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Licenciada em Biologia Celular e Molecular

Drosophila Models of Neuromuscular Disorders: From Morphology to Behavior

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

Orientador: Doutora Rita O. Teodoro, CEDOC-NMS/UNL

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Centro de estudos de doenças Crónicas

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***Drosophila* Models of Neuromuscular Disorders: From Morphology to Behavior**

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- Poster Presentation at 2018 Annual Portuguese *Drosophila* Meeting entitled " A *Drosophila* model of Pantothenate Kinase associated Neurodegeneration: characterization from morphology to behavior" Ana Cristina Fernandes and Rita O. Teodoro (Tomár, Portugal);
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- RalGTPase regulates the development of wrapping glia via the exocyst

Joana F. Silva-Rodrigues, Cátia F. Patrício-Rodrigues, Ana C. Fernandes#, Pedro M. Augusto#, Vicente Sousa-Xavier#, Beatriz Santos, Ana R. Farinho and Rita O. Teodoro*.

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Abstract

Neurodegenerative disorders are one of the most common illness manifestations worldwide. They have a chronic and progressive display, characterized by synaptic malfunction that then evolve to irreversible neuronal death. The mechanism behind neurodegeneration is not fully understood and can have several associated pathways and genetic contributions. Given that a treatment suitable for all forms of neurodegeneration is hard to get, one idea to overcome this issue is to promote neuronal complexity in the non-affected neurons, to try to delay the progression of these type of disorders. By characterizing the synaptic composition of several examples of *Drosophila melanogaster* models of neurodegenerative diseases, we intended to explore whether there is a specific target that can be targeted to slow neurodegeneration. Using cell type specific RNAi, we induce neuronal knockdown of genes associated with Pantothenate Kinase Associated Neurodegeneration and Hereditary Spastic Paraplegia. Several features of synaptic morphological and composition were addressed at the *Drosophila* NMJ. Also, behavior analyses using several assays with different sensitivities were performed in several stages of the *Drosophila* life cycle. Using neuronal or glial specific RNAi against proteins associated with Amyotrophic Lateral Sclerosis, Spinal Muscular Atrophy, PKAN and HSP, we show that all of these genotypes are associated with impaired locomotor function that seems to be dependent on the proteins that were knockdown in neurons and in wrapping glia cells.

This work contributes to the understanding of the synaptic composition of HSP and PKAN disease models, that can be used to help us target downstream pathways, that will lead to increase structural plasticity as a way to promote neuronal complexity and overcome neurodegeneration. For PKAN, as very little information is available, it contributes to the understanding of the underlying causes of the disorder and to provide insights in which are the synaptic alterations behind patients with mutations in PanK2.

Key-words: NMJ, HSP, PKAN, Neurodegeneration, Neurodegenerative disorders, synaptic activity

Resumo

As doenças neurodegenerativas, que podem originar ataxia ou demência, são doenças incuráveis e debilitantes que têm como consequência a degeneração progressiva seguida de morte neuronal. O mecanismo que provoca a neurodegeneração não é totalmente conhecido, sabendo-se apenas que está associado a múltiplas vias e contribuições genéticas. Dada a complexidade e variedade de mecanismos patológicos, um tratamento eficaz para este tipo de distúrbio é difícil de alcançar. Uma forma de contornar esta dificuldade, é através da promoção da complexidade neuronal de células ainda funcionais como meio de retardamento da progressão da doença. Ao caracterizar a morfologia e composição sináptica de vários modelos de neurodegeneração usando *Drosophila melanogaster*, o objetivo é averiguar se existe um alvo específico comum que possa retardar este sintoma. Para isto, foi utilizado como recurso um knocdown neuronal de genes associados a duas doenças neuromotoras, a Neurodegeneração associada à cinase do Ácido Pantoténico e a Paraplegias Espáticas Hereditárias. Para além disto, análises comportamentais locomotoras utilizando vários ensaios com diferentes sensibilidades foram realizadas em várias fases do ciclo de vida da mosca da fruta, através da utilização de knocdown específico para neurónio e células da glia para modelos de Esclerose Lateral Amiotrófica, Atrofia Muscular Espinhal, PKAN e HSP. Aqui, verificou-se que todos os genótipos estão associados a uma função locomotora diminuída que parece ser dependente das proteínas que foram anuladas nos neurônios e na glia.

Este trabalho contribui para a compreensão da composição sináptica dos modelos de doença acima referidos que poderá ser utilizado como fator determinante para encontrar uma via que contorne a neurodegeneração através do aumento da complexidade neuronal. Para a PKAN, dado que muito pouco se sabe acerca desta doença, este trabalho contribui para a perceção de mais uma via que induz neurodegeneração e quais as alterações sinápticas possivelmente associadas.

Palavras-chave: NMJ, HSP, PKAN, neurodegeneração, doenças neurodegenerativas, atividade sináptica.

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List of Abbreviations

A

ALS – Amyotrophic Lateral Sclerosis
AZ -Active Zones
Acyl-CoA - Acyl Coenzyme A
AD – Alzeimer Disease
AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AR – Autossomal Recessive
AD – Autosomal Dominant
ACh – acetylcholine
ATP – adenosine triphosphate
ATPase - adenosine triphosphatase

B

BRP – Bruchpilot
BBB – blood-brain-barrier
BL – Blomington

C

CNS – Central Nervous System
CoA – Coenzyme A
Cu – copper
Ca - Calcium
COASY – Coenzyme A synthase gene
CP – ceruloplasmin
CPG – Central patter generator

D

DLG – Disc Large
DABCO - 1,4- Diazabicyclo[2.2.2]octane
dsRNA - double-stranded RNA
DNA – Deoxyribonucleic acid

F

FTL – ferritin light chain

G

GluR – Glutamate Receptors
GP – Globus Pallidus
GABA – gamma-Aminobutyrc acid

H

HSP – Hereditary Spastic Paraplegia
HRP – Horseradish Peroxidase
HF – Hereditary Neuroferritinopathy
HD – Huntington Disease

I

IR – Interference RNA
ITB – Intratecal blacofen

K

Kat-p60 – Katanin p60

M

MN – Motor Neuron
mRNA – messenger Ribonucleic Acid
MRI – magnetic resonance imaging
MS – Multiple Sclerosis
ms – milliseconds
MND – Motor Neuron Disorders

N

NMJ – Neuromuscular Junction
NT – Neurotransmitter
NBIA – Neurodegeneration with Brain Iron accumulation
NADPH – nicotinamide adenine dinucleotide phosphate
NOX2 - nicotinamide adenine dinucleotide phosphate oxidase 2
nAChRs – nicotinic acetylcholine receptors

P

PKAN – Pantothenate Kinase associated Neurodegeneration
PanK1 – Pantothenate Kinase 1
PanK1α – Pantothenate Kinase 1 alpha
PanK1β – Pantothenate Kinase 1 beta
PanK2 – Pantothenate Kinase 2
Pank3 – Pantothenate Kinase 3
PanK4 – Pantothenate Kinase 4
PanKs – Pantothenate kinases
PNS – Peripheral Nervous System
PLA2G6 – pantatin-like phospholipase A2
PBS – phosphate buffered saline

R

RNAi – Interference Ribonucleic Acid
RT - room temperature
Rac1 – Ras-related C3 botulinum toxin substrate 1
RT PCR – Reverse Transcription Polymerase Chain Reaction

S

SMA – Spinal Muscular Atrophy
SMN – Survival Motor Neuron
Syt1 – Synaptotagmin 1
Spec – Spectrin
Spas - Spastin
SOD1 – Super Oxide Dismutase 1

SN – Substantia Nigra
SPG – spastic gait
SN – segmental nerves
ISN – intersegmental nerves

T **TN** – transverse nerves

U **UAS -** Upstream Activating Sequence

W **WG** – wrapping glia

V **VNC** – ventral nerve cord

Z **Zn -** Zinc

1 Introduction

Neurological disorders are one of the main concerns of today's society and medical industry worldwide. Many of these disorders are characterized by neurodegeneration that starts with synaptic loss of function that then evolve to irreversible damage, such as neuronal death. Neurodegenerative diseases are among the most common diseases, and yet, probing the pathological mechanism remains a challenge. In most of the cases, by the time patients start with disease manifestations, neuronal death has already occurred, making the development of appropriate treatments that are not chiefly symptomatic is very difficult. Also, as several genetic mutations have been identified as responsible for the pathological conditions behind some of these disorders, establishing a cure for neurodegeneration is difficult but can be guided by the function of the affected genes. In this work, we intended to understand how some mutations associated with neuromotor disorders are involved in synaptic morphology and composition by knocking down pre-synaptically a target related gene, using *Drosophila* larval neuromuscular junction. Flies share with humans many of the molecular mechanisms that govern development and drive cellular and physiological processes. Also, as the study of a phenotype-genotype interaction is relevant to the understanding of how genetic models conserved some biological mechanisms, locomotor behavior will also be analyzed using crawling and climbing features of flies behavior to evaluate the presence, or not, of locomotor impairments.

1.1 The Nervous system

The optimal function of the body is acquired when it can auto regulate and reach stable conditions, a concept called homeostasis. The maintenance of these conditions is a result of the combined action of the endocrine and nervous system that leads to wellbeing and health[68]. Although human beings are largely considered more complex than other species, all animals have fundamental physiological requirements that are fulfilled by genes with common origins that control development, function and organization of the different tissues. This implies that the majority of the physiological functions can be studied in simpler organisms[72], and the nervous system is not an exception.

The nervous system, characteristic of the animal kingdom – with the exception of sponges, placozoan and mesozoan, is the system that monitors and responds to internal

and external changes, coordinating its actions by signaling transmission from and to different parts of the body[30]. Also, this system is responsible for voluntary movement initiation, perception, behavior and memory[68]. All the information responsible for these processes is carried in neural tissue between body regions, but the integration and coordination of the information is made in the brain and spinal cord, for mammals, and in the brain and ventral nerve cord for insects, such as *Drosophila melanogaster* [13].

In humans, there is a strict division of the nervous system, which is represented in figure 1.1. The organs responsible for the information integration are called central nervous system, CNS, and the information carried and all the other neural tissue outside the brain are considered peripheral nervous system, PNS [17].

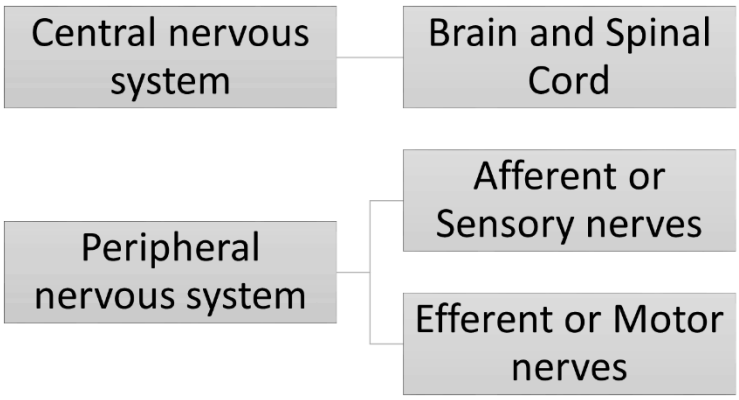


Figure 1.1. Nervous system organization. **A**. Nervous system division according to function.

1.1.1 The cells

The nervous system contains two main types of cells, in both vertebrates and invertebrates, called neurons and glia.

I. Neurons

Neurons or nerve cells are a specialized cell type that is the functional unit of the nervous system [68]. Neurons are postmitotic cells, that do not divide during the lifetime of the organism. Because in animals there is little or no neurogenesis post birth, neurons must survive for an entire lifetime, which, in the case of humans, can be up to around 100 years[21]. These cells are responsible for the reception, analysis, integration, storage and

transmission of the information, and have three main compartments: the cell body or soma, the dendrites and the axons (figure 1.2) [68]. The cell body is usually large and composed by normal eukaryotic organelles. It contains all the essential machinery for synthesis and production of proteins, energy metabolism, Ca^{2+} homeostasis and vesicle trafficking. It also produces mRNA and proteins that will ensure the dendrites, axons and synaptic terminals function. The dendrites are small highly branched outgrowths in tree like ramification that branch extensively from the cell body. Dendrites, are responsible for information reception provided by other neurons or external environment, that is then transmitted to the soma or for the axons. Axons are usually long structures rich in voltage gated sodium channels that are responsible to transmit information to other cells. Synapses locate at the axons in small round shaped structures that houses them, called boutons. These boutons are the place where axons connect with other cells for synapses to occur. These cells can be other neurons or other cell type, for example a muscle cell.

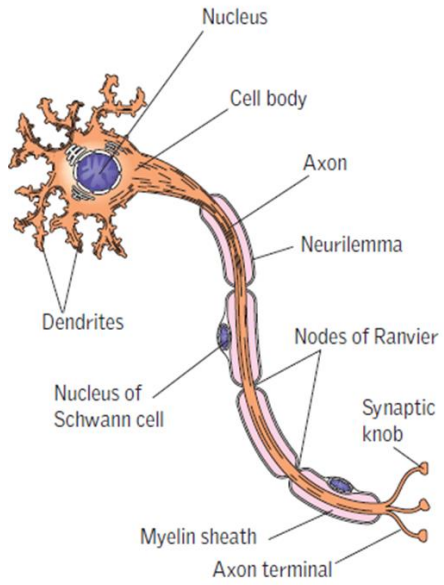


Figure 1.2. Neuron organization. A. Farley et al 2014 [68] image showing the structure of a myelinated neuron.

Although all neurons function in a relatively identical manner, during development neural stem cells, known as neuroblasts, give rise to several different neuronal subtypes that differ in morphology, neurotransmitter, neurotransmitter receptors, polarity, among others (figure1.3).

Functionally, neurons can convert body sensory or physical signals into neural signals that are directed to CNS and be called afferent or sensory. If they transmit neural signals from the CNS to the body they are therefore called efferent or motor neurons. To assure the signaling connection, interneurons connect afferent and efferent neurons.
Structurally, they are classified according to the number of projections extending from the cell body, figure 1.3.

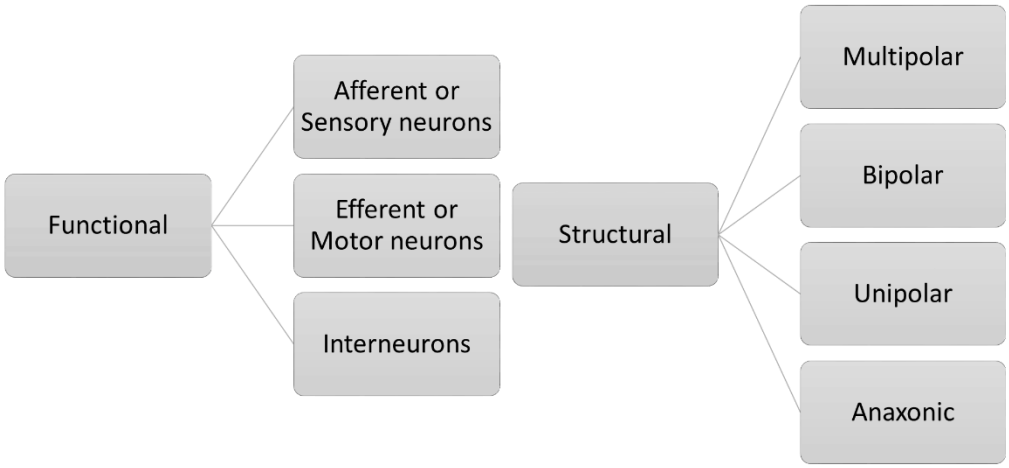


Figure 1.3. **Types of neurons according to functional and structural organization**. First scheme shows the functional neurons division and the second the structural[21].

Neurons can also be classified according to the type of neurotransmitter they produce.

II. Glial cells

Also known as neuroglial, this type of cells was discovered in 1856, as a type of tissue that connected the neurons in the brain. Presently, it is known that glial cells are present in the whole nervous system, being much more than simple support cells and more abundant than that thought at first place, for example, around 90% of the mammalian brain is composed by these cells[23]. Glia cells act as metabolic suppliers, support neuronal transmission and act as defense mechanism against pathogens and dead neurons.
During development, glia are responsible for the modulation of several biological factors of the nervous system, such as neural stem cell proliferation[15], synapse formation and maturation[4], amongst others. When the system is mature, they maintain CNS ionic

balance, re-uptake neurotransmitters after synaptic signaling[24], regulate the blood-brain barrier, BBB[1] and modulate synaptic activity[4,23,69].

Although many aspects of glia biology remains unknown, these cells are emerging as responsible for the pathophysiology of some diseases[4,2,39], that until recent times where attributed to neurons. Some authors even defend that glial cells may be the key to the understanding of complex processes such as intelligence, psychiatric disorders and brain injuries[69].

Glial cells, opposing to most neurons, have the whole machinery to undergo mitosis after nervous system maturation, but it is unclear whether they divide or not during the lifetime of the organism. Proliferation of these cells is known to occur when stroke, trauma or other damage lead to neuronal death. In these conditions, oligodendrocyte precursor cells form glia to repair the injury[2,3,69].

a. Glial cells in Humans

In humans, glial cells are present in the central and peripheral nervous system. In the CNS, there are oligodendrocytes, astrocytes, ependymal cell and microglia, while the PNS has Schwann and satellite cells (figure 1.4).

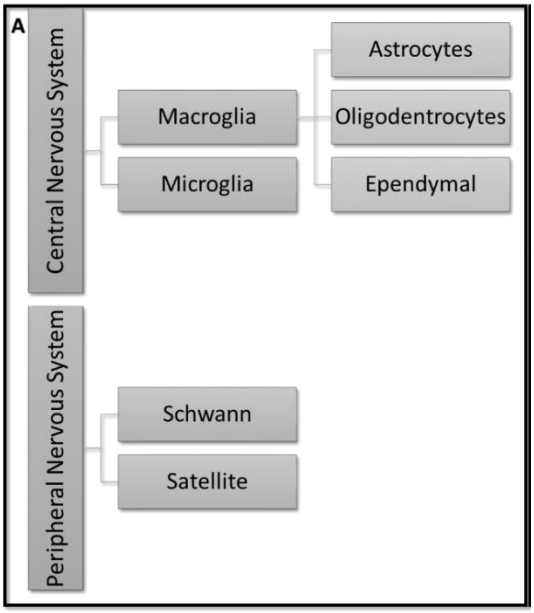


Figure 1.4. Human glial cells. A. Distribution of the glial cells according to the division of the nervous system.

b. Glial cells in *Drosophila*

Some glial functions are conserved between humans and *Drosophila*[39]. Morphologically, three main classes of glial cells can be distinguished, surface associated, cortex associated, and neuropil associated [69]. The surface associated glia is called perineurial and it forms the outer cellular sheet that covers the entire nervous system. The subperineurial (SPG) is underneath the perineurial layer[23,69]. The cortex associated function as metabolic and trophic support for neurons. The neuropil associated are wrapping, ensheathing and astrocyte-like glia. Wrapping glia (WG), is the glia cell type responsible for wrapping axonal profiles within nerve bundles[23]. Together with WG, ensheathing glia is responsible to respond to axonal injury[23]. Astrocyte-like glia, as vertebrate astrocytes, are responsible to clear neurotransmitters by expressing their transporters (figure 1.5)[23].

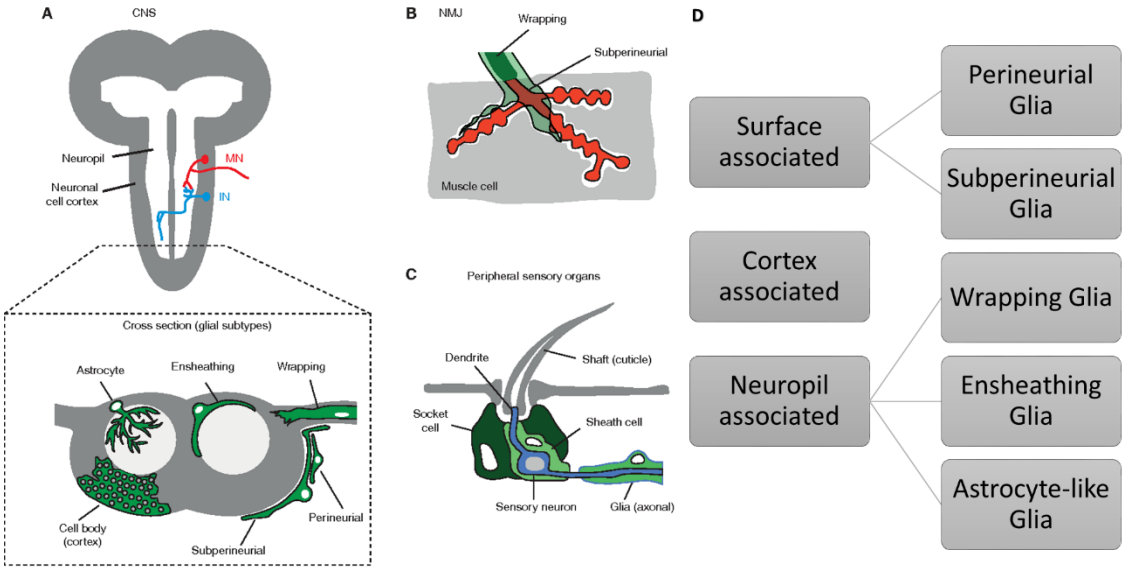


Figure 1.5. *Drosophila* melanogaster glia cells. A. B. C. Distribution of the glial cells according to the division of the nervous system[23]. D. Representation of human cell glia.

1.1.2 Mechanism of neuronal communication – nerve impulse transmission and synaptic activity

To maintain homeostatic conditions, neurons need to communicate between themselves, with other cells and with target tissues to originate specialized responses that are coordinated with the internal and external conditions of the body. For this type of

communication, neurons use specialized structures, called synapses, which allow them to send rapid and precise signals, that initiate the transmition of impulses from one cell to another. This interaction, can be between two neurons or between a neuron and another cell type, called effector. In broad terms, in a synapse, the activity of the presynaptic neuron affects the membrane characteristics of the postsynaptic cell. In the presynaptic cell, an electrical signal travels along the axons until they reach small terminal branches, where depolarization leads to the opening of voltage-gated Ca^{2+} channels[30]. Once the nerve terminal is depolarized, it induces the release of neurotransmitters that are stored in vesicles clustered at the active zones, called synaptic vesicles. These NTs are released and bind to the juxtaposed post-synaptic receptor molecules that open in response to their binding, inducing membrane depolarization and downstream signaling cascades[17,30]. This leads to the inhibition or excitation of the post-synaptic cell that is dependent of the neurotransmitter and the postsynaptic receptors[13,68].

Synapses are initially formed during development by a process called synaptogenesis. During this period, axons migrate towards their specific target, using a specialized structure called the growth cone, which upon reaching its target it suffers morphological changes, forming a functional synapse that can be remodeled [30,45]. This process that is highly regulated, is also dependent on actin and microtubule cytoskeletons that function as key effectors during this process [8,28]. Synaptic morphology and composition are very important factors for homeostasis and proper function of the nervous system. Although synapses suffer structural alterations in response to multiple forms of synaptic activation, intense morphological alterations are associated with neuronal loss, and less with synaptic plasticity. In several neurological disorders, synaptic malfunction and loss leads to neuronal cell death, that then interferes with nervous system communication. Understanding the synaptic constitution and mechanisms behind neuronal loss, can provide insights on how to manipulate synaptic activity to increase neuronal complexity and promote neuronal growth in pathological conditions.

I. Neurotransmitters

Neurotransmitters are endogenous chemicals, mostly small and derived from amino acids that normally have the size of a protein or peptide. When these molecules are released onto the synaptic cleft they can bind to the receptors of the post synaptic cells.

According to the type of neurotransmitter neurons can be classified, being the main ones, glutamatergic – release glutamate, cholinergic – release acetylcholine, GABAergic – release gamma aminobutyric acid, dopaminergic – release dopamine and serotonergic - serotonin.

II. Neuromuscular Junction – a specialized synapse

When a synapse is formed between a neuron and another cell type, is called a neuroeffector junction. A specific case of this is the communication that happens between a neuron, called motor neuron, MN, and a muscle fiber, that is called neuromuscular junction, NMJ. At the NMJ, synapses follow the same general process as described above, releasing, in the vertebrate's case, acetylcholine, ACh. This neurotransmitter binds to ligand-gated ion channels, the nAChRs, that are present on the cell membrane of the muscle fiber, the sarcolemma. This induces muscle depolarization followed by muscle contraction. The NMJ is a very important synapse because muscles need innervation to function properly, maintaining muscle tone and avoiding atrophy.

III. *Drosophila* larval Neuromuscular Junction

In contrast with the vertebrate, the *Drosophila* NMJ uses glutamate as neurotransmitter, being therefore a glutamatergic synapse similar to those at the vertebrates CNS. In the postsynaptic muscle there are, ionotropic glutamate receptors, GluRs, that are homologous to the AMPA-type GluRs present in the mammalian brain. A difference between mammalian and *Drosophila* GluRs and AMPARs is that, while the mammalian receptors are permeable to Na^+, the *Drosophila* counterparts are also permeable to Ca^{2+}[30].

NMJs are large, individually specified, morphologically stereotyped and easy to visualize and record under several experimental techniques such as immunohistochemistry, electron microscopy amongst others[45]. In terms of structure, the larva body wall is simple, having only 32 MN, in each abdominal hemisegment that are arranged in a stereotyped pattern. These motor neurons are individually specified, as well as the muscles they innervate. During embryonic development, MNs extend their axons to a genetically

determined place in the musculature via three different pathways, the segmental, SN, the intersegmental, ISN and transverse, TN, nerves. By the end of *Drosophila* embryonic development, there are already functional NMJs present on each muscle fiber that contain round shaped structures that house synapses, called boutons. These structures, after maturation, contain multiple neurotransmitter releasing sites juxtaposed to GluR clusters. When the bouton is immature, it is called ghost bouton. After embryonic development, larvae musculature grows, and NMJs get more complex. NMJs are also characterized by functional and structural plasticity that are displayed during synaptic development and maturation. Plasticity is a process by which the connection, between a neuron and its partner, is modified in response to neuronal activity. These characteristics together with the stereotyped circuitry, allows the comparation of morphological and functional aspect between larvae.

Synapses are important to maintain sensory, motor and cognitive functions. In cases where synaptic formation and function is altered, degeneration affects the organism. This can be associated with aging, but also with neurodegenerative conditions.

1.2 Neurodegenerative disorders

Characterized by a progressive degeneration of the central and/or peripheral nervous system, neurodegenerative disorders are one of the major afflictions of today's society. These are debilitating and incurable conditions that cause problems with movement, called ataxia, and/or mental functioning. Neurodegeneration normally starts with loss or malfunction of synapses and only after, neuronal cell death occurs. One relevant amount of neurological diseases, that concern investigators and doctors, are the ones associated with motor neurons. These acquired or inherited disorders, called neuromuscular disorders compromise the nervous system connection with the muscular, modifying neurotransmission that usually occurs in a regulated and controlled manner[20,75,78]. These deficits have a variety of origins, but usually result from proteins malfunctioning, leading to several atrophies and loss of quality of life [54,78]. Examples of diseases that affect motor neurons are Amyotrophic lateral sclerosis (ALS)[2], Spinal muscular atrophy (SMA)[11], Hereditary spastic paraplegias (HSP)[43] and Pantothenate kinase associated neurodegeneration (PKAN)[77].

Despite several common features between neurological disorders, the ethyologies are diverse, so we hypothesized that the characterization of the synaptic composition of some of these disease models could help us understand whether there were common features amongst them or not. The information provided by this study, could potentially help the targeting of downstream pathways or activity, that can lead to increase structural plasticity as a way to promote neuronal complexity. As several of these disorders do not have a specific and complete treatment, with this strategy, the aim is to try to delay the progression of these motor neuron disorders, and not to find a specific cure for each of them.

1.2.1 Amyotrophic lateral sclerosis

Amyotrophic Lateral Sclerosis, ALS, is the name given to a group of neurological disorders that are characterized by progressive degeneration and loss of motor neurons. Being the most common motor neuron disorder worldwide, ALS affect around 16 million individuals and is regularly distributed in the population. ALS can be inherited, but around 90% of the cases are sporadic, meaning that there is no described reason for the disease manifestation[54]. The potential risks include age, gender and race, but also several

environmental toxins and nutrition can increase the risk. Familiar ALS is mostly composed by autosomal dominant mutations, but, cases of autosomal recessive are also described [20].

I. Incidence and Causes

One of the most common familiar cases are associated with mutations in the SOD1 gene, known as ALS1[20]. This, that encompasses around 20% of the cases[63], encode a copper-zinc super oxide dismutase 1 that has more than 155 mutations described, being 80 of them pathogenic[61]. These alterations in the gene can be single base substitutions, insertions and deletions or missense and nonsense mutations. Also, mutations in SOD1 are described in 3% of sporadic ALS cases[61].

SOD 1 gene is ubiquitously expressed, and it localizes to the cytoplasm, nucleus, lysosomes and intermembrane space of the mitochondria[61]. It is a gene composed by 5 exons that form a protein with 153 amino acids, called Cu/Zn superoxide dismutase that work as body's defense mechanism against reactive oxygen species toxicity[61].

II. ALS1 mechanism of action

ALS is a neurodegenerative disorder clinically well described, but the cause behind neuronal loss still remains one of the main problems to solve regarding this disease. SOD1 is a metalloprotein that mainly binds to copper and zinc ions forming a homodimer that removes dangerous superoxide radicals by converting them into molecular oxygen and hydrogen peroxide. Also, SOD1 interferes with energy metabolism, protein nitration, phosphate activation, zinc homeostasis and immunomodulation of NO. In normal conditions, SOD1 is expressed in high levels. Although several functions are described for this protein, they are not sufficient to overcome the protein production levels, meaning that, it may well play a role in other homeostatic or even neuron-specific functions[59]. More recently, in 2A undifferentiated neuronal cell lines, using SOD1 human mutations it was proved that this protein stimulates lamellipodial protrusions by Rac1 mediated regulation[19]. SOD1 mutants have been associated with overactivation of NADPH oxidase, NOX2, due to a higher affinity

to Rac1. In these mutants, NOX2 is always in an active form, producing superoxide species, even in oxidizing conditions[19]. Rac1 is a small GTPase from the Rho family that is known to have a major role in the regulation of the actin cytoskeleton within lamellipodia. Lamellipodia is an actin projection present on the leading edge of the cells, that is believed to be critical for cell migration, for neurite formation and collateral branch outgrowth[65]. As the proper function of the nervous system depends on the complex architecture of neuronal networks, and actin projections are known to be involved in sprouting and elongation of axons and dendrites, during neurite formation[19], this can be a possible mechanism of neurodegeneration, in SOD1 mutations present in ALS1 patients.

III. Clinical features and diagnosis

ALS is a deteriorative disorder that affects upper and lower motor neurons, leading to muscle weakness and atrophy. In this scenario, voluntary movements are compromised, and patients lose their ability to perform simple tasks such as eating, moving, speaking and even breathing. This disorder has no specific age of onset, but, in all cases, start with gradual symptoms that are mostly ignored by patients. These symptoms are then intensified, leading to muscle cramps and twitches, tight and stiff muscles, muscular weakness and difficulties in chewing and swallowing. In general, no more specific symptoms appear with the disease progression, only the ones present at the onset become intensified.

Although it is one of the most common motor neuron disorders there is no specific diagnosis for ALS. Mainly this neurodegeneration is diagnosed based on medical history and intense physician observation[55]. After diagnosing, people usually die from respiratory failure in a 3 to 5 years onset, being only 10% described to survive more than 10 years.

IV. Treatments and therapy

Presently, there is no cure for ALS, just specific treatments to help controlling the symptoms and to prevent unnecessary complications. The ability of ALS patients to have an independent life is small and is normally necessary to have major supportive care with several equipment and professionals to increase quality of life and avoid early death. Basically, patients are treated for the symptoms, using several resources. Specific drugs,

such as riduzole[20], that can increase survival by a few months, physical therapy, that enhance muscle strength, speech therapy and nutritional and breathing support, that avoid weight loss and muscle weakness.

Recently, a large amount of work is being performed to try to understand the mechanism behind this devastating disease, to counter balance the degeneration. Also, several stem cell models and biomarkers are being developed to substitute the lost neurons[55].

1.2.2 Hereditary Spastic Paraplegia

Hereditary Spastic Paraplegia, HSP, also known as Hereditary Spastic Paraparesis, familial spastic paraplegia and Strumpell-Lorrain disease, refers to a diverse group of inherited disorders characterized by genetic and clinical heterogeneity, being estimated to range between 1.3 to 9.6 cases per million individuals[16]. This devastating neurological disease is a single gene disorder linked with multiple genetic *loci* that encode proteins associated with several cellular activities. Currently 60 genetic types that are encoded by several proteins are already described[14]. The diseases are distinguished by clinical features, mutated proteins and type of inheritance, being mostly called SPG, for spastic gait. For the inheritance, HSP can be X chromosome linked, autosomal recessive (AR), autosomal dominant (AD) and mitochondrial DNA linked[14].

I. Incidence and causes

Although there are several genes reported to be involved in this upper motor neuron disorder, around 40-50% of all cases are known to be associated with mutations in SPG4 gene (known as SPG4 type) that encodes a protein called Spastin. This mutation leads to upper motor neuron defects with an autosomal dominant inheritance that has a variable age of onset. Spastin is part of the "meiotic" subgroup of the AAA ATPase family, so, it is responsible to catalyse the assembly or disassembly of a variety of proteins related to microtubule dynamics and vesicle trafficking pathways, using energy provided by ATP hydrolyses. These groups of proteins are very diverse but all of them include a conserved 250 amino acid sequence that has ATP binding motifs.

II. Mechanism of action

Regulation of microtubule dynamics is the main function associated with Spastin, which is also known to be colocalized with tubulin. This protein is closely related with other highly studied microtubule severing protein, called Katanin. This last protein is composed by two subunits, being one catalytic (p60) and the other structural (p80)[44]. Although Spastin is not an essential gene, it leads to severe spasticity phenotypes and the mechanism behind its action remains controversial. Katanin was shown, by live imaging, to be a predominant microtubule severing protein in developing neurons, mainly in axonal branch formation where long microtubules of a parent axon need to be severed in order to form mobile pieces that can move into the newly formed branch[76]. More recent studies show that Spastin is also present in neurons, and unlike Katanin, is more specific to this cell type. Spastin and Katanin are conserved among several species[27,41,80], and, in *Drosophila* it was shown that these molecules localize differently during axonal branching. Although they are both responsible to sever microtubules, katanin is enhanced at the site of branch formation by local detachment from the microtubules of proteins that "cover" them. Spastin is locally detached by other proteins that accumulate closer to the branch in formation. This specific localization of these two proteins may be the reason why Spastin mutations lead to neurodegeneration and motor deficits[64] whereas Katanin does not.

III. Clinical features and diagnose

The large clinical variability associated with the several types of HSP, make generalizations very difficult, being only the spastic gait, the lower extremity hyperreflexia and a variable degree of spasticity the common symptoms[43]. This group of disorders are characterized by retrograde degeneration of the longest neurons of the spinal cord, the corticospinal tract and posterior columns, that can be caused by a disruption of a variety of processes such as membrane trafficking, organelle shaping, axon path finding, amongst other[14]. As these neurons are long, their metabolic needs are higher so, they are more likely to be affected.

For the case of mutations in the SPG4 gene, it leads to severe cognitive impairment associated with a highly variable onset that is mostly late.

The diagnosis is made by looking for structural abnormalities in the brain and spinal cord (thinning), that is then confirmed with genetic testing and clinical diagnosis[16].

IV. Treatments and therapy

HSP, has no cure and its progression cannot be slowed down, being the only available therapies a symptomatic medical management based on drugs, such as blacofen, diazepam and botulinum toxin[16].

1.2.3 Spinal Muscular Atrophy

Spinal Muscular Atrophy, SMA, denotes to a group of genetic neurodegenerative disorders that lead to degeneration of the anterior horn cells and result in muscle weakness and atrophy[6].

I. Incidence and causes

The most common form of SMA is inherited autosomal recessive encompassing about 95% of the cases. It can affect 1 in 6000 to 10.000 babies with a carrier frequency of 1 in 40, being the most common cause of infant mortality worldwide. This childhood debilitating disorder is caused by missense, nonsense or splice-site mutations of the survival motor neuron 1 gene, SMN1, leading to the impairment of the neuro-muscular system and progressive muscle weakness by loss or degeneration of motor neurons innervation. Although the disease is always caused by mutations in SMN1 gene, in the human genome, two identical copies of the SMN can be found on each allele, SMN1 and SMN2[10]. These copies, one telomeric and the other centromeric are located close together, what is a good indication of an evolutionary genomic duplication (figure 1.6). They differ in the position 840, having SMN1 a cytosine and SMN2 a thymine. This alteration results in an incomplete exclusion of exon 7 during transcription that leads to 85- 90% of non-functional, truncated and rapidly degraded protein, being that the other 15-10% encode a normal protein. This disorder is associated with a wide range of severity, that although not totally proved, is speculated to be associated with the different levels of SMN2 proteins in patients[37].

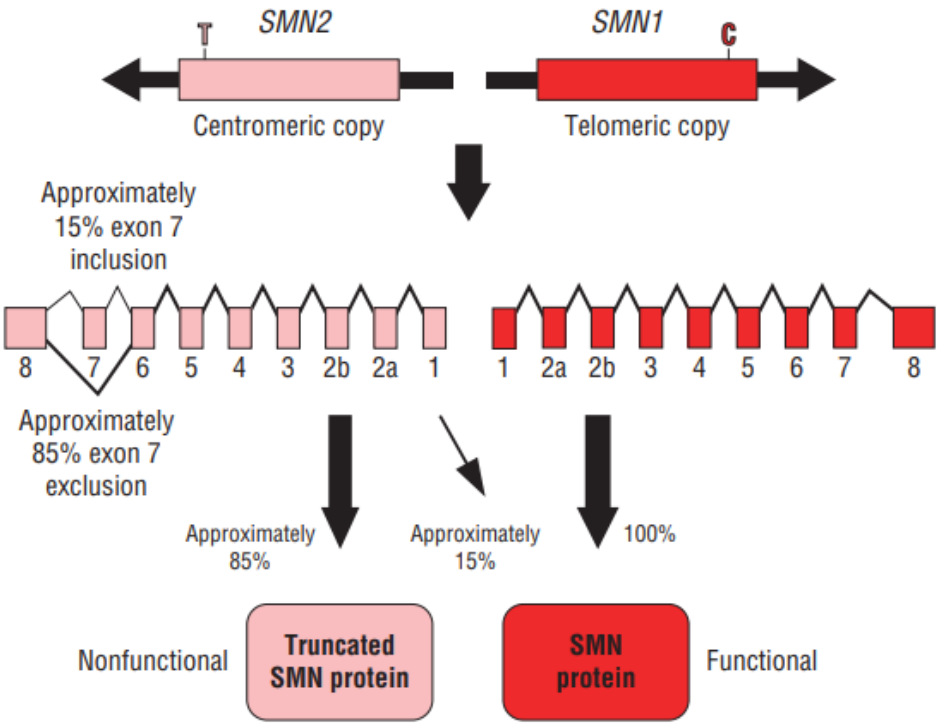


Figure 1.6. Scheme of the SMN human gene adapted from Stephen J. Kolb[37].

II. Mechanism of action

In various organisms, loss of function of the SMN gene leads to embryonic lethality, which indicates its importance for survival. Despite this, the reason why mutations in this gene selectively leads to motor neuron loss remains unknown.

The SMN protein can be found associated with other proteins, forming the SMN complex or by itself in the cell nucleus or cytoplasm. The complex is essential for mRNA biogenesis being composed and associated with nuclear riboproteins and other RNA binding proteins that are involved in the formation and transport of the spliceosomal snRNPs. Furthermore, SMN can be found associated with several proteins that are unrelated to RNA biogenesis. Some authors speculate that this potential function of SMN may be the reason why SMN protein reduction leads to neuromuscular phenotypes, but this is not clearly described. The only difference present in the nervous system cells when compared with the other cell types, is that SMN is present in the axons[38] of motor

neurons[50]. This additional cellular location, can be an indicator of a different and unique function of the protein in motor neurons, but, so far, there is nothing described[66,71].

Although the two copies of the SMN gene are only present in humans and closely related species, the sequence of this gene is highly conserved across species and present in most of the tissues described in humans. Recently, a SMA model of *Drosophila melanogaster* has been described as a powerful tool to the understanding of the mechanism behind this disorder. The *Drosophila* genome has a single homologue of the *smn* gene. In this model, the primary defects are associated with the NMJ[10].

III. Clinical features and diagnosis

According to the age of onset and range of mobility, patients with SMA can be divided into four types, present in table 1.1.

Table 1.1. Types of Spinal Muscular Atrophy and main characteristics.

	Type I – Werding-Hoffmann disease	**Type II – Dubowitz disease**	**Type III – Kugelberg-Welander disease**	**Type IV**
Age of onset	Infant onset (0-6 months)	Intermediate (7 – 18 months)	Juvenile onset (18+ months)	Late adolescent/ adulthood
Life expectancy	Less than 2 years	More than 2 years	Normal	Normal
Highest motor milestone achieved	Unable to sit	Able to sit	Able to walk independently (may lose this ability)	All

In all these types, the clinical record of the patients is diverse, where the type I cannot even do simple tasks such as crying, chewing and swallowing, and the type IV can have a normal life, but associated with tremors, gradual onset of weakness and muscle twitching.

The SMA diagnose is made by a simple genetic blood test where mutation in SMN1 gene is, in positive cases, confirmed. To be submitted to the test, patients should

show infantile impairment of mobility. In cases where the parents have an allele mutated, newborn screening can be performed, although it isn't a standard practice.

IV. Treatment and therapies

Understanding the molecular pathogenesis of the disease has been the main goal of several studies, with the objective of trying to find a specific treatment for SMA. Since 2007, the therapeutics for this disease evolved where clinicians can control some of the most severe problems associated, but no cure is available. Currently, the pharmacological strategy is to counter balance the reduced levels of SMN, by increasing the SMN2 expression, by virus mediated therapies to replace SMN1 gene, and antisense oligonucleotide-based therapies to incorporate exon 7. The development of these approaches is now proceeding at a rapid rate, with human clinical trials using RNA-based and gene therapy approaches taking place. This approach started because in mice models of SMA, it was found that animals with 2 copies of human SMN2 on a null $smn^{-/-}$ background, although with a severe disease phenotype, are viable, whereas mice with 8 copies of SMN2 on the same background do not show any phenotype[50].

1.2.4 Pantothenate Kinase Associated Neurodegeneration

Neurodegeneration with brain iron accumulation, known as NBIA, defines an heterogeneous group of neurodegenerative disorders that are characterize by axonal spheroids, typically restricted to the CNS, and an excess of iron in specific brain areas, mainly globus pallidus, GP, and substantia nigra[47], NG (figure 7). This hallmark of disorders that have a frequency of 1-3 persons per million of individuals, can affect the population from infancy to adulthood, being associated with different rates of progression (fast or slow) and stability, that are directly dependent of the genes mutated on each case. Until now, this group can count with 12 different genes that lead to the same number of disorders, being 20% of the cases unexplored and genetically undefined[46]. Within this 12, only two genes are directly associated with iron homeostasis, namely ferritin light chain, FTL, that leads to Hereditary neuroferritinopathy, HF, and ceruloplasmin, CP, leading to Aceruloplasmonaemia, (figure 1.7). Although the other 10 mutated proteins are involved in different pathways, this large heterogeneity lead to a core of proteins that share or

converge on common metabolic pathways (figure 1.7) for example COASY and PANK2 that are involved in Coenzyme A, CoA biosynthesis. Furthermore, about 33% of the genes involved encode for mitochondrial located proteins, such as PANK2, COASY, PLA2G6 and C19orf12, which can be seen as an indicator of the involvement of this organelle in the pathogenesis of this group of disorders[47].

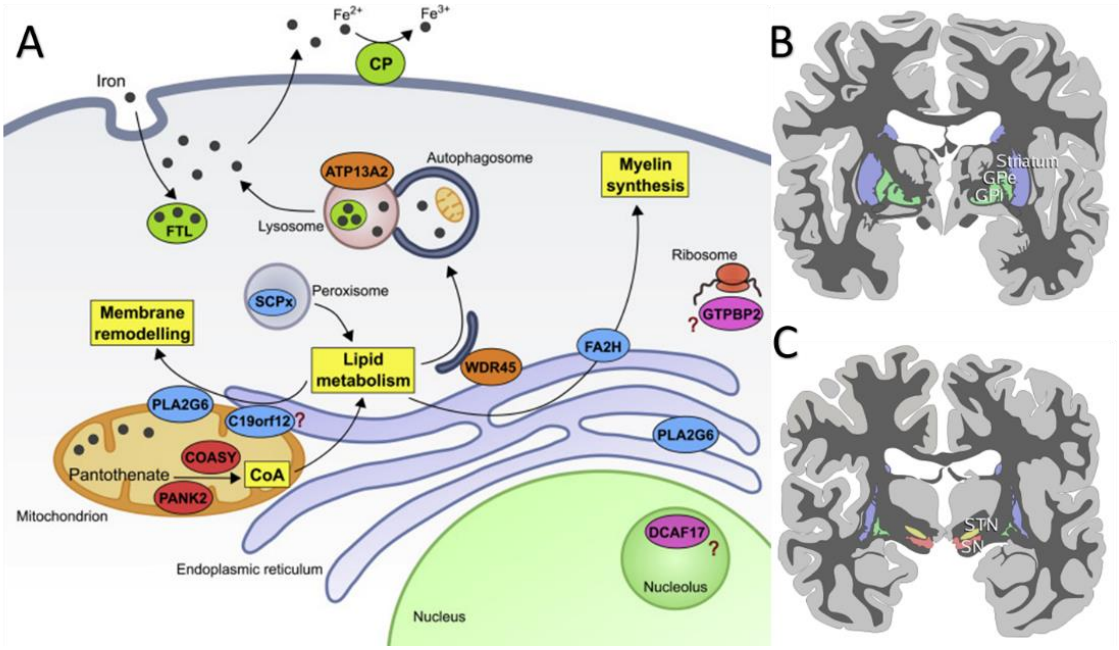


Figure 1.7. Mechanism of NBIA group of progressive disorders. **A.** Adapted from M. D. Ivano et al.[47] Schematic representation of NBIA mutated proteins localization and iron simplified metabolism. **B**. Globus pallidus represented by GPe and GPi were iron accumulates in PKAN**. C**. Substantia Nigra represented by SN where iron accumulates in several NBIA.

I. Incidence and causes

The most common form of NBIA is a neuroaxonal dystrophy associated with CoA biosynthesis, Pantothenate kinase associated neurodegeneration (PKAN), also known as Hallervorden-Spatz Syndrome[29]. Occurring in almost half of the cases of NBIA, PKAN, is an autosomal recessive disorder with a known mutation in PANK2 gene. These mutations, are mostly missense, however there are cases of duplications, deletions, splice-site and exon deletion reported[47]. In humans, there are 4 active isoforms of pantothenate kinase, PanKs, which are coded by 3 different genes, PANK1, PANK2 and PANK3, which govern the CoA biosynthesis of the whole body[26]. These, are key regulatory

enzymes in CoA biosynthesis that are responsible for the catalysis of the first step of this pathway, being regulated by CoA binding. PanK1 and PanK3 are cytosolic protein and PanK2 is specifically expressed in mitochondria. PANK1 gene by alternate initiation exons leads to the formation of PanK1α and PanK1β whose expression is higher in the liver and kidney[26]. These, are the less sensitive feedback inhibition forms of PanK, which is consistent to the fact that the levels of CoA are higher in these two organs that function as glucose providers to the body during fasting. In the case of PanK3, it is known that it is abundantly expressed in the liver and with high ubiquitous expression in the duodenum. PanK2 is ubiquitously expressed in 25 different tissues, being the bone narrow, brain, liver and testis the one's with higher expression levels. This isoform, which is the most regulated, is also the most sensitive to inhibition of CoA[26].

There is also PANK4 gene, but it does not lead to a functional protein, being likely irrelevant to the functional features of the disease.

II. Mechanism of action

PKAN is a neurodegenerative disorder with an unresolved pathophysiology, but the clinical symptoms are well described. The cause behind the degeneration, until now, is not fully understood. Several authors defend that the biggest cause of this disease is the increased oxidative stress, but it is still largely unknown how PanK2 deficits lead to neurodegeneration, being just described that it leads to reduced levels of CoA in the mitochondria[46,47]. As pointed before, this disorder is caused by mutations in PANK2 gene that mostly result in truncated proteins. This, can originate mutations that reduce or maintain enzyme activity or, in more severe cases, enzyme destabilization and degradation can occur[26].

PanK2 enzyme works as a homodimer catalysing the first limiting, energy dependent step in CoA biosynthesis inside the mitochondria. CoA, is a ubiquitous cofactor that participates in several metabolic processes, by the activation of acyl groups, such as lipid and fatty acid synthesis, ketone metabolism, fatty acid degradation and Krebs cycle[47]. Also, the organism concentrations of CoA interfere with several biological processes such as, energy production, cell growth, cell death, signal transduction, protein acetylation, epigenetics and others[47].

Using ATP as a resource, PanK2 converts pantothenate, mostly known as vitamin B5 or pantothenic acid, to 4'-phosphopantothenate, figure 1.8. Furthermore, to form CoA, there are other 4 enzymatic dependent transformations schematized in figure 1.8. These are, 4'-phosphopantothenate condensation with cysteine, decarboxylation, adenosyl group conjugation and phosphorylation[22]. This process is controlled by inhibition and activation of PanK2 by acly-CoA and acylcarnitne, respectively[26].

Additionally, the known accumulation of iron in this disease occurs in a specific brain region -the basal ganglia that is known to be responsible by the regulation of voluntary movement. Although there is not a good explanation for how PanK2 mutants, CoA deficits and neurodegeneration are correlated, the accumulation described above can be an indicator of how the degeneration happens[26,46], leaving only the cause unknown.

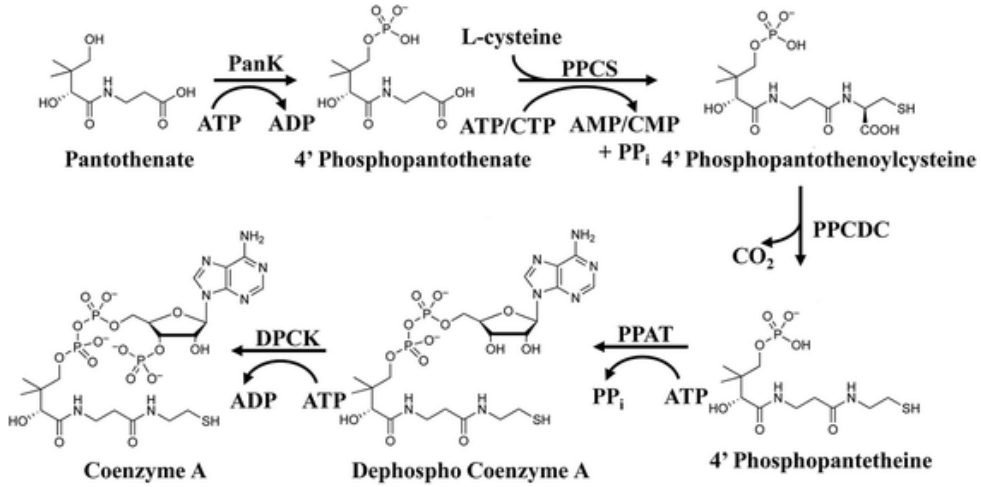


Figure 1.8. **Coenzyme A biosynthetic pathway.** Adapted from F. Sabine et al[22].

III. Clinical features and diagnosis

PKAN, a rare inborn error in metabolism, is a childhood neurodegeneration that leads to dystonia, parkinsonism, dementia and ultimately death[29,46,47]. There are two described forms of PKAN, classical and atypical, being both characterized by iron accumulation[29].

The classical form is more homogenous and with a higher incidence. Is characterized by early onset, around 6 years old, and rapid progression. Death usually occurs from secondary complications, being the most common malnutrition, pneumonia and aspiration. Also, death can occur by uncontrolled episodes of status dystonicus. Patients in early childhood manifest ataxia and postural problems that are then followed

by parkinsonism, dystonia with corticospinal tract signs, dysarthria, spasticity, mental retardation, dementia, optic atrophy and pigmentary retinopathy. Around 10-15 years, developmental delays became visible, first motor and children tend to lose ability to walk and then other developmental delays appear. In general, the disease progression is not uniform, being characterized by periods of stability and others of fast decline[29].

In the case of atypical PKAN, the disease progression and development is more variable, having a later onset, around 13-14 years old, and slower progression, with some known cases of patients that survived to their 30s to 50s. In atypical case, patients do not show any symptom until around the second or third decade of life. Neuropsychiatric signals are the firsts indicators of the disease, such as depression, schizophrenia like psychosis, impulsivity, violent outburst and obsessive-compulsive behaviour. In general, the motor deficits are less severe, but there are cases where motor tics happen in early stages, being that the ambulance loss happens between 15 and 40 years old.

Retinal degeneration deficits, known as retinopathies, are mostly associated with classical PKAN, being present in 68% of the described cases, and only in a minor percentage of atypical PKAN. Despite this, the other 32% of classic and most of the atypical cases have an abnormal electroretinographic exam. Also, intellectual impairments are described in both types of PKAN, being more severe in classical cases[29].

Mostly, PKAN is first diagnosed by brain magnetic resonance imaging, MRI, that will show a pattern of iron accumulation named “eye of the tiger” in GP. If this is identified, a molecular diagnosis is made to test for PANK2 mutations. The presence of a mutation, combining with the symptoms, make the diagnosis certain and complete. In some early cases, by MRI doctors can visualize brain iron accumulation in GP or SN, but not the typical eye of the tiger. Although these cases are mostly idiopathic NBIA, a molecular test for PANK2 gene should be performed in order to dismiss or confirm early onset PKAN[58].

IV. Treatment and therapy

Currently, there is no specific therapy for PKAN. All the treatment options that patients receive when diagnosed, are totally symptomatic, meaning that there is no therapy for the causes, only for the effects. These treatments alleviate the symptoms, but

do not slow disease progression. Example of drugs used for treatment are baclofen and deferiprone[58], that are also used for ALS and HSP patients[16,55].

Intrathecal baclofen, ITB, a GABA receptor agonist, reduces dystonia. This drug, is used since 1991 and is described to be more effective in patients with spasticity. Also, it is known that it improved vocalization, drooling, swallowing and fine motor skills, but there is still the need for more studies to improve dosage[58].

Deferiprone, an iron chelator, is used to minimize brain iron accumulation. This compound can cross the blood-brain barrier, BBB, functioning as a siderophore to iron redistribution, being first used in 2008. Although the dosage still needs to be improved, there were only small motor improvements described and most of them probably associated with neurotoxicity[58].

Nowadays, a novel clinical research is being conducted in United States hospitals, called FORT study. This will submit patients with PKAN to fosmetpantothenate, a compound that can compensate the absence of the PanK2 enzyme in the neurons mitochondria, figure 1.9. This new drug will form an intermediate compound of the CoA synthesis, selected in red in figure1.9, ideally leading to the normal formation of CoA and PanK2 compensation.

An oral fosmetpantotenate will be available for patients that would undergo negligible degradation in the stomach and that a large fraction of the dose would be available for absorption and would possibly interfere with the metabolic pathway.

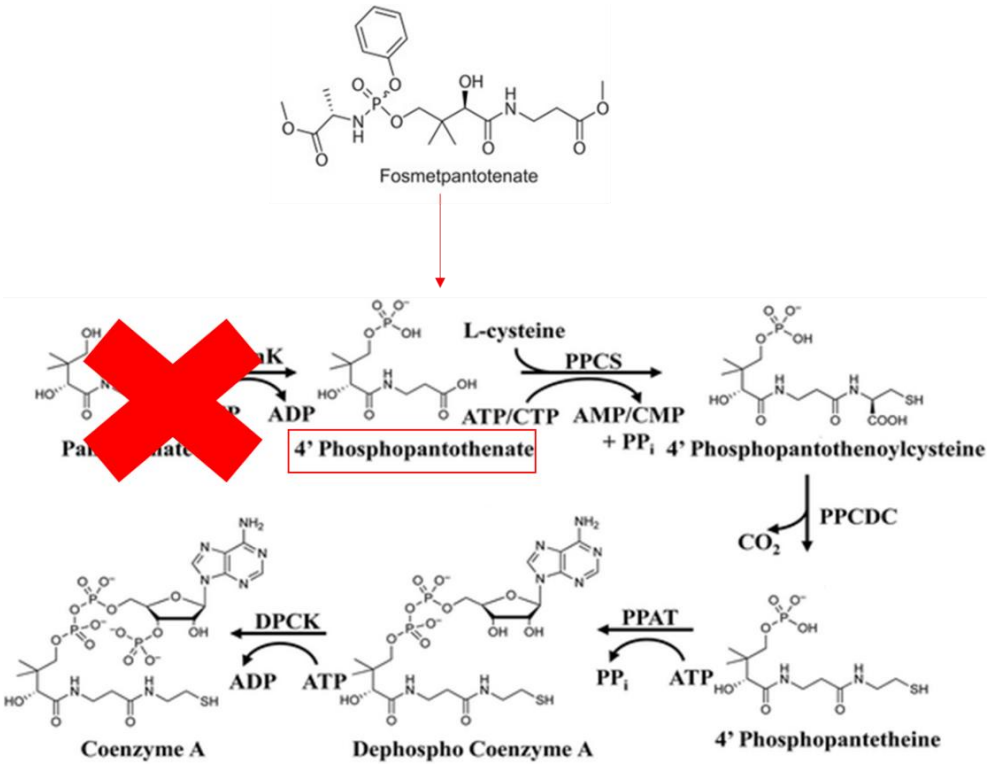


Figure 1.9. Action mechanism of the new drug fosmetpantotenate. Adapted from Sabine [22]

1.3 Commonalities in Motor Neuron disorder

Motor neuron disorders, MND, are associated with a large amount of genetic contributions that varies in the mode of inheritance. Despite this variability, the pathogenesis of these disorders is identical, being all of them conditions that affect motor neurons, leading to muscular disability and ultimately causing neuronal death.

The understanding of the several genetic mechanisms behind motor neuron disorders that all lead to a specific pathophysiology of muscle impairment, will provide important clues about common pathways involved in motor neuron degeneration.

1.4 Locomotion

Locomotion is a complex motor behaviour, that independently of the moving strategy, and that has a big importance in the animal's adaptation to the environment and survival[36]. The variability of neuronal circuits responsible for movement are the main modulators of the locomotor behaviours expressed by animals. Walking is the predominant locomotor behaviour expressed by land animals and, although it remains unknown when the essential neural circuits for this behaviour first appear, it is known that they evolved through the adaptation of a genetic regulatory network[36].

1.4.1 Basal locomotor behaviour

A variety of locomotor outputs is present in the animal kingdom, such as quadrupedal and bipedal walking, running, swimming, flying and gliding[36]. They are essential for the animal to move from one place to another, and to survive to pathological and environmental conditions. Both vertebrates and invertebrates share, comparable neuronal control mechanisms for locomotor rhythm and pattern generation and modulation[25].

Animals, from mammals to insects, move by coordinated contraction of different muscles that are produced by specialized structures called central pattern generators, CPGs. These, are composed by many neurons that exchange information between them and regulate movement. This rhythmic movement requires an intersegmental chain of synaptically connected neurons that alter between excitatory and inhibitory forms[25].

Invertebrate species, such as *Drosophila,* have enabled numerous insights into the neural basis of behavior that are broadly applicable to aspects of neural circuit function in vertebrates[512]. Despite the variety of locomotor outputs, *Drosophila melanogaster* is a widely used model system for the study of development and disease, due to the large degree of homology between human and fly genes[32]. In this work, we will use *Drosophila* as a model to characterize neurodegenerative disorders that are known to be associated with locomotive impairment, using several methods with different sensitivities and dynamic ranges.

1.4.2 *Drosophila* larvae crawling behaviour

Drosophila larvae, is a powerful model system for studying the regulation of neuronal circuits and locomotor impairments associated with genetic alterations.
The fruit fly larvae undergo 3 instar stages where the larval bodies are segmented and composed by identical copies of the same basic neuronal circuits. The larvae body wall is composed by three thoracic segments and eight abdominal segments, having on each hemi-segment about 30 muscles with longitudinal, transverse and oblique orientations[36]. The neural circuits are made up of excitatory and inhibitory neurons that regulate peristaltic movement. Larval crawling consists in waves of muscle contractions that can generate forward or reverse locomotor output. To generate these waves, segments need to relax and contract, being the contraction of one segment, in normal conditions, accompanied by the relaxation of the segment in front. This coordinate behaviour is ensured by interconnected circuits in the ventral nerve cord, VNC, and by a combination of long range inputs from the brain and local sensory feedback[25,35].

Mainly, larvae crawling consists in runs that are types of peristaltic locomotion where abdominal somatic body walls generate muscle contraction from posterior to anterior originating forward crawls and anterior to posterior originating backwards crawls (figure 1.10). However other additional behaviours are described, such as turnings that are asymmetric contractions of thoracic wall musculature, pauses and head sweeps (figure 1.10). Little is known about the identity of neurons dedicated to specific aspects of behaviour, but it is known that distinct motor programs utilize distinct subsets of interneurons.

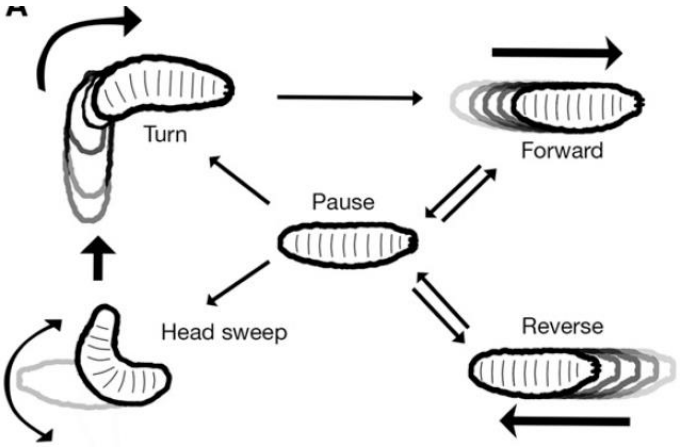


Figure 1.10 Normal *Drosophila* larvae crawling behaviour. Adapted from Clark *et al*[12].

1.4.3 Alterations in normal crawling

As different subset of neurons are responsible for different aspects of behaviour, in pathological conditions, such as neurodegeneration, the loss of function or death of specific subset of neurons can originate different behaviours and not just a slow phenotype. Examples of those are rigid paralysis, immobility, delayed paralysis and dorsal or ventral contraction (figure 1.11)[12].

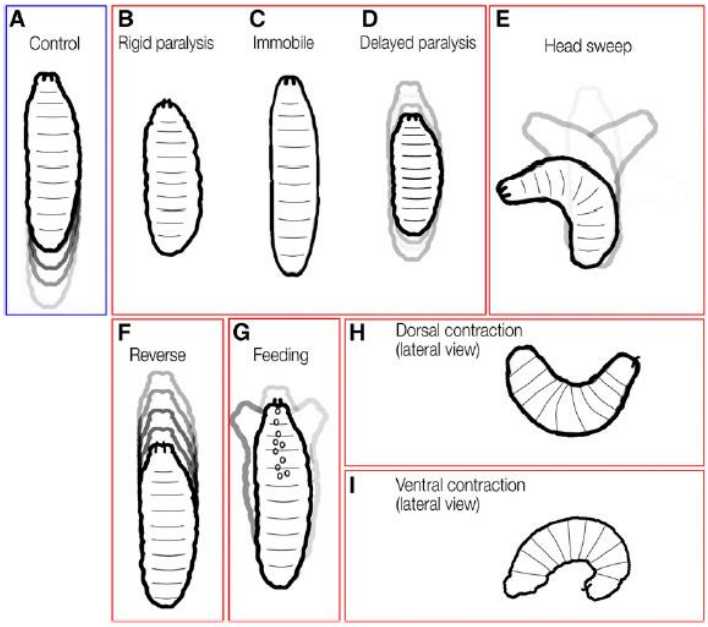


Figure 1.11. Abnormal *Drosophila* larvae behaviour. A, B, C, D, H and **I s**how abnormal behaviour during crawling**. E** shows orientation behaviour. **F** shows reverse crawl. **G** shows feeding. **F** and **G** shows normal larvae behaviour that do not characterize forward crawls. Adapted from Clark *et al*[12].

The characterization of locomotor behaviour is essential to understand the phenotype – genotype interaction, when studies are performed in animal models. Locomotion is a dynamic process that can be influenced by memory alterations, sleep deprivation, drug addiction or even certain types of disorders[73]. In this last case, the locomotor description in animal models of certain phenotypes is a good indicator of the

conservation of the mechanism that can help establish biological processes behind disorders or even neurological alterations.

1.5 *Drosophila melanogaster*: the fruit fly

Since Thomas Morgan, *Drosophila melanogaster* has emerged as one the main animal models in a variety of biological areas. Due to prone characteristics, such as short generation time and easy and inexpensive requirements, the fruit fly is one of the most studied organism in the scientific world.

Given the high conservation with the human genome, *Drosophila* is growing as an animal model for the study of human diseases, having about 70% of human disease genes a counterpart in the fly genome, including complex disorders, such as heart, mental and neurological illnesses. In the latter set of diseases, the NMJ has recently provided new insights into the roles of various proteins in neurodegenerative diseases such as ALS, Multiple Sclerosis, MS, HSP, SMA and Huntington's disease, HD. Therefore, *Drosophila* is a simple and powerful model to study neurodegeneration, given the remarkable similarities in the molecular mechanism behind neuronal function and degeneration.

The *Drosophila* neuromuscular system has a sophisticated motor behaviour while, exhibiting a relatively simple nervous system. *Drosophila* has NMJs that are large, elaborated, individually specified and simple to visualize and record from. The larval NMJ synapses are glutamatergic and are characterized by robust structural and functional plasticity. The NMJ structures are rapidly elaborated during larval development, like is represented in figure 1.12.

Additionally, several synaptic mechanisms are remarkably conserved from action potential generation, all the way through muscle contraction

Therefore, the *Drosophila* larval neuromuscular junction is an ideal system to study neurodegenerative disorders because it allows sophisticated genetic manipulations and numerous experimental techniques[5].

Glial cells, are growing as a potential cell type involved in neurodegeneration. Several authors suggest that the understanding of the glia influence in *Drosophila* can be used as a first step in the understanding of fundamental aspects of glia biology. Recently

it has been suggested that glial cells are involved in the pathogenesis and progression of some neurodegenerative disorders such as ALS[2]. Therefore, understanding the molecular influence of glia, can be a new phase towards the understanding of the mechanism of many of these extensively studied diseases[69].

1.5.1 *Drosophila melanogaster* life cycle

Drosophila has a developmental period that changes with the temperature but that encompasses several stages, embryonic, larval, pupal and adulthood represented in figure 1.12. At 25^0C this cycle takes 10 days, ranging from 7 at 28^0C, 20 at 18^0C to around 50 days at 12^0C. In adult stages males and females are easily distinguished by size, body shape, color and forelegs bristles (figure 1.12). Most of the structures present in the adult are formed from imaginal discs that grow inside the larva that then undergo morphogenetic movement in the pupal stage[33].

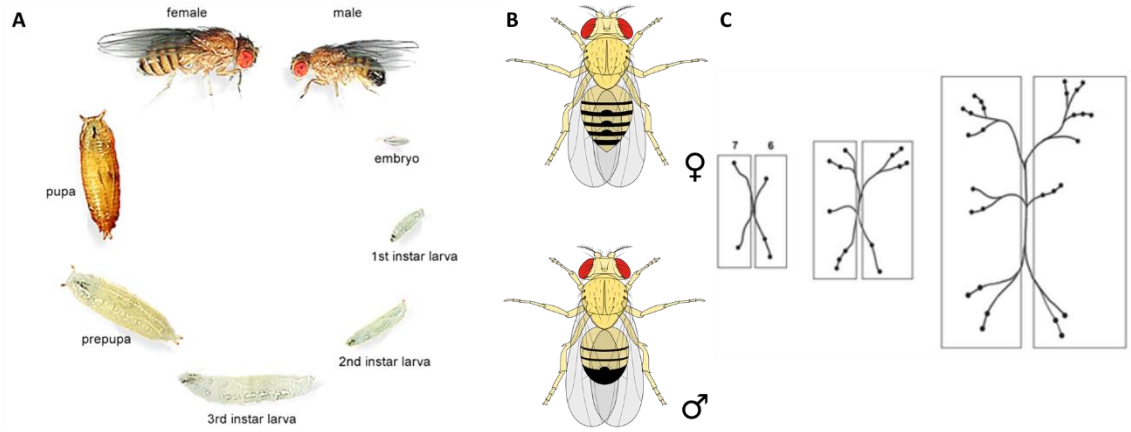


Figure 1.12. ***Drosophila* melanogaster. A.** *Drosophila* life cycle with all stages and description**. B**. *Drosophila* female (top) and males (bottom). **C**. Growth pattern of muscle 6 and 7 *Drosophila* NMJ. As muscle size increase, the number of boutons and branches of the NMJ also increase, adapted from Menon et al 2014[45].

During the past decades, genes that underlie a number of inherited MND have been identified. Clinically, identification of these mutations allows an increasing accuracy in diagnosis as well as a clue in understanding the pathogenic mechanism behind motor neurons degeneration.

In this work, using *Drosophila* NMJ as a model synapse, we will characterize NMJ morphology and composition of HSP and PKAN models of motor neuron disorders. Also, we will do behaviour analyses using *Drosophila* larvae crawling and adult climbing abilities as locomotor outputs of study.

With this work we hope that the study of these genes associated with several modes of inheritance and genetic backgrounds, will provide common clues between these group of neurodegenerative conditions.

2 Materials and Methods

2.1Drosophila melanogaster

2.1.1 Stocks and Husbandry

Drosophila fly stocks were maintained using standard diet at 18°C (apple juice, antifungicide, agarose and yeast).

For our experiments, *Drosophila* female virgins were collected into small vials and kept at room temperature or 18°C and crossed with males, with a consistent amount of flies across experiments to control crowdedness. The crosses were maintained in appropriate humidity conditions at 25°C or 29°C, depending on the experiment. Crosses were made in vials, with standard food, or in apple juice plates supplemented with a mixture of water and yeast (yeast paste). For the optogenetic experiments, trans-retinal were added in a 1:10000 dilution in the water/yeats plates mixture and flies were protected from light with aluminum foil. The stocks used in the work are described in table 1.2.

Table 2.1. **List of *Drosophila* melanogaster stocks with detailed information**.

Fly name	Genotype	Stock Number	Other information
Nsyb/TM6b	W1118; P{XP]nSybd02894/TM6B, Tb1	BL19183	Neuronal Driver
RepoG4	w^{1118}; P{GAL4}repo/TM6B, Tb1	Balanced lab line	Glia Cell Driver
Nrv2G4	W[*]; P{w[+Mc]=nrv2-Gal4.S}3	BL 6800	Wrapping Glia Driver
GcmG4	Y[1] w[*]; P{w[+Mw,hs]=GawB}gcm[Ra87.C]/CyO	BL35541	Total Glia Driver
w^{1118}	W[118]	BL5905	-
Ok6-Gal4	P{GawB}OK6	BL64199	Neuronal Driver
Spas IR	Y[1] v[1]; P{y[+t7.t] v[+t7.8] = TRiP.JF02724}attP2	BL27570	HSP model

Kat-60 IR	Y[1] v[1]; P{y[+t7.t] v[+t7.8] = TRiP.JF03012}attP2	BL28375	HSP model
Chic IR	Y[1] sc[*] v[1]; P{y[+t7.t] v[+t7.8] = TRiP.HMS00550}attP2	BL34523	ALS model
SOD IR	Y[1] sc[*] v[1]; P{y[+t7.t] v[+t7.8] = TRiP.HMS0129}attP2	BL34616	ALS model
SMN IR	Y[1] v[1]; P{y[+t7.t] v[+t7.8] = TRiP.GL00581}attP2	BL36621	SMA model
SMN IR	Y[1] sc[*] v[1]; P{y[+t7.t] v[+t7.8] = TRiP.HMS05688}attP2	BL67950	SMA model
CsChrimson	Y[1] sc[*] v[1]; P{y[+t7.t] w[+mC] = 20XUAS-IVS-CsChrimson.mVenus }attP40	BL55135	Red shifted Channel Rhodopsin
Fbl IR	Y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8] = TRiP.GL00149}attP2	BL35259	PKAN model
Fbl IR	Y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8] = TRiP.HMC05631}attP2	BL64596	PKAN model

2.1.2 ***Drosophila*** **tools**

In the last decades, the number of studies using the fruit fly as a model system has increased mostly because of the great number of possible genetic manipulations. These genetic tools allow a variety of manipulations, with the ones used in this project being described below.

I. UAS-Gal4 system

The UAS-Gal4 is a binary system with two components, the GAL4 gene, that encodes a yeast transcription activator protein, named Gal4, and an upstream activation sequence, called UAS sequence. This is based in a natural system that controls gene expression, where the Gal4 needs to bind to an upstream sequence called UAS, for a certain gene to be expressed. In *Drosophila*, the Gal4 and the UAS sequence are present in separated lines. A line contains the Gal4 and other line with a gene of interest, that is upstream of a UAS sequence. With this, the gene is only expressed when the Gal4 binds to the UAS, meaning that the two lines need to be crossed, figure 2.1. This is extremely advantageous for flies viability and also for cell specific expression.

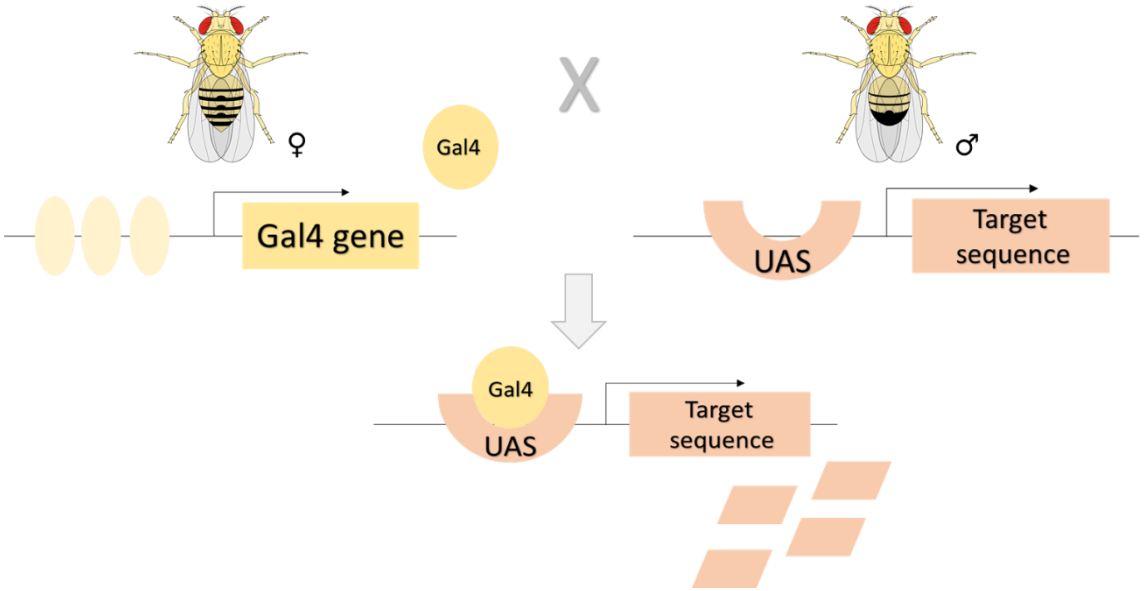


Figure 2.1. UAS-Gal4 System in *Drosophila*. Schematic representation of the UAS-Gal4 system. Gal4 expressed in a driver line leads to the expression of UAS target gene, present in other line when does two are crossed.

II. RNA interference

RNA interference, or RNAi, is a biological process where RNA molecule inhibits the translation of genes by interfering with the mRNA of a given gene. This process has been adapted as a genetic technique, which can be used in *Drosophila melanogaster* to suppress the protein synthesis of a chosen gene, using dsRNA (figure 2.12).

This tool for gene silencing is commonly used in association with the UAS-Gal4 regulation, which allows tissue-specific knock down of proteins, many time bypassing the lethality associated with the absence of some genes.

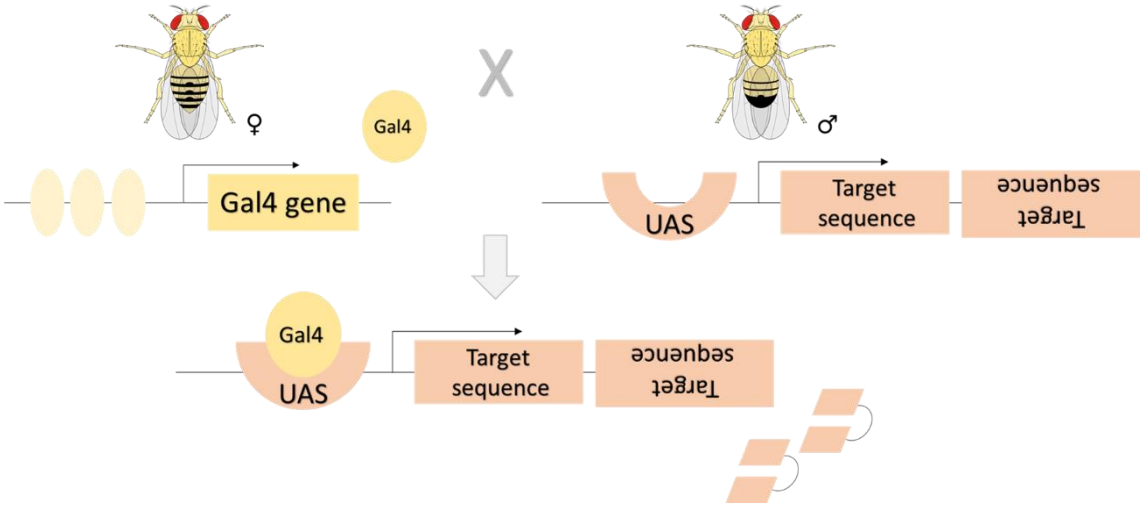


Figure 2.2. *Drosophila* ds-RNAi system. Inductive expression of Double stranded DNA with resource to UAS-Gal4 system used in *Drosophila melanogaster* genetic manipulations.

2.2 Locomotor behavior protocol

2.2.1 Setup

The experimental setup to characterize *Drosophila* larvae behavior is represented in figure 2.3. Five or six third instar larvae were placed in a circular 13.7cm arena coated with 3% Agarose (MultiPurpose Agarose – H091038 – tebu-bio), with a 10% agarose barrier concentrated with salt (normal cooking salt). They acclimatize for around 30 seconds and then are recorded for 300 000ms at 15 frames per second.

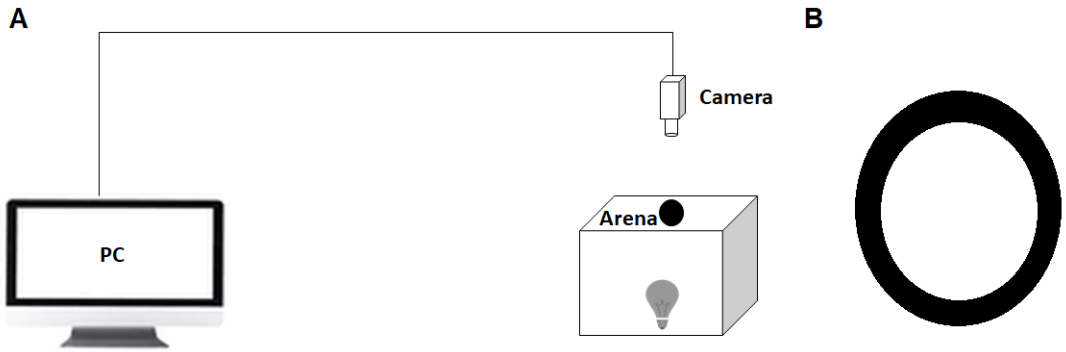


Figure 2.3. Experimental setup for locomotor behavior of *Drosophila* melanogaster larvae in open field arena. A. Open field arena. B. Agarose arena with salt barrier.

2.2.2 Tracking

Following video acquisition, a region of interest in the captured video is selected, eliminating the salt based barrier, and then larvae are track in each frame using ID Tracker. This program is based in differences of contrast, that allows to follow the larvae during the video giving a set of coordinates as final result, which can be used to calculate several parameters to characterize behavior.

I. Analyses and Corrections

With the coordinates from the tracking program, medium velocity, total distance and trajectories were calculated and represented. First, the coordinates in pixels were converted in mm by applying the following formula:

$$Xx \; or \; Yx = \frac{Coordinate \; x \; Arena \; Diameter}{Arena \; Pixels}$$

To calculate the distance, in mm, first, the distance between two points is calculates using:

$$Distance \; between \; two \; points = \sqrt{(X2 - X1)^2 + (Y2 - Y1)^2}$$

Where X and Y represent the coordinates in mm. Then, all the distances between two points are calculated and this value is the total distance of the larvae.

$$Distance \; (mm) = \sum_{1}^{1500} Distance \; between \; two \; points$$

To calculate medium velocity, the distance on each two points calculated by the formula above, is divided by the time between frames. All of these values were added, forming the velocity of the larvae, as described below:

$$Medium\ Velocity\ (mm/sec) = \sum_{1}^{1500} = \frac{\sqrt{(X2 - X1)^2 + (Y2 - Y1)^2}}{0.2}$$

For the trajectories representation, the initial coordinates were plotted as smooth line dispersion graph.

During tracking, larvae are sometimes confused with background, and the program stops following them and the coordinates are substituted by a gap. In cases where the program takes more than 20 seconds to recognize the larvae again, the larvae is ignored as the trajectory and average speed of the larvae would be significantly influencing the final result. Independently of the time that the program takes to recognize larvae again, if the larvae move during the process, the larvae is also ignored for the final result. When the gap happens for less than 20 seconds and the larvae does not move from the place where the program confound them with background until it recognize them again, we substitute these gaps with the last coordinate that the program found.

2.3 Pupation height

2.3.1 Setup

The experimental setup to quantify *Drosophila* pupae behavior is represented at figure 2.4. Six female virgins, collected between one through five days before crossing and kept at 18C, and four males were placed in a 8.5/2.5cm vial and were left for two days in a 29C temperature control incubator. Then, the adult flies were removed to another vial, and vials were maintained at control temperature until pupae appear. When pupae first appear, vials were placed near a ruler, with the zero in the beginning of the food, as described in figure 2.4, and a picture was taken and the distance they climbed annotated. For all the process, the lid of the vials was kept 6cm from the food.

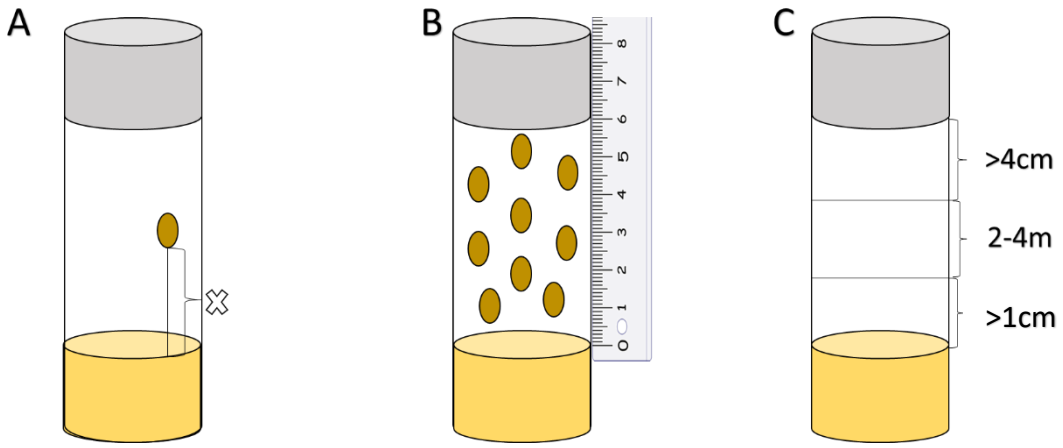


Figure 2.4. Experimental setup for locomotor behavior of *Drosophila* melanogaster pupae in narrow vials. A. Representation of the measure of distance (x) between the pupae and the food. **B.** Schematic representation of the pictures taken from a vial full of pupae and ruler measure. **C.** Vial division.

2.3.2 Behavior evaluation

For all the vials, the pupation hight index was calculated as described below.

$$Pupation\ hight\ index = \frac{Number\ of\ pupae => 3cm - Number\ of\ pupae < 3cm}{Total\ pupae\ number}$$

Also, the flies were separated in three different groups, represented in figure 2.4 C. The first group that climbed until 2cm. The second that climbed between 2 and 4cm and the third that climbed more than 4cm.

2.4 Adult climbing assay – adaptation from Rapid Iterative Negative Geotaxis (RING)

2.4.1 Setup

The experimental setup to quantify *Drosophila* adult behavior is represented in figure 2.5. Adult male and female flies of the genotype of interest, with no more than seven days, were collected from interest crosses and maintained separately at 29C. Two days before behavior analyses, ten flies of each genotype and sex were kept separately at

room temperature in a normal and fresh vial. In the day of the experiment, the flies were transferred to an empty vial, without using anesthesia (CO_2 or ice), and the lid was placed 7cm from the bottom. Each vial is marked with a ruler from the bottom to the top from 0cm to 7cm, in a 1cm intervals.

After 20 minutes of acclimatization, two vials at a time, a control and one experimental, were tapped on the bench surface three times. This tap, is hard enough to knock down all the flies to the bottom of the vial and a timer was set for three seconds simultaneously with the completion of the third tap. When the timer rings, a picture is taken with a camera (figure 2.5). Then, the flies rest for one minute, and the process is repeated six times. If during the tap, not all the flies go to the bottom of the empty vial, they must rest for 1 minute and then the protocol can be repeated for the remaining repetitions until getting to the sixth trial.

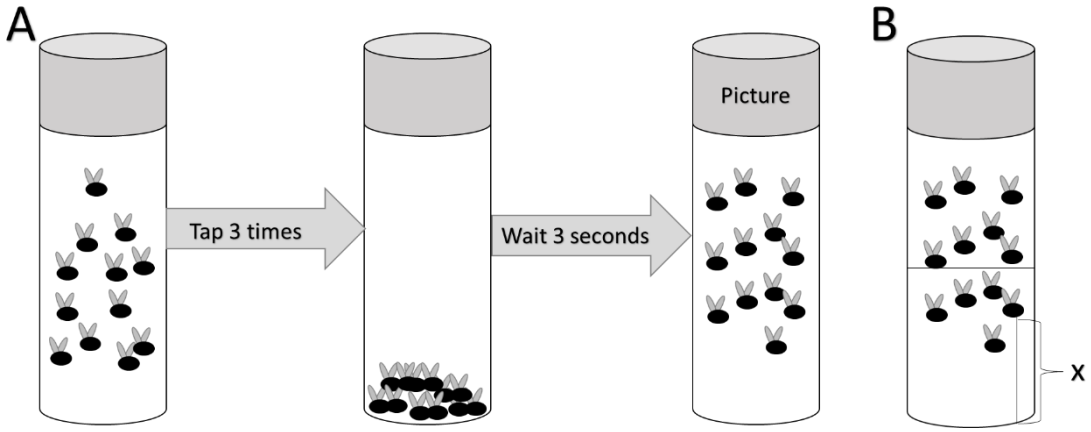


Figure 2.5. Experimental setup for locomotor behavior of *Drosophila* melanogaster adult in vials of 8.5/2.5cm. A. Schematic representation of the several protocol steps with the indication of the picture moment. **B.** Representation of the measure of distance (x) between the adult and the bottom of the vial. Also, the representation of the two groups of flies.

2.4.2 Behavior evaluation

With the pictures taken during the protocol, the number of flies within 0cm-1cm, 1cm-2cm, 2cm-3cm, 3cm-4cm, 4cm-5cm, 5cm-6cm and 6cm-7cm were count and the percentage of each genotype per group is calculated. Also, the flies were distributed in two different group, the one that climb more and less than 4cm.

2.5 Immunohistochemistry protocol

2.5.1 Larval dissection

Third instar larvae of the genotype of interest were dissected in syllgard plates in a PBS 1x solution drop. Using forceps, 0.10 mm insect pins (Austerlitz – FST# 26002-10) were placed in the head and tail of the larvae with the dorsal side up, as represented in figure 2.6. After this, an incision was made using clipper scissors in the larvae posterior part of the body (near posterior spiracles, represented on figure 2.6), creating an opening to make a vertical cut along the dorsal line, in the middle of the tracheas. Then, the organs were all removed and the body was stretch by pinning the larvae 4 times in the left-right and posterior-anterior parts, represented in figure xxx by 3,4,5 and 6.

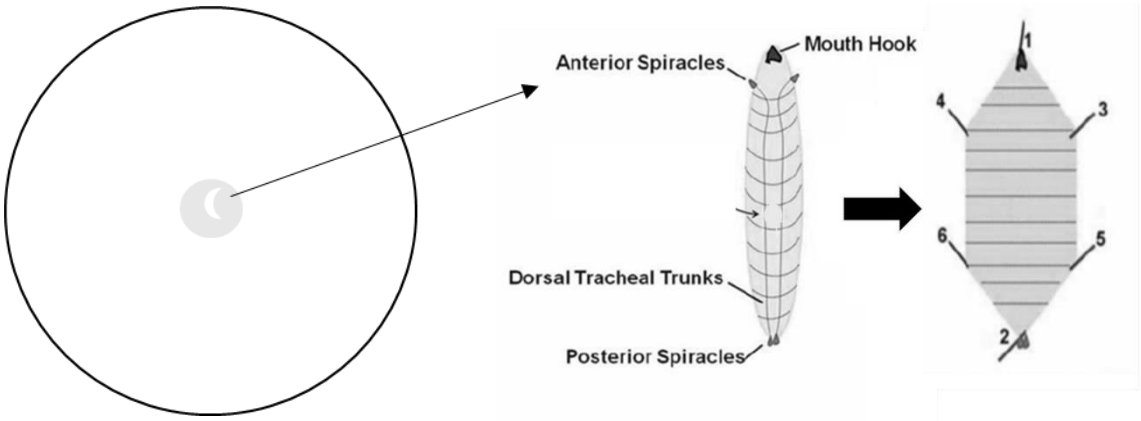


Figure 2.6. Third instar larvae dissection representation. Sylgard petri dish with a larva in a PBS drop. Intact larva in a dorsal position (tracheas up), the right position to start putting the first (1) and second (2) pins. Finish dissected larvae, before incision, cut and with the 6 insect pins.

2.5.2 Fixation

The dissected larvae were fixed with Bouins fixative for 5 minutes at room temperature and the excess was washed with PBS 1X.

2.5.3 Immunofluorescense

The fixed larvae were washed in PBT (PBS 1X with 0.3% Triton-X (Sigma-Aldrich)) for 15 minutes, 3 times, with agitation at Room Temperature. The fillets were

then incubated for 1hour in blocking solution (PBT + 5%normal goat serum- NGS (Life Technologies)), also at Room Temperature. Then larvae were incubated at 4°C, overnight, in blocking solution and the primary antibodies, present in table 2.2.

In the next day, fillets were removed from 4°C and washed 3 times followed by 1h blocking. After this, larvae were incubated in a blocking solution with secondary antibodies for 2hours, with stirring, at room temperature and covered from light. The washing step is repeated 3 times, and the larvae were put in 50% glycerol (inVitrogen) for 10 minutes. Then, larvae were mounted in slides with DABCO media (1,4-Diazabicyclo[2.2.2]octane from Sigma-Aldrich) and the head and tails were removed with a razor blade. Slides were then sealled with nail polish, and stored at 4°C, until imaging.

Table 2.2. **Primary antibodies.**

Antign	**Host**	**Dilution used**	**Supplier/Reference**	**Description/Extra information**
DLG (4F3)	Mousse	1:250	4F3	Discs-Large
SytI	Rabbit	1:100	Provided by Patrick O'Farrel	Synaptotagmin I
GluRIIIC	Rabbit	1:2000	8B4D2	Glutamate Receptors
Brp	Mousse	1:250	Nc82	Bruchpilot
Alpha spectrin	Mousse	1:500	3A9	Alpha Spectrin

Table 2.3. Secondary antibodies.

Antigen	Dilution used	Supplier/Reference	Description/Extra information
HRP -Cy3	1:500	Jackson Immuno Research	Conjugated antibodie - Red
Donkey α Rabbit A488	1:500	Jackson Immuno Research	Green
Donkey α Mousse A647	1:500	Jackson Immuno Research	FarRed
Donkey α Mousse A488	1:500	Jackson Immuno Research	Green
Donkey α Rabbit A647	1:500	Jackson Immuno Research	FarRed

2.6 Optogenetics

2.6.1 Larva preparation

For this assay, yeast paste was supplemented with trans retinal in a 1:1000 dilution. After crossing and matting, *Drosophila* third instar larvae were prepared in a sylgard plates in a HL3.1 solution drop. Using forceps, a 0.10 mm insect pins (Austerlitz – FST# 26002-10) were placed in the head and tail of the larvae with the dorsal side up. After this, an incision was made using clipper scissors in the larvae posterior part of the body, creating an opening to make a vertical cut along the dorsal line, in the middle. Then, the tail pin is removed, and the stimulation can start.

2.6.2 Setup

The experimental setup for Optogenetics is represented in figure 18. The larvae plate, with a 6 led on the top were submitted to the protocol described with the help of an arduin to connect and disconnect light, to a stimulation protocol described in figure 2.7.

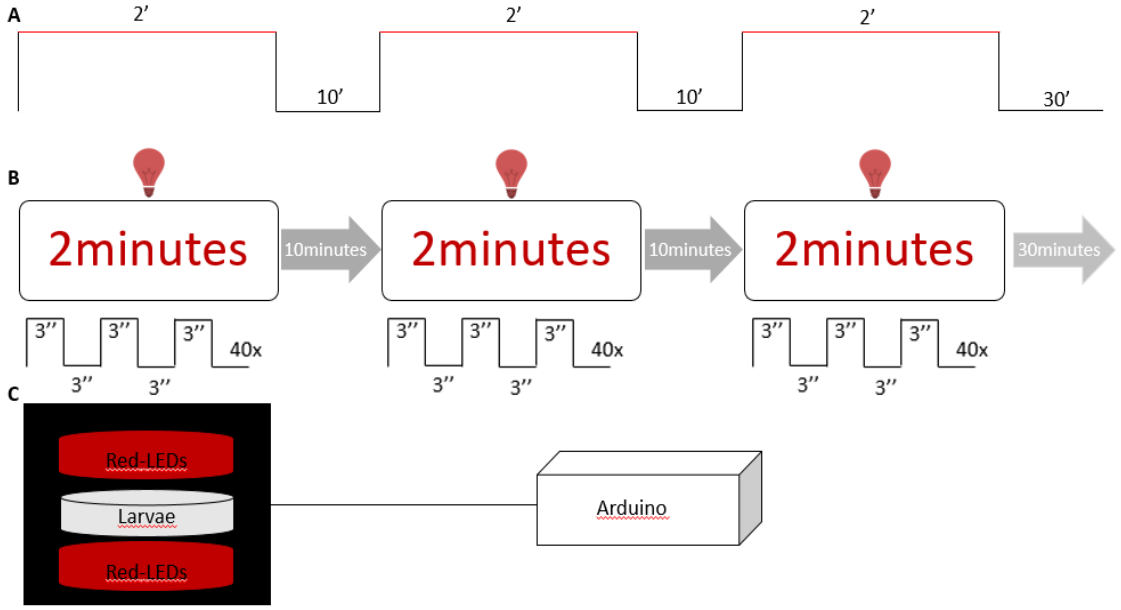


Figure 2.7. Optogenetics protocol based in Ataman et al (2008)[3]. A. Short stimulation protocol showing the light pulse of 2 minutes and resting phases of 10. 30 minutes is the time for dissection. B. Detailed description of the protocol showing the light on/off states in the 2 minutes light pulse. C. Optogenetics setup based in a dark box with two red led and a petri dish for the larvae, connected to an Arduino.

2.6.3 Larval dissection, fixation and Immunofluorescence protocol – Analysis

The larvae were then dissected, fixed and submitted to a immunofluorescence protocol. The antibodies used were the same present in the tables 2.2 and 2.3.

2.7 Image acquisition and analyses

After the Immunocytochemistry protocols, imaging from the fillets was performed using confocal microscopy (Zeiss LSM710 – Carl Zeiss), from the 6/7 muscle,

between A2 to A4 segments, of the *Drosophila* NMJ. For the image analyses and visualization FIJI-ImageJ software was used as a resource.

2.7.1 Immunohischemistry protocol – No stimulation

Using Fiji software as a resource, intensity of the antibodies of interest was calculated from confocal images without saturation. The intensity was then divided by the area of the anti-HRP staining, leading to the intensity per area parameter.

Also using Fiji, the images stained with anti-HRP, anti-DLG and anti-Synaptotagmin, were used to characterize the NMJ with the following parameters: NMJ area, NMJ perimeter, NMJ length, bouton number, NMJ height, branch number, branching points, longest branch length and number of primary branches represented in figure 2.8.

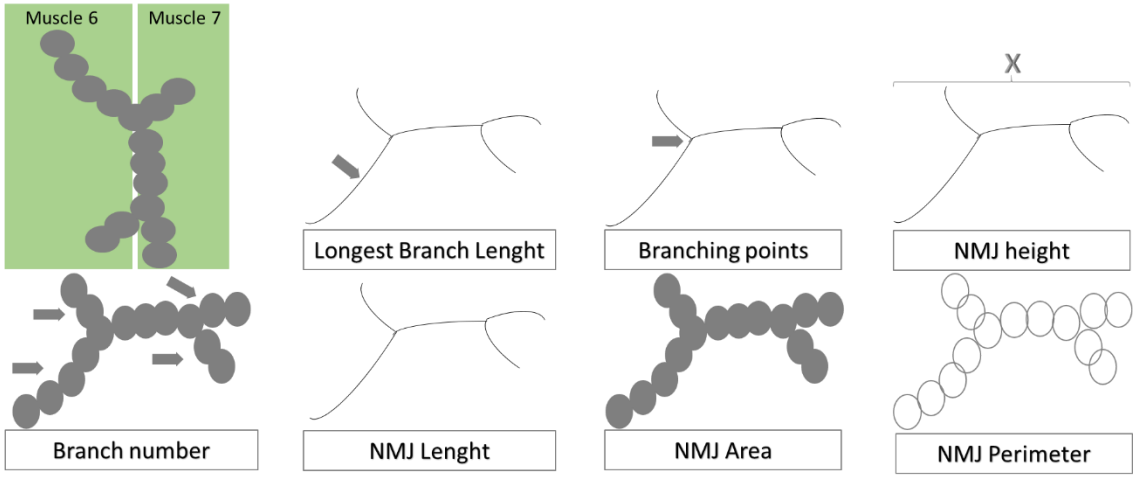


Figure 2.8. ***Drosophila*** **neuromuscular junction characterization parameters**.

2.7.2 Immunohistochemistry with the Optogenetic protocols

Using images from confocal microscopy, newly formed boutons, called ghost boutons identified by the absence of DLG, were counted.

For all experiments, graphs were made using GraphPad Prism 6 and figures were assembled using Photoshop and Microsoft Power Point.

Statistical analyses were made using GraphPad Prism 6. Statistical significance was determined by comparison to controls, using Column analysis One-way ANOVA

(and nonparametric) with multiple comparisons for behavior assays, antibodies intensity per area and bouton number count.

Controls for antibodies intensity were normalized for each experiment using the following linear equation, and values were ranged between 0 and 100.

$$Normalized\ value\ x = \frac{\big(a + (x - A) * (b - a)\big)}{B - A}$$

Where: x - is the intensity value;

a – minimum value of the range =0;

b- maximum value of the range = 100;

A – minimum intensity in the controls;

B – maximum intensity in the controls.

To test normality for the behavior experiments Kolmogorov-Smirov test was performed using Microsoft Excel as a resource tool.

3 Results and Discussion

The goal of this work is to characterize the synaptic morphology and composition of the neuromuscular synapse in several *Drosophila* models of motor neuron diseases. Additionally, by performing diversified behavior analyses, we will test whether each of the MND models have any locomotion phenotype in several life cycle stages.

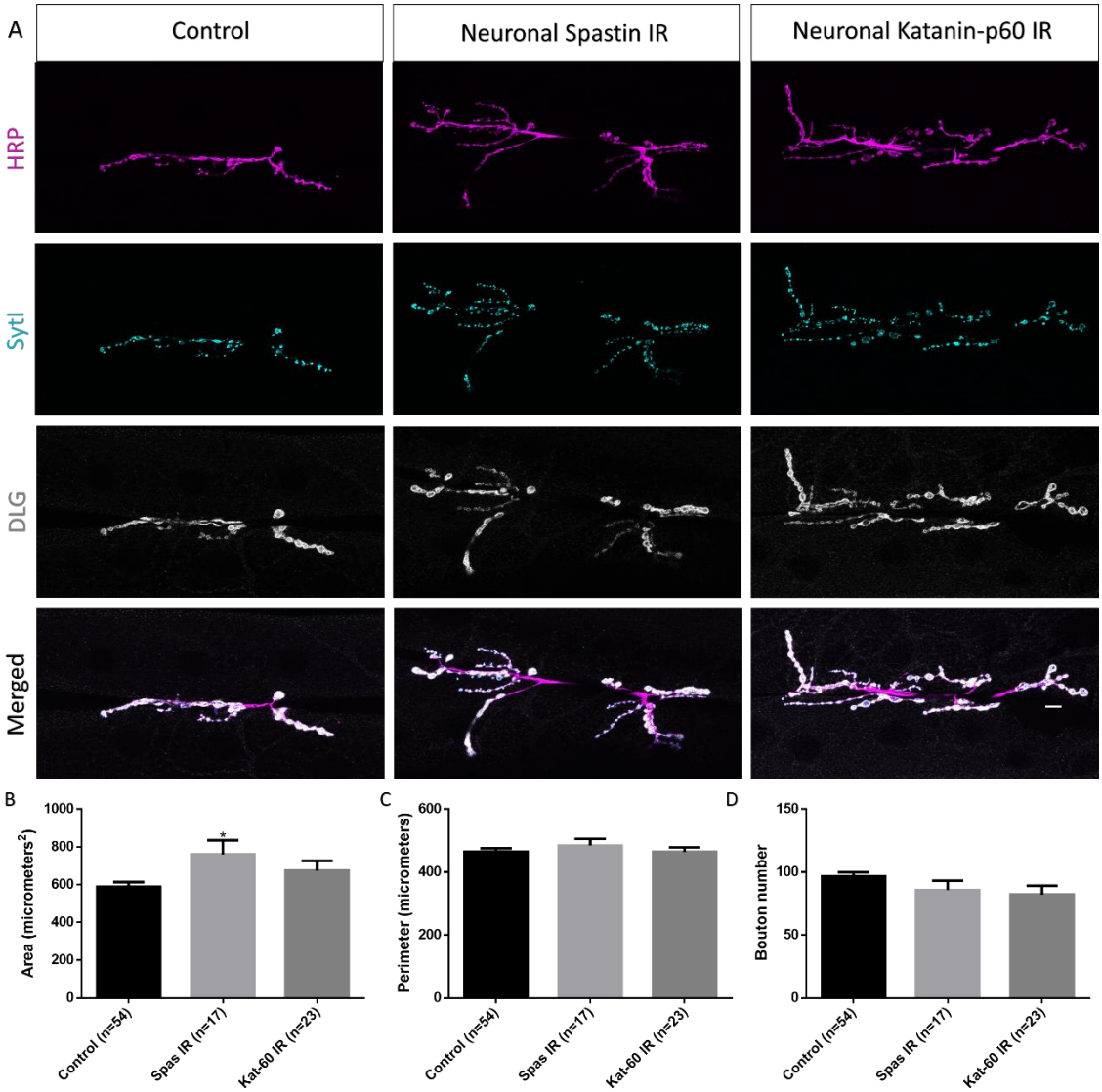
3.1 Hereditary Spastic Paraplegia

Spastin, a gene mutated in HSP, encodes for a protein that is enriched at synapses that is responsible to regulate microtubule stability, to modulate synaptic structure and function and that is required for normal motor function[64]. Katanin, which is a protein with identical function, is known to regulate dynamics of microtubules, dendritic elaboration and neuromuscular junction development[27,44,8052]. Spastin mutations are known to lead to neurodegeneration[43]. In this work we will verify how the morphology and development of third instar larval NMJ is affected in an neuronal knockdown of this protein that is known to be mutated in HSP patients.

3.1.1 Neuromuscular Junction morphology of neuronal *spastin* and *katanin p60* knockdown

Despite the existence of some evidence supporting that the cause behind Spastin mutant neurodegeneration is the involvement of this protein in the regulation of neuronal microtubule stability[52], not much is known regarding morphology. To address the question of whether *Spastin* or *katanin* leads or not to morphological alterations at the synapse, we labeled neurons with HRP, to outline the neuronal membrane, and with antibodies against DLG and Syt1 (figure 3.1) to label the postsynaptic density and synaptic vesicles, respectively. We used a neuronal Gal4 driver and two different RNAi's, one against Spastin and the other against Katanin p60 to reduce the levels of these proteins in neurons. Then we imaged muscle 6/7NMJs, segments A2 and A3 to quantify several morphological parameters. After imaging, area, perimeter, total bouton number, NMJ height, NMJ length, total branch number, branching point number, primary branch number and longest branch length were evaluated (figure 3.1). For the Katanin p60 neuronal KD, when compared with the control, it does not lead to any significant differences in the general morphological features or in the bouton number, as it can be seen in figure 3.1. *Katanin*-60 mutants are described to have increased bouton number in

muscle 4, segment A3 NMJs[42]. As this are whole mutants and we do not have any data showing that the neuronal KD of the IR is complete, we need to quantify protein levels with antibody staining or by RT PCR to take any conclusions. For the neuronal KD of *Spastin* IR, most morphological features remain the same, except the area of the NMJs that seems to be bigger. NMJs with larger areas that do not show alterations in the bouton number, can indicate a structural regulation/modulation problem, what is consistent with the involvement of this protein in synapses. *Spastin* is known to be required for the regulation of the synaptic microtubule network and is proved that loss of function of this protein leads to an increased number of MT, as the ability to sever them is lost[64]. So, the absence of spastin can be an explanation for longer branches that leads to increased NMJ areas.



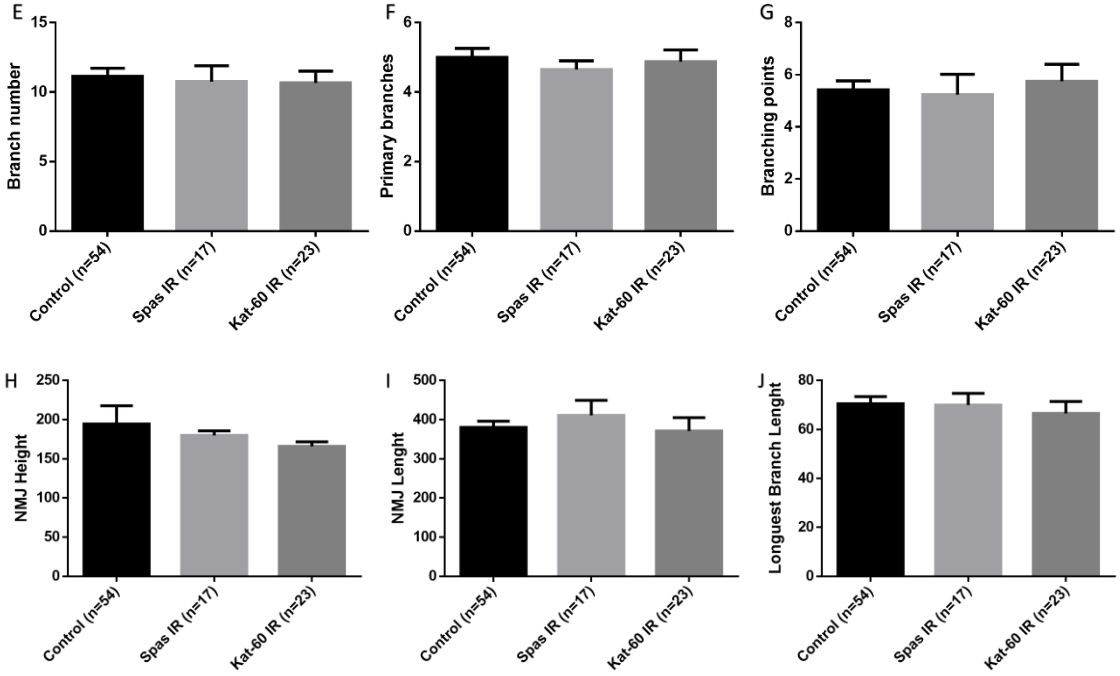


Figure 3.1. **Representative images of *Drosophila* third instar larvae NMJ morphology**. **A.** Confocal images, 40x, larvae fillets labeled with HRP (magenta), DLG (grey) and for Synaptotagmin I (cyan) of control (nSyb-Gal4/+), *spastin* neuronal IR (nSyb-Gal4/Spas IR) and *katanin p-60* neuronal IR (nSyb-Gal4/Kat60-IR). **B.** Total bouton number count. **C.** NMJ area (μm^2). **D.** NMJ perimeter(μm). **E.** NMJ height. **F.** NMJ length **G**. Total branch number. **H**. Branching point number. **I**. Primary branch number. **J.** longest branch length. Scale bar 10 μm. Error bar represent mean with s.e.m..

3.1.2 Musculature of neuronal *spastin* and *katanin* knockdown

To address the question of whether the musculature is altered in the larvae body wall, we labeled third instar larvae fillets with phalloidin, for the same two IRs against Spastin and Katanin p60 and quantified the muscle area per segment (figure 3.2) to understand if there is any muscle size influence in the previous results since it is known that an increase in muscle surface is accompanied by a synaptic increase[49]. When we compared muscle area of each muscle segment with the control, as we can see in figure 3.2, for Spastin, there are no significant alterations. This is a good indicator that the morphological changes seen at the NMJs are caused by the reduction of spastin in neurons and not related with larvae growth deficits. However, protein quantification or RT PCR

should be performed to ensure results. For Katanin-60 neuronal IR, in the segment 3, significantly differences were observed. Although there is a statistically significant difference in the muscle area, the whole NMJ morphology does not show any underdevelopment. To be sure about this reduction the number of muscles quantified need to be increased in order to take proper conclusions. If muscle area is reduced upon neuronal KD of spastin, this would suggest a non-cell autonomous role of this protein.

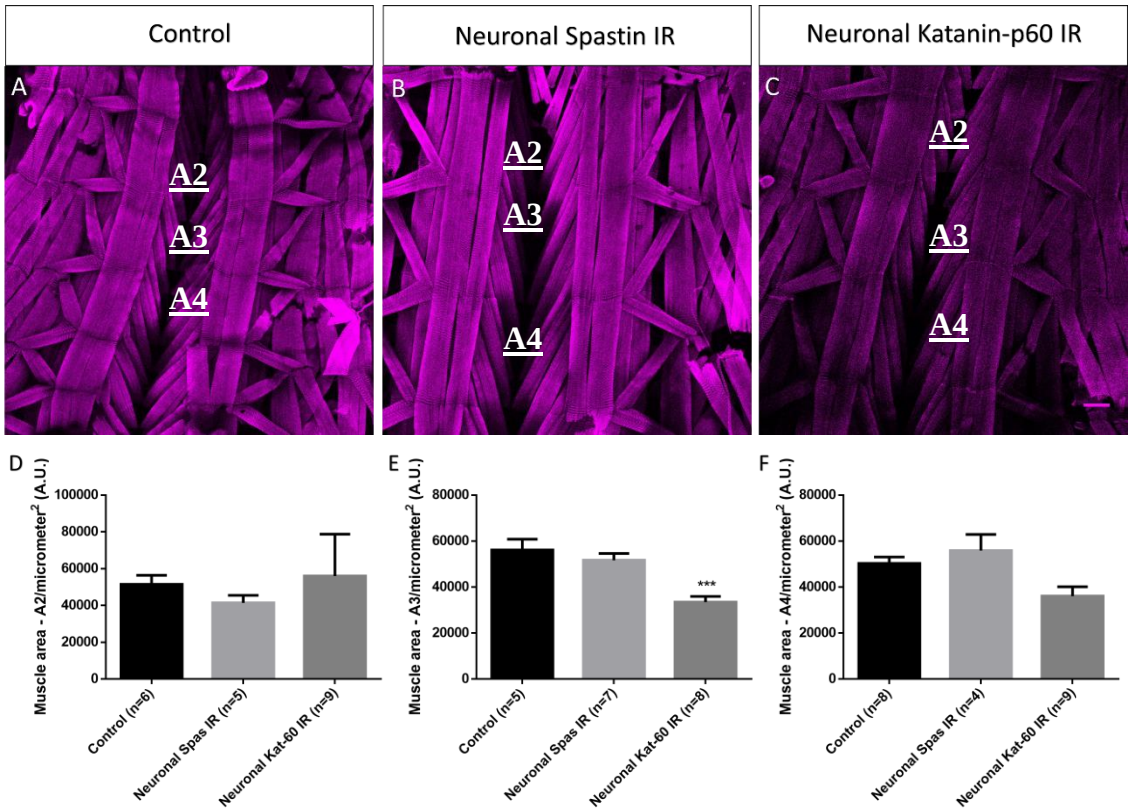


Figure 3.2. *Drosophila* third instar larvae body walls of neuronal *spastin* and *katanin* knockdown are normal. Internal view of third instar *Drosophila* fillets, labeled with phalloidin, at low magnification (10 x objective) in control, **A,** (nSyb-Gal4/+), **B** *spastin* neuronal IR (nSyb-Gal4/Spas IR) and **C** *katanin p-60* neuronal IR (nSyb-Gal4/Kat60-IR). Muscle area quantification of segment A2 **(D)**, A3 **(E)** and A4 **(F).** Scale bar 100 µm. Error bar represent mean with s.e.m..

3.1.3 NMJ synaptic composition of neuronal *spastin* and *katanin* knockdown

- ***Drosophila* alpha-spectrin intensity in neuronal KD of *spastin* and *katanin*.**

Spectrins are part of the cytoskeleton as microtubules networks, and are plasma membrane-associated cytoskeletal proteins implicated in several aspects of synaptic development and function, including presynaptic vesicle tethering and postsynaptic receptor aggregation[18]. To evaluate if S*pastin* and *katanin* IR reduction induces altered levels of alpha-spectrin, we stained larvae fillets with HRP and anti alpha-spectrin and muscle 6 /7 NMJ were imaged between segment A2 through A4 (figure 3.3) for the same two RNAis used in the morphology analysis. The intensity associated to alpha-spectrin remains unaltered for spastin, when compared with the controls (figure 3.3), suggesting that the protein is identical in both cases. For katanin-60 neuronal IR, spectrin levels are highly reduced. This can be the reason behind the synaptic deficits seen in the kat-60 IR, as spectrins are described to lead to neurotransmission reduction.

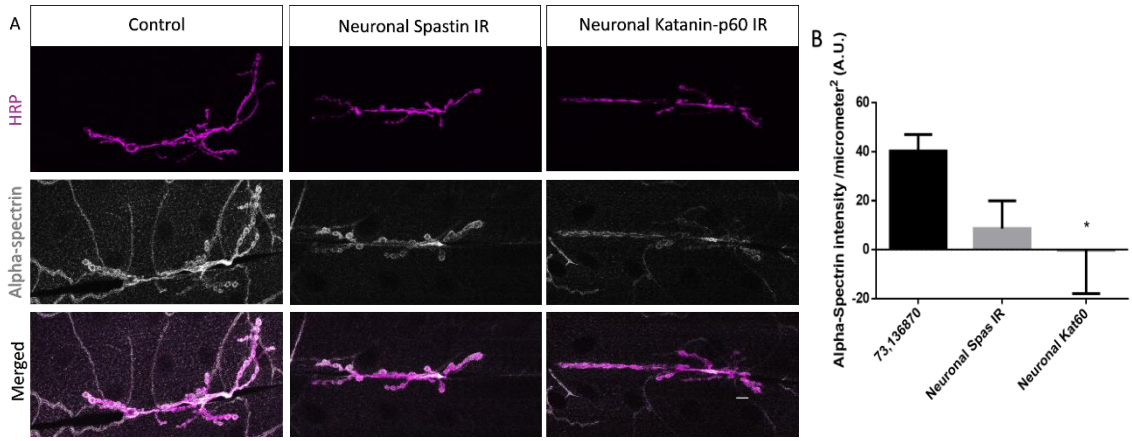


Figure 3.3. Reducing katanin in neurons leads to alterations in alpha spectrin intensity. A. Confocal images of *Drosophila* larvae fillets labeled with HRP (Magenta), alpha-spectrins (grey) of control (nSyb-Gal4/+), *spastin* neuronal (nSyb-Gal4/Spas-IR) and *katanin-p60* neuronal IR (nSyb-Gal4/Kat6 IR). **B.** Quantification of alpha-spectrin intensity per area. Scale bar 10um. Error bar represent mean with s.e.m..

- **Neuronal *Spastin* and *katanin* KD lead to decrease intensity of active zones**

The main component of active zones is Bruchpilot protein (Brp). To see if this protein is altered in the *Spastin* and *katanin-p60* knockdown, muscle 6 /7 NMJ segment A2 through A4 were imaged after staining with HRP, BRP and also for postsynaptic glutamate receptors (figure 3.4) of the same two RNAi's against *Spastin* and *katanin*. The quantification of Brp intensity per NMJ area shows a significant decrease, in both IRs, when compared with the controls. For katanin, results above show a reduction in spectrins, that is described to lead to neurotransmission impairment. This decrease in active zone intensity is in agreement with impairments in the neurotransmission, as this are the places where neurotransmitters are released. Regarding Spastin, this decrease in intensity is also concomitant with the alterations described for spastin mutants that are known to modulate synaptic structure[52].

- **Neuronal *Spastin* and *katanin* KD influence in glutamate receptors in the post synapse**

To address if Glutamate receptors (GluR) are altered, muscle 6 /7 NMJ segment A2 through A4 were imaged after fillets stained with HRP, BRP and GluR. When compared with controls, *Katanin* does not show any alteration while *spastin* shows a reduction. Given that the IR is neuronal and the GluRs are postsynaptic, this result implies that reduced spastin in the presynapse affects the postsynaptic receptor composition in a non cell-autonomous manner, possibly to match the low levels of BRP.

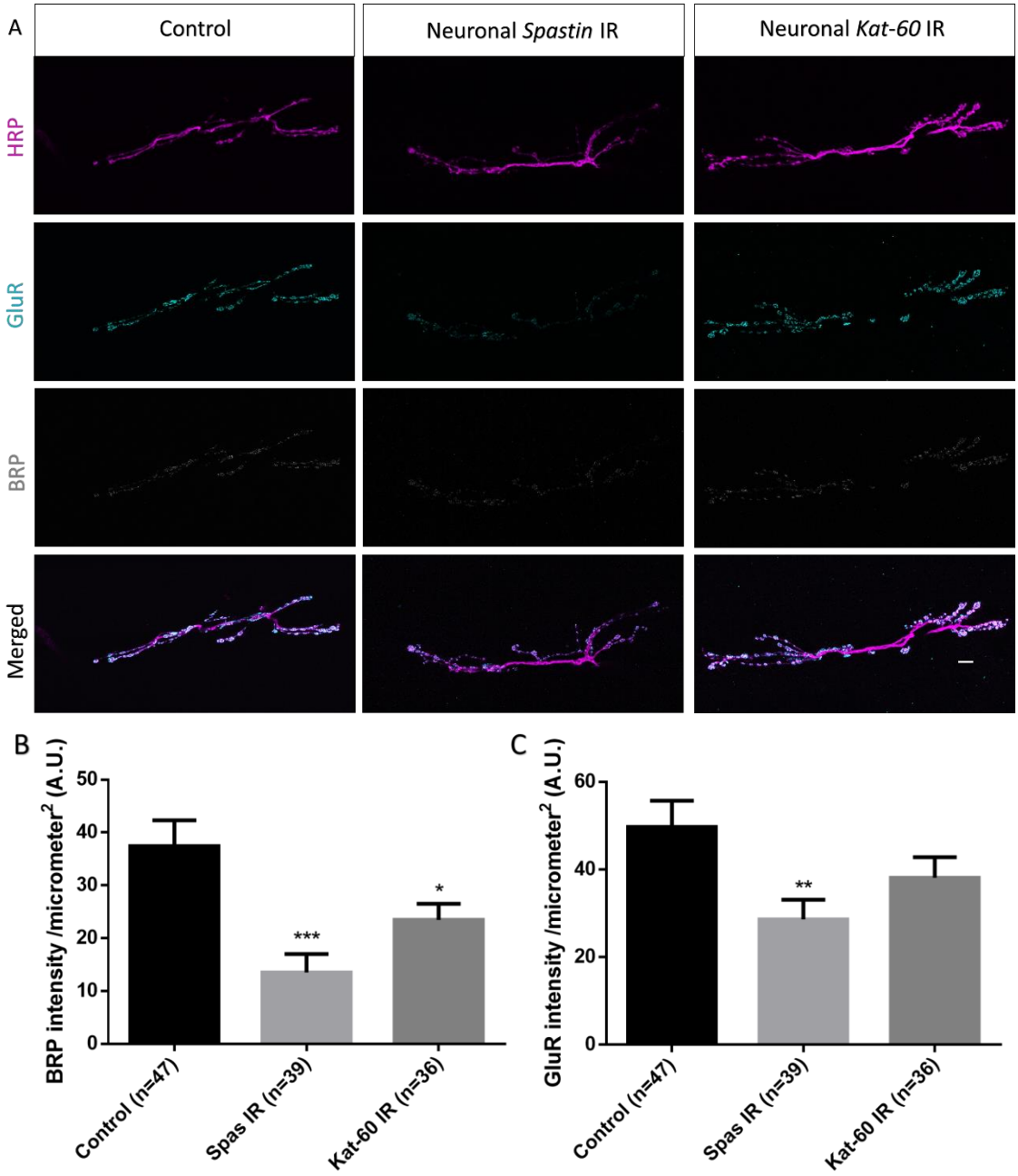


Figure 3.4. Influence of reducing *spastin* and *katanin* in neurons. A. Confocal images of *Drosophila* larvae fillets labeled with HRP (Magenta), BRP (grey) and Glutamate receptors (cyan) of control (nSyb-Gal4/+), *spastin* neuronal IR (nSyb-Gal4/Spas IR) and *katanin p-60* neuronal IR (nSyb-Gal4/Kat60-IR). **B.** Quantification of Brp intensity per area **C.** Quantification of glutamate receptors intensity per area. Scale bar 10um. Error bar represent mean with s.e.m..

- **Neuronal *Spastin* and *katanin* KD influence in synaptic vesicles intensity**

Synaptic vesicles are responsible to store neurotransmitters and are essential for the information propagation between cells. Results above show alterations in the active zones, where the main component of these structures is reduced for both IRs. Using the same NMJs from morphology analysis, intensity of synaptic vesicles was measured. When compared with the controls, *Katanin-60* neuronal IR does not show any alterations. For the *Spastin* IR, the intensity associated with the synaptic vesicles is increased, when compared with the controls. This data shows that in spastin neuronal knockdown, the intensity of synaptic vesicles, that store NTs is increased, whether, previous results show that Bruchpilot, the active zones main component intensity is diminished. This clearly show us that the synaptic machinery is altered in composition, however, the origin of those alterations is unclear. To be certain, results need to be complemented with experiments that shows the RNAi efficiency, and, in a positive case, with mutants.

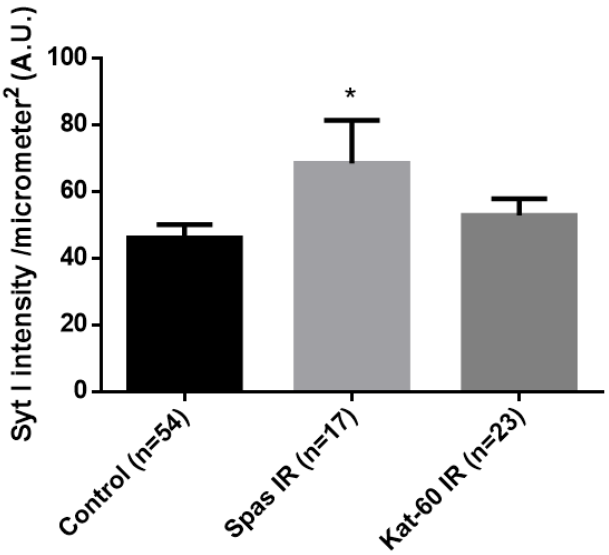


Figure 3.5. Quantification of Syt I intensity per area. Quantification of images of *Drosophila* larvae fillets labeled with HRP (Magenta), DLG (grey) and Syt1 (cyan) of control (nSyb-Gal4/+), *spastin* neuronal IR (nSyb-Gal4/Spas IR) and *katanin p-60* neuronal IR (nSyb-Gal4/Kat60-IR). Error bar represent mean with s.e.m..

3.2 Pantothenate kinase associated neurodegeneration

Fumble flies, a recently established model for PKAN, are known to have lower levels of acetylation of several proteins, such as histones and tubulin[67]. In S2 *Drosophila* cells, *fumble* mutants lead to reduced levels of CoA, that can be rescued by interfering with the CoA biosynthesis[67]. Despite this, the cause of neurodegeneration remains unknown. Because PKAN is a disease with neuronal dysfunction and loss it is important to understand how the morphology and development of larval NMJ is affected in the mutants, in order to determine how/if synapses are altered in the disease background conditions.

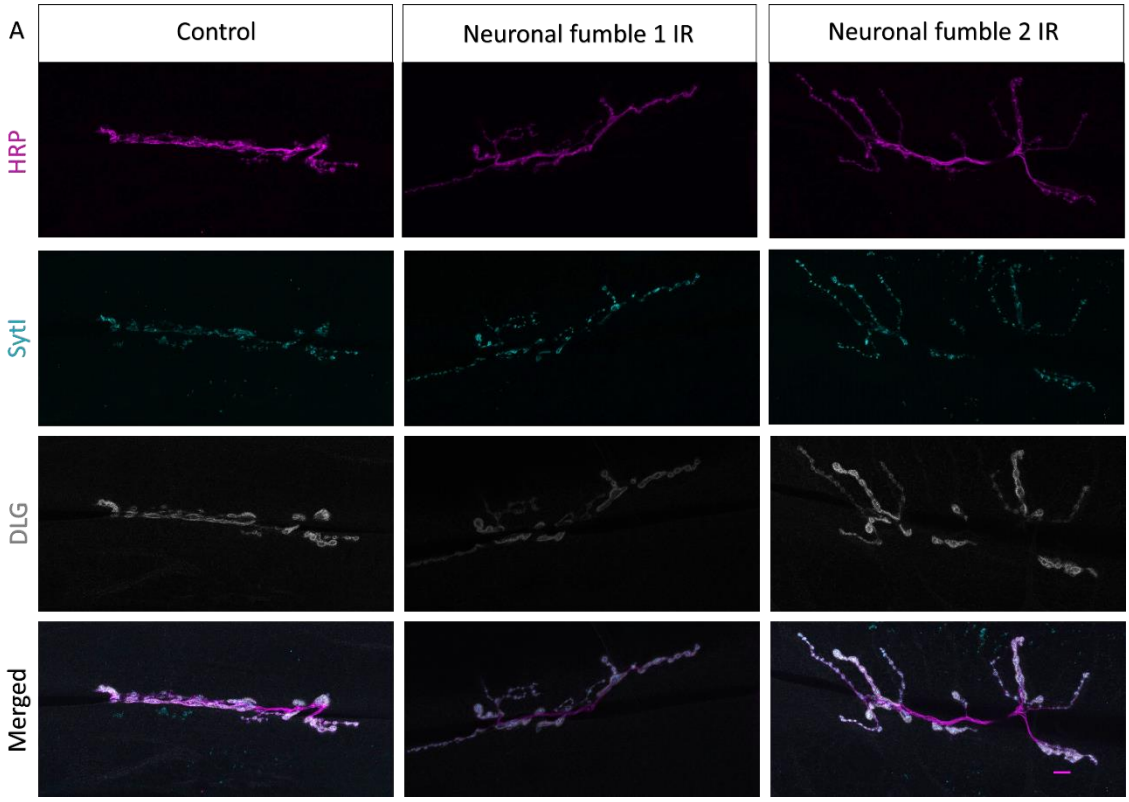
3.2.1 Neuromuscular Junction morphology of neuronal *fumble* knockdown

Morphological alterations of the NMJ are common in several neurodegenerative disorders, and the mechanism is often conserved in *Drosophila*[5]. Although there are some insights regarding how neurons function in a *fumble* background[9], very little is known regarding how their morphology at the NMJ level is affected. To address the question of whether *fumble* leads or not to morphological alterations at this synapse, we labeled neurons with HRP, to outline the neuronal membrane, and with antibodies against DLG and Syt1 (figure 3.6) to label the postsynaptic density and synaptic vesicles, respectively. We used a neuronal Gal4 driver and two different RNAi's against Fumble (Fumble-IR1 and Fumble IR2) to reduce Fumble levels in neurons. Then we imaged muscle 6/7NMJ, segments A2 and A3 to quantify several parameters. After imaging, area, perimeter, total bouton number, NMJ height, NMJ length, total branch number, branching point number, primary branch number and longest branch length were evaluated (figure 3.6) In the larvae stages, *Drosophila* undergoes rapid growth that results in increased muscle surface and also synaptic complexity, such as increased bouton number and increased NMJ size[49]. In our controls, bouton number count is identical to what is described in the literature, but, in Fumble-IR1 the values are diminished, while Fumble-IR2 had no effect (fig 3.6). These differences between the RNAis can be due to the different knockdown efficiency or non-specific effects of Fumble-IR1. To distinguish between these possibilities, we would have to assess the levels of Fumble KD by either using antibody, which is not

available, or RT PCR. If differences in Fumble KD are the cause, it suggests that losing *fumble* in neurons can lead to reduced bouton number.

To better understand if Fumble-IR has consequences in morphology, we analyzed the NMJ size and ramification, which did not reveal any significant differences in these general features, as it can be seen in figure 3.6.

In summary, Fumble RNAi knockdown in neurons does not lead to size deficits in *Drosophila* third instar larvae, but it does lead to reduced bouton number in one of the RNAi lines used. This reduction in synaptic complexity, is a common feature in several disorders that are associated with synaptic dysfunction and neuronal death, for example Alzheimer's Disease, AD[48]. This reduction could potentially explain part of the functional neuronal defects described in PKAN model of cultured human neurons[67].



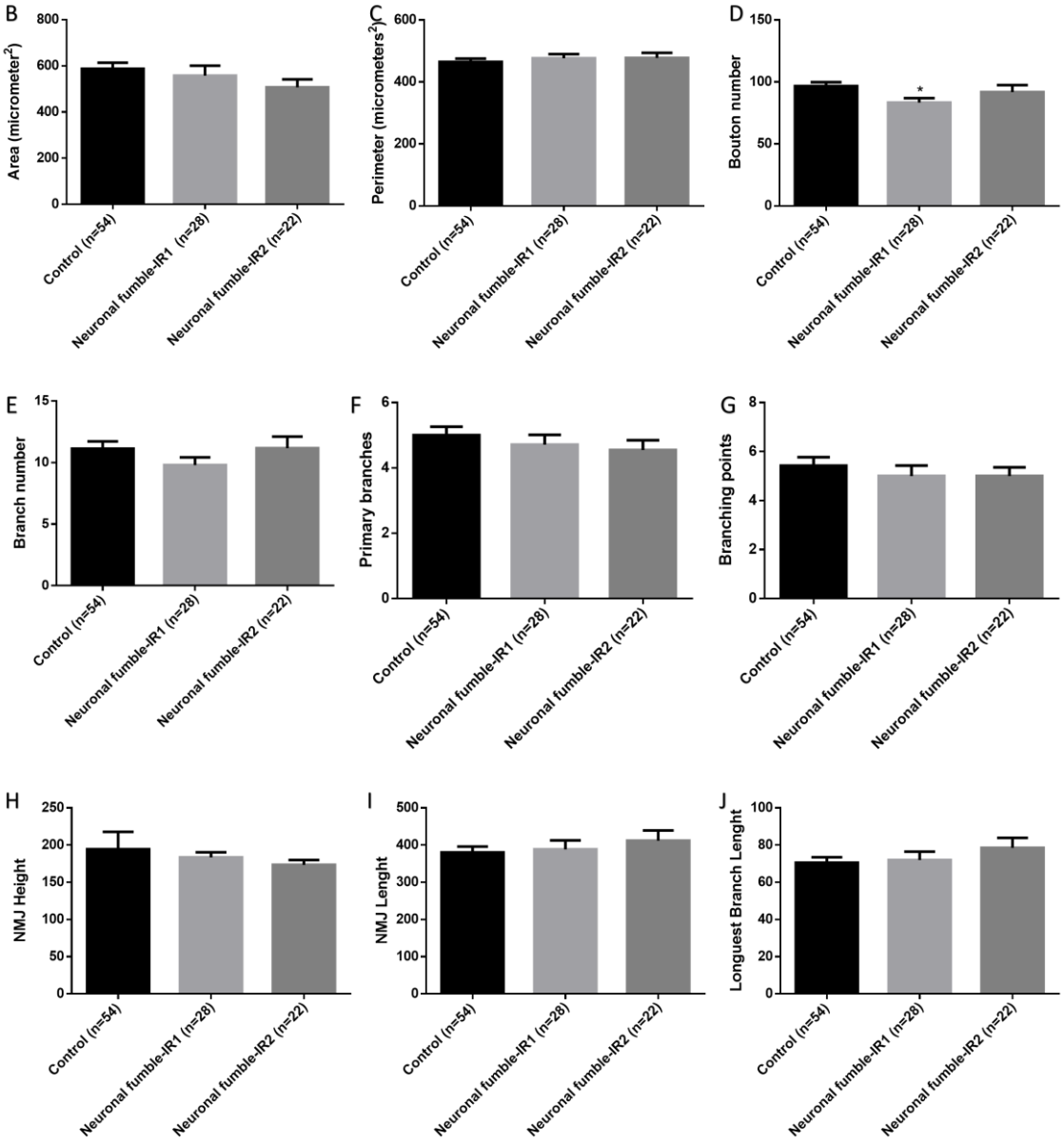
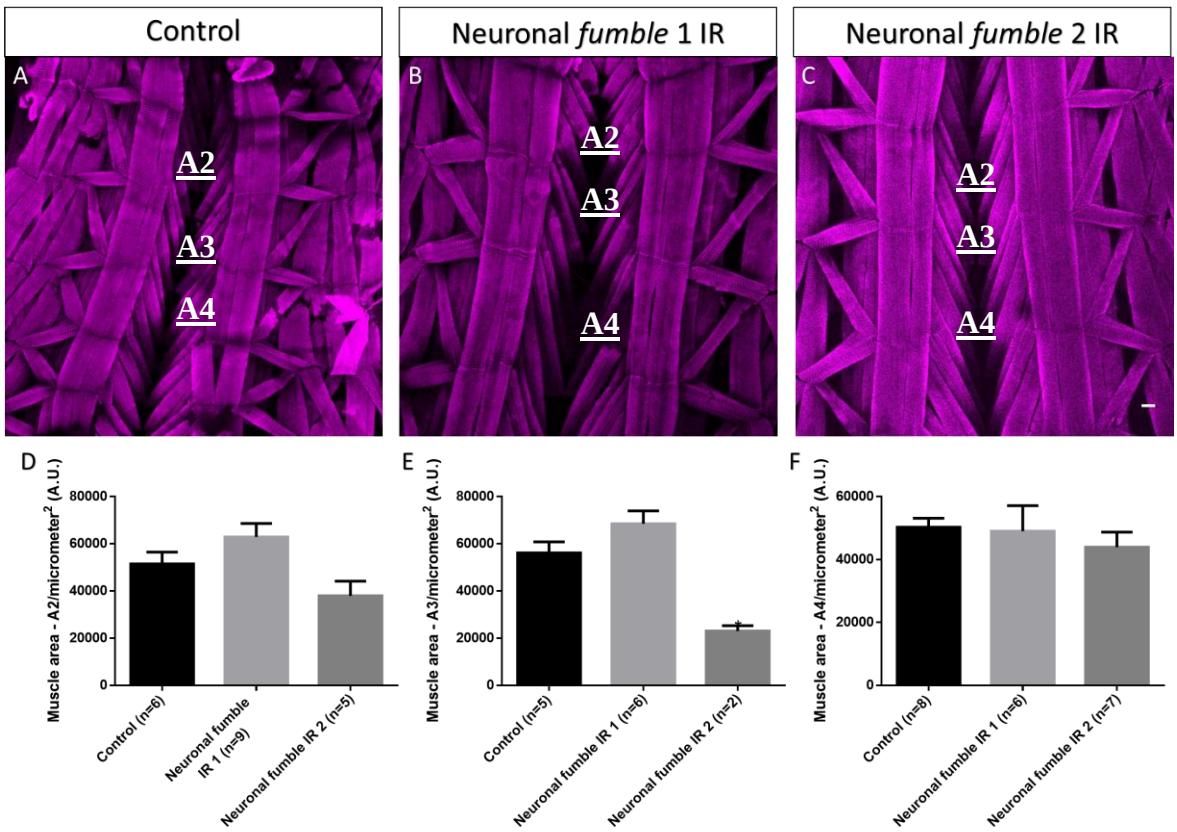


Figure 3.6. **Representative images of *Drosophila* third instar larvae NMJ morphology**. **A.** Confocal images, 40x, larvae fillets labeled with HRP (magenta), DLG (grey) and for Synaptotagmin I (cyan) of control (nSyb-Gal4/+), *fumble* neuronal IR 1 (nSyb-Gal4/Fbl-IR1) and *fumble* neuronal IR 2 (nSyb-Gal4/Fbl-IR2). **B.** NMJ area (μm^2). **C.** NMJ perimeter (µm). **D.** Total bouton count. **E.** Total branch number. **F.** Primary branch number. **G**. Branching point number. **H**. NMJ height. **I**. NMJ length **J.** Longest branch length. Scale bar 10 µm. Mean with s.e.m.

3.2.2 Musculature of neuronal *fumble* knockdown

The results described above show significant alterations in the bouton number count in one of the RNAi lines. Although the alteration of the genetic background can be sufficient to account for the bouton number alteration, in here we examine another factor that can also influence NMJ bouton count, the muscle size. This is a relevant measure because bouton number should match muscle size. By staining third instar larvae fillets with phalloidin, for the same two *fumble* IRs we quantify muscle area per segment (figure 3.7) to understand if there is any muscle size influence in the previous results. As described, the increased muscle surface area is followed by a synaptic increase. When we compared muscle area of each muscle segment with the control, as we can see in figure 3.7, only in the *fumble* 2 IR in the segment 3, significantly differences can be seen. Although there is a statistically significant difference, the number of muscles quantified is low and need to be increased in order to be sure about this reduction. If the muscles are indeed smaller in the Fumble-IR2, it is counterintuitive to have the same number of boutons, given that for a smaller muscle size, fewer boutons were expected. This paradox can only be solved by doing more experiments.



F**igure 3.7. *Drosophila* third instar larvae body walls of neuronal Fumble IR are normal**. Internal view of third instar *Drosophila* fillets, labeled with phalloidin, at low (A-C) and high (D-L) magnification in control, **A, D, G** and **J** (nSyb-Gal4/+), *fumble* neuronal IR 1 (nSyb-Gal4/Fbl-IR1) in **B, E, H** and **K** and *fumble* neuronal IR 2 (nSyb-Gal4/Fbl-IR2). **D-F** shows segment A2, **G-I** shows segment A3 and **J-L** shows segment A4. Muscle area quantification of segment A2 **(M)**, A3 **(N)** and A4 **(O).** Scale bar 10000 µm (A-C) and 10 µm (D-L). Error bar represent mean with s.e.m..

3.2.3 NMJ synaptic composition of neuronal *fumble* knockdown

Not only the morphology can affect the viability of a synapse, but also their synaptic components. Several diseases maintain the general structure of a synapse just altering the number or distribution of some of the components. Here, we show the consequence of reducing *fumble* in neurons for several synaptic proteins.

- ***Fumble* leads to increased intensity of pre-synaptic active zones**

One of the main synaptic components is BRP, the main constituent of active zones. To see if this protein is altered in the *fumble* knockdown, muscle 6 /7 NMJ segment A2 through A4 were imaged with staining for HRP, BRP and also for postsynaptic glutamate receptors (figure 3.8) of the same two different RNAis. The quantification of Brp intensity per NMJ area shows a significant increase, in both IRs, meaning that active zones intensity is higher at the synapse.

- ***Fumble* leads to increased intensity of glutamate receptors in the post-synapse**

To investigate the effect of neuronal reduction of *fumble* in the post-synapse, intensity of GluRs per area were quantified in the larvae fillets recorded from the same staining used for BRP, as presented in figure 3.8. Neuronal *fumble* leads to an increased intensity of glutamate receptors in the post-synapse, perhaps matching the increase in BRP intensity.

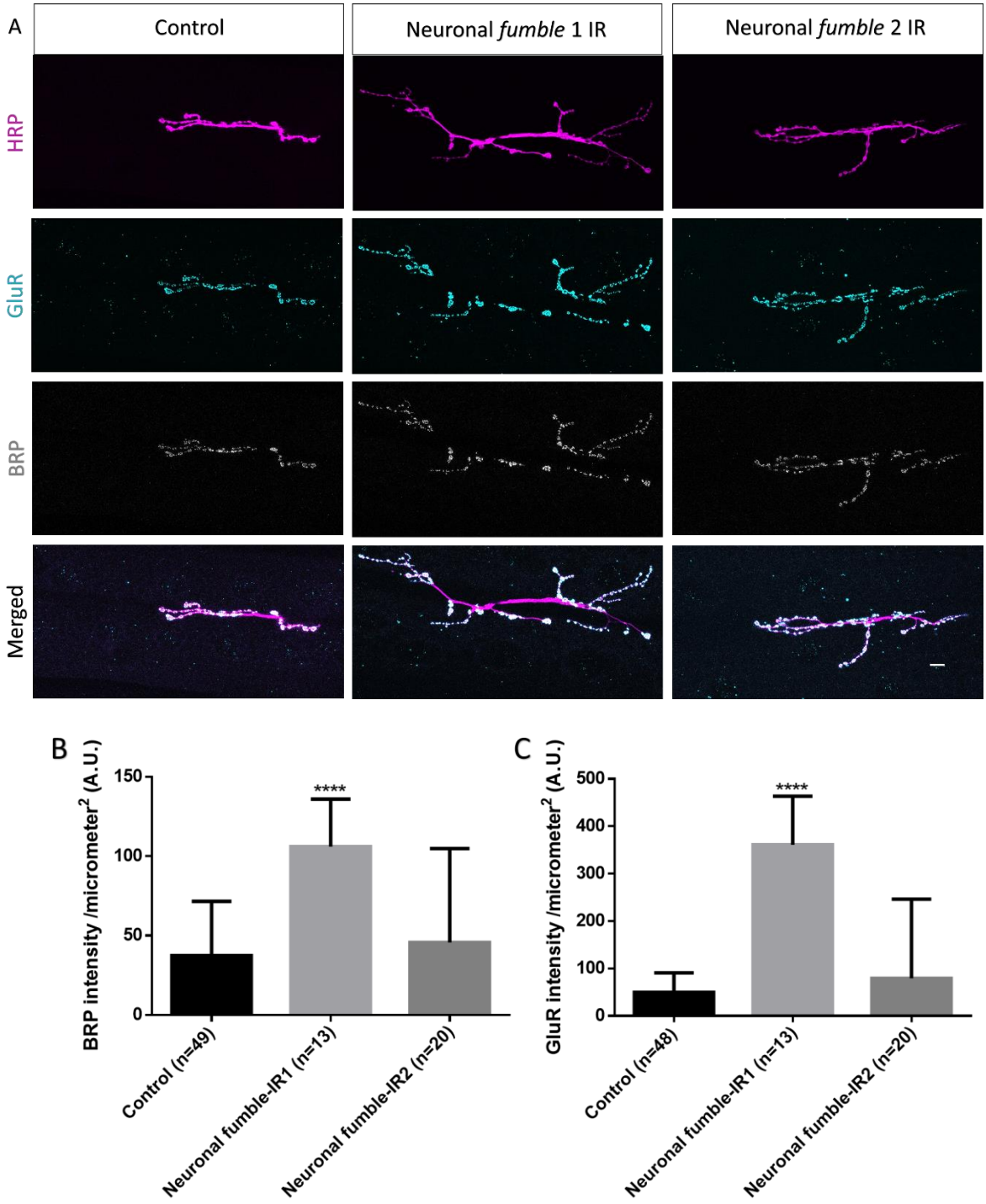


Figure 3.8. Reducing fumble in neurons lead to an increased intensity, per NMJ area, of active zones. A. Confocal images of *Drosophila* larvae fillets labeled with HRP (Magenta), glutamate receptors (cyan) and active zones protein bruchpilot (grey) of control (nSyb-Gal4/+), *fumble* neuronal IR 1 (nSyb-Gal4/Fbl-IR1) and *fumble* neuronal IR 2 (nSyb-Gal4/Fbl-IR2). **B.** Quantification of BRP intensity per area. **C.** Quantification of BRP intensity per area. Scale bar 10um. Error bar represent mean with s.e.m..

- **Synaptic vesicles are normal in neuronal *Fumble* IR**

Synaptic vesicles are responsible to store neurotransmitters and are essential for the synaptic transmission between cells. In neuronal fumble reduction, using the same staining used for morphology (figure 3.6, A), the intensity associated to Syt I remains unaltered, when compared with the controls (figure 3.9), suggesting that the number of synaptic vesicles is unchanged. However, given the high variability observed, more experiments would help clarify this result.

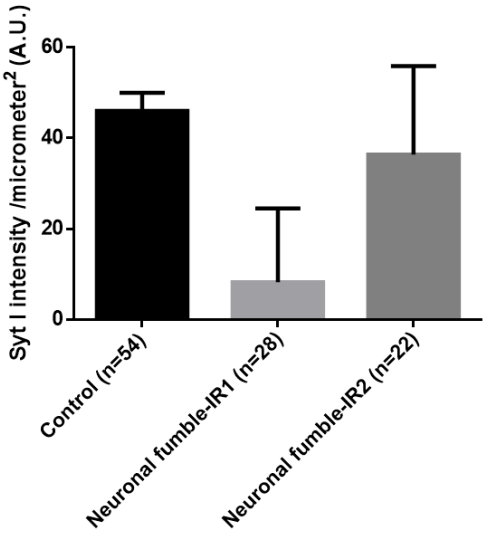


Figure 3.9. Quantification of Syt I intensity per area. Quantification of Syt1 intensity of *Drosophila* larvae fillets labeled with HRP (Magenta), SytI (cyan) and DLG (grey) of control (nSyb-Gal4/+), *fumble* neuronal IR 1 (nSyb-Gal4/Fbl-IR1) and *fumble* neuronal IR 2 (nSyb-Gal4/Fbl-IR2). Error bar represent mean with s.e.m..

- ***Drosophila* alpha-spectrin levels are not altered by *fumble***

Spectrins are a group of proteins that are part of the cytoskeleton. Although these proteins are mostly associated with a structural function, Spectrins also play a major role in *Drosophila* neurons[56]. It has been shown that knockout of alpha or beta Spectrin results in neurotransmission reduction at the NMJ, while the morphology remains unaltered. To evaluate if fumble IR reduction lead to altered levels of alpha-spectrin, we stained larvae fillets with HRP and anti alpha-spectrin and imaged muscle 6 /7 NMJ between segment A2 though A4 (figure 3.10) for the same two different RNAis. The intensity associated

to alpha-spectrin remains unaltered, when compared with the controls (figure 3.10), suggesting that the protein levels are identical to controls.

The studies presented in here, serve, not only to expand the validity of this model for PKAN studies, but also to identify potential pharmacological agents that can be used as a possible treatment complementation. More in-depth studies in this model can then lead to a more effective treatment and enhanced lifestyle for patients.

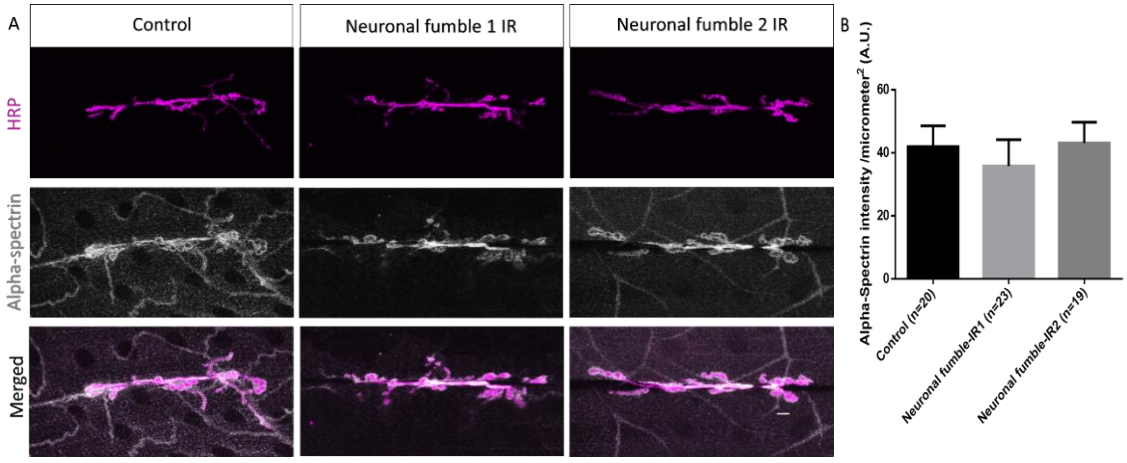


Figure 3.10. Reducing fumble in neurons do not alter alpha spectrin intensity. A. Confocal images of *Drosophila* larvae fillets labeled with HRP (Magenta), alpha-spectrins (cyan) of control (nSyb-Gal4/+), *fumble* neuronal IR 1 (nSyb-Gal4/Fbl-IR1) and *fumble* neuronal IR 2 (nSyb-Gal4/Fbl-IR2). **B.** Quantification of alpha-spectrin intensity per area. Scale bar 10um. Error bar represent mean with s.e.m..

Together, the results so far provide us a characterization of the morphology and synaptic composition of two neurological disorders. In spite of both diseases showing different features regarding their synapses, and remembering that our objective is to look for a common pathway to modulate activity, this work is still interesting for the understanding of the range and complex mechanism(s) behind neurodegeneration. To try to achieve our goal, more work needs to be developed. Firstly, to take direct conclusions, KD efficiency needs to be measured. When not described, whole mutants phenotypes should be performed to understand if results are identical to the ones obtained with RNAi. Then, as the goal is to manipulate activity, activity-dependent structural plasticity in wired

neurons in the disease context should be addressed to assess frequency and dynamics of bouton formation.

3.3 Locomotor alterations in models of neuromuscular disorders.

Motor neuron diseases can affect upper, lower or both motor neurons in humans. Although *Drosophila* has a large number of homologous with several of these disease types, it is important to assure that the *Drosophila* models, at least to a certain extent, mimic the symptoms seen in human patients. To assess whether locomotion was affected in larvae mutant for some of these disease genes, we tested several locomotor parameters by using different behavior tests and by doing cell type specific KD in neurons and wrapping glia (WG) cells. The knockdown by IR was performed for models of PKAN, ALS, HSP and SMA.

3.3.1 *Drosophila* crawling in open field arena: setting up the assay

To identify possible locomotor deficits in the background of the diseases studied in this project, knockdown by RNAi was performed separately in neurons and wrapping glia. Third instar larvae of these conditions were submitted to a crawling assay in an open field agarose arena. Because there was no behavior setup in the lab, I developed the apparatus for this assay based on previous published data. As the goal was to analyze differences between genotypes to understand the physiological relevance of certain mutations or diseases background, and not to dissect specific behavior aspects of larvae movement, an open field arena was the selected options, in contrast with more sophisticated assays[73]. The setup that I develop in the Teodoro's lab relies on filming larvae from above with an intense light from the bottom. To optimize this setup I tested with wild-type larvae different size arenas, different agarose concentrations for coating, different programs, different approaches to increase contrast and different strategies to minimize larvae exiting the arena.

For arena size, several petri dishes were tested to see how larvae behaved on each of them. For our studies, we decided by the larger plate (13.7cm) was the best option, since in the small ones larvae exited the arena very easily. For arena coats, as petri dishes have a lot of friction to facilitate larvae crawling, several agarose concentrations were tested.

In here the goal was to achieve a concentration high enough for larvae crawl without destroying it[70] and low enough to let light pass thought it. Relative to the software to analyze the data, ctrax, firm tracker and ID tracker were tested but as larvae are not highly colored, ID tracker was used, as it is based in contrast differences and provide the information that we need. To increase contrast a black pad was tested, but the video quality is higher without it. Finally, as even in the bigger dishes larvae get out of the arena[32], to avoid losing animals, we tested a salt-based barrier, that larvae naturally avoid[53], that consists is a high concentrated agarose coat with NaCl. Several concentrations were tested but we decided on using about 5g of cooking salt per 200 ml of water.

- Neuronal reduction of *spastin*, *katanin* and *smn* lead to locomotor behavior defects

Considering the larvae crawling protocol described above, 6 third instar larvae with the gut half full were placed in the center of the arena separated for more or less 1cm between them. They acclimatize 30secnds and then are recorded for five minutes, after which trajectories, average velocity and distance were calculated.

In figure 3.11, we show a typical trajectory, the distance and average velocity of each of the genotypes indicated. Looking at the trajectories, (figure 3.11 A-E) it is evident that control larvae (nSyb-Gal4/+) explore more area of the arena that the IRs. In control larvae, we can visualize fast and long forward crawls that consists of normal waves of muscle contraction that are interrupted by normal short time decision processes, such as head casts and turnings[12]. Control larvae, in general, are always moving, and do not exhibit delayed or rigid behaviors[25]. In the same conditions, when spastin (HSP model), katanin (spastin similar microtubule severing protein) and SMN 36621 and 67950 (SMA model) perform the same assay, larvae travel a shorter distance at a slower average speed (figure 3.11 F-I). Although there is a significant decrease in the parameters evaluated, there is no obvious paralysis phenotype or a striking alteration in the patterns of muscle contraction. Therefore, what we observed is that larvae crawl in a visually normal mechanistic way, but at a slower speed and with more and longer interruptions[64]. Videos on supplement.

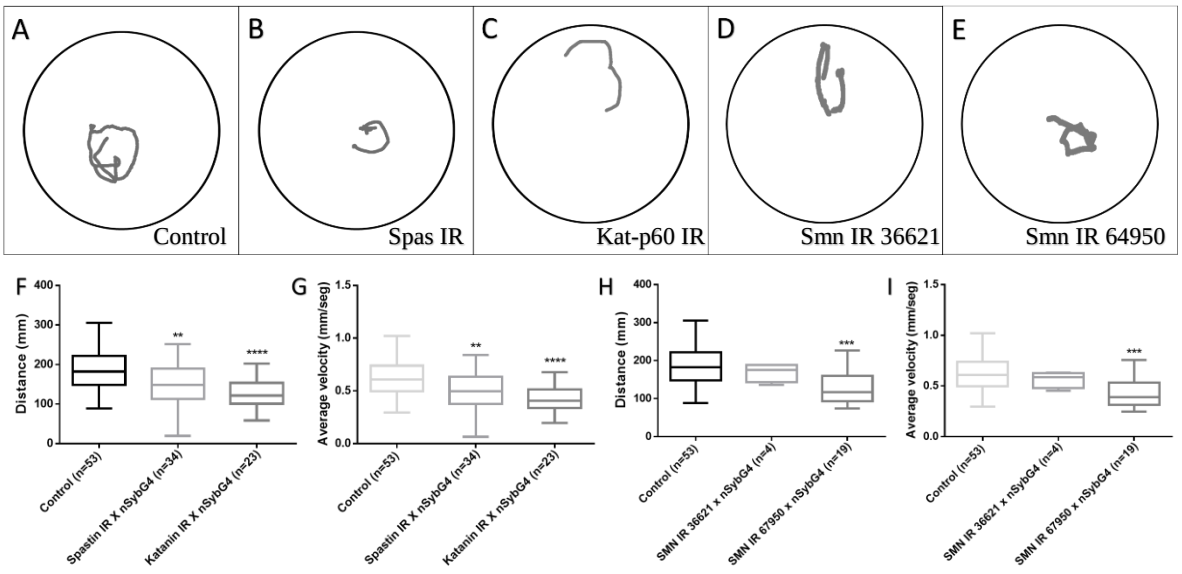


Figure 3.11. ***Drosophila*** **third instar larvae crawling patterns, distance and average speed are altered in neuronal reduction in HSP and SMA models.** Patterns of larvae crawling and arena exploration of control (nSyb-Gal4/+) (**A**), *spastin* neuronal IR (nSyb-Gal4/Spas-IR) (**B**), *katanin p60* neuronal IR (nSyb-Gal4/Kat60-IR) (**C**), *SMN 36621* neuronal IR (nSyb-Gal4/SMN-IR 1) (**D**) and *SMN 67950* neuronal IR (nSyb-Gal4/SMN-IR 2) (**E**). Distance (mm) (**F**) and average velocity (mm/seconds) (**G**) of *spastin* neuronal IR and *katanin p60* neuronal IR. Distance (mm) (**H**) and average velocity (mm/seconds) (**I**) of *smn 36621* neuronal IR and *smn 67950* neuronal IR and (H). Videos in appendix supplementary movie 1 to 5.

- Cell type specific IR reduction of *spastin* in wrapping glia lead to locomotor behavior defects

With the same conditions used for RNAi mediated neuronal reduction of *spastin*, *katanin* and *smn*, trajectories, distance and average speed were also calculated for IR mediated wrapping glia reduction of *spastin*.

In figure 3.12, we show a typical trajectory, and the quantification of the distance and average velocity of wrapping glia reduction IR and control. Looking at the trajectories, (figure 3.12 A-B) we observed that control larvae (Nrv2-Gal4/+) explore a larger area of the arena than spastin IR larvae. So, spastin reduction in wrapping glia cells

leads to a locomotor deficient phenotype, because controls, in the same conditions, crawl larger distances with higher speed. We also observed that the Gal4 line alone had an effect, but since we are comparing with this control, the effect of the Gal4 is accounted for. The normal waves of muscle contraction are still present, but we can sometimes see more evident dorsal and ventral twitches and also more immobile patterns where all the muscles are contracted, and the larvae remains in the same place, what can be seen in the videos in suplement.

- *smn* IR reduction in wrapping glia cells

When smn is reduced in the wrapping glia, we observe a significant increase of the larvae locomotor behavior, present in figure 3.12 G and H, which is counterintuitive and against the literature. However, because the number of animals filmed and quantified is too low, we will not conclude anything at this point.

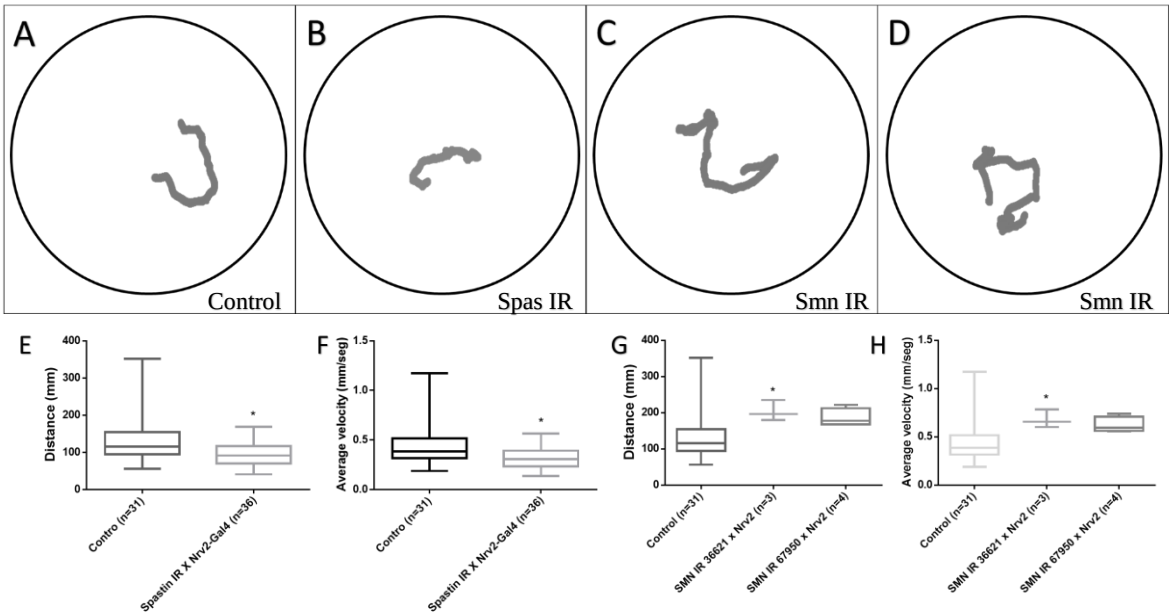


Figure 3.12. ***Drosophila*** **third instar larvae crawling patterns, distance and average speed are altered in wrapping reduction in HSP and SMA models.** Patterns of larvae crawling and arena exploration of control (Nrv2-Gal4/+) (**A**), *spastin* wrapping glia IR (Nrv2-Gal4/Spas-IR) (**B**), *smn 36621* wrapping glia IR (Nrv2-Gal4/smn-IR 1) (**C**) and *smn 67950* neuronal IR (Nrv2-Gal4/SMN-IR 2) (**D**). Distance (mm) (**E**) and average velocity (mm/seconds) (**F**) of *spastin* neuronal IR. Distance (mm) (**G**) and average

velocity (mm/seconds) (**H**) of *smn 36621* neuronal IR and *smn 67950* neuronal IR and (H). Videos in appendix supplementary movie 6 to 9.

3.3.2 Larvae pupation height assays

After embryonic development, *Drosophila* larvae remain for around 5 days in moist food. In laboratory vials, moist food stays in the bottom, keeping the bottle walls clean. Prior to pupation, mature third instar larvae became more mobile and search for a less humid place to build a pupation shelter. When these vials are kept in the same population density, temperature and humidity conditions, the farthest the larvae crawl away from the food into the pupariation place is directly related to the locomotor function of the larvae. After controlled crossing and mating (using the same number of flies and rearing conditions), W1118, controls and IR knockdown in neuronal and wrapping glia cells for ALS, HSP, SMA and PKAN, distance between food and pupariation site were measured for all the genotypes (see methods for detail).

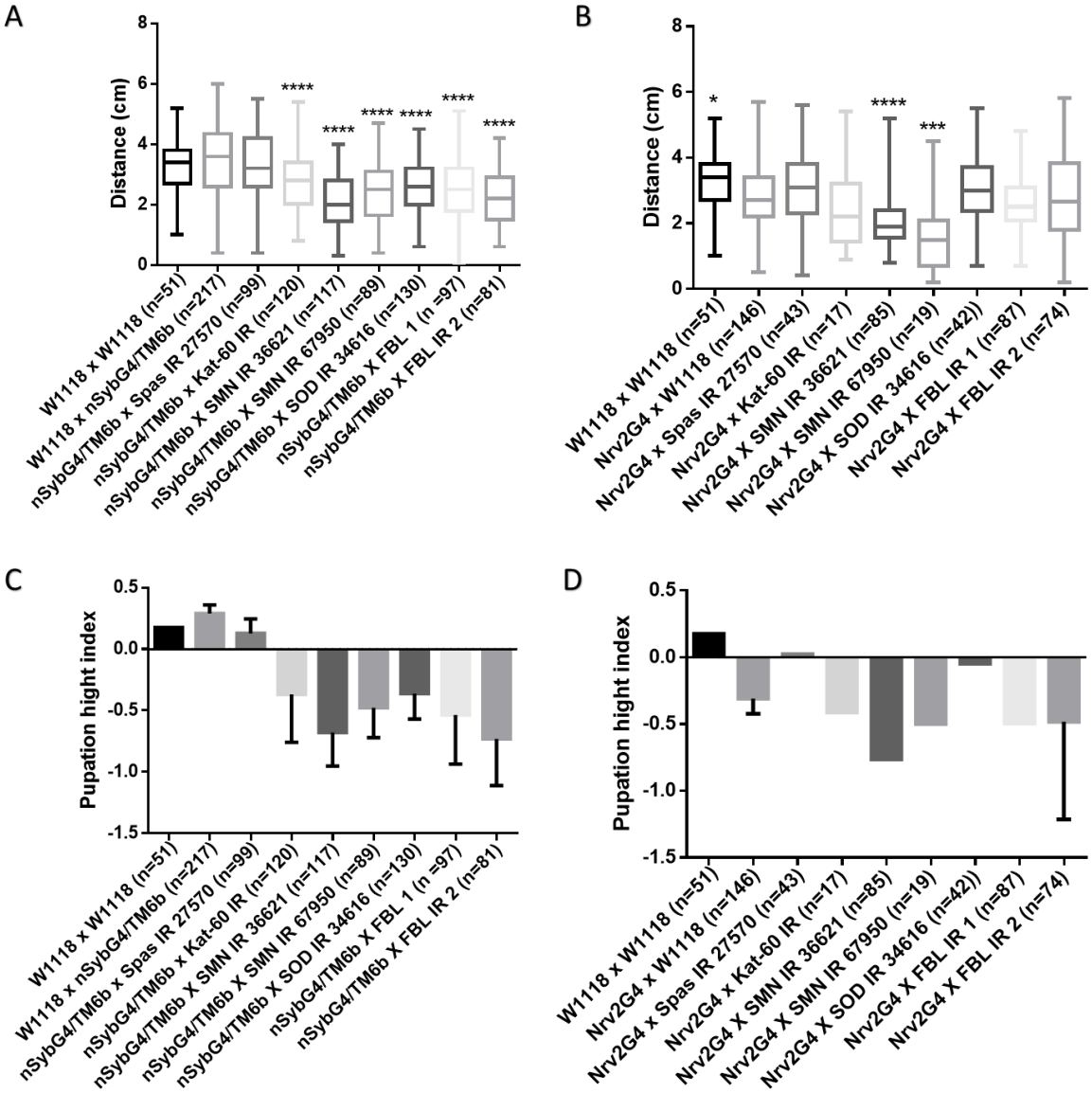
- Neuronal reduction by IR of mutated proteins in neuromuscular disorders leads to reduced pupation height index

According to Johnson Wayne et al 2012 [34] wild type larvae accumulate around four centimeters above the food in normal humidity and controlled conditions. Our data with neuronal control (nSyb-Gal4/+), and also W1118, agrees with this observation. When we look at the distribution of the pupae in the vial, figure 3.13 A, we visualized a significantly decrease in how many larvae crawl until they pupariate in the SOD1 IR (ALS), both smn IR (SMA) and fumble IR (PKAN). Looking at the pupation height index (figure 3.13, C and table 3.1), while the controls have positives values of PI, the several IR, except spastin, have negative values, meaning that in the former there are more pupae above 3cm and in the latter more pupae below that value. For spastin, this index is not statistically significant. However, having in mind that spastin mutants are described to have motor deficits[64] and that in the open field arena we can see impairment in locomotor function, we can speculate that these larvae may have a deficit associated, but this assay is not sensitive enough.

- Wrapping glia reduction by IR of mutated proteins in neuromuscular disorders leads to reduced pupation height index

For wrapping glia reduction, we can see, in graphs B and D from figure 3.13, table 3.2 that the control is significantly lower than the W1118, confirming the impairment associated with the line that was seen in the crawling protocol. When we compare the control with the other IR (figure 3.13, B and D), we can see that only smn-IR seems to have a significant impairment upon WG knockdown. Having in mind that this experiment is less sensitive than the open field arena, and that spastin show impairment in that protocol, we can say that the intrinsic impairment of Nrv2-Gal4 can be affecting the results present in table 3.2 and figure 3.13 B and D.

Although this assay does not show a path, a distance or a velocity, we can asses the locomotor behavior of the group and say that there is an impairment associated with the neuronal reduction of *sod, smn, kat-60* and *fumble* and with wrapping glia reduction of *smn*.



<u>Figure 3.13</u>. Neuronal and wrapping glia reduction of ALS, PKAN, HSP and SMA disease genes lead to altered locomotor behaviors. Pupariation distance from (**A**) neuronal reduction of W1118 (+/+), *spastin* neuronal IR (nSyb-Gal4/Spas-IR), *katanin p60* neuronal IR (nSyb-Gal4/Kat60-IR), *SMN 36621* neuronal IR (nSyb-Gal4/SMN-IR 1), *SMN 67950* neuronal IR (nSyb-Gal4/SMN-IR 2), sod neuronal IR (nSybGal4/sod IR), *fumble* neuronal IR 1 (nSyb-Gal4/Fbl-IR1) and *fumble* neuronal IR 2 (nSyb-Gal4/Fbl-IR2), and (**B**) wrapping glia reduction of W1118 (+/+), *spastin* IR (Nrv2-Gal4/Spas-IR), *katanin p60* IR (Nrv2Gal4/Kat60-IR), *SMN 36621* IR (Nrv2-Gal4/SMN-IR 1), *SMN 67950* IR (Nrv2-Gal4/SMN-IR 2), sod IR (Nrv2-Gal4/sod IR), *fumble* IR 1 (Nrv2-

Gal4/Fbl-IR1) and *fumble* IR 2 (Nrv2-Gal4/Fbl-IR2). Pupation height index for the neuronal (**C**) and wrapping glia (**D**).

Table 3.1. Pupation Height Index of neuronal reduction of several IR.

	Index 1	**Index 2**
W1118 x W1118 (n=51)	0,176471	-
W1118 x nSybG4/TM6b (n=217)	0,338462	0,2413793
nSybG4/TM6b x Spas IR 27570 (n=99)	0,043478	0,2105263
nSybG4/TM6b x Kat-60 IR (n=120)	-0,64444	-0,09333333
nSybG4/TM6b X SMN IR 36621 (n=117)	-0,48571	-0,8723404
nSybG4/TM6b X SMN IR 67950 (n=89)	-0,30612	-0,65
nSybG4/TM6b X SOD IR 34616 (n=130)	-0,21739	-0,5081967
nSybG4/TM6b X FBL 1 (n =97)	-0,25333	-0,8181818
nSybG4/TM6b X FBL IR 2 (n=81)	-0,46154	-1

Table 3.2. Pupation Height Index of neuronal reduction of several IR.

	Index 1	**Index 2**	**Index 3**
W1118 x W1118 (n=51)	0,176471	-	-
Nrv2G4 x W1118 (n=146)	-0,1875	-0,3333333	-0,40909
Nrv2G4 x Spas IR 27570 (n=43)	0,023256	-	-
Nrv2G4 x Kat-60 IR (n=17)	-0,41176	-	-
Nrv2G4 X SMN IR 36621 (n=85)	-0,76471	-	-
Nrv2G4 X SMN IR 67950 (n=19)	-0,5	-	-
Nrv2G4 X SOD IR 34616 (n=42))	-0,04762	-	-
Nrv2G4 X FBL IR 1 (n=87)	-0,49425	-	-
Nrv2G4 X FBL IR 2 (n=74)	0,034483	-1	-

3.3.3 *Drosophila* adult climbing: setting up the assay

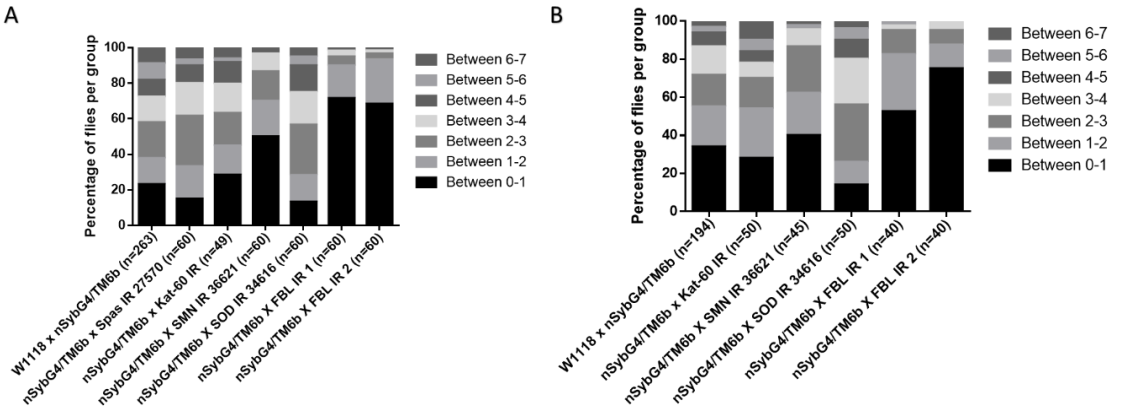
Young wild type, adult male flies are described to climb, on average, four centimeters in three seconds[51], whereas females as they are bigger the values are diminished. An excellent assay to address this, is the negative geotaxis ring assay, where flies are kept in vials without food in a ring structure that allow the knocking of all the flies in several

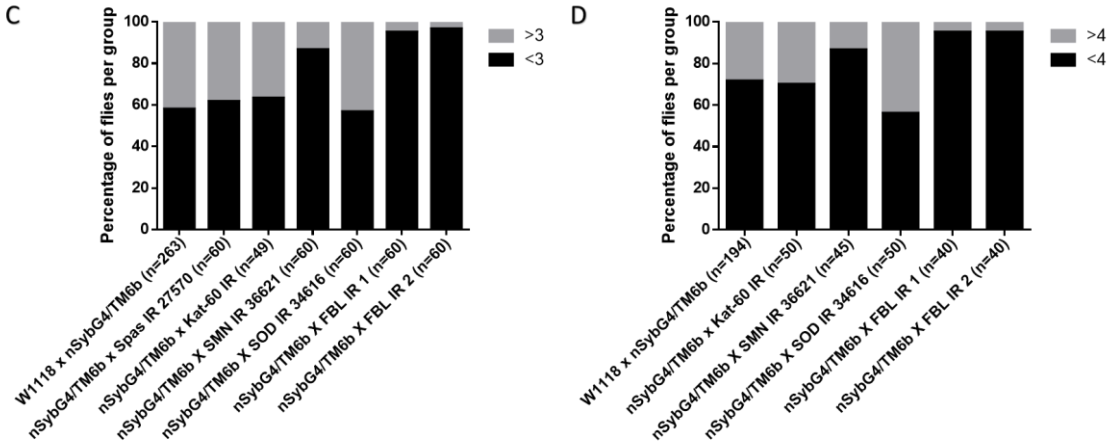
vials down and a constant recording by an associated camera. In our lab, I adapted this protocol to be performed without the ring, as described in materials and methods section.

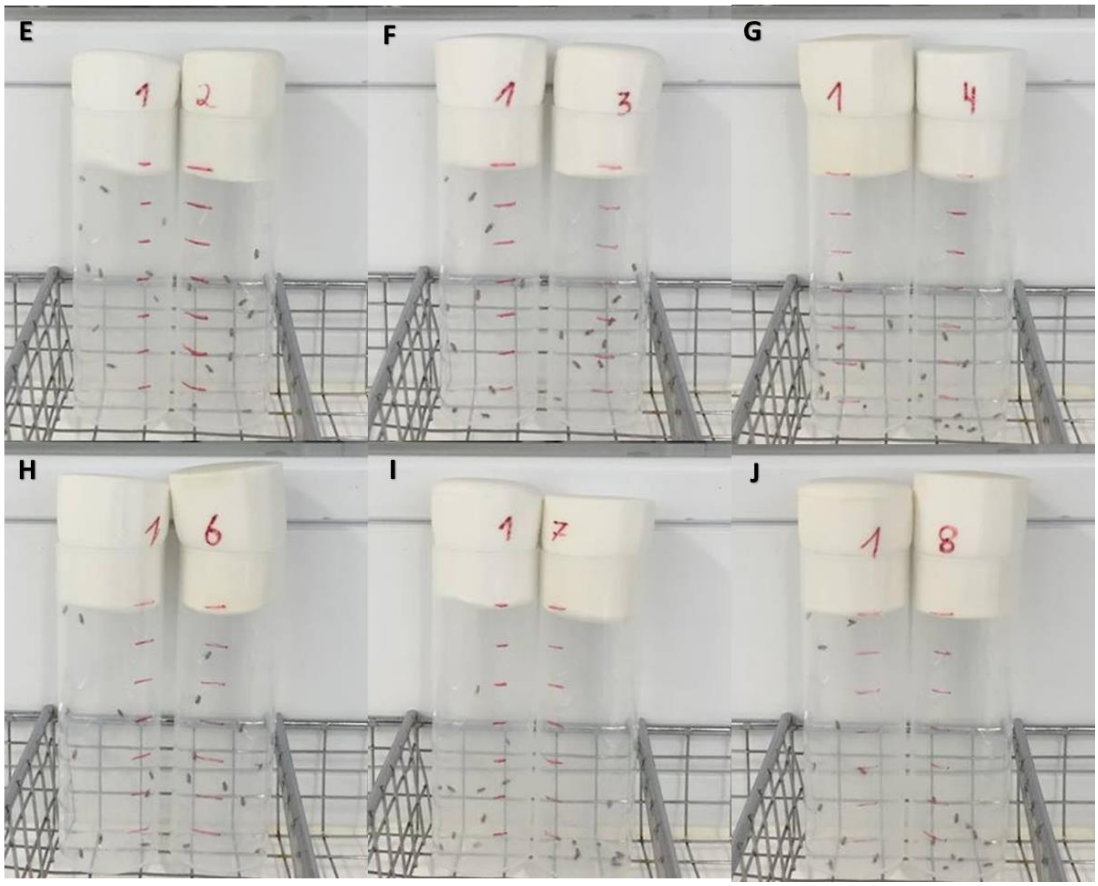
Looking at figure 3.14, we can see alterations in the flies' distribution around the vial after knocking them down. In controls, we can see, as expected, that female flies accumulate more at the lower levels at the vial, whereas males climb higher in the vial.

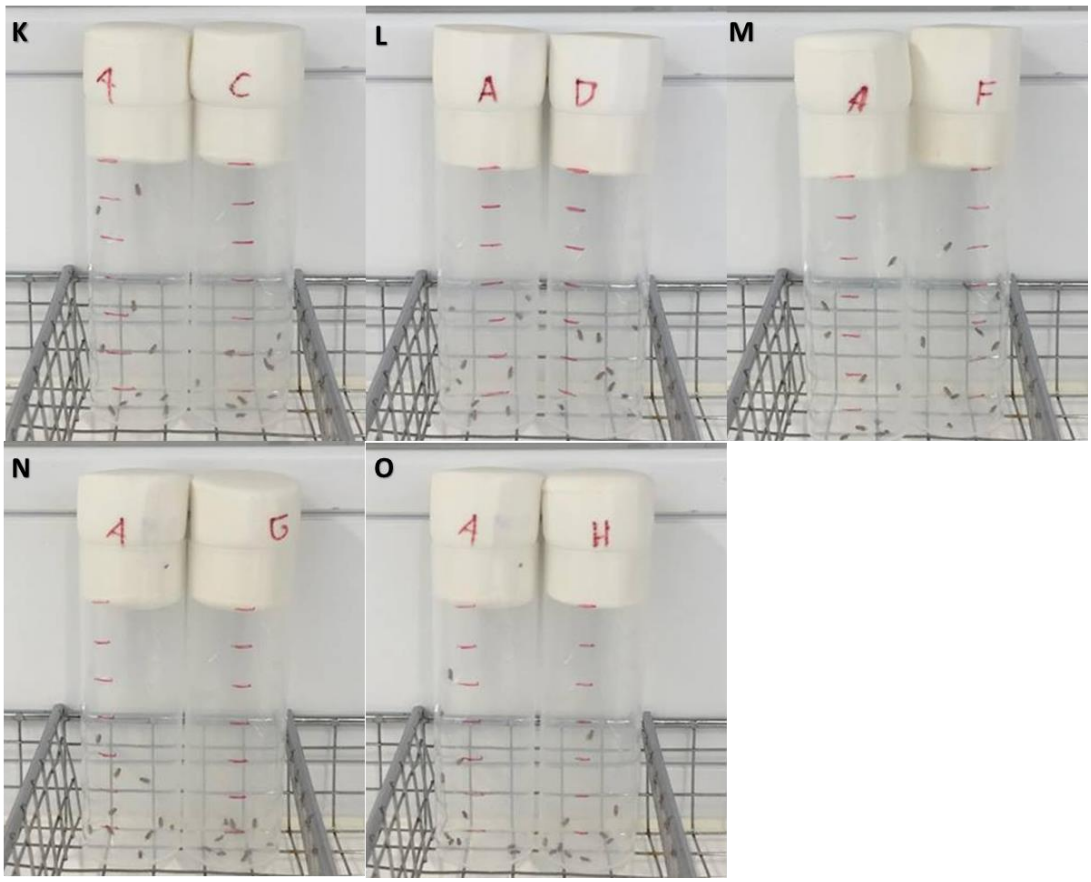
In males (figure 3.14, A and C, and table 3.3) we can visualize that the control accumulates differently between groups when compared to IR, having fumble and smn the strongest defects. In fumble IR, no more than 5 % of the flies get higher than 3 cm, and in smn around 15 whereas the control account 40%. Although sod, katanin and spastin climb also around 40%, the percentage of flies that get higher 6 cm is less, when compared to the control. With these differences, we can say that there is a decreased efficiency in the RNAis in the climbing of the flies, when compared with the controls.

For females (table 3.4 and figure 3.14), controls are distributed between all groups, but, as expected, lower percentages arrive to the top of the vial, when compared with male control. Also, fumble and smn present a stronger phenotype, but a deficiency can be seen for SOD1.




C
Percentage of flies per group
100
80
60
40
20
0
>3
<3
W1118 x nSybG4/TM6b (n=263)
nSybG4/TM6b x Spas IR 27570 (n=60)
nSybG4/TM6b x Kat-60 IR (n=49)
nSybG4/TM6b X SMN IR 36621 (n=60)
nSybG4/TM6b X SOD IR 34616 (n=60)
nSybG4/TM6b X FBL IR 1 (n=60)
nSybG4/TM6b X FBL IR 2 (n=60)
D
Percentage of flies per group
100
80
60
40
20
0
>4
<4
W1118 x nSybG4/TM6b (n=194)
nSybG4/TM6b x Kat-60 IR (n=50)
nSybG4/TM6b X SMN IR 36621 (n=45)
nSybG4/TM6b X SOD IR 34616 (n=50)
nSybG4/TM6b X FBL IR 1 (n=40)
nSybG4/TM6b X FBL IR 2 (n=40)


E
1 2
F
1 3
G
1 4
H
1 6
I
1 7
J
1 8



<u>Figure 3.14.</u> IR neuronal reduction in HSP, ALS, PKAN and SMA affect adult males and female's ability to climb. Male (**A**) and female (**B**) group division of adult climbing assay of neuronal reduction of control (nSyb-Gal4/+), *spastin* neuronal IR (nSyb-Gal4/Spas-IR) (males only), *katanin p60* neuronal IR (nSyb-Gal4/Kat60-IR), *SMN 36621* neuronal IR (nSyb-Gal4/SMN-IR 1), sod neuronal IR (nSybGal4/sod IR), *fumble* neuronal IR 1 (nSyb-Gal4/Fbl-IR1) and *fumble* neuronal IR 2 (nSyb-Gal4/Fbl-IR2). Male (**C**) and female (**D**) percentage of flies that climb more or less than 3 cm of the same genotypes. Males picture of controls, represented on each image by 1 and nSyb-Gal4/Spas-IR (**E**), nSyb-Gal4/Kat60-IR (**F**), nSyb-Gal4/SMN-IR 36621(**G**), nSybGal4/sod IR (**H**), nSyb-Gal4/Fbl-IR1 (**I**) and nSyb-Gal4/Fbl-IR2 (**J**). Females picture of controls, represented on each image by A and nSyb-Gal4/Kat60s-IR (**K**), nSyb-Gal4/SMN-IR 36621 (**L**), nSybGal4/sod IR (**M**), nSyb-Gal4/Fbl-IR1 (**N**) and nSyb-Gal4/Fbl-IR2 (**O**).

Table 3.3. Percentage of male flies per group and per genotype.

	Between 0-1	**Between 1-2**	**Between 2-3**	**Between 3-4**	**Between 4-5**	**Between 5-6**	**Between 6-7**
W1118 x nSybG4/TM6b	23,20819	14,67577	20,13652	14,33447	9,556314	9,215017	8,87372
nSybG4/TM6b x Spas IR 27570	15	18,33333	28,33333	18,33333	10	3,333333	6,666667
nSybG4/TM6b x Kat-60 IR	28,57143	16,32653	18,36735	16,32653	12,2449	2,040816	6,122449
nSybG4/TM6b X SMN IR 36621	50	20	16,66667	10	1,666667	0	1,666667
nSybG4/TM6b X SOD IR 34616	13,33333	15	28,33333	18,33333	15	5	5
nSybG4/TM6b X FBL IR 35259	71,66667	18,33333	5	3,333333	0	0	1,666667
nSybG4/TM6b X FBL IR 64596	68,33333	25	3,333333	1,666667	1,666667	0	0

Table 3.4. Percentage of female flies per group and per genotype.

	Between 0-1	**Between 1-2**	**Between 2-3**	**Between 3-4**	**Between 4-5**	**Between 5-6**	**Between 6-7**
W1118 x nSybG4/TM6b	34,02062	21,13402	16,49485	14,94845	7,216495	3,092784	3,092784
nSybG4/TM6b x Kat-60 IR	28	26	16	8	6	6	10
nSybG4/TM6b X SMN IR 36621	40	22,22222	24,44444	8,888889	0	2,222222	2,222222
nSybG4/TM6b X SOD IR 34616	14	12	30	24	10	6	4
nSybG4/TM6b X FBL IR 35259	52,5	30	12,5	2,5	0	2,5	0
nSybG4/TM6b X FBL IR 64596	75	12,5	7,5	5	0	0	0

Al together these behavior data show that there is indeed an impairment associated with the neuronal IR knockdown of the specific proteins studied. That impairment is a good indicator that the mechanism behind the disorders can be conserved in *Drosophila*, being this organism a good model for the studies of ALS, PKAN, SMA and HSP.
For the glial reduction of the proteins, the impaired locomotion deficits can also be visualized but, as the Gal4 line used has its specific phenotype associated, more specific studies should be performed in order to complement the ones presented here and confirm the conclusions made. Despite this, the results obtained with WG KD suggest that glial cells may also contribute to the phenotype observed in this disease. It will be interesting to study how the morphology of WG is affected by these perturbations: if axonal insulation is perturbed, it may lead to severe locomotor defects, akin to what is observed in disorder such as MS, where myelin is lost[57].
For HSP and PKAN model, these identified behavioral impairments upon neuronal reduction, are in agreement with the alterations seen in the morphology and composition of synapses that suggest problems with synaptic transmission.

3.4 Optogenetics protocol in third instar larvae: setting up the assay

Several optogenetic protocols are described and mainly, they are used to manipulate the activity of cells[7,31,40,60]. In here, we want to use this protocol to manipulate synaptic activity of *Drosophila* larvae NMJs and induce the formation of new activity dependent boutons using a light-induced pattern stimulation strategy[40,79].

As this technique allows a millisecond precision the first step was to elaborate a train of stimuli. Vasin *et al* 2014 [74] and Ataman *et al* 2008 [3] have established a long and short term protocols that induces the formation of new boutons: I adapted these protocols and their number of trains of stimuli and resting phases to optogenetic manipulation of activity. The trains of stimuli cannot be too long, because channels have a recovery time, so several interpulse patterns where also tested[62]. Initially, the protocol was always performed with intact larvae in a small agarose coated petri dish, with the light stimuli coming from the bottom and top. After 8 different protocols with different total durability, different number of trains of stimuli and different interpulses and resting times, a new

approach was used. Instead of using intact larvae, the animals were opened in a HL3.1 solution (that mimics hemolymph) and were kept with only one pin in the head and submitted to the light stimuli (see materials and methods for details). This protocol resulted in the formation of activity-dependent boutons, with a comparable mean with the lab established protocols for this purpose. The idea was to submit the larvae from the disease models to this protocol, and test if they had normal levels of structural plasticity, and whether these new boutons could result in motor recovery.

3.4.1 Channel rhodopsin expression in motor neurons

We use CsChrimson, a red shifted channel rhodopsin, and expressed it in motor neurons using a Gal4 driver (CsChrimson/OK6-Gal4). The food where the larvae were grown was supplemented with trans-retinal, a necessary co-factor for the channel. The larvae were dissected open and were submitted to a light stimuli protocol using a channel (CsChrimson/+) and a driver (+/OK6-Gal4) control to compare with the experimental (CsChrimson/OK6-Gal4). After light stimulation, *Drosophila* fillets were labeled with HRP and DLG, and the newly formed immature boutons were counted.

In mature neurons, in the presence of activity, new boutons are formed.These newly formed boutons, called ghost boutons, are identified by the absence of the DLG staining around them (figure 3.15 and 3.15 in cyan). Also, a control was made with all the above lines without light stimuli (unstimulated figure 3.15 A). Looking at the quantifications presented in figure 3.15 (B and C) we can see that when compared with the controls, the channel+ driver (CsChrimson/OK6-Gal4) significantly increased the number of activity dependent boutons. Also, a control experiment where all the genotypes were dissected without light stimuli shows that boutons only form in light conditions.

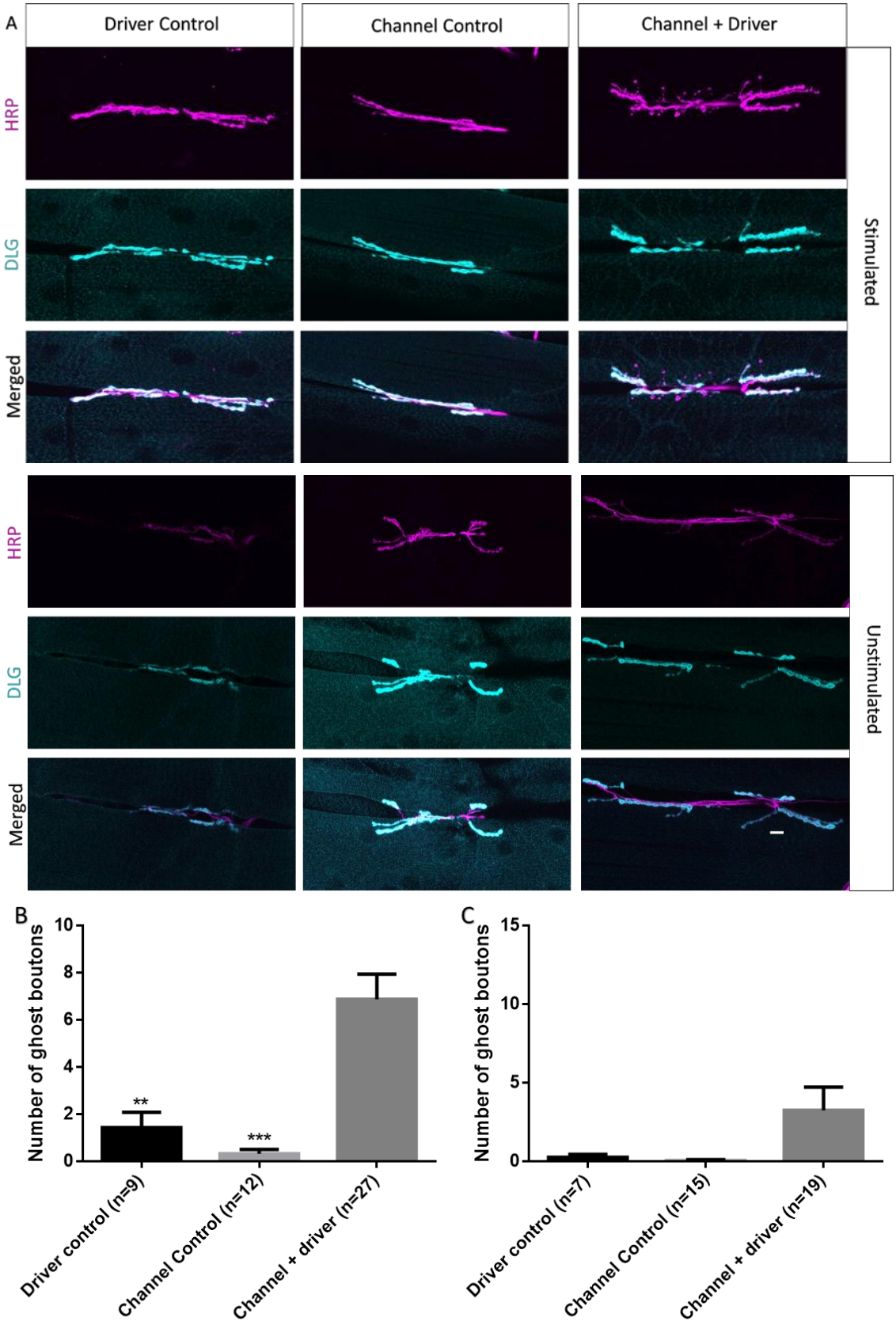

A
Driver Control
Channel Control
Channel + Driver
HRP
DLG
Merged
Stimulated
Unstimulated
B
Number of ghost boutons
10
8
6
4
2
0
**
***
Driver control (n=9)
Channel Control (n=12)
Channel + driver (n=27)
C
Number of ghost boutons
15
10
5
0
Driver control (n=7)
Channel Control (n=15)
Channel + driver (n=19)

Figure 3.15. Light stimulated and unstimulated images of neuronal expression of channel Rhodopsin . A. Confocal images of stimulated and unstimulated driver control (+/OK6-Gal4), channel control (CsChrimson/+) and channel + driver (CsChrimson/OK6-Gal4) labeled with HRP and DLG. **B.** Bouton number count of red light stimulated larvae with trans retinal supplemented food. **C.** Bouton number count of unstimulated larvae with trans retinal supplemented food.

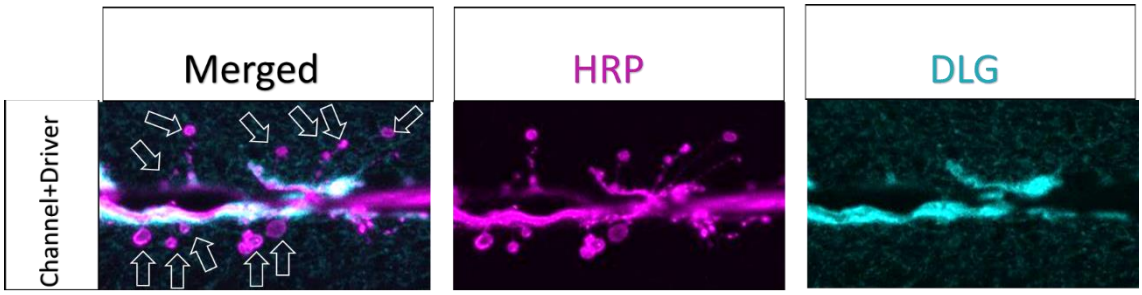


Figure 3.16. Detailed look to ghost boutons of motor neuron expression of channel Rhodopsin. Zoom in of confocal image of a channel + driver(CsChrimson/Ok6-Gal4) example.

Although this shows a prominent result for this new protocol, the number of animals needs to be increased and other experiments should be performed to complement the assay. First, experiments with light stimuli and no food supplementation should take place, in order to see if the co-factor is influencing the channel opening without the stimuli. Then, the unstimulated preparation with supplemented food, should be performed in low light conditions, to avoid some channel activation, that although not significant, can be seen in the Driver+Channel condition.

Neural plasticity refers to the ability of neurons to change their structure and function in response to alterations in external factors. Plastic changes are important for proper development and maturation of the nervous system. In adulthood, this process remains critical, not only for learning and memory processes, but also for injury recovery in the brain and peripheral system. Understanding if, in the background of some diseases,

the ability to remodel synapse remains unaltered, could be important to understand if neuronal function could be manipulated and improved.

This new protocol can help us test whether the disease models have, or not, normal levels of structural plasticity and if it can be used to induce motor recovery.

4 Conclusions

Neurodegenerative processes compromise several domains of heath, well-being and functional capacity of individuals. Counterbalancing these effects is a largely studied field of science, but, as there are multiple causes behind it, it is hard to find a suitable way of treating all these different disorders. This work is a step towards the understanding of the synaptic function behind some models of these disorders that could be used as a way of improving neuronal complexity and inducing synaptic regeneration to overcome the deficits resulting from neuronal death. Instead of trying to overcome a specific effect of a specific disorder, we aim to overcome neurodegeneration as a whole by improving synaptic complexity, using a self-neuronal characteristic, the plasticity. Neuronal plasticity and the ability of neurons to regenerate are the main keys to manipulate activity for this hypothesis to work. Naturally, synaptic defects can be retarded by manipulation of synaptic function. Recently, it was shown in animal models that voluntary physical exercise increases the synthesis of growth factors and stimulates neurogenesis. Also, exercise has been shown to provide several improvements in functional capacity, neurocognitive ability, neuroeffective status and brain plasticity in several neurodegenerative related disorders background, such as Alzheimer Disease, SMA and Parkinson disease. More specifically, it was also demonstrated that exercise causes hypertrophy of NMJs, being extremely important for their maintenance and regeneration. The visible and proved positive effects of physical exercise in wellbeing, aging and neurodegenerative backgrounds, provide some evidence that synaptic activity could indeed be modulated to maximize neuronal complexity, therefore minimizing neurodegenerative phenotypes and increase patient quality lifestyle. In this work, using *Drosophila* NMJ as a model synapse, we prove that we could use optogenetic tools to manipulate synaptic activity in a control situation. Knowing that this improved synaptic activity induced by exercise can induce a neuroprotective effect, in future, this protocol can be used in a disease background in order to evaluate how the models will behave, the effects of modulation and if the manipulation is indeed possible. To guarantee a good explanation for the results that the experiment could provide, it would be important to do a plasticity assay with the models.

The implementation of the optogenetic protocol, although some work still needs to be developed, can provide a clue that this protocol will probably show a higher efficiency when compared with established protocols for activity dependent bouton

formation. If this clue is showed to be true, as it is an easier and less expensive protocol to perform, it would be advantageous for plasticity related work, providing more efficient results using an identical concept of patterns of stimulation.

To increase our knowledge about the molecular mechanism behind the disorders and, consequently behind neurodegeneration, we characterize synaptic function and morphology of *Drosophila* NMJs. Despite all the questions that remain unanswered and the absence of a common synaptic feature that could be used to fight neurodegeneration, we think that this work is a small step forward to a better understanding of how synaptic activity could be modulated to counterbalance the severe effects of neuronal death. Also, as aging shares several features with neurodegeneration, this possible strategy that will improve neuronal complexity, could be crucial for middle aged people to maintain normal brain function and plasticity throughout life.

What we developed in here also provides a better understanding of possible synaptic alterations of the fly homologue of human degenerative disorder PKAN, *fumble*, a gene whose phenotype in the larvae were completely unknown. By using IR knockdown in neurons, we obtain an increase intensity of glutamate receptors what is concomitant with the increased number of glutamate receptors described in human patients, meaning that our model is relying the disease effects. Also about the PKAN model, we see a severe phenotype during larval stages, an early stage of development of the fly life cycle, that is also relying the earlier age of onset of this disorder.

Together, this work is a small step into a new treatment approach for the group of neurodegenerative disorders related with ataxia, that will try to overcome the synaptic deficit by improving neuronal complexity, and not by trying to treat specific disease symptoms and effects.

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6 Appendix

Supplementary information

Supplemental information includes 9 movies available in a CD.

Supplementary Movie 1. *Drosophila* larvae crawling assay video of control (nSyb-Gal4/+).

Supplementary Movie 2. *Drosophila* larvae crawling assay video of neuronal KD of spastin IR (nSyb-Gal4/Spas-IR).

Supplementary Movie 3. *Drosophila* larvae crawling assay video of neuronal KD of katanin-p60 IR (nSyb-Gal4/Kat60-IR).

Supplementary Movie 4. *Drosophila* larvae crawling assay video of neuronal KD of SMN 36621 IR (nSyb-Gal4/SMN-IR 1).

Supplementary Movie 5. *Drosophila* larvae crawling assay video of neuronal KD of SMN 67950 IR (nSyb-Gal4/SMN-IR 2).

Supplementary Movie 6. *Drosophila* larvae crawling assay video of control (Nrv2Gal4/+).

Supplementary Movie 7. *Drosophila* larvae crawling assay video of WG KD of spastin IR (nSyb-Gal4/Spas-IR).

Supplementary Movie 8. *Drosophila* larvae crawling assay video of WG KD of SMN 36621 IR (nSyb-Gal4/SMN-IR 1).

Supplementary Movie 9. *Drosophila* larvae crawling assay video of neuronal KD of SMN 67950 IR (nSyb-Gal4/SMN-IR 2).