INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

THE ROLE OF MICROTUBULES IN BOUTON INITIATION AND OUTGROWTH

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Master’s in Tecnologias Clínico – Laboratoriais

Lisbon, 2024

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Resumo

A plasticidade é o processo através do qual os neurónios adaptam a sua forma e função a estímulos externos e é um processo crítico para o desenvolvimento, aprendizagem e memória. Novas descobertas indicam que a formação de novas estruturas sinápticas imaturas – *ghost boutons* (GBs) – em neurónios motores de *Drosophila melanogaster* (*D. melanogaster*) ocorre através de um mecanismo que nunca foi reportado na remodelação neuronal, chamado *blebbing*, em resposta a estímulos agudos que mimetizam elevada atividade sináptica. Foi demonstrado que a actina não contribui para a iniciação e crescimento do processo de formação de novos botões sinápticos por *blebbing* sendo necessária em estágios posteriores de desenvolvimento, como a estabilização e maturação dos GBs. No entanto, o papel específico dos outros elementos do citoesqueleto, como os microtúbulos (MTs), neste processo permanecem pouco compreendidos. Assim, este estudo visa caracterizar a dinâmica dos MTs na formação e crescimento de GBs em *D. melanogaster*.

Neste estudo foram usadas técnicas de manipulação genética, incluindo *knockdown* e sobre-expressão de proteínas de ligação a microtúbulos, e coloração imuno-histoquímica de α- e β-tubulina. Larvas de *D. melanogaster* no terceiro estadio foram estimuladas com um protocolo intervalado rico em potássio e cálcio para induzir a formação de GBs adicionais. Para explorar melhor a dinâmica dos MTs foi utilizado o taxol, um agente estabilizador, e a vinblastina, um desestabilizador, para manipular a estabilidade dos MTs durante o desenvolvimento dos GBs.

A marcação de α- e β-tubulina revelou mudanças dinâmicas na estabilidade dos MTs dentro dos GBs. Não obstante, o tratamento com taxol levou a uma redução significativa no número de GBs, no entanto, os GBs remanescentes eram maiores e continham frequentemente uma alta intensidade da proteína estabilizadora Futsch, sugerindo que a estabilização dos MTs pode inibir a formação inicial dos GBs, mas promover o crescimento de botões maduros. Por outro lado, o tratamento com vinblastina não alterou significativamente o número de GBs, indicando que a desestabilização completa dos MTs pode interferir com o processo de maturação, sugerindo que um certo grau de estabilização dos MTs pode ser necessário para a maturação dos GBs.

EB1, uma proteína que associada ao extremo positivo dos MTs, estava presente em todos os GBs, o que provavelmente aponta para o seu envolvimento na iniciação do processo de *blebbing* e que a polimerização dos MTs pode estar a ocorrer durante o

desenvolvimento dos GBs. As proteínas Futsch e Short-Stop, que ajudam a estabilizar os MTs, exibiram resultados semelhantes em relação à sua presença nos GBs e à diminuição do número de GBs quando a sua expressão foi reduzida, destacando os seus papéis na promoção da estabilidade dos GBs e na integridade dos MTs, indicando que são cruciais para o processo de *blebbing*. Por fim, a subunidade da proteína de clivagem Katanin-60 desempenhou um papel crítico no desenvolvimento dos botões, uma vez que níveis reduzidos de Katanin-60 resultaram numa diminuição do número de GBs, sublinhando a importância da fragmentação controlada dos MTs para a formação e manutenção adequadas dos GBs.

Assim, as nossas descobertas destacam o papel crucial da dinâmica dos MTs na formação dos GBs, sugerindo que tanto os estabilizadores dos MTs como as proteínas de clivagem estão envolvidos na plasticidade sináptica. Estas descobertas podem fornecer informações relevantes sobre possíveis abordagens terapêuticas para doenças neuro-degenerativas ligadas à disfunção sináptica, como a Esclerose Lateral Amiotrófica e a Doença de Alzheimer.

Estudos futuros, incluindo *live imaging* e análise comportamental, bem como a avaliação do mecanismo compensatório entre Tau-Spastin, são necessários para elucidar os processos exatos subjacentes ao envolvimento dos MTs na formação e crescimento dos GBs.

Palavras-chave: Plasticidade sináptica; *Drosophila melanogaster;* Junção neuromuscular; *Ghost boutons*; Microtúbulos.

Abstract

Plasticity is the process through which neurons adapt their shape and function to external stimuli and is a critical process for development, learning, and memory. New findings indicate that the formation of new and immature synaptic structures – ghost boutons (GBs) – in motor neurons of *Drosophila melanogaster* (*D. melanogaster*) occurs through a mechanism that has never been reported in neuron remodeling, called blebbing, in response to acute stimuli that replicate conditions of high synaptic activity. It was shown that actin does not contribute to the initiation and outgrowth of new synaptic boutons by blebbing, being actin required in later stages of development like stabilization and maturation of GBs. However, the specific role of other cytoskeleton elements, such as microtubules (MTs), in this process remains poorly understood. Thus, this study aims to characterize the dynamics of MTs in the formation and growth of GBs in *D. melanogaster*.

This study employed genetic manipulation techniques, including knockdown and overexpression of microtubule-binding proteins, and immunohistochemical staining for α- and β-tubulin. Third-instar *D. melanogaster* larvae were stimulated with a patterned high-potassium and calcium protocol to induce the formation of additional GBs. MT dynamics were further probed using taxol, a stabilizing agent, and vinblastine, a destabilizer, to manipulate MT stability during GB development.

The labeling of α- and β-tubulin revealed dynamic changes in MT stability within GBs. Supporting this, taxol treatment led to a significant reduction in GB numbers, yet the remaining GBs were larger and often contained high intensity of the stabilizing protein Futsch, suggesting that MT stabilization may inhibit initial GB formation but promote the growth of mature boutons. Conversely, vinblastine treatment did not significantly alter GB numbers, indicating that complete MT destabilization may interfere with the maturation process, suggesting that a degree of MT stabilization may be required for GB maturation.

EB1, a plus-end tracking protein, was present in all GBs possibly indicating its involvement in initiation of the blebbing process and that MT polymerization may be happening during GB development. Futsch and Short-Stop proteins, which help stabilize MTs, exhibited similar results regarding their presence within GBs and decrease in GB numbers when their expression is reduced, highlighting their roles in promoting GB stability and MT integrity, indicating that they are crucial for the blebbing process. Lastly,

the severing protein subunit Katanin-60, played a critical role in bouton development, as reduced levels of Katanin-60 resulted in a decrease in GB numbers, highlighting the importance of controlled MT severing for proper GB formation and maintenance.

Thus, our findings highlight the crucial role of MT dynamics in GB formation, suggesting that both MT stabilizers and severing proteins are involved in synaptic plasticity. These findings might provide relevant insights into potential therapeutic approaches for neurodegenerative diseases linked to synaptic dysfunction, such as Amyotrophic Lateral Sclerosis and Alzheimer's disease.

Future studies, including live imaging and behavioral analysis, but also Tau-Spastin compensatory mechanism assessment, are needed to elucidate the exact processes underlying MT involvement in GB formation and outgrowth.

Keywords: Synaptic plasticity; *Drosophila melanogaster;* Neuromuscular junction; Ghost boutons; Microtubules.

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Abbreviations List

Ab – Antibody
ACh – Acetylcholine
AD – Alzheimer's Disease
ALS – Amyotrophic Lateral Sclerosis
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP – Action Potential
AZ – Active Zone
Ca^{2+} – Calcium
CLASP – Cytoplasmatic Linker Protein-Associated Protein
CLIP – Cytoplasmatic Linker Protein
CNN – Centrosomin
CNS – Central Nervous System
dfxr – *Drosophila* Fragile X-Related gene
Dlg – Discs-large
D. melanogaster – *Drosophila melanogaster*
DMSO – Dimethyl Sulfoxide
EB – End-Binding protein
E site – Exchangeable site
GB – Ghost Bouton
GC – Growth Cone
GDP – Guanosine Diphosphate
GTP – Guanosine Triphosphate
HL – Hemolymph-like
HRP – Horseradish Peroxidase
K^+ – Potassium
LTP – Long-term Potentiation
LTD – Long-term Depression
MACF1 – Microtubule Actin Cross-Linking 1
MAP – Microtubule Associated Protein
MN – Motor Neuron
MTBP – Microtubule-binding protein
MTOC – Microtubule Organization Center
MTs – Microtubules
NGS – Normal Goat Serum
NTs – Neurotransmitters
NS – Nervous System
NMDA – N-methyl-D-aspartate
NMJ – Neuromuscular junction
ON – Overnight
PBST – Phosphate-buffered saline 1x + 0,3% Triton X-100
PNS – Peripheral Nervous System
PTM – Post-Translational Modification
RNAi – RNA interference
RT – Room Temperature
Shot – Short Stop
SS – Short-Stim
SSR – Subsynaptic reticulum
SV – Synaptic Vesicle
UAS – Upstream Activating Sequence
+TIP – Plus-end Tracking Protein
γ-TuRC – Gamma-Tubulin Ring Complex

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1. Introduction

1.1. The nervous system

The nervous system (NS) in vertebrates, and many invertebrates like *Drosophila melanogaster* (*D. melanogaster*), is divided into two main components: the Central Nervous System (CNS) and the Peripheral Nervous System (PNS).[1] The vertebrate CNS consists of the brain and the spinal cord and serves as the primary control center for processing and transmitting information within the body. Meanwhile, the PNS comprises all the nerves (discrete bundles of axons) that branch out from the brainstem and spinal cord, connecting the CNS to the rest of the body and internal organs, facilitating communication and coordination.[1]

Two major categories of cells compose the NS: neurons and glia. Neurons are the primary information messengers, while glia provides structural, metabolic, and signaling support to the brain. A typical neuron consists of the cell body (soma) that contains the nucleus, which controls cell's activities and contains its genetic material, and two kinds of cytoplasmic extensions – dendrites and axons. Dendrites, a series of branched processes, are usually closer to the soma and receive incoming information from other neurons or sensory receptors. The long, thin axon extends far beyond the soma and sends messages from the cell. At the end of the axons are presynaptic terminals and specialized structures, called synapses.[1,2]

The synapse, a fundamental unit of communication within the nervous system, serves as a specialized junction facilitating the transmission of electrical and chemical signals between two neurons or between a neuron and an effector cell. This intricate process of synaptic transmission is crucial for the propagation of neural impulses. When an action potential (AP) reaches the axonal terminal of the presynaptic neuron, voltage-gated calcium channels open, allowing an influx of calcium ions. This triggers the release of neurotransmitters (NTs) into the synaptic cleft. The NTs then bind to receptors on the postsynaptic membrane, inducing changes in the membrane potential of the postsynaptic cell. In summary, this process converts the electrical signal of APs into a chemical signal (NTs), which is then reconverted into an electrical signal through the activation of postsynaptic receptors, ensuring the transmission of information from one neuron to another, forming the basis for neural communication.[1,3]

1.2. Neuronal growth and plasticity

During embryogenesis, neurons develop from neural progenitor cells within the neural tube, proliferating and differentiating into mature neurons.[4] As developing neurons begin to form their intricate networks, the process involves specialized structures at their growing tips. When a neurite begins to grow, a growth cone-like protrusion extends from the cell body, supported by a network of microtubules (MTs) that stabilize the neurite and assist in membrane transport necessary for its elongation. The growth cone (GC), found at the tip of the advancing axonal neurite, is a highly active and adaptable structure. It displays dynamic behaviors such as the extension and retraction of filopodia and the active reshaping of lamellipodia, which contribute to its exploration of the surrounding environment. Guided by a combination of attractive and repulsive molecular cues, the GC helps neurons navigate through the embryonic environment in a highly coordinated manner, ensuring precise connections with their targets.[5]

Once fully differentiated, most neurons enter a post-mitotic state and can no longer divide. Despite this, mature neurons must continually adapt to meet the specific demands of their targets and respond to stimuli received. This adaptability is mediated through a process called synaptic plasticity, where connections between neurons are modified in response to neuronal activity. Synaptic plasticity comprehends mechanisms such as dendritic remodeling, axonal sprouting, and activity-dependent strengthening or weakening of synapses. Initially, an excess of synaptic connections is formed, which are then pruned based on activity to ensure that only the most functional connections remain. These adaptive mechanisms are crucial for maintaining and fine-tuning neural network functionality throughout life, ensuring efficient information processing and response to environmental changes, as well as supporting learning and memory processes as it strengthens or weakens synaptic connections based on experience and activity.[1,6]

Particularly, axons respond to CNS injuries by undergoing spontaneous axonal sprouting, a form of plasticity widely observed in animal models. According to Chen *et al.* (2014), in mammals this sprouting involves the growth of new branches from intact or injured axons near the lesion site, forming compensatory synaptic boutons, essential for restoring communication with target neurons that may have been affected by the injury, allowing re-establishment of functional neural circuits. Their formation and stabilization are driven by activity-dependent mechanisms, such as neuromuscular stimulation or rehabilitation therapies like treadmill training, which encourage not only the physical creation of new boutons but also enhance their functional contribution.[7] These processes

emphasize that neuronal growth and plasticity are not static events but ongoing and finely regulated adaptations, necessary for both maintaining neural networks and facilitating recovery in the face of neural damage.

A key player in synaptic plasticity in the CNS is glutamate which facilitates excitatory signaling between neurons, being crucial for modulating synaptic strength. It binds to specific receptors on the postsynaptic neuron, like NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, playing a crucial role in modulating synaptic strength. NMDA receptors are essential for long-term potentiation (LTP), which enhances learning and memory by strengthening synaptic connections. AMPA receptors mediate rapid synaptic transmission and contribute to both LTP and long-term depression (LTD), adjusting synaptic strength. Together, these processes support cognitive functions and the brain's adaptability to new information and experiences.[8]

Failures in this plasticity mechanism can contribute to pathological conditions, leading to a variety of neurodegenerative diseases such as Alzheimer's disease (AD), where impaired plasticity affects cognitive function and disease progression.[9,10]

1.2.1. Neuromuscular Junction

Synapses typically form between neurons and are classified based on their connection points: axodendritic (axon to dendrite), axo-axonic (axon to axon), and axo-somatic (axon to soma). Additionally, synapses can occur between motor neurons (MN) and skeletal muscle fibers, forming a synaptic contact known as the neuromuscular junction (NMJ), responsible for muscle contraction.[11]

In the NMJ of vertebrates, the MN's axon terminal releases acetylcholine (ACh) packed in synaptic vesicles (SVs). Upon synaptic activation, in response to calcium influx, SVs rapidly fuse with the plasma membrane at specialized areas of the presynaptic membrane called active zones (AZs) and release ACh into the synaptic cleft. The ACh diffuses across the cleft and binds to specific postsynaptic receptors that are ligand-gated ion channels on the muscle cell membrane, causing changes in the ionic permeability and thus causing the membrane to depolarize, initiating an AP that leads to muscle contraction. This precise orchestration is essential for controlling voluntary muscle movements.[12] NMJs provide reliable yet transient excitation of muscle cells to support movement, with each MN activating target muscle cells in response to changing

nerve activity patterns that reflect the organism's functional demands. As neuromuscular transmission is highly adaptable, the architecture of this synapse is also dynamic.[13]

The NMJ is one of the best-studied and most disease-prone synapses in the NS making understanding its functions crucial for addressing disorders like Amyotrophic Lateral Sclerosis (ALS) or muscle dystonias.[14] Its fundamental role in neuromuscular signaling is highly conserved across a wide range of animal species, from invertebrates to vertebrates.[15] While much has been learned about postsynaptic morphogenesis using vertebrate NMJ models, considerably less is known about the mechanisms underlying presynaptic structural plasticity.[16]

The mechanisms of plasticity are conserved across different species and model systems have proven valuable in understanding the dissection of molecular mechanisms of neuronal growth and plasticity. Historically, *D. melanogaster* has been one of the most commonly used models for studying these molecular mechanisms in neurobiology.[17,18]

1.3. *Drosophila melanogaster*

The first scientist to adopt *D. melanogaster* (also known as fruit fly) as a research organism was William Castle in 1900, and about a decade later a few other emeritus scientists followed his lead.[19] Thomas Hunt Morgan, considered the founder of *Drosophila* genetics, later expanded on Castle's pioneer work, conducting groundbreaking research that established *Drosophila* as a model organism for genetic studies. Today, *D. melanogaster* continues to be a fundamental tool in genetic research due to its short generation time, large number of offspring, simple anatomy, cost-effectiveness, and well-characterized genetic tools, making it very useful for studying fundamental biological processes and human genetic diseases.[19,20]

The *Drosophila* genome shares around 60% homology with humans and has almost 75% similarity in the genes that cause human diseases.[19,21] In the third stage of development, the NMJ of *D. melanogaster* larvae is a stereotypical synapse, with well-discernible boutons and robust structural plasticity.[19,22–24]

As mentioned before, fruit fly has a short life cycle, with development from fertilized egg to adult taking 9 or 10 days at 25°C (or up to 19 days at 18°C). Embryogenesis takes approximately 24 hours, followed by three larval stages (first and second instar, each 1 day; third instar, 2 days).[19] Metamorphosis occurs within a puparium - a hard, protective chitin-based pupal case - where they remain for 4 to 5 days. At this stage, imaginal discs, formed during larval stages, differentiate into adult structures like eyes and wings. After

eclosion, adult flies emerge and reach sexual maturity within 8-12 hours, allowing rapid genetic studies due to their prolific reproduction and clear developmental stages.[19]

The *D. melanogaster* larval neuromuscular system is relatively simple, with each abdominal hemisegment containing only 32 MNs. Its NMJs are individually specified, and easily accessible for visualization and recording. Specifically, muscle 6/7 has stereotyped and large synapses that are easy to visualize and quantify, this happens because this NMJ has twice as many boutons since it innervates two muscles.[24,25] Additionally, this system displays robust and reproducible changes in response to genetic or environmental manipulations, making it ideal for studying the mechanisms underlying synaptic growth and plasticity.[25]

D. melanogaster's NMJs have glutamatergic synapses rather than cholinergic ones and do not include muscle voltage-gated sodium channels unlike in vertebrate's NMJs, leading to muscle contractions with varying intensity.[23,24] Instead, muscle contraction in invertebrates is graded, meaning that the number of synapses present at each NMJ needs to be carefully controlled to assure maximal contraction capacity. Still, despite its simpler structure compared to the mammalian CNS, the fruit fly NMJ retains most key synaptic molecules conserved across species and is an excellent model not only for the study of muscle contraction but also as an excellent model for mammalian CNS glutamatergic synapses.[26]

1.3.1. *Drosophila* genetic tools

The establishment of stable genetic stocks represented a major advancement in the *D. melanogaster* genetic toolkit. One of the earliest and most significant innovations was the creation of balancer chromosomes that prevent genetic recombination during meiosis, which is crucial for preserving mutations that would normally be lost due to their lethal or sterile effects. These chromosomes often carry distinct visible physical traits, making it easy to identify flies that have them. Moreover, the distinctive structure of balancer chromosomes, which includes multiple inversions, ensures that any offspring resulting from recombination events are not viable. This prevents the introduction of unwanted genetic variations and allows researchers to maintain stable populations with specific genetic traits.[19,21]

Another main tool in *Drosophila* research is the GAL4-UAS system. This method allows scientists to activate the expression of specific genes in particular tissues or at specific times. The system consists of two parts: the GAL4 protein, which binds to a

specific DNA sequence known as the Upstream Activating Sequence (UAS), and a separate genetic construct that contains the UAS sequence linked to a gene of interest. By placing, the GAL4 gene under the control of a tissue-specific promoter in one fly strain and the UAS-gene construct in another, researchers can cross these flies to create offspring in which the gene of interest, often linked to a fluorescent marker, is expressed only in the desired tissue. This system has been essential in studying the roles of individual genes in various biological processes.[19,21,27]

Furthermore, the GAL4-UAS system can be coupled with RNA interference (RNAi) by expressing short inverted hairpins that target specific genes to reduce or "knockdown" the expression of the gene's messenger RNA. Thus, researchers can study the effects of reduced gene function in a controlled and targeted way.[19]

1.3.2. Neuronal Growth and Synaptic Plasticity at the *Drosophila* NMJ

In the initial 10 hours of embryonic development in *D. melanogaster*, neuroblasts segregate and differentiate into glial cells and neurons, forming the neuroectoderm. Over the next 3 hours, MN axons leave the CNS and extend towards target muscles to make synaptic connections, through GCs.[24,28] At the larval NMJ, the presynaptic bouton becomes surrounded by a membranous structure that folds inward known as subsynaptic reticulum (SSR), which contains glutamate receptors, scaffolding proteins, and postsynaptic signaling complexes. After embryogenesis, NMJs adapt to patterns of neuronal activity that refine their final morphology (Figure 1.1A). This refinement involves increasing the number of boutons and AZs to match the rapid growth of muscle fibers. The addition of new boutons can occur by division of existing boutons, either by asymmetric budding or symmetric division, but in some cases, boutons can emerge *de novo* from the axonal membrane.[24] However, throughout NMJ development, as the terminal arbor grows and matures not all structures undergo stabilization, some synaptic connections are removed and "pruned" (Figure 1.1B).[16] Thus, by the end of embryonic development, functional NMJs, each containing a few synaptic boutons, have formed on each muscle fiber.

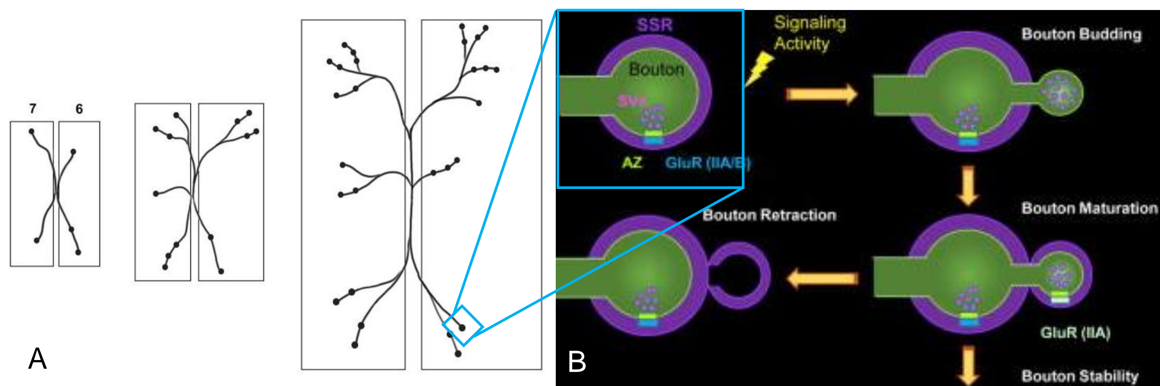

Figure 1.1 - **NMJ expansion and process of terminal bouton formation and maturation.** (A) Schematic of NMJ development to demonstrate arbor growth and bouton addition during larval development. (Adapted from Menon *et al.*, 2013) (B) Developmental signaling or synaptic activity can induce the formation of new boutons that can stabilize and mature or be retracted. (Adapted from Vactor *et al.*, 2017)

Essentially, 3 types of synaptic boutons innervate *D. melanogaster* NMJ: Type I, Type II, and Type III (represented in Figure 1.2A and 1.2B). They differ in size, morphology, physiology, type of NT released, and the amount of SSR that surrounds them. Type I boutons, which are glutamatergic, include both Ib (big) and Is (small) categories. Type II and III boutons are modulatory, lack SSR, and use different NTs. A third instar larva NMJ is composed by Type I boutons (roughly 20-50 on each muscle in a typical larvae).[24]

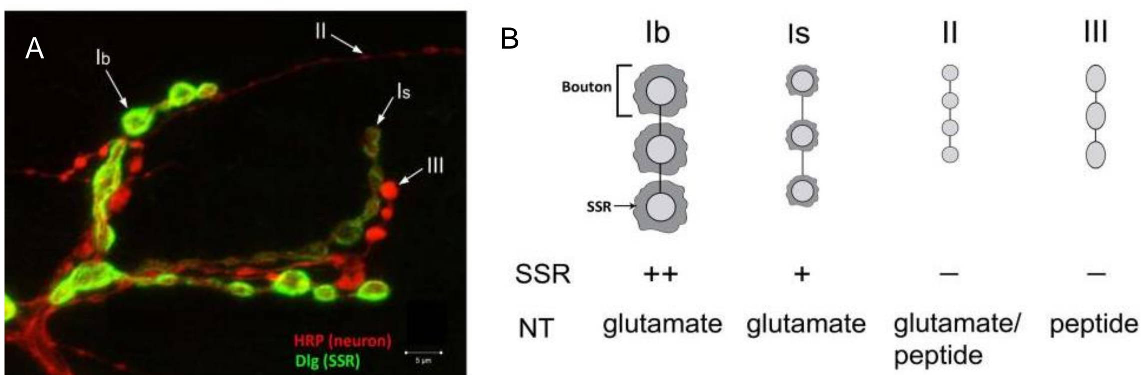

Figure 1.2 - **Types of boutons at the Drosophila larval NMJ** (A) Anti-HRP labels the presynaptic neuronal membrane and allows visualization of all bouton types. Type Ib, Is, II, and III boutons are indicated by arrows. Type Ib and type Is boutons differ in size, morphology, physiology, and the amount of SSR (Dlg staining) that surrounds them. Dlg is absent from type II and III boutons. (B) A schematic representation of differences in the bouton types seen in (A). Other than the size and morphological differences, the boutons also differ in the NT utilized. (Adapted from Menon *et al.*, 2013)

In addition to the structural changes that result from muscle size expansion, *Drosophila* NMJs can also undergo plastic changes in response to short-term perturbations of neuronal and muscle activity.[24]

1.4. Ghost boutons in *D. melanogaster*'s NMJ

Studies on *D. melanogaster*'s NMJ have revealed the rapid formation of nascent synaptic structures in response to patterned depolarization induced by high potassium (K^+) solution. These structures, known as ghost boutons (GBs), represent immature structures, that form as a result of rapid activity-dependent synaptic plasticity.[29] GBs have SVs and sometimes AZs as pre-synaptic markers, however, they lack post-synaptic machinery, and so, for confocal microscopy GBs can be recognized because they express a presynaptic neuronal membrane marker recognized by the anti-Horseradish Peroxidase (HRP) antibody but lack the post-synaptic scaffold protein Discs-large (Dlg) marker[24,30], as represented in figure 1.3A. Despite their rapid formation, GBs require further maturation to become fully functional (see Figure 1.3B). This process involves the assembly of AZs in the GB and post-synaptic specializations, including clusters of glutamate receptors, which are crucial for synaptic transmission.[24] Without proper stabilization, these newly formed boutons may be retracted or degraded by muscle and glia-mediated mechanisms.[31]

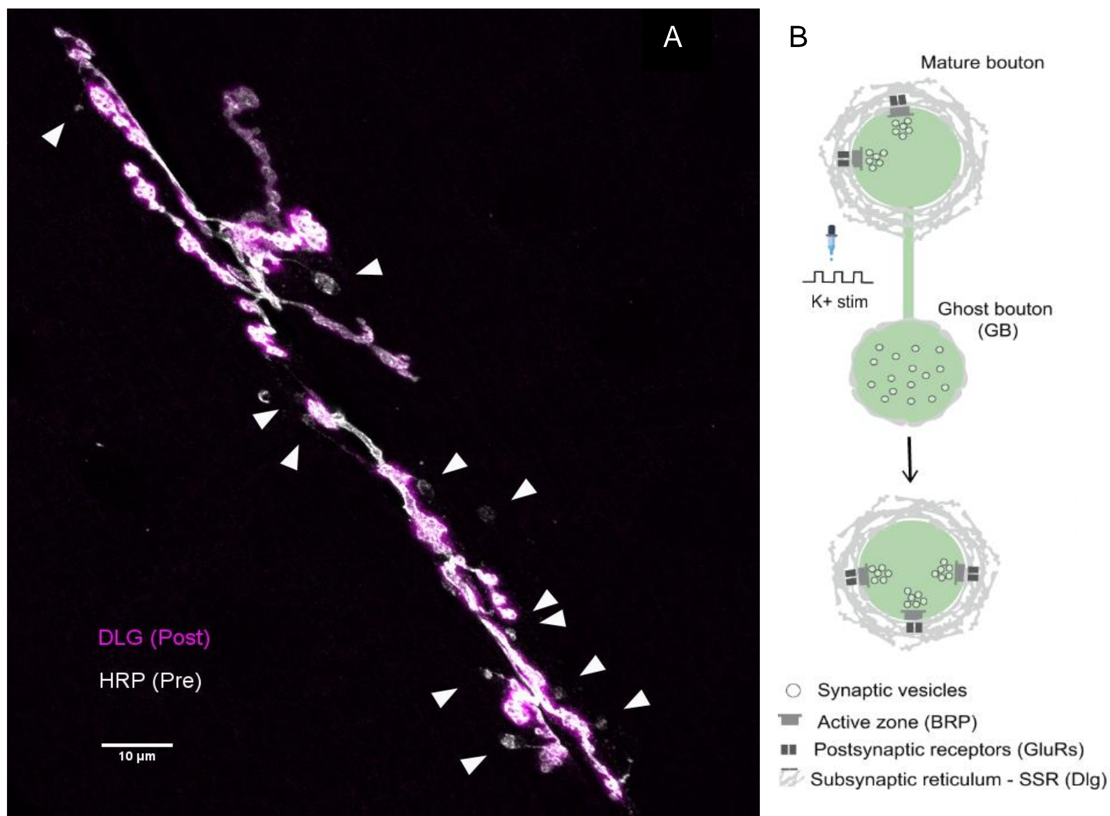

***Figure 1.3* – Ghost boutons and their maturation.** (A) Confocal image of a *D. melanogaster* NMJ. Anti-HRP labels the presynaptic neuronal membrane (white). In GBs (see arrowheads) is possible to verify the absence of anti-Dlg marker.(B) Schematic of activity-induced bouton formation and subsequent maturation progression. (Adapted from Fernandes *et al.*, 2023)

The formation of new boutons in MN of *D. melanogaster* in response to this acute synaptic activity occurs through a mechanism previously observed only in migratory cells and which has never been reported in neuronal remodeling, called blebbing. Blebs at the NMJ are pressure-induced membrane protrusions that result from the interaction of mechanical forces between the MN and the muscle (muscle contraction) that promote the growth of new boutons.[32]

The cytoskeleton plays a critical role in maintaining and shaping neuronal morphology. Components like actin filaments and MTs provide structural support and facilitate the dynamic remodeling of neurons, enabling processes such as synaptic growth, stabilization, and intracellular transport.[1] Interestingly, Fernandes *et al.* (2023) demonstrated that bouton outgrowth in response to acute synaptic activity does not rely on actin polymerization; instead, actin is required for bouton stabilization.[32] However, the mechanisms that control the development of this boutons in MN, how they interact with other cytoskeletal elements - such as MTs - in this process to facilitate the morphological changes necessary for their formation, as well as their coordination with other cell types, such as muscles and glial cells, are still open questions.[32,33]

1.5. Microtubules: Structure, Neuronal Polarity, and Neuronal Stability

Like all eukaryotic cells, neurons rely on three major cytoskeletal elements for structural integrity and motility: MTs, actin filaments, and intermediate filaments.[1] MTs, in particular, play a vital role in intracellular transport, cell division, motility, and the maintenance of cell shape. Their structure consists of dynamic polymers formed by α- and β-tubulin dimers. Each MT is composed of 10 to 15 protofilaments (13 in both *Drosophila* and mammalian cells) assembled in a cylindrical arrangement with a hollow core, forming long tubular structures with an average exterior diameter of 25nm, represented in figure 1.4A.[1,34]

MTs are polar structures that have a plus-end and a minus-end with distinct properties. The α-β tubulin heterodimer gives the MT an intrinsic polarity with a plus (β-tubulin) and a minus (α-tubulin) end. This polarity is important to determine the direction of movement along MTs.[1,34,35]

MT can be either stable or dynamic, depending on the physiological situation. Dynamic MTs, in contrast to stable MTs, have the ability to undergo stochastic transitions between phases of depolymerization and polymerization.[34] Dynamic instability,

described by Tim Mitchison and Marc Kirschner in 1984, results in rapid cycles of growth and shrinkage at the plus-ends with half-lives of only several minutes within the cell, while the minus-ends tend to shrink slowly without stabilization.[36]

When polymerization occurs, a new dimer is added to the plus-end where the α-tubulin of the new dimer contacts the nucleotide exchangeable site (E site) of the previous β-tubulin, triggering guanosine triphosphate (GTP) hydrolysis and forming a GTP cap that stabilizes the MT, as shown in figure 1.4B. Loss of this GTP cap leads to rapid depolymerization as guanosine diphosphate (GDP)-tubulin subunits are released. The MT lattice primarily consists of GDP-bound tubulin. At the minus end, the E site of the incoming dimer contacts the catalytic region of the terminal subunit, preventing a GTP cap and making the structure more prone to depolymerization.[34,37]

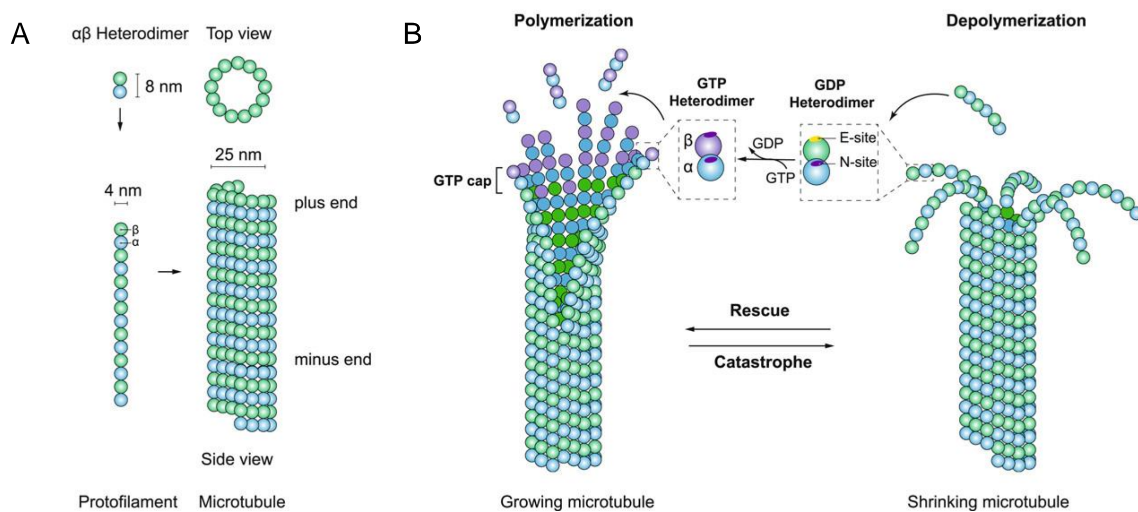

***Figure 1.4* - MT structure and dynamics**. (A) MT structure. Tubulin heterodimers with α- (blue) and β-subunits (green) assemble into protofilaments. 13 parallel protofilaments form a 25 nm hollow cylinder, with β-tubulin at the plus end and α-tubulin at the minus-end. (B) Cartoon showing MT dynamic instability intermediates. The polymerization–depolymerization cycle is driven by GTP hydrolysis and GDP/GTP exchange, leading to catastrophe and rescue events. GTP bound to α-tubulin (N site) is non-hydrolyzable and non-exchangeable, while GTP on β-tubulin (E site) can be hydrolyzed and swapped with free GDP. A GTP cap, rich in GTP-bound β-tubulin (purple), forms during polymerization due to delayed GTP hydrolysis. (Wang *et al.*, 2023)

1.5.1. Microtubule polarity in neurons

Most differentiated neurons have one long axonal process and several branches, which strongly depend on MTs for their integrity and organelle transport. In most non-neuronal cells, the more dynamic plus-ends of MTs point toward the periphery, whereas minus-ends, stabilized by a ɣ-tubulin cap, are usually at the center of the cells anchored in microtubule organization centers (MTOCs) such as the centrosome.[38] However, the

MT orientation is more complex in neurons, where it contributes to the distinction between dendrites and axons.[1,39]

While the majority of MTs in proximal dendrites have their plus-ends directed toward the cell body, distal dendrites display a more mixed orientation. This mixed polarity is essential for the bidirectional transport of molecules and organelles within dendrites, supporting synaptic plasticity and other dendritic functions.[1,37,40]

On the other hand, in axons, MTs are uniformly oriented with their plus-ends directed towards the axon terminal. This uniform orientation facilitates the directional transport of organelles, vesicles, and proteins necessary for synaptic function and maintenance. The motor proteins kinesin (plus-end directed) and dynein (minus-end directed) utilize this polarity to transport ion channels, NT vesicle precursors, and other synaptic components towards the axon terminal (anterograde transport) or the cell body (retrograde transport), respectively. In MN, proper transport is essential to support rapid synaptic signaling and the propagation of APs, as well as maintaining cellular homeostasis.[41–43]

In contrast, for some invertebrates like *D. melanogaster*, the MN is a bipolar neuron, and consequently, dendrites appear to not have mixed polarity, but rather have mostly minus-end-out MTs whereas axons, like in mammals, have plus-end-out MTs. Therefore, although details may differ, both vertebrate and invertebrate neurons share the same principle that dendrites and axons vary in their MT orientantion.[1]

During neuronal development, the axon terminal does not have well-defined boutons, but a GC instead, as mentioned before. The GC uses mostly dynamic remodeling of the actin cytoskeleton using lamellipodia and filopodia to probe its surroundings and drive it forward. Once a positive cue is sensed, dynamic MTs found in the central domain of the GC invade these actin-rich filopodia and become stabilized, forming a connection with the target. Therefore, MTs have been considered secondary players, invading the peripheral domain only where the actin cytoskeleton allows them to go.[44,45] However recent insights reveal that MT-based motor proteins, such as dynein and kinesins, may be essential as well. These motor proteins can generate forces that enable MTs to invade the peripheral domain of the GC (see figure 1.5A). By coordinating the distribution and alignment of MTs, dyneins and kinesins can ensure their polarized invasion into one side of the GC's peripheral domain, effectively instructing the cone to turn and thereby ensuring accurate axon guidance (see figure 1.5B).[46] This highlights not only the critical role of MTs in axon guidance, but also in precise neural circuit formation, which is fundamental to proper neuronal connectivity and function.

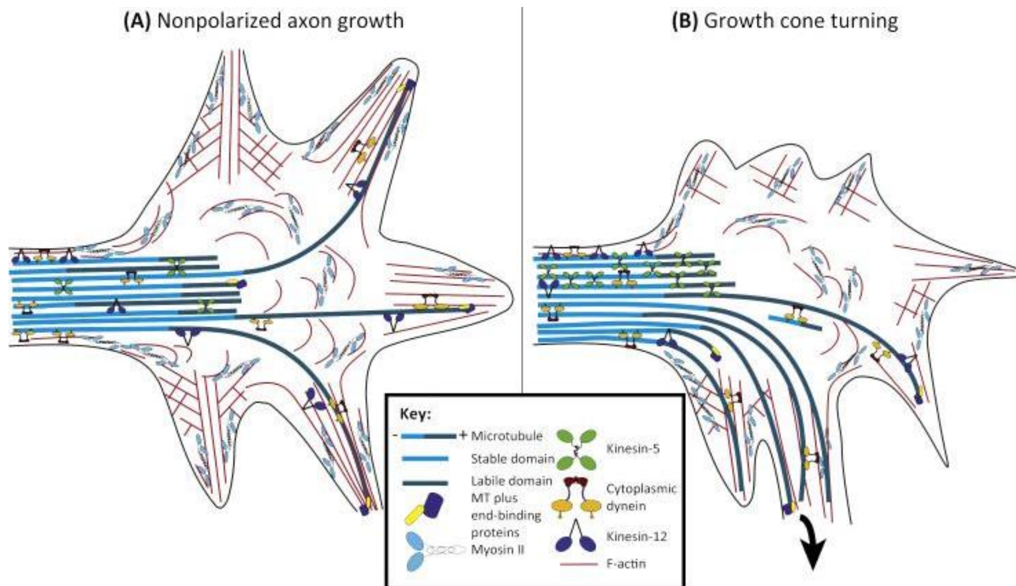

Figure 1.5 - **Motor driven forces in the growth cone.** (A) As the GC advances, cytoplasmic dynein generates forces on MTs, helping some resist the retrograde flow of the actin cytoskeleton in the peripheral domain. Kinesin-5 and kinesin-12 generate opposing forces to dynein, resisting MT entry into the peripheral domain. Kinesin-5 acts mainly in the transition zone, while kinesin-12 operates more in the filopodia. (B) During GC turning, kinesin-5 and kinesin-12 forces shift to the side opposite the turn, allowing MTs to enter the side of the GC that follows the direction of the turn. (Adapted from Khan and Baas, 2016)

Thyagarajan *et al.* (2022) elucidate the pivotal role of MT polarity in driving neuronal polarity, demonstrating that it serves as a master regulator of key intracellular processes in neurons. Their research using *Drosophila* sensory neurons revealed that altering MT polarity can significantly impact the identity and function of neuronal compartments. Specifically, dendrites that adopt an axonal-like plus-end-out MT polarity show a marked shift in cargo distribution, with a reduction in ribosomal content typical of dendrites and an increase in axonal markers. Furthermore, these dendrites exhibit enhanced MT stability and even develop ectopic diffusion barriers akin to those at the axon initial segment. Despite these profound changes in intracellular organization, the overall dendritic morphology and branching patterns remain relatively unchanged, suggesting that while MT polarity dictates functional and structural aspects of neuron compartments, the external shape of neurons is resilient to such internal shifts. This study highlighted the central role of MT polarity in coordinating the complex internal architecture that supports neuronal function and polarity.[40]

1.5.2. Microtubule stability in neurons

The balance between MT stability and instability is essential in neurons, as it supports the structural integrity required for maintaining axonal transport, neuronal shape, and synaptic function, while also allowing for the flexibility needed for growth, repair, and

synaptic plasticity. Disruptions in this balance can lead to significant neurological disorders and impairments in cognitive function.[47]

In proliferating and developing cells, most MTs are highly dynamic, but in differentiated neurons, many MTs become stabilized due to their interactions with structural Microtubule-binding proteins (MTBPs), which will be approached in more detail on chapter 1.6.1. The stability of MTs is also linked to post-translational modifications (PTMs), such as acetylation, detyrosination, and phosphorylation, with the majority of modified tubulin subunits found in the more stable, or long-lived, MT populations.[40]

A study conducted by Cappelletti *et al.* (2021) highlights the critical role of MT acetylation, particularly the acetylation of α-tubulin at lysine 40, in maintaining MT stability within neurons. The researchers found that this PTM is essential for the structural integrity of MTs, making them more resilient to mechanical stress and crucial for sustaining the complex architecture of neurons, including long axons and intricate dendritic trees. Moreover, acetylated MTs play a key role in regulating MT dynamics by reducing excessive instability, thereby preventing issues such as axonal over-branching. These findings emphasize the importance of acetylation in not only stabilizing MTs but also in supporting the overall functional and structural stability of neurons, which is particularly relevant in the context of neurodegenerative diseases where MT stability is often compromised.[48]

In addition to the effects of PTM, various drugs are known to either stabilize or destabilize MT stability in neurons. Vinca alkaloids are a class of chemotherapy drugs derived from the plant *Catharanthus roseus*. Common vinca alkaloids include vincristine, vinblastine, and vinorelbine. They work by binding to tubulin dimers, preventing MT polymerization, which is crucial for cell division. This action disrupts the mitotic spindle, halting cell division and leading to cell death, and for that reason, they are used in chemotherapy in various cancers like leukemia, lymphoma, and lung cancer. Another class of drugs that binds specifically to MTs are taxanes, like taxol (paclitaxel), but rather then inhibiting MT assembly, stabilizes it instead. By stabilizing MTs in place, taxanes disrupt the mitotic spindle, blocking cell division, leading to cell cycle arrest and apoptosis, and thus taxol is used as an anticancer agent as well as an experimental tool.[34,40] However, it is worth mentioning that both types of drugs have been observed to adversely impact peripheral nerve function when administered as part of chemotherapy treatments, with patients frequently developing peripheral neuropathy as a result.[49]

Interestingly, MT stabilization is sufficient to induce axon formation, as neurons treated with low doses of taxol extended axon-like neurites that displayed a high ratio of acetylated to tyrosinated tubulin, a proximo-distal Tau gradient and the absence of MAP2[50] (two proteins associated with MTs, explained in further detail in chapter 1.6.1.).

1.6. Microtubule-binding proteins in neurons

A protein is considered a MTBP if experimental data demonstrate its ability to bind to MTs. MTBPs can be categorized into multiple families, according to their activity and location such as stabilizers, destabilizers, cytoskeletal integrators, minus end-targeting proteins, plus-end trafficking proteins, etc.[51]

1.6.1. Stabilizers

The stabilizer proteins promote polymerization and/or slow depolymerization. Some examples of this kind of proteins in mammals are the Microtubule-Associated Proteins (MAPs) such as MAP1A, MAP1B, MAP2, and Tau. Overall, MAPs contribute to the structure and organization of MTs and are involved in regulating a range of processes associated with MTs, such as axonal transport.[51,52] MAP1A and MAP2 (which is mostly found in dendrites) have also been implicated in dendritic differentiation and maintenance.[37] However, in *D.melanogaster* there is no direct homologs for these proteins.[53,54]

Regarding MAP1B protein, it is primarily expressed in the axons of developing neurons during embryogenesis but is also present in adult neurons. Evidence from multiple studies indicates that MAP1B is a negative regulator of axon branching in both adults and embryonic neurons.[55,56] It interacts with actin filaments and MTs, with binding regulated by phosphorylation.[52] In *D. melanogaster*, Futsch, which is the MAP1B ortholog, is necessary for axonal growth and dendritic morphology. This protein associates with MTs (Figure 1.6A) and outlines cytoskeletal loops that span the edges of mature and stable synaptic boutons (Figure 1.6A and B). During synaptic plasticity and new bouton formation induction, the architecture of the MT loops in the mature boutons suffers a significant rearrangement, and research indicates that Futsch is crucial for this process. After the formation of the new boutons the structure of the mature bouton goes back to normal, with a Futsch loop in it.[57,58] Mutations in the Futsch gene lead to disorganized synaptic MTs, a decrease in the total number of boutons in the NMJ, and an increase in their size. Overexpressing a domain of Futsch related to MAP1B can

partially reverse these defects. Additionally, increasing nerve-terminal branching through genetic manipulation correlates with enhanced formation of synaptic MT loops, a process that depends on normal Futsch activity.[58]

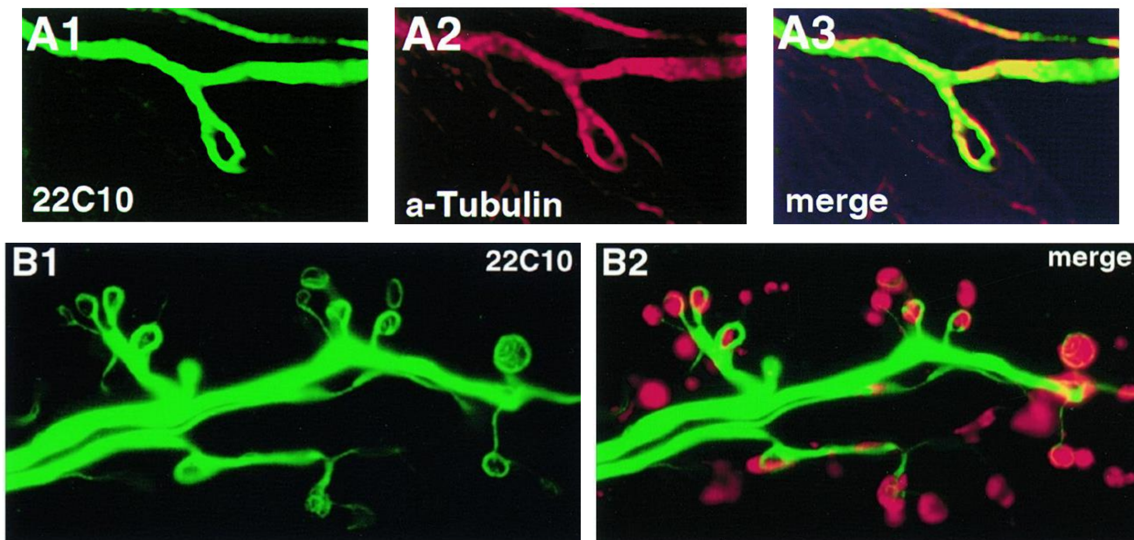

***Figure 1.6* – Futsch loops in stable boutons.** (A) Colocalization of anti-Futsch (green in A1) with anti-α-tubulin (red in A2) at a synaptic terminal (merged image in A3). (B) Futsch loops. Increase in Futsch loops (green in B1) due to a postsynaptic protein overexpression (flexin) (B2 merged image with antisynaptotagmin). 22C10 is a monoclonal antibody against Futsch. (Adapted from Roos *et al.*, 2000)

Futsch is also associated with fragile X syndrome, a genetic disorder causing mental retardation, linked to disruptions in synaptic function. In a study by Zhang *et al.* (2001), the *Drosophila* Fragile X-related gene (dfxr) was identified as a critical negative regulator of synaptic plasticity through its repression of Futsch. This relationship controls the structure of synapses at NMJs.[28] When dfxr is lost or mutated, its normal repression of Futsch is removed, leading to excessive Futsch protein levels and causing significant synaptic overgrowth[28], resembling Futsch overexpression[58]. Furthermore, dfxr Futsch double mutants restored normal synaptic structure and transmission, highlighting the importance of MT regulation in synaptic plasticity.[28]

In the mature brain, Tau is predominantly expressed in neurons, where it is preferentially located in axons. The major role of this protein in the CNS is to promote MT assembly and stability, although this interaction can be inhibited by phosphorylation at specific sites.[59,60] However, Tau has other roles in addition to its association with MTs, including regulation of neuronal plasticity, nucleolar organization, DNA protection against oxidative stress[61], and it is also involved in neurite polarity, axon elongation, and axonal transport.[60] Dysfunction of Tau has unequivocally been shown to be able to cause neurodegeneration and its abnormal accumulation leads to intracellular fibrillary deposits in neurons and glial cells of a large variety of disorders today collectively referred to as

Tauopathies that include frontotemporal dementia, AD and synucleinopathies such as Parkinson's disease.[60–62]

1.6.2. Destabilizers

Microtubule-destabilizing proteins contribute to the loss of tubulin dimers from MTs. They function by either inhibiting the addition of new dimers to the polymer, inducing catastrophes at MT ends, or severing MTs into smaller segments. These events are required within neurons to remodel the MT cytoskeleton for proper synapse formation and branching. Examples of destabilizing proteins that sever MTs in an ATP-dependent manner include Katanin and Spastin. These proteins are MT-severing proteins found in a wide range of organisms.[51,63] P60-Katanin is more highly expressed in the neuron, but Spastin is more concentrated at sites of branch formation. Thus, Spastin is particularly effective at promoting axonal branching by generating short MTs concentrated at branch sites, being that its overexpression enhances axonal branching, whereas overexpression of P60-Katanin does not.[64] Katanin is composed of P60 and P80 subunits and has a more complex role. P60 severs MTs, while P80 modulates this activity.[65] Overexpression of P60-Katanin significantly reduces MT mass and process length, particularly during early neuronal development in cultured hippocampal neurons. P80 overexpression, on the other hand, leads to milder severing effects and an increase in process number, suggesting a regulatory role in modulating P60 activity.[65] Recent findings point out that Katanin also plays a key role in synaptic plasticity by regulating both the structural remodeling of dendritic spines and the functional potentiation of synapses during LTP. Inhibition of Katanin impairs MT growth and prevents activity-induced spine remodeling without affecting overall spine density. Katanin also facilitates MT invasion into synaptic spines, a process critical for the dynamic restructuring required for synaptic strength and adaptability.[66]

Spastin, on the other hand, appears to be a negative regulator of bouton growth, where Spastin mutant NMJs show a decrease in bouton size but a slight increase in the number of boutons.[67] The work by Ozdowski *et al.* (2011) indicate that loss of Spastin reduces the distal MT network and causes bunched synaptic boutons, enhancing spontaneous NT release slightly, but reducing AP-evoked NT release, and mutations in Spastin lead to significant synaptic abnormalities, including altered bouton morphology, disrupted MT distribution, and impaired synaptic transmission.[68] A recent study revealed that Spastin has a dual role in MT dynamics. While it severs MTs into shorter pieces, it also stabilizes and promotes their growth by slowing shrinkage and increasing rescue

events. This paradoxical effect results in an overall increase in MT number and mass, as the fragments grow and accumulate. A mathematical model revealed that Spastin shifts MTs into a state with a positive net tubulin flux, which explains the observed increase in MT mass and helps resolve the apparent contradiction of severase activity leading to more MTs.[69] In *Drosophila*, overexpression of Spastin also reduces synaptic efficacy and causes loss of acetylated MTs within neurons and specifically at NMJ presynaptic terminals.[70]

Yu *et al.* (2008) reported that Tau acts as a protective agent against MT severing by P60-Katanin more so than by Spastin. Axons that are depleted of Tau display a greater tendency to form branches, supporting the idea that Tau's presence inhibits MT severing by P60-Katanin. Furthermore, in neurons lacking Tau, Spastin's effect on MT severing and axonal branching remains significant, indicating that Spastin's function is less dependent on Tau's presence compared to P60-Katanin.[64] This might suggest that there are at least two branch formation mechanisms through MT severing, one based on the local concentration of Spastin and another based on the detachment of Tau from MTs. However, this is not the only MT stabilizer protein that correlates to MT severing proteins. It might be expected that reduced Spastin activity would lead to less MT severing, resulting in higher Futsch labeling. Instead, both Futsch and tubulin staining are lower at NMJs in Spastin mutants, particularly at terminal boutons[67]. This suggests that MT severing into smaller fragments may actually enhance the transport of MTs from the axon into the NMJ.[67]

Additionally, it is worth noting that: 1) the majority of autosomal dominant hereditary spastic paraplegia - a progressive neurodegenerative disease that primarily affects the distal ends of the longest motor axons of the CNS – mutations are located in Spastin[68], and 2) downregulation of Spastin prevents loss of MTs and missorting of Tau, two key events in Alzheimer pathology. This indicates that Spastin or upstream regulators of Spastin activity could serve as therapeutic targets for AD and related Tauopathies.[71]

1.6.3. Plus end-tracking proteins

Unlike other MAPs, MT plus-end tracking proteins (+TIPs) associate specifically with the polymerizing plus-end of MTs where they act as regulators of MT dynamics and MT interactions with other structures[72], such as actin filaments (explained in greater detail in the next chapter). This group exhibits significant structural and functional diversity and includes some of the most conserved and important polymerization-MTBPs, though not

all +TIPs promote polymerization; some, like kinesin-13, induce depolymerization.[51] Based on current understanding, +TIPs association with MTs suggests that they can generally be divided into: End-binding proteins (EBs) (EB1 to EB3 in mammals); EB-dependent +TIPs, such as cytoplasmic linker protein (CLIP) 170 and CLIP-associated proteins (CLASPs), and EB-independent +TIPs[51,72], like spectraplakins which will be further explained in chapter 1.6.4.

EB proteins bind independently to the growing MT plus-ends, which they can recognize by sensing the nucleotide-bound state of β-tubulin. Once attached, EBs can recruit other +TIPs, acting as coordinators for a protein interaction network at growing MT plus-ends. Some of these recruited proteins significantly affect the dynamic properties of MTs, suggesting that disruptions in cellular MT dynamics following EB loss of function are primarily due to defects in the recruitment of these proteins.[73]

As key regulators of MTs, +TIPs are great candidates to control MTs and relay their signals during neuronal development and homeostasis. Multiple +TIPs have already been linked to neurodevelopmental functions. For instance, EBs, CLIP-170, CLASPs and spectraplakin are reported to be involved in axon outgrowth, with EBs and CLIP-170 also implicated in dendritic outgrowth and axon formation.[72]

MAPs on the MT lattice can also contribute to the regulation of +TIP binding. Research has shown that MAP1B functions as a direct regulator of EB1/3 by sequestering EB1/3 in the cytosol and anchoring them to the MT lattice, which effectively reduces the concentration of EBs at the MT plus-end, consequently fine-tuning axon growth.[74] Similarly, MAP2 can also attract EBs to the MT lattice in dendrites when synaptic stimulation occurs.[75] Additionally, recent findings suggest that Tau can also recruit and immobilize EB1/3 but to MT bundles instead, likely contributing to the stabilization of these structures, where high Tau concentrations lead to EB immobilization along the MT lattice in cell culture.[75] Conversely, MAP1B is present at one stage of neuronal development, and Tau appears at a subsequent stage. This sequential expression suggests that their functions are not overlapping but are part of a developmental progression. Nonetheless, while MAP1B was reported to increase the binding of EB1/3 to MTs plus-ends when being downregulated and thus negatively regulating these proteins with MTs, when Tau is knocked down there is a significant reduction in the number and length of EB1/3 comets at MT plus-ends.[74]

Mutations in +TIPs or their misregulation are associated to multiple neurodevelopmental and neurodegenerative diseases like AD, ALS and certain forms of autism.[72]

1.6.4. Cytoskeletal integrators

Proteins known as cytoskeletal integrators bind to and/or modulate MTs alongside other cytoskeletal elements, such as actin. Effective interaction between the actin and MT systems is crucial for processes like cell division and maintaining cell polarity. This diverse category includes scaffolding proteins like cancer-associated protein APC, and members of the plakin family.[51] In plakin family, spectraplakins are an important member of this category, which are particularly versatile cytoskeletal integrators.

Spectraplakins contain three specific domains: one that binds to actin, another that associates with the MT plus-end through EBs, which is why spectraplakin proteins can also fall into the +TIPs category, and a third that binds and stabilizes MTs, just like other MAPs and Tau. Spectraplakin deficiency causes very strong MT phenotypes correlating with neuro-development disorders as well as severe neurodegeneration in mice and humans.[76] Examples of spectraplakins include microtubule actin cross-linking 1 (MACF1) in mammals and Short-Stop (Shot) in *Drosophila*.[77]

MACF1 is known to guide MTs along actin filaments and to mediate MT capture at actin-rich sites near the membrane.[72] In neurons, MACF1 stabilizes MTs and coordinates their interactions with the actin cytoskeleton, enabling efficient axonal growth and maintenance. Loss of MACF1 leads to reduced axon length and defects in axon guidance, demonstrating its role in shaping the neural architecture. Moreover, through its association to MTs and EBs, spectraplakins regulate synaptic stability, ensuring proper synaptic transport and plasticity. In *Drosophila*, on the other hand, Shot coordinates MT reorganization necessary for synapse formation and maintenance, promoting synaptic growth during both development and aging. Shot also links MTs to the actomyosin network during important developmental processes, such as the formation of neural circuits, essential for adaptive neural plasticity.[78]

Additionally, this group can encompass even classic MAPs such as Tau, since studies have showed that Tau colocalizes with both stable and dynamic MTs as well as with actin filaments.[79,80] Furthermore, Tau neurotoxicity correlates with alterations of actin organization in animal models of AD.[81] Overall, the evidence supports that Tau acts

as a regulator of MTs and actin, suggesting that Tau is a potential linker between the two cytoskeletons.

1.6.5. Minus end-targeting proteins

During the early stages of neuronal development, MTs are attached to the centrosome, located near the nucleus, through specialized structures called gamma-tubulin ring complexes (γ-TuRCs), as mentioned before. This complex serves as a template for MT polymerization, binding to the minus-end of MTs and promoting their growth from the plus-end necessary for the motility and directionality of migrating neurons, but also their nucleation. However, in the course of neuron maturation the centrosome-dependent MT organization is lost, and so, differentiated neurons have non-centrosomal MTs with the minus-ends distributed throughout the whole cell.[38,82] Consequently, in mature neurons the minus-ends of MTs can either bind to the γ-TuRC that is relocated to other non-centrosomal MTOC where MTs continue to be nucleated and stabilized, or they can attach laterally to a set of proteins known collectively as CAMSAPs - which includes the *Drosophila* protein Patronin - that stabilizes the minus-ends of MTs in both dendrite and axon processes.[38,51]. It is known that both γ-TuRCs and CAMSAPs/Patronin can be considered capping proteins on the minus-ends of MTs, although the last ones appear to associate laterally with the minus-end[51], where specific stabilizing factors associated with these proteins can be recruited to the growing minus-ends and protect them against depolymerizing enzymes, similar to +TIPs at the plus-ends of MTs.[38] Accordingly, for CAMSAP-stabilized MTs to extend from their minus-ends, either the γ-TURC needs to be removed from these ends, or new minus-ends must be created through MT breakage or severing. In neuronal cells, this implies that γ-TURC and CAMSAPs operate in separate, yet possibly sequential, phases to produce non-centrosomal MT networks.[82]

A recent study from Gao *et al.* (2024) showed that Patronin is essential for the proper development of synaptic boutons and NMJ morphology in *Drosophila*, likely through its role in stabilizing MTs at the presynaptic terminal. It also indicated that Patronin works synergistically with Shot, to control NMJ development. This suggests that both proteins play a collaborative role in stabilizing MTs at the synapse. Additionally, the knockdown of Patronin in *Drosophila* MNs resulted in significant defects in synaptic growth, specifically reducing the size of synaptic boutons at the NMJ. This reduction in bouton size was consistent across different stages of larval development, indicating Patronin's crucial role in maintaining NMJ growth.[83]

On the other hand, a study by Qu *et al.* (2019) uncovers the critical role of activity-dependent MT nucleation at excitatory presynaptic boutons, particularly at *en passant* boutons, in regulating NT release in hippocampal neuronal cells. This study highlights that neuronal activity triggers *de novo* MT nucleation, driven by γ-tubulin and the augmin complex, which aligns MTs toward the distal axon tip. This MT nucleation enhances SV motility and bidirectional transport between boutons, crucial for efficient NT release and synaptic transmission. Furthermore, loss of MT nucleation due to γ-tubulin knockdown significantly impairs the movement of SVs between boutons, inhibiting their transport in both directions, leading to lower SVs at synapses. Consequently, this disruption severely reduces the effectiveness of neurotransmission.[84]

1.7. Justification and relevance of the topic

Although defects in synaptic boutons are linked to neurological disorders, many fundamental aspects of bouton formation - such as how, when, and where they develop - remain largely unexplored. Fernandes *et al.* (2023) found that actin polymerization is not necessary for bouton outgrowth, however, actin is required for bouton stabilization. With this finding, new questions about the involvement of other cytoskeletal elements, such as MTs, in this process had raised. While the role of MTs in dendritic trees and axonal arbors has been well established[13] and previous studies have shown that the MT cytoskeleton plays a critical role in regulating synaptic plasticity[35,85], little is known about its specific contribution to bouton blebbing at the NMJ[86].

Therefore, this research focused on understanding the dynamic behavior of MTs during the formation and growth of new GB through confocal microscopy and live imaging. A multi-faceted approach using the *Drosophila* NMJ model was used to achieve this. This NMJ is a stereotyped structure that is easily accessible through dissection but also by non-invasive imaging through the translucent larval cuticle. Moreover, it can be manipulated with sophisticated genetic mutagenesis, screening, expression, and editing strategies and is amenable to advanced biochemical, electrophysiological, ultrastructural, and light imaging techniques.[23] Genetically encoded fluorescence markers for overexpressed proteins of interest, along with immunohistochemistry staining for others allow us to visualize their localization and intensity within the GB. Additionally, RNAi to knockdown the expression of various MTBPs was used to explore the impact of their reduction in GB formation and overall synaptic plasticity. By employing these techniques, we hope to better understand how MTs undergo rearrangements during bouton formation and how these changes impact synaptic plasticity.

Consequently, this topic has significant clinical implications since synaptic dysfunction is a hallmark of several neurological and neurodegenerative disorders.[87] Thus, a better understanding of the mechanisms that regulate the blebbing process could lead to the development of new therapeutic strategies that target MTs or MTBPs and the consequent improvement of synaptic function and plasticity in these disorders.

1.7.1. Research question

The research question for this study was the following: “What is the role of microtubules in the initiation and growth of synaptic boutons at the neuromuscular junction in *Drosophila melanogaster*?”

1.7.2. Objectives

The general and main goal of this study was to characterize microtubule behavior during neuronal growth in *D. melanogaster*.

The specific aims are:

1. Determine the best lines of *Drosophila melanogaster* to visualize the dynamic of microtubules;

2. Assess microtubule localization and their dynamics in neurons before and after stimulation of synaptic activity;

3. Characterize the role of MTs in bleb formation and growth through live and confocal imaging;

4. Manipulate microtubule stability with polymerizers and depolymerizers of tubulin and assess the impact on neuronal growth and structural plasticity.

1.8. Ethical and Legal Considerations

National legislation regarding animal protection used for scientific purposes was created to ensure that the use of animals is always humane, careful, responsible, and justified.[88] However, the use of *D. melanogaster* does not raise ethical problems, being that Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, since January 1, 2013, does not apply to insects, as they are not live non-human vertebrate animals, nor live cephalopods. This legislation aims to improve the welfare of animals used for scientific purposes and promote the policy of the 3Rs (Replacement, Reduction, Refinement).[88] Additionally, NOVA Medical School is licensed

for animal experimentation by the Direção-Geral de Alimentação e Veterinária, complying with European Directive 2010/63/EU and Decree-Law nº 113/2013.

It is also worth noting that while *D. melanogaster* is not subject to the ethical restrictions outlined in Directive 2010/63/EU, we still adhered to the principles of the 3Rs by using the minimum number of larvae necessary for the scientific validity of our experiments.

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2. Materials and Methods

2.1. *Drosophila* culture and stocks

To express RNAi or overexpress specific proteins in neurons, we crossed UAS-RNAi or UAS-GFP-tagged lines with nSyb-GAL4, a pan-neuronal driver. We used 5-10 virgin females and 3-5 males, anesthetized with CO2, and maintained the crosses at 25ºC. For RNAi experiments, flies were kept at 29ºC to enhance knockdown efficiency. To ensure that phenotypes were specifically due to RNAi knockdown and not effects from the nSyb-GAL4 driver or genetic background, we used nSyb-GAL4 × w^{1118} as a control.

TM6b or GFP expressing balancer chromosomes were used to facilitate genotyping of larva, therefore only first-generation non-tubby and/or fluorescent larvae were selected for dissection.

For live imaging crosses between UAS-lines and nSyb-GAL4, CD4-Tom (where the CD4 present in the neuronal membrane is tagged with Tomato) were performed.

A list of all fly stocks used in the experiments is attached in Table 3 on Supplementary information I.

To assess the structural and functional aspects of GB formation at the NMJ in *D. melanogaster*, a systematic experimental workflow was employed (represented in Figure 2.1). This workflow encompasses the dissection of third-instar larvae, the application of a stimulation protocol to induce GB formation, and high-resolution imaging to analyze the presence or absence of specific proteins within GBs. Additionally, quantitative measurements of GBs and NMJ areas were conducted in gene knockdown contexts to unravel the molecular mechanisms underlying synaptic plasticity.

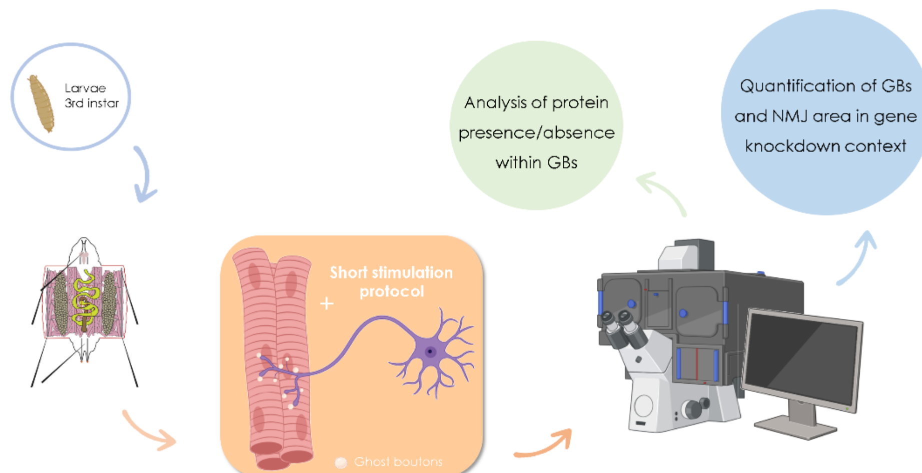

Figure 2.1 **- Experimental workflow for GB analysis in *D. melanogaster*.** The workflow includes third-instar larval dissection, stimulation protocols for GB induction, and imaging for protein localization and quantitative analyses.

2.2. Larval dissections and acute induction of activity-dependent bouton formation

The induction of new bouton formation is a critical step because it allows observation and analysis of the dynamics of MTs and their involvement in the structural changes associated with the formation, growth and stability of new boutons at the NMJ. In order to induce activity-dependent bouton formation, a well-stablished protocol described by Vasin, A. *et al.* (2014) was used to induce neuronal depolarization based on the administration of spaced High K^+ and high calcium (Ca^{2+})[30], which we call Short-Stim (SS). This protocol was performed on 3rd instar larvae.

Third instar larvae are pinned down onto Sylgard-coated plates using insect pins and partially dissected in hemolymph-like (HL) 3.1 saline (in mM: 70 NaCl, 5 KCl, 0.1 CaCl2, 4 MgCl2, 10 NaHCO3, 5 Trehalose, 115 Sucrose, 5 HEPES-NaOH, pH 7.2-7.4) using a procedure similar to Brent *et al.* (2009), a scenario equivalent to unstimulated, in which no or very few contractions were observed. Prior to being stimulated, one of the dissection pins was moved inward at approximately 60% of the original size of each larva to allow muscle contraction. The 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 minutes pulses each separated by 10-minute incubation in regular HL3.1. In these 2-minute incubations with high-K^+ and Ca^{2+} solution, patterned contractions of the body wall muscles were observed. Lastly, larvae were incubated in HL3.1 with Ca^{2+} (0,5 mM) for 30 minutes to maximize bouton formation, during which the first 10 were to rest and the other 20 minutes were to perform the remaining dissection by removing the body fat and gut, while the CNS was kept intact until the beginning of the mounting process.

For live imaging, larvae were glued to Sylgard-coated slides using topical veterinary tissue adhesive and stretched during dissection just enough to allow muscle contraction without excessive tension. Right after the stimulation protocol, larvae were imaged in HL3.1 saline for 30 minutes to 1.5 hours.

2.3. Immunohistochemistry

When the diverse experimental treatments were concluded, larval fillets were fixed in 4% paraformaldehyde at room temperature (RT) for 20 minutes. The pins were then removed, and larvae were placed on an Eppendorf tube for immunohistochemistry with

Phosphate-buffered saline 1x + 0,3% Triton X-100 (PBST) to be extensively washed 3 times for 45 minutes to 1 hour at RT with agitation, in order to permeabilize membranes.

Primary antibody (Ab) incubation was performed overnight (ON) at 4ºC in the dark with agitation, in 5% Normal Goat Serum (NGS) to minimize unspecific Ab binding, in PBST. Subsequently, larvae were extensively washed using PBST, followed by 2 hours with secondary Ab and 5% NGS at RT with agitation in the dark. After extensive washing with PBST, larvae were transferred to 50% glycerol in PBS for 5 minutes and then mounted in DABCO medium on a microscope slide where the CNS, the head, and the tail were removed. Afterward, all larvae on the slide were covered with coverslips, sealed with nail polish, and stored at 4ºC in the dark until imaging.

A list of all primary and secondary antibodies used in the experiments, along with their dilutions and sources, is provided in Table 1 and 2, respectively, in Supplementary Information I.

2.4. MT stability manipulation

In order to assess the impact of MT dynamics in GB development we used 1μM Vinblastine and 50μM Taxol dissolved in HL3.1 (0,1mM Ca^{2+}; High K^{+}/Ca^{2+}; 0,5mM Ca^{2+}) solutions, to destabilize and stabilize MT, respectively. Also, Dimethyl sulfoxide (DMSO) was also added to the working solutions of HL3.1 at the same dilution as the highest drug (50 μM), as control.

For this experiment, firstly, larvae were dissected in HL3.1 without drugs, and then they were incubated in HL3.1 with the drug for 30 minutes and after that, stimulation protocol follows as previously described with the respective drugs in the HL3.1 working solutions. Controls were done in parallel, but using DMSO in HL3.1.

2.5. NMJ imaging

Confocal images were obtained on a laser scanning confocal microscope (LSM 710) with a 63x 1.3NA oil-emersion objective (Carl Zeiss) or inverted microscope (Observer) with the same objective. All images obtained were processed in imageJ/FIJI (National Institutes of Health) software. Live imaging experiences were performed with a spinning disk confocal microscope (Andor) with a 60X 1.3 NA oil-immersion objective (Carl Zeiss) equipped with a heating stage heated to 25ºC.

2.6. Data analysis

Quantification of GB number was performed at NMJ 6/7, preferentially from abdominal segments A3-A5 were analyzed. In general, at least 12 (fixed) NMJs of each genotype were analyzed. Quantitative analyses were performed using maximum intensity projections from the Z-stacks on the image. GBs quantification was performed according to Gala, D. (2021). “The Big Confusing Book of Ghosts Boutons” unpublished.

To analyze GB number in fixed samples, maximum intensity projections from Z-stacks were used on image J. The macro used is summarized as follows:

1) Image > Color > Channels tool > Composite

2) Image > Stacks > Z-project > Max intensity projection

3) Image > Adjust > Brightness/Contrast

4) Multi-point tool (for GB count)

5) File > Save as > Tiff

In order to assess immuno-localization of the proteins of interest within GBs, a semi-quantitative scoring system was developed. The intensity scores were assigned manually by visual examination of the maximum intensity projections from Z-stacks and from the z-stacks itself. The scoring criteria are as follows:

- Score 0: No detectable protein intensity.
- Score 1: Low/Moderate intensity of protein expression.
- Score 2: High intensity of protein expression.

To analyze NMJs morphology and to identify possible phenotypes in RNAis, we measured NMJs’ areas in all RNAi and control lines. For this, the following steps were performed:

1) Image > Stacks > Z Projection > Max Intensity

2) Image > Color > Split Channels

3) Image > Adjust > Brightness/Contrast

4) Image > Adjust > Threshold

5) Analyze > Measure (to measure NMJ area)

6) File > Save as > Tiff

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

1) File > Import > Bio-formats

2) Image > Stacks > Z-project > Max intensity (all slices and all time frames)

3) Image > Stacks > Concatenate (if video was saved in separate files)

4) Image > Color > Split channels (if more than one channel)

5) Process > Enhance contrast > 0.1 to 0.3 % saturated pixels > normalize and process all slices

6) Image > Color > Merge channels (if more than one channel)

7) Stack > Animation > Animation options > Speed at 15 fps

8) Rectangular selection > Image > Duplicate (to crop in a specific range of the stack)

9) Videos were saved as AVI.

2.6.1. Statistical analysis

All statistical analysis and graph construction were performed in GraphPad Prism 9.0.0. Data was tested for normality using the Shapiro-Wilk normality test. When data sets passed the normality test, statistical significance in two-way comparisons was determined by an unpaired Student's t-test. When normality was not verified, a nonparametric unpaired t-test (Mann-Whitney) was used to compare two data sets. For MT stability manipulation experiments, in order to compare three datasets, the Krukal-Wallis analysis was used when normality was not verified.

In all figures, p-value is presented as: **** p<0.0001; *** p<0.001 ** p<0.01, * p<0.05, n.s. not significant. Statistical comparisons are with control unless noted. Sample size (n) is presented in the respective figure legend.

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3. Results and Discussion

Neuronal structure and plasticity rely heavily on MTs, which provide support to axons and dendrites while facilitating intracellular transport. This dynamic system allows neurons to modify synaptic connections and maintain effective communication.[89]

Preliminary results from Fernandes *et al.* (2023) showed that: 1) GBs were enriched with EB1, 2) Futsch was present on the majority of GBs but not as typical Futsch loops which are present in mature synaptic boutons, and it rather looked as filamentous or splattered structures, and 3) α-tubulin aggregates were identified within GBs. These findings suggest that MTs present in GBs are not stable, but dynamic. Motivated by this discovery, we decided to test the role of these proteins in GB formation. We started by confirming the presence of EB1, Futsch, and α-tubulin in GBs in our experimental system.

Given that EB1 is a key +TIP binding protein that regulates MT growth, we first sought to examine its localization in GBs to understand its potential influence on MT dynamics in the context of neuronal plasticity. For this, we conducted immunolocalization and fluorescence imaging of overexpressed EB1-GFP and live imaging of EB1. Consistent with the preliminary results described, our immunolocalization analysis confirmed that EB1 is present in 100% of GBs (see Figure 3.1, 3.2 and Figure 2 in Supplementary information III). Due to their significant role in MT regulation, EB1 is an ideal candidate to control MTs, and our results sustain the fact that this protein is a key player in GB outgrowth. Our results also point towards the idea that MTs might be polymerizing within GBs, which means that MTs are in a dynamic state at this stage of GB development. This encourages further exploration of how alterations in EB1 levels or activity might affect synaptic development and remodeling, so a future step will be to express the knockdown of this +TIP to understand if EB1 is crucial for GB development and - most importantly - formation. In parallel, we performed live imaging of this protein by expressing UAS-EB1-GFP and UAS-CD4-Tomato in neurons, using nSyb-Gal4. Unfortunately, in the movies acquired we were unable to observe GB formation events (Figure 1 in Supplementary information II). More movies need to be acquired in the future.

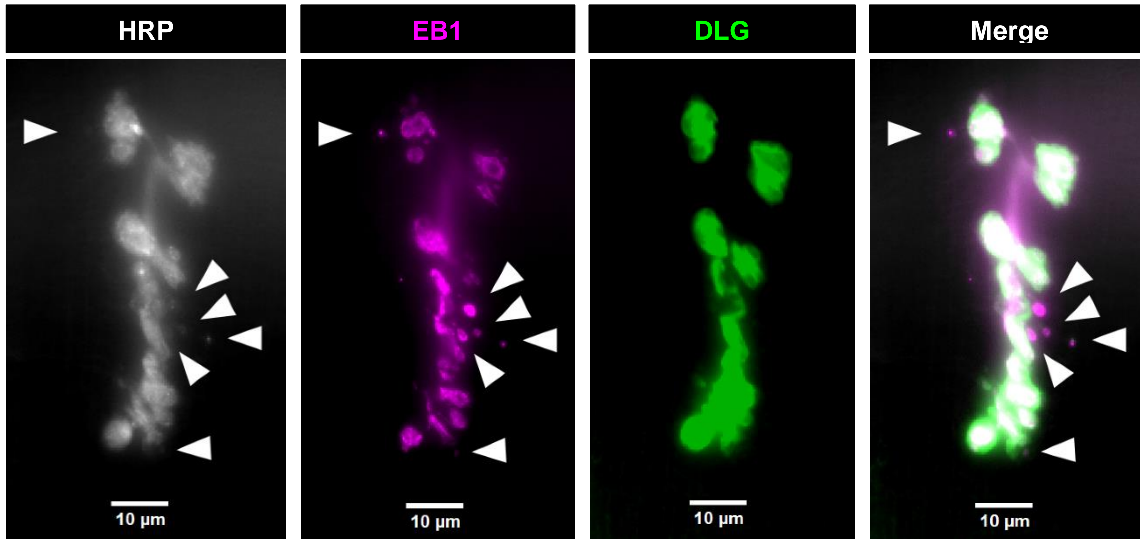

Figure 3.2 **- Localization of EB1 in ghost boutons at the *Drosophila* NMJ.** EB1 (magenta) is present in ghost boutons (arrowheads). Presynaptic and postsynaptic membranes labeled with antibodies for Horseradish peroxidase (HRP-white) and Discs-large (Dlg-green), respectively. n = 22 NMJs, genotype nSyb-Gal4;UAS-EB1-GFP

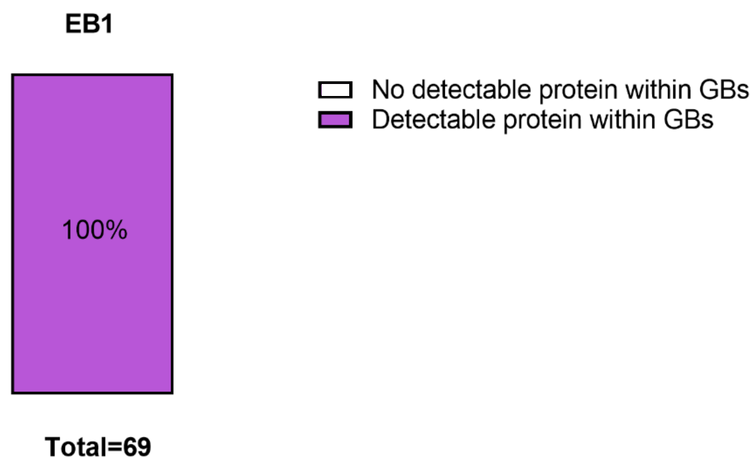

Figure 3.1 **– EB1 detection in GBs (after neuronal overexpression).** EB1 was detectable in all 69 GBs (100%). n=22 NMJs, genotype nSyb-Gal4;UAS-EB1-GFP.

To improve our understanding of MT dynamics during new bouton formation, we examined Futsch, an important MTBP with specific MT interactions that may provide deeper insights into the structural organization of GBs. To address this, we performed overexpression and knockdown of Futsch, in neurons, using the neuronal driver nSyb-Gal4 to express UAS-Futsch. In the overexpression immunofluorescence experiments, we found that most GB contained Futsch (around 74% as represented in Figure 3.3), predominantly at low intensity in the overexpression context, while a small number displayed high intensity and a few did not exhibited any Futsch within GBs (as shown in Figure 3 in Supplementary information III), however, we never observed Futsch loops. Typically, larger GBs expressed Futsch with higher intensity, whereas smaller GBs

predominantly display either no detectable signal or low-level Futsch expression (see Figure 3.4). This suggests that the developmental stage of GBs is linked to their ability to have stabilized MTs, which is dependent on the presence and intensity of Futsch.

The reduction of Futsch levels through RNAi expression did not affect the NMJ area but resulted in a significant decrease in GB number as predicted (see Figure 3.5). This data suggests that Futsch is essential for the direct stabilization of GBs and may also indirectly facilitate their initiation. The presence of varying Futsch levels within GBs, including instances where it was undetectable, and the decrease in GB number upon reduced expression levels of this protein highlights the complexity of Futsch role and hint at Futsch being dynamic during bouton formation. In other words, this variability could reflect the dynamic regulation of MTs necessary for both the stabilization and development of GBs. Considering these results, an interesting idea for future research would be to determine whether Futsch knockdown also results in an increase in GB size, paralleling the enlargement seen in typical boutons at the NMJ, which would help us support our theory that Fustch is necessary in GB stabilization.

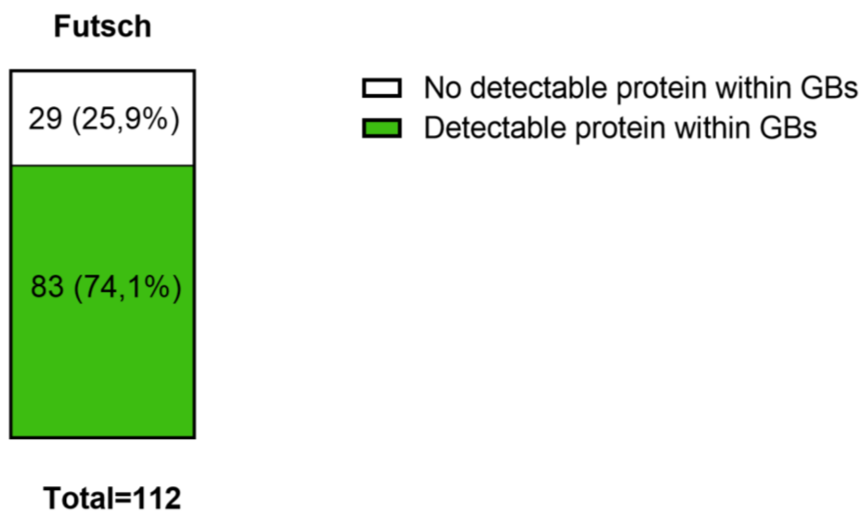

***Figure 3.3* - Futsch detection in GBs (after neuronal overexpression).** Futsch was detectable in 83 from the 112 GBs accounted (74,1%), in green. 29 GBs had no detectable Futsch within (25,9%), in white. n=18 NMJs, genotype nSyb-Gal4;UAS-Futsch-GFP.

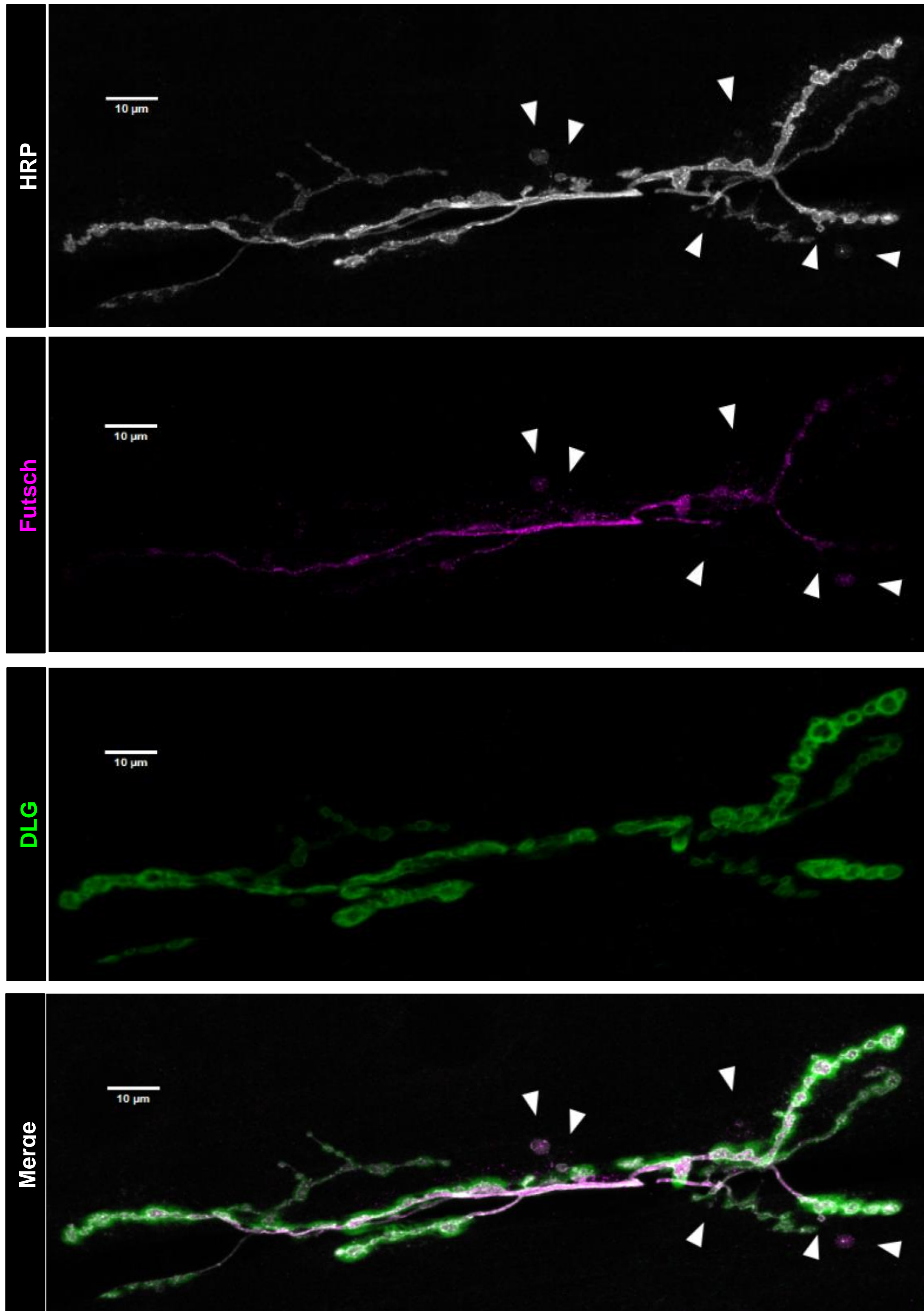

Figure 3.4 -* Localization of Futsch in ghost boutons at the *Drosophila* NMJ*. Futsch (magenta) is present in most ghost boutons (arrowheads). Presynaptic and postsynaptic membranes labeled with antibodies for Horseradish peroxidase (HRP-white) and Discs-large (Dlg-green), respectively. n = 18 NMJs, genotype nSyb-Gal4;UAS-Futsch-GFP.

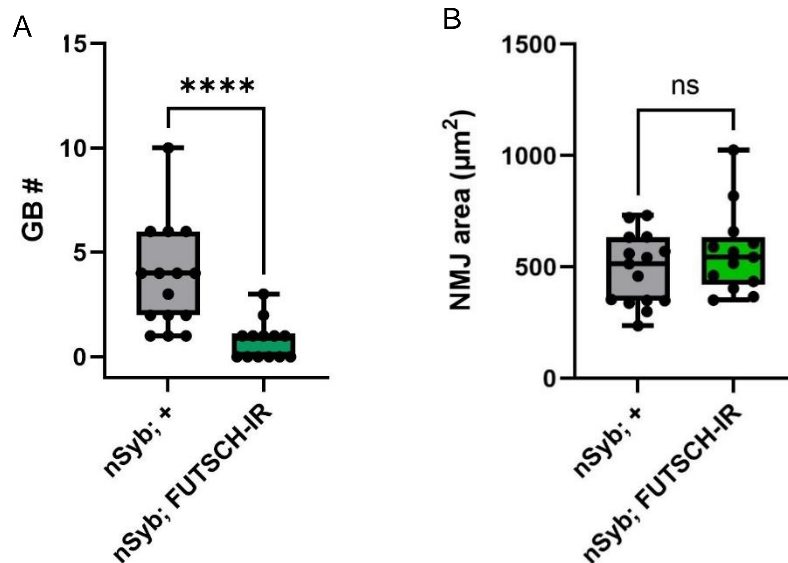

***Figure 3.5* – Quantification of GBs and NMJs area measurement after SS protocol.** (A) Number of GBs. n=15 NMJs for nSyb; + and n=13 NMJs for nSyb; Futsch-IR. Statistical significance was determined using the nonparametric Mann-Whitney test; ****p<0,0001. (B) NMJ area. n=15 NMJs for nSyb;+ and n=13 NMJs for nSyb; Futsch-IR. Statistical significance was determined using the Unpaired t-test; not significant (ns).

Having confirmed the involvement of MTBPs in GB outgrowth, we next aimed to investigate the core components of MTs, focusing specifically on their alpha- and beta-tubulin subunits, starting with alpha-tubulin. This particular subunit was predominantly identified in a dispersed manner across the majority of GBs (around 68%) (see Figure 3.6) in wild-type larvae, as represented in Figure 3.5. However, it is noteworthy that in a significant proportion of GBs we could not detect the presence of alpha-tubulin (around 32%) (see Figure 3.7). However, through alpha-tubulin analysis, we can infer that MTs are not present in GBs as a stable structure but rather in a destabilized and depolymerized state. The observation of alpha-tubulin as punctate or scattered dots suggests that tubulin is present in GBs, but in a form that reflects MT instability rather than fully polymerized filaments. EB1, being a +TIP protein that associates with growing MT ends, further supports this hypothesis. Its consistent presence in all GBs indicates that MT extension and exploration are actively occurring, confirming the idea that MTs within GBs are highly dynamic and unstable, contributing to the rapid remodeling and outgrowth characteristic of these structures.

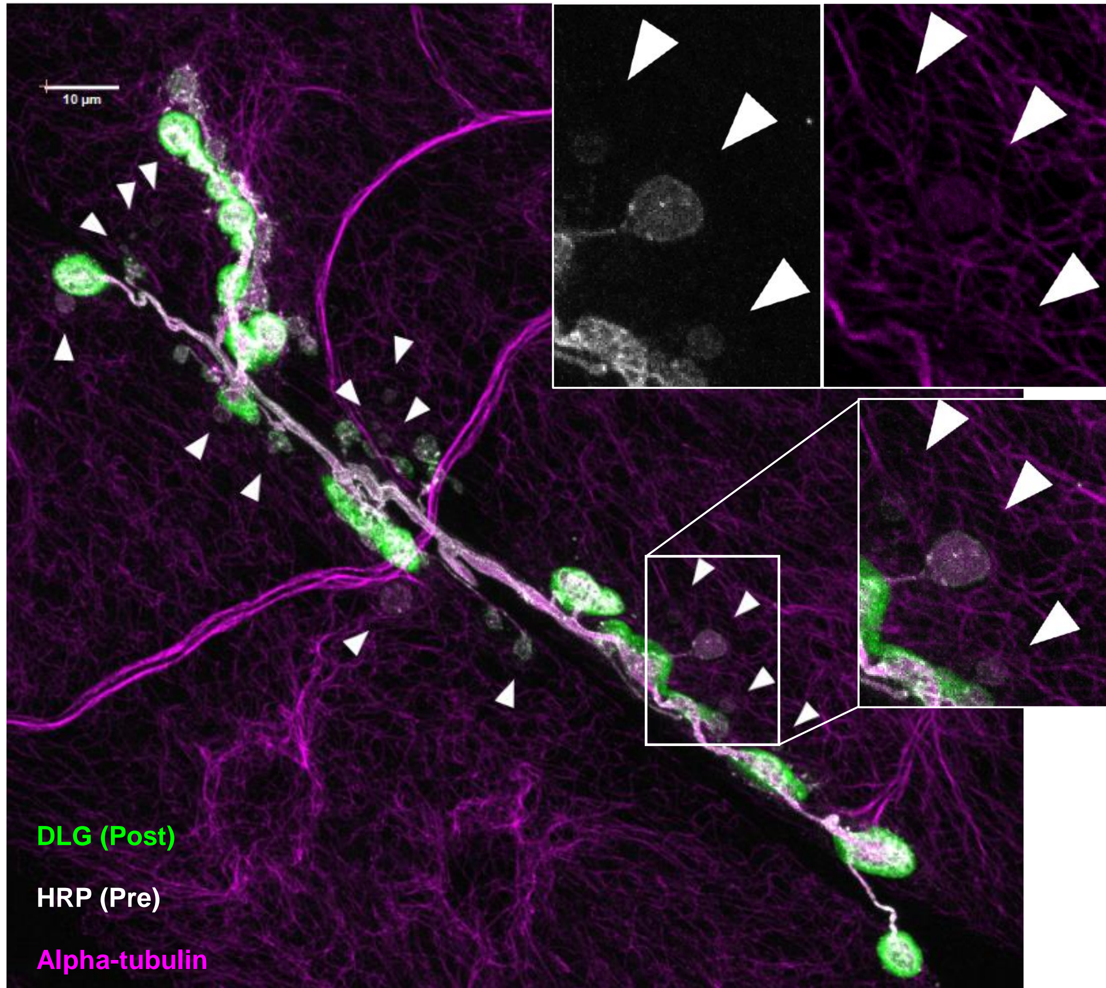

Figure 3.7 **- Localization of alpha-tubulin in ghost boutons at the *Drosophila* NMJ.** Alpha-tubulin (magenta) is present in most ghost boutons (arrowheads). Presynaptic and postsynaptic membranes labeled with antibodies for Horseradish peroxidase (HRP-white) and Discs-large (Dlg-green), respectively. n = 16 NMJs.

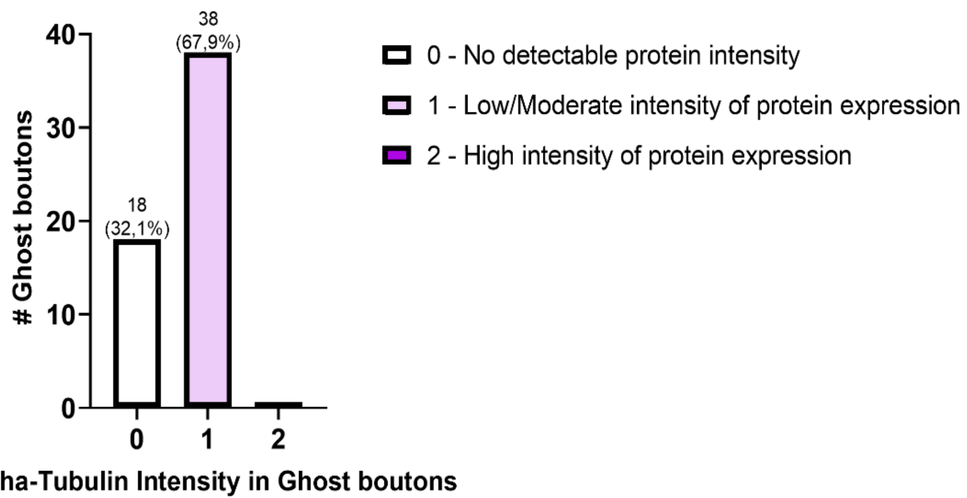

Figure 3.6 **- Quantification of alpha-tubulin intensity in GBs after SS protocol.** 18 GBs had no detectable alpha-tubulin within, which represents about 32,1%. 38 GBs had low or moderate intensity of alpha-tubulin within, which represents about 67,9%. No GBs where found with high intensity of alpha tubulin. n= 16 NMJs

To further investigate the relationship between the tubulin subunits, we examined the presence of beta-tubulin to determine whether it exhibited similar distribution patterns as alpha-tubulin. This analysis aimed to elucidate whether these subunits existed as part of a stable MT complex or whether they functioned independently. Additionally, we sought to test whether EB1 was associated with beta-tubulin, which would manifest as with a consistent presence across all GBs. However, our findings indicated that beta-tubulin was rarely detected in GBs (around 78%) (see Figure 3.8 and 3.9) in wild-type larvae, and when present, it was predominantly localized in larger GBs, raising the possibility that the monoclonal Ab used may not effectively recognize all beta-tubulin subunits or have accessibility to the epitope that it recognizes, especially when they are depolymerized and possibly at low levels. Given the uncertainty regarding the detection of beta-tubulin isolated subunits, we cannot definitively conclude that EB1 associates with GBs independently of beta-tubulin; therefore, further investigation is needed to clarify this association.

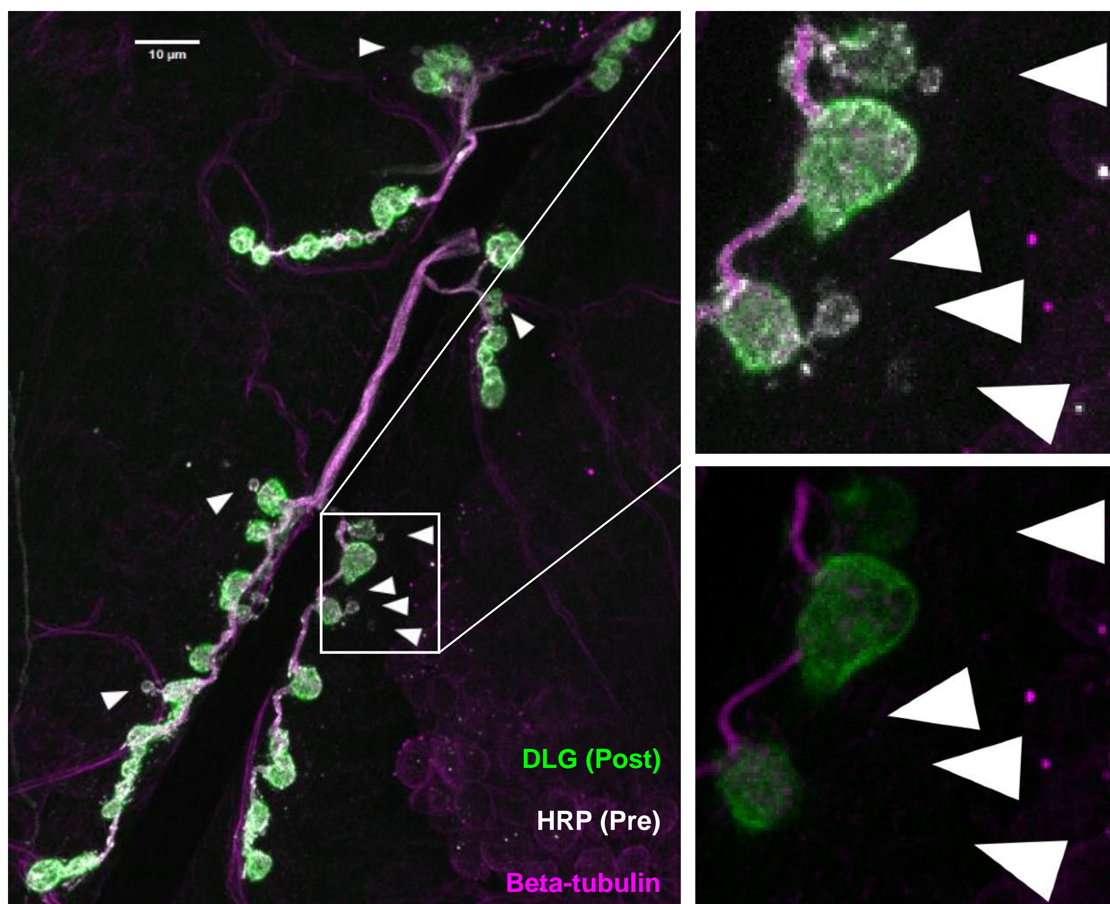

Figure 3.8 - **Localization of beta-tubulin in ghost boutons at the *Drosophila* NMJ.** Beta-tubulin (magenta) in most ghost boutons (arrowheads) is absent. Presynaptic and postsynaptic membranes labeled with antibodies for Horseradish peroxidase (HRP-white) and Discs-large (Dlg-green), respectively. n = 13 NMJs.

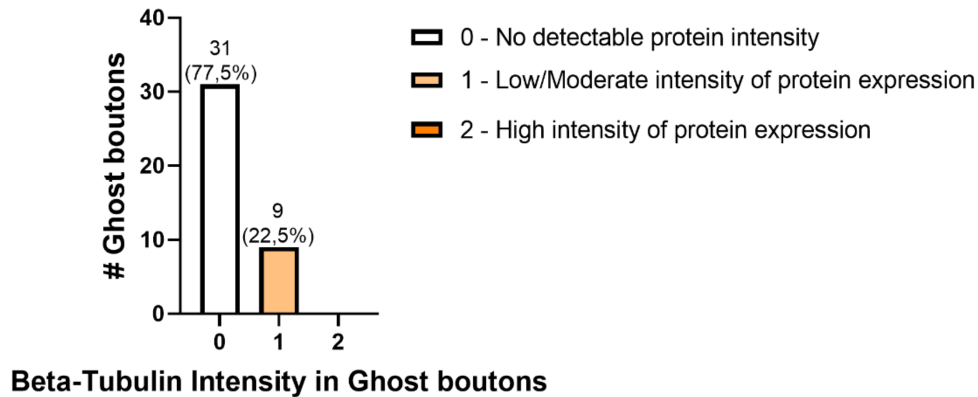

***Figure 3.9* - Quantification of beta-tubulin intensity in GBs after SS protocol.** 31 GBs had no detectable beta-tubulin within, which represents about 77,5%. 9 GBs had low or moderate intensity of beta-tubulin within, which represents about 22,5%. No GBs where found with high intensity of alpha tubulin. n= 13 NMJs

Tau was another MT stabilizer protein that we recognized as very promising due to its strong influence and importance in NMJ function, development, and plasticity, as well as its dynamic role with other MTBPs. Despite multiple attempts, Ab staining and the use of labelled Tau-lines did not yield successful results, as we were unable to visualize Tau in our preparation. However, the RNAi line targeting Tau was included in our screen for MTBPs. The analysis revealed no significant differences in either the NMJ area nor the number of GBs in the RNAi Tau condition when compared to the control (see Figure 3.10). As mentioned above, given its crucial roles in NMJ integrity and interactions with other MTBPs, we expected a decrease in the number of GB because EB1 localization depends on the expression levels and localization of Tau protein, but also because the reduction of Tau levels leads to a down-regulation between MTs and actin. Nevertheless, Tau provides a protective effect on MTs against severing proteins like Katanin-60 and Spastin. Thus, Tau depletion results in an increase in MT severing. Given these, we hypothesize that this double role of Tau may explain our results. Although a reduction in GB number would be anticipated due to Tau's role as a direct regulator of EB proteins in developing neuronal cells, the increase in MT severing could serve as a compensatory mechanism, which may account for the lack of significant change in GB count. In fact, Kuo *et al.* (2019) found that Spastin not only severs MTs but also promotes their regrowth through an ATP-independent mechanism leading to an increase in MT number and mass.[69] The reduced MT stability caused by Tau's knockdown led us to believe that the NMJs would be smaller. However, NMJ area resulting from diminished Tau levels might be counteracted by elevated severing activity, explaining the lack of difference compared

with the control. Another explanation is that the RNAi line used did not lead to high levels of reduction of Tau RNA. As such, additional RNAi lines against Tau should be tested.

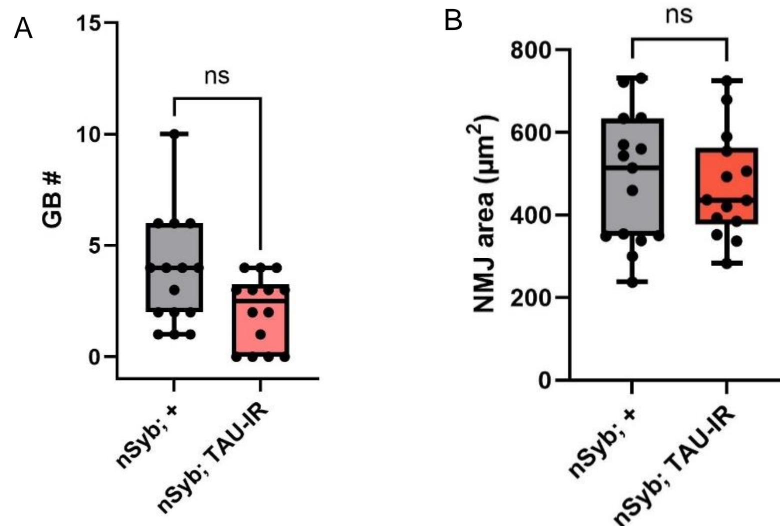

***Figure 3.10* – Quantification of GBs and NMJs area measurement after SS protocol.** (A) Number of GBs. n=15 NMJs for nSyb; + and n=14 NMJs for nSyb; Tau-IR. Statistical significance was determined using the nonparametric Mann-Whitney test. (B) NMJ area. n=15 NMJs for nSyb;+ and n=14 NMJs for nSyb; Tau-IR. Statistical significance was determined using the Unpaired t-test; not significant (ns).

With this line of reasoning and the results indicating the presence of MTBPs within GBs, the following step was to investigate whether MT severing is necessary for GB formation, as it is required for proper neuronal formation and branching. To address this, we performed knockdown experiments targeting two major MT severing proteins, Spastin and Katanin-60. Contrary to what we expected, inhibition of both proteins did not impact the NMJ area (see Figure 3.11B and D). Since the reduction in MT severing would likely lead to an accumulation of stable MT networks, we expected that this stabilization would enhance synaptic integrity and facilitate growth, thereby contributing to the expansion of the NMJ area. Nevertheless, Spastin is involved in severing MTs, which allows for remodeling and turnover, but it also contributes to the stabilization and growth of MTs by reducing their tendency to shrink and facilitating recovery events.[69] This balance may enable the NMJ area to remain stable despite its reduced expression. An alternative explanation for the observed results is that, despite the distinct mechanisms of action of these two severing proteins, both are involved in MT severing. Consequently, the knockdown of one protein may trigger compensatory mechanisms, whereby the remaining protein compensates for its loss, thereby maintaining the overall MT dynamics and preventing any significant changes in NMJ morphology. This lack of significant changes in the NMJ area can also be explained if compensatory mechanisms associated with other MTBPs, like Tau, that can maintain structural integrity despite the inhibition of

these specific proteins, as proposed previously. On the other hand, while the number of GBs in Spastin knockdown remained unaltered, the slight decrease observed in Katanin-60 knockdown may be attributed to the distinct roles that these proteins play in MT dynamics (See Figure 3.11A and C). The decrease in GB numbers with Katanin-60 knockdown likely results from impaired MT dynamics and reduced availability of MT ends needed for synaptic development and growth. Given Katanin's normal function of promoting axonal MT depolymerization and severing[90], the dynamic MT cytoskeletal environment required for synaptic plasticity is provided by their action. Although Tau serves as a protective factor against MT severing by P60-katanin (more so than Spastin), neurons exhibit higher levels of Katanin during periods of rapid axonal growth[91], which Tau may not be able to protect MTs from. This suggests that Katanin plays a critical role in maintaining dynamic MTs and promoting synaptic development, including GB formation. In contrast, Spastin knockdown does not have the same effect, potentially since Spastin functions in both severing and stabilizing MTs, which may allow for effective compensation, maintaining GB numbers despite its inhibition or through compensatory mechanisms (possibly associated with Tau) that can mitigate the loss of Spastin.

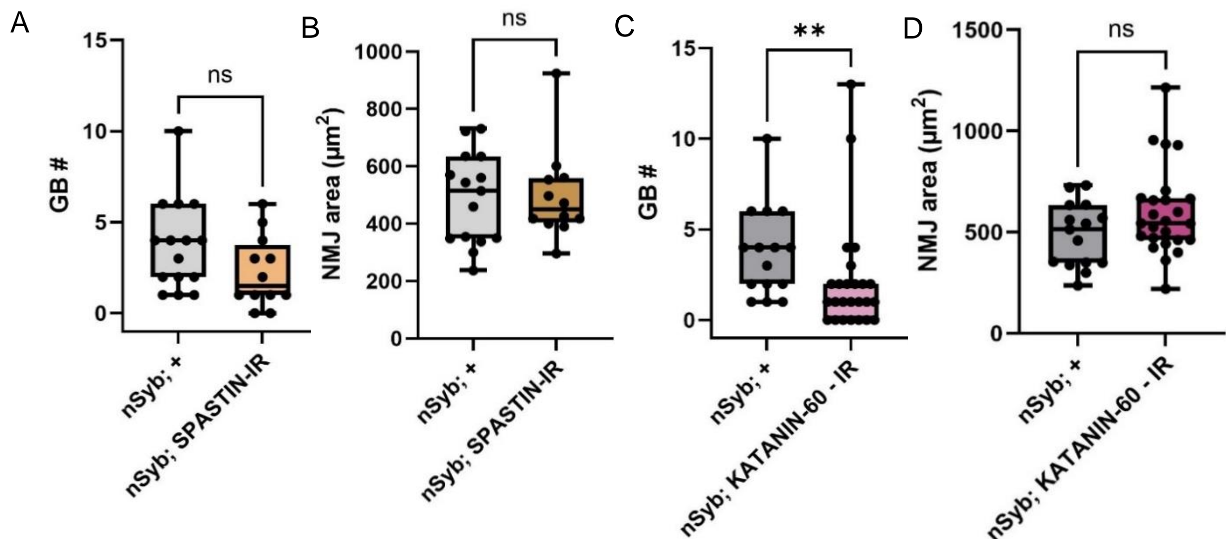

***Figure 3.11* – Quantification of GBs and NMJs area measurement after SS protocol.** (A) Number of GBs. n=15 NMJs for nSyb; + and n=12 NMJs for nSyb; Spastin-IR. Statistical significance was determined using the Unpaired t-test; not significant (ns). (B) NMJ area. n=15 NMJs for nSyb; + and n=12 NMJs for nSyb; Spastin-IR. Statistical significance was determined using the nonparametric Mann-Whitney test; not significant (ns). (C) Number of GBs. n=15 NMJs for nSyb; + and n=25 NMJs for nSyb; Katanin-60-IR. Statistical significance was determined using the nonparametric Mann-Whitney test; **p=0,0056. (D) NMJ area. n=15 NMJs for nSyb; + and n=25 NMJs for nSyb; Katanin-60-IR. Statistical significance was determined using the nonparametric Mann-Whitney test; not significant (ns).

Another interesting way to destabilize MTs is by manipulating their dynamics. To test how MT dynamics impact bouton growth we pharmacologically manipulated MTs during bouton formation using Vinblastine to induce MT depolymerization and Taxol as a MT

stabilizing drug and counted the number of GBs after stimulation in wild-type larvae. We observed that NMJs treated with Taxol exhibited a significant decrease in the number of GBs after stimulation, represented in Figure 3.12 and 3.13B. We also noticed that GBs with Taxol treatment, while fewer, they appear to be bigger and filled with Futsch, like represented in Figure 3.13C, although smaller GBs are also formed without any Futsch present inside. However, this distinctive phenotype needs to be quantified to assure that it is significantly different from controls. These findings may be attributed to the fact that for GBs to form, MTs need to be destabilized, and Taxol, as an MT stabilizer, likely inhibited the formation of GBs, leading to the observed decreased number.

Interestingly, the effects of vinblastine treatment did not lead to an increase in GB number, despite its known role in destabilizing MTs, suggesting a more complex relationship between MT dynamics and GB formation. NMJs treated with vinblastine did not show a statistically significant difference in GB number (see Figure 3.12). Based on this data, we propose that these results may be due to the necessity of MT stabilization in the long-term for GB maturation. Inadequate MT stabilization may preclude GBs from reaching full maturation, thereby increasing the likelihood of retraction or degradation of these immature boutons. Consequently, the lack of an increase in GB numbers following vinblastine treatment could reflect insufficient stability needed for these structures to mature. Additionally, the lack of significant changes in GB numbers could be attributed to the low concentration of vinblastine used in our experiments, which may not have been sufficient to induce a noticeable effect on GB dynamics. Thus, both insufficient MT stability and concentration of vinblastine may contribute to the observed results. In the future, we should test additional concentrations of vinblastine.

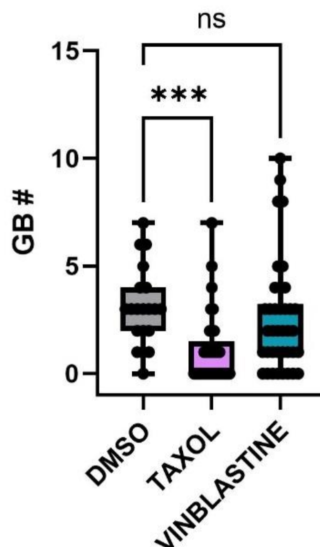

***Figure 3.12* – Quantification of GBs after SS protocol and drug administration.** Number of GBs. n=20 NMJs for DMSO, n=33 NMJs for Taxol, n=38 NMJs for Vinblastine; Statistical significance was determined using the Kruskal-Wallis; ***p=0,0001; not significant (ns)

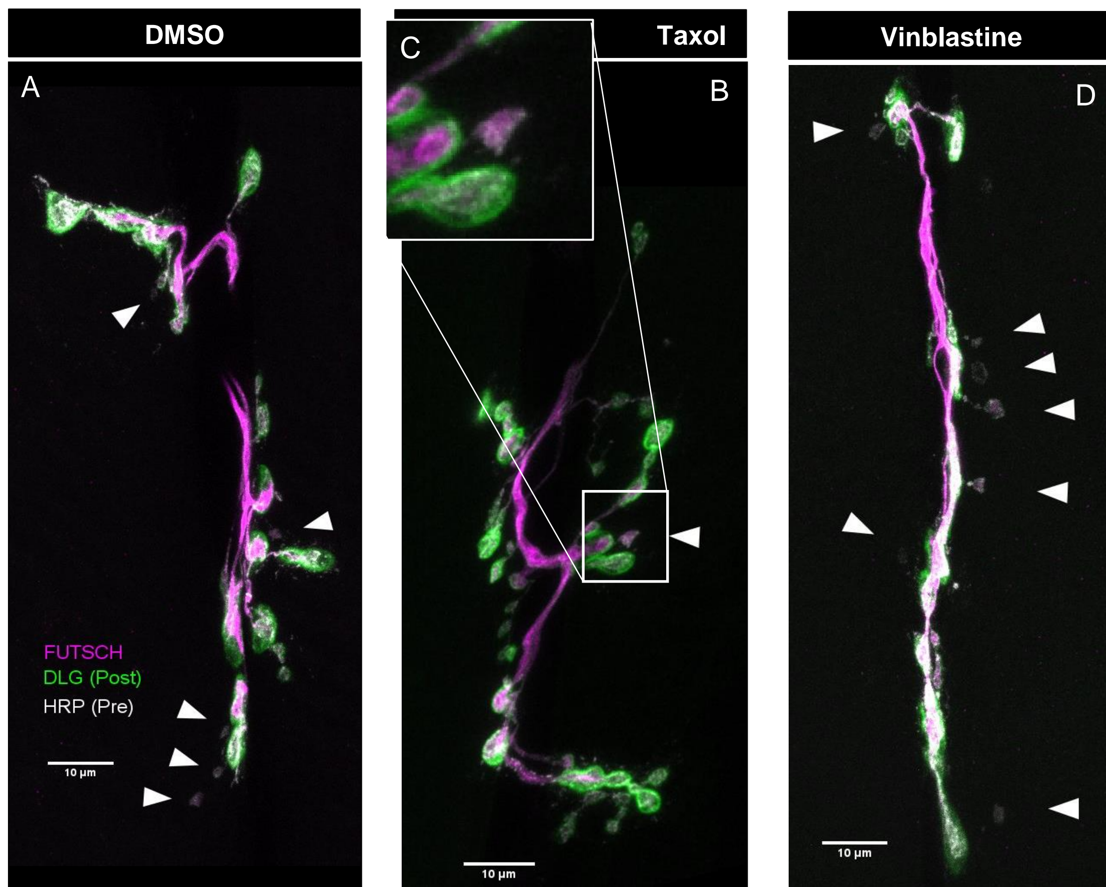

***Figure 3.13* – MT stability manipulation through drug administration at the *Drosophila* NMJs.** (A) NMJ with drug positive control (DMSO). n=20 NMJs (B) NMJ with Taxol administration. n=33 NMJs(C) Amplification of GB treated with Taxol filled with Futsch (D) NMJ with Vinblastine administration. n=38 NMJs. Futsch (magenta) in ghost boutons (arrowheads). Presynaptic and postsynaptic membranes labeled with antibodies for Horseradish peroxidase (HRP-white) and Discs-large (Dlg-green), respectively.

Lastly, we decided to try to better understand cytoskeleton interactions, motivated by the findings of Fernandes *et al.* (2023) which highlighted the role of actin in GB stabilization and maturation. To investigate this further, we conducted immunolocalization and fluorescence imaging of overexpressed Shot-GFP and knockdown of Shot. Shot is an actin-MT linker and it is interesting to analyze how the two types of cytoskeleton are coordinated. The immunolocalization assay indicated that this protein is predominantly localized within GBs (around 90%) as represented in Figure 3.15, exhibiting low intensity in most cases when we overexpressed Shot-GFP (see Figure 3.14). Notably, there were rare cases in which Shot was either absent or displayed in higher intensity (Figure 4 in Supplementary information III). These observations suggest that Shot may primarily interact with MTs and actin filaments to contribute to the structural integrity necessary for GB maturation. In light of our observations, we questioned what could happen if the expression of this protein, that makes this important

connection between two major cytoskeleton components, was knockdown. For this, we expressed Shot RNAi in neurons using nSyb-Gal4. Our results showed a significant reduction in GB number, and no alteration in NMJ area when compared with the control values (see Figure 3.16A and B), although we did identify a distinctive NMJ phenotype characterized by excessive branching and elongated branches, however this phenotype still needs to be quantified to assure that it is significantly different from the controls. Given our hypothesis that Shot functions as a crosslinker between MTs and actin, reduced levels of this protein may result in impaired recruitment or crosslinking with actin, ultimately compromising GB stability.

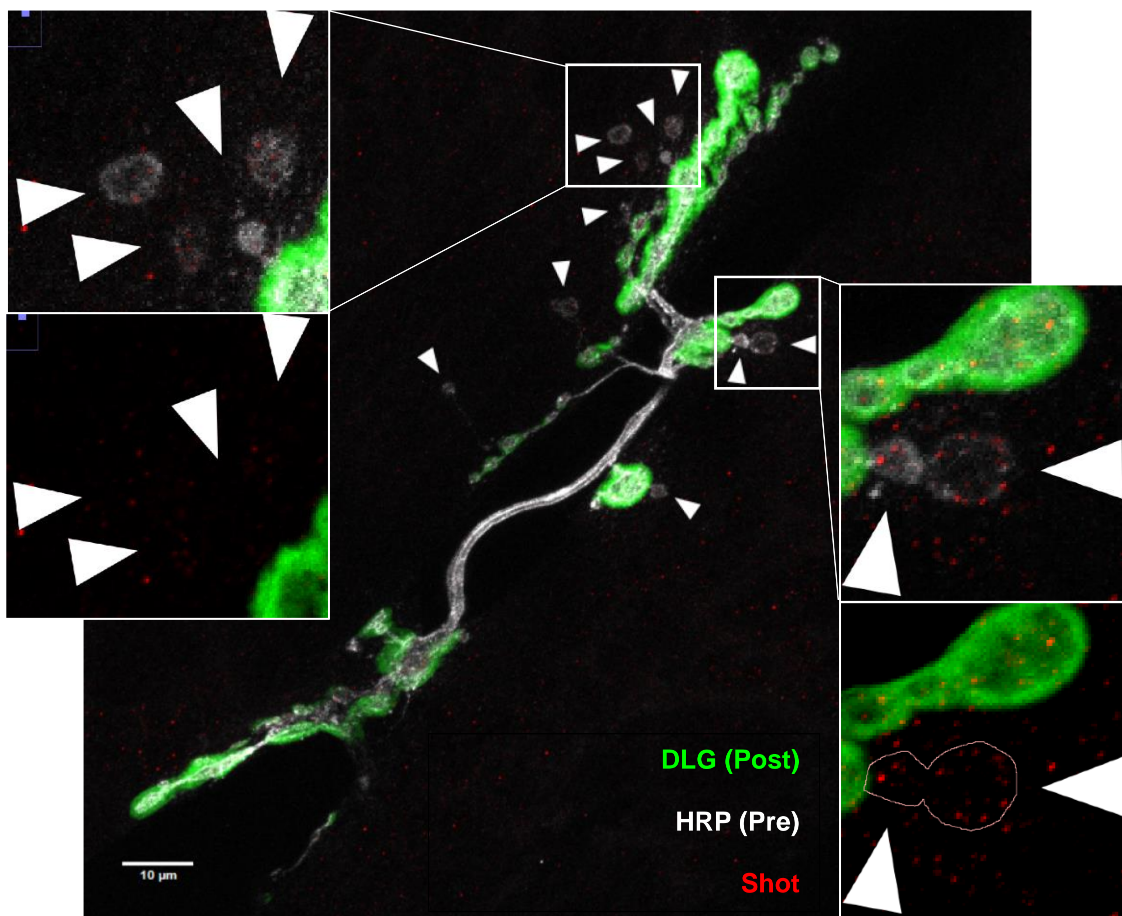

***Figure 3.14* - Localization of Shot in ghost boutons at the *Drosophila* NMJ after SS protocol.** Shot (red) in ghost boutons (arrowheads). Presynaptic and postsynaptic membranes labeled with antibodies for Horseradish peroxidase (HRP-white) and Discs-large (Dlg-green), respectively. n=13 NMJs. On the four smaller boxes we can see with greater amplification the GBs and the presence of Shot in a scattered pattern. n=13 NMJs, genotype nSyb-Gal4;UAS-Shot-GFP

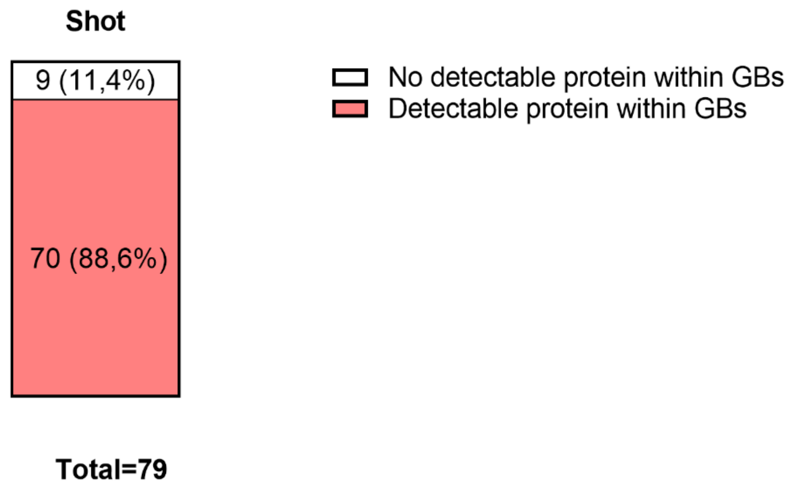

***Figure 3.15* - Shot detection in GBs (after neuronal overexpression).** Shot was detectable in 70 from the 79 GBs accounted (88,6%), in light red. 9 GBs had no detectable Shot within (11,4%), in white. n=13 NMJs, genotype nSyb-Gal4;UAS-Shot-GFP

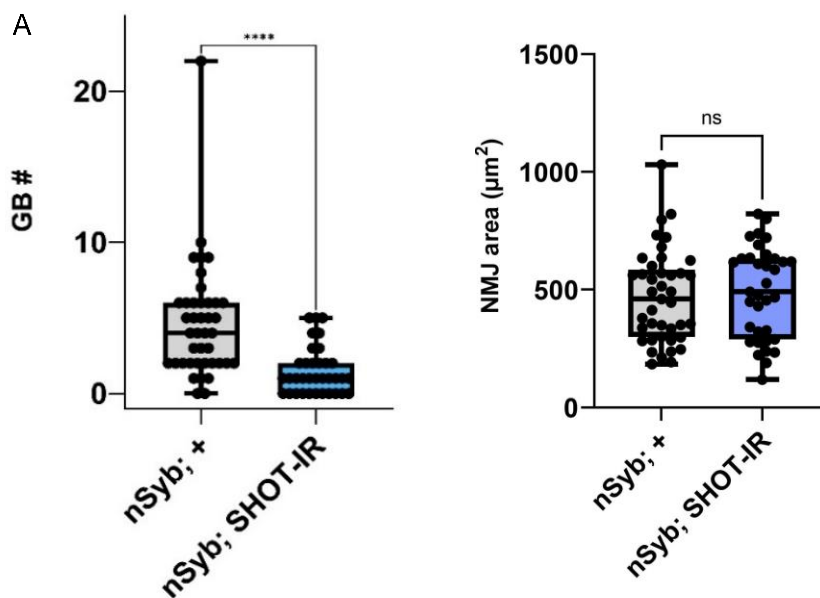

***Figure 3.16* - Quantification of GBs and NMJs area measurement after SS protocol.** (A) Number of GBs. n=41 NMJs for nSyb; + and n=35 NMJs for nSyb; Shot-IR. Statistical significance was determined using the nonparametric Mann-Whitney test; ****p<0,0001. (B) NMJ area. n=41 for nSyb;+ and n=35 NMJs for nSyb; Shot-IR. Statistical significance was determined using the Unpaired t-test; not significant (ns).

4. Final Considerations

4.1. Conclusions

Studying how the components of the cytoskeleton are regulated is essential to the understanding of the cellular and molecular mechanisms underlying synaptic development. The integrity and morphology of neurons depends on their cytoskeleton. Although there are already studies at the *D. melanogaster* NMJ on the role of actin in the formation and outgrowth of new GBs (considered precursors of mature synaptic boutons), little is known regarding the role of MTs in this process. Here, using overexpression and knockdown of MTBPs in *Drosophila* larval NMJ, visualization of the basic subunits of MTs – alpha and beta-tubulin –, and MT polymerization/depolymerization through administration of specific drugs we showed how MT dynamics and MTBPs contribute to synaptic plasticity and neuronal growth.

Regarding tubulin subunits, we discovered that alpha-tubulin was present in most GBs. Beta-tubulin however was only observed in bigger GBs. We hypothesize that this can be due to the inaccessibility of this subunit to be recognized with the Ab used, or due to some detection problem since alpha- and beta-tubulin are normally present in dimmers. In the future, this subunit must be screened with another Ab, *Drosophila* line or even overexpressed to better assess its presence.

Our analysis identified key MTBPs, notably EB1, Futsch, and Shot, as significant participants in the blebbing process. EB1 was the only MTBP screened that was present in all GBs, which reflects its role in driving the dynamic and exploratory MT behavior that is critical for synaptic outgrowth. As a +TIP protein, it facilitates MT polymerization, which we theorized may be happening during GB development. EB1's ability to link MT dynamics with synaptic signaling and its function as a scaffold for other regulatory proteins make it uniquely essential in the early, plastic stages of GB formation, explaining why it is found in every GB. Knockdown of EB1 should be performed to confirm this hypothesis. Futsch was necessary for bouton stabilization and growth, indicating that they regulate the behavior of MTs during synaptic remodeling. Both Futsch and Shot were found in the majority of GBs and knockdowns of these proteins resulted in a decrease in GB formation. Given that both proteins are considered MT stabilizing proteins, it suggests that MT stability plays a crucial role in the initiation and/or maintenance of GB formation, although for different reasons. Futsch is primarily involved in providing direct MT stability, and when expressed at low levels, GBs are more prone

to failure in stabilizing and maturing. The inability to maintain stable MTs could disrupt the structural integrity required for GB maturation. From a GB initiation perspective, reduced levels of Futsch may act as a molecular signal indicating that neuronal development and synaptic expansion are not a signaled, potentially halting the formation of new synaptic sites. Shot's role as a linker between MTs and actin filaments is crucial, particularly during the stabilization and maturation phases of GBs, when actin becomes essential. A reduction in Shot expression likely destabilizes MTs, preventing them from anchoring properly to the actin cytoskeleton. Without this connection, GBs are unable to stabilize, failing to mature into fully developed synapses. Colocalization between actin filaments and Shot should be performed to test this idea.

The results presented in this dissertation show that MT stability is essential for the formation and stabilization of new GBs. Taxol-induced stabilization of MTs prevented the formation of GBs, confirming that MT destabilization is a necessary step for bouton initiation. Conversely, destabilization with Vinblastine did not result in the possibly expected increase in GB numbers, suggesting that MT stability, while not required for GB initiation, may be crucial for their maturation. This highlights the fine balance between polymerization and depolymerization in GB formation.

In addition, we identified a differential role of MT-severing proteins. Katanin knockdown reduced GB numbers, possibly by reducing MT polymerization frequency, suggesting that MT severing is important for the blebbing process at the NMJ. Conversely, the lack of significant impact from Spastin knockdown points to the existence of compensatory mechanisms possibly Tau-associated, or to a reduced efficiency of KD with the RNAi line used.

In addition to the roles of MT-severing proteins in NMJ dynamics, the potential involvement of MT nucleation - particularly through kinetochores and centrosomes - warrants consideration due to its possible impact on GB formation. Both kinetochores and centrosomes are essential for organizing MTs during cell division, but components of these structures also influence MT dynamics in post-mitotic neurons.[92,93] Investigating these dynamics could offer valuable insights into their potential role in processes such as GB formation and synaptic function.

Preliminary results from the lab showed that MT nucleation might have a role in the blebbing process. MIS-12 protein – a member of the kinetochore complex – may play a role in the GB formation process, as its knockdown results in a decrease in GB numbers (Figure 5 in Supplementary information III). Furthermore, centrosomin (CNN), a crucial

centrosomal protein responsible for recruiting γ-Tubulin during MT nucleation, emerges as a significant player in MT dynamics within post-mitotic neurons. CNN has been found to contribute to dendritic arborization and is involved in localizing MT nucleation through an anchoring mechanism similar to that observed in dividing cells.[94] Preliminary findings from the lab suggest that CNN knockdown results in a reduction of GB numbers as well (Figure 6 in Supplementary information III). So, in the future it might be interesting to understand if MT nucleation is a crucial factor for GB formation, by conducting experiments directed to γ-tubulin.

Based on our findings, we propose the following model for MT dynamics in GB formation at the NMJ:

Initially, EB1 receives a molecular signal to localize at the site where a GB is about to form. Katanin then severs MTs at the NMJ, breaking them into smaller fragments and depolymerizing these severed MT ends. Succeeding, EB1 invades this blebbing site, promoting MT polymerization which might start in the *de novo* MT nucleation site. This polymerization activity likely occurs near the GB, starting at its base. During this polymerization process, alpha- and beta-tubulin subunits are available and scattered within the GB, due to Katanin depolymerization action, to incorporate into the polymerizing nucleated MTs. As the MTs extend, other MTBPs, such as Shot and Futsch, are recruited to provide additional stability. In the stabilization phase, actin filaments are also present and likely play a role in maintaining the structure through interactions between MTs – Shot – actin filaments. At this stage, Futsch helps to stabilize the polymerized MTs. Once stabilization is achieved, the maturation phase begins. Here, MTs are fully stabilized, with Shot mediating the connection between actin filaments and the stable MTs within the GB and Futsch contributing further to this stabilization by forming loops, completing the process of GB maturation. To complement this model, we have created a visual scheme that illustrates these dynamic processes, providing a clear and comprehensive representation of the stages described (Figure 4.1).

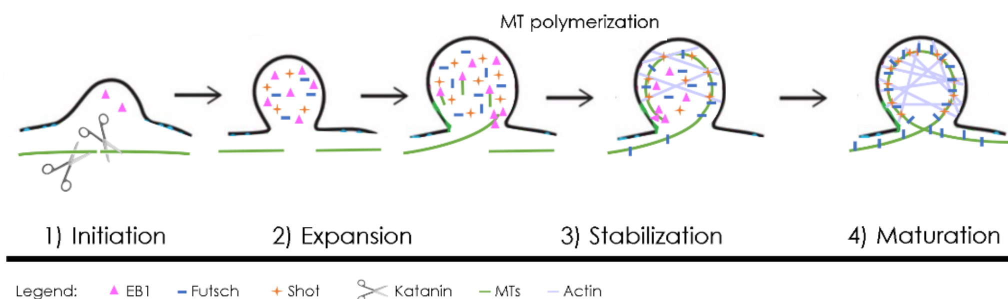

***Figure 4.1* - Schematic representation of the proposed hypothesis for GB formation and maturation.** The process involves four stages: (1) Initiation, where Katanin severs MTs, promoting local MT dynamics along with EB1; (2) Expansion, characterized by polymerization and recruitment of proteins such as Futsch and Shot; (3) Stabilization, involving actin remodeling and MT stabilization; and (4) Maturation, marked by the crosslinking between MTs and actin filaments to form a fully developed GB.

Overall, this research contributes to the broader understanding of how MT dynamics orchestrate synaptic plasticity, with both MT stability and severing playing key roles in synaptic bouton formation and stabilization. These findings provide a foundation for further exploration of MT-related mechanisms in synaptic function and dysfunction, potentially contributing to the development of therapeutic strategies for neurodegenerative diseases characterized by synaptic plasticity deficits, such as AD.

4.2. Additional research suggestions

To comprehensively characterize the role of MTs in GB formation and outgrowth, several avenues of research can be pursued. First, live imaging is a powerful tool that would enable us to analyze in real-time how MTs contribute to both the structural integrity and functional development of GBs. By labelling different MAPs, this approach would also allow us to assess the involvement of EB1 in the initiation of GB formation and to determine whether severing proteins actively contribute to this process. To further confirm the specific role of EB1 in GB formation, it would be valuable to reduce its expression levels and observe the resultant effects on MT dynamics and bouton formation, and because this +TIP can recruit other MTBPs, it will be interesting to see if lower levels of this protein have any influence on other MTBPs, like Futsch. This experiment could provide direct evidence of EB1's contribution to the processes associated with GB development.

To explore our hypothesis that compensatory mechanisms may occur, a simultaneous knockdown of Spastin and Tau should be conducted. Since both proteins have distinct but potentially complementary roles in regulating MT dynamics this approach will help determine whether a compensatory relationship exists between them. Observing the consequences of dual knockdown on GB formation and stability will offer insights into their functional interdependence.

Additionally, a straightforward hypothesis to investigate is the correlation between Shot as a linker to actin. Understanding how Shot facilitates the stabilization and maturation of GBs through its interactions with the actin cytoskeleton could provide valuable information about the interplay between MTs and actin in synaptic development. As mentioned before, a recent paper revealed that Shot and Patronin work together to regulate NMJ development, suggesting that both proteins collaborate to stabilize MTs at the synapse. Patronin knockdown in *Drosophila* MNs led to significant synaptic growth defects, notably reducing bouton size at the NMJ across different larval stages,

underscoring its key role in NMJ maintenance.[83] Thus, Patronin might also be involved in the blebbing process, even if indirectly, by manipulating MT polarity and stability.

By pursuing these research objectives, we can gain a deeper understanding of the complex mechanisms that govern GB formation and the critical roles of MTBPs in this process, along with MT severing and nucleation processes in plasticity mechanisms.

In conclusion, although many questions remain to be answered, this study highlights how the precise orchestration of MT dynamics and MTBPs are crucial to the blebbing process, marking a key step forward in understanding the molecular basis of neural plasticity.

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Supplementary information

Supplementary information I

Antibodies and *D. melanogaster* stocks

Table 1 - List of primary antibodies used in this study.

Primary Ab	Concentration used	Source
Mouse anti-Discs large (4F3)	1:500	Developmental Studies Hybridoma Bank
Rabbit anti-GFP	1:3000	
Mouse anti-alpha-tubulin (12G10)	1:100	Developmental Studies Hybridoma Bank
Mouse anti-Futsch (22C10)	1:50	Developmental Studies Hybridoma Bank
Mouse anti-beta-tubulin (E7)	1:100	Developmental Studies Hybridoma Bank
Rabbit anti-DLG	1:100	

Table 2 - List of secondary antibodies used in this study.

Secondary Ab	Concentration used	Source
Goat anti-HRP Cy3 (conjugated)	1:500	Jackson Immunoresearch Laboratories
Donkey anti-Mouse Alexa Fluor 647	1:500	Jackson Immunoresearch Laboratories
Goat anti-Rabbit Alexa Fluor 488	1:500	Jackson Immunoresearch Laboratories

Table 3 - List of *Drosophila melanogaster* stocks used in this study.

Fly name	Genotype	Source / Stock number
UAS - EB1- GFP	w[1118]; P{w[+mC]=UAS-EB1.EGFP.H}G	BL 36861
UAS - Shot - GFP	w[*]; P{w[+mC]=UAS-Shot.L(C)-GFP}3	BL 29043
UAS - Futsch - GFP	w[*]; P{w[+mC]=UAS-EGFP.futsch.LC}42A	BL 93063
W^{1118}	w[1118]	BL 3605
MHC - CD8 – GFP-sh	-	Gift from Dr. Brian McCabe
Shot - RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ23381}attP40	BL 64041 (Ana Pimenta Marques lab gift)
Tau - RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM05101}attP2	BL 28891
Futsch - RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02000}attP40	BL 40834
Katanin-60 - RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03012}attP2	BL 28375
Spastin - RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02724}attP2	BL 27570

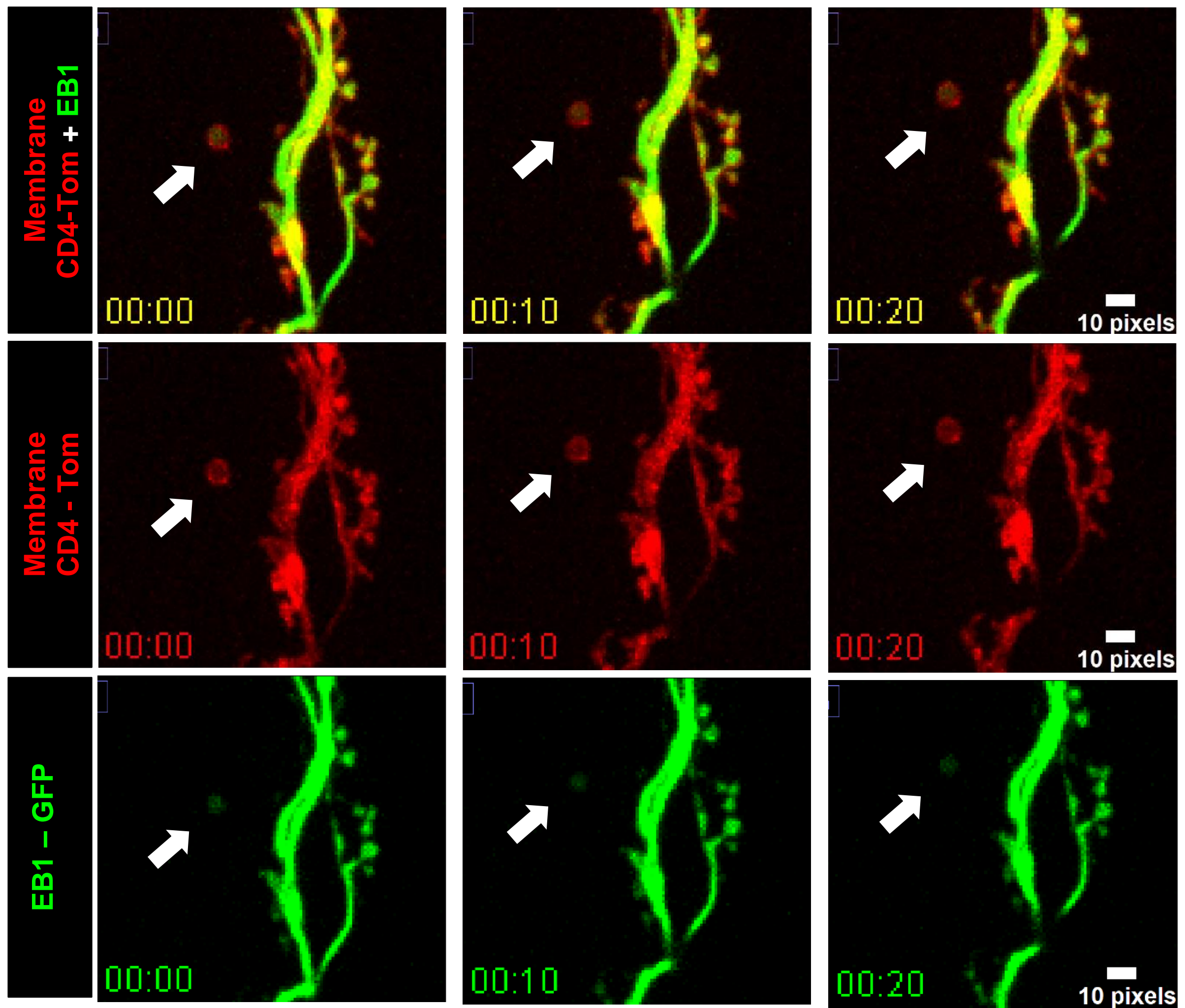
nSyb - GAL4	w[1118]; P{w[+mC]=XP}nSyb[d02894]/TM6B, Tb[1]	BL 19183
UAS - CD4 -Tom	w[1118]; PBac{y[+mDint2] w[+mC]=UAS-CD4-tdTom}VK00033	BL 35837

Table 4 – List of drugs used in this study.

Drug	Source
Paclitaxel (Taxol)	Abcam
Vinblastine	Sigma

Supplementary information II

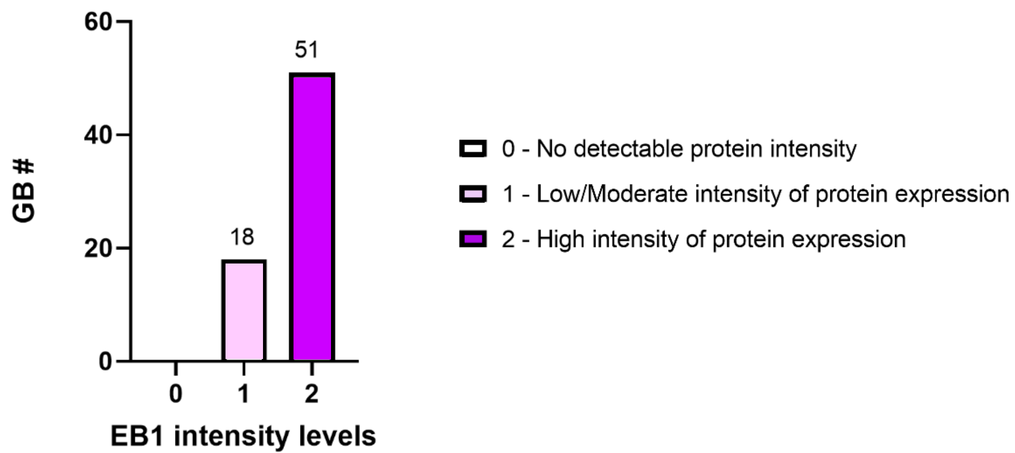
EB1 Time-lapse



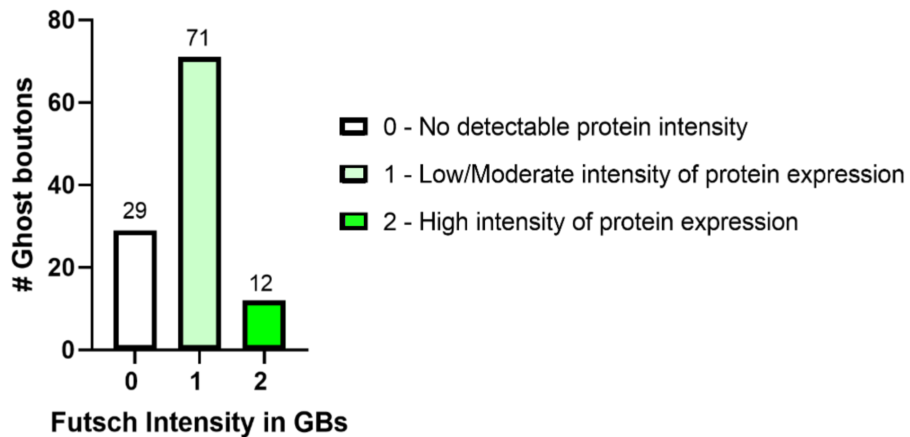
***Supplementary Data Figure 1* - Temporal time-lapse images of a potential ghost bouton (after high-K+ stimulation)**. Neuronal membrane and EB1 were labeled with UAS-CD4-GFP and UAS-EB1-Ruby, both under the control of NSyb-Gal4 (pan-neuronal driver). Arrow indicates where the potential ghost bouton with EB1.

Supplementary information III

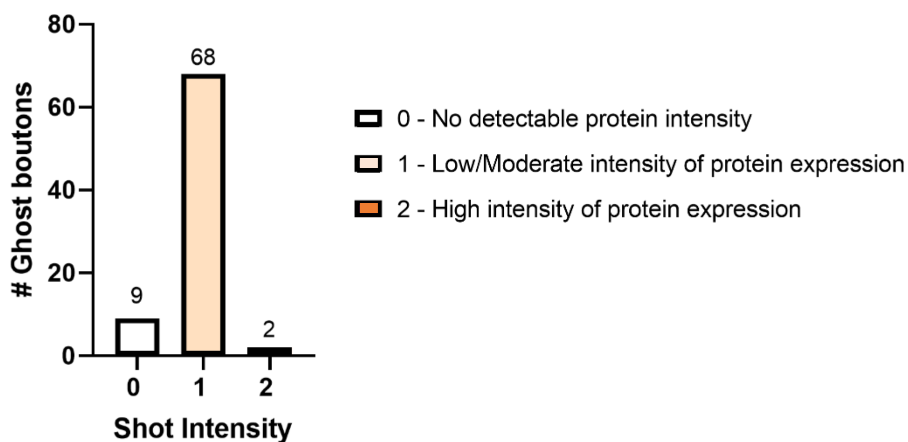
Additional Data



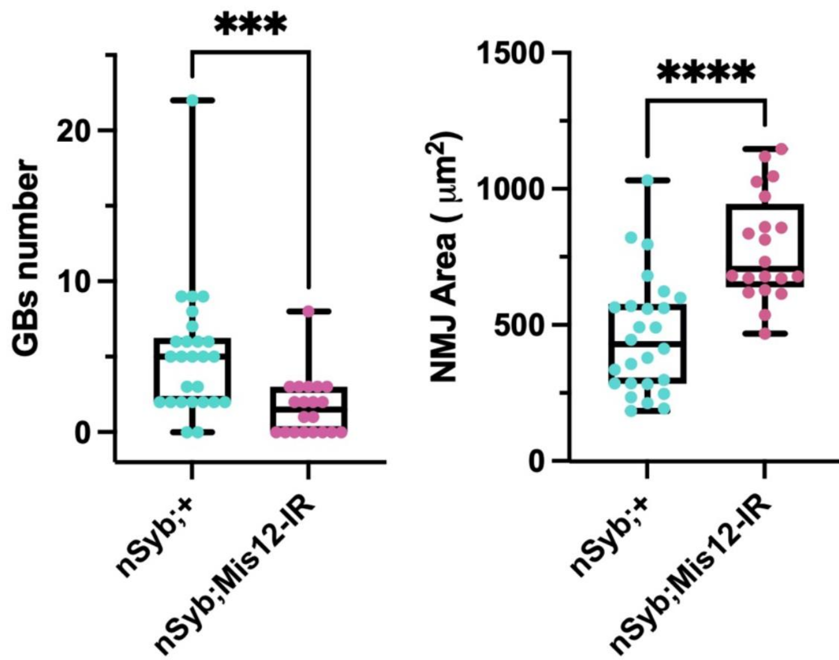
***Supplementary Data Figure 2* - Quantification of EB1 overexpression intensity in GBs after SS protocol. 18 GBs** had low or moderate intensity of EB1 within; 51 GBs had high intensity of EB1 within. n= 22 NMJs



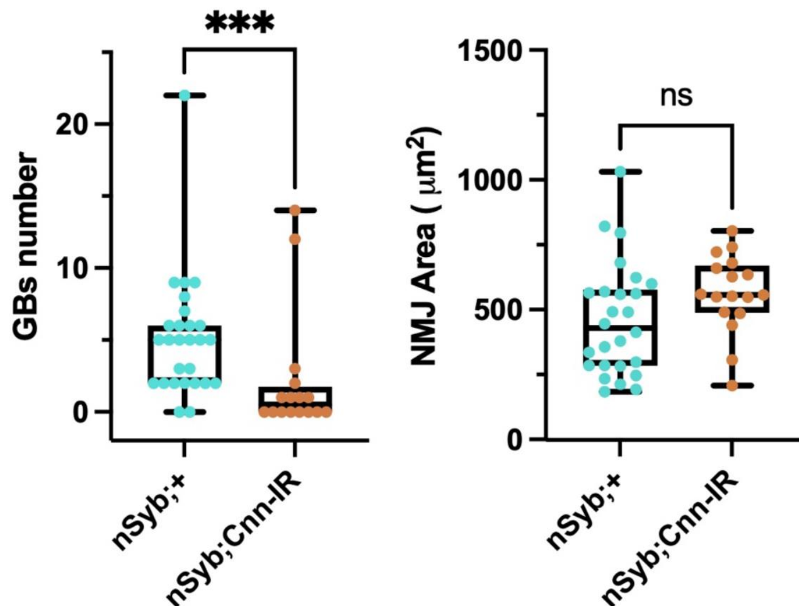
***Supplementary Data Figure 3* - Quantification of Futsch overexpression intensity in GBs after SS protocol**. *2*9 GBs had no detectable Futsch within. 71 GBs had low or moderate intensity of Futsch within. 12 GBs where found with high intensity of Futsch. n= 13 NMJs



***Supplementary Data Figure 4* - Quantification of Shot overexpression intensity in GBs after SS protocol.** 9 GBs had no detectable Shot within. 68 GBs had low or moderate intensity of Shot within. 2 GBs where found with high intensity of Shot. n= 13 NMJs



***Supplementary Data Figure 5* - Quantification of GBs and NMJs area measurement after short stimulation protocol.** On the left panel, n=27 NMJs for nSyb;+ and n=20 NMJs for nSyb;Mis12-IR. Statistical significance was determined using the nonparametric Mann-Whitney test; ***p=0.0003. On the right panel, n=27 NMJs for nSyb;+ and n=20 NMJs for nSyb;Mis12-IR. Statistical significance was determined using the Unpaired t test; ****p<0,0001.



***Supplementary Data Figure 6* - Quantification of GBs and NMJs area measurement after short stimulation protocol**. On the left panel, n=27 NMJs for nSyb;+ and n=17 NMJs for nSyb;Cnn-IR. Statistical significance was determined using the nonparametric Mann-Whitney test; ***p=0.0005. On the right panel, n=27 NMJs for nSyb;+ and n=17NMJS for nSyb;Mis12-IR. Statistical significance was determined using the Unpaired t test; not significant (ns).