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Regulation of neuronal morphology: Exploring the function of Ral GTPase in nerve thickness and in structural plasticity

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Regulation of neuronal morphology: Exploring the function of Ral GTPase in nerve thickness and in structural plasticity

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Palavras-chave

Ral GTPase, JNK, Plasticidade estrutural, Espessura do nervo, *Drosophila,* FasII, Células da Glia, Junção Neuromuscular

Resumo

Os neurónios são o tipo de célula morfologicamente mais diversificada, sendo o seu desenvolvimento e manutenção essenciais para o correto funcionamento do sistema nervoso. A estrutura primária de um neurónio é estabelecida durante o crescimento das dendrites, do axónio e na formação da sinapse, contudo está sujeita a modificações subsequentes em resposta a atividade sináptica. A Ral é uma GTPase, membro da superfamília da Ras, conhecida por desempenhar um papel importante em vários processos biológicos, como por exemplo a regulação da plasticidade estrutural no compartimento pós-sináptico. Neste trabalho, procuramos compreender o envolvimento da Ral GTPase na regulação da plasticidade estrutural pré-sináptica, ao analisar o seu papel na formação de novos botões sinápticos em resposta à atividade sináptica.

Um aspeto importante no desenvolvimento do sistema nervoso é a organização dos axónios em feixes nervosos. Ao saírem do sistema nervoso central, os axónios de vários neurónios são agrupados para inervar diferentes músculos, de uma maneira estereotipada. Com este estudo, mostramos que a Ral GTPase de *Drosophila* regula a grossura e a organização do nervo. Os mutantes de Ral têm feixes nervosos mais grossos e níveis de uma molécula de adesão celular, diminuídos, a Fasciclin II, sugerindo que, possivelmente, existe um defeito na fasciculação axonal. A Ral GTPase mostrou ser um regulador positivo ou negativo da sinalização JNK, dependendo do contexto celular, enquanto a sinalização JNK demonstrou estar envolvida na remoção do axónio através da desestabilização da proteína de adesão celular FasII. Com este estudo esperamos entender se a Ral regula a grossura do nervo via JNK através da modulação da adesão celular e se a sua função é necessária nos neurónios e/ou na glia. As células da glia também fazem parte do sistema nervoso e desempenham um papel importante na regulação do desenvolvimento e da função neuronal.

Os nossos resultados sugerem que Ral não interage com a sinalização JNK nos neurónios ou nas células da glia para regular a grossura do nervo. No entanto, a função da Ral na glia parece desempenhar um papel importante na regulação da grossura do nervo. Sendo assim, é fundamental compreender como as células da glia regulam a grossura do nervo e quais são as vias envolvidas neste processo, uma vez que os defeitos na morfologia neuronal e da glia estão envolvidos em várias doenças neurodegenerativas.

Keywords

Ral GTPase, JNK, Structural plasticity, Nerve thickness, *Drosophila,* FasII, Glial Cells, Neuromuscular junction

Abstract

Neurons are the most morphologically diverse cell type whose development and maintenance are essential for proper function of the nervous system. The primary shape of a neuron is established during axon and dendrite outgrowth and synapse formation, but is subject to subsequent modifications by physiological events. Ral is a small GTPase, member of the Ras superfamily that is known to play an important role in a plethora of biological processes such as the regulation of structural plasticity in the postsynaptic compartment. Here, we aim to understand the involvement of Ral GTPase in the regulation of presynaptic structural plasticity, by studying its role in the formation of new synaptic boutons in response to activity.

An important aspect of nervous system development concerns how axons are organized into nerve bundles. When exiting the Central Nervous System (CNS), axons from several neurons are bundled together to innervate different muscles in a stereotyped manner. Here, we show that *Drosophila* Ral GTPase regulates nerve thickness and organization. Ral mutants have thicker nerve bundles and decreased levels of Fasciclin II, a cell adhesion molecule, suggesting that possibly, there is a defect in axonal fasciculation. Ral GTPase has been shown to be a positive or negative regulator of JNK signaling, depending on the cellular context, while JNK signaling has been shown to be involved in axon pruning by destabilization of the cell adhesion protein FasII. We want to understand if Ral regulates nerve thickness via JNK, via cell adhesion modulation, and whether its function is required in neurons and/or glia. Glial cells are an integral part of the nervous system and play an important role in the regulation of neuronal development and function.

Our results suggest that Ral does not interact with JNK signaling in neurons or in glial cells to regulate nerve thickness. However, the role of Ral in glia appears to play a role in the regulation of nerve thickness. Thus, it is critical to understand how glial cells regulate nerve thickness and what are the pathways involved in this process since defects in neuronal and glia morphology are a hallmark of several neurodevelopmental and neurodegenerative disorders.

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Abbreviations

µm – Micrometer

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AP-1 – Activator protein-1

AZ – Active Zones

ATP – Adenosine Triphosphate

BBB – Blood-Brain Barrier

BDSC – Bloomington *Drosophila* Stock Center

Bsk – Basket

CAM – Cell Adhesion Molecule

CNS – Central Nervous System

DABCO – 1,4- Diazabicyclo[2.2.2]octane

Dlg – Discs large

DN – Dominant Negative

ERK – Extracellular Signal Regulated Kinase

FasII – Fasciclin II

FOXO – Forkhead Box O

GAP – GTPase activating protein

GDI – GDP dissociation inhibitor

GDP – Guanosine Diphosphate

GEF – Guanine exchange factor

Gsk3β – Glicogen synthase kinase 3β

GTP – Guanosine Triphosphate

Hep – Hemipterous

HL3.1 – Hemolymph-Like solution 3.1

HRP – Horseradish Peroxidase

JIP1 – c-Jun-amino-Terminal-interacting Protein 1

JNK – c-Jun NH_2-terminal kinase

Jra – Jun-Related Antigen

Kay – Kaya

MAP1B – Microtubule-Associated Protein 1

MAPKs – Mitogen Activated Protein Kinase

Mkk4 – MAP Kinase Kinase 4

Msn – Misshapen

NGS – Normal Goat Serum

NMJ – Neuromuscular Junction

p-JNK – Phosphorylated JNK

PBS – Phosphate-buffered saline

PBST – Phosphate-buffered saline with Triton-X

PFA – Parafolmaldehyde

PG – Perineural Glia

PNS – Peripheral Nervous System

PSD-95 – Postsynaptic density-95

Puc – Puckered

RalBP1 – Ral Binding Protein1

ROS – Reactive Oxygen Species

RT – Room Temperature

SAPs – Synapse-associated proteins

SCG10 – Superior Cervical Ganglion 10 Protein

SNARE – Soluble NSF-attachment protein

SPAKs – Stress-Activated Protein Kinases

SPG – Subperineural Glia

UAS – Upstream Activating Sequences

WG – Wrapping Glia

Chapter 1. Introduction

The development and function of the nervous system depend on the proper establishment of neuronal connections. Neurons are highly polarized cells whose morphology determines many aspects of function of the nervous system (Chung & Barres 2009; Poulain & Sobel 2010). In response to changes in the environment and in synaptic activity, neurons can alter both pre- and postsynaptic elements of the synapse (Pfenninger 2009). Defects in synaptic morphology and activity-dependent plasticity are a hallmark of several neurodevelopmental and neurodegenerative disorders. Thus, it is critical to understand the mechanisms that are regulating the shape of neurons and how they change in response to environmental perturbations. It is known that Ral GTPase is involved in postsynaptic plasticity at the *Drosophila melanogaster* (from now on called *Drosophila*) neuromuscular junction (NMJ), regulating the subsynaptic reticulum (SSR) growth in an activity-dependent manner (Teodoro et al. 2013). However, little is known about the involvement of Ral GTPase in the regulation of presynaptic structural plasticity. Therefore, we want to study whether Ral also plays a role in the pre-synaptic side, contributing to the formation of activity-dependent synaptic boutons. Besides playing a role in structural plasticity, we found that *Drosophila* Ral GTPase regulates nerve thickness at the neuromuscular junction but the mechanisms and the pathways involved in this process are not understood. Defects in axon bundling can lead to serious problems in the transduction of information between neurons (Banerjee & Bhat 2008). Our objective is to uncover if and how Ral GTPase is involved in presynaptic structural plasticity and the mechanism behind the regulation of nerve thickness, using the *Drosophila* NMJ as a model synapse.

1.1. *Drosophila melanogaster* as a model system

Drosophila melanogaster, also known as the fruit fly, started to be used as model organism in the early 1900s by Thomas Morgan and his co-workers. It is one of the most studied organisms in biological research, with research in *Drosophila* having contributed to significant discoveries in biological processes, including development (Lawrence 1992), signaling (Cadigan & Peifer 2009), cell cycle (Lee & Orr-Weaver 2003), nervous system development, function and behavior (Bellen et al. 2010; Weiner 1999), which altogether have contributed to the understanding of several developmental and neurological disorders (Bier 2005; Markow 2015). *Drosophila* is a good model system for studying various aspects of cellular biology, mainly because many of the genetic pathways that are associated with basic developmental processes are conserved during evolution, showing several similarities with higher eukaryotes. Furthermore, the fruit fly genome has approximately 75% of homology with known genes associated to human diseases, making it a powerful organism for the study of human genetics (Reiter et al. 2001; Bier 2005; Adams et al. 2000). *Drosophila* is an inexpensive animal model to maintain, has a short life cycle and is easy to manipulate, enabling many experiments in a short period of time, thus promoting the rapid advancement of research. In addition, it has a simple and accessible anatomy, a vast and powerful set of genetic tools, and is accessible to various experimental techniques (Bier 2005; Collins & DiAntonio 2007).

1.1.1. *Drosophila* life cycle

A major advantage of working with *Drosophila* is its short life cycle lasting approximately 10 days at 25°C, and this generation time doubles when kept at 18ºC. *Drosophila* life cycle consists of four stages: embryo, larva, pupa and adult fly (Figure 1.1). When fertilized, females can store the sperm for the fertilization of several eggs to be laid over the next few days. At 25°C, the embryo development in the egg occurs in approximately 21 hours, after which it hatches as a larva (Prokop 2013; Weigmann 2003).

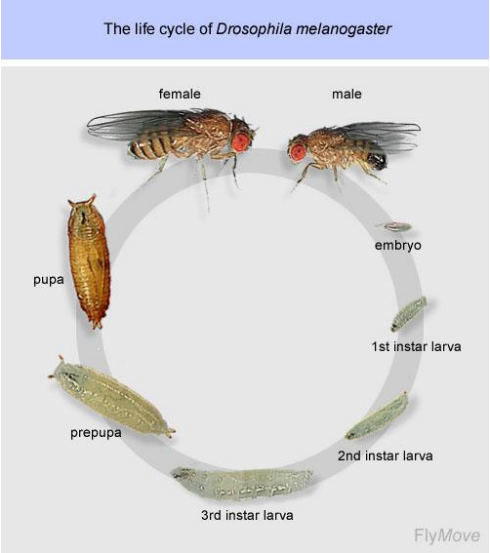

Figure 1.1. Schematics of the *Drosophila melanogaster* life cycle. At 25°C *Drosophila melanogaster* life cycle lasts 9-10 days. After 1 day of embryonic development, the hatched larvae (1st instar) spend 1 day until reaching the 2nd instar. 1 day later, the larvae proceed to the 3rd larval stage (3rd instar) which lasts for 2-3 days. In the following 5 days, the pupal stages takes place, where the organs degenerate and restructure into their adult shapes (metamorphosis). 10 days after egg laying an adult fly emerges from the pupal case. (From Weigmann et al. 2003; Prokop 2013).

The larval stage is composed of three distinct phases of development called instars. In this stage, the larva eats and grows from 1st to 3rd instar entering then in pupariation, a stage where the larva becomes an immotile pupa and metamorphosis occurs. During the pupal stage, all organs of the larva are degenerated and then restructured into adult structures. Adult flies emerge from pupal cases about 10 days after egg laying (at 25^0C). (Prokop 2013; Weigmann 2003).

1.1.2. *Drosophila* Neuromuscular Junction

Drosophila NMJ is a well characterized model system for the study of neuronal development, plasticity and function due to its stereotypical structure from animal to animal, for being one of the best studied synapses, and of course, given the availability of a wide variety of molecular, genetic and experimental techniques. The fruit fly larval NJM is a well-characterized and simple system, constituted by 32 motor neurons and 30 identified muscle cells in each hemisegment that are repeated and bilaterally symmetric (Menon et al. 2013; Collins & DiAntonio 2007). During development, each motor neuron innervates a specific muscle cell leading to synapses with stereotyped arborization and with a

relatively constant and quantifiable number of synaptic boutons, but that are different for each muscle cell. Synaptic boutons are composed by many active zones that represent the presynaptic releasing sites, where synaptic vesicles are clustered. Boutons are surrounded by a postsynaptic muscle membrane containing clusters of glutamate receptors, which translate presynaptic activity in postsynaptic depolarization, leading to activation of postsynaptic signaling cascades (Collins & DiAntonio 2007). Given that these NMJs are highly stereotyped, it makes it possible to compare the same NMJ from larva to larva. Also, it is accessible to various experimental techniques such as electrophysiology, calcium imaging, immunocytochemistry, electron microscopy and live imaging, providing a great advantage for structure and molecular anatomy studies of the synapses. Besides its stereotyped circuitry, *Drosophila* NMJ also shows robust plasticity, adapting structurally and functionally in response to changes in the environment, neuronal activity and gene function (Menon et al. 2013; Collins & DiAntonio 2007; Featherstone & Broadie 2000). The *Drosophila* NMJ synapses use glutamate as neurotransmitter, which closely resembles the vertebrate central nervous system (CNS) synapses. In *Drosophila*, larval NMJ synapses express ionotropic glutamate receptors that are similar to AMPA-Type glutamate receptors in the mammalian brain. Also, the postsynaptic scaffold protein, Discs large (Dlg) is identical to those found in mammalian postsynaptic densities, belonging to the family of PSD-95 and SAPs. Altogether, given the cellular and molecular similarities, *Drosophila* NMJ synapses are an excellent model to study excitatory glutamatergic synapses (Menon et al. 2013).

1.2. Neuronal Growth and Development

Nervous system function depends on proper establishment of complex neuronal networks determined during development (Chung & Barres 2009; Poulain & Sobel 2010). Neurons are the fundamental unit of function of the nervous system and are highly polarized cells composed by a soma, dendrites, axons and axon terminals containing synapses. During morphogenesis, neurons start to extend their axons and dendrites that are structurally and functionally different. The axon is usually a thin and long process that conducts nerve impulses through long distances, delivering specific signals to multiple cells, while dendrites are characterized by its branched projections that are important to receive and integrate synaptic inputs (Chung & Barres 2009; Poulain & Sobel 2010; Polleux & Snider 2010). When exiting the central nervous system (CNS), motor axons from several neurons are bundled together forming long nerve fibers that will establish a connection with a target cell (Araújo & Tear 2003). After reaching its target, the growth cone (the specialized structure at the tips of extending axons) contacts with dendrites of other neuron or with another cell type, like a muscle cell (neuromuscular junction) and starts to differentiate the presynaptic terminal (Chung & Barres 2009). Synapses are essential for the proper communication between neurons and other cells. Regulated formation of the synapses requires bidirectional signals between pre- and post-synaptic cells which results in the development of specialized structures important for neurotransmitter release and detection (Südhof 2012; Collins & DiAntonio 2007). After synapse formation, the continuous growth leads to addition of new synaptic branches and of synaptic boutons, which are round varicosities where synapses are located. Synaptic boutons are composed of active zones (AZ) containing neurotransmitter-filled vesicles

and the necessary machinery for their release (Südhof 2012; Harris & Littleton 2015). Opposed to that, are present other neuron or specialized cells containing postsynaptic structures with specific receptors for the neurotransmitter released by the presynaptic cell, like the muscles in case of NMJs. When an action potential reaches the presynaptic terminal, voltage-gated Ca^{2+} channels are opened which allows for Ca^{2+} ions to bind to synaptotagmins present in the synaptic vesicles, triggering neurotransmitter release to the synaptic cleft, activating the postsynaptic receptors and downstream postsynaptic cascades (Shen & Cowan 2010; Südhof 2012). Proper regulation of these various steps is necessary to avoid neuronal related disorders.

1.3. Regulation of Synaptic Growth and Plasticity

During development, neurons undergo significant remodeling processes in order to generate appropriate axonal and synaptic connections. Excitatory synapses in the vertebrate nervous system use glutamate as their primary neurotransmitter. This type of synapses shows robust plasticity, a process characterized by modifications in terminal connections in response to neuronal activity (Menon et al 2013; Harris & Littleton 2015). There are two main types of plasticity, structural and functional plasticity that are thought to be involved in learning and memory. Usually, functional plasticity is related to changes in the strength of synaptic transmission while structural plasticity is associated with changes in synaptic morphology, like alterations in the number, size and shape of synaptic elements (Shen & Cowan 2010; Griffith & Budnik 2006). In response to changes in the environment, neurons can alter both pre- and postsynaptic structures of the synapse, requiring regulated membrane trafficking and exocytosis for membrane addition (Pfenninger 2009). During the process of synaptic remodeling, coordinated dynamics and organization of actin and microtubule cytoskeleton are essential for controlling shape changes in the synapse. They are key players in the support of active transport of membranes, organelles and macromolecules required for development (Poulain & Sobel 2010; Menon et al. 2013). Regulation of these critical processes has been associated with the ability of neurons to strengthen synapses and is essential to prevent defects in neuronal structure and function. *Drosophila* has been an excellent model for studies of the synapse, given that the its NMJ is glutamatergic resembling the vertebrate central nervous system, and because NMJs are organized into branched arbors that are composed of chains of synaptic boutons with stereotyped morphology that is genetically determined but where its synaptic structure and function can be modified by extrinsic factors, such as the environment, or changes in neuronal activity (Menon et al. 2013; Collins & DiAntonio 2007). Like in vertebrates, *Drosophila* larvae synapses of the NMJ are composed by a presynaptic terminal containing active zones with pools of vesicles filled with neurotransmitters, opposed to clusters of neurotransmitters receptors in the membrane of the postsynaptic cell. The postsynaptic membrane, called subsynaptic reticulum (SSR) is formed by numerous folds and invaginations of the membrane that grows in an activity-dependent manner (Teodoro et al. 2013). During larval development, muscles and synaptic boutons grow from 1st to 3rd instar increasing the muscle area about 100 times and the number of synaptic boutons in 10 times. The addition of new boutons can occur throught different mechanisms

including, asymmetric budding of preexisting bouton, symmetric division of a bouton and *de novo* formation of a bouton from the axonal membrane (Zito et al. 1999). (Figure 1.2.)

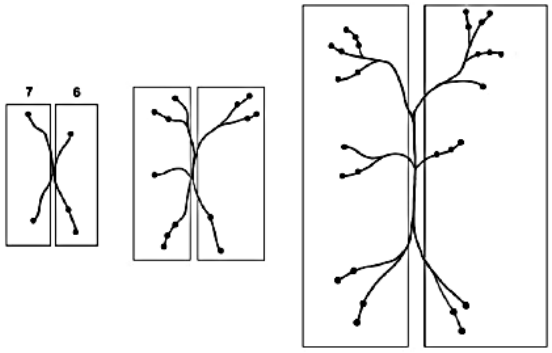

Figure 1.2. Schematic representation of the development of the Neuromuscular Junction (NMJ) in muscles 6 and 7. Drosophila larva grows from 1st (left) to 3rd instar (right), increasing the muscle size which is accompanied by the addition of new branches and synaptic boutons. (Adapted from Menon et al. 2013).

In addition to neurons, glial cells have varied functions that are important for proper development and function of both vertebrates and invertebrates nervous systems.

1.4. Glial Cells

Glial cells are part of the CNS and peripheral nervous system (PNS) in most animals. They play important roles in the regulation of neuronal development and function (Stork et al. 2012; Brink et al. 2012). Glia are involved in many processes in the development of the nervous system, like modulation of neural stem cell proliferation (Ebens et al. 1993), regulation of the differentiation of neural precursors guiding axon pathfinding (Hidalgo & Booth 2000; Sepp et al. 2001), ensheathing axon fascicles (nerves) and individual axons supplying trophic support for neurons (Barres 2008; Booth et al. 2000), function as primary immune cells by engulfing neurons and debris that are eliminated during development (Watts et al. 2004), and promoting synapse formation and maturation (Barres 2008).

The development of the nervous system in vertebrates and flies has several similarities. Due to its simplicity and the availability of robust molecular genetic tools for developmental studies, *Drosophila* proved to be an ideal model organism to study glia development (Freeman 2015; Parker & Auld 2004). There are different subtypes of glia in both central and peripheral nervous systems of *Drosophila*. The outer layer of cells is composed by perineural glia (PG), which are thought to be responsible for secreting a dense lamella that functions as a physical and chemical barrier covering the CNS and the peripheral nerves. This layer is discontinuous, however, below it there is another layer of a different subtype of glia, the subperineural glial cells (SPGs). These flattened cells cover the entire surface of the CNS that only contacts with the most superficial layer of neuronal cell bodies in the cortex and form septate junctions between them creating a blood-brain barrier (BBB). Closely associated with neurons, there are more specialized subtypes of glia as cortex glia, ensheathing glia, and astrocytes. In *Drosophila*, the ensheathment, support, modulation of the function and development of peripheral sensory neurons,

motor neuron axons and terminals are carried out by various glial subtypes. Like vertebrates, *Drosophila* peripheral nerves are covered by perineural glia and subperineural glia creating a BBB similar to the CNS, but also present other types of glia such as wrapping glia that ensheath motor and sensory axons (Figure 1.3.) (Freeman 2015; Limmer at al. 2014). Proper ensheathment of axons in *Drosophila* separate the axons from the hemolymph. This partitioning is very important because while vertebrates present a highly vascularized nervous system, *Drosophila* nervous system floats in the hemolymph that contains high concentration of potassium and other ions that could interfere with action potential propagation (Blauth et al. 2010; Banerjee et al. 2006; Banerjee & Bhat 2008). Also, defects in nerves and axon fasciculation and ensheathment could lead to diminished conduction of electrical impulses, affecting the transmission of information and thus, neuronal development (Banerjee & Bhat 2008).

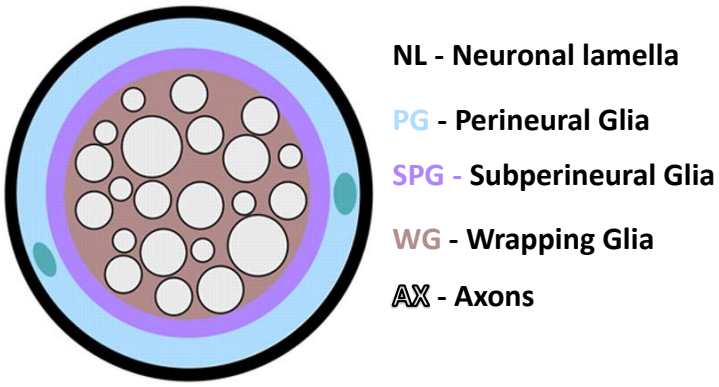

Figure 1.3. Schematic representation of the glial cell types that are part of *Drosophila* peripheral nerve. Covered by the neuronal lamella (NL) it is represented the outermost layer of glial cells which is composed by perineural glia (PG). Immediately below, there is another layer of glial cells, called subperineural glial (SPG) that is responsible for creating the blood brain barrier. Closely associated with the axons (AX) there is a layer composed of wrapping glia that enwrap the axons (WG). In green are represented two PG nuclei (From Xie & Auld 2011).

In the NMJ, subperineural glia interact with motor neuron synaptic contacts on muscles where they play an essential role in neurotransmitter recycling, in the modulation presynaptic growth by engulfing synaptic debris during development, and secreting molecules that modulate retrograde signaling between the muscle and the pre-synapse, contributing for NMJ growth (Freeman 2015; Ou et al. 2014). Glial cells have been raising an increased interest in recent years, since disruption of these cells can have many implications in the function of the nervous system, whose disruption may lead to the development of neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis and multiple sclerosis (Kurosinski and Gotz 2002; Barres 2008; Banerjee & Bhat 2008; Blauth et al. 2010; Parker & Auld 2004; Danjo et al. 2011).

1.5. Molecular Players involved in Neuronal Growth and Plasticity

During development and in response to changes in the environment, neurons undergo significant remodeling processes in order to generate appropriate axonal and synaptic connections. Many genetic pathways contribute to this process: below we summarize the ones relevant for this project.

Ral GTPase regulates numerous biological processes by interacting with effectors such as Ral Binding Protein-1 (RalBP1) and the exocyst complex. Also, it has been shown that Ral influences several pathways including the c-Jun NH_2-terminal kinase (JNK) signaling pathway. Together, Ral and the JNK pathway seem to be involved in responses to stress and cell shape changes. Besides this, JNK has been shown to be involved in axonal pruning by regulating the levels of the cell adhesion molecule Fasciclin II (Bornstein et al 2015), and in synaptic plasticity (Collins et al. 2006; Coffey 2014). Moreover, Ral GTPase has been shown to be involved in postsynaptic structural plasticity (Teodoro et al. 2013) and also, appears to have a role in nerve thickness regulation (unpublished results and this thesis).

Because Ral GTPase and JNK were shown to act together in the regulation of some remodeling processes, we want to understand if these two molecular players play a role in the regulation of presynaptic structural plasticity and in the regulation of nerve thickness.

1.5.1. Ral GTPase

Ral is a small GTPase member of the Ras superfamily, and like other small GTPases plays an important role in several biological processes, such as the regulation of vesicle and membrane transport (Figure 1.4). This protein is ubiquitously expressed in tissues, but is specially enriched in places like the brain and platelets (van Dam & Robinson 2006). It was shown that Ral is located in cellular compartments such as the plasma membrane, secretory granules and synaptic vesicles (Moskalenko et al. 2002; Sugihara et al. 2002; van Dam & Robinson 2006; Shirakawa & Horiuchi 2015). The cellular localization and activity of Ral can be regulated by post-translational events since it has distinct phosphorylation sites (Shirakawa & Horiuchi 2015; Gentry et al. 2014). In mammals, Ral GTPase has two isoforms, RalA and RalB, which share 82% of homology between them, however, in invertebrates such as *Drosophila melanogaster* and the nematode *Caenorhabditis elegans,* only one Ral gene is present, which is more similar to the RalA isoform. Ral gene orthologues are not present in the yeast genome, indicating that Ral appeared in multicellular organisms throughout evolution (Shirakawa & Horiuchi 2015). Similarly to other small GTPases, Ral also functions as a molecular switch, meaning that they have two inter-convertible forms, an active form (GTP-bound) and an inactive form (GDP-bound), cycling between them. In its active form Ral GTPase interacts with several effector proteins, triggering downstream pathways involved in actin cytoskeletal rearrangement, membrane trafficking, gene transcription, kinase cascade activation, cell survival, apoptosis and other biological processes (Shirakawa & Horiuchi 2015; Gentry et al. 2014; Sugihara et al. 2002; Carmena 2012). The cycling rate between the active and the inactive forms of Ral is very slow and weak, like in other small GTPases,

and can be significantly enhanced by guanine exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively. Thus, RalGEFs stimulate GTPase activity by releasing GDP and promoting GTP binding (activating Ral), whereas GAPs allow the hydrolysis of the bound GTP (inactivating Ral). Besides cycling between GTP and GDP form, GTPases can also change its cellular localization, from cytoplasm to membrane or the other way around, aided by GDP-dissociation inhibitors (GDIs) and GDI-dissociation factors (GDFs). Once at the membrane and in its active form (GTP-bound), GTPases can interact with their specific effectors (van Dam & Robinson 2006; Shirakawa & Horiuchi 2015; Gentry et al. 2014; Segev 2011). In *Drosophila*, Ral GTPases have specific GEFs and GAPs, that are downstream of Ras proteins and are indirectly activated by them. Rap1 and Ras lead to the activation of specific RalGEFs that subsequently activate fruit fly Ral proteins. In addition, Ral has a calmodulin (CaM) binding site, so it can be directly activated by Ca^{2+}/Calmodulim binding, in a Ras/GEF-independent manner. CaM is a conserved sensor of Ca^{2+}-dependent signaling pathways involved in the regulation of numerous biological processes. The presence of high intracellular calcium levels induces conformational changes in calmodulin proteins, allowing them to interact with the target proteins, regulating their functions. Ral GTPase has both RalGEFs and calmodulin binding sites. The activation of Ral by these two proteins leads to an interaction with various effectors and, therefore the initiation of downstream signaling events (Figure 1.4.) (van Dam & Robinson 2006; Wang & Roufogalis 1999; Feig 2003; Park 2001).

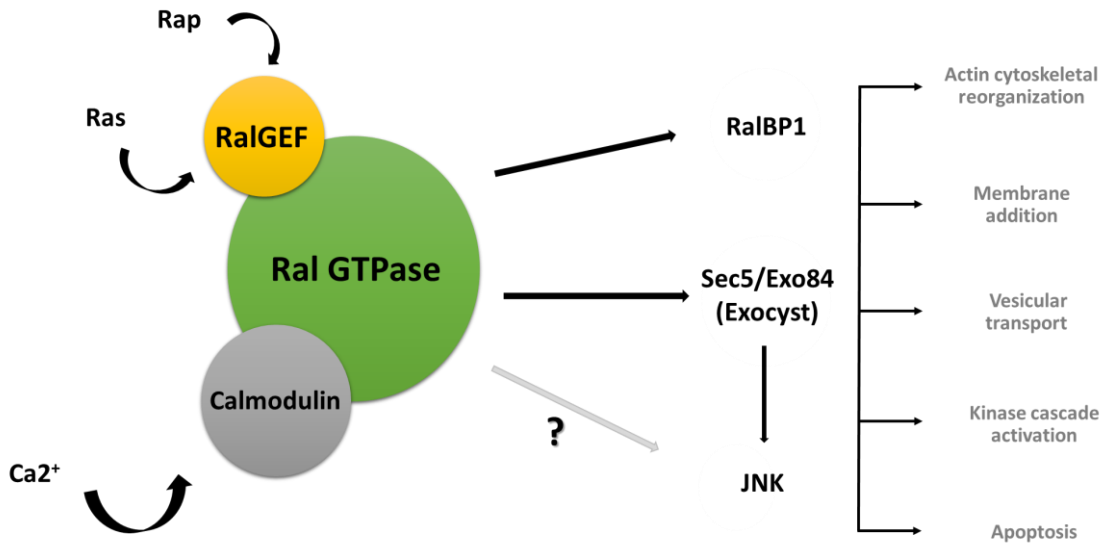

Figure 1.4. Activated Ral protein interacts with several effectors implicated in numerous cellular processes. Ral can be activated by RalGEFs (represented in yellow) which in turn are activated by Ras and Rap1, and by calmodulin binding (represented in grey) in response to calcium influx. Upon activation, Ral binds to several effectors such as RalBP1, exocyst subunits Sec5/Exo84 and JNK which are involved in multiple cell processes, such as membrane addition, actin cytoskeleton rearrangements, membrane trafficking and many other cellular responses (Shirakawa & Horiuchi 2015; Gentry et al. 2014; Sugihara et al. 2002; Carmena 2012).

Among all Ral effectors, there are two that are particularly well known: Ral Binding Protein-1 (RalBP1) and the Sec5 and Exo84 subunits of the octameric Exocyst complex. RalBP1 was one of the first to be identified as a Ral effector (Figure 1.4). It contains a GAP catalytic domain that activates the Cdc42 and Rac small GTPases, inducing actin cytoskeleton rearrangements like filopodia and lamellipodia formation, respectively. RalBP1 is also involved in receptor-mediated endocytosis (i. e. EGF receptor, insulin receptor) through interaction with two endocytic proteins, Reps1 and Reps2/POB1 (Shirakawa & Horiuchi 2015; van Dam & Robinson 2006; Gentry et al. 2014).

Another well characterized Ral GTPase effector is the exocyst (Figure 1.4.). The exocyst is an octameric complex, which is composed of eight subunits, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84, firstly identified in yeast and conserved to humans. Ral can bind directly to Sec5 and Exo84 subunits promoting the assembly of the complex, however these subunits have a competitive behavior due to the overlapping of their Ral binding sites. Together, Ral and the exocyst, are involved in intracellular trafficking and in regulation of several exocytic pathways, by tethering exocytic vesicles (i. e. Golgi-derived vesicles) to specific sites in the plasma membrane, before the assembly of the SNARE complex (van Dam & Robinson 2006) (Figure 1.5.).

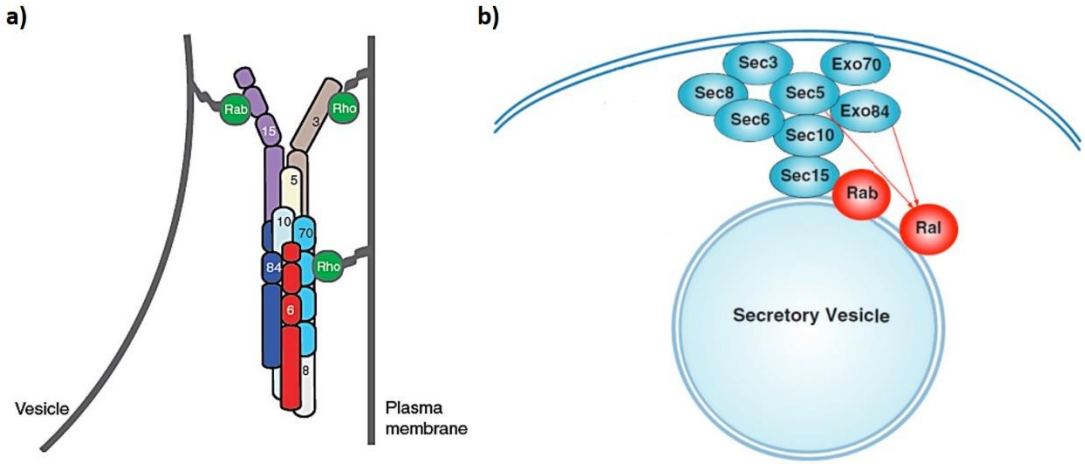

Figure 1.5. Schematic representation of the exocyst complex. a) Illustration of the structure of the assembled yeast exocyst complex, in "Y" conformation, composed by eight subunits (Adapted from Munson & Novick 2006) **b)** Schematic representation of the exocyst complex attached to a secretory vesicle. Activated Ral can bind directly to its exocyst effectors, Sec5 and Exo84; Exocyst complex binds to vesicles that have specific identity due to the presence of Rab proteins (Adapted from Liu & Guo 2012).

In absence of Ral, the assembly and stability of exocyst complex is reduced, affecting the capacity to activate many biological cascades and therefore the regulation of diverse cellular processes. In mammals, the disengagement of the exocyst, after Ral activation, is achieved through Sec5 phosphorylation at the Ral-binding site (process catalyzed by protein kinase C (PKC)), resulting in the dissociation of the exocyst subunit from active Ral. Following dissociation, Sec5 is dephosphorylated, being available for the transport of other vesicles (Chen et al 2011). Whether this inactivation mechanism is conserved in invertebrates is currently unknown. The distribution of the exocyst within a cell can be highly dynamic, however it is located in limited regions of the plasma membrane, resulting in polarized growth and secretion of essential proteins into extracellular space. The exocyst complex is also present

in the nervous system, at the ends of neuronal growth cones, axons, during neurite branching and in sites of synaptogenesis, promoting membrane addition (Heider & Munson 2012; Liu & Guo 2012). In fact, together Ral and the exocyst can modulate postsynaptic growth in *Drosophila* NMJ in an activity-dependent manner. In a previous study, Rita Teodoro and her collaborators demonstrated that, in response to activity, activated Ral in the muscle induces recruitment of Sec5 to the NMJ, which trough membrane addition promotes SSR growth (Teodoro et al. 2013). So, it is possible that the exocyst is involved in synaptic plasticity through its involvement in the regulation of tethering, docking and fusion of vesicles to specific places in plasma membrane (van Dam & Robinson 2006; Teodoro et al. 2013).

Besides, RalBP1 and the exocyst, Ral also influences other pathways, like JNK, that belongs to the mitogen activated protein kinase (MAPKs) superfamily (Gentry et al. 2014) (Figure 1.4). JNK is activated in response to different extracellular stimuli, including growth factors, cytokines, and cellular stress inducers, inducing the phosphorylation of the transcription factor c-Jun (Kim & Choi 2010).

Ral can be a positive or negative regulator of the JNK signaling pathway, depending on the extracellular stimuli (Massaro et al. 2009). In mammals, Ral has been shown to activate JNK (van den Berg et al. 2013; Essers et al. 2004), or to be a negative regulator of JNK (Balakireva et al., 2006; Beraud et al. 2014), while in *Drosophila*, Ral activity appears to antagonize the JNK pathway (Swamoto et al. 1999; Balakireva et al. 2006). Massaro *et al.* (2009) have shown that JNK signaling can be activated or inhibited by the same disruption depending on the origin of the stress, and this may explain these seemingly antagonistic results. Together, Ral and JNK pathway have been involved in responding to ROS-signaling, cell shape changes and apoptosis. Because Ral and JNK signaling could be involved in human diseases, such as cancer, it is critical to understand how they interact with each other in response to different stimuli (Shirakawa & Horiuchi 2015).

1.5.2. JNK signaling

JNK is a member of a large evolutionarily conserved MAPK family, which also integrates the extracellular signal regulated kinase (ERK) and p38 subfamilies. MAPKs are serine-threonine kinases that allow the amplification and integration of signals from a wide range of stimuli, regulating genomic and physiological responses due to changes in the environment (Weston & Davis 2002; Kim & Choi 2010). MAPKs cascades are composed by at least three components, including a MAPK kinase kinase (MAP3K), which phosphorylates and activates a MAPK kinase (MAP2K), and a MAPK that is phosphorylated and activated by MAP2K. The activation of this family of proteins is known to phosphorylate various substrate proteins including transcription factors, that are involved in the regulation of several cellular activities such as cell proliferation, differentiation, inflammatory responses, morphogenesis and apoptosis (Kim & Choi 2010; Weston & Davis 2002; Stronach 2005).

MAPKs can be activated either by interactions between kinase components, or through the aid of a scaffold protein which induces the formation of a signaling complex composed of specific kinases. The scaffold proteins facilitate the activation of MAPK signaling pathways, like the kinase suppressor of Ras-

1 (KSR) and MEK partner 1 (MP1) for the ERK pathway, JNK-interacting proteins (JIPs) are scaffold proteins for JNK signaling and β-Arrestin 2 serves as scaffold protein for ERK and JNK signaling pathways (Kim & Choi 2010; Weston & Davis 2002).

JNKs, also known as stress-activated protein kinases (SPAKs), are activated by inflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukins, or in response to cellular stress due to environmental insults (UV irradiation, oxidative stress) (Kim & Choi 2010). This subfamily of the MAPK signaling cascade transfers phosphate groups to serine/threonine residues that are flanked by carboxyl-terminal prolines (Ser/Thr-Pro) (Coffey 2014; Wang et. al 2005; Kim & Choi 2010).

In the nervous system, JNK signaling also contributes for brain morphogenesis, axon polarization, extension, synaptic plasticity, dendrite development and memory formation (Rallis et. al 2010; Coffey 2014; Weston & Davis 2002). The duration and intensity of JNK signaling, the type of stimuli, the use of different scaffolding proteins to connect certain components can induce distinct outputs. Also, the use of different upstream kinases that recognize unique signals in a specific cell type, developmental stage or spatial position contributes for the diversity of cellular responses by the JNK pathway (Stronach 2005).

In *Drosophila*, JNK pathway is composed of one JNK, Basket (Bsk), that is regulated by two JNK kinases (JNKK) Hemipterous (Hep) and MAP kinase kinase 4 (Mkk4), which in turn are controlled by six JNKK kinases (JNKKKs). Also, JNKKKs are regulated by a single upstream JNKKKK, Misshapen (Msn) (Stronach 2005; Rallis et al. 2010). In *Drosophila* Bsk activation is accomplished by phosphorylation on two predicted residues, threonine 181 and tyrosine 183, through the JNKKs, Hep and MKK4 (Rallis et al. 2010). In table 1.1. are summarized the JNK cascade components in *Drosophila* and their homologs in mammalians.

Table 1.1. Components of JNK signaling pathway and its mammalian homologs. (Adapted from Stronach 2005)

Activity	Drosophila Gene	Mammalian Homolog
JNKKKK	msn	MINK, NIK/HGK, TNIK
JNKKK	Pk92B	ASK1
	Tak1	TAK
	Tak12	TAK
	slpr	MLK
	Wallenda	DLK, ZPK
	Mekk1	MEKK 1-4
JNKK	hep	MKK7
	Mkk4	MKK4
JNK	bsk	JNK 1-3
Transcription Factor	Jra	c-JUN
	Kay	c-FOS

Activated Bsk phosphorylates the Activator protein-1 (AP-1) complex, composed of the transcription factors kayak (kay) the homolog of mammalian c-Fos and Jra (Jun-related antigen) the mammalian homolog of c-Jun. In flies, these transcription factors act either as heterodimers or Fos homodimers (Rallis et al. 2010; Stronack 2005). The strength and duration of JNK signaling is regulated

by one of its targets genes, *puckered* (puc) which encodes a JNK-specific MAPK phosphatase, inducing a negative feedback loop (Wang 2005).

In *Drosophila* JNK/Bsk is involved in a plethora of cellular processes, including dorsal closure (Zeitlinger et al., 1997), imaginal disc development (Agnes et al., 1999), wound healing (Ramet et al., 2002), apoptotic regulation (Ryoo et al., 2004), innate immunity (Delaney et al., 2006), can prolong life span and protect against oxidative stress (Wang et al., 2003). Also, in the nervous system, JNK/Bsk contributes to axon degeneration or overextension depending on the level of its inactivation at *Drosophila* NMJ (Rallis et al. 2010), regulates synaptic plasticity, growth (Collins et al., 2006; Sanyal et al., 2002) and axonal transport (Horiuchi et al. 2007). In most of these responses Bsk phosphorylates the AP1 complex (Rallis et al. 2010; Ciapponi et al., 2001). More recently, a new role for Bsk has been suggested: Bsk has been shown to be required for axon pruning, through the reduction of membrane levels of the adhesion molecule Fasciclin II (Bornstein et al 2015).

Another interesting work revealed that Ral GTPase regulates developmental cell shape changes, by acting as a negative regulator of JNK in *Drosophila*. In this study, it was reported that the loss of bristles and hairs caused by dominant-negative form of Ral was genetically suppressed by loss of function of Hep and Bsk. Also, a constitutively active form of Ral caused defects in the process of dorsal closure during embryogenesis and inhibited the JNK phosphorylation in S2 cells (Swamoto et al. 1999). In other independent study, it was suggested that Ral activity suppresses the JNK activation and induces p38 mitogen-activated protein (MAP) kinase activation. They also proposed that a molecular basis of Ral action on JNK could be mediated by the exocyst complex, influencing developmental regulatory programs (Balakireva et al. 2006). In mammals, it has been shown that Ral is activated in response to cellular stress such as reactive oxygen species (ROS) which in complex with the kinase scaffold protein JIP1 (c-Jun-amino-terminal-interacting protein 1) activates the JNK cascade resulting in FOXO (forkhead box O) activation and its nuclear translocation (van den Berg et al. 2013; Essers et al. 2004). FOXOs are transcription factors that are involved in various cellular processes including cell cycle regulation and apoptosis. Such as Ral, JNK interacts with a huge variety of signaling pathways, and its regulation could differ according to the type of stimuli, development stage, cell-type and even with the components of the cascade, generating a wide range of outputs (Figure 1.6.). Thus, it is important to decipher what is the molecular mechanism of action between Ral and JNK in the different cellular contexts to prevent pathological conditions.

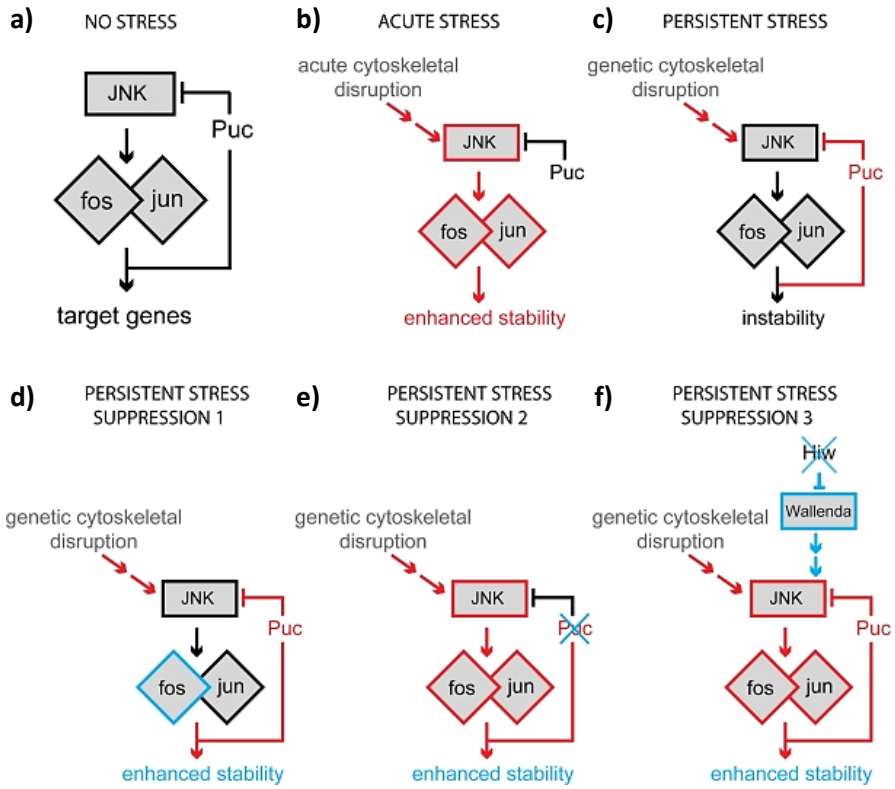

Figure 1.6. Schematic representation of different JNK outputs in response to different types of stimuli. a) In the absence of cellular stress the JNK-Fos pathway is not required for synapse stability, being present in a stable state. **b)** Upon acute cellular stress, such as cytoskeletal disruption, JNK and Fos are activated (represented in red), inducing synapse stabilization. **c)** During persistent cellular stress, the phosphatase Puckered induces a negative feedback preventing Fos reactivation, which favors synapse instability or disassembly. **d)** Fos overexpression (experimental manipulation) leads to synaptic stability in the presence of persistent cellular stress. **e)** Also during persistent cellular stress, loss of puckered (experimental manipulation), induces synaptic stability, possibly due to enhanced Fos activity. **f)** The loss of Highwire (experimental manipulation), induces upstream MAPK signaling, leading to synaptic stability through Fos activity. (from Massaro et al. 2009).

JNK also plays a role in the regulation of microtubule cytoskeleton which is a major component of neurons and is essential for numerous cellular and developmental processes, such as neuronal migration, polarity, and differentiation (Kapitein & Hoogenraad 2015). In mammals, JNK appears to stabilize microtubules by phosphorylation of substrates such as SCG10 (superior cervical ganglion 10 protein) and MAP1B (Microtubule-associated protein 1) (Chang et al. 2003; Tararuk et. al 2006; Kawauchi et al. 2003). Another study revealed that microtubule stability requires concomitant inhibition of GSK3β and activation of JNK (Ciani & Salinas 2007).

In *Drosophila*, JNK activity has been associated with regulation of FOXO through Toll-6 receptor, and this pathway has been associated to microtubule dynamics (McLaughlin et al. 2016). In addition, FOXO has been shown to negatively regulate microtubule stability, by analyzing Futsch distribution in larvae NMJ (Nechipurenko & Broihier, 2012). Futsch is the *Drosophila* homolog of MAP1B that binds to tubulin, making it an excellent marker for stable and dynamic microtubules (Roos et al. 2000; Hummel et al. 2000). Together, these studies demonstrate that JNK can regulate microtubules through the interaction with different substrates and signaling pathways.

As mentioned above, JNK signaling pathway is involved in many biological processes, and its deregulation has been implicated in the pathogenesis of many human diseases such as cancer, Alzheimer's disease (AD), Parkinson's disease (PD) and other neurodegenerative diseases. So, the understanding of how JNK regulates these numerous activities, is a critical step towards the development of new therapies for these diseases (Kim & Choi 2010).

1.6. Aims of the work

From previous studies, Teodoro *et al.* (2013) found a novel pathway that regulates neuronal morphology in response to activity through the engagement of Ral and the Exocyst complex, regulating postsynaptic membrane growth at the synapse in response to neuronal activity. Here, we want to ask whether Ral also participates in presynaptic structural plasticity. For this, we will manipulate the levels of synaptic activity by inducing acute structural plasticity and testing whether activity-dependent bouton formation remains intact.

In parallel, we observed that Ral mutants have widespread thicker nerves, suggesting a role for this GTPase in the regulation of nerve bundle structure and possibly function. We will dissect the mechanisms and pathways involved in the regulation of nerve thickness, and how Ral GTPase regulates this trait. Because Ral has been shown to regulate JNK signaling, positively or negatively, depending on the cellular context of activation of this cascade, we will test if this pathway is involved in the regulation of nerve thickness.

Like neurons, glial cells are essential for proper development of the nervous system, regulating many aspects of neuronal development, morphology and function. When exiting the CNS, axons from several neurons are bundled in fascicles that, together with glial cells, form the nerve bundle. So, we wanted to understand the contribution of these cells and the involvement of Ral and JNK signaling pathway to the regulation on nerve thickness.

With this work, we expect to have a better understanding about the mechanism of synaptic bouton formation and the pathways that might be involved in this process. In addition, we expect to understand the contribution of neurons and glial cells, as well as the mechanisms and pathways involved in the regulation of axon bundling/nerve thickness. Defects in axon bundling can lead to serious problems in the transduction of information between neurons consecutively affecting the development of the nervous system.

Chapter 2. Materials and Methods

2.1. Genetic tools

Drosophila melanogaster has the advantage of having a great diversity of genetic tools that provides the ability to study *Drosophila*, using various experimental techniques. Here are reviewed some tools that were used in this project.

2.1.1. UAS-Gal4 System

One of the main and most important genetic tools used in *Drosophila* is the UAS/Gal4 system, which allows the control of gene expression in a temporal and tissue-restricted manner. The transcriptional activation factor Gal4 was firstly identified in *Saccharomyces cerevisiae* and then used by Andrea Brand and Norbert Perrimon in 1993 to develop a method to induce gene expression regulated by Gal4. This system consists of two components present in separate fly lines, a Gal4 protein that binds directly to an Upstream Activating Sequences (UAS), which are Gal4 binding sites on DNA that are upstream of the gene of interest (Duffy 2002; Brand & Perrimon 1993) (Figure 2.1.). To activate transcription, lines under UAS control are mated with flies containing a Gal4 transgene, which is expressed in a certain pattern, called the driver. Thus, the progeny will express the gene of the interest in a pattern dictated by Gal4 expression, allowing tissue specific expression of the gene. In the absence of a Gal4 line the target gene is silent (Brand & Perrimon 1993). This system can also be regulated by temperature, since the minimal Gal4 activity is at 16°C and the maximal activity is reached at 29°C, although at this point there is a balance between the activity and minimal effects on fertility due to high temperature (Duffy 2002). One of the greatest advantages of this system is the existence of thousands of Gal4 lines available, allowing the expression of specific modified forms of a gene (e.g. dominant negative, constitutively active), inducing targeted mutations and knockdown of specific genes anywhere in the fly, *in vivo* (Elliot and Brand, 2008; Caygill & Brand 2016).

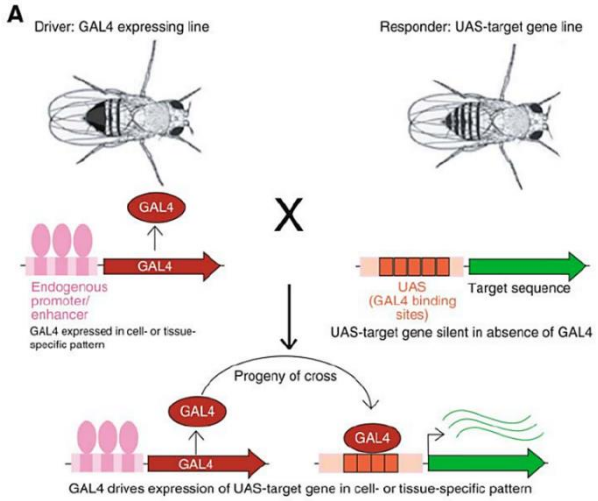

Figure 2.1. Schematic representation of the UAS/Gal4-based system for transgene expression. From (Elliott & Brand 2008)**.**

2.2. Fly stocks and husbandry

In this project, all fly stocks were maintained at room temperature (RT) in vials or bottles containing standard fly food (mixture of water, corn meal, agar, sugar, yeast and fungicides). When carrying out experiments, virgin females were collected and crosses were performed, maintaining them at 25°C in an appropriate atmosphere with controlled humidity.

All the *Drosophila* stocks used in this work were obtained from Bloomington *Drosophila* Stock Center or generated in our laboratory and are described in Table 2.1.

Table 2.1. Detailed list of *Drosophila* stocks used throughout this project. BDSC: Bloomington *Drosophila* Stock Center

Name	Description/Genotype	Stock
***W*^{1118}**	Wild-type W[1118]	BDSC#5905
***Ral*^{G0501}**	P-element disruption of ral locus; Genetic null w[67c23] P{w[+mC]=lacW}Rala[G0501]/FM7c	BDSC#12283
***Ral*^{EE1}**	Point mutation in amino acid Ser154 predicted to be the nucleotide binding site Rala[EE1]/FM7i, P{ActGFP}JMR3	BDSC#25095
nSyb-Gal4/TM6b	Expresses Gal4 in all neurons n-Syb-Gal4/TM6b	Rita Teodoro Lab
UAS-*BSK*^{DN}	Amino acid replacement; Expresses a dominant-negative form of bsk under UAS control w*; P{UAS-bsk.K53R}20.1a	BDCS#9311
Repo-Gal4	Expresses Gal4 in glia w1118; P{GAL4}repo/TM3, Sb1	BDSC#7415
***Ral*^{G0501};;nSyb-Gal4**	Ral mutant with Gal4 being expressed in all neurons *Ral*^{G0501}/ FM7i, P{ActGFP}JMR3;;nSyb-Gal4/TM3,Ser, Act-GFP	Rita Teodoro Lab
***Ral*^{EE1};;nSyb-Gal4**	Ral mutant with Gal4 being expressed in all neurons *Ral*^{EE1}/ FM7i, P{ActGFP}JMR3;;nSyb-Gal4/TM3,Ser, Act-GFP	Rita Teodoro Lab
***Ral*^{G0501};;Repo-Gal4**	Ral mutant with Gal4 being expressed in glia *Ral*^{G0501}/ FM7i, P{ActGFP}JMR3;;Repo-Gal4/TM3,Ser, Act-GFP	Rita Teodoro Lab
***Ral*^{EE1};;Repo-Gal4**	Ral mutant with Gal4 being expressed in all glial cells *Ral*^{EE1}/ FM7i, P{ActGFP}JMR3;;Repo-Gal4/TM3,Ser, Act-GFP	Rita Teodoro Lab

2.3. Larval dissection and fixation

Drosophila third instar larvae of the appropriate genotypes were selected and dissected in a drop of PBS 1x (Phosphate Buffer Saline) or of HL3.1 (hemolymph-like solution – composition described in table 2.2) using Sylgard plates (Brent et al. 2009). Larvae are placed with the dorsal side up and, using forceps (Student Dumont #5 Forceps - Fine Science Tools), a pin is placed in the anterior end of the larva, near the mouth hooks, followed by another pin inserted in the posterior end of the larva, between the posterior spiracles. Using ultra-fine clipper scissors (Fine Science Tools), a horizontal incision is made at the posterior end of the larvae and then a vertical cut is made from the incision, along the dorsal midline until the anterior pin. With the forceps, the organs are taken out and the tips of the larvae stretched, vertically and horizontally, followed by the placement of the pins, as shown in figure 2.2. After dissection, larvae are fixed in a solution of 4% of paraformaldehyde (PFA) in 1x PBS for 20 min at RT or with Bouin's fixative (Sigma-Aldrich) for 5 min at RT.

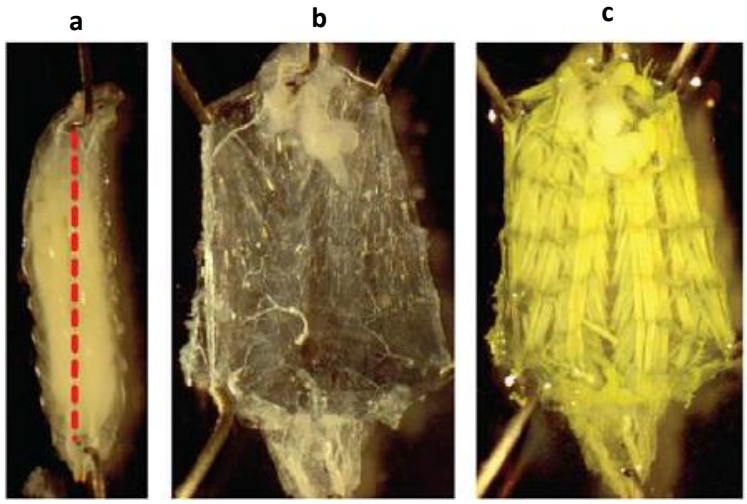

Figure 2.2. *Drosophila* NMJ dissection. The larvae are pinned in the anterior region near the mouth hooks, and in the posterior end between the spiracles. A horizontal cut is made close the posterior end of the larvae followed by a vertical incision (dotted line) **(a)** exposing the muscles and nerves **(b)**. After dissection, larva is fixed with Bouin's fixative **(c)**. From Frank et al. 2014.

Table 2.2. Composition of the solutions used for dissection and stimulation assays. (From Feng et al. 2004)

Components	HL3.1 Low Ca^{2+}/K^{+}	HL3.1 High Ca^{2+}/K^{+}
NaCl 5M	70 mM	40 mM
KCl 1M	5 mM	90 mM
$CaCl_2$ 1M	0.1 mM	1.5 mM
$MgCl_2$ 1M	4 mM	4 mM
$NaHCO_3$ 1M	10 mM	10 mM
Trehalose 0.1 M	5 mM	5 mM
Sucrose 1M	115 mM	115 mM
HEPES0.1 M	5 mM	5 mM

2.4. Immunocytochemistry protocol

After fixation, larvae are washed and permeabilized by incubating 3 times for 15 minutes with PBT [PBS 1x with 0.3% Triton-X (Sigma-Aldrich)]. Larvae are then incubated in blocking solution [PBT, 5% normal goat serum (NGS, Life Technologies)], followed by primary antibody incubation (Table 2.3.) diluted in blocking solution and PBT at 4°C, overnight. Larvae were washed 3 times for 15 minutes with PBT and blocked for 30-60 min in PBT/5% NGS, followed by incubation with secondary antibody (Table 2.4.) for 2h, diluted in blocking solution at RT. At the end of the incubation with secondary antibody, the larvae are washed again 3 times for 15 minutes with PBT and then placed in 50% glycerol solution (Invitrogen) for 10 min to exchange from a water based to a glycerol based medium. Samples are then mounted on microscope slides using mounting media DABCO (1,4- Diazabicyclo[2.2.2]octane, Sigma-Aldrich) and stored at 4°C, until its observation.

Table 2.3. Primary antibodies used in immunofluorescence assays. DSHB: Developmental Studies Hybridoma Bank; CST: Cell Signaling Technology

Antigen	Host	Dilution	Supplier	Fixative
Dlg (4F3)	Mouse	1:250	DSHB	Bouin's
Futsch (22C10)	Mouse	1:50-1:100	DSHB	Bouin's
FasII (1D4)	Mouse	1:50	DSHB	Bouin's
p-JNK	Rabbit	1:500	CST	PFA

Table 2.4. Secondary antibodies used in immunofluorescence assays. * - Conjugated atibodies

Antibody	Dilution	Supplier
Alexa Fluor 488 anti-mouse	1:500	Jackson Immuno Research
HRP Cy3 *	1:500	Jackson Immuno Research
Alexa Fluor 647 anti-rabbit	1:500	Jackson Immuno Research
HRP A488 *	1:500	Jackson Immuno Research
Texas Red-X-phalloidin *	1:500	Roche

In *Drosophila*, HRP identifies a neuronal protein, so it is used for neuronal membrane labelling.

2.5. Acute Induction of Activity-Dependent Structural Plasticity

2.5.1. Stimulation Protocol

During development and in response to activity neurons can alter the size and shape of both pre- or post-synaptic compartments. Structural plasticity of nerve terminals can involve addition, removal or remodeling of synaptic components, including the number and order of branches, the number and size of synaptic boutons and the number of active zones. Also, the postsynaptic membrane can change in size, structure and can alter the number and localization of postsynaptic molecules such as receptors and scaffolding proteins (Griffith & Budnik 2006).

In order to assay new bouton formation two different protocols were used that are known to promote morphological changes at the synapse, namely, they induce the formation of activity-dependent boutons, schematized in figure 2.3. (Ataman et al. 2008; Vasin et al. 2014). Both of protocols are composed of pulses of high K^+ and high Ca^{2+}, intercalated with a resting phase where normal K^+ and low Ca^{2+} are added to the dissected larvae. This leads to muscle contraction, inducing the formation new synaptic boutons. The high K^+ depolarizations were achieved using 90 mM K^+ and 1 mM Ca^{2+} in HL3 solution, and the resting with 0.1 mM Ca^{2+} and 5mM K^+ in HL3 solution. The solutions were the ones described in Feng et al. 2004. Prior to the stimulation, third instar larvae were partially dissected, until the vertical incision without stretching the larvae to allow body wall contractions. During the last resting time, in both protocols, the organs are removed and larvae were stretched vertically and horizontally. After that, the larvae are fixed, followed by the immunocytochemistry protocol.

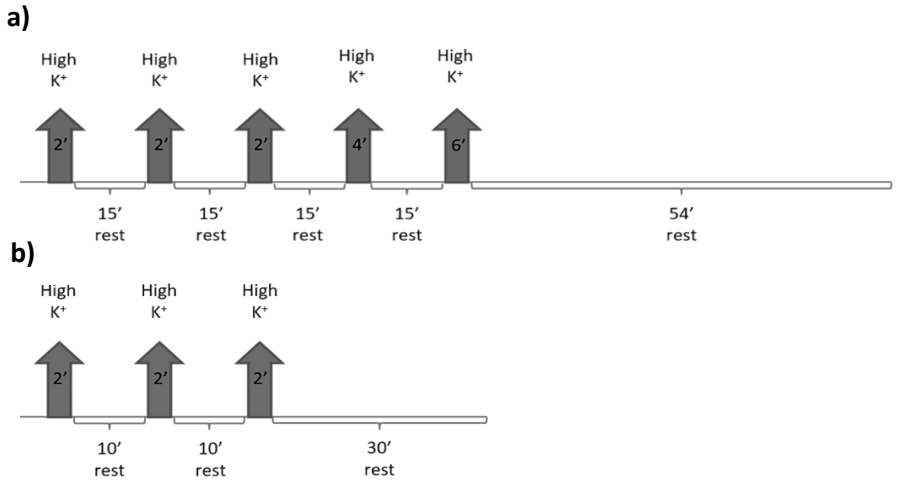

Figure 2.3. Schematic representation of the stimulation paradigms used. a) Long stimulation **-** 5 stimulations of 3 of 2 minutes, one of 4 minutes and a last of 6 minutes, all interspaced by 15 minutes of rest (Adapted from Ataman et al 2008). **b)** Short stimulation **-** 3 stimulations of 2 minutes interspaced by 10 minutes of rest (Apated from Vasin et al. 2014). At the end of both protocols, larvae are fixed and proceed for immunocytochemistry.

2.5.2. Identification of new activity-dependent boutons

New synaptic bouton formation can be identified by the presence of immature buttons (called ghost boutons) that contain synaptic vesicles but lack presynaptic active zones and postsynaptic specializations. The “ghost boutons” represent boutons that start to differentiate presynaptically but did not yet complete their maturation (Ataman et al. 2008; Vasin et al. 2014).

Ghost boutons can be characterized by the presence of Horseradish Peroxidase (HRP) antibody that is known to bind to neuronal membranes in *Drosophila* and is used as a specific neuronal marker, and the absence of Discs-large (Dlg) which is the homolog of mammalian Postsynaptic density-95 (PSD95), a scaffold protein, located in postsynaptic compartments and absent from the newly formed boutons (Ataman et al 2006).

2.6. Image acquisition and image analysis

Larval imaging was performed in a Zeiss LSM710 Confocal Microscope using 40x water or 63x oil objectives. We imaged either muscle 6/7 or muscle 4 NMJ, in segments A2-A4, with 0.7 µm between slices and the number of slices varied according to the nerve size. The images were analyzed using FIJI software and compiled using Adobe Photoshop. The result’s analysis and graphs were made using Prism GraphPad, also normality of the values was tested using D'Agostino & Pearson omnibus, Shapiro-Wilk and KS normality tests. Statistical significance was determined by comparison to controls, using a non-parametric test Kruskal-Wallis test or a parametric test ordinary one-way ANOVA.

2.6.1. Quantification of nerve thickness

To quantify the nerve thickness, we used maximum intensity projection from z-stacks. The HRP channel was used to outline the neuronal membrane and using the straight-line tool, the diameter of the nerve was measured. When necessary several measurements were taken and the average between them was performed. Notice that the measurements were not performed at the axon exit site.

2.6.2. Quantification of nerve area

To quantify the nerve area, a maximum intensity projection from z-stacks was performed. Using the HRP channel to outline the neuronal membrane, the area was measured along 80 µm of axon, using HRP threshold to define the width (notice that the bundle was always straight).

The steps used to quantify muscle 4 nerve bundle are summarized as follows:

1. Z-projection → Maximum intensity projection

2. Specify a region of interest (ROI) in a rectangle →Width: 80 µm; Height: 20 µm (this is to ensure that the entire width is within the ROI, but can be any width, since the HRP channel is what defines width)
3. Use the HRP to outline the neuronal membrane
4. Threshold, then binarize, the HRP channel
5. Using the freehand selection tool to select the region of interest and measure the area.

2.6.3. Quantification of fluorescence levels

To quantify FasII and p-JNK intensity in the nerve we used maximum intensity projection from z-stacks. The HRP channel was used to outline the neuronal membrane and the area was measured along 80 µm of nerve, followed by the quantification of FasII and p-JNK intensity in this area. The intensity was measured and then divided by the area of its respective region of interest. The quantifications are described as mean values normalized to respective controls, with standard error of the mean.

The steps used to quantify muscle 4 nerve bundle FasII and p-JNK intensity are summarized as follows:

1. Repeat the steps used to quantify nerve area (1-5)
2. Measure the FasII and p-JNK intensity (Intensity measurement limited to the region of interest set above)
3. Divide the integrated density of the channel by the HRP threshold area to get Intensity/ μm^2

Chapter 3. Results and Discussion

The main goal of this work is to understand the involvement of Ral GTPase in the regulation of presynaptic structural plasticity and, also its involvement in the regulation of nerve thickness. More specifically we aim to dissect the molecular mechanisms and the pathways that govern these two distinct aspects of neuronal development, using *Drosophila* NMJ.

In previous studies Teodoro and collaborators (Teodoro et al. 2013) established that the postsynaptic membrane of the NMJ, called the subsynaptic reticulum (SSR) grows in an activity-dependent manner, requiring the recruitment of vesicles to the membrane, a process regulated by the Ral/Exocyst pathway. Here, we want to address whether Ral GTPase also plays a role in activity-dependent bouton formation. In addition, JNK signaling has been shown to be regulated by Ral in different cellular contexts, and to be involved in cellular aspects that are important for appropriate structural plasticity, such as the regulation of microtubules (Rallis et al. 2010). Here, we will assess if Ral is operating through this signaling pathway to regulate activity-dependent bouton formation.

In parallel we want to understand the involvement of Ral GTPase and JNK signaling pathway in the regulation of nerve thickness. As explained in the introduction, Ral can act as a negative or positive regulator of JNK signaling, which depends on the cellular context of activation. In addition, JNK signaling has been shown to be involved in axon pruning through regulation of cell adhesion protein FasII (Bornstein et al 2015). Thus, we expect to understand if Ral GTPase is involved in the regulation of axon bundling/nerve thickness via JNK pathway and whether its function is required in neurons and/or glia, since glial cells are an integral part of the nervous system and play an important role in the regulation of neuronal development and function (Stork et al. 2012; Brink et al. 2012).

3.1. Activity-Dependent Structural Plasticity

Neurons are the fundamental unit of the nervous system and their morphology defines many aspects of function. Although the initial neuronal morphology is established during axon and synapses formation, it can be modified in response to physiological events. In response to activity, the structure of pre- and postsynaptic compartment of the synapse can be altered in the number, size and shape (Teodoro et al 2013). Such changes in synaptic morphology are known as structural plasticity. (Shen & Cowan 2010; Griffith & Budnik 2006). Ral is a small GTPase protein that, amongst other functions, is known to be involved in postsynaptic structural plasticity, where it is necessary to sustain membrane growth. In response to activity, Ral is activated leading to the recruitment of the exocyst to the NMJ, which promotes membrane addition and consecutively SSR growth. In the absence of Ral this process is impaired, resulting in defects in plasticity (Teodoro et al. 2013). To understand the involvement of Ral GTPase in presynaptic structural plasticity, two different protocols were used to acutely induce new bouton formation (see methods). These protocols mimic neuronal patterned depolarization, where the dissected larvae are exposed to several pulses of a high K^+ solution (Ataman et al. 2008; Vasin et al. 2014), and to which we called: “long stimulation” and “short stimulation”. The newly formed synaptic boutons can be assessed by counting the number of “ghost boutons”, which are immature boutons that contain synaptic vesicles but lack postsynaptic specializations. “Ghost boutons” are characterized by the presence of Horseradish Peroxidase (HRP) that is known to bind to neuronal membranes in

Drosophila and is used as a specific neuronal marker, and the absence of Discs-large (Dlg) which is the homolog of mammalian Postsynaptic density-95 (PSD95), a scaffold protein, located in postsynaptic compartments. It is known that newly formed boutons lack Dlg, and we therefore quantify boutons with HRP and without Dlg. To test whether *ral* mutants had defects in this process, we used two independent *ral* mutants. Ral^{G0501} that contains a P element inserted in the coding region of *ral* (Wang et al. 2008) and Ral^{EE1} that has a point mutation in the amino acid predicted to be the nucleotide binding site, in which Serine 154 is substituted by a Leucine 154, impairing the binding of the GTPase to the nucleotide, and consequently preventing the switch between GTP and GDP (Suk et al. 2007; Cho & Fischer 2011).

In both protocols, the mutant Ral^{EE1} shows a significant impairment in the capacity to form new synaptic boutons (Figure 3.1.). However, this defect is not consistent between the two *ral* mutants: while Ral^{EE1} is always defective in structural plasticity, Ral^{G0501} is not (Figure 3.1.) In the long stimulation, wild type animals have an average of 7.3±0.82 ghost boutons, while Ral^{G0501} have 7.1±0.98 ghost boutons and Ral^{EE1} have 3.2±0.55 ghost boutons. In the short stimulation, wild type animals have an average of 5.2±0.56 ghost boutons, while Ral^{G0501} have 5.5±0.78 ghost boutons and Ral^{EE1} have 2.8±0.39 ghost

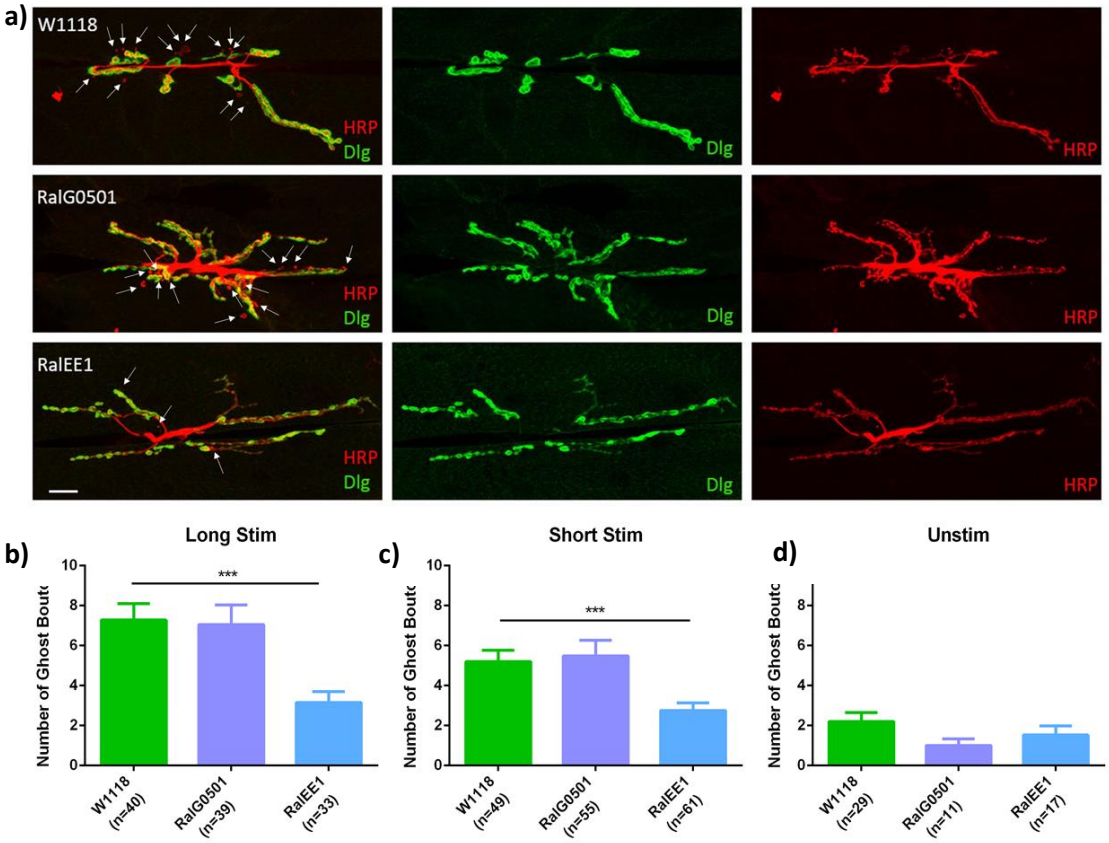

Figure 3.1. Analysis of activity-dependent bouton formation in *ral* mutants. a) Confocal images of wild type (W^{1118}), and *ral* mutants Ral^{G0501}, Ral^{EE1} NMJs of muscles 6/7 of 3rd instar larvae, segment A2-A4 after long stimulation protocol. Arrows point the new synaptic boutons, acutely induced by the depolarization protocol. HRP, in red, labels the presynaptic membrane and Dlg, in green, labels Dlg, a marker of the postsynaptic membrane. Scale bar: 10µm. **b-d)** Quantification of the number of newly formed synaptic boutons in W^{1118}, Ral^{G0501}, Ral^{EE1} after long stimulation **(b)**, short stimulation **(c)** and without stimulation **(d)**. Long Stim: W^{1118} n= 40; Ral^{G0501} n=39; Ral^{EE1} n=33. Short Stim: W^{1118} n= 49; Ral^{G0501} n=55; Ral^{EE1} n=61. Unstim: W^{1118} n= 29; Ral^{G0501} n=11; Ral^{EE1} n=17. Kruskal-Wallis test was used to determine statistical significance. Error bars represent standard error of the mean (SEM). *** p<0.001.

boutons. Without stimulation, wild type animals have an average of 2.2±0.45 ghost boutons, while *Ral*^{G0501} have 1±0.33 ghost bouton and *Ral*^{EE1} have 1.5±0.45 ghost boutons. To better understand if the differences between *ral* mutants could be related with the dynamics of bouton formation, we decided to stop the stimulation protocols at various time points, and assay new bouton formation.

The long stimulation protocol is composed of 5 high K^+/high Ca^{2+} depolarizations spaced by 15 minutes resting phases, while the short stim protocol has 3 high K^+/high Ca^{2+} depolarizations spaced with 10 minutes resting phases. To perform the time-lapse of these two protocols, we stopped the long protocol at the times indicated in figure 3.2a, that we called P1, P2 or P3.

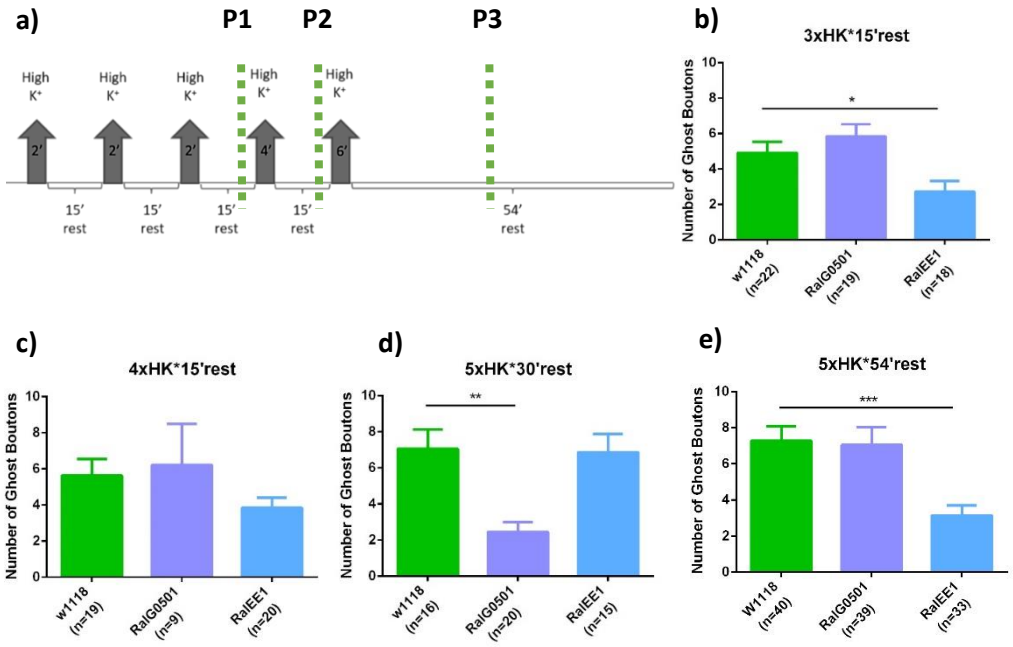

Figure 3.2. Analysis of time-lapse stimulation protocols in ral mutants. a) Schematic representation of long stimulation protocol where the dashed line represents the 3 different time points used for time-lapse study. **b, c, d, e)** Quantification of the number of newly formed synaptic boutons after 3 high K^+ and high Ca^{2+} stimulations of 2 minutes spaced by 15 minutes of rest – from now on referred as **P1 (b),** 4 high K^+ and high Ca^{2+} stimulations (3 of 2 minutes and 1 of 4 minutes) spaced by 15 minutes of rest – from now on referred as **P2 (c)**, 5 high K^+ and high Ca^{2+} stimulations (3 of 2 minutes, 1 of 4 minutes and 1 of 6 minutes) spaced by 15 minutes and 30 minutes at the end – from now on referred as **P3 (d)** and the long stimulation protocol composed by 5 high K^+ and high Ca^{2+} stimulations (3 of 2 minutes, 1 of 4 minutes and 1 of 6 minutes) spaced by 15 minutes and 54 minutes at the end **(e)**. P1 Stimulation: W^{1118} n= 22; Ral^{G0501} n=19; Ral^{EE1} n=18. P2 Stimulation: W^{1118} n= 19; Ral^{G0501} n=9; Ral^{EE1} n=20. P3 Stimulation: W^{1118} n= 16; Ral^{G0501} n=20; Ral^{EE1} n=15. Long Stim: W^{1118} n= 40; Ral^{G0501} n=39; Ral^{EE1} n=33. Kruskal-Wallis test was used to determine statistical significance. Error bars represent standard error of the mean (SEM). * p<0.05 ** p<0.01 *** p<0.001

After the P1 protocol, *Ral*^{EE1} presents a significant impairment in bouton formation. Despite the average of new boutons were slightly increased in *Ral*^{G0501} when compared to control, this difference is not significantly different (figure 3.2.b) In this stimulation protocol wild type animals have an average of 4.9±0.62 ghost boutons, while *Ral*^{G0501} have 5.8±0.69 ghost boutons and *Ral*^{EE1} have 2.7±0.61 ghost boutons. Once again, *Ral*^{G0501} did not show an impairment in bouton formation. In the protocol P2 there are no significant changes between *ral* mutants and the control (figure 3.2.c) Here, wild type animals have an average of 5.6±0.92 ghost boutons, while *Ral*^{G0501} have 6.2±2.27 ghost boutons and *Ral*^{EE1} have 3.8±0.55 ghost boutons. Performing P3 protocol, in contrary to long and short stimulation, *Ral*^{EE1}

the average of ghost boutons is similar to the control indicating that it does not have impaired bouton formation. Also, opposing to long and short stim, *Ral*^{G0501} shows a significant impairment in bouton formation (figure 3.2.d) In the P3 stimulation wild type animals have an average of 7.1±1.07 ghost boutons, while *Ral*^{G0501} have 2.5±0.55 ghost boutons and *Ral*^{EE1} have 6.9±1.02 ghost boutons.

Through analysis of the relative frequency bouton distribution (figure 3.3.) we could observe that *Ral*^{EE1} mutant form fewer new activity-dependent boutons compared to control in both protocols. In the short stim protocol *Ral*^{G0501} mutants and the *W*^{1118} have almost the same frequencies of activity-bouton formation. Regarding the long stimulation protocol, although the average of bouton formation is not significantly different between the *W*^{1118} and the *Ral*^{G0501} mutants, the *W*^{1118} animals form more frequently 9 or more boutons while the *Ral*^{G0501} form less boutons.

In summary, we see defects in structural plasticity in *Ral*^{EE1} mutants, with the 2 protocols, while with *Ral*^{G0501} we do not see any difference from control. Notice that, even if there is some reduction bouton formation, *ral* mutants still retain some capacity to respond to activity and to induce bouton formation.

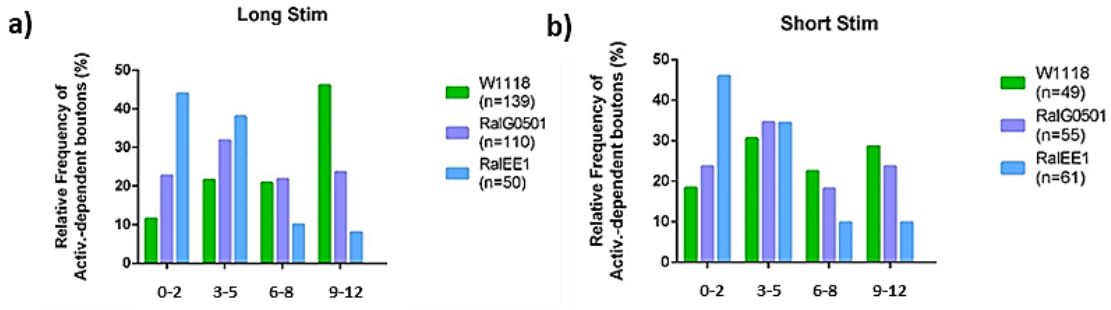

Figure 3.3. Analysis of the relative frequency distribution of activity-dependent boutons. a) Long stimulation **b)** Short stimulation. The X-axis represents the number of activity-dependent boutons formed. Long Stim*: W*^{1118} n= 139; *Ral*^{G0501} n=110; *Ral*^{EE1} n=50. Short Stim*: W*^{1118} n= 49; *Ral*^{G0501} n=55; *Ral*^{EE1} n=61.

Given these discrepancies, we questioned whether *Ral*^{G0501} is a protein null, like it has been previously reported (Teodoro et al, 2013). Additionally, we tested *Ral*^{EE1}, since nothing is known concerning Ral expression levels. To test whether these mutants are indeed protein nulls, we performed Western blot analysis on body walls of 3rd instar larvae. We observed that Ral levels are reduced in *Ral*^{EE1} mutant, and absent on *Ral*^{G0501} mutant, when compared to control wild-type as shown in figure 3.4. If we put this in context with the results obtained for these different mutations after stimulation, it appears that having a bit of protein left is more deleterious to the larvae than having none. Given that the protein made in *Ral*^{EE1} is mutated in the nucleotide-binding region, we speculate that it is possible that this dysfunctional protein may prevent that redundant pathways operate, which does not occur in *Ral*^{G0501}. It is possible that complete lack of protein could allow for compensation mechanisms or in the case of the presence of reduced protein, the translated protein could be misfolded, interacting with other proteins in the cell and could interfere with proteins or cascades necessary for bouton formation.

Alternatively, because *Ral*^{G0501} is a P-element insertion, it is possible that undetectable (by Western blot) levels of Ral are made and are sufficient to induce activity-dependent boutons.

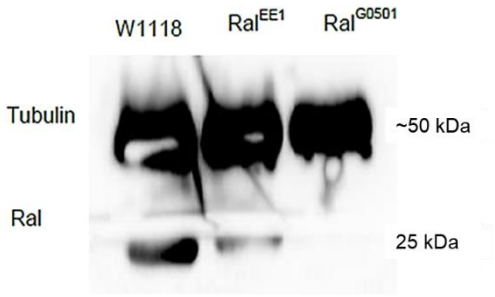

Figure 3.4. Ral expression levels in ral mutants. Western blot analysis shows that Ral levels are reduced in Ral^{EE1} mutants, and absent on Ral^{G0501} mutants, when compared to control wild-type W1118. Tubulin was used as a loading control. (Adapted from Rodrigues 2016)

3.1.1. Involvement of Ral and JNK signalling pathway in the regulation of microtubules

One of the major components of neurons is the microtubule cytoskeleton that is essential for numerous cellular and developmental processes, such as neuronal migration, polarity, and differentiation. Upon migration, development of axons and the establishment of synaptic connections, neurons undergo major developmental changes where the structural organization and dynamic remodeling of microtubule cytoskeleton plays a critical role. An appropriate synaptic architecture is required to provide the structural basis that supports synaptic transmission (Kapitein & Hoogenraad, 2015; Bodaleo and Gonzalez-Billault, 2016). It is known that microtubules are present in presynaptic boutons of *Drosophila* NMJs where they organize as loops and seem to be critical for synapse establishment and maintenance. During the formation of new synaptic boutons, microtubules undergo a dynamic reorganization where microtubule loops are splayed apart into various fibers and then are re-bundled again (Roos et al. 2000). Also, it has been shown that *Drosophila* homolog MAP1B/Futsch directly interacts with presynaptic microtubules, promoting microtubule stability at presynaptic boutons and synaptic growth (Roos et al. 2000; Godena et al. 2011). It has also been shown that JNK is involved in the regulation of microtubule cytoskeleton but it is unclear what are the molecular mechanisms of JNK action on the microtubule cytoskeleton, and the JNK substrates mediating such functions (Rallis et al. 2010).

We hypothesized that the impairment in the formation of new activity-dependent boutons could be due to defects in the regulation of microtubule cytoskeleton since microtubules have an essential role in structural plasticity. We aim to understand the involvement of JNK signaling in the regulation of microtubules in our system, since Ral has been shown to negatively regulate JNK pathway in *Drosophila*. To test this hypothesis, we overexpressed a JNK dominant-negative transgene in neurons,

using a neuronal driver, nSybGal4 in a r*al* mutant background. We focused our analysis in *Ral*^{EE1} mutants, because these were the ones that had a clear defect in structural plasticity.

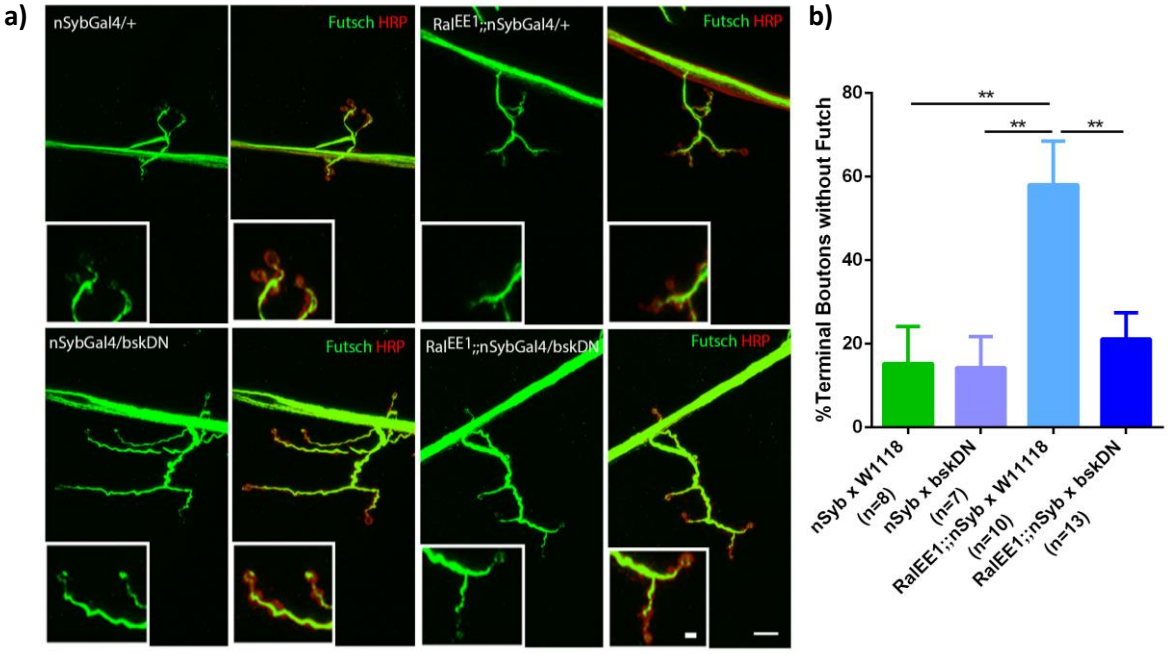

Figure 3.5. Analysis of the presence of futsch in terminal boutons. a) The panel shows confocal images of NMJ of muscle 4 of 3rd instar larvae, segment A2, A3 or A4 of neuronal diver nSybGal4 crossed with *W*^{1118} and bskDN, and the *Ral*^{EE1} mutant with the neuronal driver nSybGal4 crossed with *W*^{1118} and bskDN. Scale bar: 10µm. Zoom in scale bar: 2 µm. **b)** Quantification of the percentage of the terminal boutons without Futsch. (nSyb/+ n=8, nSyb/bsk^{DN} n=7, *Ral*^{EE1};; nSyb/+ n= 10 and *Ral*^{EE1};; nSyb/ bsk^{DN} n=13). Ordinary one-way ANOVA test was used to determine statistical significance. Error bars represent standard error of the mean (SEM). ** p<0.01.

We found that *Ral*^{EE1} mutant has an increased percentage of terminal boutons without Futsch, which indicates the absence of stable microtubules from the presynaptic terminals. Also, we could observe that the expression of the dominant-negative form of JNK in the *Ral* mutant background, could rescue the percentage of terminal boutons without Futsch (Figure 3.5) In this analysis wild type – nSyb/+ animals have an average of 15.31±8.84%, nSyb/bsk^{DN} have 14.29±7.44%, *Ral*^{EE1};; nSyb/+ have 58.09±10.40% and *Ral*^{EE1};; nSyb/bsk^{DN} have 21.13±6.35% of terminal boutons without Futsch.

Given that Ral acts as a negative regulator of JNK in *Drosophila*, in the absence of Ral, JNK could be activated leading to a decrease of stable microtubules in terminal boutons, possibly by destabilizing the microtubule network. This result suggests that JNK is negatively regulating microtubule stabilization. But more experiments are needed to ensure that Ral acts as negative regulator of JNK at the NMJ. One way to test this is by performing a western blot analysis in Ral mutants and blotting against activated form of JNK. We are expecting to see elevated levels of activated JNK in *Ral* mutants compared to control. It is also necessary to confirm that JNK destabilizes the microtubule cytoskeleton, and because as been shown that Futsch directly interacts with microtubules stabilizing them (Roos et al., 2000; Godena et al., 2011), this could be evaluated by observing the colocalization between Futsch and microtubules. If Ral negatively regulates JNK and JNK destabilizes microtubules we expect to see less

Futsch associated with microtubules in *ral* mutants, while expressing the dominant-negative form of JNK in the *ral* mutant background we expect to see more Futsch associated with microtubules.

Also, it will be interesting to understand if JNK regulates the phosphorylation state of Futsch since MAP1B as been shown to be regulated by phosphorylation (Gordon-Weeks 1997). To test this, we could use a specific antibody for the phosphorylated form of Futsch, and then assess the levels of phosphorylated Futsch in *ral* mutants and in *ral* mutants expressing a dominant-negative form of JNK.

Microtubule loops have been related to places of new bouton formation (Roos et al. 200), thus it will be interesting to compare the number of terminal loops and the capacity to form new boutons, in r*al* mutants and in *ral* mutants expressing a dominant-negative form of JNK. Here we expect to see fewer microtubule loops and new synaptic boutons in *ral* mutants, while in Ral mutants expressing a dominant-negative form of JNK, it is expected to see an increase in the number of microtubule loops in terminal boutons and in the formation of new synaptic boutons.

3.2. Contribution of Ral to the regulation of nerve thickness

When exiting the CNS, neurons extend their motor axons that are grouped in fascicles which, together with glial cells, form the nerve bundle (Lin and Goodman 1994; Araújo & Tear 2003). The axons leave the ventral nerve cord in bundles, and will defasciculate to reach their final tissue, where they will establish a connection with the target cell, which in the case of neuromuscular junctions (NMJ) is the muscle. Here, we identified a novel role for Ral GTPase in the regulation of nerve thickness and nerve fasciculation. We observed that Ral mutants have thicker nerves, possibly uncovering a new role of Ral GTPase in the regulation of *Drosophila* larval nerve bundle morphology. As it could be seen in figure 3.6., both *ral* mutants have thicker nerve bundles than the control W^{1118}. Quantification of the nerve thickness and respective nerve area indicates that Ral GTPase is involved in the regulation of nerve thickness. While in wild type animals have an average of 5.21±0.08 µm/446.89±9.86 µm^2, Ral^{G0501} have 8.01±0.26 µm /669.95±30.94 µm^2 and Ral^{EE1} have 7.82±0.14 µm /653.37±12.87 µm^2 nerve thickness and nerve area, respectively.

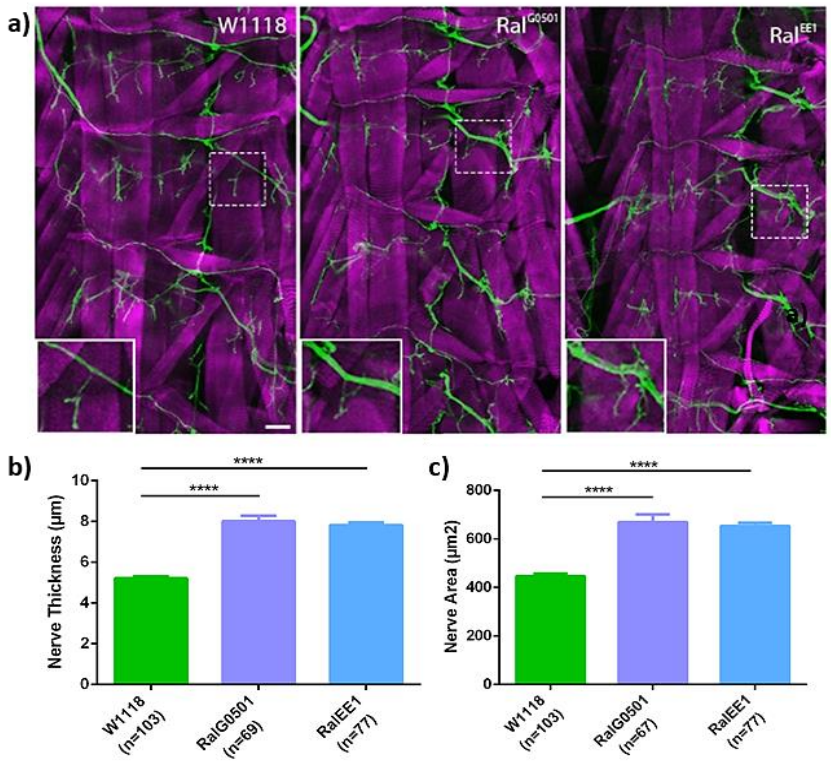

Figure 3.6. *ral* mutants have thicker nerve bundles. a) Confocal images of wild-type (W^{1118}) and *ral* mutants (Ral^{EE1} and Ral^{G0501}) larval body wall showing the muscles (in magenta) and the neuromuscular junctions (NMJ, in green). Red-muscle (phalloidin); Green – NMJ (HRP). Scale bar: 50 µm. **b)** Quantification of muscle 4 *ral* mutants and W^{1118} nerve thickness **c)** Quantification of muscle 4 *ral* mutants and W^{1118} nerve area in 80µm rectangle. W^{1118} n= 103; Ral^{G0501} n=69; Ral^{EE1} n=77. **** p<0.0001

The nerve bundle is composed of various descending motor axons and ascending sensory axons. Motor axons are long fibers that extend from the neuron's cell body and that transmit information

from the cell body to the target cell (Kevenaar & Hoogenraad 2015). Also, axons have a high density of microtubules that play an important role in the maintenance of neuronal polarity, morphology, integrity of axons and in assuring proper trafficking of cargos and organelles to (and from) the synapse (Barnes and Polleux, 2009). Closely associated with microtubules, is MAP1B/Futsch, which has been shown to influence microtubule dynamics by promoting their stabilization (Roos et al. 2000; Hummel et al. 2000). Using Futsch as our readout to assess axonal structure inside the nerve bundle, we observed that *ral* mutants have disorganized and less fasciculated axons (Figure 3.7.a). The levels of disorganization were ranked as normal (0), disorganized (1) and very disorganized (2). It can be observed that *ral* mutants, Ral^{G0501} and Ral^{EE1}, have an increased percentage of disorganized and very disorganized nerve bundles when compared to control W^{1118} (Fig. 3.7.b). Together these results suggest that Ral is involved in the regulation of nerve thickness possibly by regulating the axonal fasciculation. In other words, it is possible that the disorganization of nerve fascicles leads to a thicker bundle.

3.2.1. Is Ral regulating nerve thickness through downregulation of FasII?

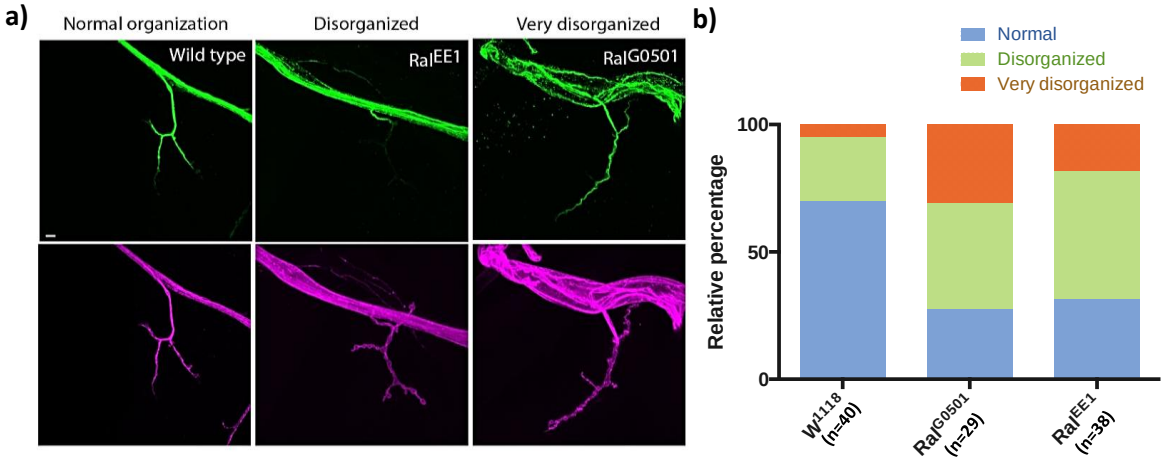

Figure 3.7. *ral* mutants have defects in neuronal fasciculation in the nerve bundle. a) Confocal images of wild-type (W^{1118}) and *ral* mutants (Ral^{EE1} and Ral^{G0501}) muscle 4. Green- Futsch, Magenta – HRP. Scale bar: 5 µm **b)** Quantification of the levels of axonal disorganization in *ral* mutants and in the control W^{1118}. The images were analyzed according to three ranks, normal, disorganized and very disorganized. W^{1118} n= 40; Ral^{G0501} n=29; Ral^{EE1} n=38. Experiments analyzed by Joana Rodrigues and Cátia Rodrigues.

Cell adhesion molecules (CAMs) play a critical role during various steps of development. In order to support the proper establishment of the neuronal network, CAMs are important for correct formation and function of the nervous system by regulating distinct types of adhesion, including axon–axon and axon-glia (Hortsch 2000) fasciculation or defasciculation (Carrero-Martıínez & Chiba, 2009). Fasciclin II (FasII) is a homophylic CAM that has been shown to be important for development, maintenance and plasticity of the NMJ, and is considered as the fly ortholog of the mammalian neuronal cell adhesion molecule (NCAM) (Sun & Xie, 2012). This protein has also been shown to be important to maintain adhesion between axons, a process called fasciculation (van Vactor et al. 1993, Lin and Goodman 1994). Fasciculation and defasciculation processes require spatiotemporal regulation to permit the

formation of highly stereotypical axon patterns, and to allow axons to reach their targets (Carrero-Martıínez & Chiba, 2009). Because we identified Ral as a regulator of nerve thickness, and because *ral* mutants showed disturbed axonal architecture, we thought that the levels of the cell adhesion molecule FasII may be altered in *ral* mutants. To test this, *ral* mutants, Ral^{G0501} and Ral^{EE1}, together with the control W^{1118} were stained using an antibody against FasII. Our quantification of FasII levels in the bundle showed that *ral* mutants have significantly decreased levels of FasII in muscle 4 nerve bundles. In wild type animals have an average of 100±3.35 Intensity/µm^2, while Ral^{G0501} have 77.61±4.86 Intensity/µm^2 and Ral^{EE1} have 77.323±3.19 Intensity/µm^2 Fas II levels. Also, we noticed that in control, larvae appeared to have FasII running along the axon as in a "cable form", but this distribution occurred less frequently in *ral* mutants, as it can be seen in figure 3.8. These results indicate that Ral is necessary for the regulation of the cell adhesion molecule FasII.

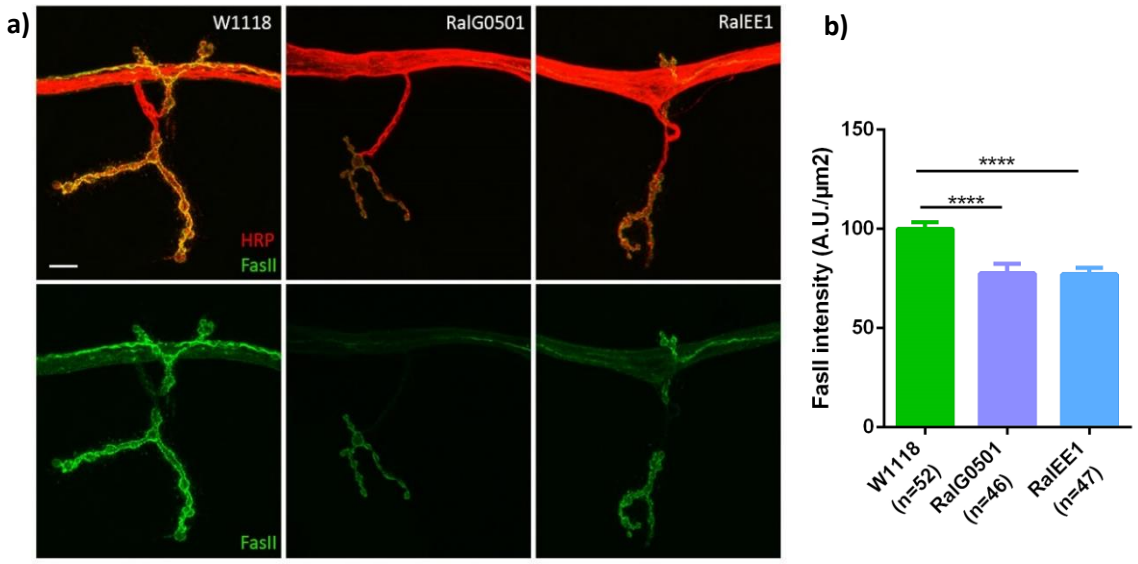

Figure 3.8. ral mutants have decreased levels of cell adhesion molecule FasII in the nerve bundle. a) Confocal images of wild-type (W^{1118}) and ral mutants (Ral^{EE1} and Ral^{G0501}) muscle 4 Green- FasII, Red – HRP. Scale bar: 10 µm **b)** Quantification of the intensity levels of FasII in ral mutants (Ral^{EE1} and Ral^{G0501}) and in the control W^{1118}. Measurements were performed in 80µm rectangle. W^{1118} n= 52; Ral^{G0501} n=46; Ral^{EE1} n=47. Kruskal-Wallis test was used to determine statistical significance. Error bars represent standard error of the mean (SEM). **** p< 0,0001.

In future experiments, to understand if cell adhesion molecule FasII is necessary for Ral GTPase regulation of nerve thickness, we can take advantage of the UAS-Gal4 system, to express UAS-FasII in neurons (using the neuronal nSybGal4 driver) in *ral* mutants. In this experiment, we would expect to see a rescue of the levels of FasII protein, and would check if nerve thickness and axonal fasciculation could be rescued in *ral* mutants.

3.2.2. Involvement of Ral in the regulation of nerve thickness via JNK signalling pathway

JNK is a member of the serine-threonine kinases family also known as mitogen-activated protein kinase (MAPK), which also integrates the extracellular signal regulated kinase (ERK) and p38 subfamilies. MAPKs are known to amplify and integrate external signals from different types of stimuli, inducing genomic and physiological responses in response to changes in the environment (Weston & Davis 2002; Kim & Choi 2010). Activation of MAPKs can be accomplished by phosphorylation of serine/threonine residues through interaction between kinases (Coffey 2014; Wang et. al 2005; Kim & Choi 2010). In *Drosophila* JNK/Bsk is activated upon phosphorylation in two amino acid residues, threonine 181 and tyrosine 183 (Rallis et al. 2010). Also, in *Drosophila* JNK/Bsk is involved in a plethora of cellular responses, such as axonal polarity (Oliva et al. 2006), axon extension (Srahna et al. 2006), and at the *Drosophila* neuromuscular junction (NMJ), JNK regulates synaptic plasticity and growth (Collins et al. 2006; Sanyal et al. 2002). Recently, it was found that JNK/Bsk is required for axon pruning, by negatively regulating the membrane levels of Fasciclin II (Bornstein et al 2015).

Because Ral GTPase act as a negative regulator of JNK signaling pathway in *Drosophila*, we wanted to understand if Ral is regulating nerve thickness via JNK. Since *ral* mutants have decreased levels of FasII, and JNK has been shown to negatively regulate membrane levels of FasII, we predicted that we should see elevated levels of activated JNK (phosphorylated JNK) in *ral* mutants. To address this question, *ral* mutants, Ral^{G0501} and Ral^{EE1}, together with the control W^{1118} were stained using an antibody against activated JNK (p-JNK). We found that Ral mutants have no significant changes in the levels of p-JNK in nerve bundle adjacent to muscle 4 (Figure 3.9), suggesting that JNK signaling is not involved in Ral nerve thickness regulation. Here, wild type animals have an average of 100±2.13 Intensity/μm^2, while Ral^{G0501} have 101.45±4.10 Intensity/μm^2 and Ral^{EE1} have 102.42±3.17 Intensity/μm^2 p-JNK levels. However, after a thorough literature search, we discovered that we cannot be sure of this result because the antibody that we used can potentially cross-react with other members of MAPKs family, ERK and p38, which can be regulated together with or in opposite ways to JNK. Therefore, a Western blot will be needed to confirm whether p-JNK is or not increased in *ral* mutants. To further circumvent this problem, we can purchase a different p-JNK antibody that does not react with the other proteins and re-test the levels at the nerve bundle. In conclusion, our initial results do not support the hypothesis that JNK signaling is increased in ral mutants, but the nature of our antibody, precluded us from having absolute certainty.

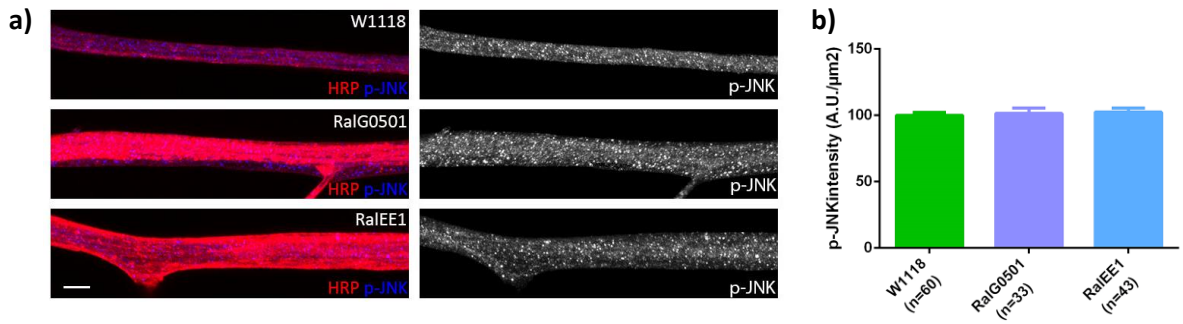

Figure 3.9. *ral* mutants have the same levels of activated JNK (p-JNK) as the control. a) Confocal images of wild-type (W^{1118}) and *ral* mutants (Ral^{EE1} and Ral^{G0501}) muscle 4. Blue/Grey- p-JNK, Red – HRP. Scale bar: 10 µm **b)** Quantification of the intensity levels of p-JNK in *ral* mutants (Ral^{EE1} and Ral^{G0501}) and in the control W^{1118}. Measurements were performed in 80µm rectangle. W^{1118} n= 60; Ral^{G0501} n=33; Ral^{EE1} n=43. Kruskal-Wallis test was used to determine statistical significance. Error bars represent standard error of the mean (SEM).

3.2.3. Is Ral regulating nerve thickness, through modulation of cell adhesion via JNK?

We show here that Ral GTPase is involved in the regulation of nerve thickness: we observed that *ral* mutants have thicker and disorganized nerve bundles. Although p-JNK does not appear to be increased in *ral* mutants, we wanted to test the possible involvement of JNK signaling using a different approach. We wanted to test if Ral is regulating nerve thickness via JNK signaling pathway, given that we observed that FasII is diminished in Ral mutants (Figure 3.8.) and that it has been shown that Ral GTPase negatively regulates JNK signaling in *Drosophila*, and that JNK negatively regulates FasII. To test this, we expressed a dominant negative form of JNK/Bsk (UAS-bsk^{DN}) in *ral* mutants (Ral^{G0501} and Ral^{EE1}) using nSybGal4 as neuronal driver, then nerve thickness and nerve area were quantified. If JNK/Bsk is involved in Ral regulation of nerve thickness, we were expecting to see thinner nerve bundles in *ral* mutants expressing bsk^{DN} when compared with *ral* mutants. We observed that *ral* mutants (Ral^{G0501};; nSyb/+ and Ral^{EE1};; nSyb/+) have thicker nerve bundles when compared to control (nSyb/+) as described above (figure 3.10.b). Also, Ral^{G0501};; nSyb/+ have increased nerve area compared to control, however Ral^{EE1};; nSyb/+ nerve area doesn't appear to be different from the control in these experiments, this could be due to smaller number of muscle 4 nerve bundles analyzed (n) (figure 3.10.c). Comparing *ral* mutants (Ral^{G0501};; nSyb/+ and Ral^{EE1};; nSyb/+) nerve thickness and nerve area with *ral* mutants expressing bsk^{DN} (Ral^{G0501};; nSyb/bsk^{DN} and Ral^{EE1};; nSyb/bsk^{DN}) we can see that there is no rescue in these parameters, since no significant changes were observed. Our results show that wild type – nSyb/+ animals have an average of 5.43±0.12 µm/499.88±13.17 μm^2, nSyb/bsk^{DN} have 6.76±0.17 µm /563.86±14.49 μm^2, Ral^{G0501};; nSyb/+ have 7.66±0.27 µm/575.83±17.56 μm^2, Ral^{G0501};; nSyb/bsk^{DN} have 7.26±0.22 µm /615.38±20.57 μm^2, Ral^{EE1};; nSyb/+ have 6.39±0.22 µm/496.72±15.09 μm^2 and Ral^{EE1};; nSyb/bsk^{DN} have 6.41±0.16 µm/553.96±14.99 μm^2 nerve thickness and nerve area, respectively. Thus, it is possible that JNK/Bsk is not required in the neurons for Ral GTPase regulation of nerve thickness.

In addition, when bsk^{DN} is expressed in wild-type background (nSyb/bsk^{DN}), both nerve thickness and nerve area values are increased compared to control (nSyb/+). Taken together this information could indicate that JNK/Bsk may play a role in nerve thickness independently of Ral, or that Ral is positively regulating JNK signaling pathway – which has also been reported to happen in several other biological situations (Massaro et al 2009). Expressing a constitutively active form of JNK in *ral* mutants should help us understanding how Ral GTPase regulates JNK. If Ral positively regulates JNK and if this pathway is involved in the regulation of nerve thickness, we expect to see a rescue in nerve thickness and nerve area in *ral* mutants expressing a constitutively active form of JNK. Also, a western blot analysis of the levels of p-JNK in *Ral* mutants, would help us to understand JNK regulation by Ral. If the levels of p-JNK are increased in *ral* mutants, possibly Ral negatively regulates JNK, in contrary, if levels of p-JNK are decreased in *ral* mutants, it is probable that Ral positively regulates JNK.

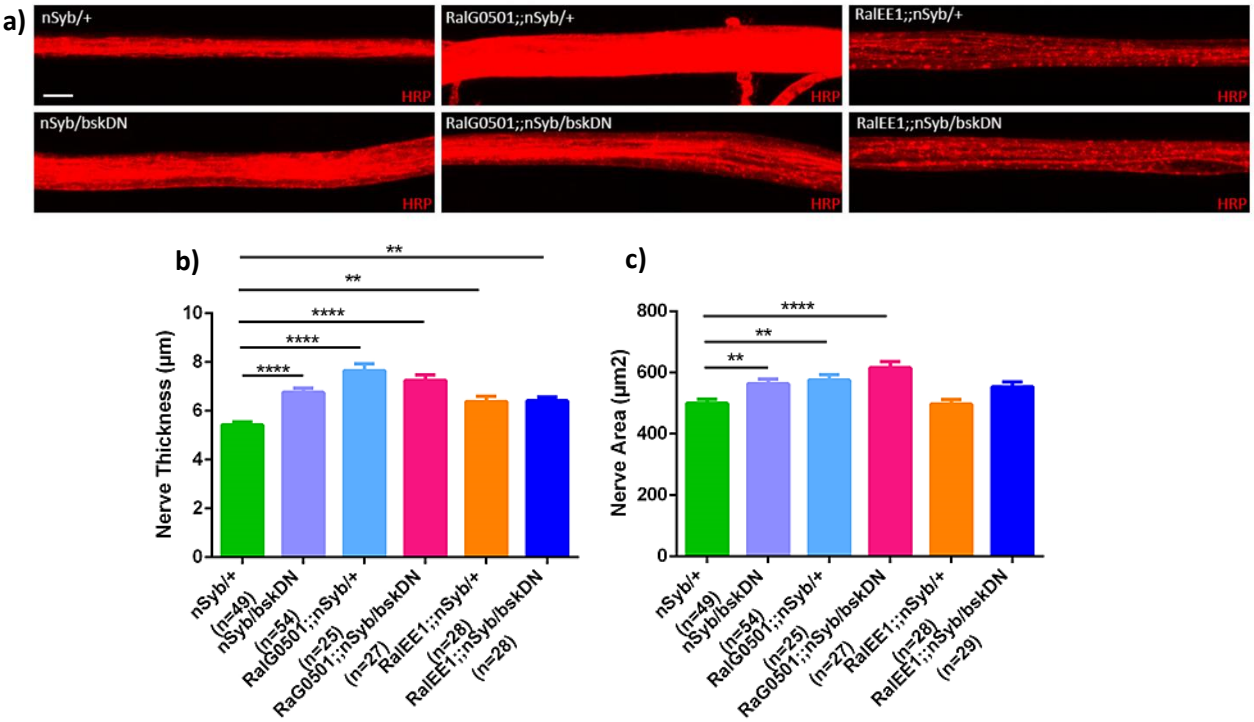

Figure 3.10. The neuronal expression of bskDN in *ral* mutants does not rescue nerve thickness nor the nerve area. a) Confocal images of nSyb/+ (control), nSyb/ bsk^{DN}, *Ral*^{G0501};; nSyb/+, *Ral*^{G0501};; nSyb/bsk^{DN}, *Ral*^{EE1};; nSyb/+ and *Ral*^{EE1};; nSyb/ bsk^{DN} in muscle 4. Red – HRP. **b-c)** Quantification of muscle 4 *ral* mutants (*Ral*^{G0501};; nSyb/+ and *Ral*^{EE1};; nSyb/+), ral mutants expressing bsk^{DN} (*Ral*^{G0501};; nSyb/bsk^{DN} and *Ral*^{EE1};; nSyb/ bsk^{DN}), wild-type (nSyb/+) and the wild-type expressing bsk^{DN} (nSyb/ bsk^{DN}) nerve thickness (nSyb/+ n=49, nSyb/bsk^{DN} n=54, *Ral*^{G0501};; nSyb/+ n= 25, *Ral*^{G0501};; nSyb/bsk^{DN} n=27, *Ral*^{EE1};; nSyb/+ n= 28 and *Ral*^{EE1};; nSyb/ bsk^{DN} n=28) **(b)** and nerve area (nSyb/+ n=49, nSyb/bsk^{DN} n=54, *Ral*^{G0501};; nSyb/+ n= 25, *Ral*^{G0501};; nSyb/bsk^{DN} n=27, *Ral*^{EE1};; nSyb/+ n= 28 and *Ral*^{EE1};; nSyb/ bsk^{DN} n=29) **(c)** in 80µm rectangle. Kruskal-Wallis test was used to determine statistical significance. Error bars represent standard error of the mean (SEM). ** p<0.01 **** p<0.0001.

In parallel, we wanted to understand if Ral GTPase is regulating nerve thickness through the regulation of cell adhesion via JNK/Bsk signaling pathway. To test this, we expressed a dominant negative form of JNK (UAS-bsk^{DN}) in *ral* mutants, *Ral^{G0501}* and *Ral^{EE1}* using the neuronal driver nSybGal4, and FasII and p-JNK (activated) intensity levels were quantified. We were expecting to see decreased levels of FasII in *ral* mutants (*Ral^{G0501}*;; nSyb/+ and *Ral^{EE1}*;; nSyb/+), as previously demonstrated, and increased levels in bsk^{DN} in wild-type background (nSyb/bsk^{DN}), since it was described that JNK negatively regulates membrane cell adhesion molecule FasII. Regarding the levels of p-JNK, as previous demonstrated we were expecting to see no significant changes between *ral* mutants (*Ral^{G0501}*;; nSyb/+ and *Ral^{EE1}*;; nSyb/+) and the control (nSyb/+). Contrary to our expectations, we did not find significant changes in the levels of FasII between all the genotypes analyzed (Figure 3.11.b). In this experiments wild type – nSyb/+ animals have an average of 100±3.28 Intensity/µm^2, nSyb/bsk^{DN} have 98.66±3.58 Intensity/µm^2, *Ral^{G0501}*;; nSyb/+ have 93.55±7.18 Intensity/µm^2, *Ral^{G0501}*;; nSyb/bsk^{DN} have 102.70±5.23 Intensity/µm^2, *Ral^{EE1}*;; nSyb/+ have 92.195.54 Intensity/µm^2 and *Ral^{EE1}*;;nSyb/bsk^{DN} have 88.42±5.84 Intensity/µm^2 FasII levels. This could be due to smaller number of muscle 4 nerve bundles analyzed (n). Therefore, we cannot conclude about the fasciculation of the nerve bundles in this experiment. Though, as we expected, the levels of activated JNK (p-JNK) in *ral* mutants (*Ral^{G0501}*;; nSyb/+ and *Ral^{EE1}*;; nSyb/+) were similar to control (nSyb/+), but we found significant increased levels of p-JNK in the genotypes where we overexpressed bsk^{DN} (nSyb/ bsk^{DN}, *Ral^{G0501}*;; nSyb/ bsk^{DN} and *Ral^{EE1}*;; nSyb/ bsk^{DN})(Figure 3.11.c). Here wild type – nSyb/+ animals have an average of 100±2.71 Intensity/µm^2, nSyb/bsk^{DN} have 181.76±3.68 Intensity/µm^2, *Ral^{G0501}*;; nSyb/+ have 125.33±4.81 Intensity/µm^2, *Ral^{G0501}*;; nSyb/bsk^{DN} have 200.68±7.56 Intensity/µm^2, *Ral^{EE1}*;;Syb/+ have 110.67±9.41 Intensity/µm^2 and *Ral^{EE1}*;; nSyb/bsk^{DN} have 171.13 Intensity/µm^2 p-JNK II levels.

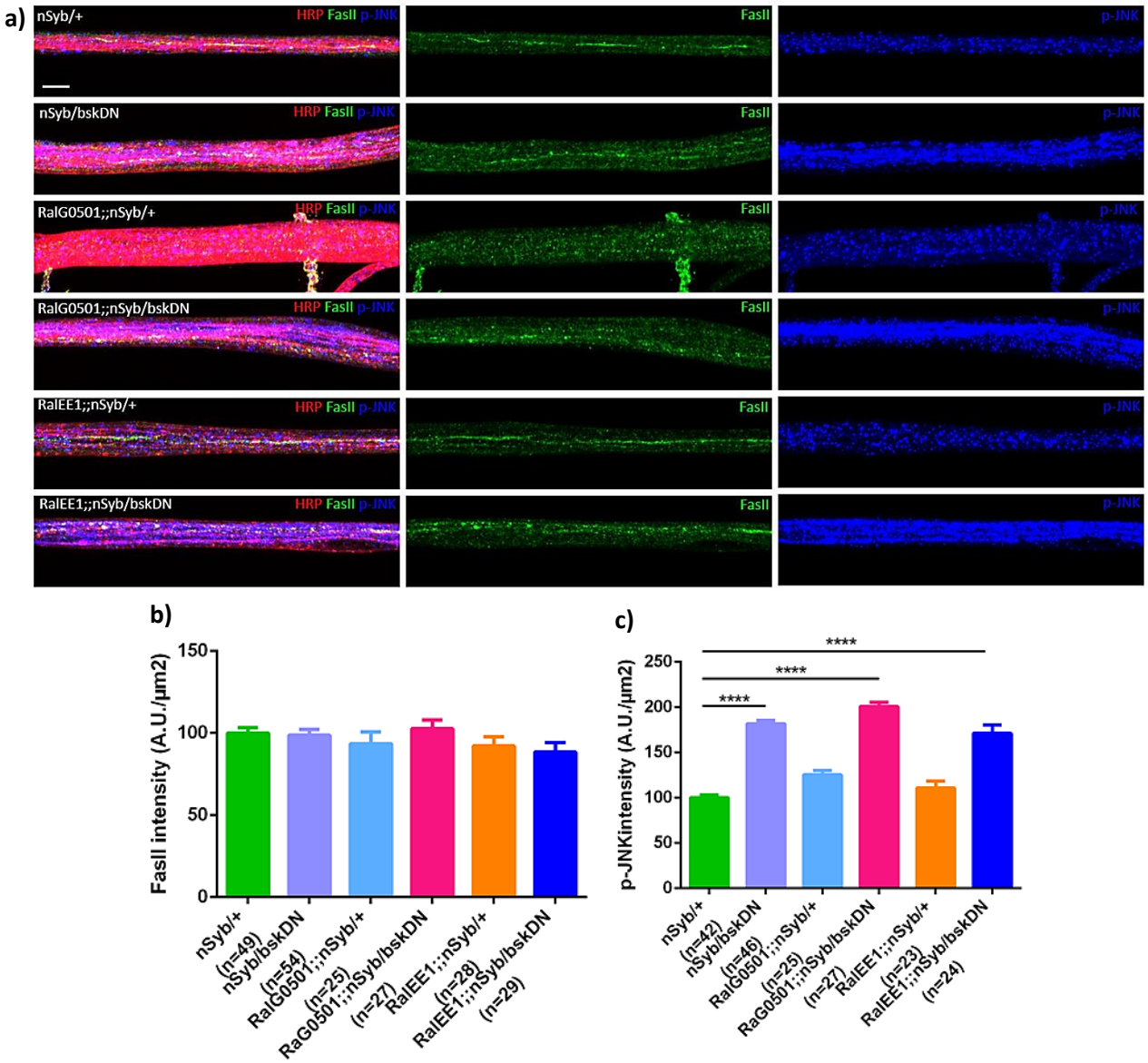

Figure 3.11. It is possible that JNK is not required in neurons to regulate Ral-dependent nerve thickness. a) Confocal images of nSyb/+ (control), nSyb/ bsk^{DN}, *Ral*^{G0501};; nSyb/+, *Ral*^{G0501};; nSyb/bsk^{DN}, *Ral*^{EE1};; nSyb/+ and *Ral*^{EE1};; nSyb/ bsk^{DN} in muscle 4. Green – FasII, Blue – p-JNK, Red – HRP. **b-c)** Quantification of muscle 4 *ral* mutants (*Ral*^{G0501};; nSyb/+ and *Ral*^{EE1};; nSyb/+), ral mutants expressing bsk^{DN} (*Ral*^{G0501};; nSyb/bsk^{DN} and *Ral*^{EE1};; nSyb/ bsk^{DN}), wild-type (nSyb/+) and the wild-type expressing bsk^{DN} (nSyb/ bsk^{DN}) FasII levels (nSyb/+ n=49, nSyb/bsk^{DN} n=54, *Ral*^{G0501};; nSyb/+ n= 25, *Ral*^{G0501};; nSyb/bsk^{DN} n=27, *Ral*^{EE1};; nSyb/+ n= 28 and *Ral*^{EE1};; nSyb/ bsk^{DN} n=29) **(b)** and p-JNK (nSyb/+ n=42, nSyb/bsk^{DN} n=46, *Ral*^{G0501};; nSyb/+ n= 25, *Ral*^{G0501};; nSyb/bsk^{DN} n=27, *Ral*^{EE1};; nSyb/+ n= 23 and *Ral*^{EE1};; nSyb/ bsk^{DN} n=24)**(c)** in 80µm rectangle. Kruskal-Wallis test was used to determine statistical significance. Error bars represent standard error of the mean (SEM). **** p<0.0001.

The line of UAS-bsk^{DN} that we used were generated by substitution of a Lysine aminoacid for an Arginine, impairing the ATP-binding site responsible for the catalytic activity of the kinase (Figure 3.12.) (Weber et al. 2000). However, the phosphorylation sites in this line are intact, so it is possible that this protein can still be phosphorylated and thus, being recognized by the antibody that we used. Because we overexpress UAS-bsk^{DN} and the antibody recognizes the phosphorylation sites, we could observe

that the dominant-negative form of JNK is being expressed but cannot conclude about its regulation by Ral GTPase.

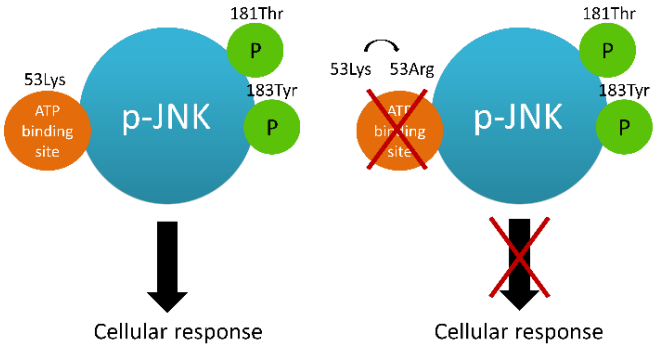

Figure 3.12. Schematic representation of activated JNK and JNK dominant-negative protein. a) Representation of activated JNK protein where in green are represented the phosphorylation sites and in orange are represented the ATP binding site. Intact ATP binding site, allows the activation of the protein and consequently regulation of cellular responses. **b)** Representation of JNK dominant-negative protein where in green are represented the phosphorylation sites and in orange are represented inactivation of the ATP binding site due to substitution of Lysine for Arginine in 53 position. Inactivation of the ATP binding site prevents JNK activation which consequently impairs the regulation of downstream cascades.

3.3. Is Ral required in glia in the regulation of nerve thickness?

Glial cells are essential for proper nervous system development regulating many aspects of morphology and function (Stork et al. 2012; Brink et al. 2012). *Drosophila* peripheral nervous system is composed by various glial cell types that are responsible for the establishment of blood brain barrier (BBB), provide metabolic support, ensheathment of descending motor neuron axons and the ascending sensory axons (Freeman 2015; Limmer at al. 2014). Proper fasciculation and insolation of axons is necessary to ensure precise conduction of nerve impulses (Banerjee & Bhat 2008). As previously described, Ral is involved in the regulation of nerve thickness and because glial cells are critical for normal nerve bundle development, we wanted to understand if Ral is necessary in these cells for proper regulation of nerve bundling and for the regulation of nerve thickness.

To address this question, we started by overexpressing a rescue construct (UAS-Ral-HA) in neurons (n-Syb-Gal4) or in glia (Repo-Gal4), in a *ral* mutant background. In this experiment, we are testing in which cell-type Ral is necessary to rescue nerve thickness. We found that Ral expression in neurons or glia rescued the thickness of the nerve in Ral^{G0501} (Figure 3.13.b) and the expression of Ral in glia also rescued nerve thickness in Ral^{EE1} (Figure 3.13.c), indicating that these two cells are involved in regulation of nerve thickness. In these experiments wild type – W^{1118}/RalHA animals have an average of 5.65±0.17 µm (Ral^{G0501} experiment) and 5.06±0.16 µm (Ral^{EE1} experiment), Ral^{G0501}/RalHA have 8.05±0.24µm, Ral^{G0501};; nSyb/RalHA have 6.99±0.30µm, Ral^{G0501};; Repo/RalHA have 6.49±0.19µm, Ral^{EE1}/RalHA have 7.41±0.25µm, Ral^{EE1};; nSyb/RalHA have 7.10±0.26µm and Ral^{EE1};; Repo/RalHA have 6.10±0.20µm nerve thickness. However, the rescue was only partial, so it will be interesting to expresses Ral in both cell types at the same time to assess if there is a total rescue of nerve thickness.

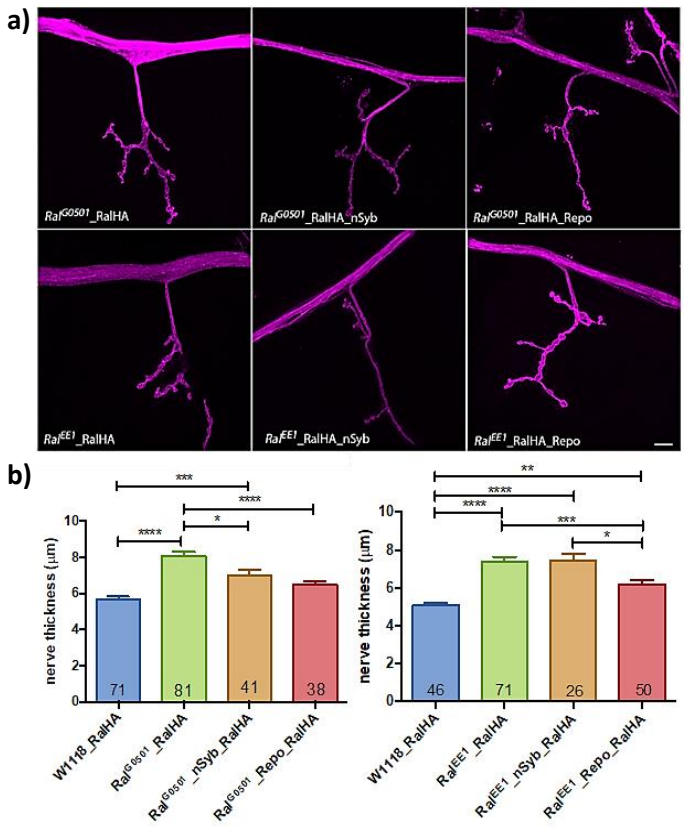

Figure 3.13. Nerve thickness is rescued by Ral in glia and in neurons. a) Confocal images of ral mutants (*Ral*^{G0501} and *Ral*^{EE1}) overexpressing Ral-HA in neurons (nSyb) and in glial cells (Repo). Scale bar: 10 µm. **b, c)** Quantification of nerve thickness in the *ral* mutant *Ral*^{G0501}, *Ral*^{G0501} overexpressing Ral-HA in neuron and in glial cells (W^{1118}/RalHA n=71, *Ral*^{G0501}/RalHA n= 81, *Ral*^{G0501};; nSyb/RalHA n=41, *Ral*^{G0501};; Repo/RalHA n= 38)**(b)** and in the ral mutant *RaE*^{EE1}, *RaE*^{EE1} overexpressing Ral-HA in neuron and in glial cells (W^{1118}/RalHA n=46, *Ral*^{EE1}/RalHA n= 71, *Ral*^{EE1};; nSyb/RalHA n=26, *Ral*^{EE1};; Repo/RalHA n= 50)**((c)**. Experiments realized and analyzed by Joana Rodrigues. Ordinary one-way ANOVA test was used to determine statistical significance. Error bars represent standard error of the mean (SEM). * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001.

Because we found that Ral GTPase is required in neurons and in glial cells to regulate nerve thickness, and although JNK does not seem to be the pathway involved in Ral GTPase regulation of nerve thickness in neurons, we wanted to test if JNK signaling pathway plays a role in glial cells. To test this, we expressed a dominant negative JNK (UAS-bsk^{DN}) in *ral* mutants (*Ral*^{G0501} and *Ral*^{EE1}) using RepoGal4 as glial driver, then nerve thickness and nerve area were quantified. If JNK pathway is involved in Ral GTPase-regulation of nerve thickness in glial cells, we were expecting to see thinner nerve bundles in *ral* mutants expressing bsk^{DN} when compared with *ral* mutants.

Through analysis of the results we could observe that *ral* mutant *Ral*^{G0501};; RepoG4/+ have thicker nerve bundles when compared to control (RepoG4/+). Despite the increased levels of nerve thickness in the *ral* mutant *Ral*^{EE1};; RepoG4/+, this value is not significant different from the control due to a high dispersion of the values (high standard deviation) (Figure 3.14.b). Contrary to our expectations, we did not find significant changes in the nerve area between all the genotypes analyzed (Figure 3.14.c). Here wild type – Repo/+ animals have an average of 6.31±0.20 µm/516.28±16.66 µm^2, *Ral*^{G0501};; Repo/+ have 7.31±0.25 µm/526.32±14.68 µm^2, *Ral*^{G0501};; Repo/bsk^{DN} have 7.41±0.32 µm /583.79±24.52 µm^2, *Ral*^{EE1};;

Repo/+ have 7.69±0.61 µm/582.92±38.51 µm^2 and *Ral^{EE1}*;; Repo/bsk^{DN} have 6.83±0.22 µm/557.04±17.91 µm^2 nerve thickness and nerve area, respectively. Also, comparing *ral* mutants (*Ral^{G0501}*;; RepoG4/+ and *Ral^{EE1}*;; RepoG4/+) nerve thickness and nerve area with *ral* mutants expressing bskDN (*Ral^{G0501}*;; RepoG4/bsk^{DN} and *Ral^{EE1}*;; RepoG4/bsk^{DN}) we can see that there is no rescue in these parameters, once that no significant changes were observed. Thus, it is possible that JNK is not involved in Ral GTPase regulation of nerve thickness in glial cells. In this experiment, however, we noticed that the differences between control and Ral mutants were smaller, and this was due to the Gal4 driver Repo. The Gal4 control has nerve thickness and nerve area values higher than the remainder of our controls, and higher than what has been published as a normal range. This can be underpinning an effect of Ral in glial cells, which we will need to test by using a different Gal4 that is also expressed in glial cells.

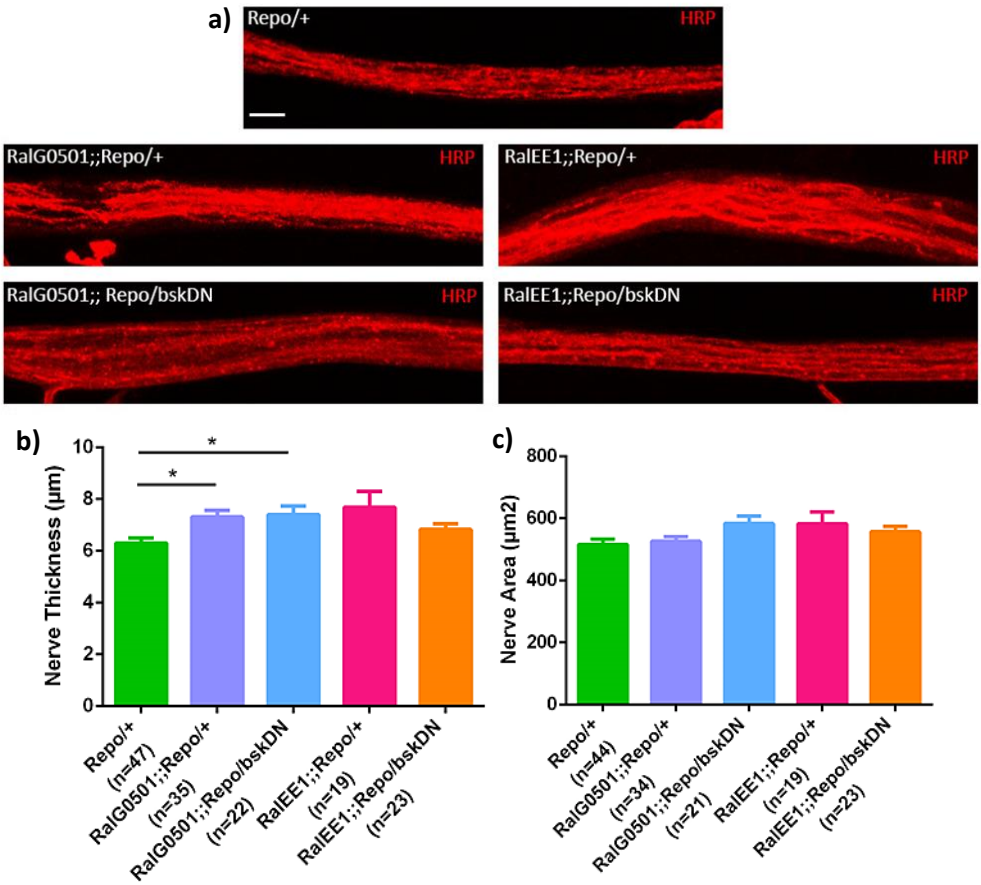

Figure 3.14. The glial expression of bskDN in ral mutants does not rescue nerve thickness nor the area. a) Confocal images of Repo/+ (control), *Ral^{G0501}*;; Repo/+, *Ral^{G0501}*;; Repo/bsk^{DN}, *Ral^{EE1}*;; Repo/+ and *Ral^{EE1}*;; Repo/ bsk^{DN} in muscle 4. Red – HRP. **b-c)** Quantification of muscle 4 ral mutants (Ral^{G0501};; RepoG4/+ and Ral^{EE1};; RepoG4/+), ral mutants expressing bsk^{DN} (Ral^{G0501};; RepoG4/bsk^{DN} and Ral^{EE1};; RepoG4/ bsk^{DN}), wild-type (RepoG4/+) nerve thickness (Repo/+ n=47, *Ral^{G0501}*;; Repo/+ n= 35, *Ral^{G0501}*;; Repo/bsk^{DN} n=22, *Ral^{EE1}*;; Repo/+ n= 19 and *Ral^{EE1}*;; Repo/ bsk^{DN} n=23) **(b)** and nerve area (Repo/+ n=44, *Ral^{G0501}*;; Repo/+ n= 34, *Ral^{G0501}*;; Repo/bsk^{DN} n=21, *Ral^{EE1}*;; Repo/+ n= 19 and *Ral^{EE1}*;; Repo/ bsk^{DN} n=23) **(c)** in 80µm rectangle. Kruskal-Wallis test was used to determine statistical significance. Error bars represent standard error of the mean (SEM). * p<0.05.

In parallel, we wanted to understand if Ral GTPase is regulating nerve thickness through the regulation of cell adhesion via JNK/Bsk signaling pathway in glial cells. To test this, we expressed a dominant negative form of JNK (UAS-bsk^{DN}) in *ral* mutants, *Ral^{G0501}* and *Ral^{EE1}* using the glial driver RepoGal4, and FasII and p-JNK (activated) intensity levels were quantified. We are expecting to see decreased levels of FasII in *ral* mutants (*Ral^{G0501}*;; RepoG4/+ and *Ral^{EE1}*;; RepoG4/+), as previous demonstrated. Regarding the levels of p-JNK, we expect to see no significant changes between *ral* mutants (*Ral^{G0501}*;; RepoG4/+ and *Ral^{EE1}*;; RepoG4/+) and the control (Repo/+).

According to our expectations *Ral^{G0501}*;; RepoG4/+ have decreased levels of FasII intensity when compared to control RepoG4 but the *ral* mutant *Ral^{EE1}*;; RepoG4/+ did not show decreased levels of FasII (Figure 3.15.b). This might be due to smaller number of muscle 4 nerve bundles analyzed (n). As we can observe there is not significant differences between *ral* mutants (*Ral^{G0501}*;; RepoG4/+ and *Ral^{EE1}*;; RepoG4/+) and ral mutants expressing bsk^{DN} in glia (*Ral^{G0501}*;; RepoG4/ bsk^{DN} and *Ral^{EE1}*;; RepoG4/ bsk^{DN}), which indicates that the levels of FasII are not rescued by the expression of bskDN. Our results show that wild type – Repo/+ animals have an average of 100±3.64 Intensity/µm^2, *Ral^{G0501}*;; Repo/+ have 85.74±3.07 Intensity/µm^2, *Ral^{G0501}*;; Repo/bsk^{DN} have 80.40±4.02 Intensity/µm^2, *Ral^{EE1}*;; Repo/+ have 93.43±6.18 Intensity/µm^2 and *Ral^{EE1}*;; nSyb/bsk^{DN} have 86.59±4.22 Intensity/µm^2 Fas II levels. However, as previously shown, the levels of activated JNK (p-JNK) in *ral* mutants (*Ral^{G0501}*;; RepoG4/+ and *Ral^{EE1}*;; RepoG4/+) were similar to control (RepoG4/+) (Figure 3.15.c). But as we already saw in experiments in neurons (using the neuronal driver nSyb) we found significant increased levels of p-JNK in the genotypes where we overexpressed bsk^{DN} (*Ral^{G0501}*;; RepoG4/ bsk^{DN} and *Ral^{EE1}*;; RepoG4/ bsk^{DN}). In this experiments wild type – Repo/+ animals have an average of 100±1.89 Intensity/µm^2, *Ral^{G0501}*;; Repo/+ have 97.90±2.64 Intensity/µm^2, *Ral^{G0501}*;; Repo/bsk^{DN} have 205.42±7.45 Intensity/µm^2, *Ral^{EE1}*;; Repo/+ have 105.85±3.87 Intensity/µm^2 and *Ral^{EE1}*;; nSyb/bsk^{DN} have 138.70±4.04 Intensity/µm^2 p-JNK levels. As mentioned before this result can be explained by the fact that the antibody recognizes the phosphorylated protein although it is not active.

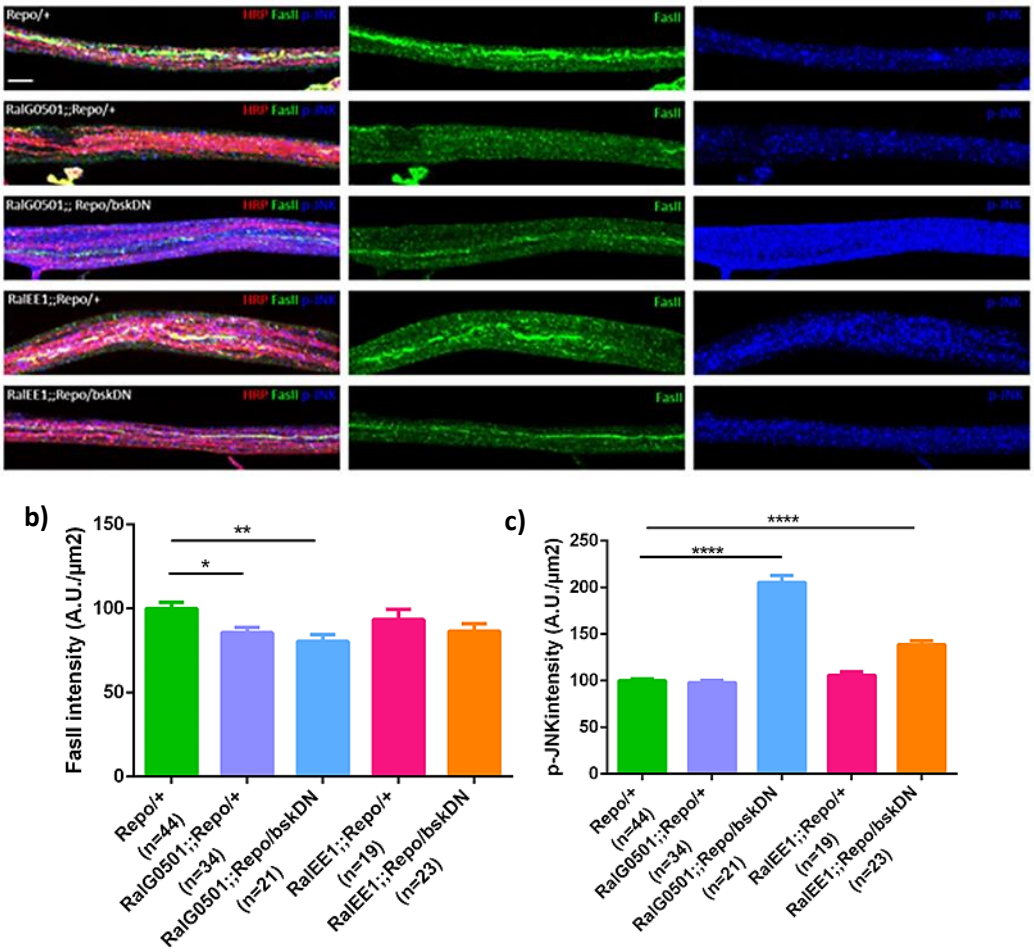

Figure 3.15. It is possible that JNK is not required in glial cells to regulate Ral-dependent nerve thickness. a) Confocal images of Repo/+ (control), *Ral*^{G0501};; Repo/+, *Ral*^{G0501};; Repo/bsk^{DN}, *Ral*^{EE1};; Repo/+ and *Ral*^{EE1};; Repo/ bsk^{DN} in muscle 4. Green – FasII, Blue – p-JNK, Red – HRP. **b-c)** Quantification of muscle 4 *ral* mutants (*Ral*^{G0501};; RepoG4/+ and *Ral*^{EE1};; RepoG4/+), ral mutants expressing bsk^{DN} (*Ral*^{G0501};; RepoG4/bsk^{DN} and *Ral*^{EE1};; RepoG4/ bsk^{DN}), wild-type (nSyb/+) FasII levels (Repo/+ n=44, *Ral*^{G0501};; Repo/+ n= 34, *Ral*^{G0501};; Repo/bsk^{DN} n=21, *Ral*^{EE1};; Repo/+ n= 19 and *Ral*^{EE1};; Repo/ bsk^{DN} n=23) **(b)** and p-JNK (Repo/+ n=44, *Ral*^{G0501};; Repo/+ n= 34, *Ral*^{G0501};; Repo/bsk^{DN} n=21, *Ral*^{EE1};; Repo/+ n= 19 and *Ral*^{EE1};; Repo/ bsk^{DN} n=23) **(c)** in 80µm rectangle. Kruskal-Wallis test was used to determine statistical significance. Error bars represent standard error of the mean (SEM). * $p<0.05$ ** $p<0.01$ **** $p<0.0001$.

Taken together our results suggest that Ral GTPase and JNK pathway may play a role in presynaptic structural plasticity through the regulation of the microtubule cytoskeleton. In addition, Ral GTPase showed to be involved in the regulation of nerve thickness through the modulation of the levels of cell adhesion molecule FasII. Also, we found that glial cells may play a role in the regulation of nerve thickness. However, JNK signaling pathway does not appear to be involved in the regulation of Ral-dependent nerve thickness neither in neurons nor in glia. We hope that additional experiments will help us to understand the mechanism by which nerve thickness is regulated and the pathways involved in this process.

Chapter 4. Conclusion and Future Perspectives

Neurons are highly complex cells that can change their shape in response to physiological events. Defects in synaptic morphology and activity-dependent plasticity can lead to problems in the development of the nervous system. It has been shown that Ral GTPase is involved in several biological processes such as the regulation of structural plasticity in the postsynaptic compartment, inducing the subsynaptic reticulum (SSR) growth in an activity-dependent manner. In this work, we wanted to understand if Ral also plays a role in presynaptic structural plasticity, by inducing the formation of new synaptic boutons in response to activity and which pathways are involved in this process. We found that *ral* mutants might have presynaptic structural plasticity defects, and that JNK may be involved through the regulation of microtubules stability. However, given that the two *ral* mutants have different phenotypes regarding structural plasticity, we cannot conclude whether Ral plays a critical role in this process, at the NMJ. Thus, it is possible that there are other molecular players involved in the structural plasticity at the pre-synapse. Because Ral and the exocyst are involved in processes of membrane addition, and since has been shown that Ral action on JNK could be mediated by the exocyst complex (Balakireva et al. 2006), it will be interesting to test this pathway in order to understand the mechanisms involved in synaptic bouton formation.

In parallel we uncovered a novel role for Ral GTPase in the regulation of nerve thickness. It was found that *ral* mutants have thicker and disorganized nerve bundles. Moreover, we detected decreased levels of Fasciclin II, a cell adhesion molecule in *ral* mutants, suggesting that there is a defect in axonal fasciculation. Additionally, we observed that JNK signaling does not seem to be involved in Ral GTPase-dependent nerve thickness regulation. However, we found that glial cells play a role in the regulation of nerve thickness. Unpublished results (Rodrigues & Teodoro) indicate that wrapping glia are underdeveloped in *ral* mutants. Although we showed that JNK signaling does not appear to be involved in the regulation of nerve thickness, other pathways could. One possibility is that Ral somehow regulates Vein, an EGF ligand that has been shown to control axonal wrapping in the Drosophila PNS (Matzat et al. 2015). Thus, it is critical to understand how glial cells regulate nerve thickness and what are the pathways involved in this process.

Chapter 5. References

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