**THESIS AND DISSERTATION**

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**Thesis title:**

# Maintaining neuronal function: the role of Gooseberry, the Pax3/7 homologue, in controlling synaptic growth, plasticity and stability.

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# CERTIFICATE OF APPROVAL

## PH.D THESIS

**This is to certify that the Ph.D. thesis of**

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**has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Biology at the December 2019 graduation.**

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Cortactin is a Regulator of Activity-Dependent Synaptic Plasticity Controlled by Wingless.
Daniel Alicea, Marizabeth Pérez[1], Carolina Maldonado, Carihann Dominicci-Cotto, Bruno Marie. The Journal of Neuroscience (Feb 22, 2017), 37(8):2203-2215. PMID: 28123080. **[1]Co-first author**.

Identification and Characterization of the V(D)J Recombination Activating Gene 1 in Long-Term Memory of Context Fear Conditioning.
Edgardo Castro-Pérez, Emilio Soto-Soto, Marizabeth Pérez-Carambot, Dawling Dionisio-Santos, Kristian Saied-Santiago, Humberto G. Ortiz-Zuazaga, and Sandra Peña de Ortiz. Neural Plasticity.Volume 2016, Article ID 1752176. 19 pages. PMID: 26843989. **Third author**.

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## ABSTRACT

Although neurons are long-lived cells little is known about the mechanisms responsible for maintaining their properties and cellular stability. Here, we investigate the pair-rule transcription factor Gooseberry (Gsb), previously known to contribute to neuroblast and neuronal fate determination during early embryogenesis. Specifically, we ask whether Gsb is responsible for the maintenance of basic neuronal properties within developed and functioning motoneurons, after fate determination has occurred. Interestingly, we find it is required at late stages of neuronal life to curtail synaptic growth and plasticity. Gsb loss of function provokes overgrown and over-plastic synapses while its overexpression generates undergrown and under-plastic synapses. We also show that it is essential for the stability and integrity of the synapse. Indeed, genetic manipulations downregulating Gsb provoke synaptic retractions, a hallmark of neurodegenerative diseases. Using transgenic combinations allowing the temporal control of Gsb under- or overexpression, we show that these phenotypes can be generated long after Gsb's requirement for fate determination. In some cases, Gsb misexpression for tens of minutes or a few hours is sufficient to provoke drastic changes in fully mature motoneuron synapses. Finally, we show that Gsb's ability to regulate growth at the synapse is the result of its antagonism to the secreted Wingless signal (Wg, the Wnt homolog). We present the first evidence that Gsb acts downstream of Wg and upstream of the protein kinase Shaggy (the Gsk3β homolog) to antagonize the Wg signaling pathway. We also describe a possible neuroprotective role for Wg at the Drosophila NMJ. Lastly, we explore the role of a Wg pathway inhibitor, Casein Kinase 1α in

synaptic growth, plasticity and stability and found that it resembles Gsb's effects at the synapse.

## PUBLIC ABSTRACT

Our brain cells allow us to learn, move and think. Our capacity to live and function depends on the communication between brain cells, also known as neurons. We are interested in understanding how certain molecules affect the structural components that allow this communication. We use the fruit fly as a model to look at changes in the morphology of neurons that connect to muscles. This connection, or synapse, is very similar to those that we have in our brains which makes the fruit fly a useful model to study brain function. In our study, we found that a protein that regulates the expression of genes during early development is also important to maintain the function of mature neurons.

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## ABBREVIATIONS

| | |
|---|---|
| ADSP | Activity-dependent synaptic plasticity |
| APC | Adematous Polyposis Coli |
| Arm | Armadillo |
| Arr | Arrow |
| BMP | Bone Morphogenetic Protein |
| Brp | Bruchpilot |
| CK1α/*ck1α* | Casein Kinase 1 α |
| CNS | Central Nervous System |
| Dlg | Discs-Large |
| Dsh | Dishevelled |
| Fz | Frizzled |
| GluR | Glutamate Receptor |
| Gsb/*gsb* | Gooseberry |
| HRP | Horseradish Peroxidase |
| MN | Motoneuron |
| NB | Neuroblast |
| NMJ | Neuromuscular Junction |
| Pan | Pangolin |
| RNAi | RNA interference |
| Sgg/*sgg* | Shaggy |
| SSR | Subsynaptic Reticulum |
| Syn | Synapsin |
| TF | Transcription Factor |
| Wg/*wg* | Wingless |

# Maintaining neuronal function: the role of Gooseberry, the Pax3/7 homologue, in controlling synaptic growth, plasticity and stability.

# CHAPTER 1

# INTRODUCTION

A major goal of the scientific community is to understand the intricacies of the nervous system. As humans, we can think, act and feel thanks to the activity of neuronal networks that control our bodily functions throughout our lifetime. The activity of these networks relies on the maintenance and functionality of contacts between neurons and other neuronal or non-neuronal cells. These neuronal contacts or synapses are the key components for the transmission of information within the nervous system and ensures its proper function. Effective synaptic transmission confers our ability to interact with the environment, do complex behaviors and ultimately live. Thus, great attention has been given to the study of structure and function of synapses in vertebrates and invertebrates.

The development of neurons and their respective synapses are the consequence of gene expression regulation by transcription factors (TFs) at the onset of nervous system assembly. Our goal is to understand how neurons maintain their functionality after the nervous system is established. We want to elucidate if TFs that were required during nervous system development are also required to maintain the functionality of mature neurons. Our findings will help us understand the molecular mechanisms that could be related to aging and neurological disorders in humans. To study the role of TFs in maintaining neuronal function we use a simple model: the synapse between a motoneuron and the muscle of a fruit fly. The purpose of this introduction is to describe the importance of transcription factors during nervous system development, to explain the

basis of neuronal properties such as synaptic growth, plasticity and stability, and illustrate how *Drosophila melanogaster* is a useful model organism to understand these properties.

## TRANSCRIPTION FACTORS DURING NERVOUS SYSTEM DEVELOPMENT

The construction and complexity of the nervous system is achieved by the activity of TFs that are deployed throughout development in a sequential manner and at different time periods. The specific gene regulation that TFs control during nervous system development defines the cellular fate and diversity of neuronal cells and determines their structural networks. These TFs act combinatorially to regulate their own expression and the expression of many other genes to commit stem cells into specific cell lineages that will differentiate into neuronal or glial cells (Miyares and Lee, 2019; Ooi and Wood, 2008; Santiago and Bashaw, 2014).

The distinct regulatory activities that TFs control during neurogenesis have been categorized to specify their function (Allan and Thor, 2015). Spatial selectors (Fig.1.1A) determine the compartments in which neuronal stem cells are going to proliferate and differentiate. These TFs define how the embryonic nervous system is going to be patterned and each developmental program that neuroblasts (NBs) will undergo in their respective cellular compartments (Bhat, 1999; Garcia-Bellido, et al., 1972; Lewis, 1978; Prokop, et al., 1998; Skeath, JB, 1999; Holguera and Desplan, 2018). Interestingly, there is conservation between *Drosophila* and vertebrates in terms of the spatial selectors that

pattern the embryo and neuroectoderm (Arendt and Nübler-Jung, 1999; Holland et al., 2013).

Another category for developmental TFs is temporal selector (Fig.1.1B). This type of TF specifies the distinct temporal identities neuronal precursor cells acquire throughout development (Bayraktar et al., 2010; Jacob et al., 2008; Li et al., 2013; Zhu et al., 2006; Holguera and Desplan, 2018). Each NB lineage has distinct developmental programs at specific time points. In this manner, NBs transform into different cell types through time until they acquire their final neuronal fate. The genetic activity that temporal selectors control is crucial for nervous system development because an impairment in such activity can lead to the loss of neuronal cell lineages or prolonged expression of some of them (Isshiki et al., 2001; Kambadur et al., 1998).

TF activity is also necessary for the final differentiation of cells into their corresponding neuronal cell type (Fig.1.1C) (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996) and are required to maintain their cell identity (Fig.1.1D) (Hobert and Kratsios, 2019; Stratmann et. al, 2019; Zhang et al., 2014). TFs define different neuronal characteristics such as neurotransmitter identity (Pym et. al., 2006; Thor & Thomas, 1997; Wolfram et al., 2012), electrophysiological properties (Wolfram et al., 2014) and axon pathfinding (Lundgren et al., 1995; Marie et al., 2002; Miguel-Aliaga et al., 2004; Thor et. al., 1999). Once neurons are spatially established, in contact with their postsynaptic partners and fully differentiated, they must be functionally competent throughout the organism's lifetime. A pending question in the field is whether TFs are required throughout the lifetime of neuronal cells to maintain their functionality. It has been shown that the maintenance of the identity of certain mature neuronal subtypes in

*Drosophila* require persistent TF activity in the adult organism (Doucet-Beaupré et al., 2015; Eade et al., 2012; Hobert, 2011; Hobert & Kratsios, 2019; Stott et al., 2013). In addition, we have shown that TF activity is required for synaptic homeostasis of fully developed motoneurons (MNs) (Marie et al., 2010). We want to explore the possibility that TFs are important post development for the maintenance of neuronal function.

## THE SYNAPSE

Neurons communicate with other neuronal or non-neuronal cells by electrical or chemical signals that originate at synapses. The basis of synaptic transmission lies in the proper alignment of presynaptic and postsynaptic structures (Fig.1.2) (Biederer et al., 2017). Presynaptic structures such as vesicles facilitate the release of neurotransmitters that will consequently interact with postsynaptic receptors (Südhof, 2018). This presynaptic-postsynaptic interaction puts in motion molecular signals that allow the transfer of information within the brain and the rest of the body. Although we have considerable understanding of the structural and functional framework of synapses (Frank and Grant, 2017; Sheng and Kim, 2011; Südhof, 2012, 2013), we still have much to learn about how they are dynamically changed and maintained.

### *Synaptic Growth, Plasticity and Stability*

The development of our nervous system depends on the proper establishment of synaptic connections between neurons and their target cells. The growth of the synapse dictates the number of connections that will be formed; thus, synaptic size is important for transmission efficacy. There is evidence that synaptic growth is determined by several factors. The size of the presynaptic nerve terminal can be influenced by the growth of the

postsynaptic cell (Balice-Gordon et al., 1990; Davis and Goodman, 1998). Competition between synapses to innervate the same postsynaptic target (Katz and Shatz, 1996; Sanes and Lichtman, 2009) and mechanisms of synaptic formation and elimination can shape the final growth of the synapse (Cohen-Cory, 2002, Eaton et al., 2002). In addition, several molecules have been implicated in synaptic growth mechanisms: growth factors (Aberle et al., 2002; Marqués et al., 2002), cell adhesion molecules (Schuster et al., 1996; Tanaka et al., 2000), neurotrophins (Huang and Reichardt, 2001), signaling molecules (Packard et al., 2002a), integrins (Beumer et al., 1999), microtubule associated proteins (Roos et al., 2000), poly (A) binding proteins (Sigrist et al., 2000), ubiquitin ligases (Wan et al., 2000), potassium channels (Budnik et al., 1990), cAMP proteins (Zhong et al., 1992), proteases (Diantonio et al., 2001), phosphatidylinositol kinases (Cantarutti, Burgess, Brill, & Dason, 2018), microRNAi's (Y.-W. Tsai et al., 2019), chaperonin interactors (Syed et al., 2019), and monoamine transporters (Sweeney and Davis, 2002). An ongoing goal in the field of neuroscience is to understand how these proteins interact to control synaptic growth and how their function is regulated.

The fact that synapses must acquire an adequate size to innervate their postsynaptic partners does not mean their size remains fixed for the entire lifetime of the neuron. Synapses must extend, but also contract and rearrange themselves in response to the environmental stimuli an organism receives. This experience-dependent synaptic plasticity is what allows an organism to adapt to external conditions and form long-lasting memories (Lee and Silva, 2009; Lisman et al., 2018). There are multiple types of synaptic plasticity that are characterized by functional and structural changes, (Caroni et al., 2012; Cheetham et al., 2014; Galimberti et al., 2006; Holtmaat and Caroni, 2016;

Holtmaat and Svoboda, 2009; Matz et al., 2010; Sigrist and Schmitz, 2011; Sugie et al., 2018; Zhao et al., 2012; Zhong et al., 1992) and these changes lead to an increase in synaptic strength (Monday et al., 2018; Hirano, 2018; Nicoll, 2017; Ho et al., 2011; Sigrist et al., 2003). The level of activity a neuron has can provoke structural remodeling of synapses in terms of bouton number and size (Monday and Castillo, 2017; Petzoldt, et al., 2016), insertion or removal of release sites (Petzoldt et al., 2016; Sigrist et al., 2003; Weyhersmüller et al., 2011), changes in postsynaptic densities (Meyer et. al, 2014; Petzoldt et al., 2016), vesicle pool redistribution (Petzoldt et al., 2016), changes in the clustering of proteins at active zones (Monday et al., 2018; Petzoldt et al., 2016; Sigrist et al., 2003; Sigrist and Schmitz, 2011; Weyhersmüller et al., 2011), modifications in conductance (Oh and Disterhoft, 2015; Kim et al., 2016; Siegelbaum et al., 1982; Yu et al., 2017), increased neuronal excitability (Lisman et al., 2018; Yu et al., 2017) and modulation of neurotransmitter release (Castillo, 2012; Yang and Calakos, 2013). It is important to elucidate how these functional and structural changes provide the cellular basis of proper brain function, learning and memory mechanisms, and disease.

Synaptic plasticity brings forth the ability to strengthen preexisting connections, and establish new ones, in order to enhance synaptic transmission, process information, conduct behavior and form memories. At the same time, synapses must remain stable to sustain long lasting connections that will maintain stored information. Thus, synaptic plasticity is required to form new memories, but synaptic stability is required to make them indelible. To establish new behavioral programs and insert new memories synapses must be labile and this includes not only the creation of new connections, but the disassembly of preexisting ones (Bailey, 1993; Lichtman and Colman, 2000). Thus, two

conflicting forces take place: one that promotes the stability of stablished synapses and one that promotes the development and/or elimination of synaptic connections. During development, these forces act to remodel and refine the formation of the nervous system (Katz and Shatz, 1996; Riccomagno and Kolodkin, 2015; Sanes and Lichtman, 2009). In mature neural circuits, processes of synaptic formation and elimination also occur to improve connections in response to stimuli (Caroni et al., 2012; De Paola et al., 2003; Eaton and Davis, 2003; Goda and Davis, 2003; Grutzendler et al., 2002; Holtmaat and Svoboda, 2009; Walsh and Lichtman, 2003). It is important then that these mechanisms of plasticity and stability are tightly regulated. The retraction of synapses is required to allow plasticity but, under a pathological context, unstable synapses may represent neurodegenerative events. Understanding the mechanisms that regulate synaptic stability will help us distinguish between synaptic elimination events that are plasticity driven, to those that are disease related.

***Synaptic Diseases***

Elucidating synaptic dynamics helps us understand how our nervous system works and find effective treatments at the onset of brain disease. Indeed, synaptic dysfunction is one of the hallmarks of neurodegenerative conditions (Lepeta et al., 2016; Luo and O'Leary, 2005; Lüscher and Isaac, 2009; Penzes et al., 2011). Several brain disorders have been linked to synaptic abnormalities such as excessive synaptic growth (Dubos et al., 2012; Nimchinsky et al., 2001), excessive remodeling of synapses (Cruz-Martín et al., 2010) and exaggerated regressive changes in connectivity (Sun et al., 2009). Genetic alterations in cell-adhesion molecules important for proper synaptic connectivity have been associated with autism (Südhof, 2008). Dysregulation of synaptic plasticity

can promote drug addiction (Mameli and Lüscher, 2011) and cognitive disabilities (Bliss et al., 2014). The most common synaptic abnormality found in neurodegenerative diseases is the loss of synaptic connections, suggesting that synaptic stability is compromised in these conditions. Studies on mental retardation, schizophrenia, Alzheimer's disease, Parkinson's disease, Huntington's disease and epilepsy have shown that a major characteristic of these pathologies is a significant degeneration of neuronal connections (Boksa, 2012; Day et al., 2006; Fuhrmann et al., 2007; Garey et al., 1998; Jiang et al., 1998; Knafo et al., 2009; H. Li et al., 2001; Selemon and Goldman-Rakic, 1999; Selkoe, 2002.; J. Tsai et al., 2004). Understanding the mechanisms that lead to axonal degeneration will advance our knowledge about these pathophysiological and neuropsychiatric diseases in order to develop better tools for diagnosis and treatment.

## *DROSOPHILA MELANOGASTER* AS A MODEL ORGANISM

The fruit fly has been used for over a hundred years as a powerful genetic tool to understand biological phenomena (Bellen et al., 2010). This model organism allows the removal or addition of genes in a very efficient and successful way, which helps us map out the gene products that are involved in different biological processes. The development of the Gal4-UAS expression system (Fig. 1.3) in this organism certainly became a breakthrough in all fields of research; a tool that provides spatial control of gene expression in a tissue-specific manner (Brand and Perrimon, 1993).

The embryonic, larval and adult stages of this organism have contributed great knowledge in the field of neuroscience in terms of mechanisms of development,

behavior, and cellular and molecular neurobiology (Bellen et al., 2010; C. A. Frank et al., 2013; Olsen and Keshishian, 2012). Fruit flies start their life cycle as embryos that eventually hatch into larvae. There are three larval stages: first instar (L1), second instar (L2) and third instar (L3). Larvae molt into each of these stages until they become pupae. The organism matures into an adult fly inside the pupal casing until it is ready to leave or eclose. Once eclosed, we can easily identify virgins to set up mating crosses that will yield the genetic backgrounds that we desire in the progeny. The development time of fruit flies varies with temperature. For example, at 25°C it takes around 5 days to acquire third instar larvae. The ease of rearing these organisms, the relatively short time to acquire larvae, and the vast availability of mutants and genetic tools at our disposal makes the fruit fly an excellent model to study biological problems.

***Morphology of the larval Neuromuscular Junction (NMJ)***

In larvae, MNs extend from the ventral nerve cord onto the muscles they innervate. Two MNs innervate abdominal muscles 6 and 7 (Hoang and Chiba, 2001), which are muscles commonly used for electrophysiological and morphological experiments. With immunohistochemistry, several components of the neuromuscular synapse can be studied. Antibodies against Discs-large (Dlg) (homolog to the mammalian PSD-95) mark postsynaptic densities (Budnik et al., 1996), Synapsin (Syn) highlights vesicles (Fdez and Hilfiker, 2006; Klagges et al., 1996), while horseradish peroxidase marks neuronal membranes (Jan and Jan, 1982). At each synaptic contact with the muscle a bouton forms. Synaptic boutons are in charge of neurotransmission (Menon et al., 2013; Schuster et al., 1996) and the sites where neurotransmitter release occurs are called active zones. The protein Bruchpilot (Brp) clusters in these active

zones which makes it useful to identify them (Wagh et al., 2006). On the postsynaptic side, glutamate receptors (GluRs) are present. These are heterotetramers that contain four different subunits (Featherstone et al., 2005; Marrus et al., 2004; Qin et al., 2005) that respond to neurotransmitter release. All the aforementioned proteins can be easily identified via immunohistochemistry to study morphological changes at the NMJ.

***Assessment of synaptic growth, plasticity and stability at the NMJ***

A common method to study changes in synaptic growth in *Drosophila* larvae is to quantify the number of boutons a synapse has (Fig. 1.4A). Synaptic growth is very stereotypical in these larvae; it does not vary in wild type animals. This makes it a useful model to identify genes that are required for synaptic growth. With this model several molecular processes have been implicated in growth mechanisms: bone morphogenetic signaling pathway (BMP), Wnt/wingless pathway (Wg), autophagy, endocytosis and changes in excitability (Budnik et al., 1990; Collins and DiAntonio, 2007; Dickman et al., 2006; Featherstone and Broadie, 2000; Keshishian, 1996; McCabe et al., 2004; Miller et al., 2012; Packard et al., 2002; Shen and Ganetzky, 2009).

Synaptic plasticity can also be assessed at the *Drosophila* NMJ. It has been shown that upon 5 cycles of spaced depolarizations, *de novo* synaptic structures form in response to changes in neuronal activity (Fig. 1.4B) (Alicea, Perez et al., 2017; Ataman et al., 2008). A significant increase in these *de novo* structures is indicative of enhanced synaptic plasticity. BMP, Wg, Syn and the actin regulator Cortactin have been found to

be important for the appearance of these activity-dependent synaptic structures (Alicea et al., 2017; Ataman et al., 2008; Piccioli and Littleton, 2014; Vasin et al., 2014).

To evaluate synaptic stability, we must focus on identifying presynaptic and postsynaptic components of the NMJ. During larval development, a series of postsynaptic membranes form into folds known as the subsynaptic reticulum (SSR). The SSR requires the presence of a presynaptic nerve terminal in order to form (Budnik et al., 1996; Featherstone and Broadie, 2000; Roos et al., 2000; Saitoe et al., 2001; Schuster et al., 1996), thus postsynaptic sites lacking their opposing presynaptic counterparts represent synaptic retractions (Fig. 1.5). Retractions are not commonly found in wild type NMJs, thus genetic manipulations that lead to an increase in the frequency of retractions will highlight genes that are required for synaptic stability. Molecules that have been implicated in synaptic stability include cytoskeleton-related proteins such as Adducin, Spectrin and Dynactin, and several kinases (Bulat et al., 2014; Eaton et al., 2002; Pielage et al., 2005; Pielage et al., 2011).

## THESIS OUTLINE

In this first chapter, we highlighted the roles that TFs have during nervous system development and we established that it is indispensable to understand their functionality post development. It has been shown that in fully grown and functional neurons some developmental TFs are still present, mostly to maintain neuronal identity and homeostasis. The primary aim of this thesis is to elucidate if transcription factors can control basic synaptic properties such as growth, plasticity and stability. With *Drosophila* we have efficient genetic tools to manipulate TF expression and good histological techniques to analyze morphological changes at the larval NMJ. Dissecting the molecular mechanisms that regulate synaptic dynamics can increase our understanding of synaptic transmission and dysfunction related to disease.

In chapter 2, we examine the role of the TF gooseberry (Gsb) in synaptic growth, plasticity and stability. We show that Gsb is an inhibitor of growth and plasticity, but a promoter of stability. If Gsb expression is perturbed exclusively after neuronal development, changes in synaptic growth, plasticity and stability are observed. These results suggest that Gsb actively controls these synaptic mechanisms post development.

Chapter 3 presents the molecular mechanisms that Gsb could be affecting to control synaptic growth, plasticity and stability. Our results suggest that Gsb antagonizes the Wg canonical pathway in synaptic growth and plasticity. It is suggested that the basis of this antagonism is the regulation of expression of a Wg pathway-related gene (or genes) by Gsb. If this is the case, our genetic interaction experiments imply that Gsb is

regulating the Wg pathway downstream of Wg but upstream of the kinase Shaggy (Sgg/homolog to mammalian GSK3β) (Cook et al., 1996).

Chapter 4 presents a possible Wg pathway-related candidate under Gsb regulation: Casein Kinase 1α (CK1α). This kinase is a known inhibitor of the Wg pathway (Liu et al., 2002). Based on our experiments, changes in CK1α expression mimics the synaptic phenotypes observed in Gsb related experiments. Thus, it is a possibility that Gsb could be regulating CK1α expression to inhibit the Wg pathway and control synaptic growth and plasticity. We also propose future experiments to answer pending questions.

Chapter 5 presents overall conclusions of our data. In appendix A and B we present additional experiments that confirm our initial findings.

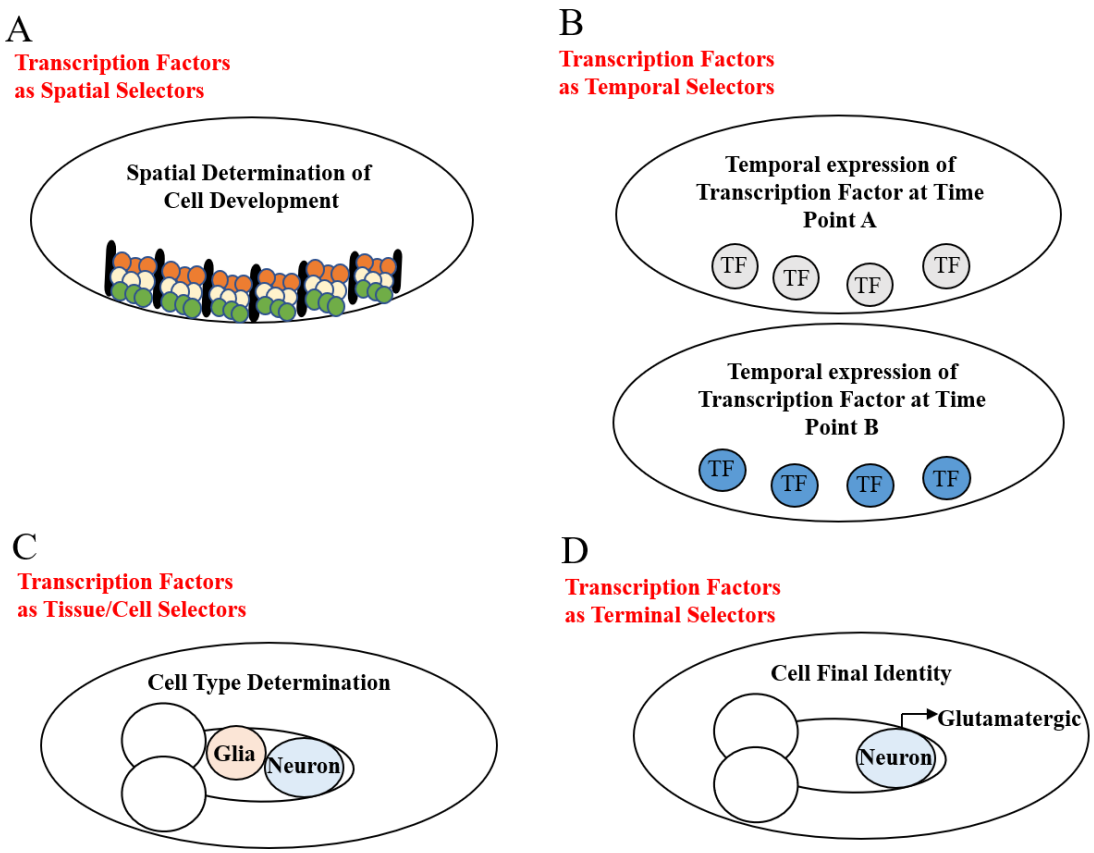


**Figure 1.1 Categories of TFs during nervous system development.**

Schematic diagrams of *Drosophila* embryos. Each diagram depicts different aspects of nervous system development that TFs control. **(A)** Patterning of the neuroectoderm by delimitating where specific NBs will differentiate (black bars). Each circle exemplifies a NB and each color represents a NB lineage that is spatially compartmentalized by a series of spatial selectors. **(B)** As NBs increase in number, TF expression changes through time to direct the development of these NBs. Here we show a group of NBs in which a transition in the expression of one TF (gray color) to another (blue color) has occurred. **(C)** As NB lineage differentiation progresses, TFs commit these cells to a glial or neuronal fate. **(D)** TFs that act as terminal selectors maintain the identity and function of postmitotic neurons.

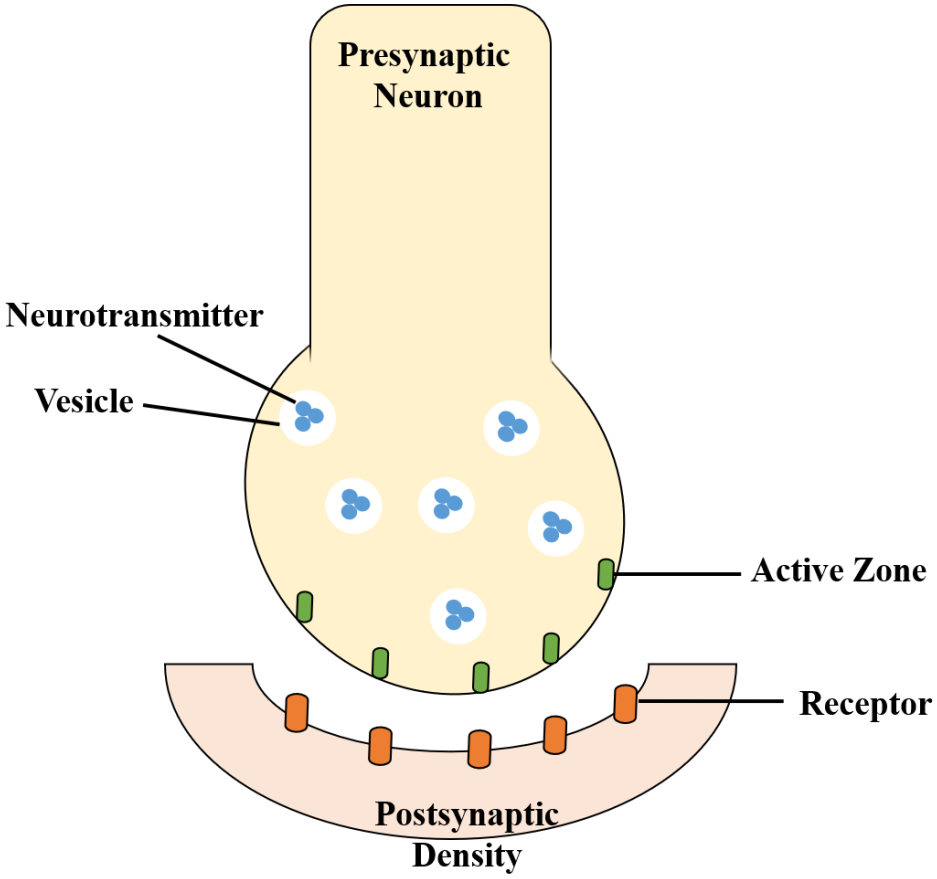


**Figure 1.2 Overview of a synapse.**

Schematic diagram of a synapse. A synaptic bouton of the presynaptic nerve terminal contains vesicles (white circles) that contain neurotransmitters (blue circles). Vesicles will dock at active zones (green rounded rectangles) to liberate neurotransmitters into the synaptic cleft. Lastly, neurotransmitters will interact with postsynaptic receptors (orange rounded rectangles) to continue synaptic transmission.

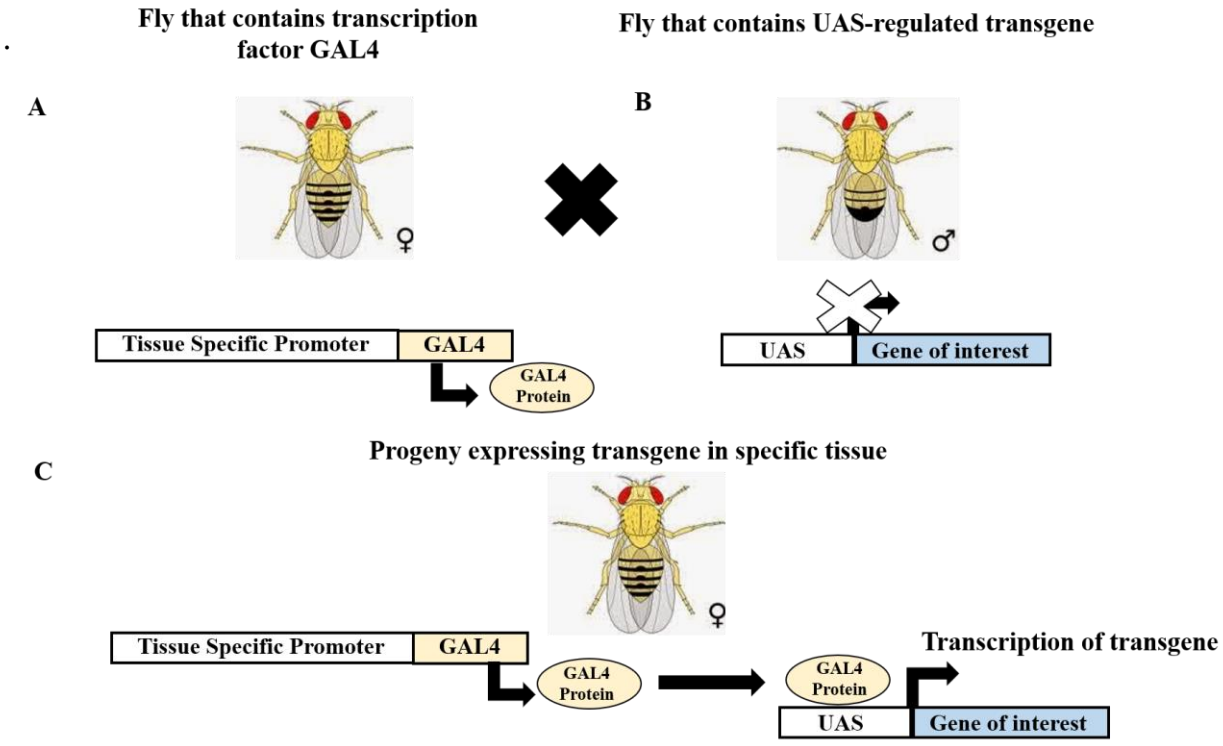


**Figure 1.3 The Gal4-UAS system.**

Diagram depicting how the Gal4-UAS gene expression system works. **(A)** In this example, a female fly expresses the TF Gal4 in specific cells. This female fly will be crossed (mated) with a male that contains the transgene of interest. **(B)** A male fly that contains the upstream activating sequence (UAS) to which the Gal4 will bind to and initiate transcription of an adjacent gene. Since this male fly does not have Gal4 expression, the UAS transgene is not transcribed. **(C)** The resulting progeny of the Gal4-UAS cross will express the tissue specific Gal4 and the UAS transgene. Cell specific transcription of the transgene will occur in this progeny.

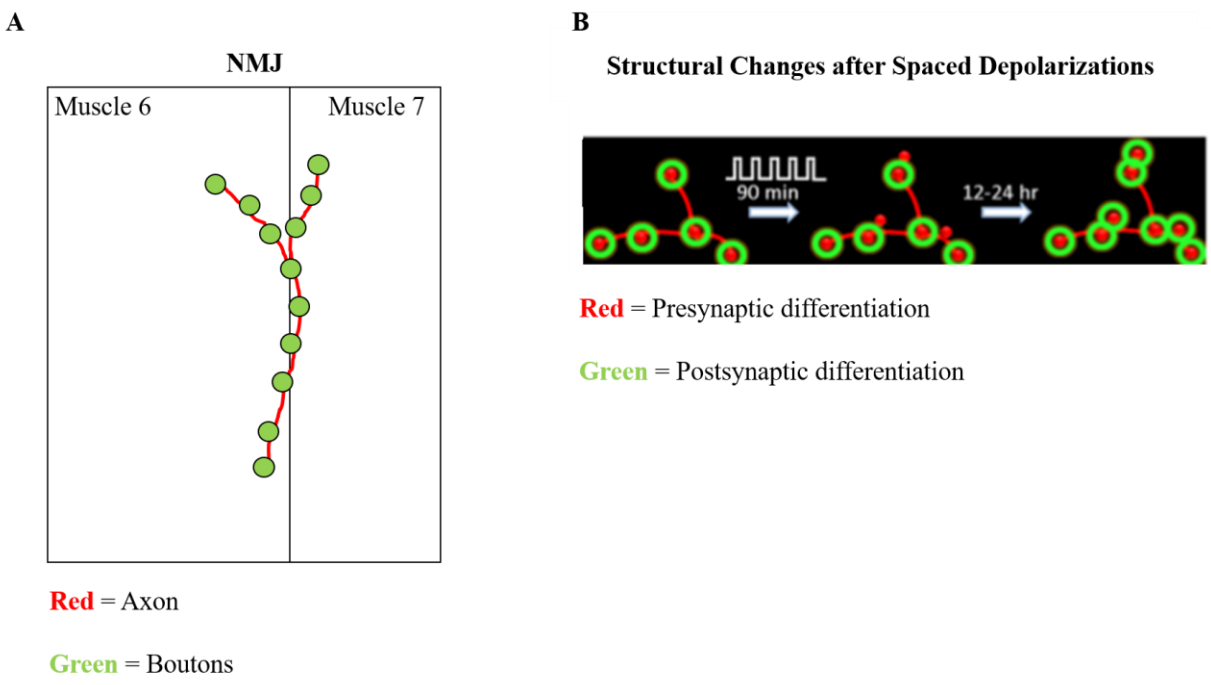


**Figure 1.4 A-B Assessment of synaptic growth and plasticity at the NMJ.**

Illustration of how growth and structural plasticity are studied at the larval NMJ. **(A)** Antibodies that mark the presynaptic membrane with HRP (red) and presynaptic vesicles with Syn (green) are used to identify the synapse of a MN that innervates muscle 6/7. The number of boutons is quantified in several animals using Syn immunoreactivity and an average is obtained for synaptic growth analysis. **(B)** A 90 minute stimulation protocol is subjected to larvae. This protocol consists of 5 spaced depolarizations with high $K^+$ concentrations. This stimulation provokes the appearance of new synaptic structures that only have presynaptic components. These structures are called ghost boutons. A significant increase in the number of ghost boutons is an indication of synaptic plasticity. Several stimulated animals are used to measure the number of ghost boutons after stimulation and average is determined to analyze the occurrence of plasticity. After a time period of 12-24 hours ghost boutons become functional boutons that have pre- and postsynaptic differentiation.

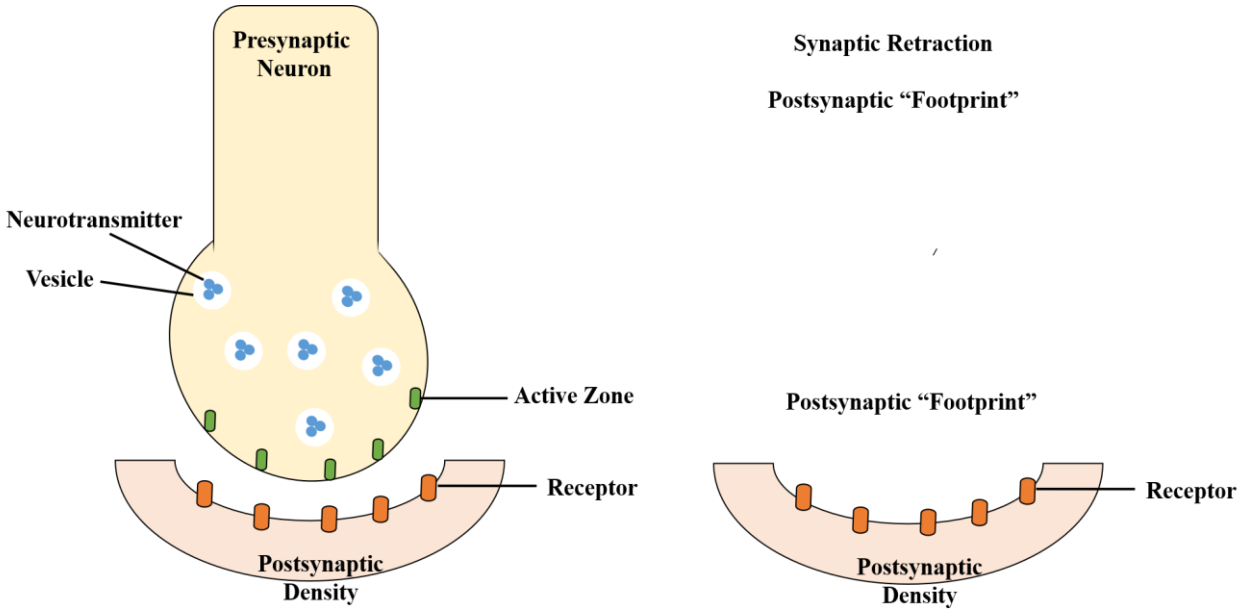


**Figure 1.5 Assessment of synaptic stability at the NMJ.**

During synaptic development, a presynaptic nerve terminal forms and reaches its target. The postsynaptic target requires the presence of its presynaptic counterpart to acquire postsynaptic differentiation. A stable synapse will have both presynaptic and postsynaptic components present; this is what is commonly observed in wild type animals. Genetic manipulations in *Drosophila* have revealed genes that are essential for synaptic stability. Events in which postsynaptic densities lack their presynaptic partners are called synaptic retractions. A significant increase in the frequency of retractions is an indication of instability at the NMJ.

# CHAPTER 2

# MAINTAINING NEURONAL FUNCTION: THE ROLE OF GOOSEBERRY, THE PAX 3/7 HOMOLOGUE, IN CONTROLLING SYNAPTIC GROWTH, PLASTICITY AND STABILITY

This thesis chapter is part of a manuscript titled:

**Maintaining neuronal function: the role of Gooseberry, the Pax3/7 homologue, in controlling synaptic growth, plasticity and stability.**

Abbreviated title: **Gsb and the maintenance of motoneuron synapses**

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## INTRODUCTION

The expression of TFs, within various cell types and at different stages of development, is essential for the setup of the nervous system. They can function as spatial or temporal selectors and form combinatorial codes that define the formation and fate of NBs and neurons (Allan & Thor, 2015; Holguera & Desplan, 2018; Miyares & Lee, 2019). TFs can also act as terminal selectors, acting just before the post-mitotic division or in post-mitotic neurons, to define and/or maintain neuronal identity and sub-identity (Hobert & Kratsios, 2019; Stratmann et al., 2019; Zhang et al., 2014). They have been implicated in determining axon pathfinding, choice of connectivity, the type of neurotransmitter and electrophysiological properties (Cho et al., 2014; Bruno Marie et al., 2002; Miguel-Aliaga et al., 2004; Pezier, Jezzini, Marie, & Blagburn, 2014; Pym et al., 2006; Thor et al., 1999; Wolfram et al., 2014). Furthermore, persistent transcription factor activity is required for the maintenance of the identity of certain mature neuronal subtypes (Doucet-Beaupré et al., 2015; Eade et al., 2012; Hobert, 2011; Hobert & Kratsios, 2019; Stott et al., 2013). This possibility that TFs could direct and maintain an array of neuronal features in long-lived animals has obvious consequences for our understanding of aging and neurological disorders. But are characteristics common to most neurons under the control of TFs? Are synaptic properties as basic and ubiquitous as growth, plasticity or stability, which would not be associated solely with a specific neuronal type or sub-identity, also controlled by TF expression? Or do they depend instead on permanent structural chromatin remodeling and/or on local synaptic signaling?

Here, we used the *Drosophila* larval NMJ to ask whether the TF Gsb, a *pax3*/*7* homolog characterized as a pair rule spatial selector during early embryogenesis (Duman-

Scheel, Li, Orlov, Noll, & Patel, 1997; X. Li, Gutjahr, & Noll, 1993), is involved in the maintenance of basic neuronal characteristics. In this system, the NMJs innervating the larval body wall muscles are set up during embryogenesis and function until the end of larval life. During larval life, fully functional NMJs expand as the muscles enlarge, and this system has been extensively used to decipher the basic mechanisms of synaptic growth. Subsequently, during the fully-grown third instar larval stage, this model synapse can be used to assess synaptic plasticity and stability (Broadie & Bate, 1995; Collins & DiAntonio, 2007; Gramates & Budnik, 1999; Harris & Littleton, 2015; H Li, Peng, & Cooper, 2002). Here we focus on the segment polarity gene *gsb* that is expressed in a distinct pattern within the neurectoderm and directs the fate of specific NBs (Deshpande, Dittrich, Technau, & Urban, 2001; Duman-Scheel et al., 1997; He & Noll, 2013; X Li & Noll, 1993; Patel, Schafer, Goodman, & Holmgren, 1989; Skeath, Zhang, Holmgren, Carroll, & Doe, 1995). After embryogenesis, very little is known about the role of *gsb*, although it continues to be expressed in the majority of the fully developed MNs (and other neurons) within the late larval CNS where it is required to maintain synaptic homeostatic compensation (B. Marie, Pym, Bergquist, & Davis, 2010).

In this study we show that Gsb functions within fully developed MNs to control synaptic growth, plasticity and stability. Interestingly, we show that it is a negative regulator of growth and plasticity while being necessary for the maintenance of synapse integrity. In particular, we show that changing *gsb* expression minutes to hours before assessing these characteristics is sufficient to reveal a phenotype, proving *gsb*'s role in maintaining these neuronal functions.

## MATERIALS AND METHODS

### *Fly stocks and genetics*

We used the *Gal4/UAS* system (A H Brand & Perrimon, 1993) to overexpress genes or express RNA interference (RNAi) constructs. To drive expression of genes in neurons at the embryonic stage we used the *elav$^{C155}$-Gal4* line [Bloomington Drosophila Stock Center (BDSC), stock #458]. To drive gene expression in neurons at the late second instar stage we used the line *D42-Gal4* (BDSC, stock #8816). The line containing *UAS-gsb* was a gift from Dr. Markus Noll (University of Zurich, Switzerland). Gsb loss of function animals were engineered by combining within the same animal a Gal4 driver (*D42-Gal4* or *elav$^{C155}$-Gal4*), a null gsb allele *gsb$^{01155}$*; BDSC stock #10999) and two gsb RNAi constructs located on the second and third chromosome (these transgenic RNAi lines were generated as described in Marie et. al 2010). The resulting genotype were *elav$^{C155}$-Gal4*/+ or Y; *gsb$^{01155}$/UAS-gsbRNAi; UAS-gsbRNAi/+* and *gsb$^{01155}$/UAS-gsbRNAi; UAS-gsbRNAi/D42-Gal4*. In some experiments, in order to add temporal control, we added a temperature sensitive *Tub-Gal80$^{TS}$* (BDSC, stock #7108) transgene. The *Tub-Gal80$^{TS}$* allele was recombined with the *gsb$^{01155}$* allele to create and animal with the following genotype: *elav$^{C155}$-Gal4/+; gsb$^{01155}$, Tub-Gal80$^{TS}$/UAS-RNAi-Gsb; UAS-RNAi-Gsb/+* and control the expression of gsb RNAi. The over-expressers were: *elav$^{C155}$-Gal4/+; Tub-Gal80$^{TS}$/+; UAS-Gsb/+*.

### *Fly rearing*

Females were dissected in experiments were the driver *elav$^{C155}$-Gal4* was used and animals of either sex were dissected when *D42-Gal4* was used. They were reared at

25°C on Jazz-mix Drosophila food (Fisher Scientific; Cat. No. AS153) following the manufacturer's instructions. For experiments requiring conditional expression, animals were reared at 18°C before being shifted to 29°C for different time periods as indicated in the study.

***Activity-dependent stimulation protocol***

Our stimulation protocol was performed as described in Alicea et al., 2017. Briefly, larvae were subjected to 5 stimulation periods that were interspersed with rest periods. The first 3 stimulations last 2 minutes, the fourth stimulation 4 minutes and the fifth stimulation 6 minutes. Each stimulation step was followed by 15 minutes of rest. The protocol lasts a total of 90 minutes. The stimulation solution consists of Haemolymph-like HL3 saline (70mM NaCl,10 mM $NaHCO_3$, 115 mM sucrose, 5 mM trehalose, 5 mM HEPES, 10 mM $MgCl_2$) containing 90 mM KCl and 1.5 mM $CaCl_2$, while the rest solution consists of HL3 saline containing 5 mM KCl and 0.1 mM $CaCl_2$.

***Experimental design, imaging and statistical analysis***

For experiments related to synaptic growth we scored m6/7 synapses at segment A3 in second or third instar larvae. Synaptic boutons revealed by the anti-Syn immunolabeling were counted and averaged. We present (in Fig. 2.1, 2.3) for each specific genotype and condition, the average, SEM and scatter plot derived from a minimum of 10 synapses from at least 5 animals.

For experiments relevant to synaptic plasticity we scored m6/7 synapses at segment A3 in third instar larvae. Ghost boutons were used to quantify the activity-dependent synaptic plasticity and were defined by the presence of anti-HRP

immunolabeling and the absence of Dlg immunolabeling (Alicea et al., 2017). Controls (*C155/+* or *D42/+*) were run in parallel to experimental animals in each stimulation protocol to account for possible variation. We used a Nikon Eclipse 80i microscope at a magnification of 400x to quantify ghost boutons. For each specific genotype and condition, the average, SEM and scatter plot derived from a minimum of 10 synapses from at least 5 animals were presented (Fig. 2.4).

For experiments dealing with synaptic stability we scored m6/7 synapses from segment A2 to A6 in third instar larvae. Synaptic retractions were defined by the presence of Dlg immunoreactivity that was not accompanied by Syn immunoreactivity. For each animal we determined the percentage of synapses presenting retraction as well as the number of boutons retracted (the sum of all boutons retracted per animal). For each specific genotype and condition, we present the average, SEM and scatter plot derived from at least 9 animals were presented (Fig. 2.5). We used a Nikon Eclipse 80i microscope at a magnification of 400x to quantify synaptic retractions.

The images reported in this article were acquired on a Nikon A1R resonant scanning confocal microscope using 40X objective. We took series of optical sections at 0.2 µm intervals prior to produce maximum intensity projections using the NIS-Elements AR 5.11.02 64-Bit imaging software (Nikon) and we converted the composite images into Tiff format using the open source ImageJ Java-based image processing and analysis program (National Institutes of Health; http://imagej.nih.gov/ij/). These images were assembled, and the contrast of the figures was adjusted using Photoshop CC2018 (Adobe Systems). Charts were created using Prism 6 (Graphpad) before being assembled using Photoshop.

To perform our statistical treatment, we first assessed whether data conformed to a normal distribution by performing a Shapiro–Wilk normality test. When the result of this test was low ($p < 0.0001$), we ran a nonparametric Kruskal–Wallis test with a post hoc Dunn's multiple comparisons test. In the other cases, we ran a parametric one-way ANOVA test. The post hoc Dunnett correction test was applied when multiple comparisons were carried out against a control value (these comparisons are indicated with asterisks over the bars in the figures), while the post hoc Tukey correction test was used for multiple comparisons between data sets (these comparisons are indicated with brackets and asterisks the graphs). When only two data sets were compared, we performed an unpaired, two-tailed students' t-test. For every experiment, the statistical tests and the P values are indicated in their corresponding figure legends.

***Immunohistochemistry and imaging***

For experiments examining synaptic growth and synaptic retraction, larvae were dissected and fixed in Bouin's fixative (Sigma) for 1 minute. For plasticity experiments larvae were fixed on 4% paraformaldehyde for 15 minutes in order to preserve the ghost boutons structure. The following primary antibodies were used overnight at 4°C: mouse anti-Syn, [1:20, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Dlg (1:20, DSHB) and rabbit anti- Dlg (1:150). The affinity purified rabbit anti-Dlg was raised (PrimmBiotech, Inc) against a recombinant protein containing the Dlg sequence stretching from amino acid 764 to amino acid 919. This polyclonal rabbit antibody revealed identical staining to the monoclonal mouse anti-Dlg (DSHB). We also used AffiniPure anti-HRP (Jackson ImmunoResearch) conjugated to Cy3 (1:300) or Cy5 (1:100) and secondary antibodies: 1:300 Alexa Fluor 488-conjugated AffiniPure goat

anti-mouse or anti-rabbit IgG, 1:300 Alexa Fluor 546 conjugated AffiniPure goat anti-mouse (Jackson ImmunoResearch). They were applied 1 hour at room temperature as previously described (Maldonado et al., 2013; B. Marie et al., 2004).

## RESULTS

### *The transcription factor Gooseberry curtails synaptic growth*

In order to assess the role of Gsb in controlling synaptic growth, we first over-expressed it using the driver (*elav$^{C155}$-Gal4*). We quantified synapse size as previously described (Alicea et al., 2017; Maldonado et al., 2013), by counting the number of synaptic boutons revealed by anti-Syn immunofluorescence (Fdez and Hilfiker, 2007; Klagges et al., 1996). We initially focused on the third instar larval NMJs innervating muscles 6/7 and found that Gsb was able to decrease synaptic size (Fig 2.1 D, E, H). Indeed, synapses over-expressing Gsb showed a significant 23% reduction in the number of synaptic boutons (57.4 ± 2.3 compared to 74.2 ± 2.6 in controls). Because the overexpression was initiated at around the end of embryogenesis, early in the development of the synapse, we wondered whether a decrease in synaptic growth would also be seen at stages earlier than the third instar. We quantified the synapse size in second instar larvae and showed that, at this stage too, Gsb overexpression was able to decrease the number of boutons by 28% (Fig 2.1 A, B, G; controls had an average of 40.2 ± 1.4 synaptic boutons while Gsb over-expressors had 28.9 ± 1.4). Hence the over-expression of Gsb in post-mitotic MNs is able to repress synaptic growth.

We then asked whether Gsb loss of function could alter synaptic arbor. Because hemizygous animals containing a strong hypomorphic allele (Duman-Scheel et al., 1997) and a wild-type copy of *gsb* (*gsb*$^{01155}$/+) did not show any synaptic growth phenotype (B. Marie et al., 2010) while an amorphic condition (*gsb*$^{01155}$/ *gsb*$^{01155}$) is embryonic lethal (Patel et al., 1989), we generated transgenic animals combining one copy of the mutation (*gsb*$^{01155}$/+) and RNA interference (RNAi) constructs against *gsb*. These transgenic animals bear two previously described RNAi constructs against *gsb* RNA (B. Marie et al., 2010) driven by *elav*$^{C155}$*-Gal4* (*elav/+; gsb*$^{01155}$*, RNAi-gsb/+; RNAi-gsb/+*). It should be noted that quantitative RT-PCR measurements showed a 43% decrease in *gsb* CNS mRNA in *gsb*$^{01155}$/+ animals and a 48% decrease in animals expressing gsb RNAi under the control of the pan-neuronal driver *elav*$^{C155}$*-Gal4* (B. Marie et al., 2010). Hence we conclude that the animal combining both mutation and RNAi constructs presents a severe neuronal loss of function for *gsb*. In this genetic context, the animals could reach adulthood and their larval nervous system could be assessed. We observed a clear increase in the average synaptic size in animals lacking Gsb in both second (57.4 ± 3.9 synaptic boutons; a 43% increase) and third larval stages (105.7 ± 3.9 synaptic boutons; 42% increase; Fig 2.1. C, F-H). Taken together these results suggest that Gsb is a repressor of synaptic growth required in post-mitotic MNs to achieve normal synaptic size.

We noticed that, despite the fact that the synapses were affected by the change in Gsb expression, they continued growing between L2 and L3. Indeed, the synapses overexpressing Gsb grew from 28.9 to 57.9 boutons (an increase of 100%) while Gsb loss of function animals increased by 84% between L2 and L3, an increase similar to controls

(85%). This could mean that the resulting differences in synapse size could be simply due to an initial early defect that is then amplified by a constant growth rate. This would imply that Gsb is required early during the establishment of the synapse and not during the growth of the mature synapse. In order to test this possibility, we decided to perturb Gsb expression at later stages.

***Gooseberry is required late for synaptic growth***

In order to ask whether Gsb was required late during the growth of the NMJ, we used a different, more temporally specific, transgenic construct, D42-Gal4 (an insertion in the *toll 6* gene; Sanyal, 2009), to drive the overexpression or the loss of function of Gsb. We first checked the expression of UAS-mCD8-GFP under the control of D42-Gal4 (data not shown) to confirm that there was no detectable expression during embryogenesis or at larval stage L1 or early larval stage L2, while there was strong expression at the late L2 and L3 stages. Not surprisingly, we first noted that at stage L2 the transgenic flies encoding for the overexpression or loss of function of Gsb had normal synaptic size (Fig. 2.3 B-D, H). Interestingly, the same genetic modifications provoked significant changes in the synaptic size of stage L3 animals (Fig. 2.3 E-G, I). Indeed, while D42-Gal4 controls show an average of 66 ± 2 synaptic boutons, the Gsb overexpressors show 46.3 ± 3.2 synaptic boutons (a 29% decrease) while the Gsb loss of function animals show 95 ± 5.5 synaptic boutons (a 44% increase). These percentage changes, which are almost identical to those seen for L3 animals with the early-onset *elav$^{C155}$-Gal4* driver (see above), show that perturbing the levels of Gsb at the end of the L2 stage, after synaptogenesis and initial substantial synaptic growth, is sufficient to

disrupt the later stages of synaptic growth. This result strongly suggests that the level of Gsb expression is an important determinant of NMJ size and that its persistence, long after development and differentiation, is essential.

To dissect further this last point, we used transgenic flies carrying a conditional expression system. We added the *Tub-Gal80$^{TS}$* transgene to the Gsb overexpressors and loss of function described previously. This transgene encodes for the Gal80$^{TS}$ protein under the control of the Tubulin promoter. Gal 80$^{TS}$ is thermo-sensitive and is only functional at low temperature where it represses the activator of transcription Gal4 (Fig 2.2 A) (Zeidler et al., 2004). As a result, the expression of gsb or Gsb RNAi is repressed at 18°C but active at 29°C (Fig 2.2 A). We then used these animals at stage L3 and changed their rearing temperature from 18°C to 29°C for 24 hours (Fig. 2.2 B and Fig. 2.3 A, J) or 2 hours (data not shown) immediately prior to ask whether their NMJ were affected. In these conditions we noticed that, in control animals, there is an increase in synaptic growth when flies are shifted from 18°C to 29°C, consistent with previous observations (Sigrist et al., 2003). Interestingly, overexpressing Gsb for 24 h is sufficient to impair synaptic growth. Indeed, the control preparations after a 24 h shift at permissive temperature presented an average of 61.8 ± 2.3 synaptic boutons while the Gsb overexpressors had 40.3 ± 2.7, a reduction of 34.8%. In contrast, the Gsb loss of function animals did not show differences to control. When we performed the de-repression for only 2 h no change was visible in preparations affected by a change in Gsb expression (data not shown).

Taken together these data show that Gsb's effects on synaptic growth are not the consequence of an early mis-determination/specification of the neuron. We showed that

changing Gsb's expression at the time when larval growth is occurring induces changes in synaptic size. For example, overexpressing Gsb for 24 hours only is sufficient to decrease synaptic growth by 35%. These experiments clearly identify a function for Gsb that is subsequent to the specification of motoneurons' identity. Our experiments did not provide evidence that Gsb loss of function for 24 hours could affect the synapse. This result is more difficult to interpret. It could be that, even though the RNAi against Gsb is functioning, the endogenous Gsb protein is not decreased within this time period, however our later experiments (see below) indicate that this is not the case. Similarly, overexpressing Gsb for 2 hours prior assessing synaptic growth did not show a phenotype. This could be explained by the fact that the control synapses do not significantly grow in 2 h: 18°C controls have 47.2 ± 2.2 boutons while they have 51 ± 3.8 when they are shifted for 2hrs at 29°C. In these conditions it is impossible to visualize a change due to the modifications in Gsb expression.

***Gooseberry controls activity-dependent synaptic plasticity.***

Because we had evidence that Gsb was required, after development, to regulate growth we wondered whether it could also influence the ability of a neuron to be plastic. Indeed, we previously showed that Gsb is required for synaptic homeostasis (Marie et al., 2010). Could activity-dependent synaptic plasticity (ADSP) be affected too? In order to test this, we submitted animals to a repeated stimulation protocol sufficient to elicit activity-dependent morphological synaptic plasticity at the NMJ (Alicea et al., 2017; Ataman et al., 2008). Indeed, upon stimulation, new synaptic boutons arise. Because for the first hours of this process these *de novo* boutons are immature (the presynaptic

differentiation is present while the post-synaptic is not), they can be detected by immunochemistry revealing the presynaptic membrane (anti-HRP; Jan and Jan, 1982) and the postsynaptic apparatus (anti-Dlg, the homolog of PSD-95; Budnik et al., 1996; Hough et al., 1997). These boutons, presenting anti-HRP immunoreactivity but lacking anti-Dlg, are called "ghost boutons" and are used to quantify the ADSP at the NMJ (Alicea et al., 2017; Ataman et al., 2008; Piccioli and Littleton, 2014; Vasin et al., 2014).

We first asked whether a constitutive change in Gsb expression could affect ADSP. We submitted preparations to a series of stimulations sufficient to provoke synaptic morphology changes in control preparations (Fig. 2.4 A, E, F). Under these conditions, unstimulated preparations present an average of 0.75 ± 0.17 ghost boutons (Fig. 2.4 A, E) while stimulated preparations present an average of 9.8 ± 1.1 (Fig. 2.4 A, F). We then asked whether preparations overexpressing Gsb or with a loss of function for Gsb would be more or less plastic. We found that animals overexpressing Gsb (Fig. 2.4 B, E, F) presented a deficit in ghost bouton formation (average of 2.69 ± 0.5 ghost boutons) suggesting that Gsb is an inhibitor of ADSP. Under these stimulation conditions the animals presenting Gsb loss of function did not present a significant change in plasticity (Fig. 2.4 F). Nevertheless, it was previously shown that an efficient way to test for over-plasticity was to submit the preparations to a subthreshold stimulation protocol (3 depolarization pulses instead of 5) (Ataman et al., 2008). Indeed, animals overexpressing wingless (wg) could show activity-dependent synaptic modifications while control preparations could not (Ataman et al., 2008). We carried out a similar experiment and showed that subthreshold stimulation was not able to provoke an increase of ghost bouton formation in controls (average of 1.5 ± 0.4 ghost boutons; Fig. 2.4 C, G)

while the animals deficient for Gsb function showed a significant amount of activity-dependent synaptic growth (average of 6.6 ± 0.8 ghost boutons; Fig. 2.4 D, G). These results strongly suggest that Gsb functions to repress ADSP at the synapse.

Having identified Gsb as a potent inhibitor of synaptic plasticity, we then turned to showing that we could perturb synaptic plasticity by manipulating Gsb expression late. Using the conditional expression system described earlier, we first showed that our system was repressed at 18˚C and that there was no difference with or without stimulation between control animals and animals bearing the transgenes (Fig. 2.4 H, K). In contrast, when we derepressed the system 24 h before testing for plasticity, both the over-expressers (reduction in plasticity to 3.1 ± 0.6 ghost boutons compared to 11.9 ± 1.6 in controls; Fig. 2.4 I) and the loss of function animals (gain in plasticity to 4.7 ± 0.9 ghost boutons compared to 1.5 ± 0.4 in controls; Fig. 2.4 L) showed a significant difference to controls. This set of experiments proves that the changes in plasticity controlled by Gsb are not a consequence of mis-development since the modifications of Gsb expression were initiated in a mature synapse. In addition, the overexpression of Gsb 15 min before stimulation was still sufficient to impede activity-dependent synaptic growth (reduction in plasticity to 3.4 ± 1.4 ghost boutons compared to 10 ± 2.5 in controls; Fig. 2.4 J). Notably, the magnitude of the reduction was identical in the three experiments, whether we over-expressed Gsb from the end of embryogenesis (Fig. 2.4 F), or 24 hours (Fig. 2.4 I) or 15 minutes (Fig. 2.4 J) before submitting the animals to spaced repeated stimulations. This shows that Gsb expression can repress synaptic plasticity in a matter of minutes. We could not however observe an increase in plasticity when we reduced Gsb expression 2 hours before the stimulation protocol (Fig. 2.4 M). This result

may be explained by the fact that, since we use RNAi to knock down the level of *gsb*, 2 hours might not be long enough for the endogenous g*sb* RNA and/or protein to be reduced. In addition, if the target(s) of Gsb is (are) a repressor of ADSP, it could remain stable after the decrease of Gsb itself. In any case, we now present evidence that Gsb acts as a repressor of activity-dependent morphological plasticity at the mature NMJ, in addition to being essential for the maintenance of electrophysiological homeostatic plasticity (Marie et al., 2010).

***Gooseberry contributes to motoneuron stability.***

Having shown that both synaptic growth and plasticity were affected by the levels of Gsb, we asked whether the stability of the MN itself might be affected. The *Drosophila* NMJ has been used extensively to characterize molecules and signaling pathways involved in synaptic stability (Eaton et al., 2002; Pielage et al., 2005; Stephan et al., 2015; Valakh et al., 2012). Indeed, while healthy synapses present a perfect apposition between pre-synaptic and post synaptic sides, a retracting synapse is indicated by a post-synaptic differentiation missing its presynaptic counterpart. These retraction events, reminiscent of what is seen in neurodegenerative diseases (Day et al., 2006; Henstridge et al., 2018; Sasaki and Maruyama, 1994; Scheff and Price, 2006), are used to quantify the level of synaptic instability. We labelled third instar larvae synapses using both a postsynaptic marker Dlg (Budnik et al., 1996; Hough et al., 1997) and two presynaptic markers: the neuronal membrane revealed by anti-HRP immunofluorescence (Jan & Jan, 1982) and the vesicle marker revealed by anti-Syn (Fdez & Hilfiker, 2007; Klagges et al., 1996). In each animal, we observed muscle 6/7 synapses from segment A2

to A6 and characterized the percentage of synapses presenting synaptic retraction and the average number of boutons retracted per animal (Fig. 2.5). We increased *gsb* expression using the *elav$^{C155}$-Gal4* driver and found that overexpressing animals showed no effects on synaptic stability (Fig. 2.5 C). In contrast, animals bearing *gsb* loss of function showed an increase in the percentage of synapses presenting retractions and in the size of these retractions. Indeed, animals lacking *gsb* displayed 15.8 ± 4.8 % of synapses with retractions, compared to 4.3 ± 2 % of control synapses (Fig. 2.5 A-A’’’, B-B’’’, C). The average size of these events was 4.3 ± 1.8 retracted synaptic boutons for animals presenting a *gsb* loss of function while it was 0.8 ± 0.4 for controls. This represents a 3.7-fold increase for the occurrence of retractions and a 5.4-fold increase for the average size of the event per animal. These results are a clear indication that Gsb is responsible for some aspects of the maintenance of synaptic stability within MNs. To ask whether this instability could be elicited by decreasing Gsb at a later stage in development we observed animals presenting Gsb loss of function under the control of the third instar D42-Gal4 driver (Fig. 2.5 D). We also observed an increase of the instability parameters. Animals with Gsb down-regulation presented 23.9 ± 6.8 % of synapses with retractions, compared to 5.6 ± 1.5 % for controls. They also showed an increase in the number of boutons retracted per animal (9.2 ± 0.9 % compared to 0.9 ± 0.3 % in controls). This represents a 4.2-fold and 10-fold increase for the occurrence and the size of the retractions respectively and shows that late perturbation of Gsb is just as effective in affecting synaptic stability. Finally, we used the conditional expression system described earlier to knock out Gsb expression only 24 h before assessing synaptic stability. Here too we found that loss of Gsb provoked the appearance of synaptic retractions (16.8 ± 2.8

% compared to 3 ± 1.5 % for controls) and that the average size of these retractions was also increased by this manipulation (2.9 ± 0.8 compared to 1 ± 0.6 for controls). It is interesting to note that our statistical treatment (ANOVA) could not detect a significant difference between the three conditions we used to knock down Gsb for either the percentage of retractions per animal or the average size of boutons retracted. This strongly suggests that Gsb expression is persistently required to stabilize the synapse. As before, decreasing Gsb expression for 2 hours did not produce any effects on synapse stability, but this is likely due to Gsb protein and/or its targets remaining stable despite the effect of the RNAi. Because this experiment, like others presented previously, is using a genetic background hemizygous for gsb in addition to a conditional expression system, this negative result shows that the reported phenotypes are due to the added time controlled expression of the RNAi constructs and not to the hemizygous condition.

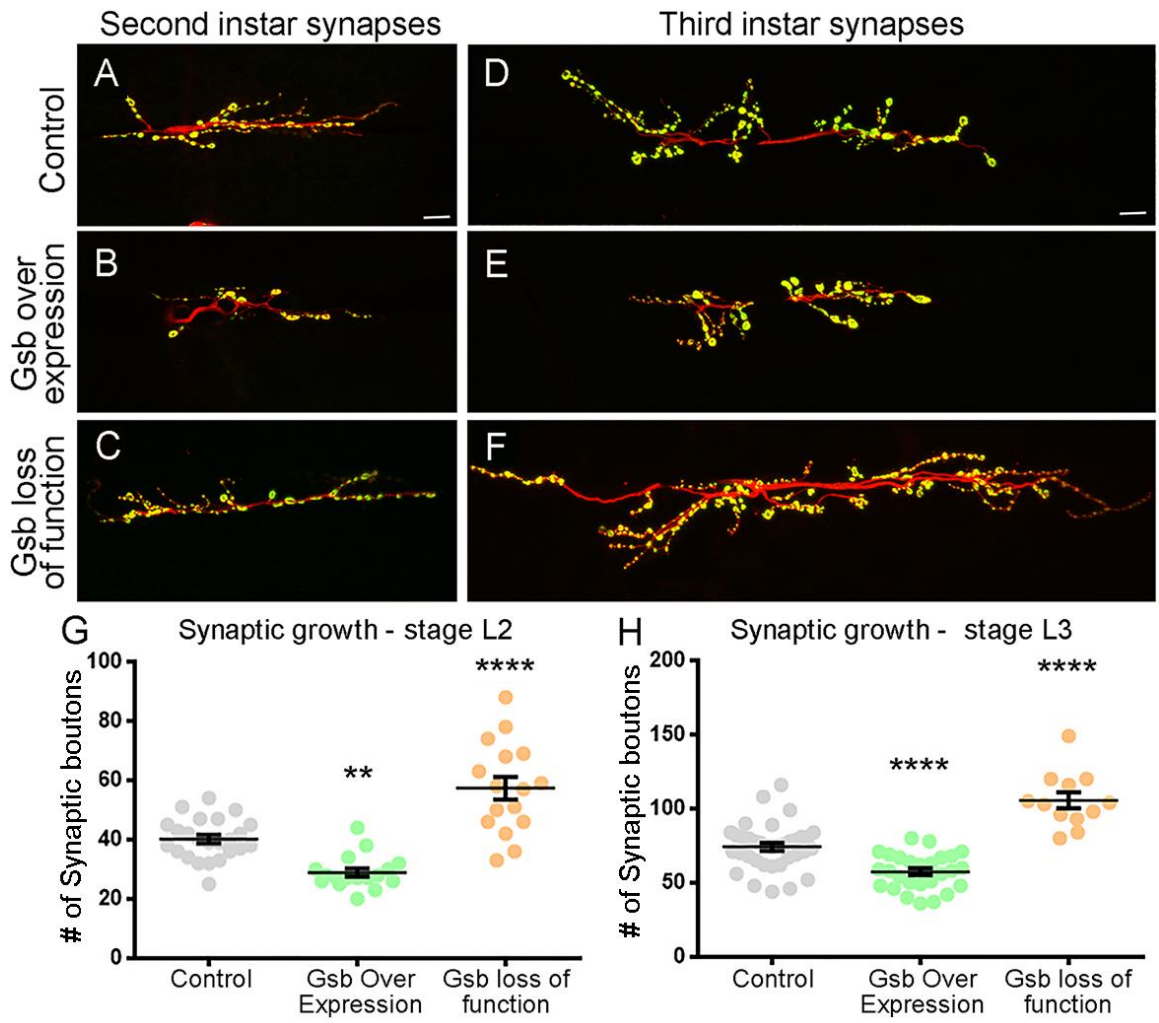


**Figure 2.1 The transcription factor Gsb curtails synaptic growth**

**(A-C)** Representative second instar and **(D-F)** third instar muscle 6/7 NMJ from control (elavC155-Gal4/+), Gsb over-expression (elavC155-Gal4/+; UAS-Gsb/+) and Gsb loss of function (elavC155-Gal4/+; gsb01155/UAS-RNAi-Gsb; UAS-RNAi-Gsb/+) animals. Antibodies staining the neuronal membrane (anti-HRP, red) and the synaptic vesicles (anti-Syn, green) were used to reveal synapse morphology. **(G)** Quantification of the number of synaptic boutons in second instar (L2) and **(H)** third instar (L3) animals from indicated phenotypes. **p < 0.01; ****p < 0.0001. ANOVA with post hoc Dunnett test. Individual data are shown as scatter plots as well as mean ± SEM. Scale bar is 10 µm.

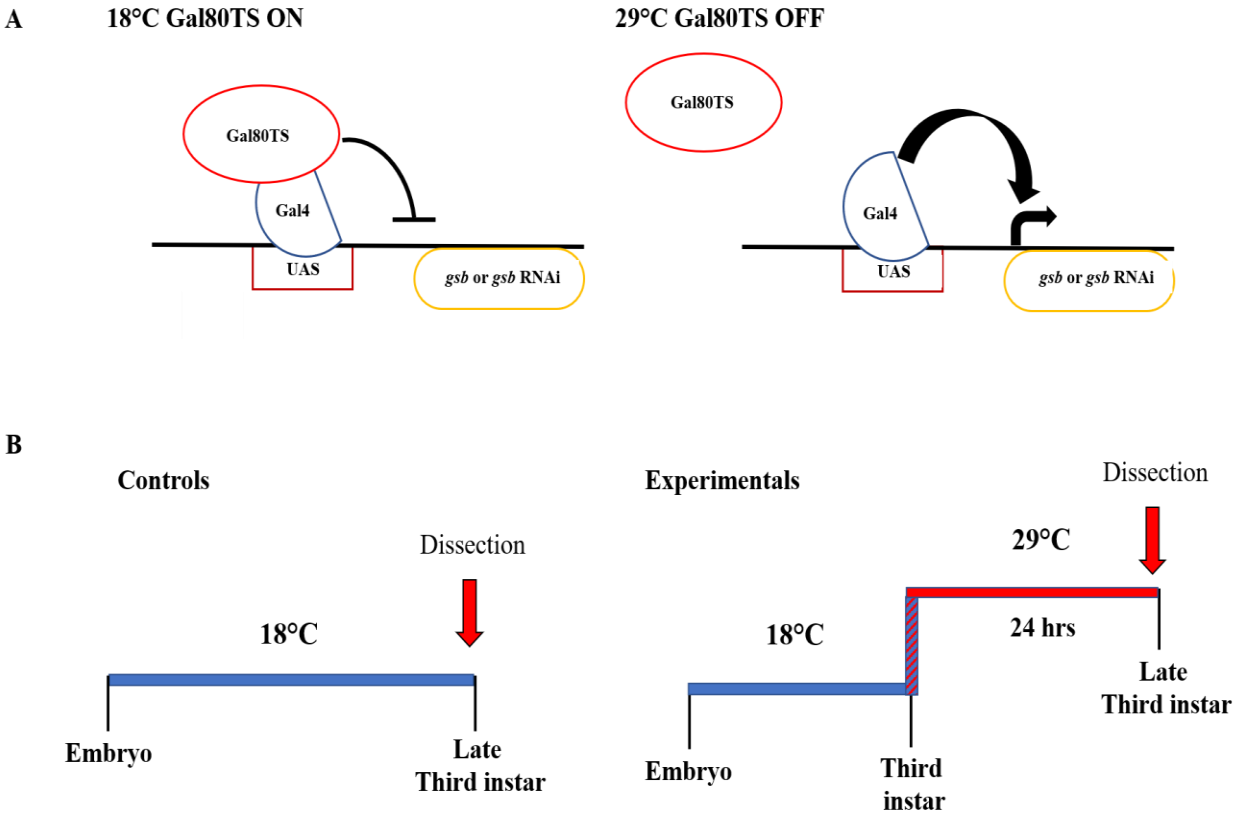


**Figure 2.2 Conditional expression system.**

Illustration describing the temperature sensitive gal80 system and our experimental design using this system. This system allows temporal control of Gal4 activity. **(A)** $Gal80^{TS}$ inhibits Gal4 at 18°C. At 29°C, $Gal80^{TS}$ is not active and Gal4 can promote transcription of its targets. We did two separate experiments. **(B)** In one experiment we reared larvae at 18°C always until the third instar stage. Larvae reared at 18°C had endogenous *gsb* expression only because the Gal4-UAS system was inactive. In another experiment we reared larvae at 18°C until the second instar stage and then shifted them to 29°C for 24 hours, and dissected them as third instar larvae. Animals reared at 29°C for 24 hrs had gain of function or loss of function of *gsb* expression for this time period.

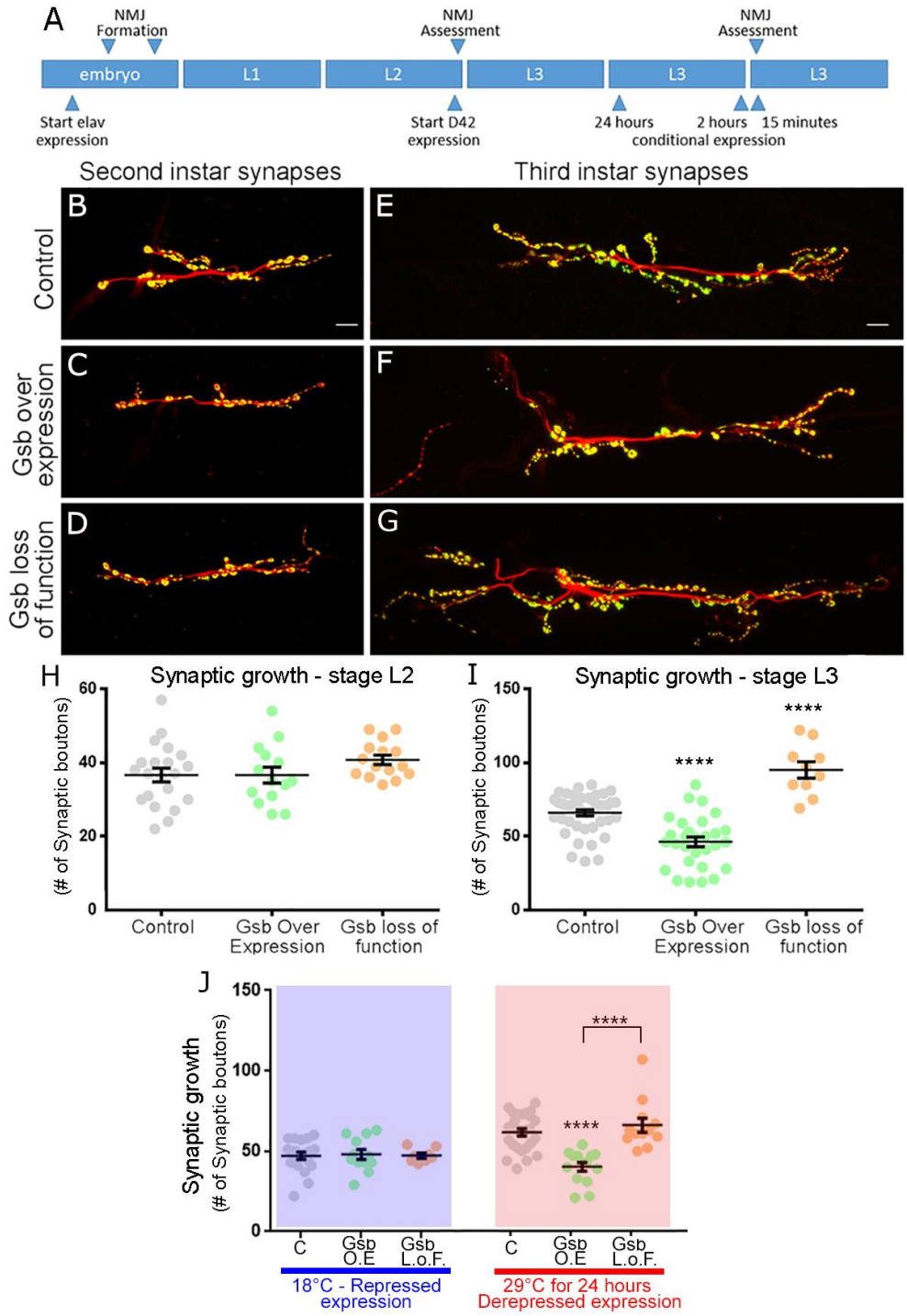


**Figure 2.3 A late change in Gsb expression provokes late changes in synaptic growth.**

**(A)** Schematic representation of the timeline of embryonic and larval development (one day/block). Arrowheads point out at the time at which events take place. **(B-D)**

Representative second instar and **(E-G)** third instar muscle 6/7 NMJ from control (D42-Gal4/+), Gsb over-expression (D42-Gal4/UAS-Gsb) and Gsb loss of function (gsb01155/UAS-RNAi-Gsb; D42-Gal4/UAS-RNAi-Gsb) animals. Antibodies revealing the neuronal membrane (anti-HRP, red) and the synaptic vesicles (anti-Syn, green) were used to characterize synapse morphology. **(H)** Quantifications of the number of synaptic boutons in second instar NMJs. **(I)** Quantifications of the number of synaptic boutons in third instar NMJs. **(J)** Quantification of the boutons number of third instar NMJ in a conditional expression system. Animals overexpressing Gsb (Gsb O.E) are elavC155-Gal4/+; Tub-Gal80TS/+; UAS-Gsb/+ while Gsb loss of function animals (Gsb L.o.F) are elavC155-Gal4/+; gsb01155, Tub-Gal80TS/UAS-RNAi-Gsb; UAS-RNAi-Gsb/+. On the left (blue) is the quantification of animals raised at 18°C. On the right (red), animals were raised at 29°C for 24 hours, allowing the expression of the different transgenes. ***$p < 0.001$; ****$p < 0.0001$. (H-J) ANOVA with Dunnett and Tukey's post hoc test. Individual data are shown as scatter plots as well as mean ± SEM. Scale bar is 10 µm.

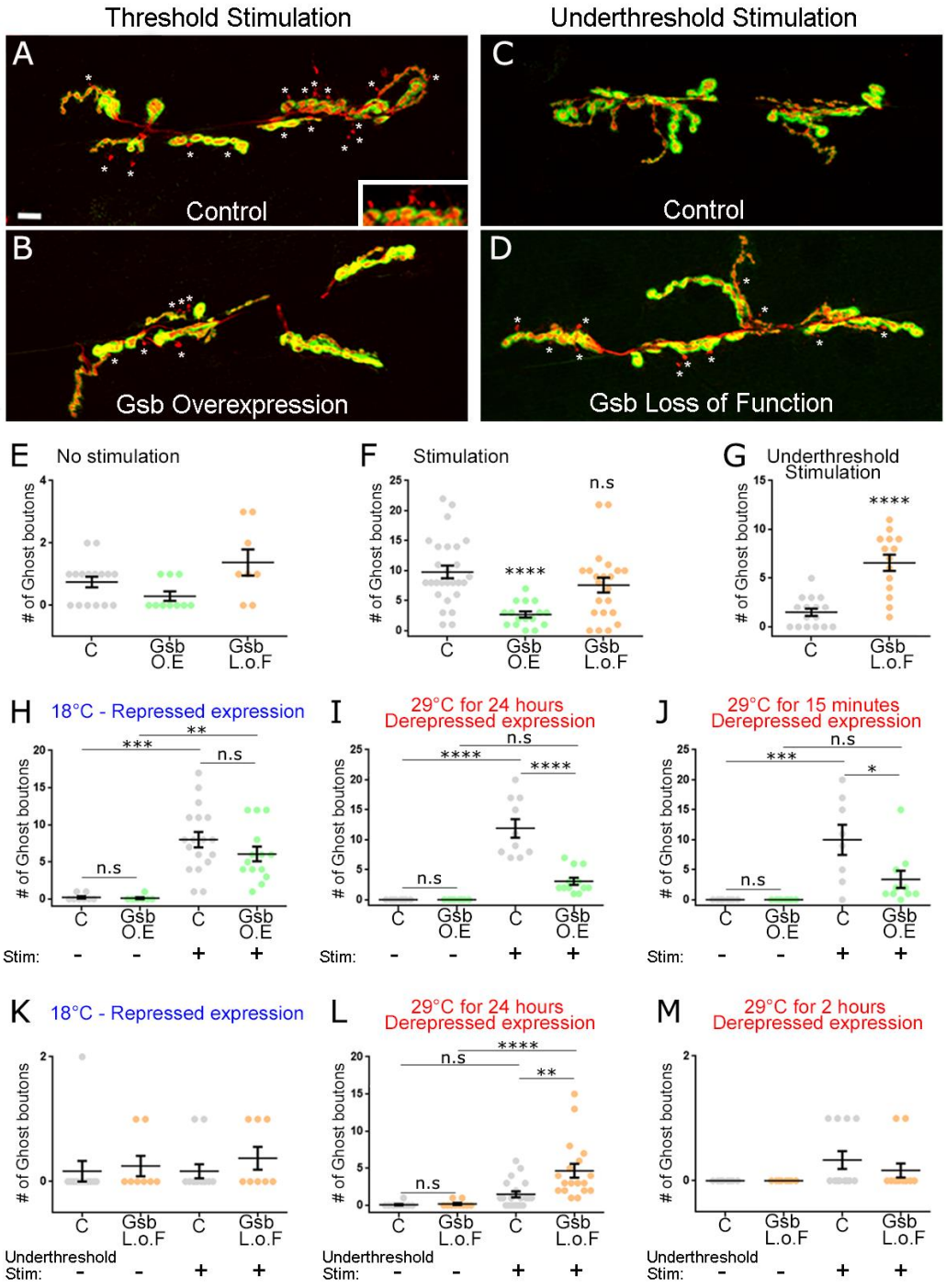


**Figure 2.4 Gsb controls activity-dependent synaptic plasticity.**

**(A)** Representative control NMJ showing ghost bouton formation (stars and inset) after threshold stimulation. **(B)** Representative NMJ overexpressing Gsb after threshold stimulation shows a decrease in ghost boutons formation. **(C)** Representative control

NMJ after subthreshold stimulation shows absence of ghost boutons. **(D)** Representative NMJ from an animal lacking Gsb shows ghost boutons formation after subthreshold stimulation. **(E-G)** Quantification of ghost boutons number in control **(C**: elavC155-Gal4/+**)**, gsb over expression (Gsb O.E: elavC155-Gal4/+; UAS-Gsb/+) and Gsb loss of function (Gsb L.o.F: elavC155-Gal4/+; gsb01155/ UAS-RNAi-Gsb; UAS-RNAi-Gsb/+) animals **(E)** at rest **(F)** after threshold stimulation and **(G)** after subthreshold stimulation. (H-M) Quantification of Ghost boutons number in a conditional expression system for control animals (C: elavC155-Gal4/+; Tub-Gal80TS/+); gsb over expression animals (Gsb O.E: elavC155-Gal4/+; Tub-Gal80TS/+; UAS-Gsb/+) and Gsb loss of function animals (Gsb L.o.F: elavC155-Gal4/+; gsb01155, Tub-Gal80TS/UAS-RNAi-Gsb; UAS-RNAi-Gsb/+). **(H-J)** data for preparations at rest or after threshold stimulation. (K-M), data for preparations at rest or after underthreshold stimulation. **(H, K)** display experiments at 18°C that do not express any transgenes while **(I, J, L, M)** display experiments at 29°C allowing for robust transgene expression. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. (E, H, K-M) Kruskal-Wallis test with Dunn's multiple comparison test. **(F)** ANOVA with post hoc Dunnett test. **(G)** Unpaired t-test with Welch's correction **(I, J)** ANOVA with Tukey's post hoc test. Individual data are shown as scatter plots as well as mean ± SEM. Scale bar is 10 µm.

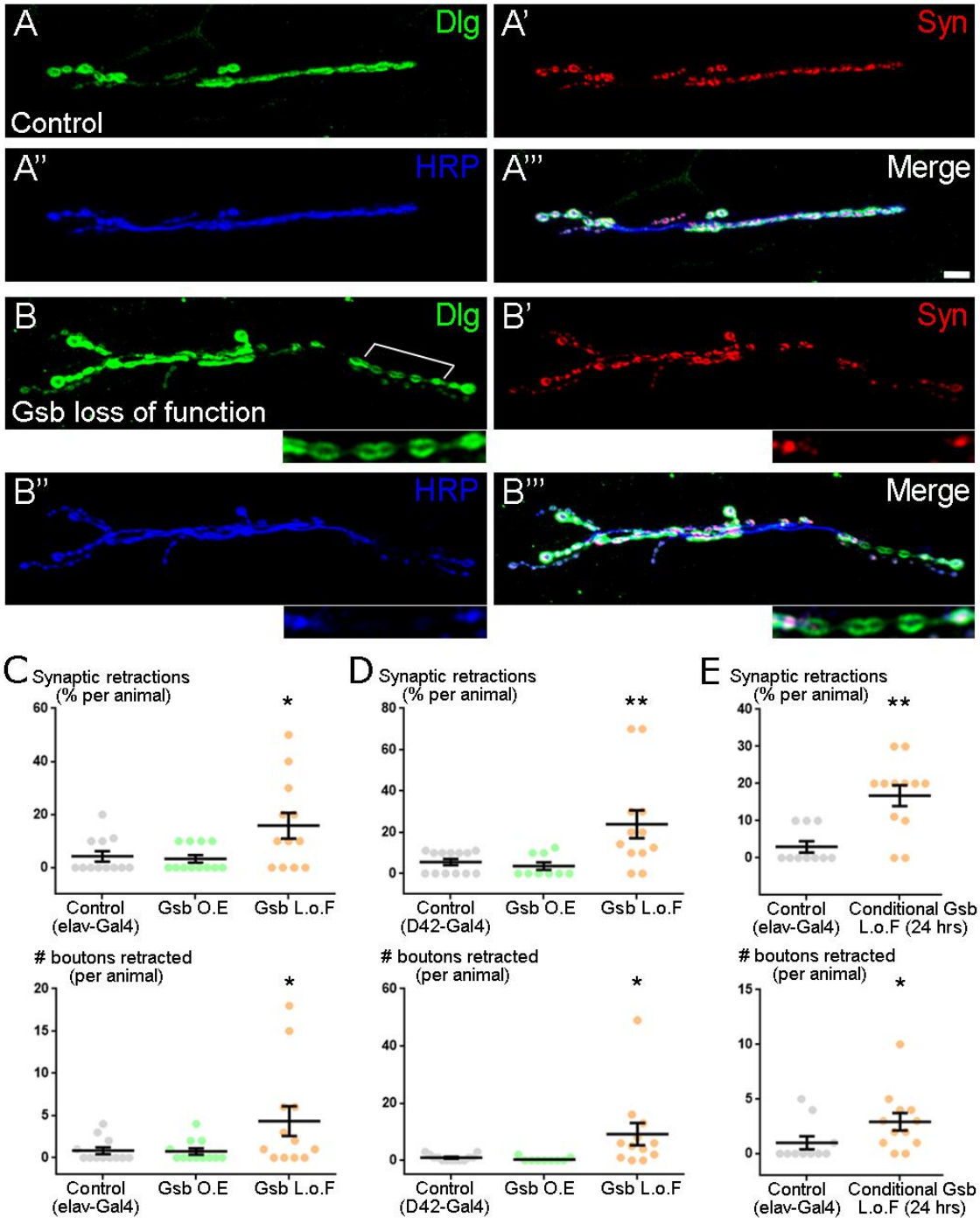


**Figure 2.5 Gsb modulates synaptic stability.**

**(A)** Control and **(B)** Gsb loss of function synapses showing typical immunoreactivity for the post synaptic marker Disc large (Dlg), the synaptic vesicle marker Syn and the neuronal membrane marker HRP. **(C-E)** Quantification of the % of synaptic

retractions (occurrence) per animal and of the number of boutons retracted (severity) per animal in control, Gsb over expressors and gsb loss of function animals. Transgenes are driven by **(C)** elav-Gal4C155; **(D)** D42-Gal4 and **(E)** conditional expression at 29°C (as previously described). *$p < 0.05$; **$p < 0.01$. **(C**, **D** - % synaptic retractions**)** ANOVA with post hoc Dunnett test. **(D** – number of boutons retracted**)** Kruskal-Wallis test with Dunn's multiple comparison test. **(E)** Mann-Whitney test. Individual data are shown as scatter plots as well as mean ± SEM. Scale bar is 10 μm.

## DISCUSSION

We have shown that the transcription factor Gsb is required in mature motoneurons to maintain an array of basic synaptic properties such as growth, plasticity and stability. We have shown that over-expression of Gsb is sufficient to inhibit synaptic growth and plasticity. In addition, our loss-of-function experiments have shown that Gsb is required to inhibit synaptic growth and plasticity as well as to maintain synaptic stability. It is interesting that both over-expression and loss-of-function conditions affect the same synaptic characteristics but in opposite directions. Indeed, a loss of function of Gsb provokes phenotypes of over-growth and over-plasticity while its over-expression provokes under-growth and under-plasticity. The phenotypes observed in Gsb over-expressing synapses could be the consequence of Gsb affecting new transcriptional targets that are usually not affected at its basal level. Nevertheless, the fact that loss-of-function genetic conditions present phenotypes that are the mirror image of the Gsb-over-expressing synapses suggests that these phenotypes reflect a dose-dependency of Gsb and not simply an "on/off" switch or the activation of unspecific targets. In addition, because the gsb/+ heterozygote condition does not show phenotypes (this work and Marie et al., 2010), it suggests that Gsb, like other transcription factors (Li et al., 2004; Stott et al., 2013; Whittington et al., 2015), must be reduced to a certain critical threshold to show a phenotype and that it functions in a dose-dependent manner. Gsb loss of function synapses also show instability (presence of synaptic retractions). While it is not possible to assess whether a synapse over-expressing Gsb is over-stable, it is tempting to think that over-stable synapses would be impeded in their ability to perform plastic morphological changes, as was observed in Gsb-over-expressers. This idea supposes that

a transition from synaptic stability to some state of disassembly is required in order to achieve the addition of *de novo* synaptic material.

If the Gsb phenotypes observed seem to be dose-dependent, they do not appear to be time-dependent. We have used different genetics strategies to show that affecting Gsb expression late during motoneuron's life can also affect its properties. Amazingly, we noted that there was no gradation in the magnitude of the observed phenotypes whether they were provoked by an early change in Gsb expression (driven by the post-mitotic embryonic construct elav$^{C155}$-Gal4) or later (driven by the late second instar larval construct D42-Gal4 or 24 to 2 hours before assessing the synapse when we used our conditional expression system). Indeed, the inhibition of the ADSP is similar in animals overexpressing Gsb in post-mitotic embryonic motoneurons or in fully grown NMJs within functioning and mobile animals (only 24 hours or 15 minutes prior experimental test). Similarly, Gsb loss of function can increase activity-dependent synaptic plasticity in a similar way whether Gsb activity is reduced 24 hours before the test or during embryogenesis. The same observation is valid regarding the stability of the synapse. We obtained the same results showing synaptic instability (for both occurrence and severity) whether we perturbed Gsb function in the embryo, at the end of the second larval stage or at the fully grown larval stage (24 hours before carrying out our test). We also showed that over-expression of Gsb late (24 hrs before assessment) can inhibit synaptic growth. These observations show that Gsb's effect on ADSP and stability are not due to mis-development or mis-specification of the neuronal precursors but reflects a constant and sustained requirement for the transcription factor. In addition, this suggests that Gsb controls the expression of molecules with short half-lives, allowing us to observe

phenotypes in short periods of time. The rapidity (15 min of expression) of the inhibitory effect of Gsb on activity dependent synaptic plasticity also suggests that it controls a potent synaptic inhibitor of plasticity rather than represses an activator; the latter option requiring the disappearance of the activator mRNA and protein, a process demanding a longer time.

The role of the transcription factor Gsb so late during the development/life of a neuron is not unique. Some transcription factors, linked to the fate of neuroblasts and neurons, are also involved in the maintenance of neuronal fate. They have been termed terminal selectors and are defined as capable of activating a series of specific genes required for specific neuronal sub-types or sub-routines (Allan and Thor, 2015; Hobert and Kratsios, 2019). While there is no doubt that the characterization of Gsb's targets will be of prime importance for our understanding of motoneuronal maintenance, the question remains as to whether Gsb fits the terminal selector gene definition. Because terminal selectors control the expression of markers underlying neuron-type specific features (Kratsios Paschalis , Stolfi Alberto , Levine Michael, 2011; Zhang et al., 2014) and because Gsb is expressed late in all (but not exclusively) fully developed motor neurons (Marie et al., 2010), it is difficult to imagine that Gsb is a terminal selector gene specific to the motoneuron sub-identity. In addition, our experiments suggest that strong loss of function of Gsb or ectopic expression of Gsb do not change neuronal identity, a characteristic of terminal selector genes. It is maybe more likely that Gsb is part of a network of pan-neuronal regulators (Stefanakis et al., 2015) responsible for maintaining some features shared by several neurons and determining the magnitude of growth and plasticity/stability at the synapse.

# CHAPTER 3

# THE TRANSCRIPTION FACTOR GOOSEBERRY, A PAX3/7 HOMOLOGUE, INTERACTS WITH WINGLESS TO CONTROL NEURONAL FUNCTION

This thesis chapter is part of a manuscript titled:

**Maintaining neuronal function: the role of Gooseberry, the Pax3/7 homologue, in controlling synaptic growth, plasticity and stability.**

Abbreviated title: **Gsb and the maintenance of motoneuron synapses**

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## INTRODUCTION

Wnt/Wg is a secreted signaling molecule that is essential for the formation of the body axis and for the differentiation and proliferation of cells in vertebrates and invertebrates (Siegfried and Perrimon, 1994; van Amerongen and Nusse, 2009). Wg activates several signaling cascades within the cell. The most characterized pathway is the Wg canonical pathway. In this pathway, inactivated Wg signaling promotes the assembly of a protein complex called "β-catenin (armadillo/Arm) destruction complex" in the cytosol. This complex is composed of proteins Axin, Adenomatous Polyposis Coli (APC) and the kinases Sgg and CK1α. The destruction complex phosphorylates the TF Arm, which leads to its ubiquitination and subsequent degradation by the proteasome. In this scenario, the Wg pathway is inhibited because its target genes are not transcribed. When Wg binds to the receptor Frizzled (Fz) and co-receptor LRP 5/6 (arrow/arr), the destruction complex is disrupted by the recruitment of Dishevelled (Dsh). Dsh sequesters Axin, APC, Sgg and CK1α, which impairs Arm phosphorylation. Arm is able then to translocate to the nucleus to associate with the TF TCF/LEF (Pangolin/Pan) to initiate transcription of Wg target genes (Fig. 3.1) (Komiya and Habas, 2008).

In vertebrates, Wg promotes axon pathfinding, dentritic development, synaptogenesis, synapse differentiation and plasticity (Ahmad-Annuar et al., 2006; Budnik and Salinas, 2011; Speese and Budnik, 2007; Varela-Nallar et al., 2010). Wg is also important for the regulation of neuronal excitability (Cerpa et al., 2011). In *Drosophila*, NMJs without Wg fail to expand properly during larval development (Budnik and Salinas, 2011; Franco et al., 2004; Miech et al., 2008; Packard et al., 2002; Speese and Budnik, 2007). At the NMJ, Wg is secreted presynaptically and it is

important for bouton differentiation (Ataman et al., 2008; Mathew et al., 2005; Miech et al., 2008; Packard et al., 2002), and it also regulates postsynaptic differentiation (Mathew et al., 2005; Mosca and Schwarz, 2010; Speese, 2012). In addition, it controls actin dynamics as a result of increased synaptic activity (Alicea et al., 2017).

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Interestingly, during NB differentiation, NBs expressing *gsb* show antagonism to *wg* signaling and this property is essential in determining NB fate (Fig. 3.2) (K M Bhat, 1996; Bhat KM; van Beers EH; Bhat P, 2000; Duman-Scheel et al., 1997). In mature MNs, this antagonism persists during synaptic homeostasis (Marie et al., 2010). Here we provide evidence that an antagonistic relationship between Wg and Gsb is responsible for directing synaptic growth and plasticity at the NMJ and that Gsb functions downstream of Wg but upstream of the kinase Sgg.

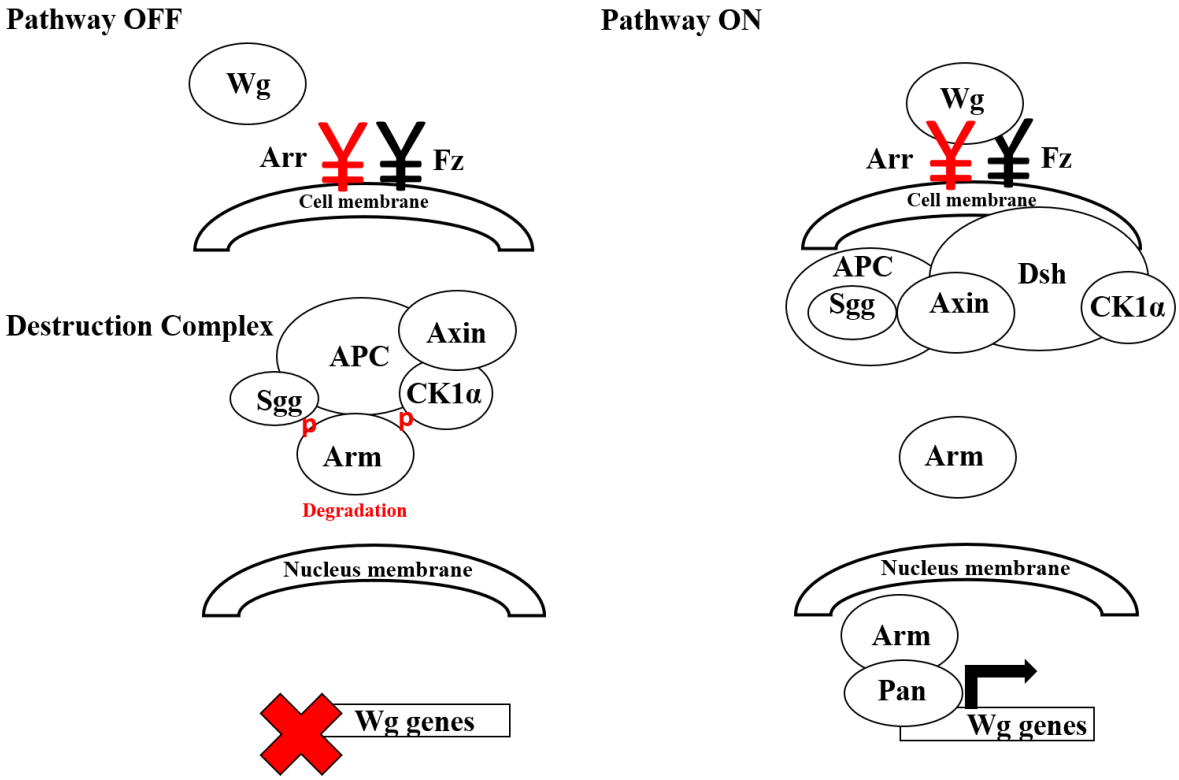


**Figure 3.1 The canonical Wg pathway.**

Schematic diagram of the Wg signaling cascade. (Left) When Wg is not bound to the receptor Fz, the destruction complex phosphorylates Arm which targets it for degradation. (Right) When Wg binds to the receptor Fz and co-receptor LRP 5/6, Dsh is recruited to the membrane along with the members of the destruction complex. Arm accumulates and translocates to the nucleus to associate with another TF (Pan). Both TFs start the transcription of Wg target genes.

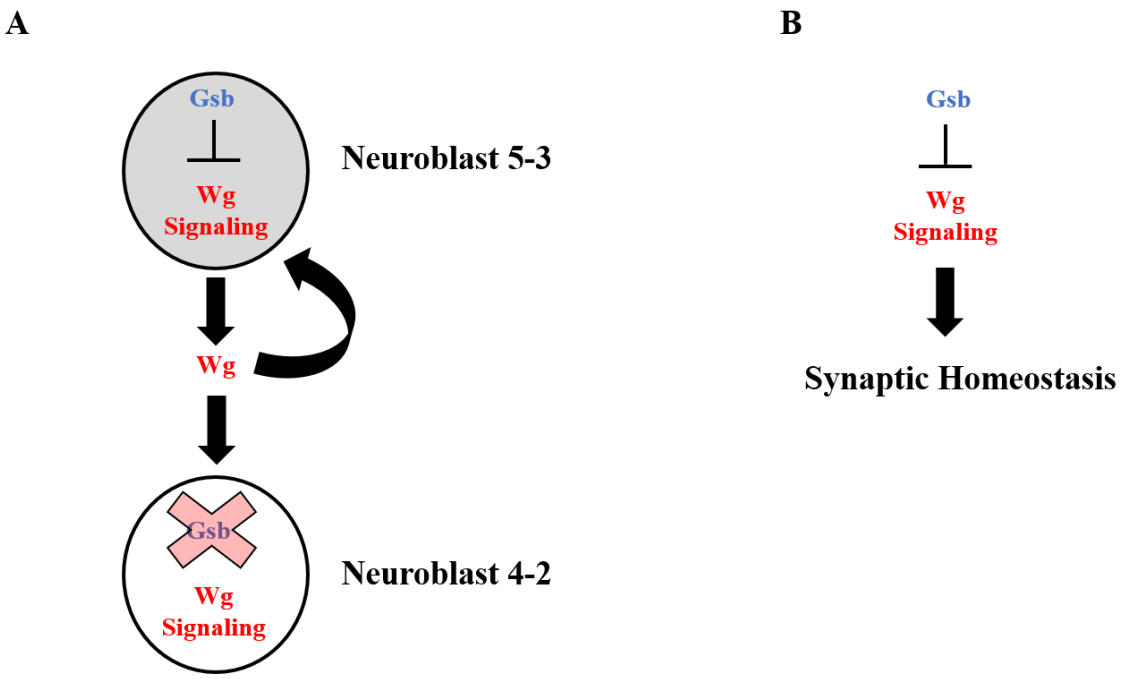


**Figure 3.2 Antagonism between gsb and wg during embryogenesis and synaptic homeostasis.**

Adapted from Marie, et. al 2010. **(A)** Schematic diagram showing *wg-gsb* interaction during neuroblast differentiation. Briefly, *wg* signaling secretes to both cells, allowing differentiation to NB 4-2. However, if *gsb* is expressed, *wg* autocrine signaling fails to differentiate the cell into NB 4-2 and it differentiates into NB 5-3. **(B)** In a similar antagonistic fashion, it is suggested that *gsb* controls synaptic homeostasis in motoneurons by inhibiting *wg* signalling

## MATERIALS AND METHODS

### *Fly stocks and genetics*

We used the *Gal4/UAS* system (Brand and Perrimon, 1993) to overexpress genes or express RNA interference (RNAi) constructs. To drive expression of genes in neurons at the embryonic stage we used the *elav$^{C155}$-Gal4* line [Bloomington Drosophila Stock Center (BDSC), stock #458]. The line containing *UAS-gsb* was a gift from Dr. Markus Noll (University of Zurich, Switzerland). Gsb loss of function animals were engineered by combining within the same animal a Gal4 driver (*elav$^{C155}$-Gal4*), a null gsb allele *gsb$^{01155}$*; BDSC stock #10999) and two gsb RNAi constructs located on the second and third chromosome (these transgenic RNAi lines were generated as described in Marie et. al 2010). The resulting genotype were *elav$^{C155}$-Gal4*/+ or Y; *gsb$^{01155}$/UAS-gsbRNAi; UAS-gsbRNAi*/+. The over-expressers were: *elav$^{C155}$-Gal4/+; Tub-Gal80$^{TS}$/+; UAS-Gsb/+*. For experiments related to wg we used the *wg$^{1}$* allele (BDSC, stock #2978) and *UAS-wg* transgene (BDSC, stock #5918). The *wg$^{1}$* allele was recombined with the *gsb$^{01155}$* allele to allow the removal of wg in a gsb loss of function background: *elav$^{C155}$-Gal4/+; gsb$^{01155}$, wg$^{1}$/UAS-RNAi-Gsb; UAS-RNAi-Gsb/+*. The *sgg* dominant negative transgene used was *UAS-sgg$^{A81T}$* (BDSC, stock #5360).

### *Fly rearing*

Females were dissected in experiments were the driver *elav$^{C155}$-Gal4* was used and animals of either sex were dissected when *D42-Gal4* was used.. They were reared at 25°C on Jazz-mix Drosophila food (Fisher Scientific; Cat. No. AS153) following the manufacturer's instructions. For experiments requiring conditional expression, animals

were reared at 18°C before being shifted to 29°C for different time periods as indicated in the study.

***Activity-dependent stimulation protocol***

Our stimulation protocol was performed as described in Alicea et al., 2017. Briefly, larvae were subjected to 5 stimulation periods that were interspersed with rest periods. The first 3 stimulations last 2 minutes, the fourth stimulation 4 minutes and the fifth stimulation 6 minutes. Each stimulation step was followed by 15 minutes of rest. The protocol lasts a total of 90 minutes. The stimulation solution consists of Haemolymph-like HL3 saline (70mM NaCl,10 mM $NaHCO_3$, 115 mM sucrose, 5 mM trehalose, 5 mM HEPES, 10 mM $MgCl_2$) containing 90 mM KCl and 1.5 mM $CaCl_2$, while the rest solution consists of HL3 saline containing 5 mM KCl and 0.1 mM $CaCl_2$.

***Experimental design, imaging and statistical analysis***

For experiments related to synaptic growth we scored m6/7 synapses at segment A3 in second or third instar larvae. Synaptic boutons revealed by the anti-Syn immunolabeling were counted and averaged. We present (in Fig. 3.3 D, 3.4) for each specific genotype and condition, the average, SEM and scatter plot derived from a minimum of 10 synapses from at least 5 animals.

For experiments relevant to synaptic plasticity we scored m6/7 synapses at segment A3 in third instar larvae. Ghost boutons were used to quantify the activity-dependent synaptic plasticity and were defined by the presence of anti-HRP immunolabeling and the absence of Dlg immunolabeling (Alicea et al., 2017). Controls (*C155/+* or *D42/+*) were run in parallel to experimental animals in each stimulation

protocol to account for possible variation. We used a Nikon Eclipse 80i microscope at a magnification of 400x to quantify ghost boutons. For each specific genotype and condition, the average, SEM and scatter plot derived from a minimum of 10 synapses from at least 5 animals were presented (Fig. 3.3 E, 3.5).

For experiments dealing with synaptic stability we scored m6/7 synapses from segment A2 to A6 in third instar larvae. Synaptic retractions were defined by the presence of Dlg immunoreactivity that was not accompanied by Syn immunoreactivity. For each animal we determined the percentage of synapses presenting retraction as well as the number of boutons retracted (the sum of all boutons retracted per animal). For each specific genotype and condition, we present the average, SEM and scatter plot derived from at least 9 animals were presented (Fig. 3.3 F). We used a Nikon Eclipse 80i microscope at a magnification of 400x to quantify synaptic retractions.

The images reported in this article were acquired on a Nikon A1R resonant scanning confocal microscope using 40X objective. We took series of optical sections at 0.2 µm intervals prior to produce maximum intensity projections using the NIS-Elements AR 5.11.02 64-Bit imaging software (Nikon) and we converted the composite images into Tiff format using the open source ImageJ Java-based image processing and analysis program (National Institutes of Health; http://imagej.nih.gov/ij/). These images were assembled, and the contrast of the figures was adjusted using Photoshop CC2018 (Adobe Systems). Charts were created using Prism 6 (Graphpad) before being assembled using Photoshop.

To perform our statistical treatment, we first assessed whether data conformed to a normal distribution by performing a Shapiro–Wilk normality test. When the result of

this test was low ($p < 0.0001$), we ran a nonparametric Kruskal–Wallis test with a post hoc Dunn's multiple comparisons test. In the other cases, we ran a parametric one-way ANOVA test. The post hoc Dunnett correction test was applied when multiple comparisons were carried out against a control value (these comparisons are indicated with asterisks over the bars in the figures), while the post hoc Tukey correction test was used for multiple comparisons between data sets (these comparisons are indicated with brackets and asterisks the graphs). When only two data sets were compared, we performed an unpaired, two-tailed Students' t-test. For every experiment, the statistical tests and the P values are indicated in their corresponding figure legends.

***Immunohistochemistry and imaging***

For experiments examining synaptic growth and synaptic retraction, larvae were dissected and fixed in Bouin's fixative (Sigma) for 1 minute. For plasticity experiments larvae were fixed on 4% paraformaldehyde for 15 minutes in order to preserve the ghost boutons' structure. The following primary antibodies were used overnight at 4°C: mouse anti-Syn, [1:20, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Dlg (1:20, DSHB) and rabbit anti- Dlg (1:150). The affinity purified rabbit anti-Dlg was raised (PrimmBiotech, Inc) against a recombinant protein containing the Dlg sequence stretching from amino acid 764 to amino acid 919. This polyclonal rabbit antibody revealed identical staining to the monoclonal mouse anti-Dlg (DSHB). We also used AffiniPure anti-HRP (Jackson ImmunoResearch) conjugated to Cy3 (1:300) or Cy5 (1:100) and secondary antibodies: 1:300 Alexa Fluor 488-conjugated AffiniPure goat anti-mouse or anti-rabbit IgG, 1:300 Alexa Fluor 546 conjugated AffiniPure goat anti-

mouse (Jackson ImmunoResearch). They were applied 1 hour at room temperature as previously described (Maldonado et al., 2013; Marie et al., 2004).

## RESULTS

### Decreasing Wg function at a synapse lacking Gsb is sufficient to restore normal growth and plasticity but not stability.

Interestingly, the phenotypes we characterized linking Gsb activity to synaptic growth and plasticity are the mirror image of those observed when the Wg signaling is affected. Indeed, a loss of function of Wg provokes, like a gain of function of Gsb, a decreased synaptic growth and plasticity. Similarly, Wg gain of function or Gsb loss of function give rise to the same phenotypes - increased synaptic growth and plasticity (Ataman et al., 2008; Miech et al., 2008; Packard et al., 2002b). In addition, it was previously shown that there is an antagonistic relationship between the two molecules in neuroblast determination (Bhat, van Beers, and Bhat, 2000; Bhat, 1999; Duman-Scheel et al., 1997) and, later, for the prolonged expression of synaptic homeostasis (Marie et al., 2010). We therefore asked whether this antagonism could be regulating synaptic growth, plasticity and/or stability.

We first asked whether the increased synaptic growth observed in Gsb loss of function animals could be restored in *wg* mutants. We examined synapses expressing Gsb loss of function and, as in Fig. 2.1, characterized an over-growth (Fig. 3.3 A, A'; the synapses have 105.7 ± 5.4 boutons compared to controls that have 75.9 ± 2.5). In contrast, the synapses of animals hemizygous for *wg* presented an undergrowth

phenotype (Fig. 3.3 A'', D; 50 ± 4.5 boutons) in accordance with what was previously published (Miech et al., 2008; Packard et al., 2002). Interestingly, synapses presenting a deficiency in both Gsb and Wg activity showed growth at control levels (Fig. 3.3 A''', D; 74.3 ± 3.6 boutons). This shows that Wg signaling is required for the expression of the overgrowth at synapses missing Gsb and suggests that, like in the embryo, these two molecules might be exercising antagonistic functions determining the NMJ growth.

Wg is a key molecule for ADSP. Indeed, upon repeated stimulation Wg increases at the synapse, leading to morphological changes (Ataman et al., 2008). Upon subthreshold stimulation, preparations hemizygous for *wg* did not present a difference in the number of ghost boutons produced (2.7 ± 0.8 while controls are 1.5 ± 0.4; $p > 0.999$; Fig. 3.3 B, B'', E). In contrast, synapses lacking Gsb activity showed higher morphological changes after subthreshold stimulation (6.1 ± 0.8 ghost boutons; Fig. 3.3 B', E). Again, in this genetic context, the removal of 50% of Wg was sufficient to reverse the over-plastic phenotype associated with the lack of Gsb expression (1.8 ± 0.5 ghost boutons; Fig. 3.3 B''', E). This shows that Wg signaling is required for the expression of the over-plasticity at synapses missing Gsb and suggests that Wg and Gsb are antagonists in determining synaptic plasticity.

We then asked whether we could detect the same antagonistic relationship between Gsb and Wg in the context of synaptic stability. We observed the percentage of synapses presenting retractions in controls, Gsb loss of function, *wg* hemizygous animals, as well as in animals with a loss of both Gsb and Wg activity. We noticed that animals hemizygous for *wg* did not show more synaptic retractions (6.4 ± 2.2 % of synapses presented retractions compared to 6.2 ± 1.9 % in control), while the synapses from

animals lacking Gsb did (18 ± 2.9 %). In addition, removing 50% of Wg in the Gsb loss of function background did not restore synaptic stability. On the contrary, the instability of the synapse was increased since this animal presented a higher percentage of synaptic retractions (30.9 ± 4.8 %; Fig. 3.3 C-C''', F). We argue that this enhancement illustrates a different function of Gsb that is not related to its antagonism to Wg signaling. In addition, this might illustrate a function of Wg that could be essential in maintaining synaptic stability at stressed synapses. Indeed, there is growing evidence that perturbing Wg/Wnt signaling might be linked to synapse dysfunction and the onset of neuropathologies (Oliva et al., 2018; Purro et al., 2014).

**The Expression of a Dominant-Negative form of Sgg/Gsk3β but not Wg can rescue the phenotypes associated with the over-expression of Gsb.**

In order to better understand the antagonistic relationship between Wg and Gsb we decided to study animals that were overexpressing both signals. Within a Gsb overexpression background, we overexpressed Wg or a dominant negative form of Sgg ($Sgg^{DN}$; Hazelett et al., 1998). Both forms of genetic manipulation are sufficient to activate the Wg pathway and, like others (Ataman et al., 2008; Miech et al., 2008; Packard et al., 2002b), we have shown that they affect synaptic growth and plasticity (Fig 3.4 C, E, G). We reasoned that if Gsb antagonizes Wg signaling by acting on the same transcriptional targets, then activating Wg signaling at any level (*wg* or $sgg^{DN}$) should be able to hinder Gsb's effects. Similarly, if Gsb and Wg signaling act on totally distinct sets of genes (having antagonistic effects on growth and plasticity) then activating Wg signaling at the level of Wg or Sgg should equally hinder Gsb's effects. On the contrary,

if Gsb exercises its antagonist effect by affecting the Wg transduction signal then overexpressing Wg or expressing $Sgg^{DN}$ could have different consequences on synapses overexpressing Gsb.

We first examined synaptic growth (Fig 3.4) and reiterated our results showing that overexpressing Gsb reduced synaptic size (57.5 ± 2.3 synaptic boutons) while over expressing Wg (103.1 ± 7 synaptic boutons) or $Sgg^{DN}$ (120 ± 9.3 synaptic boutons) led to increased synaptic growth compared to control (74.2 ± 2.6 synaptic boutons). We then over-expressed both Gsb and Wg and observed that the synaptic growth phenotype (48.1 ± 10 synaptic boutons) was as affected as in the Gsb overexpressors. Indeed, there was no difference between the two genotypes ($p = 0.59$) and the expression of Wg in the Gsb over-expressers showed absolutely no rescue towards control values (the difference between controls and Gsb/Wg over expressers is highly significant; $p < 0.0001$). In contrast when Gsb and $Sgg^{DN}$ were overexpressed the synaptic size was different from the Gsb over-expressers ($p = 0.0002$) and rescued towards control values (88.3 ± 6.5 synaptic boutons). This shows that expressing Wg or $Sgg^{DN}$ have drastically different effects on mitigating the overexpression of Gsb. This is consistent with Gsb acting on the Wg signal transduction pathway downstream of Wg and upstream of Sgg.

We then focused our attention on the phenotypes linked to ADSP. We performed stimulation of the synapse and showed that the overexpression of Gsb inhibited structural plasticity (Fig 3.5 B, G; 2.1 ± 0.4 ghost boutons) while the overexpression of Wg (Fig 3.5 C, G; 7.2 ± 0.5 ghost boutons) or the dominant negative form of Sgg (Fig 3.5 E, G; 8 ± 0.8 ghost boutons) produced ghost boutons at control levels (Fig 3.5 A, G; 8.1 ± 0.9). When we overexpressed Gsb and Wg together the ability of the synapse to be plastic was

just as inhibited as with Gsb overexpression alone (Fig 3.5 D, G; 2.3 ± 0.5 ghost boutons; $p > 0.99$). Again, while over-expressing Wg could increase activity-dependent synaptic plasticity during subthreshold stimulation paradigms (our unpublished results; Ataman et al., 2008), it appears that the overexpression of Gsb renders the synapse "immune" to Wg. In contrast, overexpressing Gsb and the dominant negative form of Sgg together led to synaptic plasticity back to control levels (7.4 ± 1 ghost boutons), showing again that the expression of $Sgg^{DN}$ is capable of reversing the effect of the Gsb over-expression towards control levels.

These experiments show that overexpressing Wg has no effect on the phenotypes generated by the overexpression of Gsb. In contrast, overexpressing $Sgg^{DN}$ does reverse the effects that overexpressing Gsb has on synaptic growth and plasticity. Taken together these results strongly suggest that Gsb is antagonizing the Wg signaling pathway by acting upstream of the kinase Sgg.

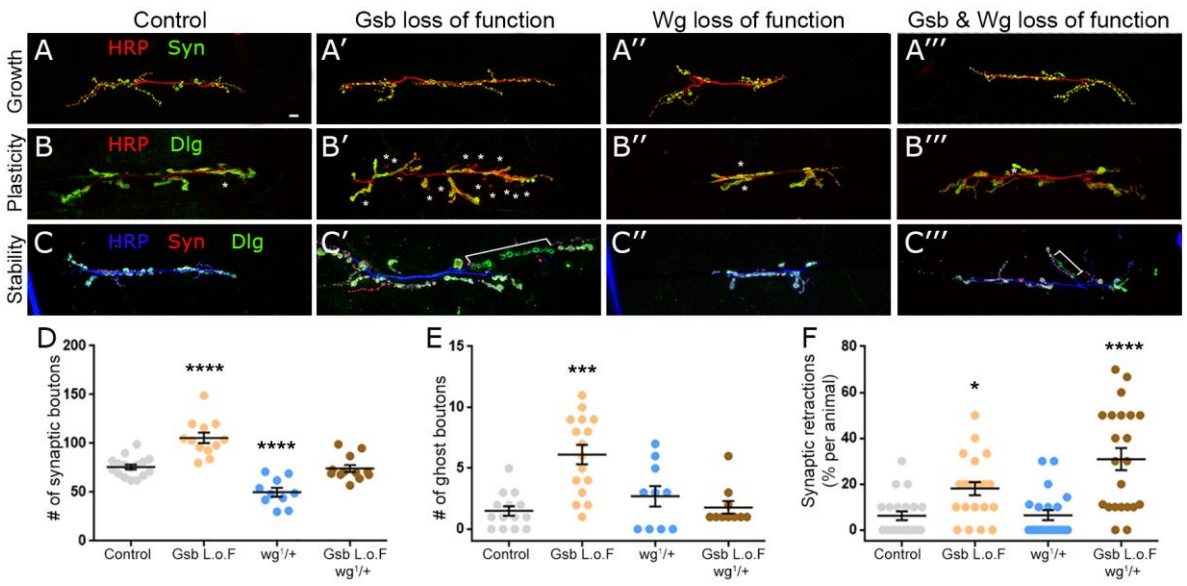


**Figure 3.3 An antagonistic relationship between Gsb and Wg defines synaptic growth and plasticity but not synaptic stability.**

**(A-C)** Representative NMJs for control, Gsb loss of function, wg loss of function, and gsb and wg loss of function show typical growth, plasticity and stability revealed by immunoreactivity to HRP, Syn and Disc large. Quantification of **(D)** synaptic boutons number **(E)** Ghost boutons number and (F) % of synaptic retractions per animal. *p < 0.05; ***p < 0.001; ****p < 0.0001. ANOVA with Dunnett and Tukey's post hoc test. Individual data are shown as scatter plots as well as mean ± SEM. Scale bar is 10 µm.

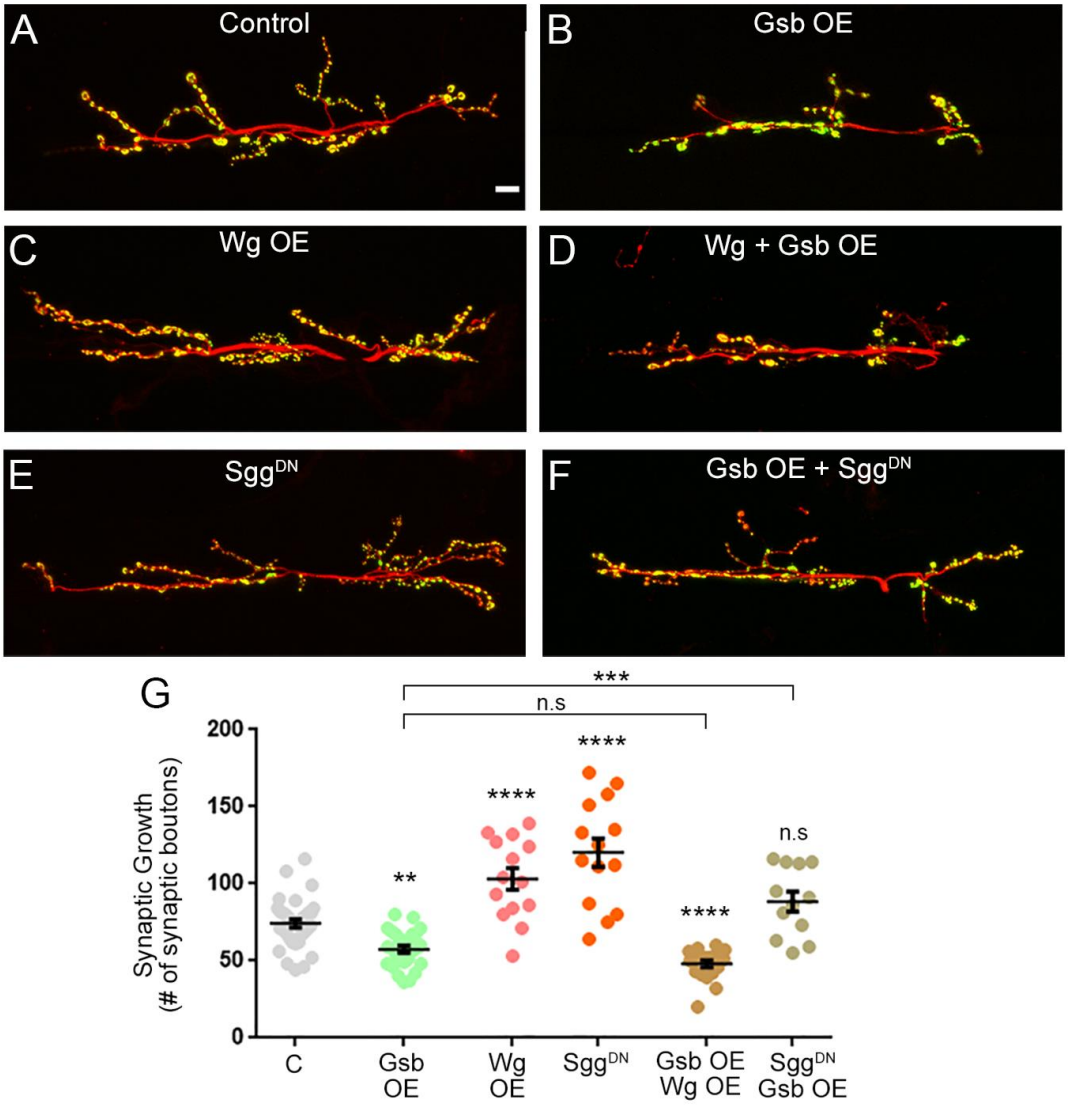


**Figure 3.4 Gsb antagonizes Wg signaling downstream of Wg and upstream of Sgg to define synaptic growth.**

**A-F**, Representative NMJs showing anti-HRP (red) and anti-Syn (green) immunoreactivity from control (elavC155-Gal4/+), Gsb over-expression (Gsb OE: elavC155-Gal4/+; UAS-Gsb/+); Wg over expression (Wg OE: elavC155-Gal4/+; +; UAS-Wg/+); Wg and Gsb over expression (Wg +Gsb OE: elavC155-Gal4/+; UAS-Gsb/+; UAS-Wg/+); the expression of a dominant negative form of Sgg (SggDN: elavC155-Gal4/+; +; UAS-sggA81T/+); Gsb over expression and the expression of a dominant negative form of Sgg (Gsb OE + SggDN: elavC155-Gal4/+; UAS-Gsb/+; UAS-sggA81T/+). G, Quantification of synaptic growth in the different genotypes. **p < 0.01; ***p < 0.001; ****p < 0.0001. ANOVA with Dunnett and Tukey's post hoc test. Individual data are shown as scatter plots as well as mean ± SEM. Scale bar is 10 µm.

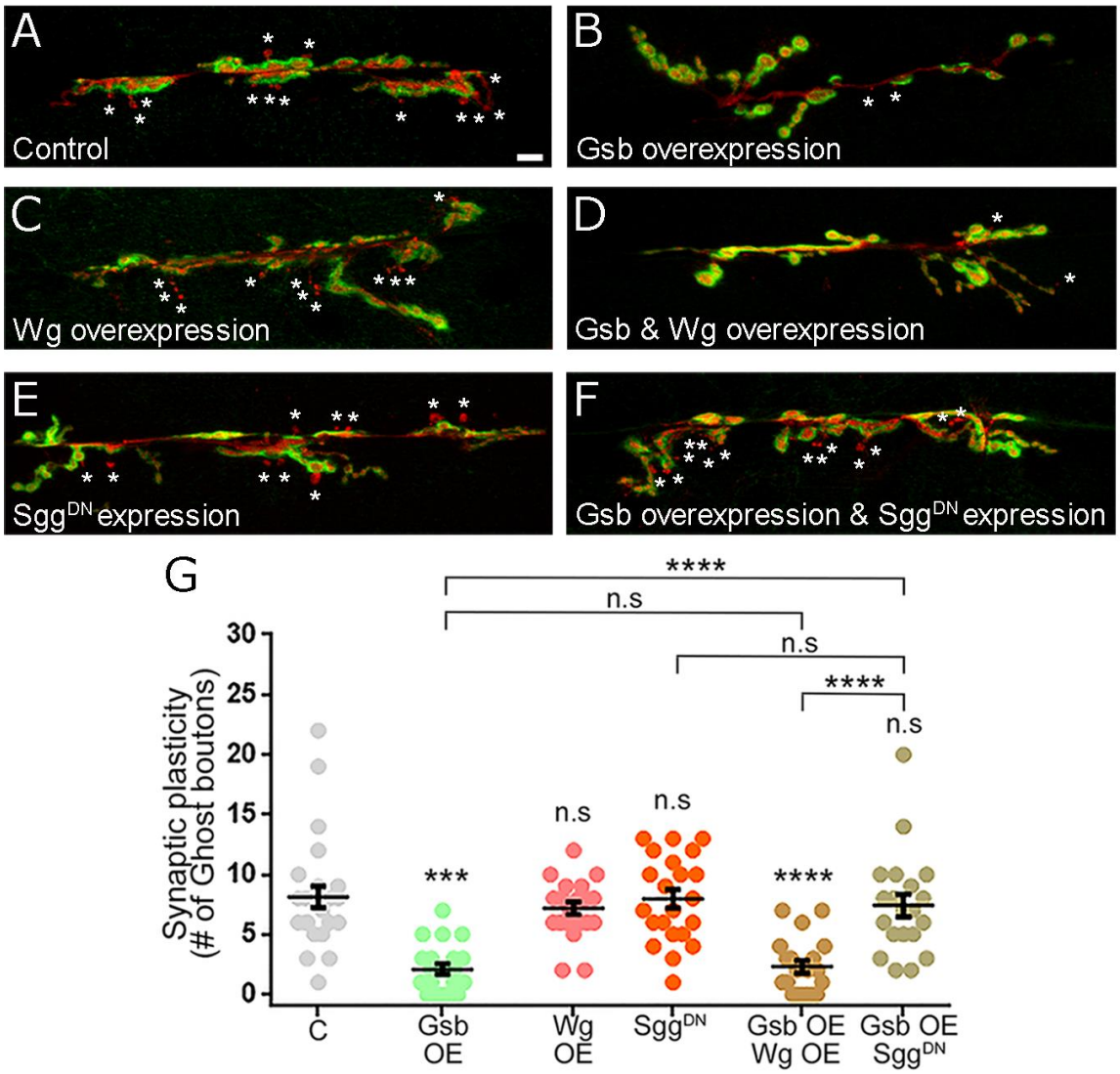


**Figure 3.5 Gsb antagonizes Wg signaling downstream of Wg and upstream od Sgg to define synaptic pasticity.**

**(A-F)** Representative NMJs showing anti-HRP (red) and anti-Dlg (green) immunoreactivity from control (elavC155-Gal4/+), Gsb over-expression (Gsb OE: elavC155-Gal4/+; UAS-Gsb/+); Wg over expression (Wg OE: elavC155-Gal4/+; +; UAS-Wg/+); Wg and Gsb over expression (Wg +Gsb OE: elavC155-Gal4/+; UAS-Gsb/+; UAS-Wg/+); the expression of a dominant negative form of Sgg (SggDN: elavC155-Gal4/+; +; UAS-sggA81T/+); Gsb over expression and the expression of a dominant negative form of Sgg (Gsb OE + SggDN: elavC155-Gal4/+; UAS-Gsb/+; UAS-sggA81T/+). **(G)** Quantification of synaptic plasticity in the different genotypes. **p < 0.01; ***p < 0.001; ****p < 0.0001. ANOVA with Dunnett and Tukey's post hoc test. Individual data are shown as scatter plots as well as mean ± SEM. Scale bar is 10 µm.

## DISCUSSION

It is not uncommon to find transcription factors that are determinant in setting up fate during early development being recruited for a later function. It is somehow more striking that the antagonistic interaction between the transcription factor Gsb and the secreted signaling molecule Wg, which has been characterized during neuroblast fate determination (Bhat, 1996; Bhat et al., 2000; Duman-Scheel et al., 1997), is still involved in defining the ability of a mature motoneuron to grow and be plastic. The similitudes of this interaction at different times and in different cells are remarkable. At the NMJ, the overexpression of Gsb provokes phenotypes similar to those provoked by Wg loss-of-function and opposite to those provoked by Wg overexpression. When both molecules were over-expressed, the phenotypes for growth and plasticity were similar to the phenotypes of Gsb overexpression alone. It seems that, in these over-expression conditions, Gsb is able to render the motoneuron insensitive to the Wg signaling at the NMJ. Elegant experiments showed that it is the relative dosage between Wg signaling and Gsb expression that determine NBs fate (Bhat et al., 2000). In our experiments, the phenotypes provoked by the loss of function of Gsb and affecting the NMJ growth and plasticity, are rescued by a 50% reduction in the Wg signaling. This shows that, like in early NBs, it is the imbalance between the two molecules that is responsible for the mutant phenotypes at the NMJ. Since it was shown that, to achieve plasticity, an increase of Wg at the NMJ is necessary, one possibility is that Gsb expression is constant and is required to determine a set point for both growth and plasticity. In this scenario, it is the varying amount of Wg that would create the imbalance resulting in differential growth and plasticity. Alternatively, Gsb expression might vary and, in turn, affect the expression

of synaptic molecules. A precise study of Gsb levels of expression at different stages of development and under different contexts of synaptic activity/plasticity will be required to shed light on the role of the Gsb/Wg interaction in regulating growth and plasticity at the synapse.

The decrease of Gsb expression within fully developed and functioning motoneurons provokes synaptic retractions similar to those observed in neurodegenerative diseases. This result should advocate for an increased interest in the role of transcription factors during aging and/or in the context of neurological disorders. Indeed, it is quite clear that transcription factors, once considered as developmental factors, are expressed in mature brains. One well studied example is the expression of the mammalian *engrailed* (*en*) gene within dopaminergic neurons and its link to Parkinson's disease (Rekaik et al., 2015). It is interesting to note that the human homolog of Gsb, the pax 7 gene, is expressed in an array of neurons within the vertebrate adult brain (Bandín et al., 2014; Shin et al., 2003). It will be important to find out whether transcription factors' late neuronal mis-expression is a common feature of neurological diseases. The present work also shows a role for Wg in the stability of the NMJ. Although this role is not detectable in Wg loss of function conditions, removing 50% of Wg is sufficient to significantly enhance the synaptic instability provoked by the loss of Gsb expression. This is consistent with the recent reports characterizing Wnts having essential neuroprotection functions (Cerpa et al., 2010; Galli et al., 2014; Marzo et al., 2016). It will be interesting to ask, in our system and others, whether Wg/Wnts expression increases when the stability of the synapse is challenged.

# CHAPTER 4

# THE ROLE OF CASEIN KINASE 1α (CK1α) IN SYNAPTIC GROWTH, PLASTICITY AND STABILITY

## INTRODUCTION

As mentioned and illustrated in chapter 3, Ck1α is an inhibitor of the Wg canonical pathway (Fig. 3.1) (Komiya and Habas, 2008; Legent et al., 2012). Ck1α phosphorylates Arm, leading to subsequent phosphorylation by Sgg (Liu et al., 2002). This dual-kinase mechanism is required for complete targeted degradation of Arm (Liu et al., 2002). In chapter 3, we showed data that suggest that Gsb antagonizes the Wg pathway downstream of Wg, but upstream of Sgg to control synaptic growth and plasticity. We hypothesize that Gsb is controlling the expression of a gene (or genes) that promote the inhibition of the Wg pathway. Proteins of the Wg-inhibiting destruction complex that are upstream of Sgg include: APC, Axin, and Ck1α. The expression of these proteins could be under the regulation of Gsb to control synaptic growth and plasticity. Preliminary transcriptomics data (results not shown) from central nervous system (CNS) samples of Gsb overexpressing larvae show an increase of Ck1α RNA, while CNS samples of Gsb loss of function larvae show a decrease of Ck1α transcripts. In addition, stimulated CNSs show a decrease of Ck1α, while non-stimulated CNSs show an increase. These preliminary data suggest that Ck1α is potentially an inhibitor of ADSP, similar to Gsb, and that Ck1α expression could be under the transcriptional control of Gsb. In addition, others have found that the loss of function of Ck1α provokes

synaptic instability at the NMJ (Bulat et al., 2014) similar to what we observe with Gsb loss of function animals. Thus, we hypothesize that Ck1α could be the Wg pathway inhibiting gene that is under the control of Gsb to regulate synaptic growth and plasticity. This chapter focuses on demonstrating that Ck1α has the same effects at the synapse as Gsb: it restricts growth and plasticity but stabilizes the synapse.

## MATERIALS AND METHODS

### *Fly stocks and genetics*

We used the *Gal4/UAS* system (Brand and Perrimon, 1993) to overexpress genes or express RNA interference (RNAi) constructs. To drive expression of genes in neurons at the embryonic stage we used the *elav$^{C155}$-Gal4* line [Bloomington Drosophila Stock Center (BDSC), stock #458]. Ck1α overexpression animals were produced using *elav$^{C155}$*-Gal4 and UAS-*Ck1α* (BDSC, stock #5506). Ck1α loss of function animals were produced with *elav$^{C155}$*-Gal4 and UAS-*Ck1αRNAi* (BDSC, stock #25786).

### *Fly rearing*

Females were used in these experiments. They were reared at 25°C on Jazz-mix Drosophila food (Fisher Scientific; Cat. No. AS153) following the manufacturer's instructions. For experiments requiring conditional expression, animals were reared at 18°C before being shifted to 29°C for different time periods as indicated in the study.

### *Activity-dependent stimulation protocol*

Our stimulation protocol was performed as described in Alicea et al., 2017. Briefly, larvae were subjected to 5 stimulation periods that were interspersed with rest

periods. The first 3 stimulations last 2 minutes, the fourth stimulation 4 minutes and the fifth stimulation 6 minutes. Each stimulation step was followed by 15 minutes of rest. The protocol lasts a total of 90 minutes. The stimulation solution consists of Haemolymph-like HL3 saline (70mM NaCl,10 mM $NaHCO_3$, 115 mM sucrose, 5 mM trehalose, 5 mM HEPES, 10 mM $MgCl_2$) containing 90 mM KCl and 1.5 mM $CaCl_2$, while the rest solution consists of HL3 saline containing 5 mM KCl and 0.1 mM $CaCl_2$.

***Experimental design, imaging and statistical analysis***

For experiments related to synaptic growth we scored m6/7 synapses at segment A3 in second or third instar larvae. Synaptic boutons revealed by the anti-synapsin immunolabeling were counted and averaged. We present (in Fig. 2.1, 2.3, 3.3 D, 3.5, 3.6) for each specific genotype and condition, the average, SEM and scatter plot derived from a minimum of 10 synapses from at least 5 animals.

For experiments relevant to synaptic plasticity we scored m6/7 synapses at segment A3 in third instar larvae. Ghost boutons were used to quantify the activity-dependent synaptic plasticity and were defined by the presence of anti-HRP immunolabeling and the absence of Dlg immunolabeling (Alicea et al., 2017). Controls (*C155/+* or *D42/+*) were run in parallel to experimental animals in each stimulation protocol to account for possible variation. We used a Nikon Eclipse 80i microscope at a magnification of 400x to quantify ghost boutons. For each specific genotype and condition, the average, SEM and scatter plot derived from a minimum of 10 synapses from at least 5 animals were presented (Fig. 2.4, 3.3 E, 3.5, 3.7).

For experiments dealing with synaptic stability we scored m6/7 synapses from segment A2 to A6 in third instar larvae. Synaptic retractions were defined by the presence of Dlg immunoreactivity that was not accompanied by synapsin immunoreactivity. For each animal we determined the percentage of synapses presenting retraction as well as the number of boutons retracted (the sum of all boutons retracted per animal). For each specific genotype and condition, we present the average, SEM and scatter plot derived from at least 9 animals were presented (Fig. 2.5, 3.3 F, 3.8). We used a Nikon Eclipse 80i microscope at a magnification of 400x to quantify synaptic retractions.

To perform our statistical treatment, we first assessed whether data conformed to a normal distribution by performing a Shapiro–Wilk normality test. When the result of this test was low ($p < 0.0001$), we ran a nonparametric Kruskal–Wallis test with a post hoc Dunn's multiple comparisons test. In the other cases, we ran a parametric one-way ANOVA test. The post hoc Dunnett correction test was applied when multiple comparisons were carried out against a control value (these comparisons are indicated with asterisks over the bars in the figures), while the post hoc Tukey correction test was used for multiple comparisons between data sets (these comparisons are indicated with brackets and asterisks the graphs). When only two data sets were compared, we performed an unpaired, two-tailed students' t-test. For every experiment, the statistical tests and the P values are indicated in their corresponding figure legends.

***Immunohistochemistry and imaging***

For experiments examining synaptic growth and synaptic retraction, larvae were dissected and fixed in Bouin's fixative (Sigma) for 1 minute. For plasticity experiments

larvae were fixed on 4% paraformaldehyde for 15 minutes in order to preserve the ghost boutons structure. The following primary antibodies were used overnight at 4°C: mouse anti-Syn, [1:20, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Dlg (1:20, DSHB) and rabbit anti- Dlg (1:150). The affinity purified rabbit anti-Dlg was raised (PrimmBiotech, Inc) against a recombinant protein containing the Dlg sequence stretching from amino acid 764 to amino acid 919. This polyclonal rabbit antibody revealed identical staining to the monoclonal mouse anti-Dlg (DSHB). We also used AffiniPure anti-HRP (Jackson ImmunoResearch) conjugated to Cy3 (1:300) or Cy5 (1:100) and secondary antibodies: 1:300 Alexa Fluor 488-conjugated AffiniPure goat anti-mouse or anti-rabbit IgG, 1:300 Alexa Fluor 546 conjugated AffiniPure goat anti-mouse (Jackson ImmunoResearch). They were applied 1 hour at room temperature as previously described (Maldonado et al., 2013; Marie et al., 2004).

## RESULTS

### *The kinase Ck1α impairs synaptic growth*.

We overexpressed Ck1α in all neurons using the driver *elav$^{C155}$-Gal4*. We quantified synaptic size by the number of boutons as previously described in chapters 2 and 3. This quantification was done on third instar larval NMJs innervating muscles 6/7. We found that Ck1α overexpression decreases synaptic growth, similar to what we observed in Gsb overexpressors (Fig 3.5). We then asked whether Ck1α loss of function could change synaptic size. We observed a significant increase in synaptic growth in

animals with Ck1α knockdown. These results suggest that Ck1α restricts the growth of synapses in larval NMJs.

***Ck1α inhibits activity-dependent synaptic plasticity.***

Since we found that Gsb controls synaptic plasticity (Chapter 2), we wondered if Ck1α also controlled this process. To test this, we submitted animals to the repeated stimulation protocol we described before (Chapter 2), which is sufficient to elicit activity-dependent structural changes at the NMJ. We asked whether preparations overexpressing Ck1α would be less plastic or if preparations with a loss of function Ck1α would be more plastic. We found that animals overexpressing Ck1α (Fig.4.2) presented a decrease in ghost bouton formation suggesting that Ck1α is a repressor of ADSP. In addition, we showed in chapter 2 that if we submit larvae to a subthreshold stimulation protocol (3 depolarization pulses instead of 5) Gsb loss of function animals showed a significant increase of ADSP. In a similar manner, knockdown of Ck1α increases the number of ghost boutons after this subthreshold stimulation (Fig.4.2), suggesting that this kinase is an inhibitor of ADSP at the NMJ.

***Ck1α controls synaptic stability.***

Ck1α has been shown to be required for the maintenance of synaptic stability (Bulat et al., 2014). Indeed, in our hands Ck1α loss of function shows a significant increase in the frequency of retractions and in the number of boutons retracted per animal (Fig. 4.3). These findings reflect the Gsb loss of function phenotype described previously. We found that overexpression of Ck1α had no detrimental effects on synaptic stability

(Fig. 4.3), just like Gsb overexpressors. These data suggests that both Ck1α and Gsb are required to stabilize the synapse.

## DISCUSSION AND FUTURE DIRECTIONS

It is interesting to find that Ck1α controls synaptic growth, plasticity and stability in a similar fashion as Gsb. In our experiments, Ck1α and Gsb overexpression provoke a decrease in synaptic growth while the loss of function of these molecules lead to overgrown synapses. In addition, Ck1α and Gsb overexpression impair synaptic plasticity while their knockdown enhances it. Our data also suggest that both Ck1α and Gsb expression is essential for synaptic stability. The roles of both Ck1α and Gsb in synaptic growth and plasticity antagonize the Wg pathway in these same processes. It is not surprising that Ck1α provokes opposite changes in synaptic structure compared to Wg. It has been shown that Wg is required for normal synaptic growth and ADSP (Ataman et al., 2008; Packard et al., 2002b). Since Ck1α blocks the transcription of Wg target genes (Komiya & Habas, 2008), it is reasonable to expect that changes in Ck1α expression would have opposite effects at the synapse compared to Wg. Gsb and Ck1α have similar effects on the synapse. This makes us wonder if Gsb regulates Ck1α expression to antagonize Wg and control synaptic growth and plasticity. To answer this question we would need to do genetic interaction experiments in which we alter Gsb and CK1α expression simultaneously to determine whether they are interacting genetically in synaptic growth and plasticity. In detail, we would decrease CK1α expression in a Gsb overexpression background to determine if the reduction in growth and plasticity that is observed in Gsb overexpressors depends on CK1α expression. Also, we would increase

CK1α expression in Gsb loss of function animals to analyse if the increase in synaptic growth and plasticity depends on CK1α. These experiments would allow us to determine whether the growth and plasticity phenotypes that we observe in Gsb gain of function or loss of function larvae are consequence of CK1α expression. Another essential experiment to answer our hypothesis would be to measure CK1α RNA levels and/or protein levels in Gsb overexressors and Gsb loss of function animals. We would expect an increase of CK1α expression in Gsb overexpressors and a reduction of CK1α expression in Gsb loss of function. Lastly, experiments to identify Gsb targets such as chromatin immunoprecipitation would allow us to determine if CK1α is a gene regulated by Gsb.

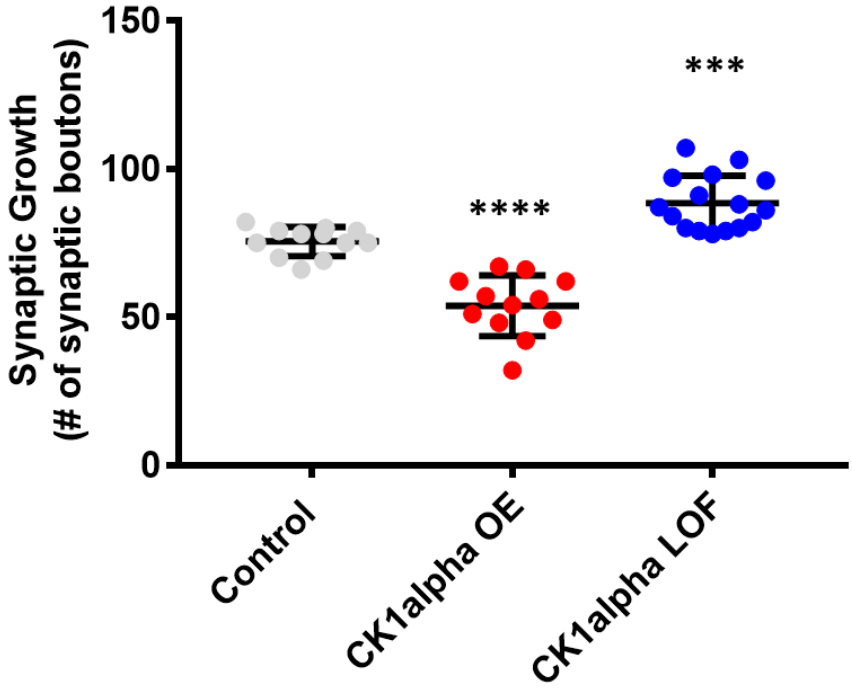


**Figure 4.1 The kinase Ck1α impairs synaptic growth**.

Quantification of the number of synaptic boutons in third instar larvae with the following genotypes: control (elavC155-Gal4/+), Ck1α overexpression (elavC155-Gal4/+; UAS-Ck1α /+) and Ck1α loss of function (elavC155-Gal4/+;+; UAS-RNAi- Ck1α /+) animals. ***$p < 0.0001$; ****$p < 0.0001$. ANOVA with post hoc Dunnett test. Individual data are shown as scatter plots as well as mean ± SEM.

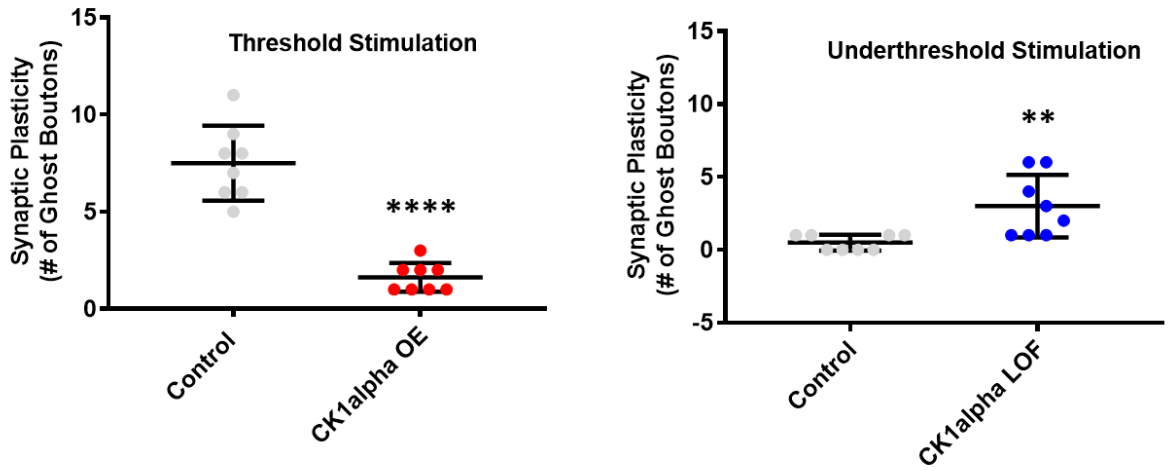


**Figure 4.2 The kinase Ck1α impairs activity-dependent synaptic plasticity.**

Quantification of the number of ghost boutons in third instar larvae with the following genotypes: control (elavC155-Gal4/+), Ck1α overexpression (elavC155-Gal4/+; UAS-Ck1α /+) and Ck1α loss of function (elavC155-Gal4/+;+; UAS-RNAi- Ck1α /+) animals. (Left) Quantification of ghost boutons number after threshold stimulation and (right) after underthreshold stimulation. An unpaired t-test with Welch's correction was done between genotypes; **p < 0.01; ****p < 0.0001. Individual data are shown as scatter plots as well as mean ± SEM.

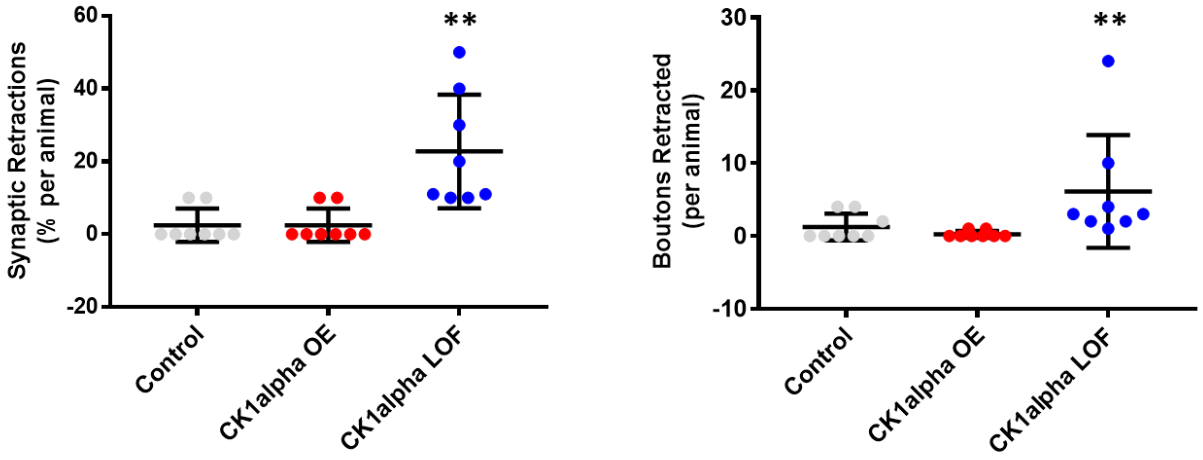


**Figure 4.3 The kinase Ck1α controls synaptic stability.**

Quantification of the % of synaptic retractions (occurrence) per animal and of the number of boutons retracted (severity) per animal in control, (elavC155-Gal4/+), Ck1α overexpression (elavC155-Gal4/+; UAS-Ck1α /+) and Ck1α loss of function (elavC155-Gal4/+;+; UAS-RNAi- Ck1α /+) animals. **p < 0.01; **p < 0.01. Kruskal-Wallis test with Dunn's multiple comparison test. Individual data are shown as scatter plots as well as mean ± SEM.

# CHAPTER 5

# CONCLUSIONS

It is established that TFs are important for nervous system development in terms of neuronal differentiation, proliferation, migration and identity. Less is known about what they do in fully functional and mature neurons. With our work we wanted to elucidate the role that TFs have to maintain basic neuronal properties such as synaptic growth, plasticity and stability. We focused our study on Gsb, a pair rule developmental regulator that controls the differentiation of a subset of neuroblasts. Interestingly, its role during early development has been linked to its ability to antagonize Wg signaling. Here, we hypothesized that Gsb is required in mature MNs to maintain general neuronal properties, also by antagonizing Wg.

To assess the role of Gsb in the mature nervous system we manipulated its expression at different stages of MN development and asked whether the growth, stability and plasticity of the neuromuscular junction was affected. Perturbing Gsb expression at early (post-mitotic; embryo) and late (after initial synaptic growth; larval stages 2 and 3) stages of synapse development affected synaptic growth, stability and plasticity suggesting that Gsb is not only an early fate determinant but is also required late to control MNs synaptic properties. In addition, we analyzed these synaptic properties after manipulating Gsb and Wg expression simultaneously. We found that Gsb antagonizes Wg to control synaptic growth and plasticity but controls synaptic stability independently of Wg. Gsb overexpression reduces synaptic growth and impairs synaptic plasticity, while Wg overexpression leads to overgrown and overplastic synapses. When Gsb and

Wg are overexpressed simultaneously, the phenotypes are identical to the Gsb over expressors suggesting that Gsb renders the synapse resistant to Wg. In contrast, when we overexpress Gsb and activate the Wg pathway by expressing a dominant negative form of the kinase Sgg, we found that both growth and plasticity phenotypes are restored to control levels. This finding strongly suggests that Gsb inhibits the Wg signaling pathway upstream of Sgg and downstream of Wg. We conclude that Gsb and Wg, two molecules essential to nervous system development, interact to control mature neuronal function. We hypothesize that the nature of this interaction is due to Gsb's regulation of Wg pathway inhibitors. CK1α is a kinase that inhibits the Wg pathway and we think it could be under the control of Gsb to antagonize Wg in synaptic growth and plasticity. We found that CK1α mimics Gsb's phenotypes at the synapse by repressing growth and plasticity, but maintaining stability. Experiments in which Gsb and CK1α expression are changed simultaneously are needed to draw conclusions in terms of interactions. In addition, CK1α level of expression in Gsb gain of function or loss of function genotypes should be addressed.

Overall, our data provide a strong foundation to recognize that TFs that are normally recruited during nervous system development can also have distinct roles in the mature neuron to maintain its function. Also, we provide new insights about how the Wg pathway can be regulated in mature neurons to regulate synaptic growth, plasticity and stability. Wg and problems in synaptic function and structure have been implicated in neurodegenerative diseases such as Alzheimer's disease, schizophrenia, bipolar disorder, and William's syndrome (Caricasole et al., 2005; De Ferrari et al., 2007; Hoseth et al., 2018; C. Zhao et al., 2005). Our work suggests a genetic interaction between Gsb and Wg

that could be relevant to the understanding of neurodegenerative diseases. Gsb, a TF that interacts with Wg during embryogenesis still antagonizes this signaling molecule in mature neurons. Gsb is possibly a modulator of the Wg canonical pathway, which makes it a focus of study to further understand synaptic diseases. Our study highlights the importance of investigating genetic interactions of the same molecules in different developmental contexts. TFs of the developing nervous system may have critical roles in the function and maintenance of mature and aging neurons.

# APPENDIX A

## ADDITIONAL GOOSEBERRY EXPERIMENTS

### RESULTS

In chaper 2 we explored Gsb's role in synaptic plasticity at early and late timepoints in 3rd instar development (Fig 2.4). To manipulate Gsb expression late we used the Gal80 temperature sensitive system. With these conditional expression experiments we were able to conclude that Gsb controls synaptic plasticity late in mature neurons. We also did late expression experiments using the driver D42 but did not include them in the manuscript because our conditional expression experiments suffice for our arguments. Nonetheless we include in this appendix our results using D42-Gal4 (Fig. A1). With these experiments our argument that Gsb impairs synaptic plasticity and that is a function that it has in mature neuronal life still stands.

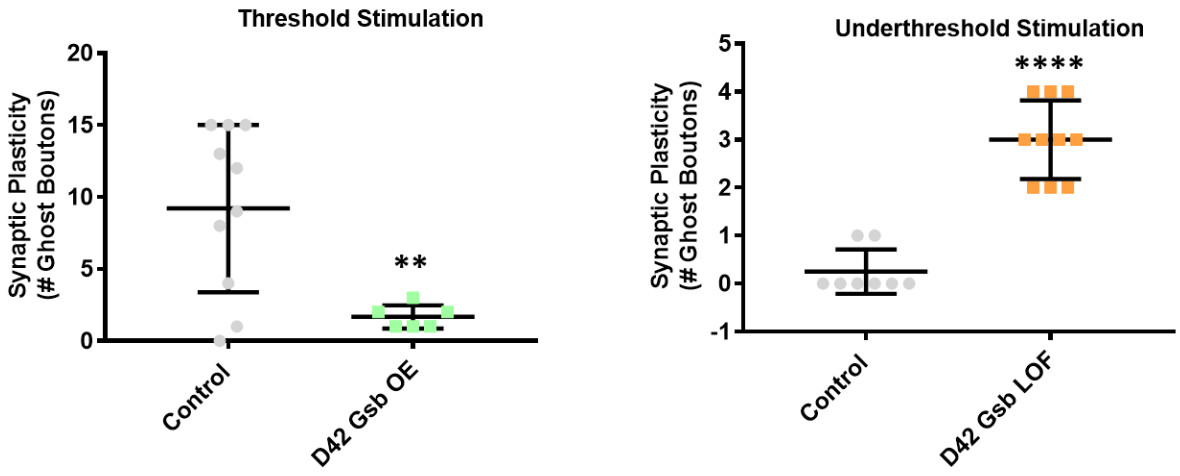


**Figure A.1 Late changes in Gsb controls activity-dependent synaptic plasticity.**

Quantification of ghost boutons number in third instar muscle 6/7 NMJ from control (D42-Gal4/+), Gsb over-expression (D42-Gal4/UAS-Gsb) and Gsb loss of function (gsb01155/UAS-RNAi-Gsb; D42-Gal4/UAS-RNAi-Gsb) animals. (Left) Quantification of ghost boutons number after threshold stimulation and (right) after underthreshold stimulation. Unpaired t-test with Welch's correction **$p < 0.01$; ****$p < 0.0001$. Individual data are shown as scatter plots as well as mean ± SEM.

# APPENDIX B

## ADDITIONAL CK1α EXPERIMENTS

### RESULTS

In chapter 4 we presented results that suggest that Ck1α controls synaptic growth, plasticity and stability. In these experiments we changed Ck1α expression using the pan-neuronal driver *elav*-Gal4. We also reproduced these experiments with the late driver D42. In these conditions we also found that overexpression of Ck1α provokes undergrown synapses, but interestingly Ck1α synapses do not show an increase in bouton number as previously seen with the *elav*-Gal4 data. This suggests that at late stages the removal of Ck1α does not control synaptic growth (Fig. B.1). Overexpression of Ck1α D42 shows non-plastic synapses (Fig. B 2) similar to what we saw with *elav*-Gal4, and Ck1α loss of function at late stages increases synaptic instability (Fig. B 3).

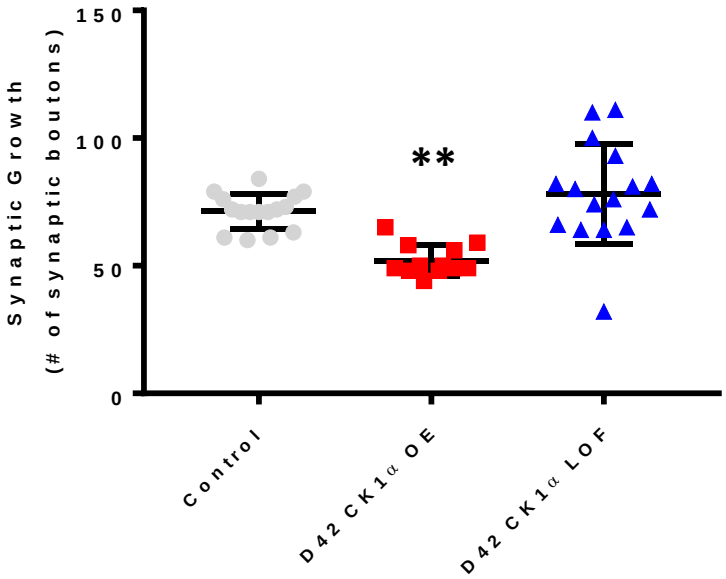


**Figure B.1 Late changes in Ck1α controls synaptic growth.**

Quantification of the number of synaptic boutons in third instar larvae with the following genotypes: control (D42-Gal4/+), Ck1α overexpression (D42-Gal4/+; UAS-Ck1α /+) and Ck1α loss of function (D42-Gal4/+;+; UAS-RNAi- Ck1α /+) animals. **p < 0.001. ANOVA with post hoc Tukey's test. Individual data are shown as scatter plots as well as mean ± SEM.

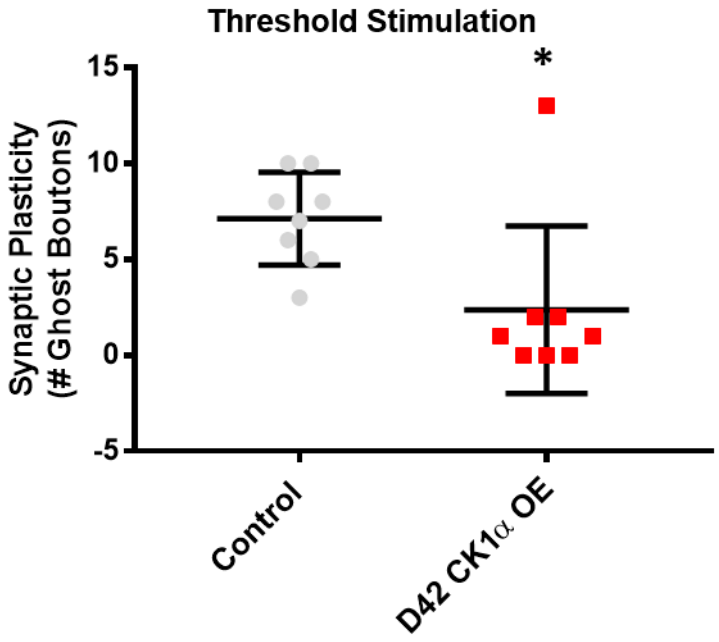


**Figure B.2 Late changes in Ck1α impairs activity-dependent synaptic plasticity.**

Quantification of the number of ghost boutons in third instar larvae with the following genotypes: control (D42-Gal4/+) and Ck1α overexpression (D42-Gal4/+; UAS-Ck1α /+). Threshold stimulation was performed. An unpaired t-test with Welch's correction was done between genotypes; *$p < 0.01$. Individual data are shown as scatter plots as well as mean ± SEM.

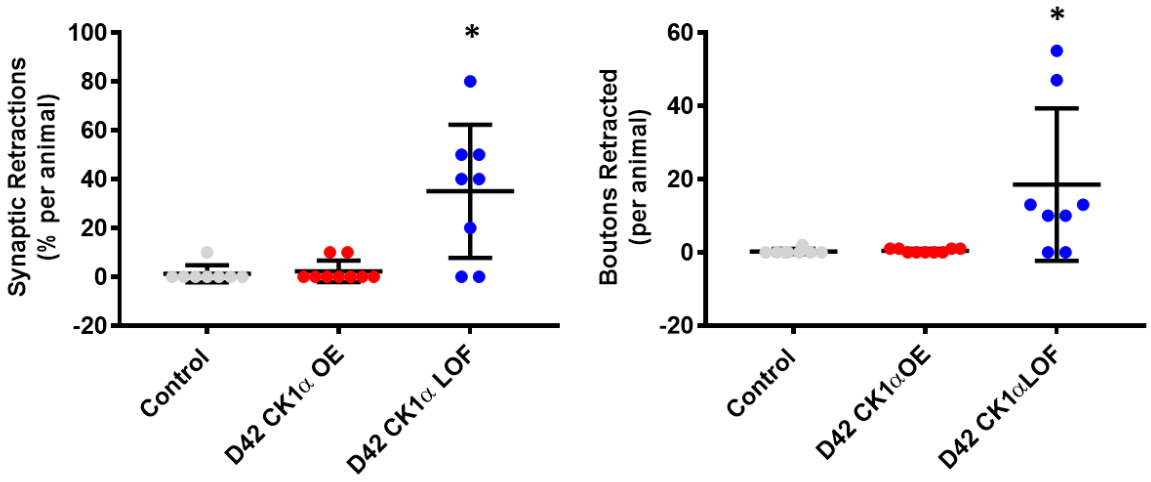


**Figure B.3 Late changes in Ck1α controls synaptic stability.**

Quantification of the % of synaptic retractions (occurrence) per animal and of the number of boutons retracted (severity) per animal in control, (D42-Gal4/+), Ck1α overexpression (D42-Gal4/+; UAS-Ck1α /+) and Ck1α loss of function (D42-Gal4/+;+; UAS-RNAi-Ck1α /+) animals; *p < 0.01; *p < 0.01. Kruskal-Wallis test with Dunn's multiple comparison test. Individual data are shown as scatter plots as well as mean ± SEM.

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