Calcium role in activity dependent bouton formation

Pedro Miguel Matias Augusto

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Biomedical Research

Dissertação para obtenção do grau de Mestre em Investigação Biomédica

at Faculdade de Ciências Médicas | NOVA Medical School of NOVA University Lisbon

September, 2019

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Supervisors: Rita O. Teodoro, Prof. Auxiliar Convidada and Group Leader at CEDOC

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Acknowledgments

I cannot imagine a better person to start my thanks than my supervisor Dr. Rita O.Teodoro. When I was looking for a lab to be just a trainee and have some contact with neuroscience, you welcomed me with opened arms. You motivated me to stay in the lab, and deciding to work with you was one of the best decisions of my life. Your kindness and enthusiasm are contagiating, putting everyone in a good mood and eager to work. The naturality with wich you discuss science has really inspired me to a better job every day. More than just my supervisor, you have been a mentor both in the lab and in my personal life. I am grateful every day for the moment you accepted me in the lab; my only wish is that this thesis and future work make you proud.

I would also like to thank all the team in the lab for the amazing support. Cátia e Joana, you were the first ones to teach me everything in the lab, from matting flies, to dissect larvae, to imunos. Your patience was never ending, even when I asked for help from 10 in 10 minutes. João, you are a really altruistic person and have always gave me good advices. Mariana, you are a really introspective person and I have always enjoyed our talks. Margarida, for being an outstanding rotation student who helped me a lot during a few weaks. Andreia, your work rhythm is just unbelievable and I admire your passion for your project; it is one of the reasons I chose to be part of it. You have been not only a great teacher, but also a good friend. Vicente, my good man, for all the presentations, talodalinas, reports, crawling assays, arenas, idTrackeraiai, and many more; I salute you. I think you will agree when I write that it has been a pleasure to work with you for the last two years. If you do not agree, oh well, I will remove these sentences in a future version. I could always count on you and for that I thank you. I wish you all the best of luck and success.

A special thanks to everyone organizing/teaching/attending the Master's NBR, it has exceled all my expectations. To Paulo Pereira for taking this initiative of such a challenging but rewarding master degree. You have been always ready to help the students the best you could. To all my colleagues that have help me to grow not only as a scientist but also as a person, to the best class of the NBR of all time.

Special thanks to other people in CEDOC. I would like to thank Telmo for his supreme capacity of solving almost any tech problem that comes in harms' way of our experiments. I thank also César Mendes and Susana Ponte for critical discussion of the project

I would like to thank to all other people in CEDOC/NMS that have contributed directly or indirectly to this work.

Truth is CEDOC has been my second home for along time and work can sometimes pull people apart. Good friends were lost, but life carries on just the same. Nevertheless, to those who helped me to unburden all my worries and problems, you have my profound gratitude. Thanks to the Jean-Pierres who were the best to provide a fun time and forget about work. Thanks to Narsos who have been with me almost as long as I remember and have helped me to overcome adversities in life.

Although a little beat cliché, I must really say that my family has been my number one fan. Without a dough I dedicate this thesis to my parents. Their support is something I will cherish for all my life that I can only hope to retribute in a near future. They have provided more than any son could ever want and they are the reason I am pursuing my childhood dream of becoming a scientist. To my grandmother and grandfather that have been my beacons of inspiration to keep up the hard work no matter the adversities.

Last but not the least, a huge thanks to my girlfriend Mariana Guarda. What an adventure this has been, and what a bigger one I hope this will be. You stood by me when moments were most difficult, even though sometimes you had bigger issues going on. You taught me so much along this year that I can only say I wish it didn´t passed so quickly. The strength you have, moves me every time. For all the nights staying up late to help me with my figures, I cannot thank you enough. I am so proud of you.

Abstract

Synaptic plasticity is an intrinsic mechanism of the nervous system essential for modifying existing and creating new synaptic connections, which underlie complex processes like learning and memory. Neuronal connectivity is mostly genetically determined; however, neurons can also reshape their morphology by adding or eliminating synaptic structures in response to activity. Defects in this structural plasticity have been reported to be the cause of several neurodegenerative disorders. Although some molecular players have been identified, how acute activity induces structural changes and how deficiencies in these mechanisms can lead to disease is far from being understood. Therefore, it is our goal to study how neurons change their structure in response to activity and to identify the pathways that regulate this process.

We use the neuromuscular junction of *Drosophila melanogaster* as a model to understand mechanisms of structural plasticity, because it has stereotypical morphology but exhibits robust plasticity, allowing the study of how neurons grow and remodel. Previous data from the lab indicates that new pre-synaptic structures (synaptic boutons) emerge rapidly (in seconds) with a bulky round morphology, associated with intense muscle contraction. We further showed that, unlike a growth cone mediated migration, formation of activity-dependent boutons much more resembles membrane blebbing. Blebs are pressure-driven membrane protrusions resulting from the contraction of the actomyosin cortex through myosin motors. Some studies showed that high contractibility is accompanied by cyclic variations of intracellular Ca^{2+} and even in some cases this increase in Ca^{2+} precedes blebbing. Considering this, we asked if Ca^{2+} could be the signal initiating local bouton formation by providing temporal and space specificity.

To test this hypothesis, we did live imaging of 3rd instar larvae expressing a neuronal membrane tag together with the genetically encoded calcium sensor (GCamP6f) and performed acute patterned depolarization protocols using High K^+ High Ca^{2+} solutions. These protocols are well described to induce presynaptic remodeling a few minutes after application. We observed that every event of bouton formation and retraction is preceded by a sudden very large increase in intracellular Ca^{2+}. The difference between these two phenomena was that while in bouton formation, GCaMP6f signal is sustained in the new bouton, in bouton retraction the signal dissipates rapidly after the burst. Since it is known that Ca^{2+} elevations can regulate actomyosin contraction, these results suggest that bouton formation and retraction might have a common trigger mechanism through actomyosin regulation. The fact that new boutons have a sustained elevated GCaMP6f signal (as opposed to retracted ones) might reflect ongoing cytoskeleton remodeling or vesicle recruitment, which have been proved to be essential processes for activity-dependent bouton formation.

To dissect the source of the Ca^{2+} signal, we manipulated extracellular Ca^{2+} concentrations and Ca^{2+} channels in motor neurons, always in the context of acute activity protocols. There are several channels that have been described to be crucial not only for neuronal migration, but also for neuronal activity. In addition, cytoplasmic Ca^{2+} is also influenced by organelles such as the endoplasmic reticulum and mitochondria. Our data suggests that downregulation of voltage-gated Ca^{2+} channels in motor neurons does not significantly affect new bouton formation. On the other hand, intracellular Ca^{2+} increase by downregulation of an ER Ca^{2+} ATPase increases bouton formation, which implies that different origins of Ca^{2+} might contribute differently to presynaptic structural alterations during activity.

This research further expands our understanding of the mechanisms that control presynaptic growth in a wired and *in vivo* neuron. Potentially, these findings can contribute to understand how neuronal structural plasticity can be manipulated and used as a neuroprotective strategy to target diseases that show neuronal loss.

Resumo

A plasticidade sináptica é um mecanismo intrínseco do sistema nervoso, essencial para modificar e criar novas conexões sinápticas, suportando processos neuronais como a aprendizagem e memória. A conectividade neuronal é maioritariamente geneticamente determinada; no entanto, os neurónios também podem remodelar a sua morfologia adicionando ou eliminando estruturas sinápticas em resposta à atividade. Defeitos nesta forma de plasticidade estrutural estão descritos como sendo a causa de várias doenças neurodegenerativas. Embora algumas vias de sinalização envolvidas neste processo já tenham sido identificadas, como é que a atividade induz mudanças estruturais e como é que deficiências nesses mecanismos podem levar à doença, está longe de ser compreendido. Desta forma, o nosso objetivo é estudar como os neurónios alteram sua estrutura em resposta à atividade e identificar as vias que regulam esse processo.

Utilizamos a junção neuromuscular da *Drosophila melanogaster* como modelo para plasticidade estrutural, pois a sua morfologia é estereotipada, mas exibe uma plasticidade estrutural robusta, o que permite o estudo do mecanismo pelo qual os neurónios crescem a alteram a sua forma. Resultados recentes do laboratório indicam que novas estruturas pré-sinápticas (botões sinápticos) emergem rapidamente (em segundos) com uma morfologia arredondada, associados a intensa contração muscular. Foi também demostrado que, ao contrário dos processos de migração mediada por cones de crescimento, a formação de botões em resposta a atividade assemelha-se mais com um mecanismo diferente, chamado de *blebbing* . *Blebs* são protusões de membrana induzidas por pressão, e resultantes da contração do córtex de actina através de motores de miosina. Alguns estudos mostraram que a alta contratilidade é acompanhada por variações cíclicas de Ca^{2+} intracelular e, mesmo em alguns casos, esse aumento no Ca^{2+} precede o aparecimento de *blebs*. Tendo isto em consideração, colocámos como hipótese se o Ca^{2+} poderia ser o sinal que inicia a formação local de botões sinápticos, fornecendo especificidade temporal e espacial.

Para testar esta hipótese, fizemos vídeos de microscopia de larvas de *Drosophila* expressando um marcador de membrana neuronal juntamente com um sensor de cálcio geneticamente codificado, GCamP6f. Para induzir atividade realizamos protocolos de despolarização neuronal usando soluções com alta concentração em K^+ e Ca^{2+}. Estes protocolos estão bem descritos como suficientes para induzir a remodelação pré-sináptica em apenas alguns minutos após a estimulação. Observamos que todos os eventos de formação e retração de botões são precedidos por um aumento repentino e de grande magnitude no Ca^{2+} intracelular. A diferença entre esses dois fenómenos foi que, enquanto na formação de botões, o sinal GCaMP6f é sustentado no novo botão, na retração do botão o sinal dissipa-se rapidamente após o aumento repentino. Uma vez que se sabe que elevações de Ca^{2+} podem regular a contração do córtex de actina, estes resultados sugerem que a formação e a retração de botões podem ter um mecanismo de iniciação comum através da regulação da miosina. O facto de os novos botões terem um sinal GCaMP6f elevado e prolongado (em oposição às estruturas retraídas) pode refletir a remodelação do citoesqueleto celular ou o recrutamento de vesículas sinápticas.

Para entender qual a fonte do sinal de Ca^{2+}, manipulamos as concentrações extracelulares deste ião e os canais de Ca^{2+} nos neurónios motores, sempre no contexto de protocolos de atividade. Existem vários canais que estão descritos como cruciais, não apenas para a migração neuronal, mas também para a atividade neuronal. Além disso, o Ca^{2+} citoplasmático também é influenciado por organelos como o retículo endoplasmático e mitocôndrias. Os nossos dados sugerem que a regulação negativa dos canais de Ca^{2+} dependentes da voltagem nos neurónios motores não afeta significativamente a formação de novos botões. Por outro lado, o aumento intracelular de Ca^{2+} pela regulação negativa de uma ATPase de Ca^{2+} do retículo aumenta a formação de botões, o que implica que diferentes origens de Ca^{2+} podem contribuir de maneira diferente para alterações pré-sinápticas durante a atividade.

Este estudo expande a nossa compreensão dos mecanismos que controlam o crescimento pré-sináptico num neurónio num sistema *in vivo*. Potencialmente, este trabalho pode contribuir para entender como a

plasticidade estrutural neuronal pode ser manipulada e usada como estratégia neuroprotetora para tratar doenças em que haja perda neuronal.

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1. Introduction

1.1 A little bit of history of synaptic plasticity and towards my question

The ability of the nervous system to adapt and give rise to the complex human behavior from which we build our daily life has fascinated scientists for generations. As early as the 19th century, researchers were already trying to understand how neurons change in response to new stimuli so that we can learn and form new memories, but also how these cells can regenerate during aging or trauma **(G. Berlucchi and H. A. Buchtel 2008)**. In fact, the first time the term "plasticity" was put to use was in a book entitled *Principles of Psychology* (1890) by William James, where he referred that "*the phenomena of habit in living beings are due to the plasticity of the organic materials of which their bodies are composed*", referring in particular to the nervous system **(James W. 1890)**. Although there was no concrete evidence or experiments that supported most of James' ideas, the notion that "neuronal modifiability" was an intrinsic biological property essential for human development was well routed in the scientific community. Contemporary to James, S. Ramon y Cajal studies on the architecture of different regions in the brain gave rise to the first picture of what this plasticity might be originated from: small but numerous neuronal connections **(Ramon y Cajal S. 1894a)**. He defended that mental activity, intelligence or even brain stimulation in general would be more related to adding/maintaining neuronal connections, as opposed to aging, or mental illness being more related to the absence of new structures or elimination of existing ones **(Ramon y Cajal S. 1894b)**. Along the same line of thought of Cajal, and motivated by the discovery of the chemical neuronal synapse by Sherrington **(Sherrington CS. 1897)**, Eugio Tanzi and Ernesto Lugaro developed an hypothesis that repetitive learning of a motor task could depend on localized synaptic facilitation as well as coincidence of activity between the neurons responsible for learning of that motor task **(Tanzi E. 1893, Lugaro E. 1898)**.

Although these theories started to shape what we call the beginning of modern neurobiology, many neuropsychiatrists were still sceptic and criticized these new ideas. One of these critics is actually one of the most difficult questions that scientist still struggle today to find a good answer: "Why does the protoplasm stretch towards one neighboring neuron when the organism happens to be in one situation, towards another neuron when the organism is in another situation?" **(Meyer M. 1912).**

For some years after these critics, synaptic plasticity lost relevance as a scientific research theme. However, mostly due to the technological development, more detailed experiments using animal models started to reignite the interest in this field. Important concepts were later introduced by Jerzy Konorski who defined neuronal plasticity as "formation and multiplication of new synaptic junctions between the axon terminals

of one nerve cell and the soma (i.e. the body and the dendrites) of the other" **(Konorski J. 1948)**, and which Donald Hebb followed, resulting in his famous postulate "neurons that fire together, wire together" **(Hebb DO 1949).** At this time, psychiatry and neurobiology started to diverge. Ultimately, this would lead to a period where multiple experiments were done not only to validate many of the intuitive concepts established in the past, but also in the endeavor of answering many questions left behind **(von Bernhardi R. et al 2017)**. Important landmarks include the discovery of critical developmental periods, discovered by David Hubel and Torsten Wiesel with the monocular eye cat closure experiments **(Hubel DH., Wiesel TN. 1965)**, along with M. Merzenich (and others) studies on adult brain plasticity. **(Kaas JH. Merzenich MM. et al 1983)**

Presently, we known that regulation of both neuronal function and structure are important to achieve proper nervous system development, but it is also unquestionable that activity-dependent processes are very important throughout our life. However, after more than 100 years from the critics of the plasticity theory, we still understand very little about how neurons coordinate activity-dependent synaptic transmission and the production of new synaptic structures. Therefore, it is the goal of our lab and of this thesis to elucidate what are the cellular signals responsible for these morphological alterations, which are a hallmark of structural plasticity.

1.2 Fundamental concepts in Neurobiology

Before getting into the details related to my thesis, I consider important to describe some basic concepts of neuroscience. In particular, I will focus on neuronal polarity, how neuronal morphology relates to function and the synapse as the unit of neuronal communication.

Neurons are considered the fundamental cell type of the nervous system, and when rearranged in specific patterns form circuits with specific functions such as sensory feedback or motor output. Considering that neurons must perceive, process and send information in these varied conditions, these cells have developed an asymmetric organization of cellular components that ultimately contributes to the flow of information. This asymmetric organization is also designated as polarity. (**Fig 1A) (Banker G. 2018, Iden S. and Collard JG. 2008).**

Initial neuronal polarity is established during development either by the sensing of extracellular cues or by accumulation of a certain transcription factor that will break the symmetry and start neuronal differentiation (**Yogev S. and Shen K. 2017).** After several cytoskeleton modifications and trafficking of specific synaptic machinery, polarity can be interpreted in two ways:

- From the cellular perspective, one neurite in specific will form the axon, while the remainder neurites (initial neuronal protrusions) will form the dendrites (structures that receive and integrate information).

- From the synaptic perspective, polarity can be seen as the place where a synapse is established between a presynaptic neuron that releases a specific type of neurotransmitter in the synaptic cleft, and a postsynaptic cell that expresses the corresponding receptors.

Neuronal morphology is extremely diverse (**Fig 1B)** with neurons acquiring shapes that range from small but dense regions in the brain surrounded by thousands of cells, to elongated axons in the spinal cord that can reach several meters in large sized mammalians. This diverse morphology is intimately connected to function **(Kasai H. et al 2010)**. Some brain neurons need to receive many inputs to process complex information (like a memory in the hippocampus) and therefore develop highly complex dendrites. On the other hand, some motor neurons need to specifically innervate few muscle fibers to achieve proper contraction, so they develop few but precise axon terminals.

Neuronal communication mechanisms are relatively stereotypical and conserved across species. The most common type of communication between a neuron and another cell is the chemical synapse, which converts chemical signals (the neurotransmitters) into electrical information (membrane depolarization). Electrical pulses are fundamental for the rapid transmission of information within the neuron. This electrical ability is achieved by alterations in the concentration of different cations and anions within the cell, which results in alterations in the membrane potential. **(Liqen Luo 2016)** When membrane depolarization reaches the threshold for the opening of voltage-gated sodium channels, an action potential is produced. In a physiological situation, when the action potential reaches the pre-synaptic terminal it depolarizes the membrane activating voltage-gated Ca^{2+} (Ca^{2+}) channels concentrated at presynaptic regions called active zones (AZ). Active zones are dense regions with several scaffolding proteins which anchor the Ca^{2+} channels and organize different pools of synaptic vesicles filled with neurotransmitters **(Ghelani T. and Sigrist SJ. 2018)** These vesicles are maintained in a ready releasable state in close proximity with the presynaptic membrane by several synaptic vesicle proteins. Once Ca^{2+} channels are activated, Ca^{2+} flows into the cell, binds to the Ca^{2+} sensor present on synaptic vesicles (SVs) - synaptotagmin I, which leads to conformational changes of the SNARE proteins within SVs, thereby inducing SV fusion with the plasma membrane, and consequent release of the neurotransmitters **(Waites CL. And Garner CC. 2011).** The ability to rapidly release neurotransmitter and to modulate the rate of neurotransmitter release is absolutely critical for normal function but also for plastic alterations (**Fig 1C).**

The main function of neurotransmitters is the rapid activation of postsynaptic receptors to promote depolarization of the postsynaptic membrane and activation of activity-dependent pathways. These postsynaptic receptors must be aligned with presynaptic release sites to ensure minimal neurotransmitter dispersion in the synapse cleft. In addition, activation of postsynaptic metabotropic receptors can also lead to secondary signaling cascades that promote more prolonged changes such as transcription and cytoskeleton rearrangements **(Cohen S. and Greenberg ME. 2008)** (**Fig 1C).**

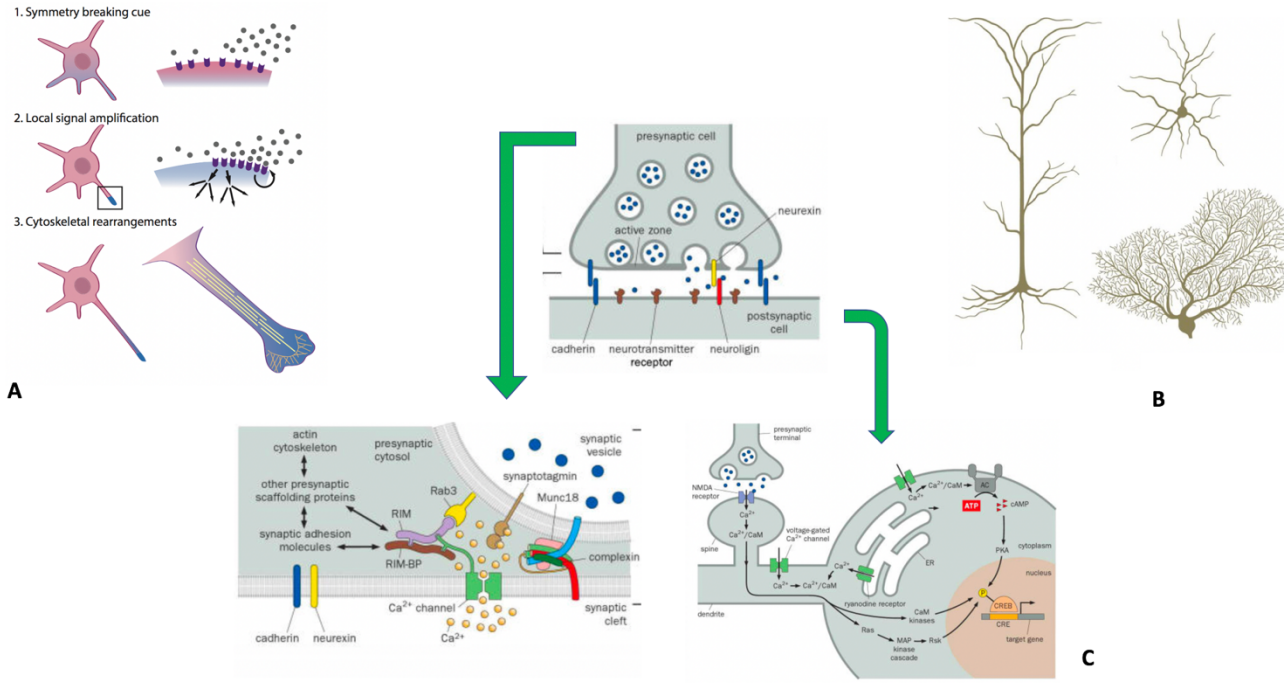

Figure 1 – Principles of neurobiology

(A)- Neuronal polarity. External symmetry breaking cues are amplified in the newly born neuron and induce the growth of one neurite that will become the axon. **Adapted from Yogev S. and Shen K. 2017 (B) –** Morphological neuronal diversity in the brain cortex of the mouse **(C) –** Neuronal communication: pre- and postsynaptic compartments and the main molecular components**. Adapted from Liqun Luo 2016 Principles of neurobiology**

1.3 The synapse as the *center stage* of neuronal plasticity

Once it was understood that the synapse was the key target in neurons to achieve major modifications, several laboratories dwelled in the search of the molecular mechanisms of plasticity. One of the first observations was that plastic changes involved both changes in the frequency of release of existing synapses (functional plasticity) and by selective formation or deletion of synaptic compartments (structural plasticity) **(Power JD and Schlaggar 2017, Citri A. and Malenka RC. 2008)**. Functional and structural plasticity are deeply correlated and the mechanisms that regulate both processes are very often shared **(Raphael Lamprecht and Joseph LeDoux 2004; Lisman J. 2017).**

Both of these alterations are involved in positive feedback responses to neuronal activity (high activity leads to higher synaptic efficacy and vice-versa) and in negative feedback responses (high activity leads to decreased synaptic efficacy). Positive feedback can be seen as Hebbian plasticity since the *maxima* "neurons that fire together, wire together" applies, while negative feedback can be denominated as homeostatic plasticity since the objective is to counter the disturbance due to excessive activity alterations, therefore resetting circuits **(Fauth M. and Tetzalff C. 2016).**

While Hebbian plasticity occurs in a short time window after stimulation (seconds to minutes) **(Regehr WG. 2012**) homeostatic plasticity tends to occur after longer periods of time (hours to days), therefore resetting circuits to homeostatic conditions.

Considering structural plasticity, the vast majority of the research has focused on the analysis of dendritic spine (postsynaptic compartment) formation and retraction, in response to high and low activity, respectively **(Moser MB. Et al 1994; Engert F and Bonhoeffer T. 1999).** However, it is also known that presynaptic boutons are added or retracted to axonal terminals in response to activity **(Piccioli ZD. and Littleton JT 2014; Monday HR. and Castilo PE 2017; Ninan I. et al 2006)**, but much less is understood regarding how presynaptic boutons respond rapidly to activity, and even if the mechanisms that regulate this process are similar to the ones required for postsynaptic structural plasticity **(Fig 2).**

Therefore, it is the interest of the lab to investigate how presynaptic structures remodel in response to activity. In particular, we want to unravel the molecular mechanisms required for the remodelling of presynaptic structures in an activity-dependent context

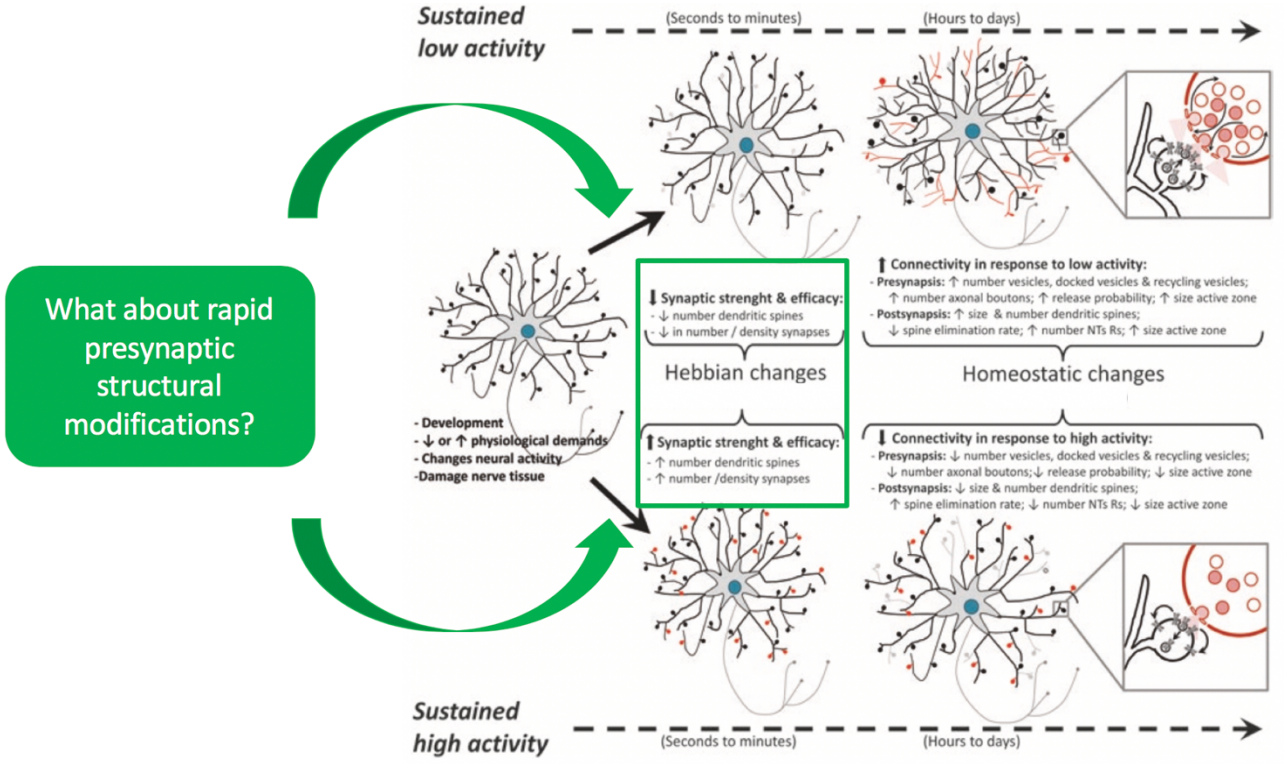

Figure 2 – Different types of plasticity are used in activity- and time-dependent manner
Hebbian changes correspond to synaptic strengthening/weakening or synaptic addition/removal in response to rapid changes of high versus low activity, respectively. Homeostatic changes correspond to strengthening/weakening or synaptic addition/removal in response to low/high activity to restore baseline levels of activity. **Adapted from Von Bernhardi et al 2017**

1.4 Synaptic formation and differentiation

Before neurons are able to engage into complex synaptic processes they must first connect to their proper targets. At the beginning of neurogenesis, neurons start by establishing their main compartments – the axon, dendrites and the soma. Once these structures are formed, synaptic connections can be stabilized through cell adhesion molecules (CAM) and the pre- and postsynaptic machinery is organized to ensure proper neuronal communication **(Sudhof TC. 2018; Yun Kyung Park and Yukiko Goda 2016)**. The great majority of the pre-synaptic compartments are "canonical" meaning that the release machinery is essentially the same, ranging from scaffolding proteins to voltage-gated or ligand-gated channels in the membrane **(Sudhof TC. 2012)**. Interestingly, the presynaptic machinery is specific to neurons, which represents an important feature of the way these cells communicate. Unlike its juxtaposed partner cell, the postsynaptic machinery is diverse with a wide range of different receptors but common to other cells such as the muscle. In addition to canonical neuronal communication, glial cells are also able to release gliotransmitters and modulate neuronal activity (even though they do not possess active zones) **(Ho VM. 2011).**

Synapse formation and differentiation take place mostly during embryonic and post-natal development under tight genetic regulation and are often activity-independent since blocking neurotransmitter release in knockout mice during this period results in mice with normal synapse number **(Sigler A. et al 2017).** Neuronal activity becomes crucial mostly (with exceptions) during developmental critical periods and throughout life since it contributes to the release and postsynaptic receptor machinery. This fine tuning involves neuronal growth but also physiological synapse elimination of less fit or misconnected synapses **(Bourgeois JP. et al 1993; Petanjek Z. et al 2011)**. However, it is still unclear if presynaptic sites determine the location of synapse formation, or if the reverse occurs. There are three main models that describe how synapses are first born: one that defends that presynaptic machinery dictates the formation of the post and as consequence the synapse, another defends that it is the post synaptic machinery location the signal for establishment of the presynaptic bouton, and yet a more complex model where "preferential" presynaptic sites are predetermined when the neuron is formed which will eventually lead to the formation of the postsynaptic part (**Fig 3) (Pinto MJ and Almeida RD 2016)**.

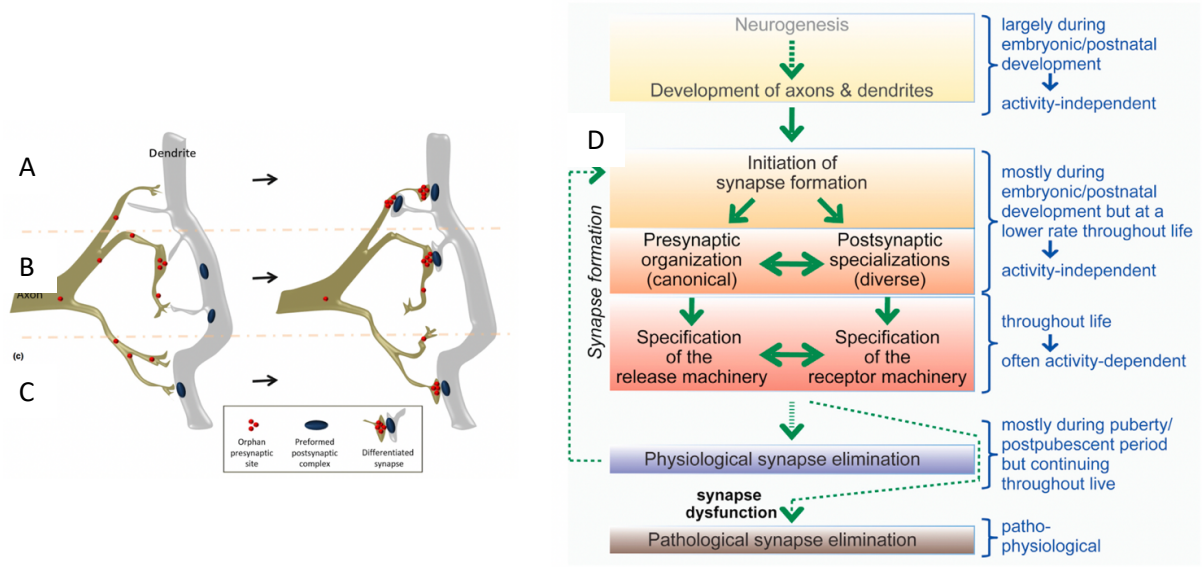

Figure 3 - Synaptic differentiation models and synapse formation

(A) – Neuronal filopodia form growth cones that extend towards the postsynaptic compartment, creating cell adhesion contacts which will promote differentiation of the pre and post synaptic machinery. **(B) –** Presynaptic sites are predefined in early development and will promote differentiation of the postsynaptic counterpart upon contact. **(C) –** Postsynaptic complexes are localized in specific locations of the neuron and will promote differentiation of the presynaptic part when contact is established. **Adapted from Pinto MJ. And Almeida RD. 2016 (D) –** Most of the developmental neuronal processes leading to differentiation are activity-independent. However, activity becomes crucial during the adult life of organisms, where the modulation of the receptor and release machinery leads to the establishment of healthy synapse addition or elimination of dysfunctional or less active ones. Many neurological disorders originate not only from excessive synapse elimination, but also from the overabundance of dysfunctional synapses in response to activity. **Adapted from Sudhof TC. 2018**

1.5 Synaptic innervation and the growth cone

The establishment of a global neuronal network is a complex process, being therefore important to understand the initial steps, namely, what are the mechanisms that guide neurons towards their target destination. At the extremity of the axon of each developing neuron there is the formation of a highly dynamic structure that probes the extracellular environment for cues that guide the axon to its correct target tissue: the growth cone **(Lowery LA. And Vactor DV. 2009)**. The famous "hand" shape of this structure is crucial for the navigation process since there are several filipodia protrusions that actively search the environment, intercalated with lamellipodia veils to increase surface area **(Dent EW1 and Gertler FB 2003)**. In addition, the microtubule dense core assures that the growth cone is able to steer in any direction of movement. Specific adhesion and extracellular matrix molecules shape concrete paths by which growth cones must attach when moving as they also provide important cues for correct target guidance. Nonetheless, guidance is majoritarily determined by chemotropic cues that can either be attractive or repulsive to growth depending on the type of receptors expressed in the tip of the filopodia protrusions **(Jay DG. 2000).** Ultimately, the combinatorial actions of the different cues induce several cytoskeleton rearrangements (mainly actin related, although there are also microtubule alterations) through a diversity of transducer molecules, being the most well characterized the Rho family of GTPases. RhoaA, CDC42 and Rac1 are a few examples of well known molecules that integrate diverse signals to coordinate cytoskeleton effectors into different actin modifications, in response to specific upstream signals **(Ridley AJ. 2011).**

Most of actin modifications can be seen as cycles of removal of actin monomers (to which actomyosin contraction and F-actin disassembly contribute) and addition of actin monomers forming filaments (F-actin polymerization). This continuous intrinsic cyclic activity of the actin cytoskeleton (also called threamilling), in combination with the F-actin retrograde flow from leading edge towards the center of the growth, allows for the high flexibility of the growth cone (**Fig 4) (Athamneh AI. and Suter DM. 2015).**

In spite of growth cone guidance being under a strict genetic program, activity plays an indispensable role. One well known example is a subclass of neurons in the visual cortex that require that the eye-projecting neurons are activated (by retinal activity), or otherwise start to atrophy and eventually dye (even if correctly targeted) **(Briggman KL et al 2011)**. This necessity not only starts at embryonic stages (mammalian) with random spontaneous retinal activity, but continues during the post-natal period with visual experience strengthening the connection in this region of the cortex. How do neurons in the visual cortex know they are receiving input from neurons in the eye? Activation of NMDA receptors in the neurons from the visual cortex ensures that this circuit is active, since NMDAR act as "coincidence detectors" of both pre and postsynaptic activity **(Penn AA. and Shatz CJ. 1999)** . Another good example, more related to axonal guidance rather than

neuronal strengthening, are the olfactory receptor neurons where high or low odorant activity of different odor receptors leads to the expression of different guidance receptors in the front of the growth cone

It is thought that many of the mechanisms that underlie neuronal migration in development are also present in activity-dependent plasticity in already wired networks. However, the molecular pathways that regulate these neuronal modifications and the dynamics of these structures during activity are still unknown.

In addition, numerous neurological diseases like Alzheimer's, Amyotrophic Lateral Sclerosis or even psychiatric disorders like schizophrenia and autism, present synaptic innervation defects much later after development **(Monday HR. et al 2018).** Therefore, these conditions might be originating from the coordination between activity and synapse formation or elimination. Understanding how neuronal activity induces structural plasticity might helps us to prevent some neurological disorders in the future.

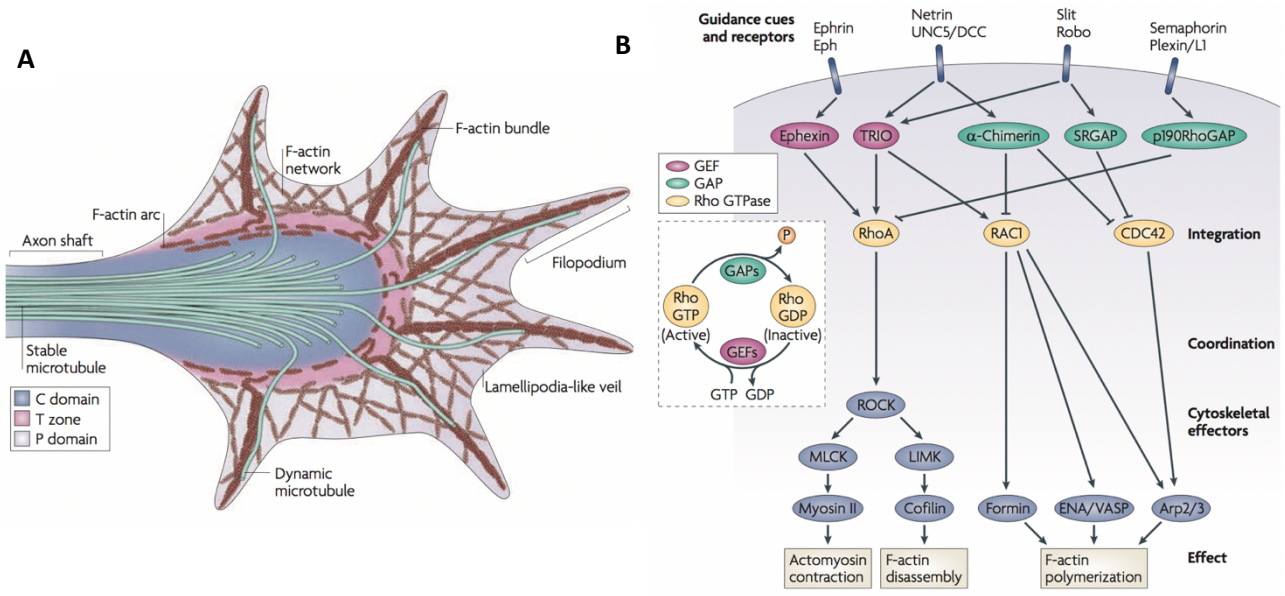

Figure 4 -Synaptic innervation and the growth cone
(A) – Hand-like shape of the growth cone allows extensive environmental exploration. Different zones concentrate different cytoskeleton rearrangements. P domain is enriched in actin filaments forming filopodium finger like structures and lamellipodia veils. T domains connect the actin cytoskeleton with the microtubules. C domain concentrate the microtubules core to steer the growth cone tip. **(B) –** Guidance cues activate different receptors that induce cytoskeleton effectors to coordinate different cytoskeletal responses. **Adapted from Lowery LA. And Van Vactor D. 2009)**

1.6 Drosophila NMJ as a model system for neuronal plasticity

How neurons grow, remodel and communicate with each other are well-conserved cellular processes. Despite the multiple examples on vertebrate CNS synapses, the inaccessibility that they represent, especially if considering *in vivo* analysis, makes mechanistic studies with genetic and neuronal activity manipulation quite challenging. Moreover, functional and structural post-synaptic plasticity has been in the spotlight for several years, with an unbalance in knowledge regarding pre-synaptic structural plasticity, which is equally important to the study of neuronal plasticity. This may have resulted from the fact that presynaptic terminals, characterized by the presence of roundish synaptic boutons, are much smaller than dendritic spines, and therefore harder to visualize.

A model synapse used for several years in neuroscience (both in vertebrate and invertebrate animals) to study presynaptic terminals and structural plasticity is the neuromuscular junction (NMJ), since it is not only a simple robust circuit easy to manipulate, but it also allows the visualization of large individualized synaptic boutons **(Personius KE. and Gordon RJB. 2002; Petralia RS. 2017).** These characteristics make the NMJ a very good model to study activity-dependent synaptic structural plasticity.

Invertebrate NMJs such as *Drosophila melanogaster* and *Caenorhabditis elegans* are very used in research because they allow extensive genetic manipulations of synaptic proteins with known homologs in mammalian and are usually simpler and more accessible than its vertebrate counterparts, making their study simpler **(Wu H. et al 2010).** To study bouton formation however, *Drosophila* is better suited given the larger size of these elements.

Drosophila has been used for over 100 years in several research areas from cancer, developmental biology, tissue repair and, of course, neurobiology. The first and foremost advantage of *Drosophila* is the vast genetic toolkit that allows spatial and temporal control of gene expression, disruption, but also expression of fluorescent tags **(Bellen HJ et al 2010)**. These tools have been developed by several labs that engineered numerous fly lines, including binary systems like the Gal4/UAS or lexA/lexAop systems that allow the expression of genes or constructs of interest under the control of cell specific drivers. This regulation can be further fine tuned by using temperature sensitive repressors that provide temporal control (like the use of Gal80) **(McGuire SE. et al 2004; del Vale Rodriguez A. et al 2011)** (**Fig 5).**

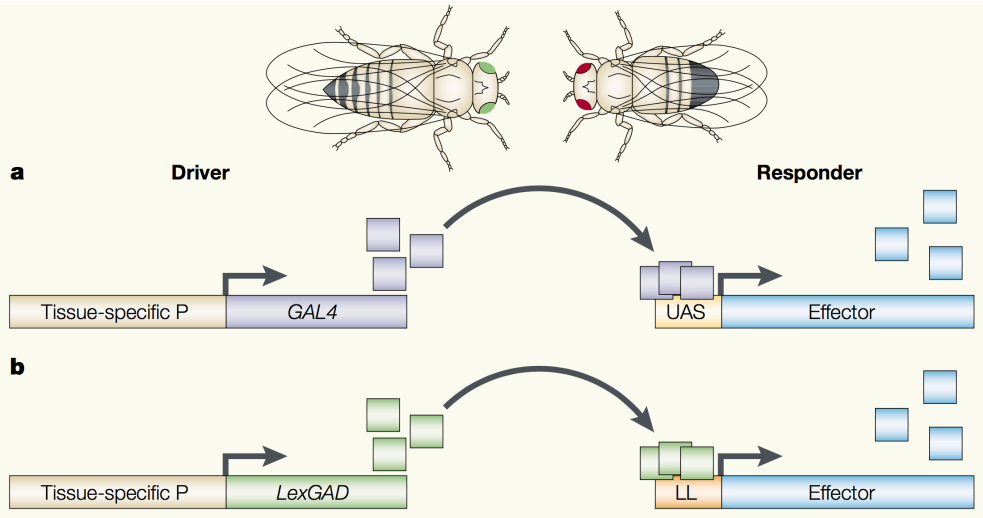

Figure 5 – Binary expression systems in Drosophila melanogaster
(A) - Tissue specific driver drives the expression of Gal4. Being a transcription factor, Gal4 will directly bind to the upstream activating sequence driving the expression of the genomic sequence of interest (effector). **(B) –** A similar system is the lexA/lexAOp (here represented as lexGAD/LL but it is the same system). **Adapted from del Vale Rodriguez A. et al 2011**

Therefore, to uncover the molecular pathways that govern neuronal remodeling one needs to be able to test candidate genes and manipulate their levels, while monitoring NMJ morphology. The Drosophila NMJ fulfills all the criteria to be a good model to study the pathways that regulate structural changes in a activity-dependent manner.

Drosophila has been extensively used in neuroscience to study the mechanisms of synaptic function responsible for memory, behavior, neurodegenerative diseases, and many more **(Frank CA. et al 2013)**. In our lab we use the NMJ of *Drosophila* third instar larvae to study molecular and cellular mechanisms that regulate synaptic growth and plasticity, since it has a well characterized stereotypic morphology that is plastic upon exposure to different protocols of synaptic stimulation. **(Koon AC. et al 2011; San Martin A. et al 2017)**

The larva has 10 segments (seven abdominal and three thoracic) with 30 muscles per hemisegment. Motor neurons descend from the ventral nerve cord in six bundles and project to the different muscles in well organized morphogenic fashion **(Kohsaka H. et al 2012)**. During embryonic development, axons grow with the growth cones extending filopodia that contact the corresponding muscle protrusions (named myopodia). Upon contact and, by yet unclear mechanisms, the growth cone differentiates in round shaped presynaptic structures along the neurons designated synaptic boutons, each one with multiple active zones. At the *Drosophila* NMJ there are 3 types of motor endings, I-III. Type I boutons can be divided in Ib (big) and Is (small)

boutons that are completely imbedded in the muscles, where the postsynaptic site matures. The presynaptic type I boutons are surrounded by a multilayer membrane structure called that subsynaptic reticulum (SSR) which contains clutters of glutamate receptors juxtaposed to the active zones **(Menos KP 2013).** Drosophila NMJ is therefore glutamatergic (and excitatory), which contrasts with mammalian NMJs that are cholinergic (using acetyl choline as the neurotransmitter), but resembles mammalian CNS synapses in the cortex, where most mechanistic studies on plasticity were done **(Budnik V. 1996; Keshishian H. et al 1996) (Fig 6).**

Akin to the mammalian, *Drosophila* NMJ also suffers activity-dependent synaptic refinement and extensive growth. From embryo hatching to third instar larvae the muscle grows about 100 times, which means that boutons must be continually added to the NMJ in order to assure adequate innervation and proper muscle contraction **(Van Vactor D. and Sigrist SJ. 2017)**. The most characterized NMJs are the ones innervating muscle 6/7, 4, and, 12/13. Throughout the years several protocols to induce structural plasticity have been developed, mostly based on electrophysiological stimulation (using directly electrodes in the nerves), optogenetics (using light activation of specific ion channels) and chemically (using solutions with ion concentrations known to induce neuronal depolarization) **(Martin AS. et al 2017)**. Pattern stimulation elicits the formation of new boutons **(Budnik V. et al 1990)**. When these boutons form, they are still immature neuronal structures lacking any post-synaptic markers like Disc large proteins (DLG, scaffolding postsynaptic protein) or even glutamate receptors, being only possibly to visualize with neuronal membrane markers or synaptic vesicle markers. Therefore, it is thought that new boutons resulting from activity can undergo maturation and originate full stable synaptic boutons with the postsynaptic machinery (glutamate receptors and DLG), but they can also retract, if they fail to mature (**Fig 6).**

Drosophila larvae can be easily dissected, exposing the musculature and NMJs of interest for imaging in fixed or live samples. While fixed samples have contributed deeply to the understanding of different molecular players in activity-dependent bouton formation, live imaging as been a quite a defying task for reasons that will be explored further ahead. Nevertheless, the few studies that tried to characterize this process of bouton addition based on live imaging, which were initially summarized in three models: new boutons are added by budding (in a mechanism resembling yeast budding), by *de novo* (the new bouton forms anew) and the division model (a mature bouton divides into two boutons) **(Zito K. et al 1999)**. While revolutionary at the time, this study was done using large time intervals (several hours) between imaging time points and used a postsynaptic marker to visualize new boutons, which only allows the analysis of mature boutons. More recently, two studies **(Ataman B. et al 2008, Piccioli ZD. et al 2014)** have demonstrated that acute spaced stimulation is able to induce new bouton formation in a time window of seconds or minutes. Remarkably, growth cone like structures were never seen in these studies, pointing to the premise that NMJs have

adapted other strategies for rapid growth in activity dependent plasticity in post-embryonic periods. The model that has been reported to be more similar with *in vivo* studies is in fact bouton budding, but many of the molecular factors that mediate activity and the formation of a new presynaptic structure are still unknown.

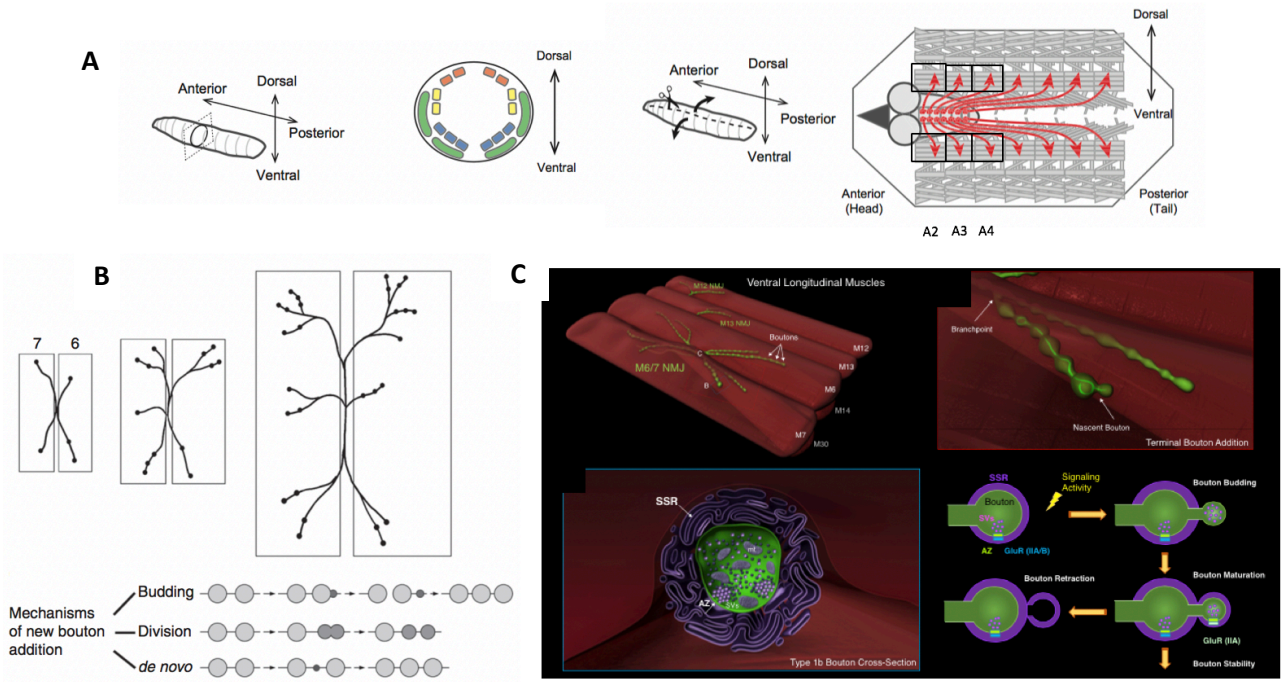

Figure 6 - Drosophila NMJ as a model system for neuronal plasticity
(A) – Cross-sectional view of the *Drosophila Melanogaster* 3rd instar larvae. Dorsal dissection exposes musculature of interest: m6/7 segments A2,A3 and A4. **Adapted from Kohsaka H. et al 2012 (B) –** Proposed mechanisms of new bouton addition to match muscular growth: Asymmetric division from a mature bouton (Budding), equal division from a mature bouton (Division), new initial synapse **formation** in the axon and posterior growth (de novo). **Adapted from Menon KP. Et al 2013 (C) –** The presynaptic mature bouton is filled with synaptic vesicle that concentrate near active zones. Surrounding the bouton is a complex membrane structure called the sub synaptic reticulum (SSR) that holds glutamatergic receptors juxtaposed to each active zone. Neuronal activity is able to induce the formation of immature presynaptic structures that can either mature and stabilize, or retract. **Adapted from Van Vactor D. and Sigrist SJ. 2017**

1.7 Pathways regulating activity-dependent neuronal growth

There are several known genes that, when affected (by overexpression or loss of function), contribute to NMJ development, but the cytoskeletal changes and the genes specifically required for new bouton formation in response to acute activity are still far from understood. Nevertheless, it is known that many of the molecules

and pathways necessary for neuronal development, also play a role in activity-dependent processes. So the big question is how does the neuron transduce activity into the formation of a new synaptic bouton?

1.7.1 Membrane Growth

Similar to what happens during development, growth of the neuronal membrane and cytoskeleton modifications are essential to the formation of activity-dependent boutons.

Wg (Wnt/Wingless family proteins) is one neurotrophic factor that is secreted to the synaptic cleft by the neuron after patterned activity. Wg functions by binding to its receptor frizzled, in an autocrine manner and in an anterograde fashion. Wg binds to Frizzled in the postsynaptic side, where it will induce activity-dependent transcription in the muscle, thereby promoting bouton maturation. In addition, it binds to its receptor in the presynaptic terminal inducing new bouton formation in an autocrine fashion, by directly regulating the cytoskeleton **(Ataman B. et al 2008; Alicea D. et al 2017).**

This crosstalk between muscle and neuron has been observed in other pathways and has proven to be essential to coordinate time specific bouton budding.

Rapid activity-dependent synaptic growth also requires retrograde BMP signaling, which has two main functions: promote activity-dependent transcription and cytoskeleton changes. These cytoskeleton changes are regulated by LIM kinase (LIMK), which stabilizes mature boutons by inhibiting depolarizing actin factors like cofilin (Tsr) in non-stimulated conditions. However, upon patterned depolarization, Glass bottom boat (Gbb), is secreted from the muscle and binds to Wishfullthinking (Wit) neuronal receptor. Wit activation will inactivate LIMK, thereby releasing inhibition on Tsr. Once cofilin (Tsr) is active, it can promote local actin depolymerization, which is essential for bouton budding **(Piccioli ZD. and Littleton JT. 2014).**

1.7.2 Presynaptic machinery modulation

In order to form new functional boutons, the neuron must detect ongoing specific patterned activity, and regulate the presynaptic machinery to be present in these newly formed structures.

Ion channels are crucial for modulating different levels of activity and vesicle release. In fact, double loss-of-function (LOF) mutants that have reductions in the levels of two voltage-gated K+ channels, Ether-a-go-go (Eag) and Shaker (Sh) have hyperexcitable neurons and as a consequence, more boutons. **(Mosca TJ. et al 2005)** One possible strategy by which the neuron can respond to increased activity is by increasing neurotransmitter release.

In contrast to potassium channels, Ca^{2+} channels mutations disrupt the rapid intracellular Ca^{2+} concentration needed for vesicle release and lead to reduced neuronal branches and boutons. (**Rieckhof GE. Et al 2003)**

Synaptic vesicles are one of the few components of the synapse that have been described to be more homogenously present in activity-dependent new boutons. A recent study has revealed that new boutons may actually form in a more progressive (but less common) fashion initially devoid of synaptic vesicles, but also by a more rapid (and more often) budding of the neuronal membrane that is filled with synaptic vesicles **(Vasin A. et al 2019)**. This rapid budding depends on synapsin vesicle clustering, which can be regulated by several kinases. PKA and CaMKII are important kinases that respond to activity and could therefore connect neuronal activity with vesicle clustering and new bouton formation **(Nesler KR. Et al 2016).**

1.7.3 Adhesion and confinement

Despite the importance of growth and presynaptic machinery modulation, adhesion and confinement are also crucial factors to consider for synaptic plasticity. During embryonic development, adhesion is crucial for establishing correct pre-post synaptic innervation of different neurons but also of different synapses. In accordance, new bouton addition during acute activity is dependent on reshaping these numerous contacts. To accomplish that, activity can immediately and locally downregulate integrin and focal adhesion molecules like laminin to allow membrane plasticity for bouton budding **(Tsai-PI et al 2012)**. Receptors for ECM proteins like tenectin can even regulate neurotransmission and bouton architecture **(Qi Wang et al 2018)**. Nevertheless, *Drosophila* NMJs need to secrete integrin activators like Shriveled in response to high activity so that new boutons can stabilize and mature by acquiring postsynaptic specializations. (**Lee JY. et al 2017)**

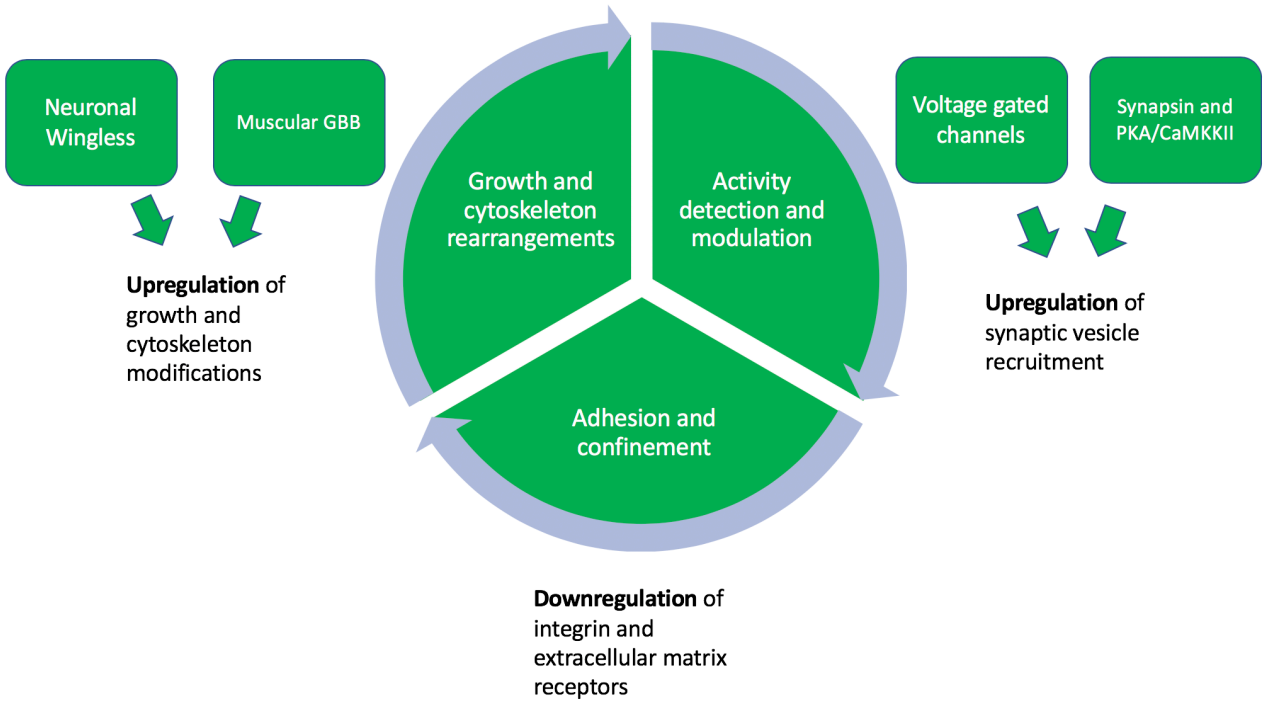

Figure 7 - Pathways regulating activity-dependent neuronal growth

1.8 Membrane blebbing in activity-dependent bouton formation

There are many pathways known to be important for the regulation of bouton formation in response to activity, but the cytoskeleton changes and the mechanism by which these structural changes occur remain a mystery. Data from the lab has shown that new boutons emerge rapidly with a bulky round morphology without actin supporting the plasma membrane, a process very much like membrane blebbing. Blebs are pressure-driven membrane protrusions resulting from the contraction of the actomyosin cortex through myosin motors, favored in conditions of high confinement and low adhesion. Despite being first identified in apoptotic cells, this type of membrane protrusion is widely used by healthy cells for cell migration, especially in 3D environments **(Ridley AJ. 2011; Charras GT. 2008; Gordarzi M. et al 219)**

Localized blebbing can occur due to detachment of the cortex from the plasma membrane or cortex rupture due to strong myosin contractions. Either of these scenarios creates a weakness in the cell cortex, which can no longer cope with the normal hydrostatic pressure of the cell cytoplasm. This cortex weakening results in membrane expansion and stretching, while being filled with cytoplasmatic fluid. When expansion starts to slow down, actin polymers as well as actin bundling proteins are recruited to reform the cortex. After the cortex is reformed, myosin is recruited together with RhoA/ROCK to promote cortex contractions that retract the bleb. The retraction phase is slower than the expansion phase and does not always occur – in fact, in motile cells rather than retracting, sequential blebbing is often observed **(Fackler OT. and Grosse R. 2008; Paluch EK. And Raz E. 2013; Charras G and Paluch E. 2008)**

A major molecular player during blebbing is the activation of non-muscle myosin II (NMII) that promotes actin rearrangements including contraction of the cellular cortex **(Chi Q. et al 2014, Betapudi V. 2014)**. Data from the lab indicates that bleb-like boutons occur more frequently when NMII is reduced in neurons by doing cell-type specific RNAi. Although counterintuitive at the first, it has been shown that cells with NMII KD are able to bleb more often if pressed between agar surfaces **(Sugiyama T. et al 2015).** This observation implicates that, by promoting confinement (squeezing force applied with agar surface), cells can bleb efficiently even in the absence of NMII. If we consider the NMJ, it is interesting that we observed that the majority of bouton formation events is highly correlated with strong muscle contraction. Given that presynaptic boutons are completely imbedded in the muscle fibers, this suggests that bouton formation can be, not only dependent on neuron pressure and cell autonomous regulation of bouton formation, but also dependent on external pressure derived from muscle contraction that can facilitate the budding of the new bouton. Our current hypothesis is that NMJs have adapted blebbing to quickly modulate bouton formation in response to high levels of activity and intense muscle contraction.

Despite having identified membrane blebbing as a novel mechanism used by neurons to remodel, it is still not understood which pathways are involved in bleb formation (in neuronal context) neither how the neuronal network can so rapidly produce these events in response to activity.

Actin cortex contractions are one of the main features of blebbing and are usually caused by NMII. There are two pathways that have a direct effect or the myosin light chain and consequent NMII activation: Rho/Rock pathways and the MLCK pathway. While Rho/ROCK can be activated by a wide variety of different membrane receptors, MLCK can be activated by increase in intracellular Ca^{2+} concentration **(Kapustina M. et al 2008)**. Some studies described that high contractibility is accompanied by cyclic variations of intracellular Ca^{2+}, but whether this molecule is cause or consequence of this cytoskeleton process has not been established. Accordingly, other authors have stated that localized increases in Ca^{2+} levels in zebrafish migrating germ cells briefly precede blebbing of these cells **(Blaser H. et all 2006)**. Whether Ca^{2+} can serve as a signal transducer molecule that can coordinate synaptic activation with activity-dependent structural changes, is an open question.

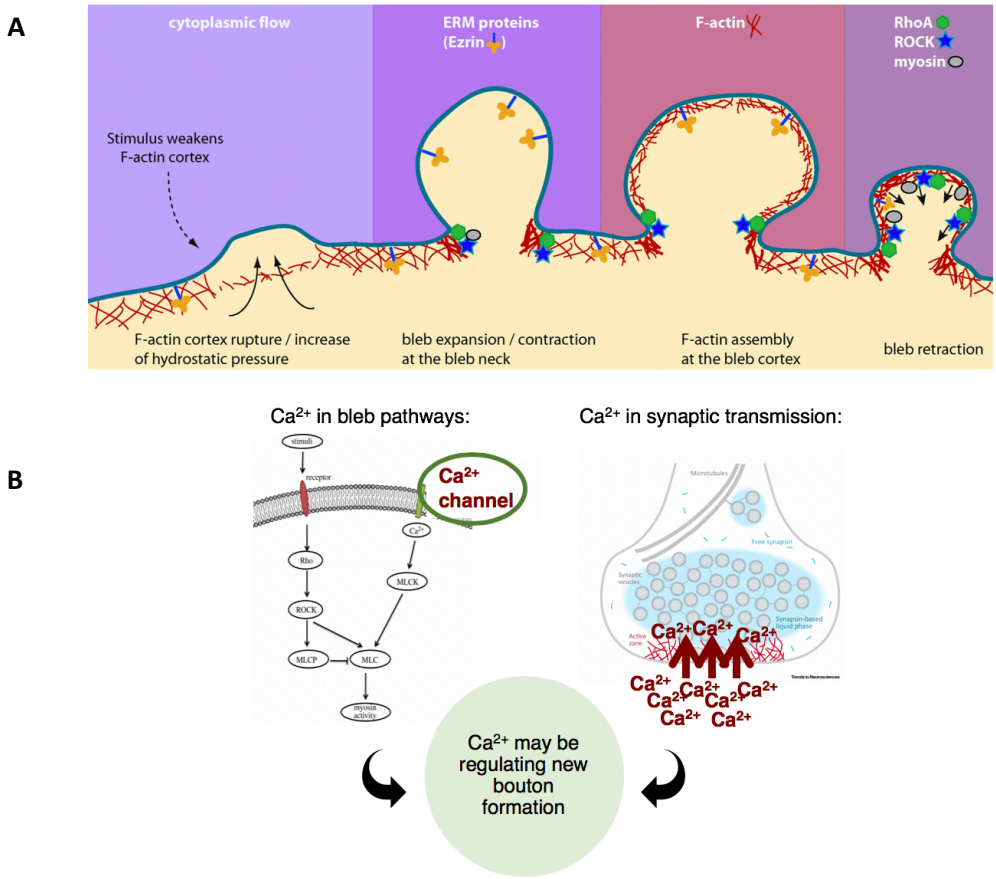

Figure 8 – Bleb migration in activity-dependent bouton formation

(A) – Detachment of the cortex from the plasma membrane or cortex rupture due to myosin contractions induce local hydrostatic pressure. Plasma membrane together with the bleb neck expand and cytoplasmic fluid fills in the bleb. When bleb growth ceases, the F-actin cortex can reform. Myosin is recruited to the newly formed cortex and, in some blebs, induces new contractions that promote bleb retraction. **Adapted from Fackler OT. and Grosse R. 2008 (B) –** Our hypothesis: Since Ca^{2+} is important for neurotransmitter release and structural plasticity and at the same time can regulate blebbing through actomyosin contractions, could Ca^{2+} be the signal regulating in a time and space specific manner new bouton formation? **Adapted from Chi Q. et al 2014**

1.9 Ca^{2+}as an important cellular transducer

Before exploiting the role of Ca^{2+} in neurons, one must first understand why and how is Ca^{2+} (and other signaling molecules) so important as multifunctional cell transducer(s).

Protein modifications like phosphorylation/dephosphorylation are well known to regulate a multitude of biological processes. These types of modifications rely on the addition or removal of a phosphate group to proteins, using ATP as a donor molecule. Therefore, phosphate is an important anion that can regulate many cellular functions. Classical biology books describe molecules like ATP and GTP to be carriers of this important ion that binds or releases hydroxyl groups of serine, tyrosine and threonine residues in specific proteins, thereby altering protein's conformation/interactions and consequently their function. Protein kinases and proteins phosphatases are enzymes that are able to add and remove, respectively, phosphate from these residues; which interestingly display a significant part of eukaryotic genomes (about 2 %), likely reflecting their importance in cell function **(Westheimer FH. 1987).**

Similar to phosphate, Ca^{2+} is a bivalent cation with diverse cellular regulatory functions across many different organisms, from the most simple and unicellular archaebacterium to a complex multicellular eukaryote, like humans. Remarkably, its atomic properties make this ion not easily diffusible in water and also an excellent phosphate precipitator. Therefore, free Ca^{2+} inside the cell can destabilize the interchange of phosphate between several proteins, ultimately being able to compromise cellular function. In addition, Ca^{2+} cations are extremely difficult to be chemically modified, which means that cells evolved different strategies to regulate this cation **(Clapham DE. 2007).**

To maintain Ca^{2+} levels low, cells can for example, chelate Ca^{2+}, extrude it to the extracellular environment or even compartmentalize it in specific organelles. In fact, concentration differences of Ca^{2+} in cells can achieve 20,000-fold between intracellular compartments (nM) and the extracellular medium (mM). These properties resulted in evolution acting on eukaryotic cells to adapt to Ca^{2+} first as something to remove, but

later as an important molecule for signal transduction due to the elevated binding affinity and capacity to form intracellular micro-domains **(Rizzuto R. and Pozzan T. et al 2006).**

Throughout evolution, cells have selected proteins that are either able to mobilize Ca^{2+} or to use it as the trigger for activating signaling networks ranging from cell death, metabolism and gene expression. One of the proteins that has specifically adapted to bind Ca^{2+} is calmodulin. Calmodulin drastically changes conformation upon Ca^{2+} binding, exposing a bulky hydrophobic structure able to interact with different target sites in many different proteins leading to dimerization, active site reshaping and loss of protein autoinhibition **(Abzhanov A. et al 2006)** Curiously, in agreement with the theory described above, the majority of these target proteins constitute kinases and phosphatases. The most important ones for the context of neuronal plasticity are MLCK **(Lamprecht R. et al 2006)**, CaMKII **(Shonesy BC. et al 2014)**, PKC and PKA **(Boehm J. et al 2006)**

Given the characteristics of Ca^{2+}, it is understandable that cells dedicate several proteins or protein complexes to regulate cytoplasmic concentrations of this ion. Moreover, organelles are also important quenchers of intracellular Ca^{2+}, such as the case of mitochondria and endoplasmic reticulum (ER). To maintain normal low Ca^{2+} concentrations, ATPase pumps actively expel Ca^{2+} to the extracellular medium through PMCA and to the ER through SERCA **(Parys JB. and Guse AH. 2019).**

Therefore, there are two possible sources of intracellular cytoplasmic Ca^{2+}: the extracellular environment and internal stores (in most healthy/physiological conditions, the ER). In order for signaling to occur, Ca^{2+} must enter the cytoplasm in a spatial and time specific manner. For this, cells have developed several channels with different affinities, kinetics and permeabilities that are recruited to specific places in the cell depending of the cellular process initiated **(Berridge MJ. et al 2003).**

There are two main classes of channels: voltage-gated and non-voltage-gated. These are not present uniformly in all cell types since they are highly dependent on the cell function. The best example is voltage-gated neuronal presynaptic Ca^{2+} channels that are crucial for quick and transient neurotransmitter release into the synaptic cleft. These voltage-gated channels are the fastest Ca^{2+} signaling proteins, as they can achieve 100-fold increase in intracellular Ca^{2+} concentration in a matter of milliseconds **(Worrell JW. and Levine RB. 2008; Guéguinou M. et all 2013)**. On the other hand, non-voltage Ca^{2+} channels are slower and do not increase as much intracellular Ca^{2+} concentrations. They include ion exchangers (which exchange sodium or potassium with Ca^{2+}), ligand-gated channels and store operated Ca^{2+} channels. TRP channels are the most well characterized ligand-gated channels since they respond to mechanical distortion, multiple G-

coupled receptors and growth factor receptors, making them not only important for sensing physical cellular stress but also for Ca^{2+}regulation during development **(Ramsey IS. et al 2006).**

While all other channels mediate quick transient cytoplasmic Ca^{2+} increase, store operated channels like the STIM/Orai complex allow for more sustained concentration changes overtime. These sustained Ca^{2+} changes start with the activation of IP3 receptor in the ER, resulting in Ca^{2+} depletion in the cytoplasm. This abrupt Ca^{2+} release activates the ER Ca^{2+} sensor STIM, which while still included in the ER membrane, travels closer to plasma membrane. Activated STIM is able to contact Orai and promote Ca^{2+} influx from the extracellular space into the cytoplasm. This process is crucial for creating local specific microdomains of high Ca^{2+} concentrations since Ca^{2+} will only entry in the locations where STIM binds Orai **(Qiu. R and Lewis RS. 2019; Zhang SL. et al 2005).**

Altogether these channels with distinct properties explain how Ca^{2+} variations can influence in a time and space specific manner, several molecular transducers and in consequence many cellular processes.

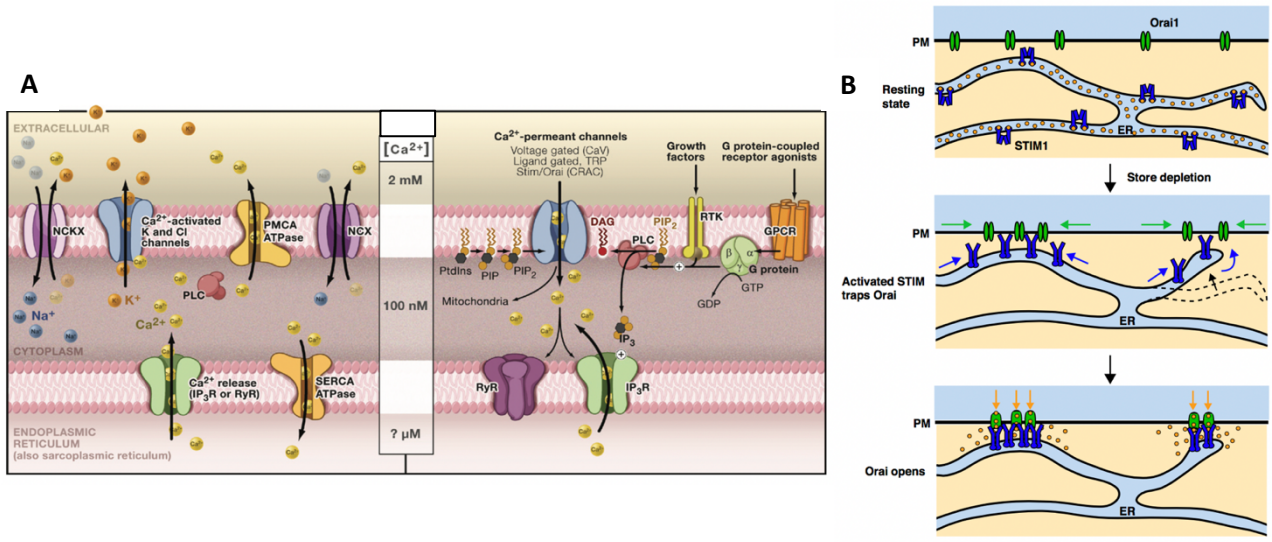

Figure 9 - Different channels and pumps that regulate Ca^{2+} concentration. Schematic representation of STIM/ORAI activation

(A) – In resting physiological conditions Ca^{2+} must be maintained in low intracellular concentrations. Plasma membrane Ca^{2+} ATPase (PMCA), smooth endoplasmic reticular Ca^{2+} ATPase (SERCA), Sodium Ca^{2+} potassium exchanger (NCKX) and Sodium Ca^{2+} exchanger (NCX) contribute to low intracellular Ca^{2+} concentrations. Voltage-gated Ca2+ channels (VGCC), Non voltage-gated channels (like TRP and STIM/ORAI), ryanodine receptors (RyR) and inositol (1,4,5) trisphosphate (IP_3) receptors (IP_3R) increase locally Ca^{2+} in the cytoplasm. G protein-coupled receptor (GPCR) or receptor tyrosine kinase-mediated activation of phospholipase C (PLC) cleaves PIP_2 into IP_3 that is essential for IP_3R function. PLC is also responsible for regulating lipid rafts associated with Ca^{2+} channels, thereby regulating their locations in membranes.

Adapted from Clapham DE. 2007 (B) – Store operated Ca2+ entry through STIM/ORAI. STIM is a Ca^{2+} sensor in the ER and ORAI is a Ca^{2+} channel in the plasma membrane. When there is Ca^{2+} store depletion from the ER or even local increase in cytoplasmic Ca^{2+}, STIM is activated and induces ER dislocation to the plasma membrane. Activated STIM can bind ORAI and this interaction opens ORAI channels that allow massive amounts of Ca^{2+} to flow into the cytoplasm. **Adapted from Qiu R. and Lewis RS. 2019.**

1.10 The role of Ca^{2+} in synaptic plasticity

Neurons dispend much of their energy in regulating Ca^{2+} since it has the ability to modulate normal synaptic function by mediating vesicle fusion as well as regulate synaptic plasticity **(Golowasch J. 2019).** Considering synaptic plasticity, the classical (and probably the best) textbook example that has been studied for many years is the mechanism of long-term potentiation or LTP. Long-term potentiation, LTP, induction is dependent on presynaptic neurotransmitter release and postsynaptic depolarization. In excitatory, glutamatergic synapses, presynaptic neurotransmitter (NT) release will activate AMPA and NMDA receptors. However, because NMDA receptors have magnesium blockade, after initial NT release, only AMPA receptors will open, making possible for sodium ions to enter and depolarize the postsynaptic partner **(Diering GH. and Huganir RL 2018)**. Depolarization of the postsynaptic neuron leads to the removal of the magnesium blockade from NMDA receptors. If neurotransmission is still occurring at the same time that the magnesium blockade is relieved, NMDA receptors will allow the entrance of sodium, but more importantly, of Ca^{2+}. For this reason, NMDA receptors are called coincident detectors, since glutamate activation and postsynaptic depolarization must both occur to allow for their opening. Ca^{2+} concentration increases in the postsynaptic neuron, leading to cAMP production and kinase activation like PKC and CamKII , which can regulate several signaling cascades **(Zucker RS. And Regehr WG 2002).**

Focusing on CamKII, when Ca^{2+} enters the cell and activates calmodulin by direct binding, calmodulin itself can bind to CamKII catalytic domains, leading to transient CamKII activation. However, CamKII has also auto-inhibitory domains that rapidly remove calmodulin binding. Therefore, if the increase in Ca^{2+} concentration in the postsynaptic neuron is slow or simply not enough, CamKII is only active during brief and almost insignificant moments. To overcome this self-regulation, Ca^{2+} increase must be rapid or prolonged so that multiple calmodulins can bind to different CamKII subunits, thereby promoting a chain reaction of rapid phosphorylation of these different subunits. This property of CamKII (together with high Ca^{2+} concentration) allows the surpassing of the auto-inhibitory effect and allows CamKII to be fully active when Ca^{2+} concentration decreases afterwards (and calmodulin detach from the kinase). CamKII has multiple target that contribute to synaptic plasticity **(Lisman J. et al 2002).**

Interestingly, low frequency stimulation also activates NMDA receptors but leads to a small sustained increase in Ca^{2+} concentration which is thought to promote activation of phosphatases. Due to this opposing effect, low frequency stimulation leads to long term depression (LTD).

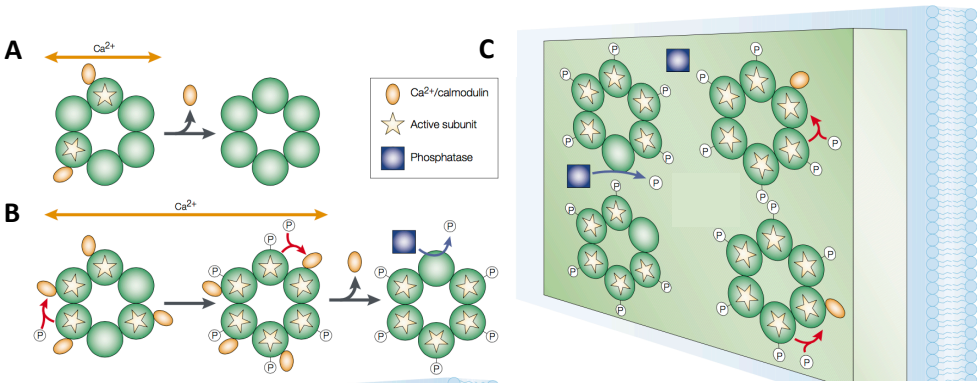

Figure 10 - Different Ca^{2+} concentrations regulate differently CaMKII
(A) – CaMKII is activated by Ca^{2+}/calmodulin binding because CaMKII subunits can be phosphorylated by Ca^{2+}/calmodulin. When intracellular Ca^{2+} concentration is low, the magnitude of phosphorylation is also low and is not able to induce the cascade of autophosphorylation of the remaining CaMKII subunits. **(B) –** When intracellular Ca^{2+} concentration is not only high in amplitude, but also quick in time, autophosphorylation is promoted and CaMKII subunits are activated. **(C) -** These phosphorylations also propagate to other CaMKII molecules. **Adapted from Lisman J. et al 2002**

LTP and LTD are mutually exclusive phenomena when one considers a single window of time in a single synapse. Therefore, distinct regulation is imperative. Studies on Ca^{2+} synaptic plasticity have tried to develop some basic rules that should predict what is called the direction of plasticity: meaning that plasticity either follows LTP or LTD **(Fauth M. and Tetzlaff C. 2016).**

Considering Ca^{2+} concentration over time, the first property of neuronal Ca^{2+} signaling is amplitude. For the same period of time, a high Ca^{2+} peak promotes LTP whereas a low Ca^{2+} peak results in LTD (as described in the previous example).

The second level of complexity is the duration of the Ca^{2+} signal. In addition to amplitude, LTP is promoted when Ca^{2+} concentration increases greatly and rapidly, while LTD is promoted when Ca^{2+} concentration increases modestly and slowly.

At last, the final layer of complexity is location. Neurons can regulate channel trafficking and diffusion across the plasma membrane. These different Ca^{2+} channels have different kinetics when transporting Ca^{2+}, making

some more suitable for LTP (quick and high) and other for LTD (slow and low). Therefore, the neuron can create micro and even nanodomains of Ca^{2+} entry in specific regions in the cell to coordinate multiple LTP and LTD phenomena (**Evans RC and Blackwell KT 2015).**

In addition to synaptic strength modulation, LTP also involves the formation of new neuronal structures. This can also be explained by the fact that CamKII phosphorylates other targets, such as cytoskeleton regulators and transcription factors like CREB, essential for the formation of more spines and boutons.

Despite the vast knowledge that has been gathered from LTP/LTD studies and in how postsynaptic plasticity (both functional and structural) occurs, much less is known about the presynaptic structural alterations that neurons undergo during these plastic events. Presynaptic alterations represent another important layer of neuronal flexibility since structural modifications are crucial for allowing the establishment of new active zones and of synaptic boutons, that can sustain increased activity. The importance of structural plasticity is further evidenced by genome-wide analysis studies with disruptions in genes mostly related to presynaptic mechanisms underlying susceptibility to psychiatric disorders like schizophrenia and autism **(Monday HR. et al 2017, Nanou E. et al 2018).**

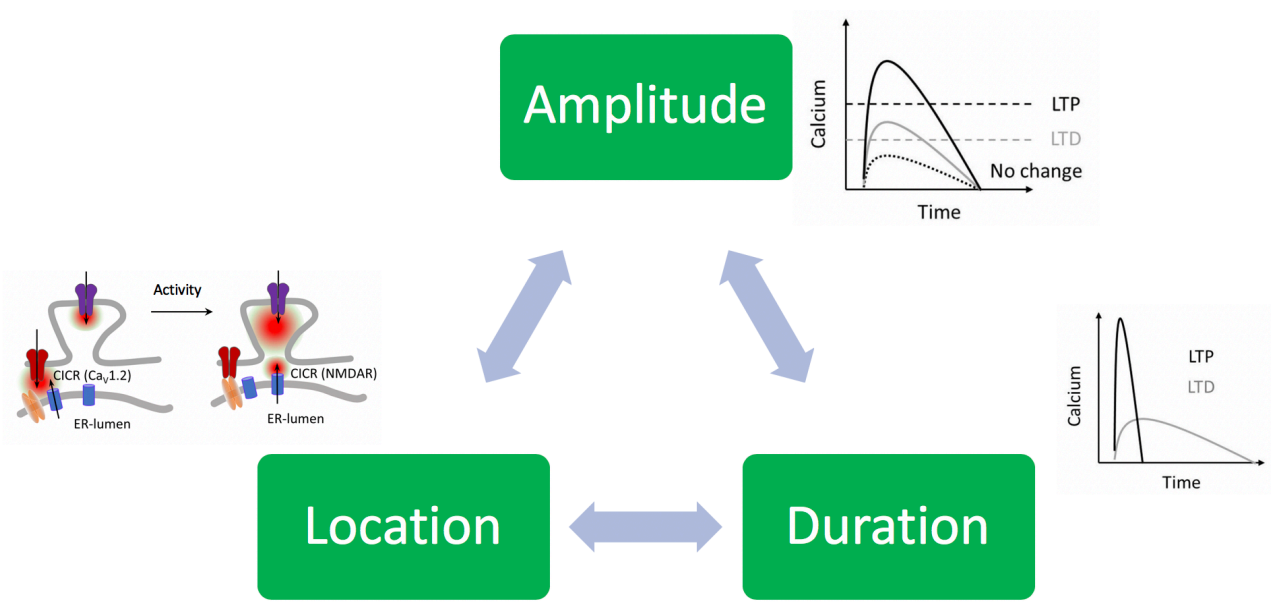

Figure 11 - The triangle of Ca^{2+} regulation
Adapted from Evans RC. and Blackwell KT. 2015

1.11 Ca^{2+} role during axon pathfinding and synaptogenesis

Ca^{2+} is essential for synaptic plasticity, but if it plays a direct role in neuronal structural plasticity is not known. Is Ca^{2+} important during structural changes in neurons, particularly is it important in the remodeling of presynaptic boutons?

Cell migration plays an important role in many cellular functions from tissue organization to homeostasis (**Manzanares MV et al. 2005, Trepat X. 2012**). Ca^{2+} fluctuations are crucial for time and spatial modulation of the cytoskeleton architecture and adhesion in order to achieve directionality of movement **(Wei C. 2012, Tadross MR. et al 2008)**. Normally, high Ca^{2+} microdomais are seen in the front of a migrating cell in response to membrane tension and chemoattractive cues. Both of these triggers recruit either membrane Ca^{2+} channels (like TRPs or voltage channels) or ER channels (IP3 receptor or ryanodine) to promote Ca^{2+} variations in the cell cytoplasm **(Wei C. 2009**). These Ca2+ pulses lead to cycles of lamellipodia retraction and adhesion modifications, which eventually lead to cell movement towards one direction or another (**Tsai FC. 2012**).

This rationale also applies during nervous system development, more concretely in the steering of the growth cone. Different guidance cues will induce different Ca^{2+} dynamics in the neuron leading to the activation of different downstream targets **(Rosenberg SS. And Spitzer NC. 2011, Heine M. et al 2019)**. For example, when Ca^{2+} signal is low in amplitude and slow in time it favors the activation of phosphatases like Calcineurin (CaN) and protein phosphatase 1 (PP1), that by removing phosphate groups from important signal transducers (kinases and other transducers) will lead to lamellipodium and filopodium retraction. However, when Ca^{2+} signal has an intermediate amplitude but rises quickly in time, kinase activation like CamKII and PKC will instead be promoted. Interestingly, this is very similar to what happens in LTP. Kinase activation will then modify crucial actin cytoskeleton regulators, such as Rac1 and Cdc42, resulting in lamellipodium protrusions and filopodial forward movements **(Gomez TM. and Zheng JQ. 2006, Kholmanskikh SS. Et al 2006).**

Taking into account that Ca^{2+} is a well described regulator of cell migration, in particular of growth cone migration, it is plausible that activity-dependent processes also use Ca^{2+} as a major regulator of the presynaptic cytoskeleton to form new boutons.

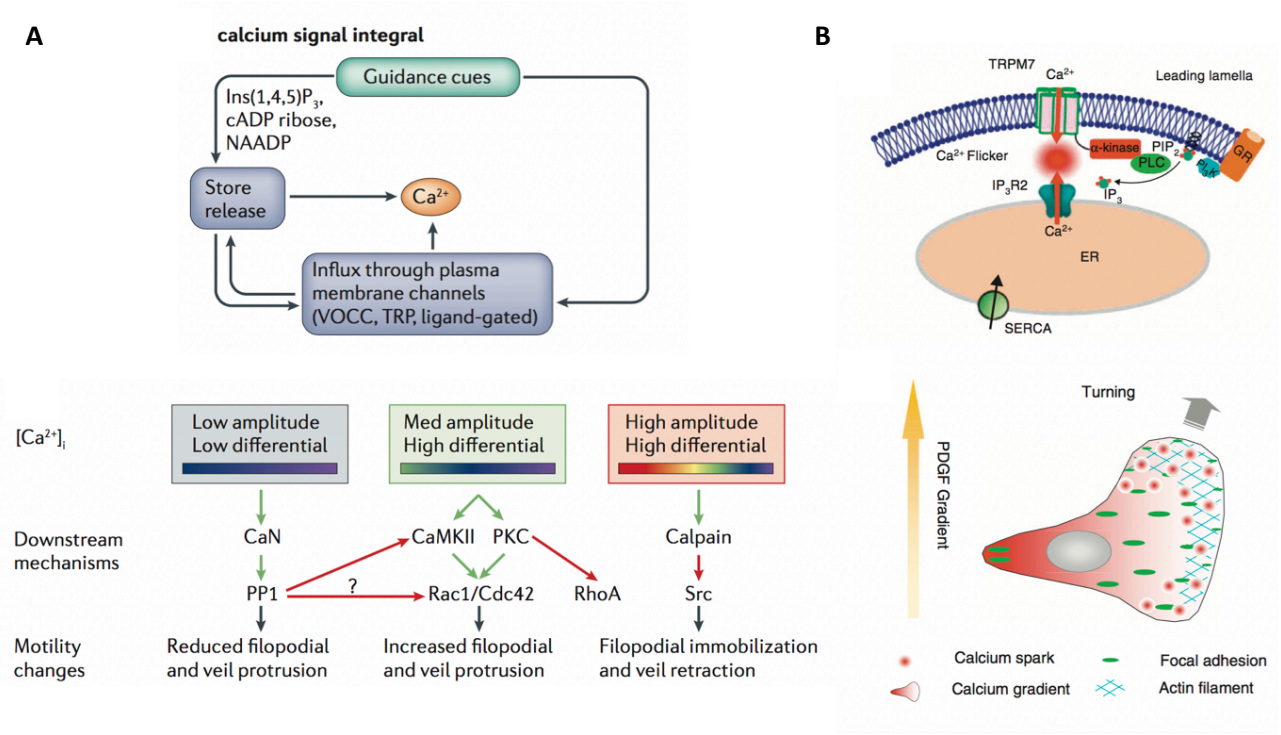

Figure 12 – Ca^{2+} role during axon pathfinding and migration

(A) - Several sources contribute to increase intracellular Ca^{2+}. Different Ca^{2+} concentrations contribute to distinct downstream activators that lead to different motility changes. **(B)** – Local Ca^{2+} gradients activate different downstream pathways to regulate cytoskeleton and adhesion in cellular microdomains. **Adapted from Gomez TM. and Zheng JQ. 2006; and Wei C. et al 2012**

1.12 Ca^{2+} role in Drosophila structural plasticity

While in growth cones there is a free axonal end that guides migration, how wired neurons grow and add new synaptic structures in response to activity, remains an open question.

It is well established that neuronal function and functional plasticity require Ca^{2+}. While Ca^{2+} uptake can be regulated at the level of the presynapse to regulate neurotransmitter release **(Akbergenova Y. et al 2018)**, glutamate receptor activation in the postsynapse also leads to Ca^{2+} entry in the postsynaptic terminal. **(Choi BJ. et al 2014)** However, how and whether this cation is required during or prior to structural changes of synapses is not very well understood. Additionally, it is unknown if the Ca^{2+} needed for NT release and the pathways that regulate structural remodeling are co-activated, if they crosstalk or if they are independently regulated.

The first studies interested in characterizing Ca^{2+} dynamics at the Drosophila NMJ plasticity where done by manipulating presynaptic voltage gated Ca^{2+} channels (VGCC). The most important VGCC for neurotransmitter release is the N-type receptor cacophony (cac), also designated as Dmca

Littleton and colleagues showed that a loss of function mutant of this Ca^{2+} channel ($Dmca^{NT27}$) in third instar larvae m6/7 NMJs had fewer presynaptic structures than wild-type larvae, along with disrupted neurotransmission **(Rieckhof GE. Et al 2003).** However, the presynaptic undergrowth observed was not due to neurotransmission defects since affecting neurotransmission only, did not lead to the same presynaptic defects. Despite these important observations, all the presynaptic structure analysis took only into account developmental defects since no activity-dependence was tested.

In addition, Robin L. Cooper and colleagues performed a similar study with a different mutation (Cac^{ts2}, loss of function) and observed identical presynaptic defects. Moreover, by doing electrophysiology recordings, the authors added that presynaptic boutons exhibit some level of presynaptic transmission, meaning that these boutons are not fully dysfunctional (otherwise the larvae would not reach third instar – cac null mutants die as late stage embryos). Altogether, and considering the role of Ca^{2+} in growth cone migration, the authors hint to the possibility that reduced cytoplasmic Ca^{2+} could lead to decreased membrane dynamics therefore resulting in reduced synaptic connectivity. Again, these authors do not analyze activity-dependent presynaptic structural alterations **(Xing B. et al 2005).**

2. Objectives

Altogether, in my thesis I aim to understand:

- Is Ca^{2+} involved is structural plasticity?
- How is Ca^{2+} regulating synaptic structural plasticity in a space and time specific manner using a bleb like mechanism?
- What is the source of Ca^{2+}?

The study of how Ca^{2+} can regulate activity-dependent processes, will help the understanding of the possible pathways that might be in interplay during presynaptic structural plasticity. Since many neurological disorders have plasticity defects as a common hallmark, we also expect that understanding mechanisms that promote plasticity, one can contribute to the development of novel strategies that could eventually promote synapse formation and disease recovery.

3. Materials and methods

Fly stocks and husbandry

Flies were cultured at 25ºC and maintained on normal media. For 3rd instar larval collection, eggs were laid and grown on vials with yeast paste and apple juice for 25ºC. The w1118 line served as a control genotype, as the stocks used are in this genetic background. Neuronal-specific transgene expression of Nsyb-Gal4 (Pan-neuronal driver) was used to drive UAS-constructs' expression. UAS lines are listed below. UAS-mitoGFP/TM6b line was kindly given by Susana Ponte.

For RNAi experiments, flies expressing the neuronal driver and the UAS-RNAi were mated at 29-30ºC to maximize the efficiency of knockdown.

Fly lines used

Line name	Description	Reference
Cyo/Sco ;Dvglut>mcherry/TM6	Expresses a red fluorescence CAAX internal membrane tag mcherry in glutamatergic neurons 3rd chromossome	Beak M. et al 2013
13XLexAop2-IVS-GCaMP6f	Expresses the genetically Ca^{2+} sensor GCaMP6f under the control of lexAop2	BDSC #44277
UAS-Cac RNAi	Expresses RNAi against the voltage gated Ca^{2+} channels pore subunit Cac	BDSC #27244
UAS-Stj RNAi	Expresses RNAi against the voltage gated Ca^{2+} channel auxiliary subunit Stj	BDSC #25807

UAS-SERCA RNAi	Expresses RNAi against the ER ATPase SERCA	BDSC #44581
UAS-mitoGFP/TM6b	Expresses a GFP with a mitochondrial tag	
W_{1118}	Wild type line	BDSC #3605

Immunohistochemistry

For immunohistochemistry of the neuromuscular junction, 3rd instar larvae were dissected in PBS (phosphate-buffered saline) or HL 3.1 (Brent JR 2009). Gut and fat body were removed, while the CNS was kept intact until after fixation. The resulting larval fillets were fixed with PFA (4% paraformaldehyde diluted in 1x PBS) for 20 min. After fixation, larvae are washed in PBT (1x PBS + 0,3% Triton) to permeabilize membranes and remove fixative. Blocking of unspecific antibody binding was done incubating 30 min-1 hour with 5% NGS (Normal Goat Serum) diluted in PBT. Primary antibody incubation was performed in blocking solution overnight at 4ºC. Subsequently, larvae were washed in 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.

Primary antibodies

Antigen ID	**Species**	**Concentration**	**Fixative**	**Reference/Source**
DLG(4F3)	Mouse	1:250	PFA	Hibridoma bank
GFP (A-11122)	Rabbilt	1:1000	PFA	Life technologies

Conjugated antibodies

Antigen	**Concentration**	**Source**
Cy3-conjugated goat anti-HRP	1:500	Jackson Immunoresearch

Secondary antibodies

Antigen	Concentration	Source
A488-Donkey anti-Mouse	1:500	Jackson Immunoresearch
A647Donkey anti-Mouse		
A488-Donkey anti-Rabbit		

Stimulation of larval NMJs

Third instar larvae were pinned down in 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 to the stimulation the dissection pins were moved inward to allow muscle contraction. We used two well-established protocols to induce activity dependent bouton formation using spaced High K+ depolarization that we call Short-Stim (SS) **(Piccioli ZD. and Littleton JT. 2014)** and Massed Stim (MS) **(Vasin A. et al 2019),** and both induce new boutons at the *Drosophila* larval NMJ (in our hands at NMJ muscle 6/7 ± 5.5 new boutons). Relaxed fillets were subjected to incubations in high K^+ (90 Mm) and High Ca^{2+} (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 a single massed pulse of 16 min with High K^+ High Ca^{2+}. For immunohistochemistry larvae were fixed after 30 min and SS was always used **(Fig 13).**

Since these solutions maintain the viability of the larvae for about 2 hours, for live imaging larvae we used MS to have more imaging time in the spinning disk confocal. Larvae were previously selected in a fluorescence stereoscope to be Dvglut>CAAX-mcherry and GCaMP6f positive.

In addition, larvae for live imaging were dissected as described before but with much smaller pins to allow a lamella on top of the larvae. Slides and lamella are filled with HL3.1, sealed with tape and imaged. For live imaging, only the gut and fat cells are removed, leaving intact the ventral nerve cord and all the central nervous system.

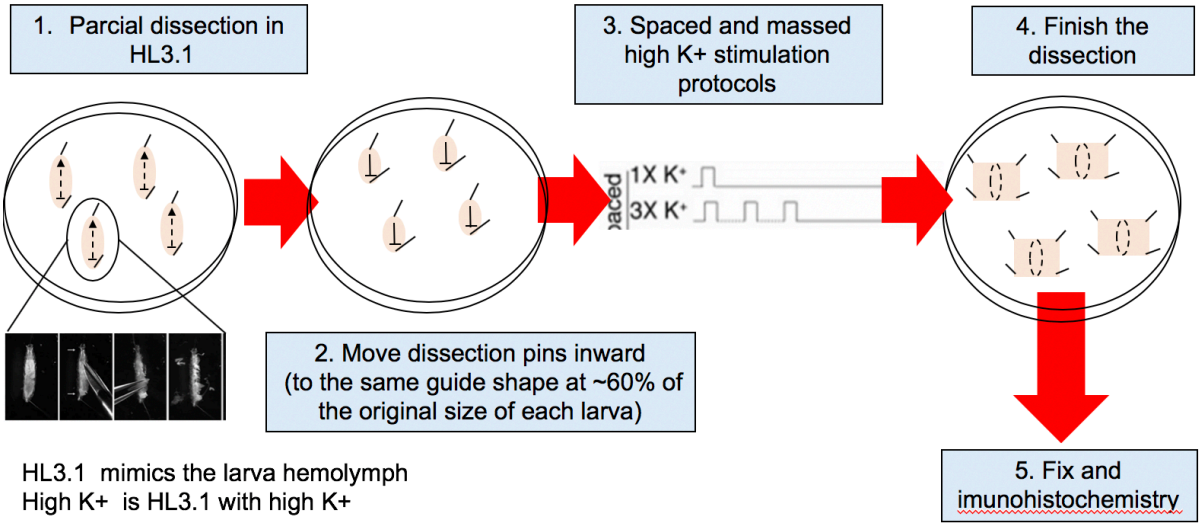

Figure 13 - Workflow of the dissection and stimulation protocol

Identification of new activity-dependent boutons:

New boutons can be identified by direct observation of live events OR they can be inferred from fixed larvae post stimulation. In fixed animals, we stain larvae with a neuronal membrane marker to visualize MNs (usually HRP) and with the postsynaptic marker Discs Large (Dlg), which has been shown to be absent from the newly formed boutons (and present in mature boutons). The logic is that when new boutons form they are still immature and devoid of Dlg. These new boutons can either be maintained and matured, or be transient and pruned. In either case, we can identify new structures based on this approach. This method is routinely used in the field and has been shown to accurately identify acutely formed boutons. The analysis of activity-dependent bouton formation in live samples versus fixed samples is a powerful approach to extract both the dynamics (from live) and the quantitative information (from fixed) from larvae subjected to acutely induced structural plasticity.

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. Live-imaging experiments 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, from abdominal segments A2-A4.

All confocal images were analyzed using Fiji image analysis software. For analysis of bouton formation and immuno-localization in fixed samples, maximum intensity projections from z-stacks were used.

Live imaging analysis

For live imaging analysis, maximum intensity projection from Z-stacks were also used. To upload videos on Image J, BioFormats plug in was used. Max intensity projections were performed together with contrast enhancement (0,3% saturated pixels, all slice normalized)

1 - Ca^{2+} levels in stimulated vs unstimulated *Drosophila* larvae

We performed unstimulated versus stimulated analysis using different NMJs from different larvae from different imaging sessions. Acquisition settings were adjusted depending on the stimulated larvae expression. Unstimulated larvae were imaged according to stimulated larvae settings.

To have a quantification of the GCaMP6f intensity values, a Region of Interest (ROI) generally surrounding the NMJ was delimitated. Mean intensity values were extracted from 3 time points representing an initial, a middle, and a final arbitrary time point of the video. This temporal analysis was performed to demonstrate that during one imaging session, GCaMP6f signal is relatively stable. A different ROI representing background levels was also measured and subtracted to the NMJ GCaMP6f intensity values. Similar analysis done in Gregory T. Macleod 2015

2 - Ca^{2+} and membrane dynamics in new bouton formation and retraction

After identifying videos with new sites of bouton formation or retraction, rigid registrations across time using the maximum thresholds of the GCaMP6f channel was performed to remove possible XYZ drift that could interfere with further analysis. For more details on registration see **Preibisch S. et al 2010.**

To analyze local intensity signals, a ROI was defined based on the site previous to the Ca^{2+} burst and on the location of the structure after the Ca^{2+} burst. Mean intensity values were measured frame by frame for both channels with the same area. In addition, the same ROI was used to measure background levels which were subtracted to the GCaMP6f levels in the bouton in each frame.

To analyze signal dislocation, we traced a single line ROI starting in the furthest point of the original bouton and ending in the furthest point of the secondary structure (either a new bouton or a retracted one). With this ROI, we used Plot profile to generate a graph with the intensity signal (Y axis) across the specified distance (X axis). To have the temporal perspective, we did this ROI plot profile analysis frame by frame. All analysis was done in ImageJ/Fiji software

Statistical analysis

All analysis was completed in GraphPad 8.0. Data was 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, Kruskal-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 controls. Sample size (n) is presented in the figure legends.

4. Results and Discussion

4.1. Ca^{2+} and structural Plasticity

4.1.1 Ca^{2+} imaging at the *Drosophila* NMJ

Activity-dependent plasticity is essential for learning and memory but the molecular pathways that contribute to this process are still poorly understood. Our lab has identified membrane blebbing as a novel mechanism by which motor neurons remodel in a wired network, in response to acute activity. However, how motor neurons coordinate specific sites of new bouton formation with synaptic transmission is still unknown. Presently, we know that Ca^{2+} can regulate a multitude of neuronal functions from neurotransmitter release, to plasticity and cell migration. It is also known that actomyosin contractions present in initiation and retraction of blebbing protrusions can be regulated by increase in intracellular Ca^{2+}. Therefore, our goal is to study if Ca^{2+} could be the signal mediating new bouton formation in the context of neuronal activity and activity-dependent bouton remodeling.

In order to study Ca^{2+} dynamics, one must be able to visualize the presence of this ion inside the cell. Initial studies imaging Ca^{2+} in *Drosophila* motor terminals used florescent molecular probes that allowed quantification of Ca^{2+} concentration already with single bouton resolution. Even though type Ib boutons are larger than Is boutons and usually contain more active zones, they have slightly lower synaptic transmission efficacy **(Karunanithi S. et al. 1997)**. Nevertheless, Ca^{2+} concentration accumulation is directly proportional to the frequency of the stimulation protocol in both terminals, which is an important factor when studying Ca^{2+} intensity and dynamics, since it might provide information about the ongoing activity of the neuron **(He T. and Lnenicka GA. 2011).** In addition, Ca^{2+} concentration inside the terminal dissipates shortly after the end of the terminal being stimulated (about 60ms), suggesting the existence of efficient quenching or extrusion mechanisms of cytosolic Ca^{2+} clearance **(Macleod GT. Et al 2002).** This is agreement with the physiological role of motor terminals, since intense muscle contraction needs a high frequency of firing rate, which is only possible if variations in Ca^{2+} concentrations are quickly regulated.

Despite the general requirement of Ca^{2+} regulation in presynaptic boutons, there is a significant heterogeneity in synaptic transmission across boutons of the same NMJ. This is evident upon stimulation where Ca^{2+} influx (as well as exocytosis) is higher in distal boutons compared to proximal ones (in relation to the branch center) **(Guerrero G. et al 2005; Akbergenova Y. et al 2018; Newman ZL. Et al 2017).** However,

how Ca^{2+} levels and dynamics are regulated in presynaptic terminals, and whether it is required for structural changes, remains an open question.

4.1.2 Ca2+ dynamics: using molecular probes

The original Ca^{2+} indicators used in different studies were small organic synthetic molecules that bind and unbind to Ca^{2+} (emitting fluorescence) with different affinities and kinetics. Although these probes represented a gold standard technique for many years that allowed the characterization of neuronal Ca^{2+} dynamics, they demand severe nerve manipulation and/or damage (since they need to be injected in the cytoplasm of neurons). Additionally, these indicators bound with excessive affinity to Ca^{2+}, which may result in significant Ca^{2+} buffering interfering with normal neuronal functions **(McMahon SM and. Jackson MB 2018; Karunanithi S. et al. 1997).** To overcome such problems, several labs have developed genetically encoded indicators of neuronal activity (GINAs) that can report on vesicle release (like pHfurins), changes in neurotransmitter concentration (like glutamate sensors), membrane voltage and of course Ca^{2+} dynamics. The most important advantage of GINAs (besides the previously mentioned) is cell type specificity since they are genetically encoded and can be integrated in binary system like Gal4/UAS or lexA/lexaOp **(Lin MZ and Schnitzer MJ. 2016; Broussard GJ. Et al 2014).**

GINAs structure design is crucial for temporal reporting since fluorescence only occurs when binding of one specific molecule. Focusing on genetically encoded Ca^{2+} indicators (GECI) – the most used across neurobiology studies, is the GCaMP family. There are three major divisions in the structure of GCaMPs: a sensing domain from calmodulin (CaM), an intermediate peptide from smooth muscle myosin light chain kinase and a cyclically permuted florescence protein. The main result of this engineered probe is that when Ca^{2+}+ is in low concentration, the amount of reporter proteins that will be activated is also low, resulting in low fluorescence signal. Conversely, when Ca^{2+} concentration is high, there will be more protein reporter's activation, and a higher fluorescence signal. This property makes signal intensity quantifiable **(Adapted from Sun XR. Et al 2013).**

How exactly can the particular structure of GCaMP provide such properties?

The sensing domain of CaM is split in the N-terminus, which has low affinity for Ca^{2+} and the C-terminus, which high affinity for Ca^{2+}. Between N- and C-terminus, there is an intermediate protein (also called RS20) which connects to the cyclically permuted fluorescence protein. Equally important, the N-terminus also directly connects to the fluorescence protein **(Fig 14A).**

When there is a slow Ca^{2+} step (difference between any two time points) Ca2+ will bind primarily to the C-terminus due to its high affinity, but not to the N-terminus. C-terminus activation will activate posteriorly the N-terminus through the RS20, resulting in an increase Ca^{2+} affinity of the N-terminus. Once N-terminus binds to Ca^{2+}, the entire CaM-RS20 complex shifts conformation, activates the fluorescence protein, making it available to be excited by the correct light wavelength **(Fig 14B).**

When the Ca^{2+} step is high, Ca^{2+} can directly bind to the N-terminus. Since the N-terminus has a connection to the fluorescent protein, the conformation shift occurs and rapidly induces its activation, making excitation possible. In this scenario, C-terminus is almost dispensable but can be posteriorly activated to give a more robust conformational change. After Ca^{2+} is released, the CaM-RS20 complex dissociates and the sensor goes back to the initial stage **(Fig 14C).**

Several GCaMPs have been developed to improve the signal to noise ratio, taking into account different population of neurons, imaging techniques and stimulation protocols. Altogether, the best sensor that has been validated among many studies is GCaMP6f.

GCaMP6f (GCaMP6 fast) has an excellent signal to noise ratio (better than any previous versions) and fast low affinity properties. This last property allows this Ca^{2+} indicator not only to have a quick fluorescence readout to easily identify Ca^{2+} pulses, but also to be an efficient probe without quenching too much Ca^{2+} for normal cellular functions **(Lin MZ and Schnitzer MJ. 2016; Chen TW et al 2013; Xing X. and Wu. CF. 2018).**

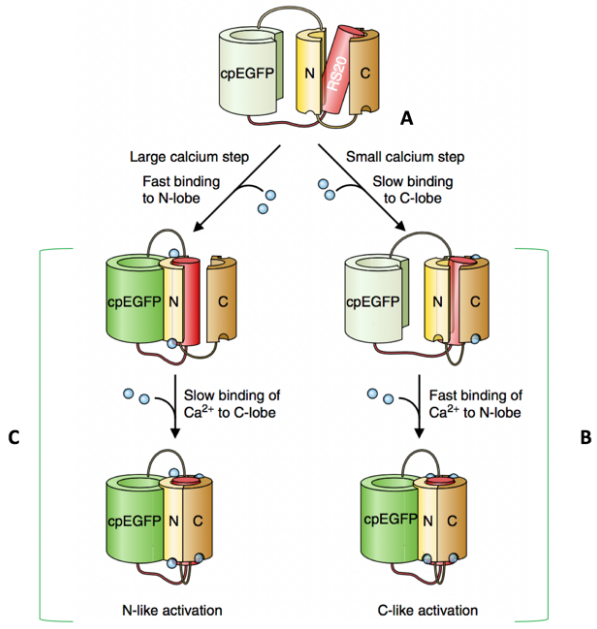

Figure 14 – GcaMP molecular mechanism

(A) GCaMP6f general structure. CaM is split into the N-terminus and the C-terminus. Between N- and C-terminus, there is an intermediate protein (also called RS20) which connects to the cyclically permuted fluorescence protein. **(B) -** When there is a slow Ca^{2+} step (difference between any two time points) Ca^{2+} will bind primarily to the C-terminus due to its high affinity, but not to the N-terminus. C-terminus activation will activate posteriorly the N-terminus through the RS20. Once N-terminus binds to Ca^{2+}, the fluorescence protein is activated. **(C) -** When the Ca^{2+} step is high, Ca^{2+} can directly bind to the N-terminus and activate the fluorescence proteins. After Ca^{2+} is released, the CaM-RS20 complex dissociates and the sensor goes back to the initial stage. **Adapted from Sun XR. Et al 2013**

4.1.3 Imaging presynaptic Ca^{2+} dynamics at the NMJ

To study the dynamics of Ca^{2+} during activity-dependent structural plasticity we expressed GCaMP6f in glutamatergic neurons and performed live imaging of stimulated larvae. We focused our analysis on the NMJs innervating muscle 6/7, segments A2-4. In order to visualize neuronal membrane remodeling together with Ca^{2+}, the glutamatergic driver used also expressed a mcherry transgene tagged to the plasma membrane (Dvglut>mCherry), which allows simultaneous visualization of GCaMP6f with the neuronal membrane. We analyzed Ca^{2+} dynamics in basal and stimulated conditions. Non stimulated larvae are dissected in haemolymph-like saline solutions (HL3.1) and imaged. To induce activity-dependent bouton formation, we dissect third instar larvae in HL3.1 and then added high K^+ high Ca^{2+} solutions (either in short pulses or in a single massed pulse) to depolarize the neurons and induced activity-dependent bouton addition. Following stimulation, larvae are kept in HL3.1 and imaged up to 2 hours post-dissection in a spinning disk confocal (see methods for a more detailed description), which allows fast acquisition of both GCaMP6f and mCherry signals (same imaging procedure for unstimulated larvae).

4.2 Ca^{2+} levels in stimulated vs unstimulated Drosophila larvae

The first important observation is that unstimulated larvae have a more uniform and lower GCaMP signal when compared with stimulated larvae (after stimulation), which have a generally higher signal in the entire NMJ with a few distinct boutons showing a really elevated Ca^{2+} signal **(Fig 15).** Since stimulation protocols are known to induce higher rates of neurotransmitter release, the GCaMP6f signal may reflect the increased general neuronal firing activity in the motor neuron. However, since the imaging session is posterior to the stimulation, this high Ca^{2+} must reflect a generalized alteration in the basal state of the terminal.

Note that this analysis was performed comparing NMJs imaged with different acquisition settings suited to the different expression levels of each NMJ. Ideally, one should perform analysis between unstim vs stim of

the same NMJ. Nevertheless, the objective is not to compare absolute values, but just to compare one condition relative to the other.

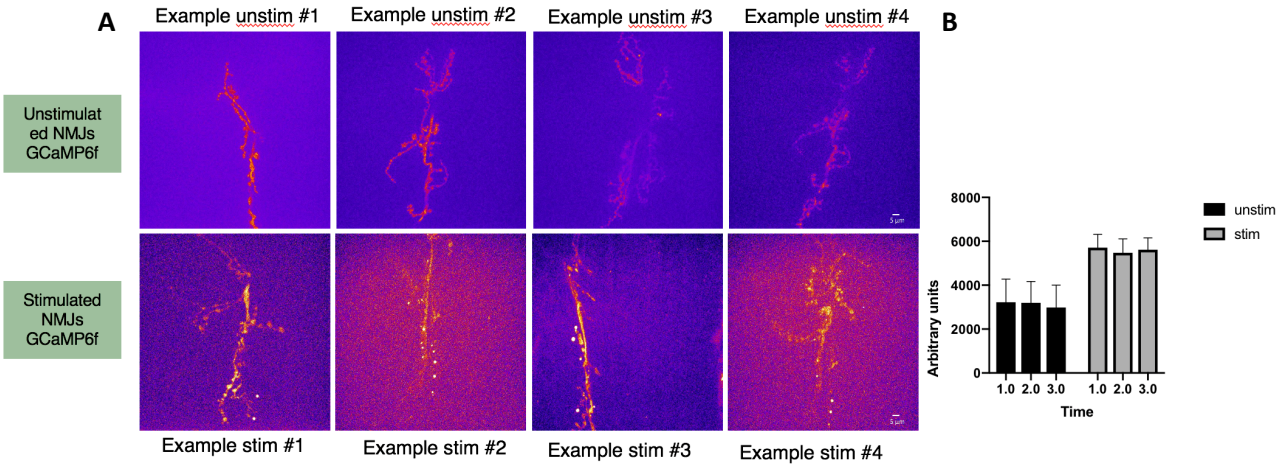

Figure 15 - GCaMP6f signal intensity analysis between unstimulated and stimulated NMJs

(A) – Fire imaging filter on stimulated and unstimulated Dvglut>mcherry and GCamP6f expressing larvae. For analysis purposes, only the GCaMP6f signal is shown. Unstimulated NMJs have a general lower signal. **(B)** Plotting signal intensity across 3 arbitrary time points that represent the beginning (t=1) the middle (t=2) and the end of the video (t=3). Data collected from 4 unstimulated NMJs and from 7 stimulated NMJs. Error bars represent SEM. Scale bar is 5μm

4.3 Ca^{2+} compartmentalization at the NMJ

Some stimulated NMJs show some easily identifiable boutons with very high GCaMP6f signal, which is in agreement with the heterogenicity in synaptic transmission that some authors reported, as mentioned previously. However, it is interesting to note that the very high signal is rather local **(Fig 16)**. In addition, these boutons with high Ca^{2+} signal have a morphology compatible to what it is described to be a new bouton. Even though, new boutons can only unequivocally be identified as boutons that lack postsynaptic labelling (DLG signal), there are few structural presynaptic characteristics that are common to new boutons. One of such characteristics is that new boutons are very round, big and, more often than not, are connected to the main branch by a thin thread that is almost imperceptible. In addition, new boutons generally have low membrane signal when compared with the main arbor. Some studies rely only on these characteristics to identify new boutons **(Ataman et al 2008, Vasin A. et al 2014).**

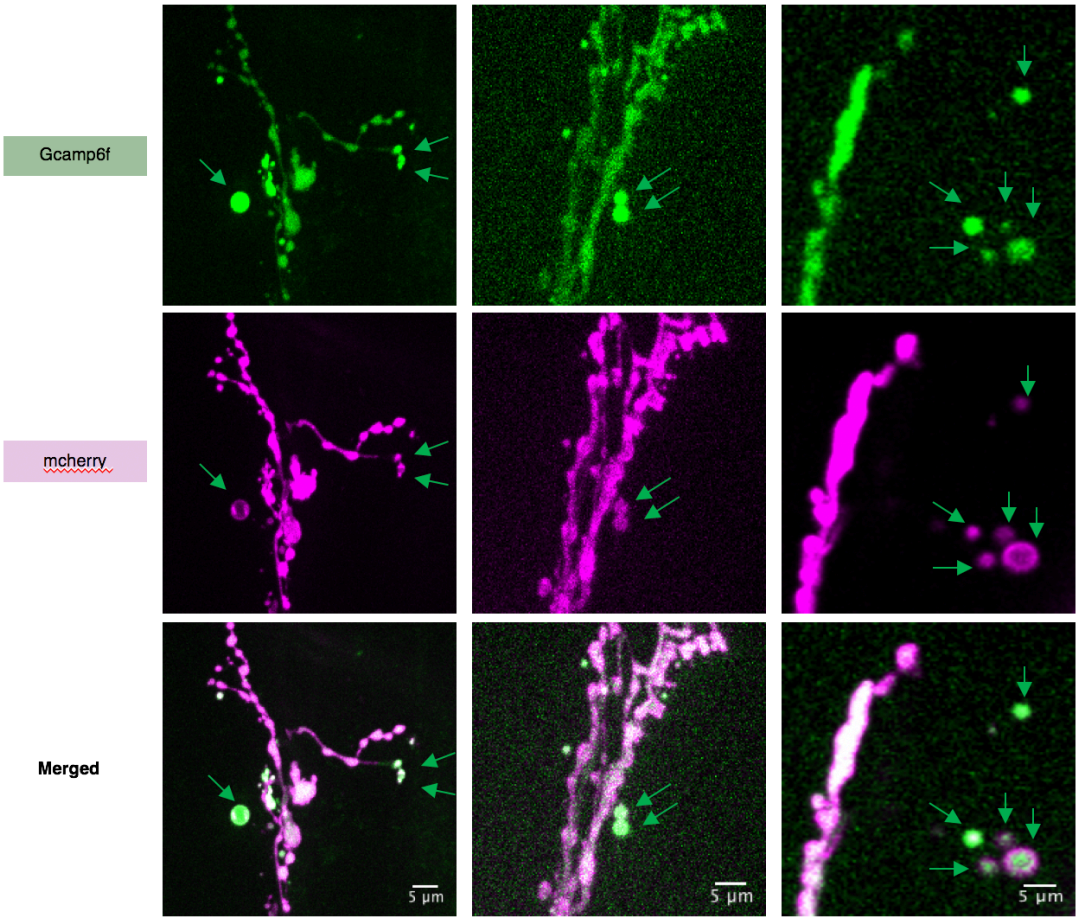

Figure 16 – Ca^{2+} compartmentalization at the NMJ

Stimulated NMJs expressing mcherry and GCaMP6f under the control of Dvglut. Neuronal membrane corresponds to the mcherry signal. Arrows point towards new like boutons that generally have high GCaMP6f signal comparing with rest of the NMJ, in response to activity. In these 3 examples all the boutons are connected to main arbor although in this saturation level it is not perceptible. Scale bar is 5µm

4.4 Ca^{2+} increase precedes new bouton formation

In addition to inducing synaptic transmission, stimulation protocols have been demonstrated to induce presynaptic structural rearrangements. Unlike previous live imaging studies, in our lab, we have demonstrated that bouton formation in response to acute activity leads to cytoskeletal rearrangements and plasma membrane remodeling that resemble membrane blebbing mechanisms. These remodeling events occur in a matter of seconds or minutes, usually associated with muscle contraction.

By doing imaging of neuronal GCaMP6f, we observed that prior to the appearance of new boutons there is a massive burst of GCaMP6f signal, which is sustained in the new bouton for several minutes, sometimes until the end of the video (which usually lasts for 60 min). Ca^{2+} increased more than 10 fold during the burst, while membrane intensity signal remains relatively stable throughout the formation of the new bouton **(Fig 17).**

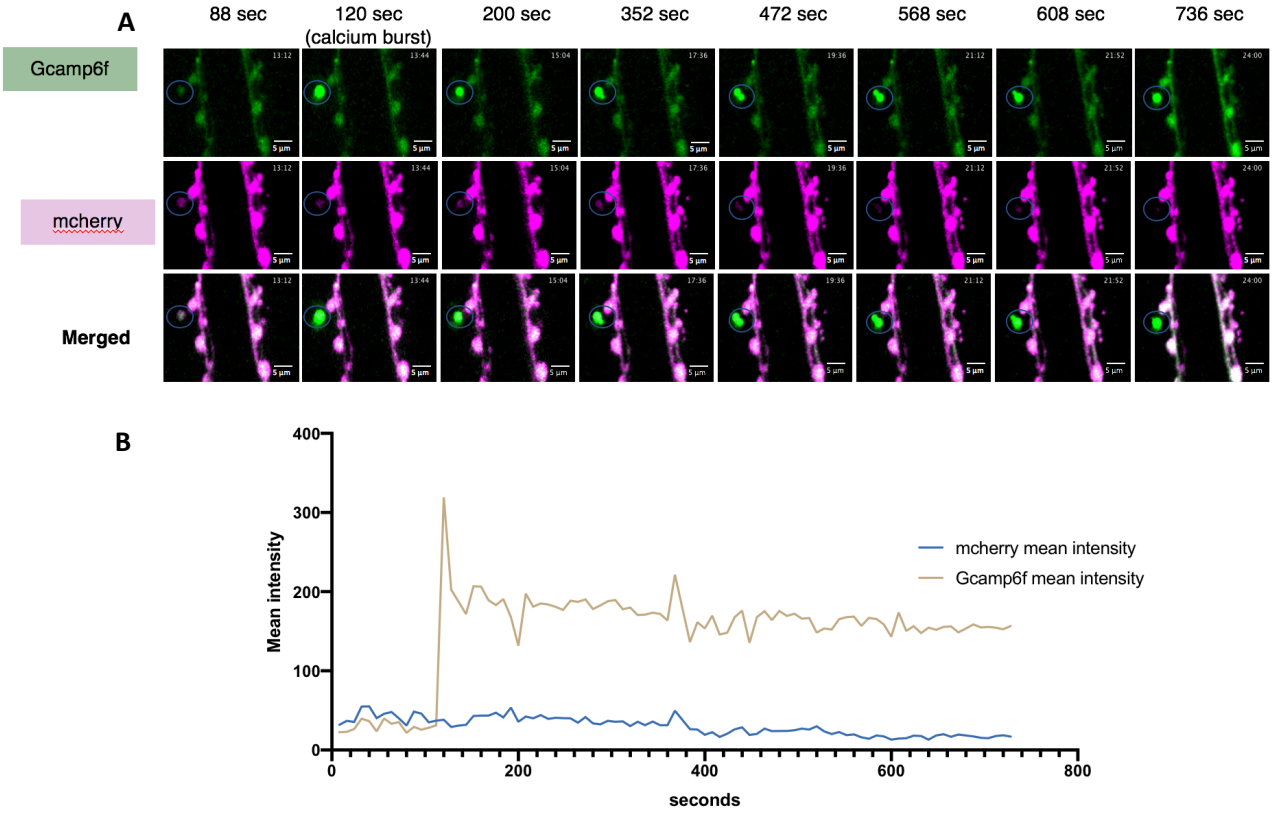

Figure 17- Ca^{2+} increase precedes new bouton formation

(A) –Stimulated Dvglut>mcherry and GCamP6f expressing NMJs. Neuronal membrane corresponds to the mcherry signal. We observed that GCaMP6f signal rapidly increases and posteriorly a new bouton starts to form. The membrane signal is very deemed. A schematic of the ROI used for mean intensity analysis is also represented **(B) –** Mean intensity plot of both channels, frame by frame, within the previous ROI. Background subtraction was done with the same ROI but in another region of the video. We observe that GCaMP6f rise more than 10-fold while membrane signal is more less stable. Scale bar is 5µm

To ask whether the Ca^{2+} burst precedes the membrane or vice-versa, we performed a single line ROI plot profile analysis (more details in Methods), which allows to follow the signal within a specified distance across time. This analysis revealed that the Ca^{2+} burst precedes membrane expansion, which only occurs after a few seconds of the increase in GCaMP6f signal. Furthermore, we observed that the Ca^{2+} signal accompanies bouton outgrowth and is displaced towards the site of the new bouton **(Fig 18).**

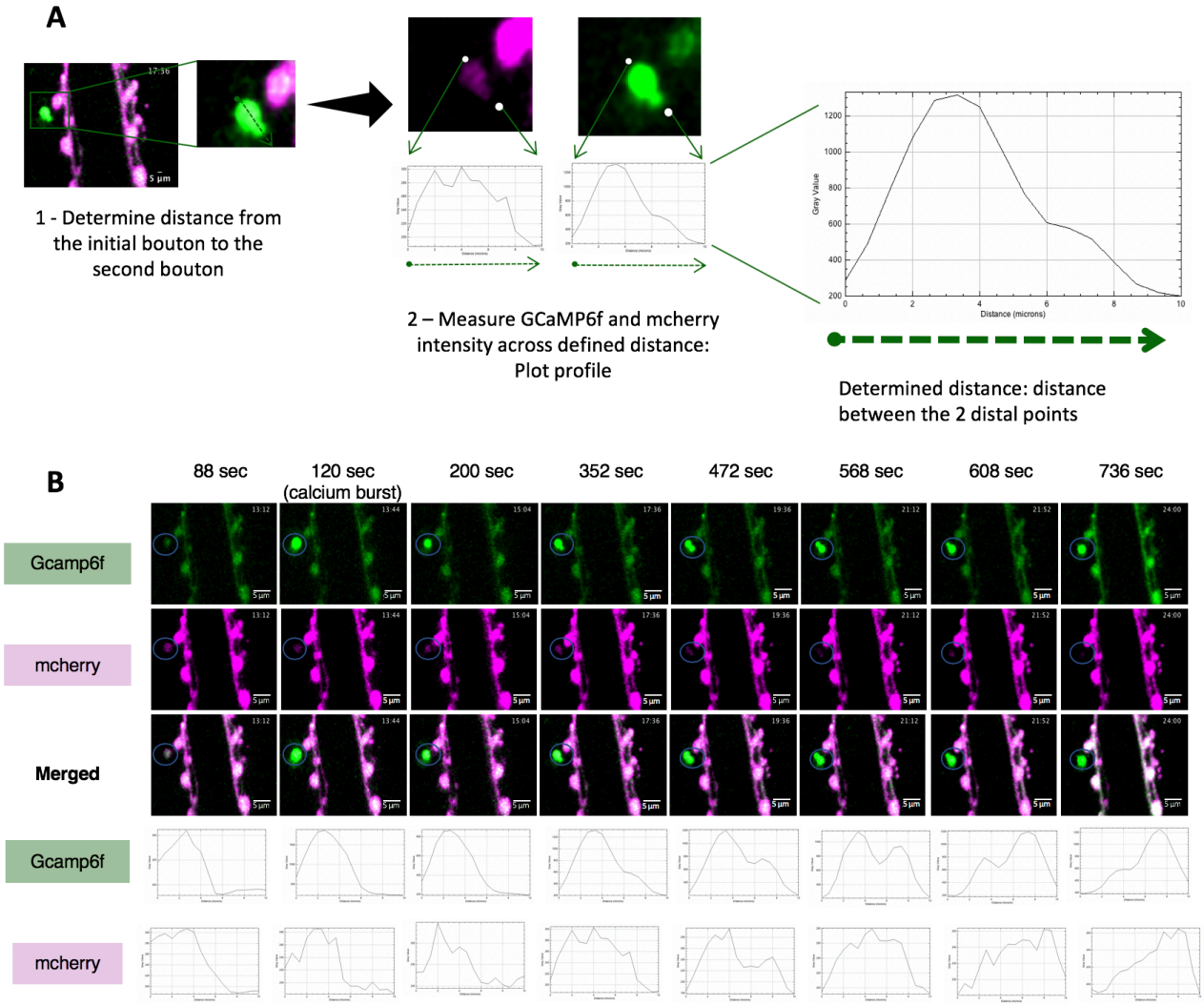

Figure 18 – Single line ROI Plot profile analysis

(A) – Stimulated NMJs expressing mcherry and GCaMP6f under the control of Dvglut. Flow diagram of the analysis. First, distance between the furthest point of the original bouton and the furthest point of the second bouton is determined. This defines the ROI of analysis. After, we plot the signal intensity profile, that is the mean intensity of each channel across the previous distance. **(B) –** When we do this profile analyses frame by frame (represented here in frames of interest) we can observe the signal across distance and across time. The second column indicates the Ca^{2+} burst. By doing this, it is possible to see that Ca^{2+} burst really occurs before any signal (GCaMP6f or membrane) dislocation shown in the plot profiles. Scale bar is 5µm. See supplementary video "Bouton formation"

From all the bouton formation events observed, there was always a rapid increase in Ca^{2+} inside the presynaptic bouton with sustained elevated Ca^{2+} signal in the new bouton. However, it is not true that all presynaptic Ca^{2+} bursts were followed by the formation of a new structure. This means that although rapid increase in Ca^{2+}+ levels could be a trigger to locally induce new bouton formation, other factors need to be

present to induce the formation of a new presynaptic bouton. In fact, data from colleagues in the lab showed that muscle contraction and glial contacts are also likely important contributors to the formation of new boutons **(Fig 19).** In summary, a Ca^{2+} burst is a good predictor of bouton formation, but other yet unidentified factors must co-occur.

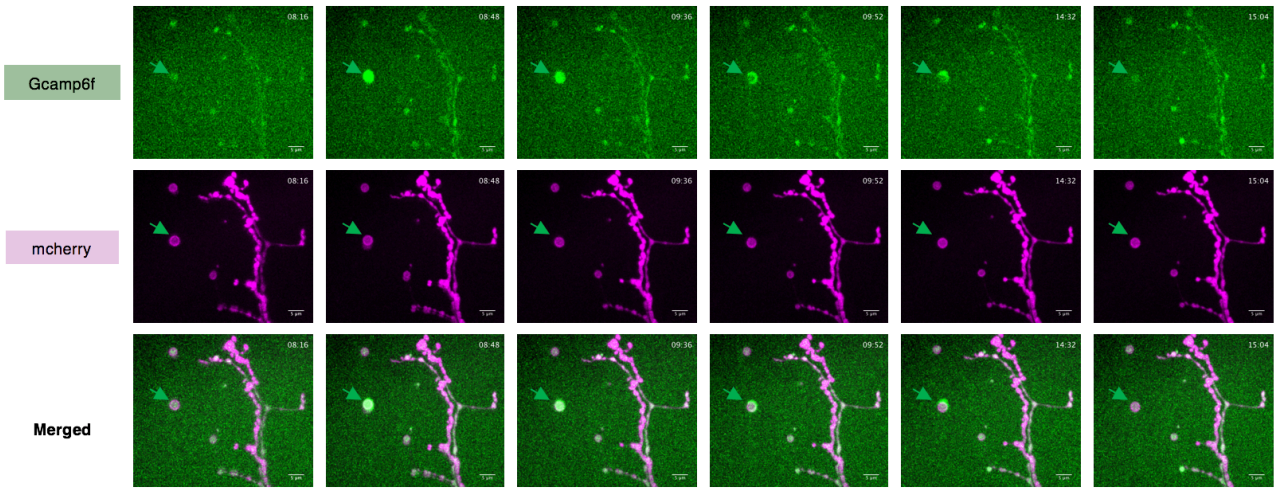

Figure 19 – Not all presynaptic Ca^{2+} bursts were followed by the formation of a new structure
Stimulated NMJs expressing mcherry and GCaMP6f under the control of Dvglut. Some boutons have moments of local increase in GCaMP6f signal but do not give rise to any structure alteration. One of the reasons for that might be the fact that detachment (like probably this case) from the main arbor makes structural modifications not possible due to lack of trophic support. Arrows indicate bouton of interest. Scale bar is 5µm.

4.5 Ca^{2+} increase signals local membrane remodeling

In addition to Ca^{2+} preceding bouton outgrowth, we also observe that some Ca^{2+} bursts are followed by a loss of definition in the delimitation of the membrane of boutons. These include not only new bouton formation events but also other events where there is no formation of boutons, suggesting that Ca^{2+} can lead to some local membrane alterations. Increases in Ca^{2+} concentration is associated, in the neuronal context, with flipase and lipase activation, which can both alter the membrane lipid composition **(Segawa K. et al 2014; Lauwers E. et al 2016).** Altering neuronal membrane lipid composition is one of the strategies to regulate voltage gated Ca^{2+} channels localization and neurotransmitter release in response to activity **(Khuong TM. et al 2013; Mochida S. 2017).**

Since the membrane tag used in our experiments has an mCherry CAAX marker (intracellular tag), rapid Ca^{2+} entry could activate flipase/lipases thereby removing some CAAX modifications from the tag, alter neuronal membrane composition and leading to the modification of the membrane profile.

Another possibility could be the increase in neuronal membrane surface area during bouton formation either by stretching or by vesicle addition, which would result in a "dilution" of the membrane signal. Although in our videos it is not possible to quantify if bouton membrane area is increasing, it is known that blebbing formation requires a source membrane, either by unfolding of membrane from intracellular invaginations or by exocytosis of secretory vesicles **(Goudarzi M. et al 2017)**. If vesicle fusion resulting from exocytosis occurs in response to the increase in intracellular Ca^{2+}, it would eventually lead to the dilution of the internal mCherry CAAX tag and to the alteration of the membrane profile **(Fig 20).** But which of these models is correct requires further experiments.

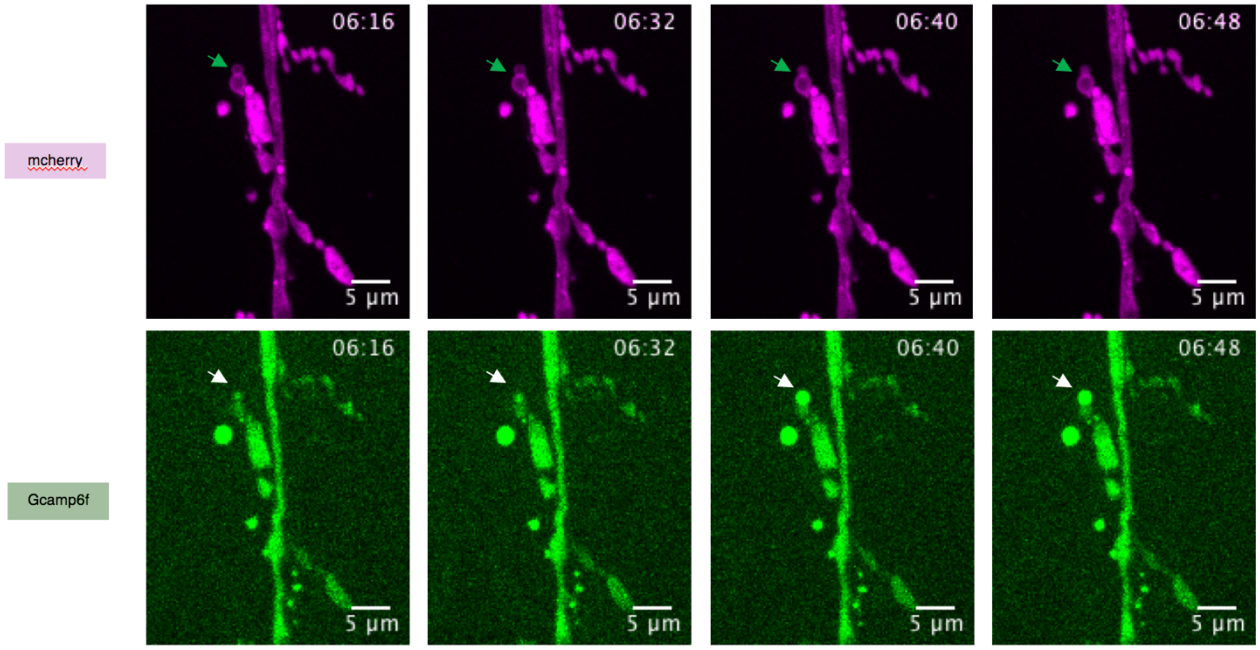

Figure 20 – Ca^{2+} increase signals local membrane remodelling
Stimulated NMJs expressing mcherry and GCaMP6f under the control of Dvglut. In other cases, although rapid GCaMP6f signal occurs and no structural alterations (formation or retraction) are detected, membrane signal alters drastically. This might be due to modifications in the internal mcherry tag or mcherry tag dilution due to vesicle fusion promoted by Ca^{2+}. Arrow indicates bouton of interest. Scale bar is 5µm.

4.6 Ca^{2+} rise precedes bouton retraction

It is known that new boutons not always stabilize and often retract due to mistargeted connections, insufficient metabolic support, or by active pruning signaling. We therefore asked if Ca^{2+} also plays a role during bouton retraction or pruning. Interestingly, during development this process is also mediated by activity, which can induce bouton retraction using Ca^{2+} as a major signal transducer **(Vonhoff F. and**

Keshishian H. 2017). However, this was never seen *in real time* in the Drosophila NMJ and in the context of increased activity.

During our imaging we observed events that resemble bouton retraction. The retracted structures do not have round shapes like new boutons do. Importantly, in retraction events there is also a Ca^{2+} burst before membrane displacement. Unlike bouton addition events, the GCaMP6f signal rises and decays rapidly to levels similar to pre-burst, and only then the membrane is retracted **(Fig 21).**

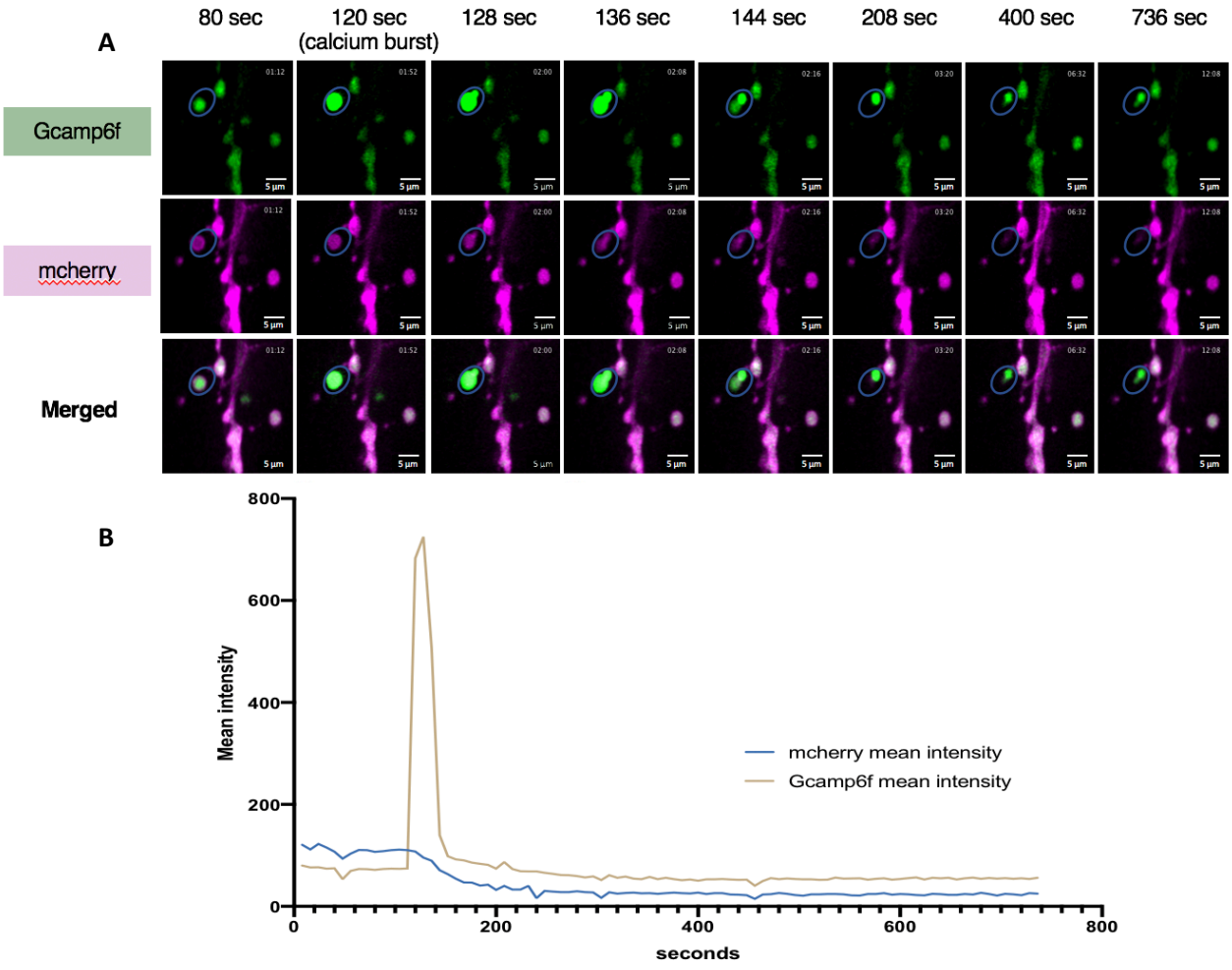

Figure 21 – Ca^{2+} rise precedes bouton retraction

(A) – Stimulated NMJs expressing mcherry (neuronal membrane) and GCaMP6f under the control of Dvglut. We observed that GCaMP6f signal rapidly increases and posteriorly the bouton retracts. The membrane signal is again very deemed in the retracted structure. A schematic of the ROI used for mean intensity analysis is also represented **(B) –** Mean intensity plot of both channels, frame by frame, within the previous ROI. Background subtraction was done with the same ROI but in another region of the video. Scale bar is 5µm

Single line ROI plot profile analysis shows again that Ca^{2+} bursts precedes membrane movement **(Fig 22).** It is interesting to note that even though Ca^{2+} is involved in bouton addition and retraction, the dynamics are different and may be what determines if a bouton is to be added or removed.

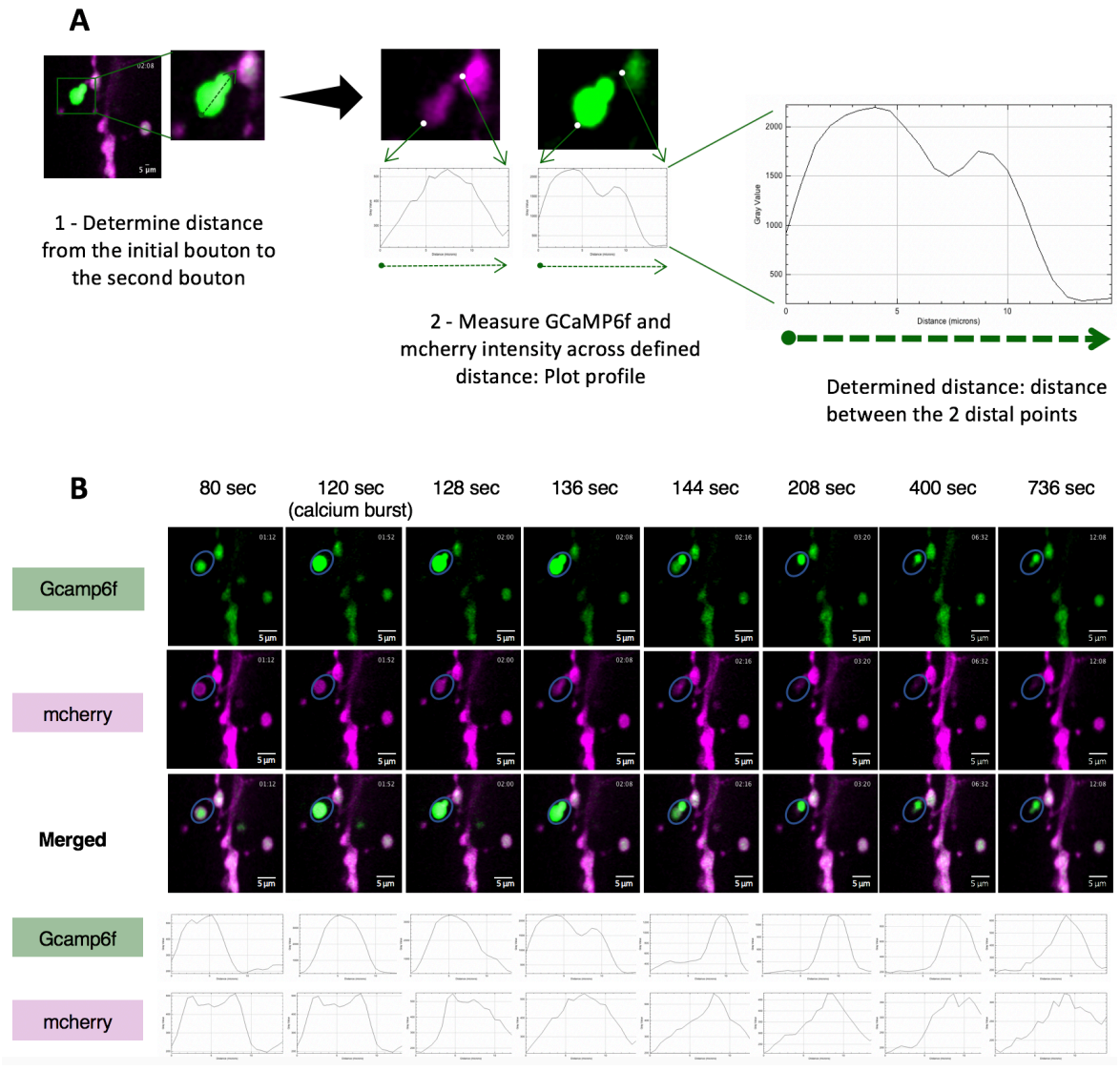

Figure 22 - Single line ROI Plot profile analysis

(A) – Stimulated NMJs expressing mcherry (neuronal membrane) and GCaMP6f under the control of Dvglut.Flow diagram of the analysis. First, distance between the furthest point of the original bouton and the furthest point of the second bouton is determined. This defines the ROI of analysis. After, we plot the signal intensity profile, that is the mean intensity of each channel across the previous distance. **(B) –** When we do this profile analyses frame by frame (represented here in frames of interest) we can observe the signal across distance and across time. The second column indicates the Ca^{2+} burst. By doing this, it is possible to see that Ca^{2+} burst really occurs before any signal (GCaMP6f or membrane) dislocation shown in the plot profiles. Scale bar is 5µm. See supplementary video "Bouton retraction"

In conclusion, live imaging of motor neurons expressing GCaMP6f showed that Ca^{2+} is highly increased in individual presynaptic boutons during structural plasticity. This rapid increase in GCaMP6f signal occurs exclusively in places that are remodeled and is not spread throughout the neuronal arbor, although some boutons have already high levels of GCaMP6f (which may have been induced during the stimulation protocol). This data suggests that Ca^{2+} is the signal by which neurons actively regulate sites to be structurally remodeled, either by the formation of new boutons or by the retraction of unstable ones.

4.7 How can Ca^{2+} mediate both bouton formation and retraction?

We saw that Ca^{2+} increases both during bouton addition and bouton retraction, raising the question of what is the mechanism by which Ca^{2+} mediates these two distinct events?

In other cellular contexts it is known that blebbing requires extracellular Ca^{2+} and that Ca^{2+} intracellular flux occurs before blebbing events **(Blaser H. et all 2006; Kapustina M. et al 2008)**. The molecular pathway(s) that are reported to regulate blebbing, converge on the activation of non-muscle myosin II (NMII), which leads to the contraction of actin filaments in order to promote changes in the direction of migration. In the initial stages of blebbing, NMII activity can be also required but to promote ruptures in the cortex, increasing locally intracellular pressure and dislocating the membrane **(Betapudi V. 2014).** Therefore, the Ca^{2+} burst identified in these videos could mediate this actomyosin contraction resulting in blebbing formation.

Although important in the initial steps of blebbing, NMII activity also occurs when there is reformation of the cortex leading to membrane collapsing and bleb retraction. This step does not always occur when there is bleb formation, but can also be mediated by increased cytoplasmic Ca^{2+} since actomyosin contraction is equally important. The most well characterized kinase, responsible for myosin activation, is myosin light chain kinase (MLCK) **(Chi Q. et al 2014)**., which is phosphorylated by calmodulin in response to increased intracellular Ca^{2+} levels. However, it is known that other kinases such as CaMKII are important to bouton formation through unknown mechanisms. One possibility is that CaMKII could phosphorylate directly the myosin light chain and promote cortex contraction. Alternatively, CaMKII could phosphorylate MLCK and indirectly promote cortex contraction as well. In future experiments it will be crucial to do RNAi against these kinases and evaluate the frequency of bouton formation, as well as Ca^{2+} dynamics to assess if they are downstream of the intracellular Ca^{2+} increase.

Blebbing is also known to be promoted in low adhesion conditions, and we showed in the lab that new bouton formation is promoted by down regulation of adhesion molecules and integrins **(Tsai-PI et al 2012, Qi Wang et al 2018)**. Importantly, adhesion can also be regulated by Ca^{2+} and is crucial for the degree of movement in

a migration context. Increased Ca^{2+} concentration intracellularly can promote reduction of focal adhesions and integrins in the cell surface through several kinases (like CaMKII) and phosphatases (like calcineurin and calpain), which cleave focal adhesion complexes **(Sheng L. et al 2013; Carlson SS. Et al 2010)**. In addition, cell adhesion molecules and integrin activation in growing neurites have been reported to increase intracellular Ca^{2+}, by modulating VGCC and/or Ca^{2+} release from internal stores **(Hansen SM et al 2008)**. This mutual relationship could explain why both formation and retraction have a rapid increase in intracellular Ca^{2+}, since both type of events require low adhesion.

4.8 What is the physiological function of sustained intracellular Ca^{2+}?

An additional question that our data raised is why is the Ca^{2+} increase sustained during bouton outgrowth and not in bouton retraction?

Due to dual channel imaging (GCaMP6f plus membrane) and technical limitations of the confocal spinning disk, we found challenging to perform live imaging at low intervals, regardless of other acquisition parameters. Since GCaMP6f is a low affinity but high kinetic Ca^{2+} indicator with decay times inferior to 100ms, the observed sustained signal could reflect several but summed Ca^{2+} bursts.

Both signal intensity profiles in new bouton formation (high GCaMP6f peak with sustained signal) and in bouton retraction (high peak with low signal afterwards) have some similarities with LTP and LTD, respectively. For example, LTP phenomena are known to induce addition of postsynaptic structures (spines) with high frequency stimulation protocols **(Monday HR. and Castilo PE 2017)**. Therefore, one possible origin of the observed sustained GCaMP6f signal could be the high frequency stimulation, since it can induce multiple uptakes of $Ca^{2+}$and induce new structure formation. On the other hand, LTD is known to induce elimination/retraction of postsynaptic structures with low frequency stimulation **(Citri A. and Malenka RC. 2008)**. Therefore, the absence of the sustained GCaMP6f signal could reflect the low firing frequency of the neuron, which will result in the retraction of that bouton. This could be an efficient strategy that motor neurons have developed to code distinct remodeling mechanisms in response to activity.

One of the few components reported to be present in new boutons are synaptic vesicles. These vesicles are a common target of several signaling molecules including PKA and Synapsin **(Vasin A. et al 2019; Nesler KR. Et al 2016).** Ca^{2+} influx can control cAMP levels and thereby the levels of PKA **(Berridge MJ. Et al 2003)**. Therefore, sustained Ca^{2+} measured by sustained GCaMP6f signal could be promoting vesicle recruitment to achieve bouton maturation, continually adding membrane.

Additionally, sustained Ca^{2+} signal is highly correlated with neurotransmitter release. Although in new immature boutons it is very unlikely that there are active zones or juxtaposed postsynaptic receptors, exocytosis mediated by Ca^{2+} entry could continuously add membrane to allow bouton remodeling. An additional experiment to test this would be to express a genetically encoded glutamate sensor that could report on the presence of glutamate being released during bouton formation. Also, during bouton outgrowth, several pathways converge in cytoskeleton alterations, specifically related to actin dynamics. Likewise, Ca^{2+} dynamics are well characterized to modulate actin modifications. Since many "new like boutons" also have the sustained GCaMP6f signal, it could suggest that sustained Ca^{2+} is promoting an initial reorganization of the cortex to stabilize the new bouton.

4.9 Source of Ca^{2+} during synaptic structural plasticity

Live imaging experiments with GCaMP6f have uncovered a novel role of presynaptic intracellular Ca^{2+} during NMJ bouton remodeling. While during bouton formation there is fast Ca^{2+} increase that remains relatively elevated in the new bouton, in bouton retraction the Ca^{2+} signal fades away after the initial burst. Since GCaMP6f expression is cytosolic, it is not possible to identify what is the Ca^{2+} source (intracellular vs extracellular). Identifying the Ca^{2+} source would clarify the mechanism by which motor neurons differentially regulate formation and retraction resorting on the same cation, but with distinct temporal and amplitude dynamics.

More importantly, our experiments are performed in an activity-dependent context and Ca^{2+} is well known to be required for neurotransmitter release. Therefore, we also aim to evaluate if the rise in intracellular Ca^{2+} could be a mechanism to couple presynaptic structural plasticity with normal synaptic activity.

Ca^{2+} is absolutely required for synaptic transmission but also during structural plasticity, since performing the stimulation in zero Ca^{2+} conditions inhibits bouton formation **(Ataman B. et al 2008).**

Neurons are constantly changing the concentration and localization of Ca^{2+} channels and pumps within different compartments to create microdomains of localized elevated intracellular Ca^{2+}, in order to coordinate multiple functions such as neuronal communication or cytoskeleton rearrangements **(Catterall WA. And Few AP 2008; Heine M. et al 2019; Rosenberg SS. And Spitzer NC. 2011)**. Activity plays a major role in this regulation since voltage-gated channels are required to be enriched in active zones to promote rapid, but transient neurotransmitter release through vesicle fusion. In mammals, voltage-gated Ca^{2+} channels can respond to high voltage and to low voltage levels. The basic subunit of a voltage-gated Ca^{2+} channel (the

alpha/α subunit) is a four transmembrane heteromultimeric complex which forms a barrel like structure, forming a pore through which Ca^{2+} can pass. While low voltage-gated Ca^{2+} channels are only composed by this pore forming subunit, high voltage Ca^{2+} channels have additional subunits like alpha2delta/$\alpha 2\delta$, gamma/γ and beta/β. These auxiliary subunits allow, not only for more regulated membrane turnover of Ca^{2+} channels across different cellular compartments, but also increase the interactions with different molecular players **(Guéguinou M. et al 2014) (Fig 22A).**

There are six major classes of voltage-gated Ca^{2+} channels (L, N, P, Q, T, and R subtypes), which differ in conductance and other pharmacological properties, like inhibitor resistance. In *Drosophila*, there are four genes for α subunit but that are essentially homologs of the L, T and N Ca2+ channels subtypes **(Littleton JT. And Ganetzky B 2000).**

Both in vertebrates and invertebrates, N-subtype is enriched in the nervous system making it the major subtype responsible for neurotransmitter release and synaptic communication. Nevertheless, there is a gene *locus* that encodes the α subunit common to all voltage-gated Ca^{2+} channels called *cacophony* (*cac*). Defects in *cac* gene are long known to disrupt not only neurotransmission, but also to cause synaptic atrophy. However, disruption of neurotransmission in a more specific manner (SNARE mutants) does not mimic the phenotype of synaptic undergrowth (**Rieckhof GE. et al 2003**). Therefore, it has been argued by some studies that Ca^{2+} influx mediated by voltage-gated Ca^{2+} channels were required independently of its function in vesicle release.

Moreover, *Drosophila* also has a $\alpha 2\delta$ (*straightjacket, stj*) subunit that is able to regulate *cac* activity and traffic to the plasma membrane. Similar to *cac* mutants, $\alpha 2\delta$ mutants display neurotransmission defects **(Bauer CS. Et al 2010; Ly CV. Et al 2007).** However, NMJs of $\alpha 2\delta$ hippomorphic mutants have increased number of satellite boutons (but with fewer active zones per bouton), in contrast with null mutants that die at the end of embryogenesis, without being able to form synaptic boutons – neurons are correctly targeted, but boutons fail to round up and develop **(Kurshan PT. et al 2009).**

4.10 Voltage gated channels subunits and new bouton formation

Although some studies report alterations in synaptic growth when *cac* and $\alpha 2\delta$ are affected **(Rieckhof GE. et al 2003; Dickman DK. Et al 2007)**, this analysis never contemplated new boutons resulting from acute activity-dependent structural changes. To assess whether these Ca^{2+} channels were required for structural plasticity, we performed the same activity protocols, known to promote bouton formation.

As a proof of concept, we wanted to confirm that extracellular Ca^{2+} was in fact necessary for bouton formation. Therefore, we started by performing the same stimulation protocol but in low Ca^{2+} concentration during the High K^{+}-induced depolarization. As mentioned previously, new boutons can be identified by the presence of a presynaptic marker, but absence of postsynaptic labelling. Wild type NMJs respond to the stimulation protocols in a robust and stereotypical fashion forming in average 6 new boutons per NMJ, which is in contrast with 0.5 immature boutons in an unstimulated condition. Based on this, we stipulated that experiments in which the stimulation did not induce a minimum of 3.5 new boutons in controls, were dismissed, and considered as failed experiments. All results from fixed tissue were done using the patterned stimulation protocol (see methods).

In agreement with other studies, low Ca^{2+} concentration throughout the stimulation protocols diminishes the ability to form new synaptic boutons. Moreover, if Ca^{2+} is removed only in the last pulse of stimulation, it is as effective in diminishing bouton formation as having always low Ca^{2+} concentration, suggesting that Ca^{2+} is required not only to elicit synaptic vesicle fusion, but also directly during structural changes. **(Fig 23B)** The average number of new boutons after stimulation was 5,86 in w1118 control larvae, whereas reducing Ca^{2+} during the all protocol was 2,23 and reducing Ca^{2+} only in the last pulse was 1,84. This result shows that extracellular Ca^{2+} is essential to induce new bouton formation in response to activity.

To analyze the role of *cac* and $\alpha 2\delta$ in activity-dependent structural plasticity, we expressed RNAi against these subunits specifically in neuros using a Nsyb-Gal4 line. Surprisingly, neither of the disruptions seem to affect new bouton formation. However, one needs to be careful when interpreting these results since the stimulation efficacy of control NMJs was low, especially when considering $\alpha 2\delta$ since new bouton formation in controls did not reach the minimal value of 3.5 new boutons in average. Further experiments with more efficient stimulations will help to clarify if these results have a true biological significance. **(Fig 23 C + D)**

Despite the loss of efficiency in these experiments, it is possible to have other mediators of Ca^{2+} entry to the cell cytoplasm. In fact, besides voltage-gated Ca^{2+} channels there are good candidates, like TRP channels **(Wong CO. et al 2014)** and STIM/ORAI **(Tsai FC. Et al 2014)** that could mediate Ca^{2+} amplification within the synaptic bouton. TRP channels, for example, will be a very interesting candidate because they are mechanoresponsive. Since previous data from the lab describes muscle contraction as one of the possible driving forces of new bouton formation, TRP channels could couple muscle contraction with a local intracellular rise in Ca^{2+} concentration, and ultimately in new bouton formation.

However, Ca^{2+} rise through voltage-gated Ca^{2+} channels could couple ongoing synaptic activity with new bouton formation. Considering that this is one of the most important questions of this project and that the

experiments showed here are insufficient to answer it, we will try to validate them with more efficient stimulations protocols.

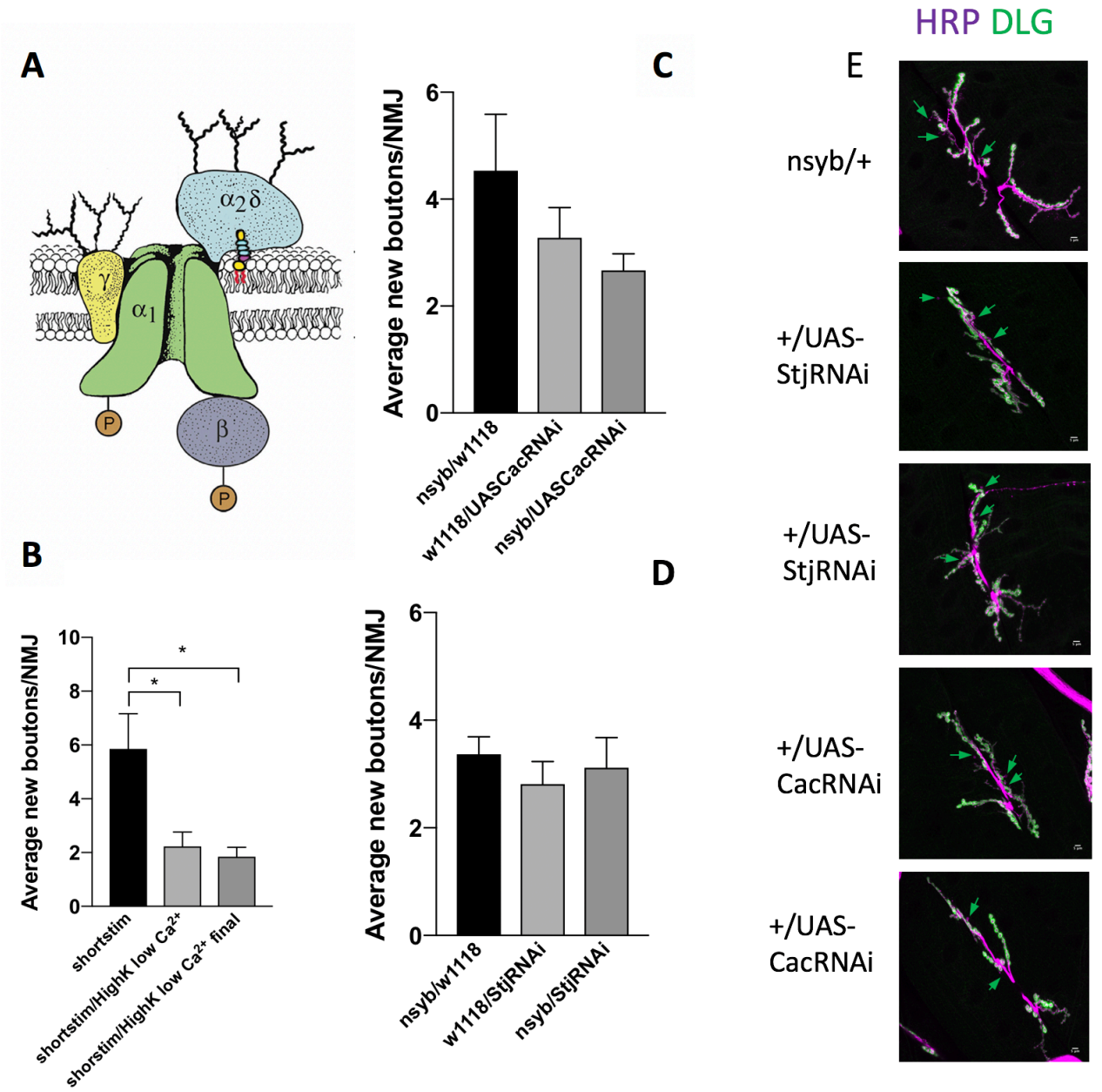

Figure 23 – Downregulation of voltage gated channels does not significativily affect new bouton formation

(A) – Scheme representing the subunits of voltage gated Ca^{2+} channels. In *Drosophila* $\alpha 1$ is *cac* and $\alpha 2\delta$ is straighjacket (Stj) **Adapted from Nanou E. and Catterall WA. 2018 (B) –** Both removing Ca^{2+} during the stimulation pulses and or only in the last 30 resting minutes after the stimulation pulses is able to decrease new bouton formation in W_{1118} larvae. Error bars represent SEM. Data collected from 15 (shortstim), 31 (shortstim/High K^+ Low Ca^{2+}), 32 (shortstim/High K^+ Low Ca^{2+} final) NMjs. More than 4 larvae per line and per condition. *p<0.05. **(C + D)** Downregulation of voltage gated channels subunits *cac* and stj by neuronal expression of RNAi against each subunit did not affec significantly new bouton formation, although control levels of new boutons are very low. Error bars represent SEM. For *cac*, data collected from 15 (nsyb/+), 29 (+/UAS-CacRNAi), 33 (nsyb/UAS-CacRNAi) NMjs. More than 4 larvae per line and per condition. For *cac*,

data collected from 71 (nsyb/+), 37 (+/UAS-StjRNAi), 42 (nsyb/UAS-StjRNAi) NMjs. More than 7 larvae per line and per condition. **(D)** NMJs with neuronal label (HRP) and postsynaptic label (DLG). Arrows indicate new boutons. Scale bar is 5μm

4.11 Ca^{2+} stores and synaptic plasticity

In addition to membrane Ca^{2+} channels that regulate Ca^{2+} entrance from the extracellular environment, Ca^{2+} can also be uptaken or released by several organelles within the neuron. The most important organelles that are able to store and extrude Ca^{2+} are the mitochondria and the endoplasmatic reticulum (ER) **(Zucker RS. 1999; Fitzhohn SM. And Collingridge GL. 2002).**

The ER is a dynamic organelle that, in addition to its conventional roles in protein synthesis, is used by neurons to regulate membrane trafficking for membrane addition during neuronal growth **(Ramirez OA and Couve A. 2011)**, as well as to regulate intracellular Ca^{2+} levels **(Juan Sanz J. et al 2017).** The ER regulates Ca^{2+} levels essentially by removing it from the cytoplasm through the ATPase SERCA, or by releasing Ca^{2+} to the cytoplasm through channels like Ryanodine receptors (RyRs) or IP_3Rs.

Since Ca^{2+} regulation and vesicle transport are key factors for synaptic transmission, the ER, in particular the smooth ER, is relatively ubiquitously distributed among all synapses including at the NMJ **(O'Sullivan NC et al 2012)**. There are many studies describing the contribution of the same individual pump or channel in different contexts of synaptic plasticity such as LTP and LTD. This means that Ca^{2+} ER transporters are redundant and can function differently, depending on the synaptic context. Nevertheless, the most important property of these channels or pumps is that they are highly depend on the pattern of neuronal activity **(Padamsey Z. et al 2018).**

In presynaptic boutons, high-frequency stimulation can also promote Ca^{2+} release from intracellular stores, through RyRs, increasing Ca^{2+} baseline levels and thereby increasing probability of release **(Cabezas C and Buno W. 2006).** Conversely, Ca^{2+} uptake by the ER through SERCA **(Fig 24A)** helps Ca^{2+} concentrations to return to baseline levels within the synaptic bouton so that another round of vesicle release can be performed. Synaptic growth is also associated with the ER Ca^{2+} regulation, since overexpression of *extended synaptotagmin* (a ER Ca^{2+} regulator associated protein) induces NMJ overgrowth **(Kikuma K. et al 2017)**. However, whether Ca^{2+} regulation by the ER is required during activity-dependent presynaptic structural plasticity is unknown. Importantly, in one study it was found that bleb formation is promoted when cells are treated with a specific drug (thapsigargin) that inhibits SERCA function **(Jansen C. et al 2019)**. Given that ER

Ca^{2+} is important for synaptic transmission and at the same time regulates blebbing, we hypothesized that increasing presynaptic Ca^{2+} levels in presynaptic boutons could increase the possibility to form new boutons during stimulation.

To evaluate the role of increased intracellular Ca^{2+} levels, we performed neuronal RNAi against the ER pump SERCA using the neuron specific driver Nsyb-Gal4. Interestingly, downregulation of SERCA in an activity-dependent manner increased new bouton formation. The average number of new boutons after stimulation was 5,15 in nsyb/+ driver control larvae, 4,37 in the +/UASRNAiSERCA control and 8,49 in the nsyb/UASRNAi. **(Fig 24 B + C)**

This result suggests that promoting increased intracellular Ca^{2+} during stimulation by diminishing the ER buffering ability, increases the probability of activity to induce structural remodeling. This is also in agreement with the fact that “new like boutons” in our GCaMP6f movies have a higher Ca^{2+} signal, pointing towards the idea that higher Ca^{2+} levels are indicative of boutons that are undergoing remodeling or that have just been formed. Having more Ca^{2+} for longer periods of time inside a presynaptic terminal could lead to more opportunities to promote NMII activation and/or down regulation of adhesion. Ultimately, reductions in SERCA levels could be sensitizing motor neurons to activity. Live imaging of SERCA RNAi NMJ expressing GCaMP6f would confirm that, not only general presynaptic Ca^{2+} would be increased, but also that more bouton formation should be observed.

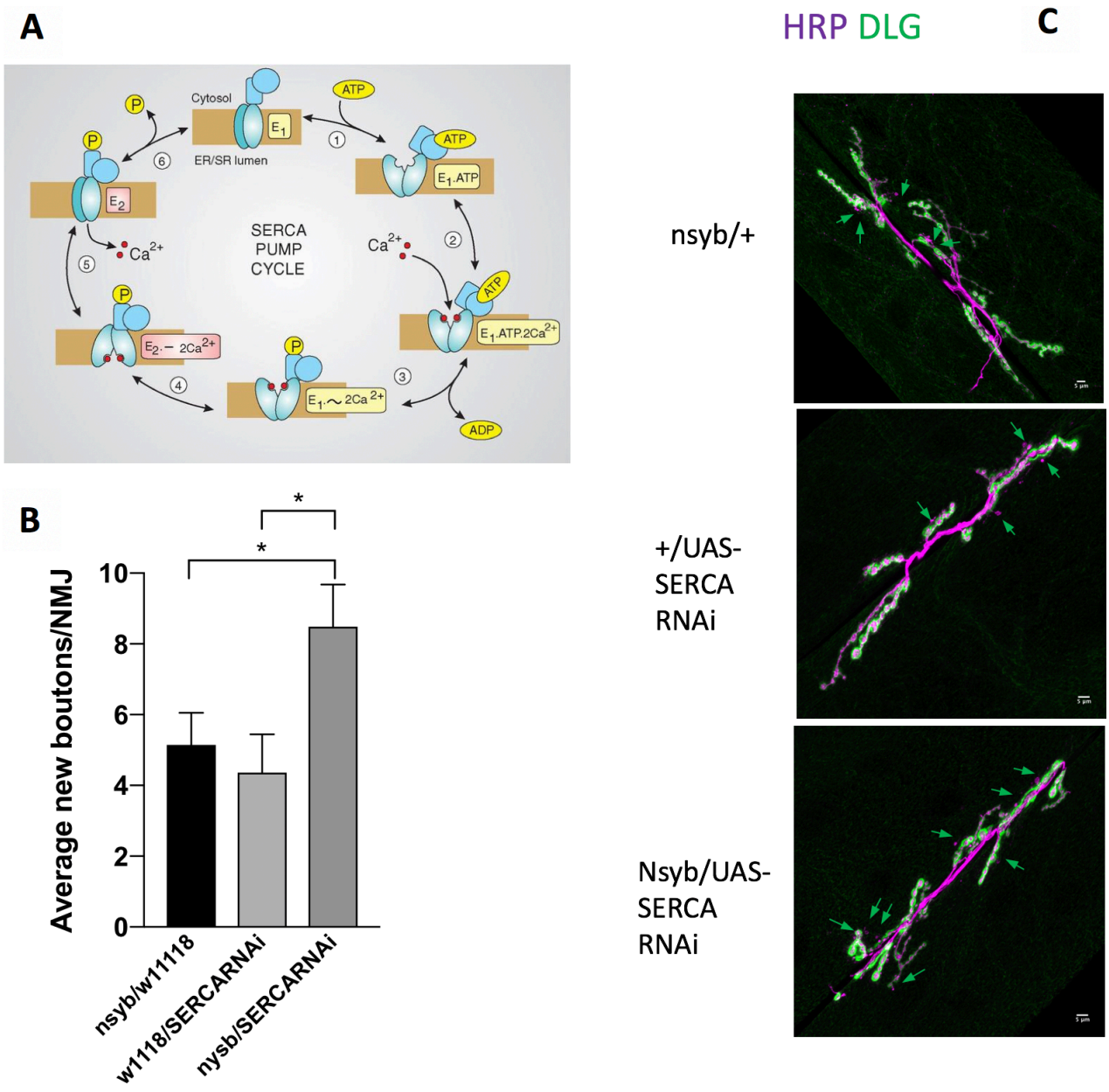

Figure 24 – Downregulation of ER ATPase SERCA promotes new bouton formation

(A) – Schematic representing SERCA regulation by ATP. Upon ATP binding, SERCA binds to Ca^{2+}. When there is phosphorylation, SERCA internalize Ca^{2+} ions into the ER. **(B)** Neuronal expression of SERCA RNAi promotes new bouton formation. Error bars represent SEM. Data collected from 27 (nsyb/+), 19 (+/UAS-SERCA RNAi), 31 (nsyb/UAS-SERCA RNAi) NMJs. More than 4 larvae per line and per condition. *p<0.05. **(C) –** NMJs labeled for neuronal membrane (HRP) and the postsynaptic density. Arrows indicate new boutons. Scale bar is 5µm.

4.12 Organelle localization and presynaptic function

Neuronal morphology is highly diverse, with numerous protrusions extending significantly into the environment when comparing to any other cell type. This feature makes subcellular compartments like presynaptic boutons to be distant from the cell body, where all the essential survival machinery of the cell is typically located. Therefore, neurons have developed efficient trafficking strategies to locally distribute not only specific proteins, but also organelles **(Heine M. et al 2019).**

Mitochondria are one of such organelles whose transport is tightly controlled in order to spatially provide ATP and to buffer Ca^{2+}. These organelles are in constant movement to "sense" the intracellular environment for compartments with higher demands of their function **(Devine MJ. and Kitler JT. 2017)**. Nevertheless, they can halt their movement in response to increased local levels of glucose and Ca^{2+}, by modifying adaptor proteins that connect them to the motor proteins along microtubules. Neuronal activity is known to generally promote more motile mitochondria and increase mitochondria concentration in presynaptic sites with increased synaptic transmission **(Mattson MP. Et al 2008)**. At the Drosophila NMJ, it is known that prolonged activity leads to the recruitment of mitochondria to provide ATP for mobilization of the reserve pool **(Patrik Verstreken 2005; Chouhan AK. Et al 2012)**. However, and unlike some mammalian models, mitochondria do not seem to sequester significative levels of Ca^{2+} from presynaptic boutons even in response to both high and low frequency stimulation **(Chouhan AK et al 2010)**. These studies highlight the fact that although many mechanisms are conserved between model organisms, different types of neurons and their activity patterns influences deeply some neuronal functions.

There are several studies describing how mitochondria regulate Ca^{2+} levels and how they contribute to functional plasticity **(Padamsey Z. et all 2018).** However, how or if this regulation is maintained during structural plasticity is not known. Therefore, we hypothesized that mitochondria could be somehow important to regulate new bouton formation in response to activity.

To determine if mitochondria were localized to new boutons, we overexpressed a UAS-GFP with a mitochondria tag, under the control the neuronal driver Nsyb-Gal4. Dissected larvae were stimulated with the same protocol as in previous experiments.

Our data shows that the large majority of mature boutons (even though not quantified) had also mitochondrial GFP inside, suggesting that, as described before, mitochondria are normally present at synapses. In fact, some studies have already characterized this feature of Drosophila motor neurons and even used mitochondrial markers to clearly identify presynaptic boutons **(Mosca TJ. Et al 2012).** In contrast,

analysis of new boutons (labeled with HRP but no DLG) showed that from 112 new boutons analyzed **(Fig 25A)**, only 9 had mitochondria GFP in them **(Fig 25B)**. This result suggests that mitochondria might be more associated with bouton maturation or function, rather than with the initiation of new boutons. In agreement with this result is the previous observation that "new like" boutons have high GCaMP6f levels. Since mitochondria can quench Ca^{2+}, one possible strategy to assure new boutons with a higher plastic capacity could be preventing mitochondria from entering new boutons. This would allow Ca^{2+} to regulate cytoskeleton remodeling in the initial stages of bouton formation, without it being buffered by mitochondria.

Despite this result, it would be interesting to do live imaging of new boutons that acquire mitochondria because retractions events lose the high GCaMP6f signal right after the Ca^{2+} burst. Therefore, mitochondria could be either related to providing trophic support for the maturation of the neuron, or rapidly quenching Ca^{2+} to promote bouton retraction. It is also possible that very dynamic mitochondria enter new boutons but that this type of dynamics can only be caught in *in vivo* recordings of structural plasticity.

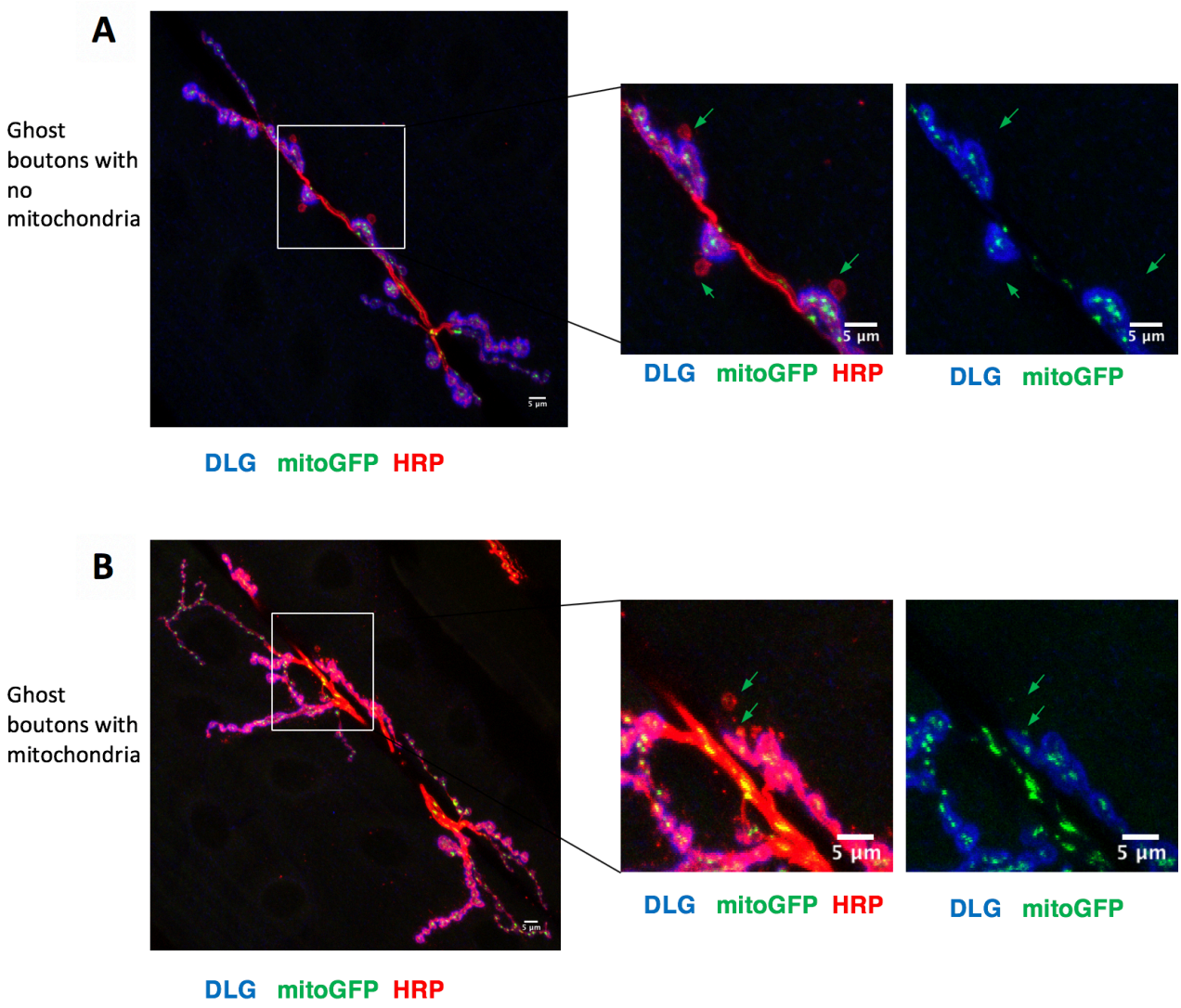

Figure 25 – Mitochondria are more often NOT present in new boutons

(A) – The big majority of new boutons do not have mitochondria. Stimulations did not work, therefore the graphs were omitted. NMJs were labeled with neuronal HRP, postsynaptic DLG and antiGFP to strengthen the mitochondrial signal. Arrows point to new boutons. **(B) –** Very few new boutons have mitochondrial, suggesting that these organelles can be actively restricted from new boutons. NMJs were labeled with neuronal HRP, postsynaptic DLG and antiGFP to strengthen the mitochondrial signal. Arrows point to new boutons. Scale bar is 5µm

5. General discussion and major conclusions

The ability of synaptic boutons to morphologically change in response to activity is one key component of synaptic plasticity crucial for higher order processes, such as learning and memory. However, the mechanisms that contribute to structural modifications and how these are coordinated with neuronal activity still remain a mystery. The Drosophila 3rd instar NMJ is a powerful model to study structural plasticity since acute stimulation protocols robustly induce the formation of new immature boutons. While one can extract information from fixed samples to determine the frequency at which these events occur in different genetic backgrounds, live imaging informs on the dynamics of the presynaptic membrane and Ca^{2+} while bouton addition is occurring.

Although the initial developmental presynaptic connections are known-to-be mediated by exploratory growth cones, the mechanism(s) that regulate NMJ growth after connectivity being established was less clear. In our lab we have identified a novel mechanism used by motor neurons to promote activity-dependent bouton formation - membrane blebbing. Ca^{2+} was identified as one of the candidates that could be regulating the initials steps of this migration process through actomyosin contraction. In addition, Ca^{2+} is long known to regulate other forms of migration through cytoskeleton modifications, as well as to be indispensable for synaptic transmission. Therefore, motor neurons could have adapted Ca^{2+} signaling as a strategy to coordinate neuronal activity with the blebbing of a new bouton in a time and space specific manner.

By expressing the genetically encoded Ca^{2+} sensor GCaMP6f we observed cytoplasmic Ca^{2+} dynamics in response to activity. Pattern stimulation is able to increase intracellular Ca^{2+} generally throughout the motor neuron when compared with unstimulated NMJs. In addition, it was possible to identify distinguishable presynaptic boutons, morphological similar to new boutons, with high GCaMP6f signal. These preliminary results already informed us that Ca^{2+} could be somehow important for structural plasticity.

More interesting, we also observed that Ca^{2+} bursts preceded new bouton formation and this GCaMP6f signal remains relatively elevated in the new bouton. In addition to new bouton formation, we observed that bouton retraction was also preceded by a Ca^{2+} burst. However, this signal rapidly dissipated in the retracted structure, making the GCaMP6f signal intensity profile very much different from bouton formation.

Important to note is also that not all events of Ca^{2+} bursts induced presynaptic structural modifications, which might hint to the fact that other surrounding factors contribute to bouton formation or retraction and neuronal Ca^{2+} is not enough to promote such modifications. Even when there are no structural modifications

(*de novo* formation or retraction) sometimes it is also possible to see membrane signal remodeling after the Ca^{2+} burst, suggesting that Ca^{2+} might play a role in lipid modification or vesicle addition to promote membrane growth.

Actomyosin contractions can influence bleb initiation and collapsing. Considering that Ca^{2+} can regulate actomyosin contractions, the fact that both new bouton formation and retraction are dependent on an initial Ca^{2+} burst is in agreement with the bleb life cycle.

Although having a common initial Ca^{2+} burst before movement, formation and retraction are clearly differentiated by the sustained/not sustained signal afterwards, respectively. This result might reflect a high frequency state of the bouton when it is forming, like LTP, versus a low frequency state of the bouton when it retracts, like LTD.

Similar to LTP, sustained Ca^{2+} signal during bouton formation may recruit several kinases (like PKA or CaMKII) that potentiate growth, synaptic vesicle recruitment and low adhesion.

In contrast, decreased Ca^{2+} signal during bouton retraction may recruit phosphatases (like calpain and calcineurin) that inhibit vesicle recruitment and growth.

To understand where is this intracellular Ca^{2+} originating from and if this rise in Ca^{2+} is associated with neuronal activity, we disrupted VGCC subunits *cac* and *stj*. To our surprise, even with low efficient stimulation protocols, downregulation of these subunits did not seem to affect new bouton formation, suggesting that other source might be providing Ca^{2+} during activity dependent bouton formation. But it is also possible that the RNAi was not efficient therefore not revealing any defects. Exploring the idea of different Ca^{2+} sources, the ER is well described to be a master regulator of Ca^{2+} levels within neurons. In fact, when we disrupted ER capability to quench Ca^{2+} by downregulating the ATPase SERCA, frequency of bouton formation increased. This result really strengthens the idea that the Ca^{2+} elevated signal detected in live imaging movies can derive from an intracellular source. Even though we do not discard the role of VGCC in activity dependent bouton formation, the ER seems to have a considerable contribution to this process.

The importance of having microdomains with high Ca^{2+} concentrations inside the new bouton is further evident when we observed almost no mitochondria inside newly formed boutons. Considering that these organelles can effectively quench Ca^{2+}, but are also extremely necessary for bouton maintenance, some molecular mechanism might be actively preventing mitochondria from entering new presynaptic boutons.

Overall, this thesis identifies Ca^{2+}, apart from its normal function in neurotransmitter release, as a master regulator of activity dependent structural plasticity. Taking into consideration previous data from the lab identifying blebbing as a new mechanism for bouton formation, we suggest that temporal and spatial Ca^{2+} regulation might be the trigger signal to remodel presynaptic structures.

6. Future perspectives

From the beginning of the study of neuroplasticity, scientists wonder how neurons establish such complex and numerous connections between each other. It is curious that after more than 100 years we still do not know the answer to the initial question: “Why does the protoplasm stretch towards one neighboring neuron when the organism happens to be in one situation, towards another neuron when the organism is in another situation?”.

Although much of the knowledge that exists on synapse innervation has been characterized from developmental studies, neuronal plasticity during adult life is crucial for almost every action of our daily lifes but is much less comprehended. In addition, how neurons alter their function (neurotransmitter release) in response to activity is much better understood than how neuros change their shape, even though both processes are equally important and interdependent. Therefore, one of the goals of out lab is to unravel the mechanisms that regulate presynaptic structural plasticity during activity. This work, together with our previous observations that new boutons form in the context of muscle contraction through a bleb like mechanism, could possibly describe in the future a new pathway that coordinates mechanical and biochemical factors to remodel presynaptic structure in response to activity.

Many neurological disorders are characterized by severe reduction on presynaptic structure and disruption of presynaptic release machinery. In fact, some neurological disturbances usually report toxic hyperexcitability due to hyperfunction or overexpression of voltage-gated Ca^{2+} channels **(Nanou E. and Catterall WA. 2018)**. Although many available treatments consist on administrating VGCC inhibitors to patients, medications are only able to halt these disorders **(Zamponi GW 2015)**. This works suggests that one possible strategy to induce presynaptic structural plasticity could be to modulate Ca^{2+} regulation from de ER. Therefore, future therapeutics could combine not only specific drugs that inhibit VGCC to prevent cytotoxicity, but also strategies that could enhance Ca^{2+} release from the ER to produce more presynaptic boutons in the surviving neurons and induce neuronal recovery.

7. Funding

H2020-GA661543-Neuronal 634 Trafficking (ROT), IF/00392/2013/CP1192/CT0002, FCT – Portugal (ROT)

635 UID/Multi/04462/2013 from iNOVA4Health (co-financiado: FCT-FEDER-PT2020).

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