Marta Contreiras da Silva
Licenciada em Química Aplicada

In vivo mechanisms of synaptic bouton formation: Dissecting the role of the exocyst

Dissertação para obtenção do Grau de Mestre em
Bioquímica para a Saúde

Orientador: Rita Teodoro, PhD, CEDOC – FCM/UNL

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H2020-GA661543-Neuronal Trafficking (ROT)
IF/00392/2013/CP1192/CT0002, FCT – Portugal (ROT)
UID/Multi/04462/2013 from iNOVA4Health (co-funded by FCT-FEDER-PT2020

Júri:

Presidente: Prof. Doutor António Sebastião Rodrigues
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Bibliographic Elements

Poster presentation at the 2018 Annual Portuguese *Drosophila* Meeting entitled "*In vivo* mechanisms of synaptic bouton formation: Dissecting the role of the exocyst" – Marta C. Silva and Rita O. Teodoro – Tomar, Portugal

Acknowledgements

First and foremost, I would like to express my gratitude to my supervisor Dr. Rita Teodoro, for the incredible opportunity to work in her lab and for all the help given throughout this process. I truly feel like I evolved as a scientist throughout this past year. To my lab colleagues, Ana Fernandes, Andreia Fernandes, Cátia Rodrigues and Joana Rodrigues, a very big thank you for everything, both scientific and personal. You turned this experience into something immensely more pleasant, by always being helpful and fun. I would like to single out my colleague, Andreia Fernandes, for an extra thank you. You listened (and answered!) all my dumb (and every once in a while, smart) questions, with insane amounts of patience. This wouldn't have been the same without your friendship.

Eunice, Marta and Patrícia, thank you for accompanying me in this voyage and by making it extra hard to explain to people who you were ("they're my colleagues, but not from the same lab. Well, we work in the same physical lab, but we have different bosses, so it's the same lab but not the same lab, get it?). Then we left and moved to another building and now it's easier: I just introduce you as my friends.

To the girls that have been by my side since we were wee lasses fresh out of high school: Daniela, Inês, Marina, Vanessa and Zara, a huge thank you because you have always been there for me and never refused to make plans with me even when all I did was complain about how my experiments weren't going perfect. I'm convinced that I am the person and scientist that I am today, because of your unbelievably positive influences. And to Bárbara who, in just two years, managed to become indispensable in my life with her positive outlook on everything. I'd be much grumpier without your motivation and support.

Thank you to my grandparents for supporting me and asking about my work, always wanting to know if everything was going well. Special thanks to by grandmother for telling everyone that asked about my endeavours: "my granddaughter kills flies".

And last, but not least, thank you to my parents and my sister. I wouldn't be here if it weren't for you. I know I can always count on you and on your unconditional support, especially in telling me the tough things that I don't want to hear. I hope I make you proud.

Abstract

Regulation of synaptic structure is critical for proper neuronal function. Neuronal morphology is genetically determined but can be modified by changes in synaptic activity, a process named neuronal structural plasticity. Synaptic boutons are conserved presynaptic specializations where synapses are located. Despite its importance, the mechanisms behind their formation are not fully understood. The neuromuscular junction of the *Drosophila melanogaster* is a well-characterized stereotyped synapse, where acute structural plasticity can be induced. Upon induction, new boutons emerge quickly as rounded structures, leading to an increase in neuronal surface, but whether this process requires membrane addition or simply a rearrangement remains elusive.

The exocyst is a multi-subunit complex that is involved in a variety of mechanisms. It targets secretory vesicles to a specific place at the membrane and interacts with cytoskeletal components. Given this, we asked whether this complex plays a role in activity-dependent bouton formation. By using a protocol that induces new bouton formation in response to activity, with RNAi against each of the subunits or mutations, we assess whether each of the subunits contributes to this process and whether knock down of each of the subunits has different effects. We found that exocyst subunits are present in new synaptic boutons, and that Sec3 appears to be important for this process, while Sec6-IR larvae show a strong locomotor phenotype. Two other subunits, Sec8 and Exo70 may also be involved in the formation of new synaptic boutons, but further analysis will be required.

Understanding the mechanism by which activity-dependent bouton formation occurs can provide insights onto how to manipulate the number of synaptic structures. Considering that neurodegenerative diseases are usually characterized by neuronal simplification and loss, an alternative to increasing the number of neurons would be to stimulate pathways that result in increased number of synaptic structures, perhaps ameliorating the function.

Resumo

A regulação de estruturas sinápticas é crítica para o normal funcionamento neuronal. A morfologia neuronal é geneticamente determinada, mas pode ser modificada por alterações de actividade sináptica, um processo chamado plasticidade estrutural. Botões sinápticos são especializações presinápticas conservadas onde se localizam as sinapses. Apesar da sua importância, os mecanismos que regulam a sua formação são desconhecidos. A junção neuromuscular de *Drosophila melanogaster* é uma sinapse estereotipada e bem caracterizada, onde plasticidade estrutural pode ser induzida. Após indução, novos botões emergem rapidamente, levando a um aumento da superfície neuronal, mas se este processo requer adição de membrana ou simplesmente rearranjo ainda não é conhecido.

O exocisto é um complexo de várias subunidades que se liga a vesículas secretórias e transporta-as para um local específico da membrana plasmar, interagindo com diversos efectores do citoesqueleto. Tendo isto em conta, questionámo-nos se este complexo teria um papel na formação de botões. Usando um protocolo que induz a formação de novos botões em resposta a actividade, com RNAi contra cada uma das subunidades ou mutações, tentámos saber se alguma das subunidades contribuía para a formação de botões e se a eliminação de cada uma das subunidades teria efeitos diferentes no processo. Este trabalho permitiu concluir que as subunidades estão presentes nos novos botões, e que Sec3 parece ser importante para o processo, enquanto que as larvas de Sec6-IR demonstra um fenótipo locomotor observável. Duas outras subunidades, Sec8 e Exo70, são promissores para estarem envolvidos no processo de formação de novos botões.

Compreender este mecanismo através da formação de botões pode informar-nos acerca da manipulação do número de estruturas sinápticas. Considerando que as doenças neurodegenerativas são usualmente caracterizadas por perda ou simplificação de neurónios, uma alternativa ao aumento do número de neurónios seria estimular vias que resultassem num aumento de estruturas sinápticas.

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List of Abbreviations

ADP – Adenosine diphosphate
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARF – ADP-ribosylation factor
Arp – Actin related protein
AZ – Active zone
BDSC – Bloomington *Drosophila* Stock Centre
Cdc42 – Cell Division Control protein 42
Cdk1 – Cyclin-dependent Kinase
CNS – Central Nervous System
Cryo-EM – Cryo-Electron Microscopy
D2 – Dicer2
DABCO – 1,4-Diazabicyclo[2.2.2]octane
Dlg – Discs-Large
EGF – Epidermal growth Factor
ERK1/2 – Extracellular Signal-regulated Kinases
G4 – Gal4
GFP – Green Fluorescent Protein
GluR – Glutamate Receptor
GTP – Guanosine-5'-triphosphate
HA – Hemagglutinin
Hrp – Horseradish Peroxidase
ITPR3 – Type 3 inositol 1,4,5-triphosphate Receptors
Lgl – Lethal Giant Larvae
Longstim – Protocol described by Ataman *et al.* in 2008
Munc18 -- Mammalian uncoordinated-18
NGS – Normal Goat Serum
NMDAR – N-methyl-D-aspartate Receptor
NMJ – Neuromuscular Junction
nSyb – Neuronal Synaptobrevin
Par6 – Partitioning-defective protein 6
PBS – Phosphate Buffer Saline
PBT – Phosphate Buffer Saline with Triton
PDZ – Initialism of PSD-95, Dlg, and zonula occludens-1 protein (zo-1)
PFA – Paraformaldehyde
$PI(4,5)P_2$ – phosphatidylinositol 4,5-bisphosphate
PICT – Protein interactions from Imaging Complexes after Translocation
PSD – Post Synaptic Density
PSD-95 – Post Synaptic Density protein-95
RFP – Red Fluorescent Protein
RhRx – Rhodamine RedX
RNAi or IR – Ribonucleic Acid Interference
SEM – Standard Error of Mean
Shortstim – Protocol described by Vasin *et al.* in 2014
siRNA – Small interfering RNA
SNAP-25 – Synaptosomal-associated protein 25
SNARE – Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor
SSR – Subsynaptic Reticulum
UAS – Upstream Activating Sequence
UDP-glucosyltransferase – Uridine 5'-diphospho-glucuronosyltransferase
VAMP – Vesicle-Associated Membrane Protein
VDRC – Vienna *Drosophila* Resource Center

1. Introduction

1.1. The neuron and synaptic transmission

Living organisms have neuronal circuits that allow them to interact with the world and react to stimuli. Neurotransmission across neurons, the fundamental unit of the nervous system (Figure 1 - A), and from neurons to other cells is essential for several processes, such as locomotion and learning. This communication between neurons is achieved mainly through a conserved structure called synapse. At the synapse (Figure 1 - B), neurotransmitter molecules are released, which then bind to neurotransmitter receptors in the postsynaptic cell, ensuring that neuronal transmission is propagated. And this is the basic event that allows us to move, talk, learn and solve mathematical problems[56].

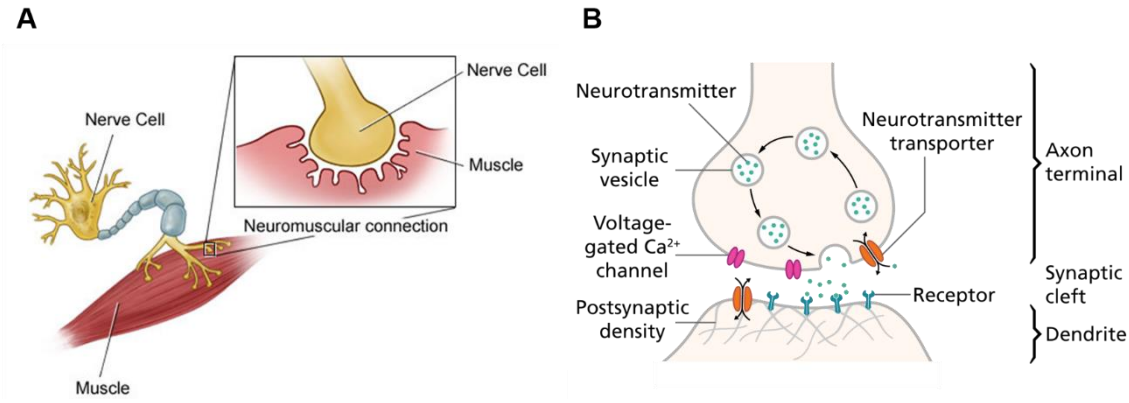

Figure 1 – Neuronal structures: the neuron and the synapse (**A**) Schematic representation of a neuron connected to a muscle fibre, and detail of the neuromuscular connection through a synapse. Adapted from Stanford Children's Health. (**B**) Schematic representation of a synapse labelled with diverse synaptic components. Image designed by Thomas Splettstoesser.

An important component of the nervous system is its ability to adapt to environmental cues, by altering its structure and functional properties to fit the needs of the organism and, because of this, the system remains plastic throughout life. This means that in addition to the changes brought on during early development, neuronal structures can still be altered in response to different factors throughout life. Diseases such as Alzheimer's or other neurodegenerative disorders can impact on this *status quo* by changing the capacity of neurons to be plastic leading to the simplification or even loss of neurons, which will then impair the proper function of the nervous system in these patients. In

addition to diagnostic tools and a cure for these disorders, another important goal is the management of the symptoms that result from the neurodegeneration. One idea towards this goal is if one could find a strategy to compensate for neuronal loss, perhaps by assuring that neurotransmission and plasticity still occurred as needed. This could be achieved by promoting neurogenesis and increasing the number of neurons or, another possible way, would be to promote the increase in the synaptic transmission ability of existing structures or by manipulating neuronal complexity.

Along or at the terminal end of axons, are synaptic boutons, where synapses assemble. These specialized and conserved round structures incorporate active zones, which will be the place where neurotransmitter release occurs.

Understanding how these structures work and how they are formed, may allow the manipulation of neuronal function. Despite conserved, there are many unanswered questions regarding their genesis, namely, the molecular cascade and cytoskeletal changes that occur during new bouton formation. In this work we will use *Drosophila melanogaster*, or the fruit-fly, as a model system to investigate the mechanism of synaptic bouton formation.

1.2. Drosophila melanogaster as a model

Akin to humans (Figure 2), *Drosophila* synaptic boutons are present clearly visible and contain active zones with clusters of synaptic vesicles. Juxtaposed to active zones are clusters of glutamate receptors (GluRs), homologous to AMPA-type vertebrate GluRs. The postsynaptic densities from mammalian synapses resemble those present in *Drosophila*, including the presence of one synaptic component called Discs Large (Dlg) that is ortholog to the mammalian Post Synaptic Density protein 95 (PSD-95)[60]. In addition to the molecular conservation, the fly model also offers other advantages such as the simplicity to handle in the laboratory, the fact that its nervous system is accessible for stimulation and imaging, and neuronal morphology is highly stereotyped. Furthermore, there is a wide array of genetic tools available for this model, allowing for the manipulation of the animal in very specific ways.

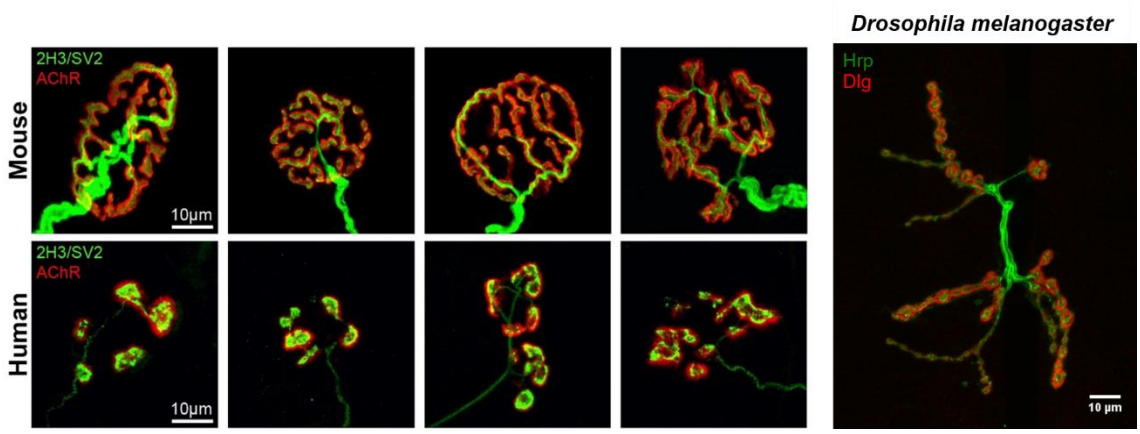

Figure 2 – Confocal images of the Neuromuscular Junction of mouse, human, and *Drosophila melanogaster*. Images for the mouse and human NMJ were obtained by Jones *et al.* in 2017 and are stained for acetylcholine receptors in red and for nervous tissue (2H3, neurofilament, and SV2, synaptic vesicle 2) in green. The *Drosophila* image was obtained during this work and is stained for horseradish peroxidase (Hrp) in red and Discs-Large (Dlg) in green. For all images presented, the green staining is representative of presynaptic membrane and the red for postsynaptic membrane. Scale bars are 10µm.

1.3. The neuromuscular junction and its synaptic boutons

The neuromuscular junction (NMJ) of the *Drosophila* is the synapse that connects motor neurons to their muscle target. In the embryo, the motor neuron axon starts to search for a specific and genetically determined muscle fibre, using a specific type of protrusion, called the growth cone[82]. At the end of the embryonic stages, the growth cone constricts and, by a still unclear maturation mechanism, differentiates into a functional neuromuscular junction with few boutons (about 20 at muscles 6/7's NMJ)[60]. As the larva grows and in parallel with the 100-fold increase in surface area of the muscle fibre, the NMJ increases about 10x, adding boutons and active zones to its structure to accompany this larval growth[2,88]. Besides adding new boutons, there is also elimination of these structures. This dynamism ensures that in the end there is a correct number of active zones and receptors, necessary for the normal function of the muscle.

Normal development of neuronal structures during growth is very important and the process of addition and elimination of synaptic boutons is essential to normal neuronal function. But this dynamic doesn't occur solely during development. The synapse can also add and remove boutons in response to acute synaptic activity, an adaptation process known as structural plasticity. This process allows the neuron to adapt to the environment, in response to a variety of stimuli. Despite its importance, the mechanism of formation of synaptic boutons is not yet fully understood. So how do synaptic boutons form?

To try to this question Zito *et al.* (1999)[108] performed time-lapse imaging of live larvae, which resulted in the genesis of the hypothesis described in Figure 3[95]. In this study, the authors suggested budding, division and *de novo* formation, as possible mechanisms for bouton addition to the neuronal arbour. Since then, not many studies have looked at real time bouton formation during development, but during activity-dependent bouton addition, the data seems to support the notion that the process is more similar to budding.

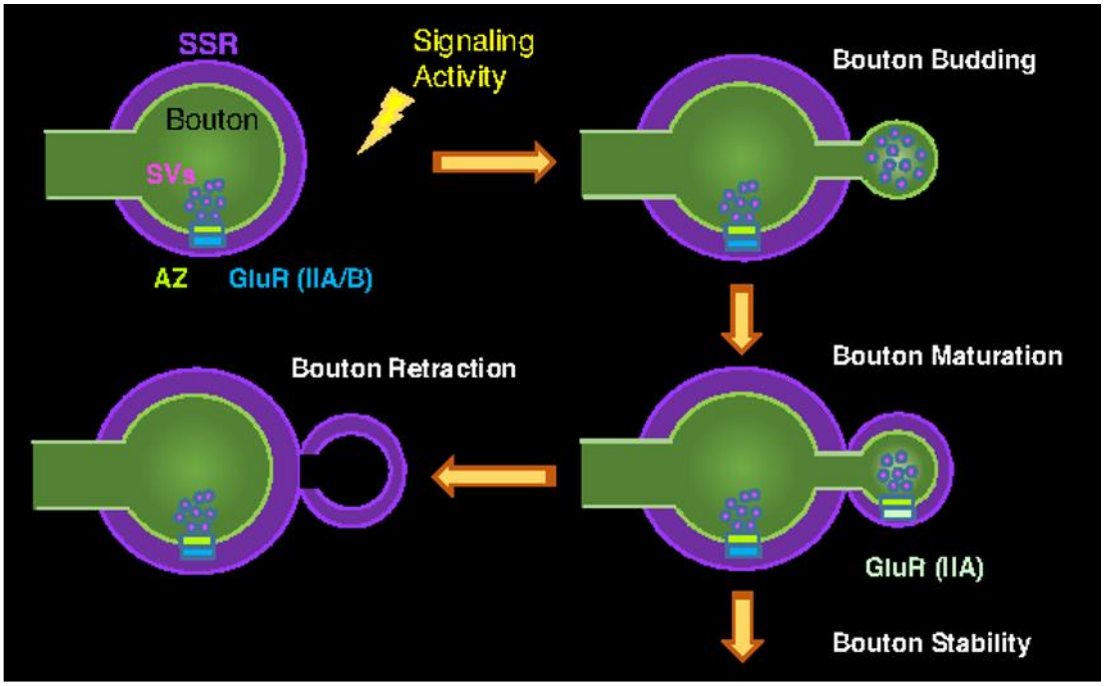

Figure 3 – Formation of a new synaptic bouton. Schematics designed by Vactor and Sigrist in 2017 depicting the formation of a new bouton in response to signalling activity and its putative retraction, if maturation fails. A mature bouton containing synaptic vesicles (SVs) and active zones (AZ) is surrounded by subsynaptic reticulum (SSR) containing glutamate receptors (GluR). After activity a new bouton is formed (labelled bouton budding) with just presynaptic membrane at first. Afterwards there is a process of bouton maturation, where the new structure develops an SSR complete with GluRs. After this there are two possibilities either the bouton stabilises, or it retracts.

In our lab, by doing *in vivo* and in real time imaging we were able to watch bouton formation occurring (Figure 4). The observation of these events in real time, allowed us to propose a new mechanism of bouton formation.

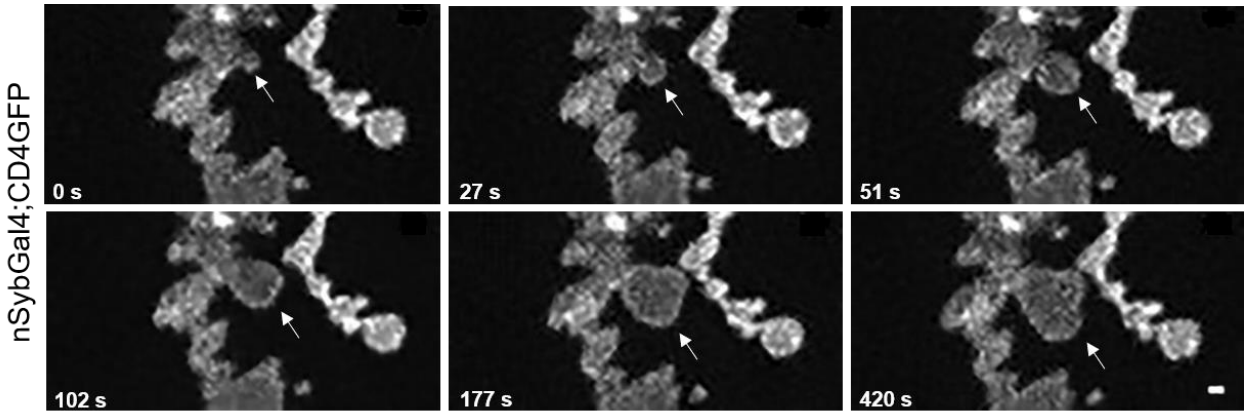

Figure 4 – Time lapse of a new synaptic bouton being formed in the NMJ of Drosophila. Sequence from a live imaging video depicting a new synaptic bouton being formed. Neuronal membrane is marked with GFP, using a neuronal driver (nSybGal4) with the UAS/Gal4 system and a CD4GFP, leading to the expression of this fluorescent protein in neuronal tissue. From a very small rounded structure, it quickly grows to a large bulky morphology that is still rounded. The morphology of the rest of the NMJ is not disrupted by this event. In the final image it appears that a second bouton is being formed sequentially from the first, due to the appearance of a slight constriction in the body of the bouton that looks like a second round structure. The new bouton is marked by an arrow and the images are in sequence from left to right and from top to bottom. Time is represented in seconds. (Andreia Fernandes, 2017, unpublished)

We observed that boutons form rapidly and often in a sequential manner, growing rapidly to a bouton size within the range of mature boutons. For this rapid increase in size, some membrane alteration is necessary, with three options being proposed: membrane rearrangement, membrane unfolding and membrane addition.

Membrane rearrangement implies that the existing membrane reshapes to form the secondary structure. This would happen by pushing and pulling the membrane to reposition it, creating the new bouton.

The unfolding, however, presupposes the presence of membrane ruffles inside the mature bouton. During the process of formation, these membrane invaginations would unfurl and stretch to create the new structure. This hypothesis was proposed by Goudarzi *et al.* in 2017 for the process of bleb expansion in migrating cells[26]. However, at synapses, this mechanism is less likely to occur because electron microscopy images of synaptic boutons do not show any ruffling of the plasma membrane – but it remains as a formal

possibility.

Membrane addition, at least for the example presented, appears to be essential. The new bouton grows quickly to a mature bouton size, and this increase appears to be dependent on the addition of new membrane.

Although these mechanisms of bouton growth appear to be very diverse, there is one molecular player that might be involved in all three possibilities of bouton growth: the exocyst complex. This conserved protein complex involved in polarized vesicle exocytosis, has been shown to be involved in mechanisms similar to the three proposed. It is involved in several processes involving the cell's cytoskeleton, such as creation of a membrane curvature[107], accumulation at the leading edge of migrating cells[79], and it has established interactions with membrane phospholipids[6,15,51], with microtubules[48,98], with actin binding GTPases[65,101] and with the Arp2/3 complex[54,109] (a complex that has been shown to be important for synaptic bouton formation[42]).

The exocyst complex has also been shown to localize at the plasma membrane for polarized membrane growth. In yeast budding, for example, it has been shown to be important for bud-site selection[35], and it localizes to the budding tip of the 'daughter' cell where active membrane growth is occurring[15,30,61,103]. In mammals, the exocyst localizes to places of neurite outgrowth[36,97] and branching[45].

Considering all the described interactions for the exocyst, we wondered if this complex might also be involved in synaptic bouton formation, a process that appears to be dependent on either alteration of membrane morphology, membrane growth or both.

1.4.The exocyst complex

The exocyst is a multi-subunit protein complex composed of eight subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (Figure 5).

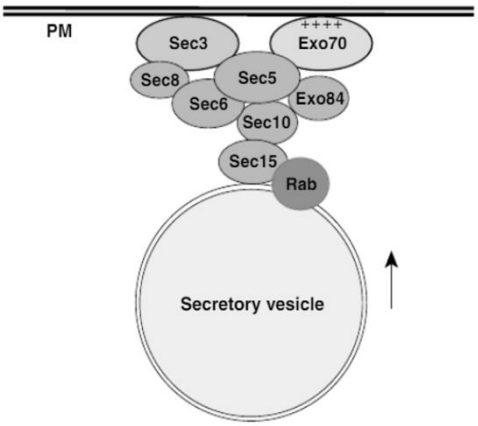

Figure 5 – The exocyst complex, with its subunits and interactions with a secretory vesicle and the plasma membrane. From He *et al.* 2007. Schematic representation of the exocyst complex with its eight subunits: Exo70, Exo84, Sec3, Sec5, Sec6, Sec8, Sec10 and Sec15. Demonstration of the interactions between Sec3 and the plasma membrane, Exo70 and the plasma membrane, Sec15 and Rab than then binds to the secretory vesicle.

Initially, it was thought that the whole complex was necessary for its specific functions. In more recent years, data has accumulated suggesting that, besides working as a whole complex, different sub-complexes can form and are responsible for an array of functions. Below, I will summarise the data for each of the subunits and will then discuss how this complex may participate in bouton formation.

1.4.1. Exo70

Exo70 has been shown to interact with phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), a phospholipid component of cell membranes, which indicates that this subunit is involved in the attachment of the exocyst to the plasma membrane[37,52]. This subunit has been shown to have membrane deformation properties[107], by being capable of inducing the formation of membrane protrusions, as well as inducing negative curvature in membranes. While there is a strong interdependence between all the subunits in yeast, Exo70 is one of the subunits that is resistant to the disruption of the actin cytoskeleton[6], indicating a degree of independence, in part confirmed by its ability to function independently in the plasma membrane[51]. Three proteins from the Rho family have been shown to interact with Exo70: Rho3, Cdc42 and TC10. Mutants of Rho3 and Cdc42 result in an accumulation of secretory vesicles in yeast[80,101], and TC10 has been shown to localize to the axonal growth cone with this subunit, an interaction necessary

for membrane addition[13]. Exo70 also interacts with the complex Arp2/3, involved in the branching of actin in migrating cells[72,76]. This interaction is necessary for the formation of lamellipodia and to maintain direction during migration by regulating actin[54], and abolishing it prevents the formation of actin-based membrane protrusions[53,109]. Most importantly the Arp2/3 complex is involved directly in the formation of new synaptic boutons at the *Drosophila* NMJ[42]. In the end, these interactions with four different actin-interacting molecular players, strongly suggests that Exo70 might be involved in changes in morphology of the cell's cytoskeleton, perhaps important for the initial deformation in the process of synaptic bouton formation.

Likewise, the overexpression of Exo70 in rat cells disrupted the microtubule network and formed protrusions in the membrane similar to filopodia[98]. Knockdown of Exo70 using RNAi does not significantly disrupt the traffic of vesicles[75], but the lack of integrity of the subunit blocks the fusion of secretory vesicles[52]. Dominant negative mutants of Exo70 also impede the fusion of AMPA receptors at the postsynaptic membrane[23]. Exo70 has been identified as a direct substrate of the extracellular signal-regulated kinases (ERK1/2), and phosphorylation by this kinase promotes the assembly of the complex in response to signalling by the epidermal growth factor (EGF)[75].

This subunit has a strong interaction with cytoskeleton and membrane components, indicating that it might be involved in the alteration of the morphology of cell's plasma membrane.

1.4.2. Exo84

Exo84 interacts with the GTPase RalA in its active form[63] and is necessary for neuronal polarization, which is agreement with the observation that depletion of Exo84 leads to unpolarized neurons[44]. This GTPase is also known to mediate the interaction between the Exo84 and the PDZ domain of Par6, a protein recruited by active Cdc42[40] and a potential connection to the subunit Exo70. Exo84 also has been shown to interact with Sro7/77, the yeast homologues for Lethal Giant Larvae (Lgl)[104]. Lgl associates with the t-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (t-SNARE) protein syntaxin 4, mediating vesicle fusion[70] and the yeast homologues interact directly with Sec9p, also a t-SNARE protein[46], establishing a connection between the exocyst and the SNARE machinery. Lehman *et al.* suggest, based on genetic analysis, that Sro7 and

Sec9 work in tandem downstream of Rho3, which would establish yet another added connection to Exo70. Exo84 can be phosphorylated in several places by cyclin-dependent kinase (Cdk1), leading to a disruption of the complex[29], which is consistent with the observation that inhibition of Exo84 reduces the formation of a complex between Sec6 and Sec10[64], and that Sec10p, Sec15p, and Exo70p depend on this subunit to polarize properly[105]. *Drosophila* mutants of Exo84 showed a reduced rate of surface area growth during anaphase cell elongation, indicating they might mediate some type of membrane addition required for cell elongation[25].

The interaction of this subunit with the Ral GTPases is a good indication that it might be involved in the process of synaptic bouton formation, in addition to its interactions with SNARE machinery components. The evidence that Exo84 might be needed for the assembly of the complex is also relevant.

1.4.3. Sec3

Similarly to Exo70, Sec3 binds to PI(4,5)P_2, indicating a direct interaction with the plasma membrane, and to Cdc42[106]. Disruption of either interaction will result in a defect in exocytosis. Sec3 interacts with another Rho GTPase, Rho1, which has been reported to regulate the localization of Sec3[31]. A yeast double mutant is lethal, and the genetic interaction suggests that the N-terminal of Sec3 becomes indispensable in a *exo70* mutant background[106]. Just like Exo70p, Sec3p is resistant to the disruption of actin[6]. In yeast, by expressing the different subunits on the surface of mitochondria, all of them were able to recruit other exocyst subunits but only Sec3 was able to recruit secretory vesicles[55]. There is also a connection between Sec3 and Sso2, a yeast t-SNARE protein, and addition of the subunit accelerates the interaction *in vitro* between Sso2 and Sec9, mentioned earlier in connection to Exo84[102]. In maize, a mutant of Sec3 exhibits a phenotype of impaired root hair elongation, a process that is dependent on polar exocytosis and tip growth[99].

Overall, this subunit shows interactions with both membrane and actin binding GTPases. The interactions with SNARE machinery components might also be relevant for the process of bouton formation.

1.4.4. Sec5

Sec5, akin to Exo84, binds to RalA[22] and has a region sufficient for interaction with Exo84, suggesting that they may associate independently with each other and with RalA[64]. RalA-Sec5 binding is required for cell migration, and loss of this interaction led to cells with no lamellipodia and extended spindle shape[34]. In *Drosophila*, neurite outgrowth fails once maternal contribution is depleted but synaptic vesicle fusion is independent of Sec5[66]. During larval growth, wild-type larvae show an increase in synaptic bouton number of 2.5 at the NMJ but *sec5* mutants show no increase[66], suggesting that this subunit is essential for the mechanism associated with bouton number formation during larval development. Additionally, postsynaptic membrane growth is impaired in *sec5* mutants or upon postsynaptic knock down[94]. *Drosophila* embryos with a temperature sensitive mutation in *sec5* have been shown to be unable to complete cellularization – a specialized form of cytokinesis, likely because of defects in membrane addition[68], and, in the ovary, this subunit has been shown to be essential for directed membrane traffic and the establishment of polarity[69]. *Drosophila* null mutants are lethal[66].

Defects in Sec5 have been described to impair growth and traffic, with strong neuronal phenotypes. This appears to indicate that Sec5 is an important subunit for the proper function of neuronal processes.

1.4.5. Sec6

Sec6 has been reported to anchor the exocyst complex at sites of secretion[92]. Additionally, this subunit has been shown to interact with several SNARE proteins:Snc2, Sec9, and Sec1, which are a yeast v-SNARE[89] required for fusion of secretory vesicles in the plasma membrane[74], a plasma membrane t-SNARE[62,91] that is a homologue of SNAP-25[32], and a SNARE regulator of the Sec1/Munc18 family[62] involved in synaptic transmission, respectively. Sec6 is also involved in the formation of the ternary Sec9-Sso1-Snc2 SNARE complex, which connects Sec9 to the t-SNARE Sso1, to then bind the v-SNARE Snc2 to drive membrane fusion[12]. The mammalian equivalents would be the syntaxin family, the SNAP-25 family and the VAMP/synaptobrevin family, respectively. In *Drosophila sec6* mutants, Sec5 is mislocalized indicating an interaction between the two subunits[67]. Also, similarly to Sec5 mutants described by Murthy in 2004, *sec6* mutants caused lethality during the first instar stage (due to depletion of maternal

contribution), their growth was debilitated and there were trafficking defects[67].

Overall, this subunit has strong interactions with the SNARE machinery, which makes it a strong candidate to be involved in the formation of new boutons, accounting for the need for polarized exocytosis.

1.4.6. Sec8

Sec8, similarly to Exo70, was shown to play a role in the formation of invadopodia in tumour cells and in the secretion of matrix metalloproteinases[53]. Both these characteristics are important for the effective invasion of tumour cells, indicating that Sec8 may be a relevant player in the process of tumorigenesis. Regarding cell migration, depletion of Sec8 from carcinoma cells resulted in suppression of migration via reduced phosphorylation of cytokeratin 8, a structural cytoskeleton protein often used as a cancer biomarker, that has also been shown to play a role in the function of tumour cells[27]. In mice embryos, Sec8 was shown to be important for the formation of the paraxial mesoderm and subsequent body plan elaboration[21]. This subunit has also been shown to play a role in the polarized growth of pollen tubes, for both initiation and maintenance[10]. Sec8 has been shown to bind to PSD-95[77], a homologue of the *Drosophila* Dlg. It has also been shown to form the complex Sec8-SAP102-NMDAR[86], binding a PSD protein with an expression parallel to new synapses[85] and glutamate receptors. Sec8 coimmunoprecipitated with type 3 inositol 1,4,5-triphosphate receptors (ITPR3), actin, Sec6, and the plasma membrane Ca^{2+} pump in rat cells[28,90]. A mutant of Sec8 was identified in a screen for genes involved in GluR expression and localization in *Drosophila*, with a phenotype for a decrease in immunocytochemically detectable GluRIIA[49]. Using the previously mentioned Sec8 mutants, another study showed that these mutants developed more branches and presynaptic terminals during larval development, had increased microtubules, and again showed a mild disruption of glutamate receptor trafficking[48]. Another set of mutants for this subunit showed a reduced rate of surface area growth, just like previously mentioned in Exo84, showing also that this subunit is involved in some way with the membrane addition necessary for elongation during anaphase cell progression[25]. *Drosophila* null mutants are lethal at 1st instar larval stage[48] and depletion of this subunit leads to unpolarized neurons[44].

1.4.7. Sec10

Sec10 has a dynamic and/or flexible C-terminal domain[47], and overexpression of mutants of this subunit blocks neurite outgrowth[97]. Regarding the terminals of this subunit in yeast, overexpression of the carboxy terminal domain results in an elongated bud, and overexpression of the amino terminal two-thirds blocked exocytosis[81]. Transfection of human Sec10 to canine cells resulted in the increase in synthesis of several proteins (one basolateral plasma membrane protein, and an array of apical and basolateral secretory vesicles) and increased tubulogenesis[50]. An interaction has been described between Sec10 and ADP-ribosylation factor (ARF), a GTP-binding protein that regulates membrane recycling to regions of plasma membrane[73]. Sec10 in *Caenorhabditis elegans* was shown to tether apical recycling vesicles to the plasma membrane, and in its absence the basolateral endosomal tubules were mostly fragmented into small ring-like structures[9].

1.4.8. Sec15

One of the first interactions described for the exocyst was the binding of the Sec15 subunit to activated Sec4, a Rab-GTPase present in the surface of secretory vesicles in yeast[28,83,84], which allowed to infer one of the canonical functions of the exocyst – the transport of secretory vesicles to be targeted at the plasma membrane. It has also been shown that Sec15 co-localizes with the Sec4p *Drosophila* homologue, Rab8[14]. There is also co-localization between this subunit and Rab11[39], a regulator of recycling vesicle exocytosis[93]. Sec15 has also been shown to co-migrate with Snc2, a yeast v-SNARE that works as a vesicle marker[28], which establishes an indirect connection to Sec6. Co-immunoprecipitation shows that Sec15 binds directly to Sec10, and that the latter is needed to establish a connection to the complex via Sec5[28,81]. Loss of Sec15 is not cell lethal, unlike Sec5 and Sec6, but the null mutants also die during 1st instar[57]. However, in the eye, the photoreceptors of Sec15 mutants are viable but have targeting effects, uncovering a function for this subunit in this biological process. In addition, in a *sec15* background, the exocyst subunits Sec5 and Sec8 are mislocalized[57].

In summary, the majority of Sec15's interactions are with secretory vesicle components. This indicates that Sec15 is the subunit that connects the vesicle to the rest of the complex.

1.4.9. The structure of the exocyst

The exact structure of the exocyst has been hard to discern but recently two models have been proposed, obtained using different methods (Figure 6). The first method, used by Picco *et al.*, is named Protein Interactions from imaging Complexes after Translocation (PICT)[71], and the other structure, by Mei *et al.*, uses Cryo-electron microscopy (Cryo-EM)[58]. These two approaches resulted in similar results, with a few small differences, possibly explained by the use of different methodologies and *in vivo* vs. *in vitro*.

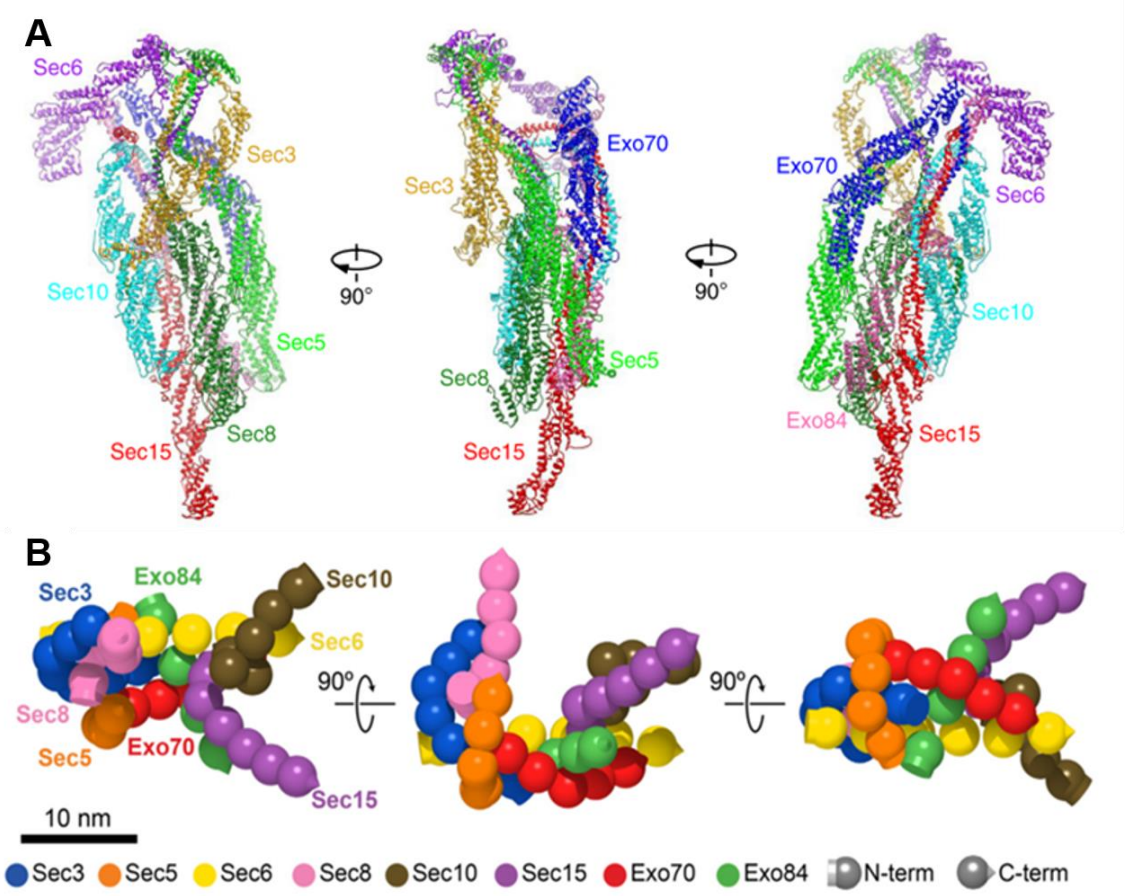

Figure 6 –Structure of the exocyst complex obtained using two differet methods by Mei *et al.* 2018 and Picco *et al.* 2017 (**A**) Cryo-EM structure obtained by Mei *et al.* in 2018. Each subunit is labelled a different colour and the complex is rotated by 90º in three sequential images. The structure of the subunits is represented according to its secondary structure. Exo70 is dark blue, Exo84 is pink, Sec3 is yellow, Sec5 is light green, Sec6 is purple, Sec8 is dark green, Sec10 is light blue and Sec15 is red. (**B**) PICT structure obtained by Picco *et al.* in 2017. Each subunit is labelled a different colour and the complex is rotated by 90º in three sequential images. The structure of the subunits is represented by small beads, with indication of the N-terminal and the C-terminal. Exo70 is red, Exo84 is green, Sec3 is blue, Sec5 is orange, Sec6 is yellow, Sec8 is pink, Sec10 is brown and Sec15 is purple. The N-terminal is represented by a round tip, and the C-terminal by a pointed tip.

Overall, both studies concluded that the positioning of the subunits in the complex is agreement with the interactions that have been described in the literature. Considering that, for example, Sec6 interacts with the SNARE machinery, Sec15 with the vesicle and Sec3 and Exo70 with the membrane, the complex must be organized in a way that allows for all these interactions, while also having room for the vesicle to fuse with the membrane. Both models allow those interactions.

There are however some differences between the models. Most of them are small differences in spacing and angles, that can be very easily accounted by the different methods used and the state of the protein complex. There is one that appears to be more significant. The positioning of Sec6 and Sec15 on the same side of the complex, in the structure determined by Picco *et al.* (2017), while these subunits were identified as being on opposite ends in Mei *et al.* (2018), which directly influences the hypothesis over the interactions between the vesicle and Sec15, and Sec6 and the SNARE machinery. Despite these conflicting results, as stated before, the interaction between SNAREs, secretory vesicles and the plasma membrane are still plausible in both models (Figure 7), with a change in angle between the two models being enough to accommodate the three interactions: vesicle, SNAREs, membrane.

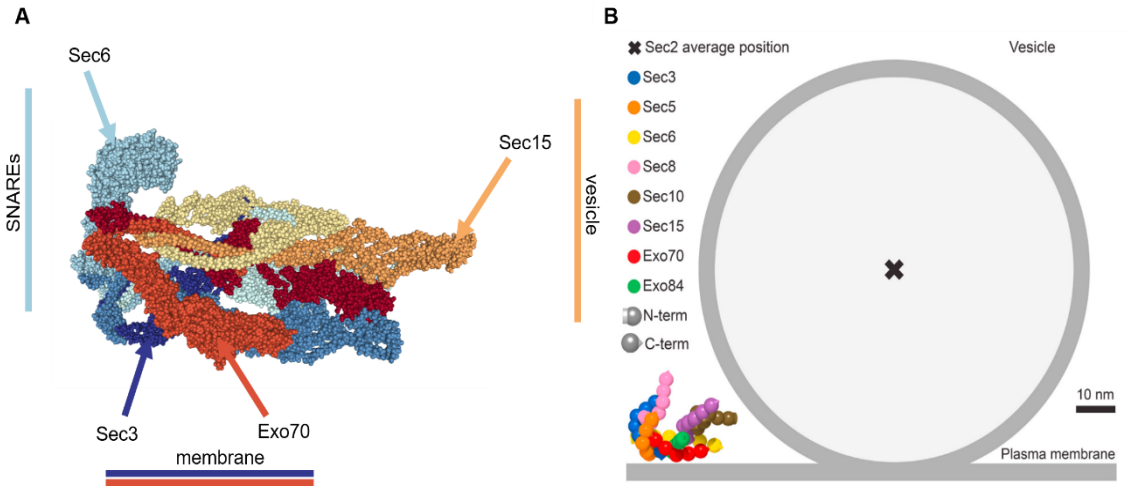

Figure 7 – Exocyst structure arranged to accommodate the interactions with the SNARE machinery, the secretory vesicle and the plasma membrane (**A**) Exocyst complex oriented to allow for the interactions with the SNARE machinery, the secretory vesicle and the plasma membrane. The vesicle should be positioned behind the complex as it is shown. Indication of the subunits Sec6 in light blue, Sec15 in yellow, Sec3 in dark blue and Exo70 in orange. Approximation of the place where the SNARE machinery, the secretory vesicle and the plasma membrane should be, regarding their interaction with the complex, is indicated with the colours corresponding to the subunits. Adapted from Mei *et al.* 2018 (**B**) Schematics of the orientation of the exocyst complex, when interacting with the secretory vesicle and the plasma membrane, from Picco *et al.* 2017. The exocyst complex is shown next to the plasma membrane and the secretory vesicle, with an approximation of the possible position regarding these two interactions. Sec2 average position, that was used as a vesicle marker, is marked in the centre of the vesicle drawing.

The exocyst complex has been shown to be involved in many cellular processes. However, in the majority of the studies, only one or a few subunits are analysed, which means that the questions of whether the complex functions as one or in sub-complexes, remains mostly undefined.

In yeast there is literature supporting the hypothesis that the exocyst does not disassemble prior to fusion, meaning that the complex stays together as whole throughout its functioning[3,11,92]. There is however also data supporting the hypothesis that Sec3, and perhaps also Exo70, stay in the plasma membrane permanently and that these two serve as spatial landmarks, to induce a directional movement to a specific point[6,15,51].

This might be explained by the different methodologies used in these studies, the state of the protein and the process of isolation. Some influence can also be due to whether *in vivo* methods were used or *in vivo*. One cannot also disregard the possibility that both hypotheses co-exist. Perhaps the complex can be in both conformations, whole or in sub-complexes, depending on the task at hand.

In other organisms there have been experiments suggesting the existence of functional sub-complexes[38,43,67,78], but no proof that it remains fully assembled throughout its function.

The sub-complexes can be a way of further defining the function of the exocyst. Perhaps in some situations the first step necessitates only a sub-complex that can then attach to rest of the subunits, if those functions are needed. Even if it is the case that the whole complex is needed to complete the process, the subunits might assemble throughout time, instead of all at the same time, which would lead to the presence of sub-complex at a certain time-point.

The data available suggests two different sub-complexes: Sec3-Sec5-Sec6-Sec8 and Sec10-Sec15-Exo70-Exo84[59].

These sub-complexes appear to be able to function effectively enough, based solely on the interactions of the subunits that constitute them. Both can account for interactions with the plasma membrane, Ral GTPases, and secretory vesicle components. This indicates that the idea of functional sub-complexes is not as unrealistic as once thought.

1.4.10. The exocyst in synaptic bouton formation

The expression of synaptic plasticity requires polarized traffic of vesicles to the membrane, carrying the diverse cargo that is needed for this process. There are several lines of evidence that suggest the involvement of the exocyst complex in the formation of synaptic structures. One of the most immediate parallels that can be suggested is that the exocyst targets secretory and/or synaptic vesicles to the place of neuronal expansion. It was demonstrated by Hazuka *et al.* in 1999 that the presence of the exocyst precedes the accumulation of groups of synaptic vesicles in rat hippocampal neurons, arguing that this complex may play a role in signalling of the location where a synapse will form[36]. In addition, the reported association of exocyst with microtubules might also be important for the initiation of growth in neurites[97].

Furthermore, there are known interactions between the exocyst and the GTPases Ral. The exocyst/Ral pathway has been implicated in several functions related to neuronal growth, namely: 1) RalA, associates with synaptic vesicles[4], 2) this pathway is activated by Ca^{2+} and induces postsynaptic membrane growth[94], 3) disruption of the interaction between the two, impaired the ability of RalA to promote neurite branching[45]. From these studies, the hypothesis that emerged is that RalA and the exocyst function together to regulate the direction of synaptic vesicle movement or of other cargo required for growth.

In yeast, a specific model for the mechanism of vesicle trafficking has been described. Using a myosin II motor, the exocyst 'walks' across actin cables transporting a vesicle attached by its interaction with Sec15 (Figure 8). In *Drosophila* instead of actin cables, this role would be fulfilled by microtubules, resembling vertebrate mechanisms[33], if a similar mechanism exists.

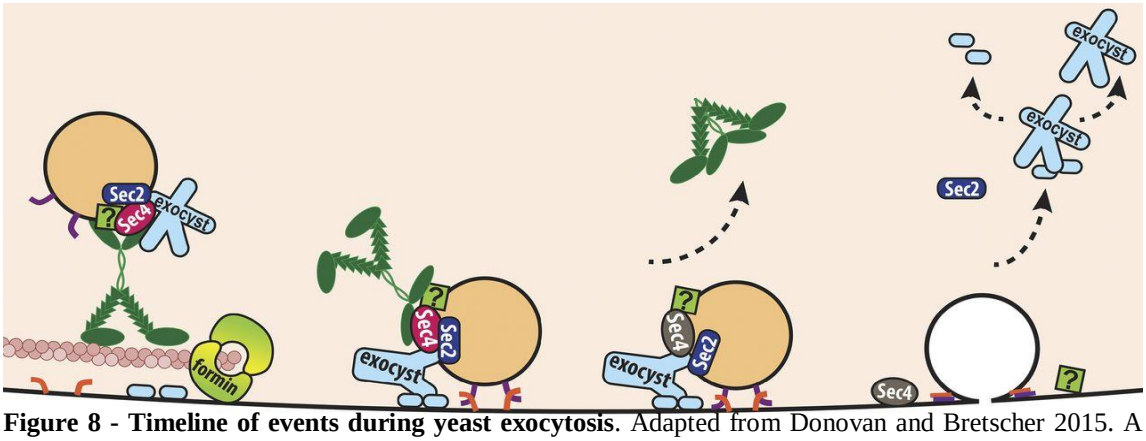

Figure 8 - Timeline of events during yeast exocytosis. Adapted from Donovan and Bretscher 2015. A secretory vesicle is transported across microtubules, using a myo2p motor and the exocyst complex. The myo2p binds to the Sec4 present in the surface of the vesicle, while the exocyst complex binds to both the Sec4 and the Sec2 present in the surface of the vesicle. The exocyst aids in the binding of the vesicle to the membrane, while the myo2p motor detaches. After fusion, the exocyst complex detaches from both the vesicle and the plasma membrane.

Overall, the participation of the exocyst in the traffic of vesicles is well established and can account for a possible role in the initiation of the process, by marking the place where a new bouton is going to form and clustering vesicles there. This is in agreement with several authors that consider Sec3 and Exo70 as spatial landmarks to target exocystosis[28,37,51].

In addition to the previously described interaction, there are also reported interactions between the exocyst and members of the cytoskeleton, most notably with actin. The interaction with Rho GTPases and the Arp2/3 complex can account for the manipulation of the morphology of the plasma membrane. By interacting with the cytoskeleton, the exocyst can actively manipulate the structural part of the formation of a new bouton. The actin cortex of the bouton is an essential structural component, and the formation of a new one appears to imply the disruption of the cortex to allow for the morphing of the membrane, possibly without the need for membrane addition.

The exocyst has been localized to points of membrane addition in diverse organisms and in diverse processes. Membrane addition does not always depend on the exocyst but considering it has been localized to points of neurite outgrowth[36,97], among others, it is conservative to assume that it might also be involved in membrane addition in new bouton formation. That is, of course, if membrane addition is indeed necessary for the formation of new synaptic boutons.

Overall there are several places where we can envision the involvement of the exocyst in this process.

1.4.11. …and now what?

The exocyst complex has been shown to be involved in many interactions and diverse processes. Accounting for all this, there is the hypothesis that this complex might be involved in the specific process for synaptic bouton formation. When a bouton forms, there is a rapid increase in size that leads to a bulky morphology immediately resembling a mature bouton. Considering this, three hypotheses arise. The exocyst complex might be involved in changes in membrane morphology, accounting for its interactions with membrane cytoskeleton components, meaning that it pushes and pulls the existing membrane or unfurls membrane ruffles. If membrane growth is necessary for the process of formation, the exocyst has been shown to localize to places of active membrane addition, meaning that the transport of cargo to the place of the new bouton might hinge on exocyst function. Another possibility is that, while not involved solely on one process, the exocyst might be at the interface between rearrangement, unfolding and addition. This complex, with its various interactions, could be responsible for the interchange between these mechanisms.

Altogether, the exocyst is a good candidate to be involved in the formation of new boutons. If not by its specific interactions with different components, then at least by its canonical function of transporter of secretory vesicles.

To confirm (or disprove) a role for the exocyst in the formation of new synaptic boutons, we have first tested its localization during activity-dependent bouton formation, and then assessed the importance of each of its subunits. This allowed us to verify the presence of the exocyst in the newly formed boutons and to establish the importance of some of the subunits of the exocyst.

2. Materials and Methods

2.1. Genetic tools

2.1.1. The UAS/Gal4 system

Tissue-specific expression of the Gal4 protein will induce the activation of the transcription of a specific gene[7], leading to the overexpression of this gene in a defined tissue of the animal (Figure 9).

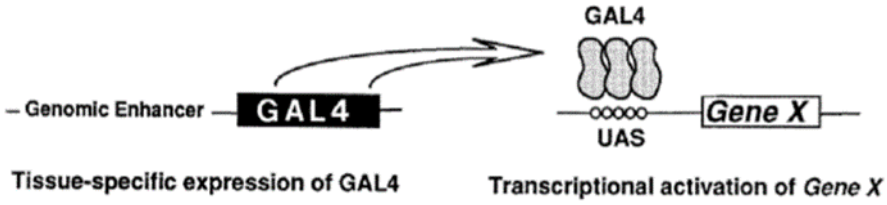

Figure 9 – The UAS/Gal4 system. Schematic representation of the UAS/Gal4 system. The Gal4 coding region is present in genomic sequence that when crossed with the upstream activating sequence (UAS) will induce the overexpression of the gene next to it. Image obtained from Brand and Perrimon, 1993.

2.1.2. RNA interference

RNA interference (RNAi) is a process where small strands of RNA bind to target genetic sequences. After this binding, either the messenger RNA is fragmented, or the ribosomes cannot initiate transcription due to blocking by the RNAi. This leads to a decrease in the expression of the targeted protein, for example.

When RNAi is being generated there is often a hairpin at the end, a structural component that decreases its efficiency. To counteract this, RNAi lines are often crossed with lines that express components capable of cutting this hairpin. In this work, the protein used was Dicer2, an endonuclease capable of cleaving double stranded RNA into small interfering RNA (siRNA)[19] (Figure 10).

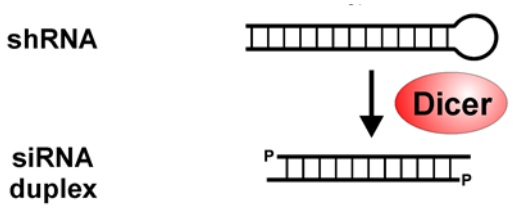

Figure 10 – Dicer activity in the RNAi processing. Schematic representation of the action of the protein Dicer cutting the hairpin at the end of a sequence of RNAi. Adapted from an image by the Hartig group of Konstanz University.

2.1.3. Mutant line for Exo84

For the subunit Exo84, a mutant line was used. This line was identified in a screen for male sterile mutants[24] and was later predicted to have a mutation that led to a truncated protein[5]. This mutant was crossed with a deficiency line for this gene, described in the same screen. In this deficiency line, there is a deletion of a section of the genome corresponding to the subunit Exo84 to avoid the occurrence of a different mutation in the second copy of the gene. The mutant is referred throughout this work as *onr*.

2.2. Drosophila melanogaster stocks and genetics

Fly stocks were maintained on standard media at either 25ºC or room temperature.

Crosses were done with a minimum of 7-10 virgins and 4-6 males of the lines of interest. Depending on the experiment, the crosses were done in cages with apple juice plates and yeast paste, or in wide vials with standard media.

In a cage system, the plates where the eggs are deposited are removed, ensuring that there are no flies from a second generation. In vials, the 'parent' flies are flipped to a new vial after a few days, ensuring that again there is no interference from second generation flies.

RNAi experiments were grown in apple juice plates at 29ºC, and remaining crosses were kept in vials at 25ºC. Controls and experimental sets were always processed and analysed under the same conditions.

The lines used throughout this study are referenced bellow divided into the following categories: UAS/Gal4 system, RNAi lines, and additional lines. There is a brief description and the stock's reference (BDSC, stands for Bloomington *Drosophila* Stock Centre and VDRC, stands for Vienna *Drosophila* Resource Centre).

Table 1 – Gal4/UAS system lines used. Indication of the name of the line, a brief description and the reference of the line.

	Description	**Reference**
nSyb-Gal4	Expression of Gal4 in the neuron.	BDSC #51635
UAS-Sec15-GFP	Overexpression of GFP-tagged Sec15 under UAS control.	BDSC #39685
Elav^{C155}G4;D2	Expression of Gal4 in the neuron and of UAS-Dicer2.	BDSC #43351
Elav-Gal4, UAS-Sec8-HA	Neuronal overexpression of the subunit Sec8 of the exocyst tagged with HA.	
Elav-Gal4, UAS-Sec3-HA	Neuronal overexpression of the subunit Sec3 of the exocyst tagged with HA.	

Table 2 – RNAi lines used. Indication of the name of the line, a brief description and the reference of the line.

	Description	**Reference**
Exo70 IR	RNAi for the subunit Exo70 of the exocyst	VDRC #103717
Exo84 IR	RNAi for the subunit Exo84 of the exocyst	BDSC #28712
Sec3 IR	RNAi for the subunit Sec3 of the exocyst	VDRC#35806
Sec5 IR	RNAi for the subunit Sec5 of the exocyst	BDSC #50556
Sec6 IR	RNAi for the subunit Sec6 of the exocyst	VDRC #22077
Sec8 IR	RNAi for the subunit Sec8 of the exocyst	BDSC#57441
Sec15 IR	RNAi for the subunit Sec15 of the exocyst	VDRC #105126

Table 3 – Additional lines used. Indication of the name of the line, a brief description and the reference of the line.

	Description	**Reference**
***onr*$^{142.5}$**	Missense mutation in the Exo84 gene.	Giansanti *et al.* 2004
Df(3R)Espl3	Deficiency associated with the mutant *onr*. The gene for Exo84 is absent from the genotype	BDSC #5601
W^{1118}	Wild type. Since all lines were produced in a W^{1118} background, we chose this background as the wild type control.	

2.3.Neuronal Spaced Pattern Stimulation

To study the formation of new boutons, it is necessary to induce this process. In the case of this work, it is necessary to induce the formation of a new synaptic bouton, to try to understand the mechanism that leads to boutongenesis.

To achieve this, different protocols can be used, but the ones chosen for this work were the neuronal spaced pattern stimulations that are described in Ataman *et al.* (2008) and Vasin *et al.* (2014).

These two protocols use two solutions: HL3.1 and a High K^+/High Ca^{2+} solution (Table 4), to induce membrane depolarization and achieve the formation of new synaptic varicosities[1,96,100]. They do this by using patterns of stimulation and rest with specific time intervals and with a specific duration. In the end, if the stimulation worked the larvae should have developed new synaptic boutons, that can be visualized and studied.

To start the protocol 3rd instar larvae were dissected[8] on a Sylgard surface in HL3.1 solution, that mimics the haemolymph of the larvae allowing for a 'resting' phase. The dissection was partial, cutting open only the ventral part of the animal after pinning it and then approximating the pins to about half of the distance to allow the larvae to contract.

Table 4 –Preparation of the solutions HL3.1 and High K^+, High Ca^{2+}. Concentrations of each solution used in mM and indication of the final pH.

	NaCl	**KCl**	**CaCl2**	**MgCl2**	**NaHCO3**	**Trehalose**	**Sucrose**	**HEPES**	**pH**
HL3.1 (mM)	70	5	0,1	4	10	5	115	5	7,2-7,4
↑K^+, ↑Ca^{2+} (mM)	40	90	1,5	4	10	5	115	5	7,2-7,4

The protocol described by Ataman *et al.* in 2008 follows a pattern of 5 moments of stimulation (2', 2', 2', 4', 6') with resting phases of 15' in between, ending with a final resting period of 54'. This protocol is 2h10 and is referred to as Longstim. The second protocol was described in 2014 by Vasin *et al.* and follows a pattern of 3 pulses of stimulation (2', 2', 2') with resting phases of 10' in between, ending with a final resting period of 30' (Figure 11). This protocol is 56' and is referred to as Shortstim. During the final resting phases of both protocols the dissection is finished, ending with the larvae stretched by 6 pins to expose the entirety of the musculature. According to the literature,

the average new bouton number described for Longstim[1] is higher than for Shortstim[96], averaging 6 and 5,5, respectively.

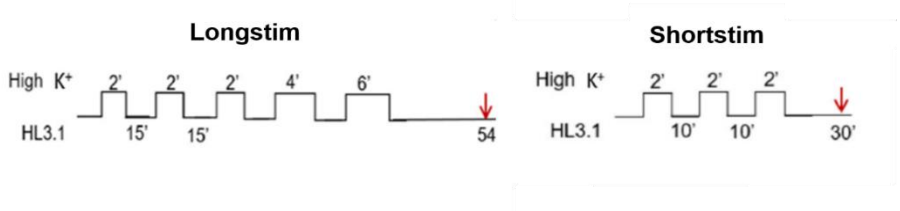

Figure 11 – Protocols for neuronal spaced pattern stimulation, Longstim and Shortstim. Schematic representation of the protocols for Neuronal Spaced Pattern Stimulation. Indication of the time in minutes spent in each phase, either resting or stimulating.

2.4.Immunocytochemistry

After the stimulation protocol, larvae were fixed with different solutions, according to the staining that was to be done afterwards. The fixative used for each of the experiments is described in Table 5. If there was no previous stimulation, larvae were dissected in either HL3.1 or PBS (phosphate-buffered saline) and then fixed immediately.

Table 5 – Fixatives used in the experiments. Depending on the antibody used for the experiment, specification of the fixative used, composition and time of fixation.

Staining for	Fixative
Hrp, Dlg	5mins of Bouin's fixative (picric acid, formaldehyde, glacial acetic acid)
Hrp, Dlg, GFP **Hrp, Dlg, HA-tag** **Hrp, Sytanxin**	20mins of PFA (4% paraformaldehyde diluted in 1x PBS)

Following the removal of the fixative with PBS, larvae were washed with PBT (PBS 1x, 0,3% Triton) and then blocked with NGS (Normal Goat Serum) for 30min to 1h. Incubation with the primary antibody (Table 6) was done overnight at 4ºC followed, the next day, by another sequence of washes with PBT. Another period of blocking with NGS for 30min to 1h was done before the incubation with the secondary antibody (Table 7) and/or the conjugated antibody (Table 8). After being again washed extensively with PBT, the larvae were transferred to 50% glycerol and mounted in slides using a mounting medium (DABCO, PBS 1x, 100% glycerol). After sealing the slides, they were placed in

cold storage. The protocol was performed at room temperature except when otherwise stated.

Table 6 – Primary antibodies used in the immunofluorescence staining. Indication of the antigen and code (when applicable), species, concentration and reference/source.

Antigen	Species	Concentration	Reference/Source
Dlg (4F3)	Mouse	1:250	Hibridoma Bank
GFP	Rabbit	1:1000	Life Technologies A11122
Syntaxin (8C3)	Mouse	1:100	Hibridoma Bank

Table 7 – Secondary antibodies used in the immunofluorescence staining. Indication of the antigen and code (when applicable), concentration and reference/source.

Antigen		Concentration	Reference/Source
Anti-Mouse A488			
Anti-Rabbit A488	Donkey	1:500	Jackson ImmunoResearch
Anti-Mouse RhRx			

Table 8 – Conjugated antibodies used in the immunofluorescence staining. Indication of the antigen and code (when applicable), concentration and reference/source.

Antigen	Species	Concentration	Reference/Source
anti-Hrp Cy3			
anti-Hrp A645	Goat	1:500	Jackson ImmunoResearch
anti-Hrp A488			

2.5. Imaging and Image Analysis

Confocal images were obtained using a Zeiss LSM710 with two objectives (C-APOCHROMAT 40x/1.2 W Corr and C-APOCHROMAT 63x/1.2 W Corr) and lasers for the wavelengths of 488nm, 561nm and 633nm (green, red and blue, respectively). Microscope hardware was controlled using the ZEN software provided.

Widefield images were obtained using an Axio Imager.Z2 with a N-ACHROPLAN 40x/0.45 objective and lasers for wavelengths 488nm and 561nm, for green and red, respectively. Microscope hardware was controlled using the ZEN software provided.

Throughout the work, imaging was always conducted in muscles 6 and 7 from the segments A2 to A4 of 3rd instar larvae. To process the images obtained for immuno-

localization and quantification of new boutons, the open-source software Fiji[87] was used.

For the quantification of new boutons, the images were imported to Fiji as composites, and were then stacked using a macro that followed the pathway Image > Stacks > Z Project and selected for maximum intensity. In this final image the boutons were counted using the multi-point tool provided by the software, and then the result was saved as Tiff.

For the immuno-localization, images were opened in Fiji and then stacked using the macro described above. Afterwards the channels were split using the pathway Image > Color > Split Channels, and then were merged using the pathway Image > Color > Merge Channels. Afterwards these final images were saved as Jpeg.

2.6. Quantification of new synaptic boutons

After imaging each NMJ, the new boutons were counted for each NMJ and genotype. If the average of new boutons for the controls was lower than 4, the experiment was assumed to have failed and was discarded (control plus experimental genotypes). If the average was above 4, new boutons were quantified. In the results averages are presented with the respective Standard Error of Mean (SEM).

Afterwards, the number of newly formed boutons was plotted in a box plot graph with whiskers depicting the maximum and minimum values, and a line representing the median. Sample size is indicated in the X axis, beneath the genotype.

2.7. Statistical analysis

The statistical analysis of the bouton quantification was done in GraphPad Prism 6. First the data sets were analysed for normal distribution using a D'Agostino & Pearson omnibus normality test. If the population followed a normal distribution, it was compared for significance using either a one-way ANOVA analysis or a Student's t-test, for three-way and two-way comparisons, respectively. Data-sets that didn't pass the normality test were analysed for statistical significance using nonparametric tests, either a Mann-Whitney test or a Kruskal-Wallis test, depending on whether there were two or more data sets being studied, respectively. Statistical differences are represented directly on the graph, according to following key: **** $p<0.0001$; *** $p<0.001$ ** $p<0.01$, * $p<0.05$. If the difference is not significant, there is no annotation to the results.

3. Results and Discussion

3.1. Understanding the formation of new synaptic boutons

To study the formation of new synaptic boutons, one must induce this process. The neuronal spaced pattern stimulation protocol induces this process but then it is necessary to identify these new structures.

New synaptic boutons are characterised by the presence of presynaptic membrane but no postsynaptic membrane. This is due to the fact that when the bouton initially forms, there is budding of the presynaptic membrane that only later matures and acquires Dlg (Figure 3). It is well characterised that immature boutons are marked only by antibodies for presynaptic components, and then some will mature acquiring postsynaptic components, and establishing the subsynaptic reticulum[95]. An example of one NMJ with several new activity-dependent synaptic boutons is shown in Figure 12.

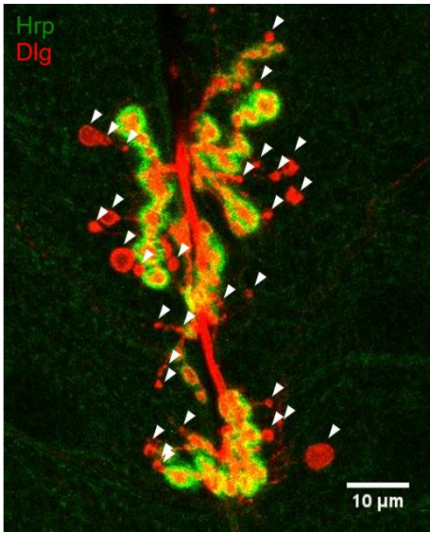

Figure 12 – Example of a larval NMJ after induction of activity-dependent bouton formation. The NMJ is labelled with anti-Hrp (red) and anti-Dlg (green). There are numerous new synaptic boutons (white arrows) that, because they have just been formed, are marked only by anti-Hrp that stains the presynaptic membrane. The remainder of the boutons, the mature ones, are both stained by anti-Hrp and anti-Dlg. Scale bar is 10µm.

These new boutons have been stained by anti-Hrp, a presynaptic membrane marker, but they show no staining by anti-Dlg, which is the postsynaptic membrane marker for the Discs-Large protein[60], a postsynaptic component. This indicates that this structure has been recently formed, and therefore is a new synaptic bouton. Throughout the results presented hereafter, these are the structures that are identified, marked by arrows and counted.

Given the nature of the protocols used to form new synaptic boutons, one can name these structures as activity-dependent. Meaning that induced membrane depolarization leads to an increase in neuronal transmission and to structural plasticity. In response to

this increase in neurotransmission, the neuronal structure adapts by creating more boutons to increase the capacity of the NMJ and match activity. To increase the number of active zones, responsible for neurotransmission, the system also creates more synaptic boutons that house these sites. It is unknown whether these differ from the other synaptic boutons created during development and larval growth, be it by their mechanism of formation or molecular composition and function.

Two different protocols were used to induce the formation of new synaptic boutons. As described in the Methods Section, one is longer with more pulses and wider intervals, while the other lasts less time and has smaller and more consistent intervals. They both have very similar averages, with 6 and 5,5, but only the Longstim is thought to depend on new protein synthesis. However, in the Shortstim, the authors concluded that acute bouton formation was independent of protein synthesis without blocking it, meaning that their conclusions may be inaccurate. Longstim, however, is thought to be protein synthesis dependent, because blockage of this process had an impact on activity-dependent bouton formation. Due to this, these protocols are not interchangeable and while both can be used to study synaptic bouton formation, they may provide complementary information regarding protein synthesis dependence. Because the main goal of this work was to study the formation of new boutons, the Shortstim protocol was preferentially used, save for one exception presented in the section ***3.3.2 Exo84***.

3.2. Is the Exocyst complex present in new synaptic boutons? – Localization of subunits of the complex in the new structures

If the exocyst complex is necessary for the formation of new boutons, it is expected that it is present in these structures during their formation. Therefore, the first step to understand if the exocyst may play a role during bouton formation is to assess its localization in the new boutons. If images are obtained where the exocyst is present in new boutons, the conclusion is not definitive, since its presence does not correlate with function. There is a possibility that it is indeed present, but it is not playing a role in any of the mechanisms. But if the exocyst does not localize to new boutons, it is unlikely to be required for their formation

Imaging the whole complex at once is not possible, so an alternative is to tag one of the subunits which, while not truly a whole complex marker, is a good approximation.

For this, three different subunits were tagged: Sec15 (nSyb-Gal4, UAS-Sec15-GFP), Sec3 (elav-Gal4, UAS-Sec3-HA) and Sec8 (elav-Gal4, UAS-Sec8-HA. These images were obtained after larvae went through a neuronal spaced pattern stimulation protocol and were then stained appropriately and imaged.

We expressed GFP-tagged Sec15 in neurons using nSyb-Gal4 and performed the Shortstim protocol on this background. Staining of NMJs with anti-Hrp and anti-Dlg allowed us to visualize new boutons. To asses if Sec15-GFP was present in newly formed boutons, we used an antibody against GFP (Green Fluorescent Protein) to amplify the signal in Figure 13 – A. New synaptic boutons are marked with white arrows.

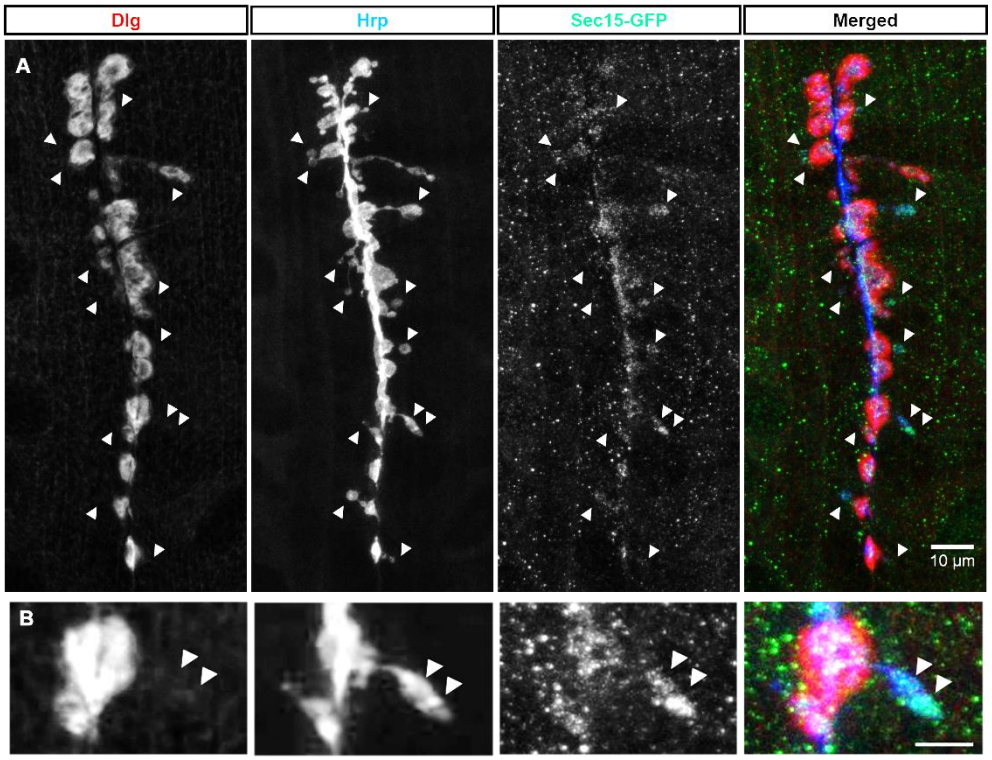

Figure 13 – The exocyst subunit Sec15 is present in acutely formed boutons. (**A**) The genotype of the larvae is nSybG4, UAS-Sec15-GFP, leading to the overexpression of the subunit Sec15 tagged with GFP in neuronal tissue. The NMJ is stained with anti-Dlg (red), anti-Hrp (blue), and anti-GFP (green). New synaptic boutons are stained by both anti-Hrp and anti-GFP and are marked by white arrows. Sec15 is ubiquitous in the NMJ and is present in new synaptic boutons. Scale bar is 10µm. (**B**) Detail of the image presented in A). Two new synaptic boutons formed in sequence with an accumulation of Sec15 at the tip of the second bouton. Scale bar is 5µm.

We observed that the subunit Sec15 is present in the new synaptic boutons, which indicates that it may play a role in this process. Given that Sec15 is an assumed exocyst marker, one can extrapolate that the exocyst (or a subcomplex that includes Sec15) is present in new synaptic boutons, and therefore may play some role, although exactly

which role is still unknown.

A careful analysis of these images showed what appears to be a sequential formation of new boutons in the right side of the NMJ (Figure 13 - B).

These two boutons appear to have been formed in sequence, and there is an accumulation of the subunit Sec15 at the leading edge of the second synaptic bouton. This shows a high resemblance to images obtained by Rivera-Molina and Toomre in 2013, when performing live-imaging of migrating mammalian cells (Figure 14)[79]. Although the authors tagged the subunit Sec8 and not Sec15, the visual appearance of the accumulation of Sec8 in the migrating cells is very similar to the appearance of the accumulation of Sec15 in the sequential synaptic boutons.

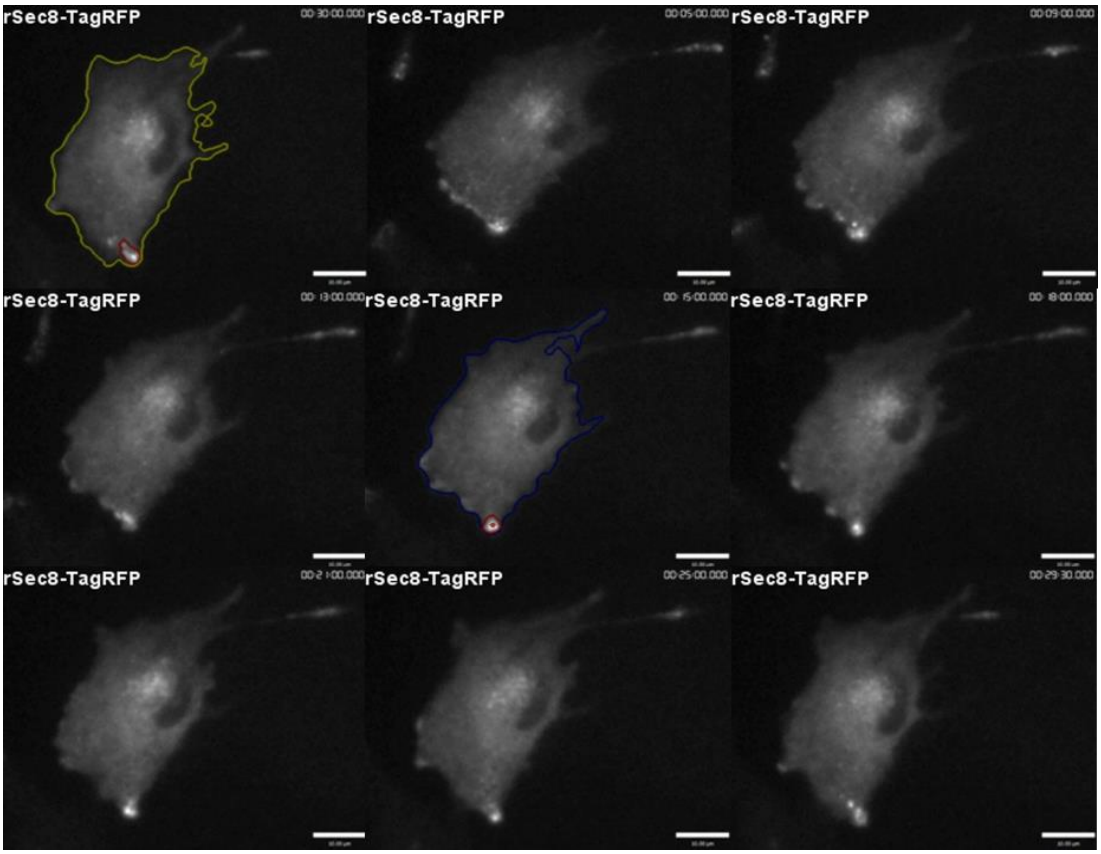

Figure 14 – The exocyst subunit Sec8 accumulates at the leading edge of a migrating cell. From Rivera-Molina and Toomre 2013. The subunit Sec8 is tagged with RFP (Red Fluorescent Protein). The time-lapse image shows the outline of the migrating cell in yellow and the rough outline of the accumulation of Sec8 at the leading edge in red. The fifth still again shows the rough outline of the accumulation of Sec8 at the leading edge in red. The accumulation of the subunit is persistent and strong throughout the whole process of migration.

This similarity might indicate a similar spatial-temporal accumulation for both processes. However, in the images obtained for synaptic boutons, this accumulation is not verified in all the structures. In fact, this instance presented here is the only one found in all the images obtained for the subunits of the exocyst. Meaning that, although the similarity is very interesting, it might have been a coincidence.

To further confirm the presence of the complex, the subunit Sec3 was tagged with HA and expressed in neurons, and after the Shortstim protocol the NMJs were marked with an antibody against this epitope to identify Sec3-positive structures (Figure 15 – A).

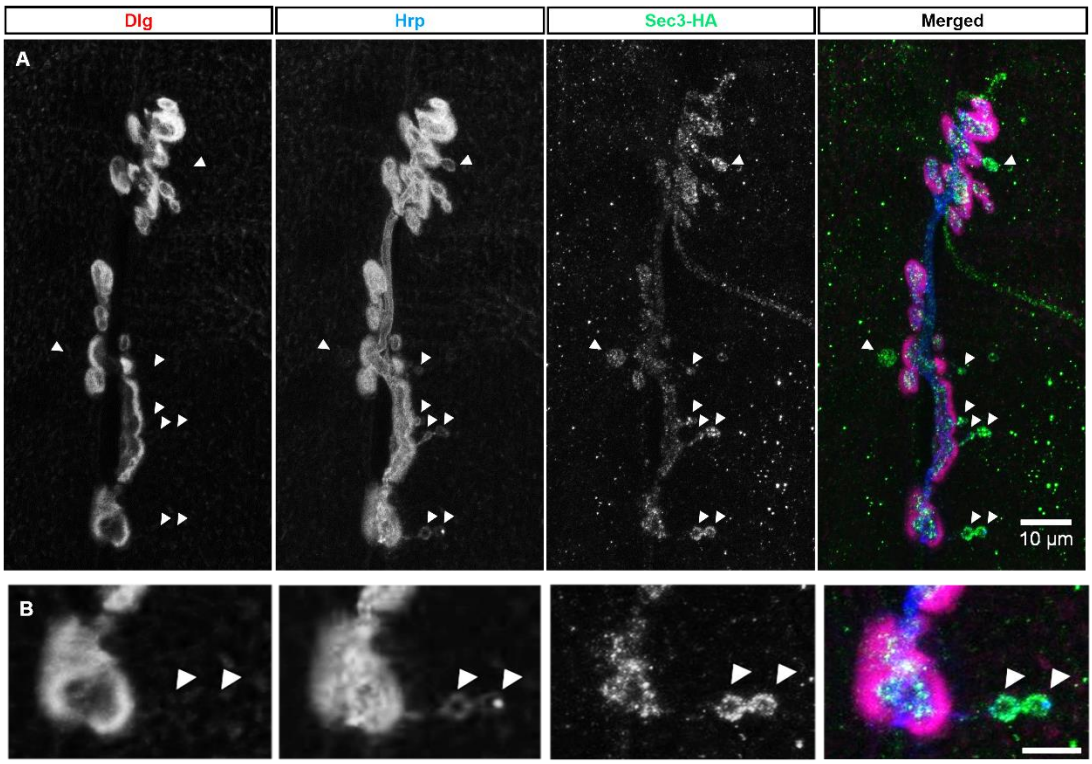

Figure 15 – **The exocyst subunit Sec3 is present in acutely formed boutons.** (**A**) The genotype of the larvae is elav^{C155}G4, UAS-Sec3-HA, leading to the overexpression of the subunit Sec3 tagged with HA in neuronal tissue. The NMJ is stained with anti-Dlg (red), anti-Hrp (blue), and anti-HA (green). New synaptic boutons are stained by both anti-Hrp and anti-HA and are marked by white arrows. Sec3 is ubiquitous in the NMJ and is present in new synaptic boutons. Scale bar is 10µm. (**B**) Detail of the image presented before. Two new synaptic boutons formed in sequence with an accumulation of Sec3 in the outline of the bouton. Scale bar is 5µm.

Like with Sec15, the exocyst component Sec3 is present in the newly formed boutons, indicating that perhaps it is playing a role in the process. For this subunit, there was also an interesting detail (Figure 15 - B) in the two boutons in the right bottom corner.

These two boutons appear to have an accumulation of Sec3 only at the membrane,

forming the outline of the new structures. This is particularly interesting considering that Sec3 is one of the subunits know to interact with the plasma membrane. But again, similarly to Sec15, this is not true for all the structures that appear in the images, meaning that more examples need to be found to claim that there is a conserved spatial-temporal positioning of the exocyst.

For the subunit Sec8, again the HA tag was used, and respective antibody (Figure 16).

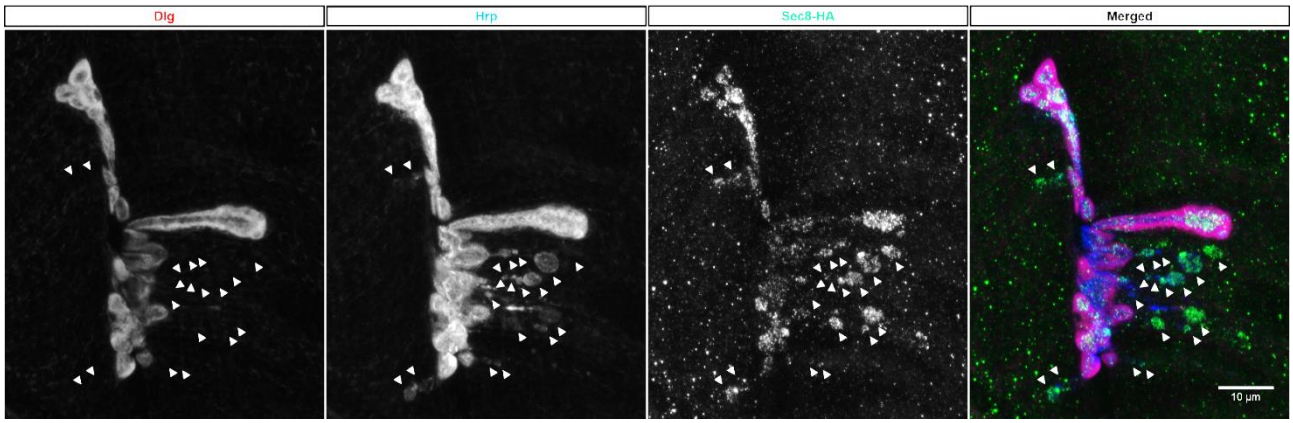

Figure 16 – **The exocyst subunit Sec8 is present in acutely formed boutons.** (**A**) The genotype of the larvae is elav^{C155}G4, UAS-Sec8-HA, leading to the overexpression of the subunit Sec8 tagged with HA in neuronal tissue. The NMJ is stained with anti-Dlg (red), anti-Hrp (blue), and anti-HA (green). New synaptic boutons are stained by both anti-Hrp and anti-HA and are marked by white arrows. Sec8 is ubiquitous in the NMJ and is present in new synaptic boutons. Scale bar is 10µm.

In accordance to our previous results, this subunit is present in the new synaptic boutons, confirming the presence of three different components of the complex that are present in the newly formed structures.

Overall, there are several new structures formed and marked with different subunits, and they do not seem to all have the same quantity of these subunits. This indicates that there is a dynamic range of exocyst. This might mean that at different points of the formation of new synaptic boutons, different amounts of exocyst, or at least specific subunits, are required. Considering the different ways that the exocyst complex might be involved in this process, this could indicate that its involvement is spatial-temporally locked. A high concentration of the complex might be necessary for the initiation of the process, and then it decreases; or a high concentration outlining the bouton might be needed for stabilization of the membrane; or it might be even more dynamic than that, increasing and decreasing at more than one time point.

However, one can argue that, since the exocyst complex is present ubiquitously in the neuronal tissue, that when a new bouton forms the exocyst is transported to this new structure by default regardless of function or use.

Contradicting this, our lab has been able to image other synaptic components and verify that not all proteins localize to new boutons when they are formed. A concrete example of this is the filamin protein (Figure 17). The short neuronal spaced pattern stimulation protocol was applied to larvae to induce the formation of new boutons, and the NMJ was then stained for filamin and imaged. Filamin is a protein that crosslinks the membrane to the actin cytoskeleton and was therefore expected that this protein would be present in the new boutons that bud from pre-existing ones. However, as it can be seen in Figure 17, new boutons are not marked by the *Drosophila* homologue Cheerio, a 100% penetrant observation.

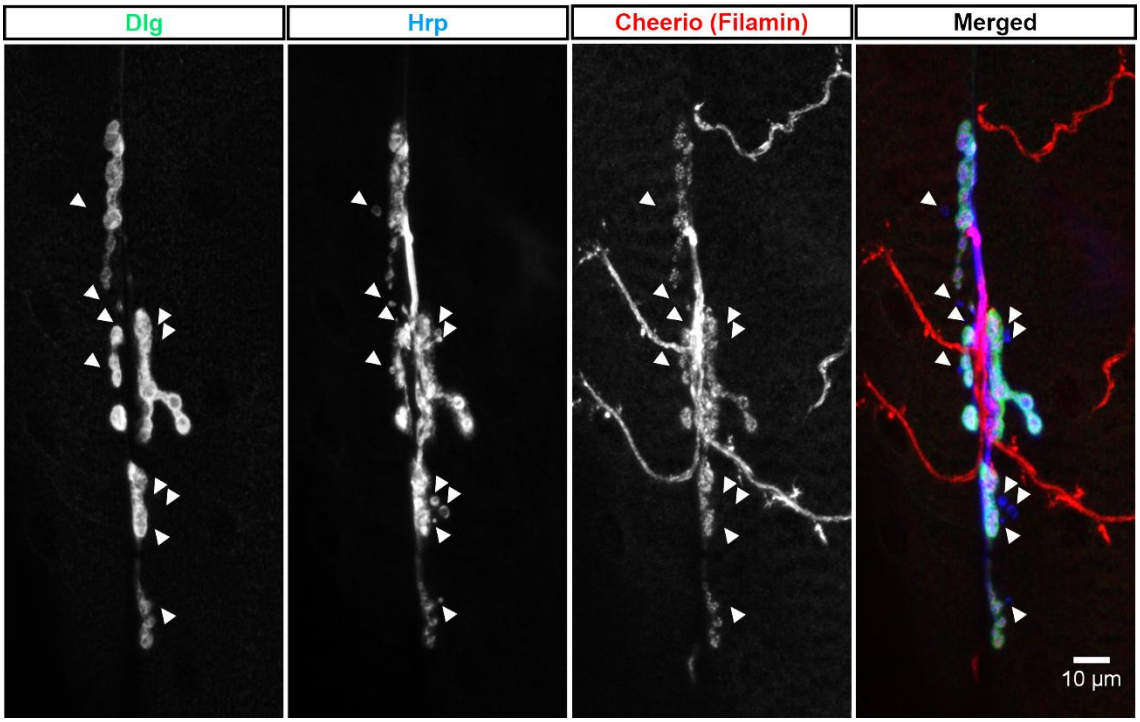

Figure 17 – **Filamin does not accumulate in newly formed activity-dependent boutons.** (**A**) The larvae are wild-type like with the genotype W^{1118}. The NMJ is stained with anti-Dlg, anti-Hrp, and anti-Cheerio. In the merged image, Dlg is marked in green, Hrp in dark blue and filamin in red. New synaptic boutons are stained by anti-Hrp and are marked by white arrows. Filamin is ubiquitous in the NMJ but the newly formed boutons show no staining for filamin. Scale bar is of 10µm.

In the images, the new synaptic boutons formed in response to the stimulation protocol are marked by white arrows. These new boutons are easily distinguishable by the fact that they are marked by Hrp (a pre-synaptic membrane marker in blue) but not

by Dlg (a post-synaptic membrane marker in green).

This suggests that there is a regulation of the transport of proteins to the new boutons, leading to the conclusion that if the exocyst complex is present in the new synaptic boutons then it is likely necessary for the process.

To really understand the dynamic of this complex while the new boutons are being formed, live imaging would be necessary. This would allow us to visualize the spatial-temporal localization of the subunits. This possible dynamic increase and decrease of the exocyst complex could be directly observed in concurrence with the alterations of the neuronal membrane. These experiments will be done in the future.

3.3. Is the Exocyst required for activity-dependent bouton formation? - Neuronal Spaced Pattern Stimulation of Exocyst RNAi and mutant lines

The exocyst is a complex composed of eight subunits, all with different described interactions with several distinct molecular players. In recent years, it has become clear that not all subunits are important for the same processes, with knock downs of each being disruptive in different ways for the mechanisms and leading to distinctive phenotypes. This implies that some of the members of the complex may be required for new bouton formation, while others may be dispensable. Given this, we decided to have an unbiased approach and test the putative requirement of each of the subunits for the process of bouton genesis. To do so, we used RNAi to specifically knock down each of the subunits in neurons. To induce bouton formation, we stimulated the larvae using the Shortstim patterned depolarization protocol in controls and in knock downs of the exocyst subunits. If any of the exocyst components is required for this process, the RNAi larvae will have lower numbers of new boutons formed during the stimulation period, meaning that the process was unable to occur correctly.

For this set of experiments, two controls were used in the RNAi experiments, one for the neuronal driver (elav^{C155}Gal4;D2 or nSyb-Gal4) and another for the RNAi construct, both crossed with the wild-type background w^{1118}. In theory, both of these controls should behave as wild-type and should not be different between themselves. The neuronal driver elav^{C155}Gal4;D2 is present in the X chromosome. Because of this all the larvae selected

were males, to maximize Gal4 expression. In *Drosophila*, males double the expression of X-linked genes to equal the two X chromosomes present in females, meaning that in male larvae the RNAi is expressed at higher levels when compared to females.

Because RNAi only allows for the reduction rather than complete elimination of the protein, a lack of effect cannot be interpreted alone as that subunit not playing a role. It is possible that the disruption might be at a lower level than necessary to draw conclusions about the interactions, meaning that there is still a significant amount of protein available that is able to function as normal, without manifesting a phenotype. Testing the levels of reduction or using a mutant would help drawing conclusions. Despite this, the RNAi is still one of the best strategies to assess if a gene is required in a given tissue or cell type.

3.3.1. Exo70

Exo70 is one of the subunits that has been shown to interact with the membrane[37,52], which puts it in a privileged place to affect the morphology of this compartment. In fact, some of the interactions of the subunit do seem to support that hypothesis, specifically its interaction with Rho GTPases[13,80,101] and the Arp2/3 complex[72,76]. In theory Exo70 could be relevant in the first moments of the formation of the new bouton, possibly by being involved in morphological alterations. This subunit also seems to be important for the fusion of the vesicles, but not the traffic[52,75]. Additionally, Exo70 has been shown to play a postsynaptic role, where null mutants have defects in AMPA receptors insertion[23]. Finally, the phosphorylation of this subunit seems to promote the assembly of the complex, in opposition to Exo84 where phosphorylation promotes the disassembly[75].

For this subunit, its role in boutongenesis may be related to the cell membrane morphology, accounting for its interactions with the plasma membrane and with cytoskeleton components and actin-binding molecular players.

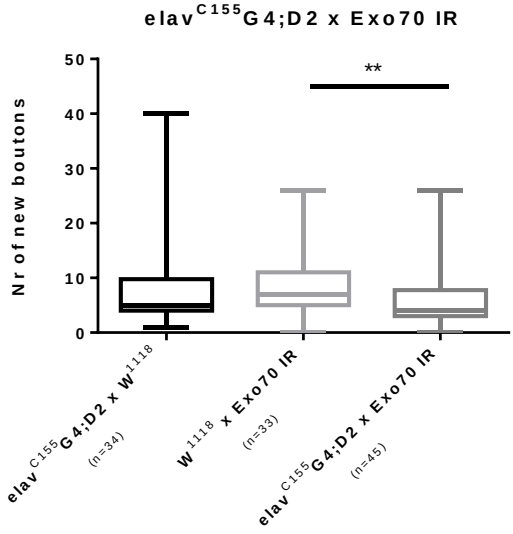

Figure 18 - Neuronal reduction of Exo70 impairs activity-dependent bouton formation. Genotypes represent: Gal4 control (elav^{C155}G4;D2 x W^{1118}, n=34), RNAi control (W^{1118} x Exo70 IR, n=33) and neuronal reduction of Exo70 (elav^{C155}G4;D2 x Exo70 IR, n=45). The median is shown as a line in the box, along with the maximum and minimum values as the whiskers of the box plot. ** $p<0.01$

After stimulation, both controls have an average number of new boutons within the median range, with 7,2±0,71 for the neuronal driver control and 8,4±0,65 for the RNAi control. When Exo70 RNAi larvae are analysed, the average is 6,2±0,61 (Figure 18). There is a significant difference between the RNAi larvae and the RNAi line control, but no difference to the Gal4 control, suggesting that there may be no difference, just a large variability. Another explanation is that the RNAi control might be slightly higher, resulting in a statistic difference or the driver control might be slightly lower, obscuring a difference that should be there. Considering the averages of other controls performed for other subunits, the value of 8,4 for the RNAi control is higher than expected. This leads me to believe that the observed statistical difference is more likely to be created by this higher value, rather than representing a real difference, although alternative explanations are still possible. To better conclude, the experiment would have to be repeated a few more times, in order to better understand if this difference is real or not.

The interactions of the subunit Exo70 with actin-binding molecular components and the plasma membrane makes it a good candidate to be involved in the process of new bouton formation, however, this is not fully supported by the results obtained. In addition to Exo70, the subunit Sec3 also interacts with actin-binding GTPases and with the plasma

membrane. Furthermore, mutants for both these subunits are lethal, leading to the hypothesis that perhaps disruption of the function of only one of them will be compensated by the subunit that conserves its integrity. However, if there is indeed a difference in the number of new boutons, then the lack of Exo70 results in effects in activity-dependent bouton formation.

3.3.2. Exo84

This subunit has been shown to interact with RalA[63] (a small GTPase involved in several cellular processes), to have an indirect connection to a t-SNARE[70,104] and an actin regulating Rho GTPase[40,46] in *Drosophila* and yeast, and to interfere with surface area growth during cell division[25]. In addition, the assembly of the complex seems to be impeded by the phosphorylation of this subunit[29]. In the formation of new synaptic boutons, it is possible to conceive that Exo84 could be needed for the assembly of the complex, and then perhaps the establishment of polarized direction and finally a change in conformation for the interaction with the SNARE machinery. RalA has also been described to be involved in actin dynamics, which can perhaps establish an indirect connection between the exocyst and the cytoskeleton.

Overall, this subunit has described interactions with actin dynamics and SNARE machinery. Its involvement in the assembly of the exocyst complex is also interesting.

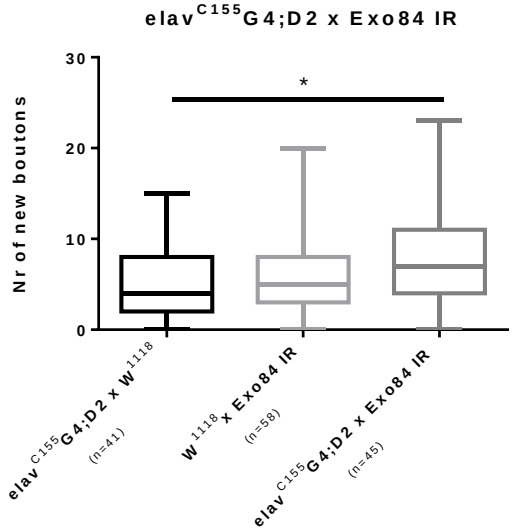

Figure 19 - Neuronal reduction of Exo84 does not impair activity-dependent bouton formation. Genotypes represent: Gal4 control (elav^{C155}G4;D2 x W^{1118}, n=41), RNAi control (W^{1118} x Exo84 IR, n=58) and neuronal reduction of Exo84 (elav^{C155}G4;D2 x Exo84 IR, n=45). The median is shown as a line in the box, along with the maximum and minimum values as the whiskers of the box plot. * $p<0.05$

After stimulation, the average activity-dependent bouton number for the controls for this experiment are overall lower than for the other subunits, with 5,2±0,60 and 5,8±0,58 for the Gal4 and RNAi, respectively, which makes it harder to draw conclusions (Figure 19). However, the RNAi larvae show an average of 7,8±0,74 new boutons, which is within the normal number for controls of other experiments, indicating that the Exo84-IR is able to form new synaptic boutons at roughly the same rate as wild type-like larvae. Therefore, the statistical difference observed between the Exo84-IR and the control for the Gal4, is probably due to a large variability that lead to a lower average bouton number for this specific control. To further confirm this hypothesis, the experiment would have to be repeated to increase the sample size.

In the case of this subunit, in addition to RNAi, a mutant line that survives to the 3rd instar stage is available and could also be tested[24]. Exo84, or *onr* in *Drosophila,* mutants were crossed with a deficiency that uncovers this gene, with the *onr* mutation leading to a truncated protein. For this experiment we used W^{1118} larvae as controls because the mutant line was produced in this background.

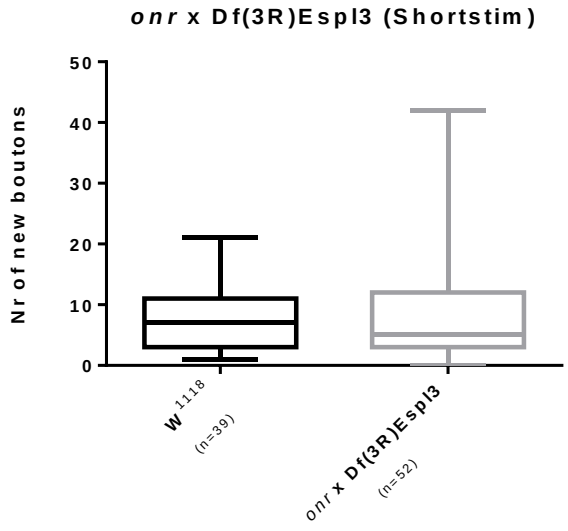

Figure 20 - Using the Shortstim protocol with a mutation in the Exo84 *onr* gene does not impair activity-dependent bouton formation. The control used was W^{1118}, n=39 for the *onr*;Df(3R)Espl3 mutant of the subunit Exo84, n=52. The median is shown as a line in the box, along with the maximum and minimum values as the whiskers of the box plot

After the Shortstim protocol, the averages were 7,7±0,79 and 8±1,1 for wild-type and the mutant, respectively (Figure 20). This average is actually higher than the expected,

given that the reported average in literature for Shortstim at 25ºC is around 5,5[96]. Despite this, once again there was no difference, confirming the results obtained for the RNAi. Therefore, it appears that Exo84 is not an essential subunit for the process of activity-dependent synaptic bouton formation.

Considering that the mutation results in a truncated protein, we hypothesised that the truncated protein could function during short periods of time but, if the stimulation was stronger and for longer periods, there would be an increase in demand for trafficking of cargo, and possibly a defect could be observed. To address this question, we used the protocol Longstim that is longer and has been shown to depend on new protein synthesis.

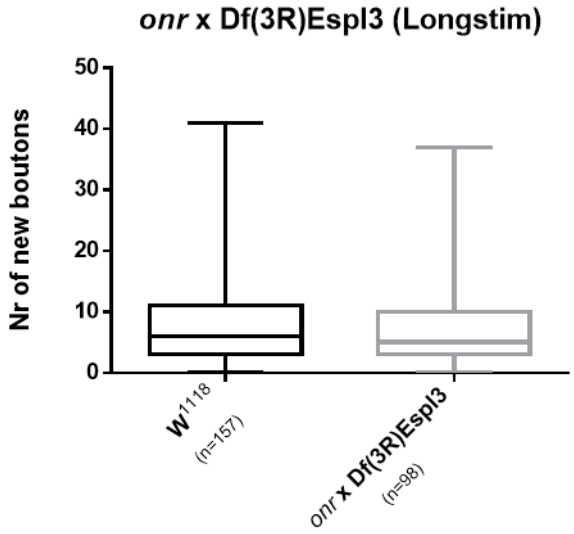

Figure 21 - Using the Longstim protocol with a mutation in the Exo84 *onr* gene does not impair activity-dependent bouton formation. The control used was W^{1118}, n=157 for the *onr*;Df(3R)Espl3 mutant of the subunit Exo84, n=98. The median is shown as a line in the box, along with the maximum and minimum values as the whiskers of the box plot.

Again, no statistical difference between the two conditions was observed. The averages reported for Longstim are barely higher than for Shortstim, with a value of around 6[1]. Both wild-type and the *onr* mutant have higher averages, with 8,2±0,59 and 7,2±0,63, respectively, and there is no statistical difference (Figure 21).

In conclusion, our experiments with both RNAi and the mutant, and using two different stimulation protocols, show no differences between control larvae and larvae with Exo84 defects. This appears to confirm that lack of Exo84 will not impair the process of formation of new synaptic boutons. This could be further tested with other mutants and other RNAi lines, but there is a good degree of confidence in the results obtained.

This result suggests that the assembly of the exocyst in *Drosophila* does not actually depend on Exo84 like in yeast or that another subunit can compensate and take on the same role, for example the phosphorylation of Exo70 by ERK1/2. Regarding RalA, the subunit Sec5 also interacts with it, which perhaps can mean that there is compensation. In terms of neuronal polarization, other subunits have been shown to be important for these mechanisms, so perhaps the exocyst works as a whole in that direction with no specific subunit being essential. Other subunits, such as Sec6 and Exo70, also have described interactions with the SNARE machinery and Rho GTPases, so perhaps their functions might compensate for the disruption of Exo84.

In conclusion, Exo84 does not appear to be an essential subunit for this process, regardless of the synaptic demands after the exposure to a longer or shorter stimulation protocol.

3.3.3. Sec3

Sec3 also interacts directly with the membrane and with actin regulating Rho GTPases[31,106]. Besides that, it also interacts with t-SNAREs, and plays a role in facilitating the assembly of certain components of the SNARE machinery[102]. The absence of Sec3 was shown to interfere with tip growth in maize[99], and it has been hypothesised that it functions as a spatial landmark[15,51], although there is no consensus on that.

This subunit can then be involved in both the regulation of membrane morphology and in SNARE mediated fusion.

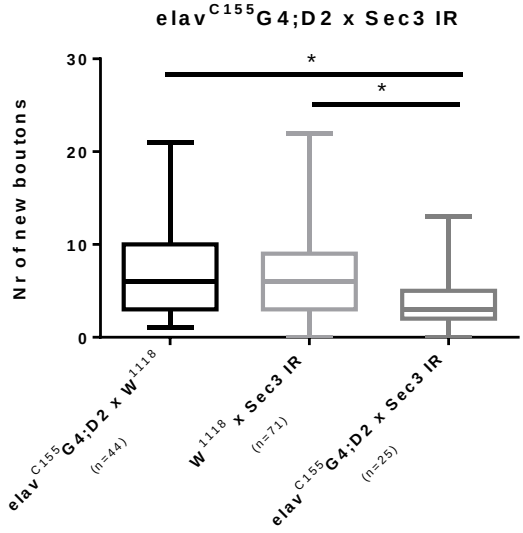

Figure 22 - Neuronal reduction of Sec3 impairs activity-dependent bouton formation. Genotypes represent: Gal4 control (elav^{C155}G4;D2 x W^{1118}, n=44), RNAi control (W^{1118} x Sec3 IR, n=71) and neuronal reduction of Sec3 (elav^{C155}G4;D2 x Sec3 IR, n=25). The median is shown as a line in the box, along with the maximum and minimum values as the whiskers of the box plot. * $p<0.05$

When we subject larvae to the Shortstim protocol, we observed that Sec3 reduction in neurons gives rise to significantly fewer boutons than both the controls, with an average of 7,0±0,75 and 7,1±0,65 for the controls and of 4,0±0,69 for the RNAi (Figure 22). This indicates that Sec3 is important for the process of formation of new boutons, although exactly which interactions are important cannot be concluded.

Sec3 is not the only subunit that interacts with the membrane and with Rho GTPases, Exo70 also shares some of these interactions. Therefore, it is interesting to observe that results for Sec3 indicate that this subunit is essential for the formation of new synaptic boutons, while the results for Exo70 do not. This could indicate that, despite sharing similar interactions, a functional Sec3 is more important for the process of new synaptic bouton formation than the subunit Exo70.

Overall, Sec3 is a good candidate to be a molecular player in the process of formation of new boutons and, given its known interactions with actin-binding components and with the plasma membrane, it is likely that it may have a role related to remodelling, rather than membrane addition.

3.3.4. Sec5

Sec5 has been shown to interact with RalA[22], similarly to Exo84. The fusion of synaptic vesicles is independent of Sec5 functionality[66], but it does play a role in the establishment of polarity[69], cell migration[34], and membrane addition[68]. Most importantly, null mutants of this subunit are lethal at 1st larval stages, and maternal contribution is the only reason they make it to that stage[66]. Relative to the NMJ of *Drosophila*, mutants have been shown to not increase bouton number during development[66], and to have defects in postsynaptic membrane growth[94]. The polarity establishment and role in membrane addition could also play a role in the formation of new boutons, which makes Sec5 a good candidate to be involved in the process.

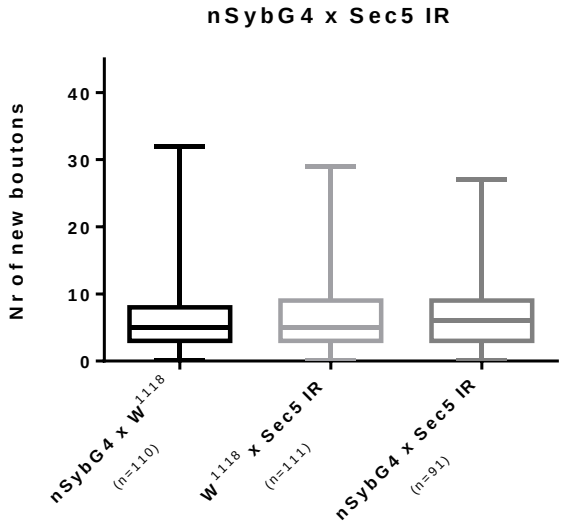

Figure 23 - Neuronal reduction of Sec5 does not impair activity-dependent bouton formation. Genotypes represent: Gal4 control (nSybG4 x W^{1118}, n=110), RNAi control (W^{1118} x Sec5 IR, n=111) and neuronal reduction of Sec5 (nSybG4 x Sec5 IR, n=91). The median is shown as a line in the box, along with the maximum and minimum values as the whiskers of the box plot.

However, after induction of activity-dependent bouton formation, we observed no difference between the controls and RNAi larvae, with the averages being 6,5±0,56, 7,0±0,59 and 6,5±0,51, for the two controls and Sec5-IR respectively (Figure 23). The fact that there is no phenotype is somewhat unexpected given all the neuronal phenotypes already described for this subunit. However, to completely discard a role, we would have to analysed null mutants. If we assume that the RNAi was efficient, this could mean that bouton formation processes are different during development *vs.* in response to acute activity.

In conclusion, these results need to be confirmed by using other RNAis or mutants, because the lack of disruption of bouton formation is not in agreement with a plethora of data that would suggest a role for this subunit with all the data that is described for this subunit. But it would be interesting if acutely formed boutons rely on a different molecular machinery than the one used by the organism during development.

3.3.5. Sec6

Almost all the interactions described for Sec6 are with components of the SNARE machinery: Snc2, Sec9, Sec1, Sso1[12,62,89]. The other interactions are with the subunits Sec5 and Sec8[67,90]. This seems to indicate that the role of Sec6 is likely connected with a function in the mediation of the attachment of the secretory vesicle to the plasma membrane SNAREs, by correctly positioning the complex (and therefore the vesicle) at the site of secretion. Even though other subunits show some interactions to SNARE components, Sec6 is by far the one with more members and therefore more direct.

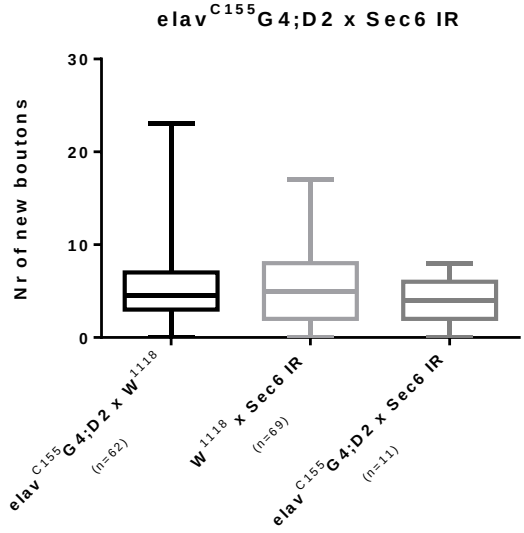

Figure 24 - Neuronal reduction of Sec6 does not impair activity-dependent bouton formation. Genotypes represent: Gal4 control (elav^{C155}G4;D2 x W^{1118}, n=62), RNAi control (W^{1118} x Sec6 IR, n=69) and neuronal reduction of Sec6 (elav^{C155}G4;D2 x Sec6 IR, n=11). The median is shown as a line in the box, along with the maximum and minimum values as the whiskers of the box plot.

In our experimental setup, the controls induced the formation of 5,3±0,52 and 5,7±0,47 boutons for the driver and the RNAi respectively. These values are in a normal/low range of values. Surprisingly, the Sec6-IR larvae have an average of 3,8±0,74, which is not statistically different from the controls (Figure 24). However, Sec6-IR larvae have a very distinctive phenotype that is observable by the naked eye, and that explains

the marked difference in the n of animals analysed in Figure 24, between controls and RNAi larvae. In the plates used to grow the larvae, it is normal to see the agar revolved and the larvae digging into the food; however, in the case of the RNAi the agar was almost intact, meaning that the larvae were not digging into it (Figure 25). All plates, in groups of three, represent both controls and the RNAi larvae, originating from crosses that were made in the same day with the same numbers of initial flies of the respective genotypes. They were all kept in cage systems at 29ºC and flipped at the same time points.

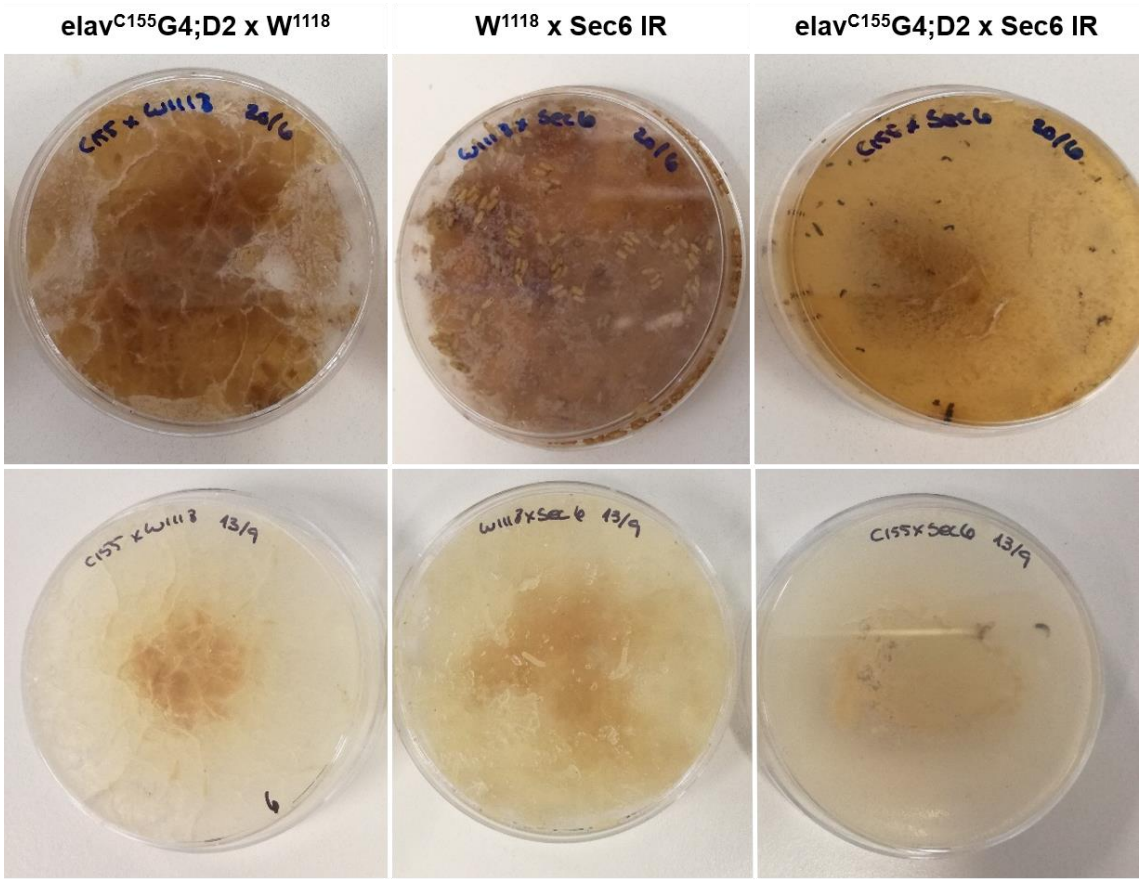

Figure 25 - Neuronal reduction of Sec6 results in larval defects. The depicted agar plates are part of two groups of three plates (two controls and IR larvae). The first column has the genotype of elav^{C155}G4;D2 x W^{1118}, as a control for the Gal4. The second column has the genotype of W^{1118} x Sec6 IR, which functions as a control for the RNAi. The third column has the RNAi larvae, with the genotype elav^{C155}G4;D2 x Sec6 IR. In both plates for the controls, the food is heavily disturbed and destroyed from the larvae feeding and burrowing. The plate for the IR larvae has almost no tracks and in the first one, there are several dead larvae in the surface of the food.

After some days, the food from the control plates (first and second) was destroyed and loose, from all the digging the larvae did. The food from the RNAi larvae was almost intact and there were also a lot of dead larvae on the surface, which might be either a

phenotype by itself or a result of the lack of burrowing.

In addition to this phenotype, selecting males was harder for these larvae, suggesting an associated lethality. Among all the larvae that developed on the plates only a very small number was male (approximately 10%). Females, although easier to find, were also not present in numbers that matched the controls or even the RNAi larvae for other subunits. This appears to indicate that disruption of expression of Sec6 in neurons is enough to induce a strong phenotype and perhaps lethality.

These three observations do not match the lack of significance seen with statistical analysis. However, it is possible that the difference in the sample size renders the statistics less powerful, and we will need to increase the n before concluding. It is also possible that the larvae analysed were escapers. One hypothesis is that, since Sec6 binds to several components of the SNARE machinery, this subunit is responsible for the guidance of the complex in the orientation of the vesicle and, if this process is defective, there is no orientation to the movement then perhaps the fusion cannot occur properly.

Another possibility is that the RNAi for Sec6 used is interfering with other molecular targets. This would mean that both Sec6 and another target are being interfered with, and the results obtained might be due to off-target disruption.

Finally, to be able to conclude if Sec6 is required for new bouton formation, we will need to test with other RNAis and mutants, so that we can draw clear conclusions.

3.3.6. Sec8

Sec8 hypomorph mutants have increased number of boutons in the NMJ and more microtubules[49]. If the mutants have more boutons than the controls, that would indicate that somehow this process is enhanced by the lack of Sec8 or that the mutation somehow made the subunit more efficient. This last option is unlikely considering that these same mutants die before they pupate, meaning that the disruption of Sec8 is lethal at a later stage. Considering that during development Sec8 hypomorphs create more synaptic boutons, it would be expected that the same would happen in Sec8-IR when stimulated; however, this is not what our results show.

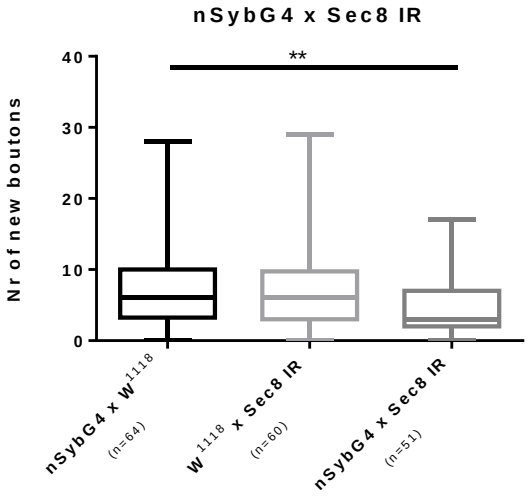

Figure 26 - Neuronal reduction of Sec8 impairs activity-dependent bouton formation. Genotypes represent: Gal4 control (nSybG4 x W^{1118}, n=64), RNAi control (W^{1118} x Sec8 IR, n=60) and neuronal reduction of Sec8 (nSybG4 x Sec8 IR, n=51). The median is shown as a line in the box, along with the maximum and minimum values as the whiskers of the box plot. ** $p<0.01$

After stimulation, both the controls are within the expected interval of new bouton formation, with averages of 7,2 for both (±0,64 for the Gal4 control, and ±0,82, for the IR control). The RNAi larvae show a value of 4,6±0,53 new boutons post stimulation, which is statistically lower than the control for the Gal4 (Figure 26).

In the end, we are left with the question about the difference between the previous results for hypomorph mutants and these RNAi results. The mutants create more boutons during larval growth when compared to controls, but then Sec8-IR is not capable of forming new boutons at the same rate as its controls. This indicates that perhaps the mechanism for developmental formation of new boutons is different from the activity-dependent bouton formation, which is in agreement with the hypothesis put forward in the results for the subunit Sec5.

In conclusion, Sec8 is a good candidate to be involved in the process of formation of new boutons, and further testing, by inducing activity-dependent bouton formation on the hypomorph mutants, for example, would confirm whether this subunit plays a role in this process.

3.3.7. Sec15

The role of Sec15 can be roughly divided in two main aspects: its interaction with the vesicle and its interaction with Sec10. The direct binding to Rab GTPases present on the vesicle surface[14,28,83,84], indicates that Sec15 is responsible for binding the secretory vesicle and connecting it to the complex via Sec10[28,81]. Given this, we hypothesised that if membrane addition is required for bouton formation, this subunit would likely be required to target secretory vesicles to the place of bouton formation.

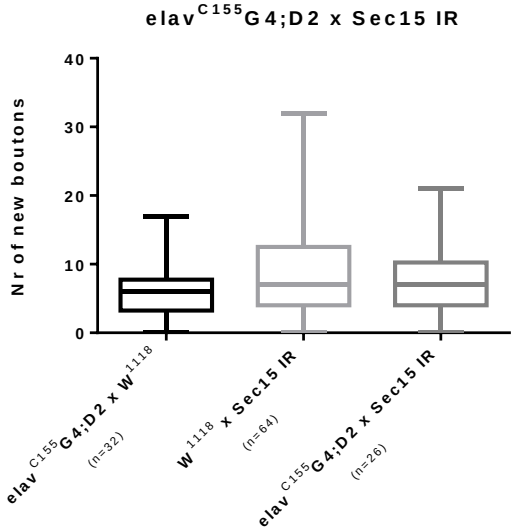

Figure 27 - Neuronal reduction of Sec15 does not impair activity-dependent bouton formation. Genotypes represent: Gal4 control (elav^{C155}G4;D2 x W^{1118}, n=32), RNAi control (W^{1118} x Sec15 IR, n=64) and neuronal reduction of Sec15 (elav^{C155}G4;D2 x Sec15 IR, n=26). The median is shown as a line in the box, along with the maximum and minimum values as the whiskers of the box plot.

However, after stimulation, there is no difference between the three sets of larvae studied, with the control for the neuronal driver forming on average 6,1±0,68 boutons, while the control for the RNAi formed 8,7±0,86. For the RNAi larvae the average was 7,5±0,99 which between both of the controls (Figure 27).

Assuming that the results are representative, the RNAi larvae for Sec15 had no defects in making new boutons in response to stimulation, which indicates that, despite the seemingly exclusive interaction of Sec15 with the secretory vesicle, it is not essential for this process. Perhaps the connection of the subunit Sec6 to the SNARE components of the vesicle is sufficient to chaperone the vesicle to the plasma membrane, or there are other interactions that have not been described yet. Overall, Sec15 does not appear to be necessary in the process of synaptic bouton formation.

Due to time constraints, the subunit Sec10 was not tested, leading unfortunately to a slightly incomplete picture of the exocyst complex.

3.3.8. Potential issues with RNA interference

The use of RNAi comes with some caveats, that must be addressed.

As mentioned previously, the lack of significant difference between the controls and the neuronal knock down of each of the exocyst subunits is not necessarily indicative of a lack of function, since the RNAi only reduces the expression of the protein and does not completely eliminate it. As an additional step, the levels of protein expression can be tested, confirming that the percentage of targeted protein is indeed decreased. However, even if the protein levels are decreased there might still not be a phenotype, meaning that the remaining percentage of the protein might be sufficient to fulfil its role in the process.

Another problem can arise if the RNAi is indeed working but interfering with the wrong target. This would mean that there will be a phenotype, but due to knock down of the wrong target.

However, the off-targets of the RNAi can be predicted, and Table 9 summarises the information provided by the stock centres.

Table 9 – Described off-targets for the RNAi lines used. Indication of the subunit of the eoxcyst, predicted off-targets provided by the stock centres with a brief description, and the reference of the RNAi line

	Off-targets and brief description	**Reference**
Exo70 IR	No off-targets	VDRC #103717
Exo84 IR	No off-targets	BDSC #28712
Sec3 IR	CG5999 – UDP-glucosyltransferase[17] CG7718 – Phosphatidylcholine-hydrolysing phospholipase D[18] CG8213 – Membrane-anchored trypsin protease present in the tracheas[20] CG15336 – Zinc finger[16]	VDRC#35806
Sec5 IR	No off-targets	BDSC #50556
Sec6 IR	No off-targets	VDRC #22077
Sec8 IR	No off-targets	BDSC#57441
Sec15 IR	No off-targets	VDRC #105126

Of all the RNAis, only the line used for Sec3 has predicted off-targets. Meaning that of the results obtained for this subunit are the only ones that might be due to disruption of other components. Taking in account the fact that Sec3 was one of the subunits that showed an impairment in the process of forming new synaptic boutons, confirmation of the results is essential.

In the end, despite the issues that might arise from the use of RNAi, a good degree of confidence was assumed for the results obtained.

4. Conclusions

The results present here are not the whole story, far from it. They have allowed us, however, to advance some steps in the direction of understanding the mechanism by which activity-dependent boutons form at the *Drosophila* NMJ.

The fixed imaging of the NMJ with different subunits tagged, allowed us to determine the localization of three separate subunits to the new synaptic boutons. Assuming that indeed only necessary components are trafficked to the new boutons, we can conclude that the exocyst is likely playing some role in the process, despite us not being able to exactly determine which role that is. This is important because it not only supports our hypothesis, but it is also encouraging to further move in this direction. Considering the panoply of processes in which the exocyst is involved, gathering all the information and constructing a clear image might be a laborious task but not an impossible one.

To further study this complex it would be interesting not only to know that it is present in the new bouton, but to see its dynamics using live imaging. With this we could potentially see the moment when it concentrates on a specific place, which one would assume would be the point where a new bouton is going to form, right before it occurs. With live-imaging, it would also be interesting to tag two different subunits and follow them throughout the process. This could enlighten us regarding the assembly of the exocyst, and its arrangement as the two subcomplexes described for mammalian cells. This is however challenging from a technical point of view, but if we manage to identify a group of fluorophores that would allow imaging of the membrane together with two distinct exocyst components, while ensuring that there was no bleed-through, it would be a very informative experiment. Co-localizing the exocyst with synaptic vesicles would also be an interesting exercise in molecular timing, to confirm the conclusions of Hazuka *et al.* in 1999 that the exocyst precedes the accumulation of synaptic vesicles[36]. Considering that synaptic vesicles are one of the first components to be localized to new synaptic boutons, these results would give us some insight into a timeline of events.

The RNAi experiments performed are not complete enough to draw some concrete conclusions. Unfortunately, the effectiveness of the RNAi was not tested, and this is a big drawback. Besides testing protein expression levels, it would also be relevant to test mutants of the subunits just like it was done for Exo84. There have been mutants reported

for almost all the subunits, which would allow us to have an additional set of results to compare to the ones that already exist. In the end, using all the lines available to us, to test and retest each of the subunits, would allow us to have more sturdy results that could confirm the importance of each subunit during the process of bouton formation. However, given that the majority of these mutants is 1st instar lethal, we would have to characterize activity-dependent bouton formation in these early stages, or do our analysis in a clonal manner.

Overall, the subunits that show more promise to be involved in the formation of new boutons are: Sec6, Sec3 and, probably, Sec8 and Exo70. They account for interactions with the SNARE machinery, the membrane and postsynaptic components, and again the membrane, respectively. Other subunits showed no signs of being essential to the process, but it cannot be concluded that they do not play a role, because we do not know how efficient the RNAi was. It is interesting however, to ponder whether each subunit is differentially important for distinct processes. Also, because we do have an effect with a few of the subunits that are reported to be in a subcomplex, it is unlikely that all the other subunit's RNAi was less efficient than the one for the subgroup that had an effect.

Perhaps for the specific case of activity-dependent synaptic bouton formation, there are interactions that are essential while others are not. For example, the fact that both Sec3 and Exo70 appear to play a role in the process but not Sec15, might indicate that interactions with cell membrane morphology are more important than the connection to the vesicle, or that the connection between Sec6 and the vesicle is enough for the task at hand. One can envision a scenario where membrane rearrangements are critical for bouton initiation, while membrane addition can be used for, but not essential, for bouton growth.

Further experiments are needed in order to confirm or refute the results obtained from the previous experiments. This would allow for clear conclusions to be drawn regarding not only the role of each of the subunits in the formation of new synaptic boutons but also how interchangeable they are in their functions, and the relative importance of their specific interactions for different processes.

Interesting as well, would be to knock down pairs of subunits with the same type of interactions to see if that further abolishes function. For example, both Sec3 and Exo70 interact with the membrane, and disrupting both eliminates the possibility that one might be compensating for the other. This would allow us to have some knowledge, not specific

for each subunit, but specific to each type of interactions.

In summary, the exocyst is present in the new synaptic boutons and 4 subunits of the exocyst: Sec3, Sec6, Sec8 and Exo70, appear to play a role in the process of forming these new neuronal structures.

In the end, this is a step, however small, in the direction of understanding the molecular mechanism that orchestrates the formation of new synaptic boutons.

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