeabilize membranes. Blocking of unspecific binding was done incubating 30 min-1 hour with NGS (Normal Goat Serum) dissolved in PBT. Primary antibody incubation was performed overnight at 4ºC, in blocking solution. Subsequently, larvae were extensively washed using PBT, followed by blocking for 30min-1h and incubated for 2 hours with secondary antibody at room temperature. After extensive washing using PBT larvae were transferred to 50% glycerol in PBS for 5 min and then mounted onto to slides in DABCO (100% glycerol in PBS) as mounting medium, covered with coverslips which were then sealed using nail polish. Slides were stored at -20ºC until ready to image.

Table 4 - Primary antibodies used

Antigen and clone ID	Species	Concentration	Fixative	Reference/Source
Dlg(4F3)	Mouse	1:250	Bouin's	Hibridoma bank
Dlg	Rabbit	1:10000	PFA	Vivian Budnik Lab
Filamin (N terminus/aa 189-482)	Rat	1:800	Bouin's	(Külshammer and Uhlirova, 2013) / M. Uhlirova Lab
GFP	Rabbit	1:1000	For SqhGFP PFA	Life Technologies A11122
Myosin 2	Rabbit	1:1000	PFA	Gift of Christine Field (Harvard Medical School)

Table 5 –HRP conjugated secondary antibodies used

Antigen	Concentration	Source
Cy3-conjugated goat anti-HRP	1:500 (initial dilution in 50% glycerol, according to manufacturer indications)	Jakson Immunoresearch
A488-conjugated goat anti-HRP		
DL647-conjungated goat anti-HRP		

Table 6 – Secundary antibodies used

Antigen	Concentration	Source
A488-Donkey anti-Mouse (used for Dlg)	1:500 (initial dilution in 50% glycerol, according to manufacturer indications)	Jakson Immunoresearch
RhRx-Donkey anti-Mouse (used for Dlg)		
A647-Donkey anti-Mouse (used for Dlg)		
A488-Donkey anti-Rabbit (used for MyoII)		
A647-Donkey anti-Rabbit (used for NMII or GFP)		
A488-Donkey anti-Rabbit XR (used for Dlg)		
Cy3-Donkey anti-Rat (used for filamin)		

iii. Acute-induction of activity dependent boutons

Third instar larvae were pinned down onto Sylgard-coated plates using insect pins and partially dissected in HL3.1 saline solution (in mM: 70 NaCl, 5 KCl, 0.1 CaCl2, 4 MgCl2, 10 NaHCO3, 5 Trehalose, 115 Sucrose, 5 HEPES-NaOH, pH 7.3-7.4) at room temperature. Importantly, prior stimulation the dissection pins were moved inward to the same guide shape at 60% of the original size of each larva to allow for muscle contraction. We used two well-established protocols to induce activity-dependent bouton formation using spaced High K^+ depolarization paradigms that we call Short-Stim (SS) and Long-Stim (LS). These paradigms were developed by Vasin et al., 2015 and Ataman et al., 2008, respectively, and both induce new boutons at the Drosophila larval NMJ (in our hands at NMJ muscle 6/7 SS ±5.5 and LS±8 new boutons). Briefly, relaxed fillets were subjected to incubations in high K+ (90 Mm) and High Ca2+ (1.5 Mm) HL3.1 adjusted for osmolarity changes with 2, 2, 2 minute pulses each separated by 10 min incubation in normal HL3.1 (SS) or 2, 2, 2, 4 and 6 min pulses each separated by a 15 min incubation in normal HL3.1. For immunohystochemistry larvae were fixed after 30 min (SS) or 54 min (LS) to maximize bouton formation in the respective paradigms.

Control larvae (Non-Stim, NS) are dissected and incubated using the same protocol as above but with only normal-HL3.1. The protocol for acute stimulation of boutons used for live imaging is identical to the ones described above. Briefly, third instar larvae are dissected and glued directly onto a Sylgard

coated slide and imaged right after (or during) stimulation. During the dissection larvae are stretched but not excessively still allowing for muscle contraction. The ventral nerve cord and the CNS were maintained intact, unless otherwise stated.

iv. Confocal imaging and data analysis

Confocal images were obtained on a Laser scanning confocal microscope (LSM 710) with a 40x 1.3 NA water-immersion objective or a 63x 1.3NA oil-emersion objectives (Carl Zeiss). Images were processed in image J (National Institutes of Health) and Adobe Photoshop software. The live-imaging experiences were performed with a spinning Disk confocal microscope (Andor) with a 60X 1.3 NA oil-immersion objective (Carl Zeiss). Quantification of bouton number was performed at NMJ 6/7, the most studied, from abdominal segments A2-A4 were analyzed. In general, at least 12 (fixed) or 10 (live) NMJs of each genotype were analyzed for each time-point.

Quantification of imunohystochemistry

For analysis of bouton formation and immuno-localization in fixed samples, maximum intensity projections from z-stacks were used. The process was semi-automated using macros (for one, two or three channels) on image J. The macro used is summarized as follows:

1) Image > Stacks > Z-project
    a. Max intensity projection
2) Image > Color > Channels tool
    a. Make composite
3) Images were saved as JPEG

Images used in this thesis were cropped and mounted in Photoshop (bouton quantification) or in Power-Point (immuno-localizations).

Video analysis

For analysis of live bouton formation, maximum intensity projections from z-stacks were used. The stacks were imported into image J using a plugin called Bio-formats for video mounting and editing, as follows:

1) File > Import > Bio-formats
    a. View stack with Hyperstack
2) Image > Stacks > Z-project
    a. Max intensity projection
    b. All slices and all time frames
3) Image > Stacks > Concatenate (if video was saved in separate files)
4) Image > Color > Split channels (if more than one channel)
5) Process > Enhance contrast
    a. 0.1 to 0.3 % saturated pixels
    b. normalize and process all slices
6) Image > Color > Merge channels (if more than one channel)
7) Stack > Animation > Animation options
    a. Speed at 15 fps
8) Rectangular selection > Image > Duplicate (to crop in a specific range of the stack)
9) Videos were saved as AVI.

To make the time-lapse images from the videos, selected frames were duplicated and saved as TIF. A scale bar was added in the last frame. Then, images were edited in Photoshop as it follows:

1) Image > Image size
    a. Resolution 300 Pixels/Inch

b. Resample: automatic

2) Crop tool > 1X1 Inch
3) Brightness/Contrast (optional)
4) Images were copy and mounted on a new document
    a. U.S. paper with resolution of 300 Pixels/Inch
5) Time-lapse was saved as JPEG.

v. Drug Administration

To assess the effects of muscle inactivation in new bouton formation we used a 100 mM solution of 1-Naphtylacetil spermine (NASPM) trihydrochloride dissolved in H_20 that was diluted to the desired concentration (mostly 300 uM NASPM solution) in HL3.1 and the same was done for High K^+. The solution HL3.1 containing NASPM was incubated with the preparation in the dark for 30 min prior to stimulation protocol. We also used NASPM (Sigma, N193) during the stimulation protocol. During stimulation the microscope light was turned off to minimize light exposure to the drug. Muscle contraction was also blocked with a 100 uM Blebbistatin solution (-) (Sigma) which was prepared from a 5 mM stock solution dissolved in DMSO, diluted to the desired Blebbistatin solution in HL3.1. In this case DMSO was also added to the control solution of HL3.1 at the same dilution. Blebbistatin was incubated with the preparation for 30 min prior to stimulation protocols and it was also present during the stimulation protocol.

vi. Statistical analysis

All analysis was completed in GraphPad Prism 6.0. Data were tested for normality using D'Agostino & Pearson omnibus normality test. When data sets passed the normality test, statistical significance in two-way comparisons was determined by a Student's *t*-test, while ANOVA analysis was used when comparing more than two datasets. When normality was not verified for all comparing data sets, statistical significance in two-way comparisons was determined by Mann-Whitney test, while Krukal-Wallis analysis was used when comparing more than two datasets. In all figures, the data is presented as mean ± standard error of the mean (SEM); **** $p<0.0001$; *** $p<0.001$ ** $p<0.01$, * $p<0.05$, n.s. not significant. Statistical comparisons are with control unless noted. Sample size (n) is presented in figure legend.

II- Future research

i. Dissect the pathway(s) used for activity-dependent bouton formation

Several pathways have been reported to play a role in bleb initiation, some of which converge on NMII activation (Fig 3.1). We found that NMII contributes to bouton formation, but we need to uncover what activates NMII and whether this is the only pathway that contributes to bouton formation. We are already testing these genetic cascades and we will continue to explore core components involved in blebbing and assay their effect in bouton formation. By systematically interfering with these pathway, we will isolate if there is a preferred route to bouton formation. Since not much is known about the localization and regulation of the genes in the bleb pathway at the NMJ, we have to analyze this. We will do antibody staining for the relevant players in blebbing, before and after stimulation. By using fluorescently-tagged versions of these proteins, we will observe their behavior live, after stimulation. We will also score the participation of genes linked to activity-dependent bouton formation by stimulating the larvae and quantifying different genotypes compared to control. Genes showed to be involved in this process will also be analyzed for the dynamics. We will follow-up these studies by genetic and pharmacologic manipulation of key pathways to evaluate how neuronal growth can be perturbed. This will allow us to complete our understanding of the pathways that regulate blebbing in

the context of bouton formation (specially if the mechanism adapts only parts of bleb mechanism or uses different players to achieve a similar output) and may establish blebbing as a novel mechanism of activity-dependent bouton formation. This understanding will be relevant to design strategies to manipulate bouton formation and increase neuronal complexity while maintaining an operational system.

ii. Explore the contribution of MNs and muscle to activity-dependent plasticity

Our data suggested a dynamic interplay between MNs and the muscle in the regulation of bouton formation in response to activity. This MN-muscle interplay poses interesting possibilities, given that the factors required for blebbing can be regulated by this interplay. Further analysis of how this interplay can influence synaptic growth at the NMJ will allow us to systematically dissect the contribution of MNs and muscle for activity-dependent bouton formation. Specfically, we want to answer: i) Is muscle activation/contraction required for this mechanism? ii) Can neuronal cortical actin rupture/displacement be coupled with internal pressure or muscle contraction? iii) How does the muscle membrane accommodate the displacement imposed by the presynaptic bouton? iv) Can we alter the mode/frequency of bouton formation by changing the levels of the neuronal adhesion molecules? To test these hypothesis, we will manipulate confinement, contractibility, and adhesion independently in the MN and in the muscle to access how the system evolves, and whether we can predict how boutons will be formed in response to protocols that acutely induce structural plasticity. We manipulated contractibility in MN by either reducing the levels of NMII (NMII-IR) or expressing an activate form of NMII (NMII-CA). To influence MNs confinement we already blocked muscle contraction (using GluRs and MHC inhibitors) and promoted neuronal activation (High K^+ stimulation). To further manipulate MNs confinement we will: a) activate muscle contraction (CsChrimson in muscle) and block neuronal activation (Anion Channelrhodopsin or Tetrodotoxin (TTX) in MN); b) use MHC mutants with altered functional properties leading to hypo (less confinement) and hypercontractile (more confinement) muscles; c) test mutants with bigger and smaller postsynaptic membrane elaboration, to assess whether the SSR facilitates or obstructs bouton addition. We will manipulate adhesion by changing the levels of the neuronal adhesion molecule Fasciclin II (FasII), in a MN or muscle specific manner. We chose FasII because hyperexcitable mutants downregulate FasII levels, leading to increased number of boutons. Also, small changes in pre-postsynaptic ratios of adhesion have a big impact on structure. To manipulate adhesion, we will test: a) FasII mutants (less adhesion); b) FasII over-expression in MN, in muscle, in MN and muscle; c) MN or muscle specific FasII overexpression in FasII mutant-background.

iii. Test the coupling between mechanical forces and biochemical signaling in the induction of activity-dependent boutons

Our model for presynaptic structural plasticity at the NMJ is based on a balance of mechanical forces and biochemical signaling between the MN and the muscle. To test this we will combine factors possibly associated with the bleb nucleation phase, namely factors that promote local actin disassembly (local activation of NMII and possible role of ADF / Cofilin) or cortex displacement (reorganization ERM proteins like moesin and actin crosslinking proteins such as filamin) with different levels of muscle activity and test their contribution to the process. Considering that bleb nucleation is not so well characterized, and neurons may have adopted a similar strategy using other pathways, we will test the genes linked to activity that scored positive for bouton formation at the NMJ has candidate genes for the initiation of the biochemical events in coordination with the confinement of the muscle. These results will be relevant to the understanding of the process not only mechanically but how it is regulated biochemically and will allow the development of strategies to manipulate neural structure that can possibly be adapted to other systems.