

CHARACTERIZATION OF *CG13531* KNOCKOUT MUTANTS IN *DROSOPHILA*
MELANOGASTER AS A
MODEL FOR *SPG11*-ASSOCIATED MOTOR NEURON NEUROPATHIES

by

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For those who survived life without regret...

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ABSTRACT

CHARACTERIZATION OF *CG13531* KNOCKOUT MUTANTS IN *DROSOPHILA MELANOGASTER* AS A MODEL FOR *SPG11*-ASSOCIATED MOTOR NEURON NEUROPATHIES

Motor neuron diseases (MNDs) are a large group of degenerative disorders with multiple genes involved in pathogenesis. These diseases often have complex contributing factors, which can influence the effectiveness of treatment. Patients diagnosed with the same MND but carrying different mutations may respond differently to the same treatment. While high-frequency causative gene mutations in populations are studied with well-defined models to uncover disease progression and treatment, less-frequent gene mutations and their associated mechanisms remain poorly understood. The *Spastic Paraplegia Gene 11* (*SPG11*) is one such gene associated with motor neuron diseases that currently lacks a *Drosophila melanogaster* model. *SPG11* encodes for the spatacsin protein, which plays a role in various cellular processes. The loss of *SPG11* function causes motor neuron degeneration, movement difficulties, and thin corpus callosum in patients. The *Drosophila melanogaster CG13531* gene is the only ortholog candidate of *SPG11*, but a fruit fly model for this gene has not yet been established. This study aimed to establish a well-defined *Drosophila* model for *SPG11*-related diseases that could be used in drug repurposing and screening studies. A knockout model of *CG13531* was generated using the CRIMIC method and thoroughly examined. Results demonstrated that the knockout mutant had reduced locomotor ability and survival rates. Additionally, motor neuron morphology was altered, confirming the observed locomotor deficits. An assessment of midline-crossing neurons was performed to understand whether the knockout model reflects the thin corpus callosum phenotype observed in patients. Overall, these findings suggest that the *CG13531* knockout model can effectively reflect *SPG11*-related diseases, providing a valuable tool for understanding the disease mechanisms and testing therapeutic approaches.

ÖZET

***CG13531* NAKAVT MUTANTLARININ MEYVE SİNEĞİNDE *SPG11*-İLİŞKİLİ MOTOR NÖRON NÖROPATİ MODELİ OLARAK KARAKTERİZASYONU**

Motor nöron hastalıkları (MNH'ler), patogeneizde birden fazla genin rol oynadığı büyük bir dejeneratif hastalık grubudur. Bu hastalıklar genellikle tedavi etkinliğini etkileyebilen karmaşık faktörlere sahiptir. Aynı MNH tanısını almış ancak farklı mutasyonlarına sahip hastalar, aynı tedaviye farklı tepkiler gösterebilir. Popülasyonlarda sık görülen neden gen mutasyonları, hastalık ilerleyişini anlamak ve tedavi geliştirmek için iyi tanımlanmış modellerle çalışılmaktadır. Ancak daha nadir görülen gen mutasyonları ve bu genlerin ilişkili mekanizmaları genellikle yeterince anlaşılmamaktadır. Spastik Parapleji Gen 11 (*SPG11*), motor nöron hastalıklarıyla ilişkili böyle bir genidir ve şu anda *Drosophila melanogaster* modeline sahip değildir. *SPG11*, çeşitli hücresel süreçlerde rol oynayan spatacsin proteini için kodlama yapar. *SPG11* işlevinin kaybı, motor nöron dejenerasyonuna, hareket güçlüklerine ve hastalarda ince korpus kallozuma neden olur. *Drosophila melanogaster CG13531* geni, *SPG11*'in bilinen tek ortolog adayıdır, ancak bu gen için henüz bir meyve sineği modeli oluşturulmamıştır. Bu çalışma, *SPG11* ile ilişkili hastalıklar için iyi tanımlanmış bir *Drosophila* modeli oluşturmayı ve bu modeli ilaç yeniden konumlandırma ve tarama çalışmaları için kullanmayı amaçlamıştır. CRIMIC yöntemiyle bir *CG13531* nakavt modeli oluşturulmuş ve detaylı bir şekilde incelenmiştir. Sonuçlar, nakavt mutantın azalmış lokomotor yeteneğe ve hayatta kalma oranlarına sahip olduğunu göstermiştir. Ayrıca motor nöron morfolojisi değişmiş ve bu durum lokomotor bozukluğu doğrulamıştır. Orta hattı geçen nöronların değerlendirilmesi yapılmış ve bu modelin hastalarda görülen ince korpus kallozum fenotipini yansıtır yansıtmadığı incelenmiştir. Genel olarak, bu bulgular, *CG13531* knockout modelinin *SPG11* ile ilişkili hastalıkları etkili bir şekilde yansıtabileceğini ve hastalık mekanizmalarını anlamak ve terapötik yaklaşımları test etmek için değerli bir araç sağlayabileceğini göstermektedir.

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LIST OF SYMBOLS

bp	Base pair
g	Gram
kb	Kilobase pair
L	Liter
ml	Milliliter
mm	Millimeter
M	Molar
n	Number of samples
ng	Nanogram
nM	Nanomolar
rcf (g)	Relative centrifugal force (gravity)
rpm	Revolutions per minute
v	Volume
w	Weight
μg	Microgram
μL	Microliter
μm	Micrometer
μM	Micromolar
°C	Degree Celsius

LIST OF ACRONYMS / ABBREVIATIONS

AL	Antennal Lobe
ALS	Amyotrophic Lateral Sclerosis
cDNA	Complementary DNA
CMT	Charcot-Marie-Tooth
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
GFP	Green Fluorescent Protein
gRNA	Guide RNA
HSP	Hereditary Spastic Paraplegia
MB	Mushroom Body
NC	Negative Control
NMJ	Neuromuscular Junction
nt	Nucleotide
OR	Olfactory Receptor
ORN	Olfactory Receptor Neuron
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
pH	Power of hydrogen
PN	Projection Neuron
RNA	Ribonucleic Acid
RNAi	RNA Interference
RNA-seq	RNA Sequencing
RT	Room Temperature
RT-qPCR	Real Time Quantitative PCR
<i>SPG11</i>	Spastic Paraplegia Gene 11
UAS	Upstream Activating Sequence

1. INTRODUCTION

1.1. Modeling of Motor Neuron Diseases in *Drosophila*

1.1.1. Motor Neuron Diseases

Motor neuron diseases (MNDs) encompass a wide range of disorders characterized by the degeneration of motor neurons. They are a heterogeneous group of diseases that can broadly be categorized into two main types: upper motor neuron (UMN) disorders and lower motor neuron (LMN) disorders. While ‘motor neuron disease’ is an umbrella term to describe the conditions affecting motor neurons in the brain and spinal cord, ‘motor neuron neuropathy’ (MNN) or ‘motor neuropathy’ generally refers to diseases of the peripheral nervous system. Disease progression and symptoms of these diseases vary but generally lead to motor impairments, muscle weakness, and atrophy (Hobson et al., 2016; Pinto et al., 2019).

Motor neuron degeneration arises due to genetic and environmental factors, and the disease onset depends on these factors as well as the specific genetic mutations involved (National Institute of Neurological Disorders and Stroke, 2019). Among the most common motor neuron diseases is amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, which is characterized by the degeneration of both upper and lower motor neurons. This degeneration may result from cellular stress factors such as impaired protein homeostasis, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, as well as functional impairment in neurons, including demyelination, impairment in axonal transport, glial dysfunction, and neuroinflammation (Mead et al., 2023).

ALS can be classified based on factors such as family history (familial or sporadic) and the site of disease onset (upper or lower). Mutations in over 100 genes have been identified as ALS-causing, many of which also overlap with other MNDs (Ghasemi and Brown, 2018).

Spinal muscular atrophy (SMA) is another group of motor neuron diseases, referring to autosomal recessive neuromuscular disorders characterized by the degeneration and dysfunction of α -motor neurons in the spinal cord and brainstem. SMA results from mutations in a single gene, *SMN1*, and is classified based on age of onset and severity. The disease causes progressive muscle atrophy and weakness. Additionally, patients often exhibit weaknesses in trunk, bulbar, and respiratory muscles (Mercuri et al., 2022).

Hereditary spastic paraplegia (HSP) is another group of motor neuron diseases that are genetically and clinically heterogeneous, with all modes of inheritance. Patients show a range of symptoms represented. While patients exhibit a range of symptoms, the hallmark features are lower limb weakness and spasticity (Salinas et al., 2008). Many subtypes also present cognitive impairments or cerebral signs. In HSP, motor neuron degeneration is caused by various genetic mutations in approximately 80 genes. These mutations lead to impairments in axonal transport and myelination or to cellular stress, such as mitochondrial dysfunction and protein folding defects (Lo Giudice et al., 2014; Shribman et al., 2019).

Charcot-Marie-Tooth (CMT) is an inherited peripheral nervous system disease that causes sensory and motor neuropathies. The disease is heterogeneous regarding its mode of inheritance, progression, severity, and phenotypic characteristics (Szigeti and Lupski, 2009). The CMT1 group is characterized by demyelination events and decreased nerve conduction velocity (NCV), while the CMT2 group shows a reduced number of Schwann cells, yet nerve conduction velocity remains normal. Other groups primarily affect motor neurons (hereditary motor neuropathy, HMN) or sensory neurons (hereditary sensory neuropathy, HSN) (Salinas et al., 2008).

CMT is a highly studied peripheral nervous disease, with many affected pathways and the responsible mutations identified. Impairments in vesicle trafficking, intracellular transport, cellular stress responses, and tRNA synthetase dysfunctions are among the known mechanisms. Similar to other MNDs, many patients show muscle weakness (Kitani-Morii & Noto, 2020).

Other motor neuropathies arise from autoimmune diseases, one of which is multiple sclerosis (MS). MS is an autoimmune and neurodegenerative disease characterized by inflammation, neural loss, demyelination, and gliosis. The disease results from complex gene-

environment interactions, and primarily affects the central nervous system (Filippi et al., 2018).

In addition to the already mentioned diseases, there are several other neuropathies, and many disease-causing mutations and therapeutic targets are identified. It is now understood that vulnerabilities at the neuromuscular synapse may contribute to disease progression, making them excellent targets for therapeutic interventions (Murray et al., 2010). However, many MNDs are multigenic and multifactorial. Rare forms of these disorders are often not extensively studied or well understood. Consequently, there is a crucial need to investigate the role of each gene and the contribution of each mutation to these disease pathologies.

1.1.2. *Drosophila melanogaster* as a Motor Neuron Disease Model

The common fruit fly, *Drosophila melanogaster*, has been extensively studied for over 100 years, paving the way for many discoveries in genetics and advancing disease research. Besides the obvious advantages of using *Drosophila* as a model organism -such as ease of maintenance and handling, high progeny number, short lifespan, and complex behaviors (Hirth, 2010)- the fruit fly also possesses a remarkably intricate brain structure with more than 140,000 neurons and over 50 million synapses (Schlegel et al., 2024). Furthermore, there is significant conservation between the genomes of *Drosophila melanogaster* and humans, with approximately 60% overall similarity and up to 75% of human disease-causing genes having orthologs in *Drosophila* (Adams et al., 2000; Fortini et al., 2000). Additionally, it has been shown that 76% of synaptic genes in humans have orthologs in *Drosophila* (Lloyd et al., 2000).

High accessibility to neuromuscular junctions (NMJs) in *Drosophila* and the ability to perform rapid screens have allowed researchers to identify critical synaptic genes and study neurotransmitter release and signaling (Walters et al., 2019). Sophisticated genetic tools, such as the Gal4/UAS binary expression system, are widely used in *Drosophila* research, enhancing the study of phenotypic changes in disease modeling. The availability of RNA interference (RNAi) tools also facilitates faster in vivo screenings with fruit flies (Bulus et al., 2020; Walters et al., 2019).

The *Drosophila* NMJ is a well-established model for studying development and function, providing valuable insights into MNDs. Electrophysiological and immunohistological assays allow researchers to investigate NMJ morphology and defects, while behavioral assays for locomotion help elucidate the consequences of genetic modifications. The short lifespan of *Drosophila* further facilitates the observation of disease progression over time (Bulus et al., 2020; Walters et al., 2019).

In summary, *Drosophila melanogaster* is an excellent model organism for studying motor neuron diseases, offering rapid, high-throughput insights into disease mechanisms and progression. Its genetic tools, conserved pathways, and versatility in experimental designs make it an invaluable resource in MND research.

1.2. *SPG11* Gene, Its Function, and Its *Drosophila* Ortholog Candidate *CG13531*

1.2.1. *SPG11* Gene, Its Expression, and Function

The *Spastic Paraplegia Gene 11* (*SPG11*) is located on chromosome 15 in humans and consists of 42 exons. The gene transcribes for five different transcripts, encoding three isoforms of the spatacsin protein. Among these, the 2443-amino-acid-long isoform is the canonical form most frequently studied (Figure 1.1a, b) (Stevanin et al., 2008). Spatacsin is a transmembrane protein with several predicted functional domains, including a short coiled-coil (CC) domain, a glycosyl hydroxylase F1 signature, a leucine zipper, a Myb domain, transmembrane (TM) domains (Paisan-Ruiz et al., 2008), and a potential scaffold domain indicating interaction with Adaptor Protein-5 complex (Hirst et al., 2013). The predicted structure of spatacsin, as analyzed in the InterPro database, supports the presence of these domains and highlights similarities with other known structures (Figure 1.1b) (Blum et al., 2024). This multifaceted domain architecture indicates that spatacsin plays a role in various cellular processes, including vesicular trafficking, membrane dynamics, and possibly intracellular signaling pathways. Its interaction with the

Adaptor Protein-5 complex further suggests a role in endosomal and lysosomal functions.

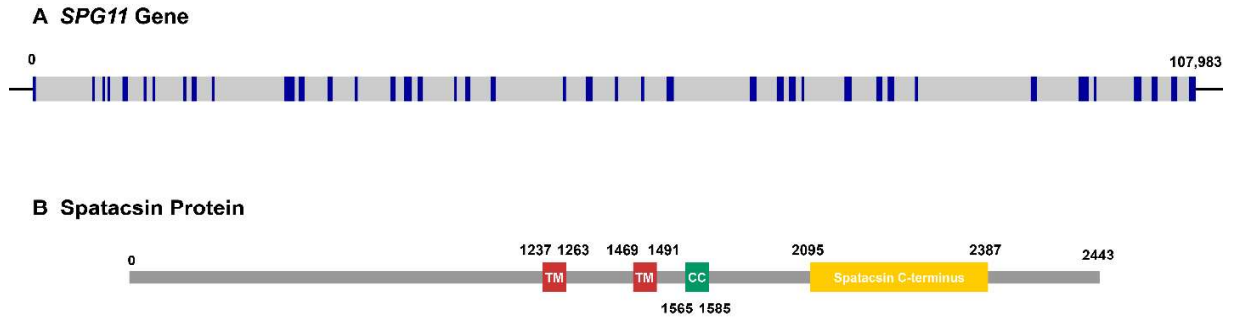


Figure 1.1. Diagram of the *SPG11* gene and Spatacsin protein structure. (A) Schematic representation of the *SPG11* gene. The exonic regions (blue) span a total genomic length of approximately 107,983 base pairs. (B) Domain structure of the Spatacsin protein. Transmembrane (TM) domains at residues 1237–1263 and 1469–1491 (red). Coiled-coil (CC) domain at residues 1565–1585 (green). Spatacsin C-terminus at residues 2095–2443 (yellow).

The *SPG11* gene is ubiquitously expressed in the central nervous system (CNS) throughout development in many species. Expression data from human patients indicate high expression in brain regions with high neuronal density, such as the cerebellum and cerebral cortex (Pozner et al., 2020). Subcellular localization studies have detected spatacsin predominantly in dendrites and axons (Pérez-Brangulí et al., 2014). Furthermore, studies using neuronal-like cell lines suggest its presence in endosomes, lysosomes, and along microtubules, indicating a potential role in vesicular trafficking (Murmu et al., 2011).

While the precise role of *SPG11* in development remains unclear (Pozner et al., 2020), existing research suggests that it plays a critical role in axon maintenance and anterograde transport (Pérez-Brangulí et al., 2014). Experimental evidence supports these functional roles. Knockdown of *SPG11* in neuronal cell lines has been shown to reduce axon length and branching, dendrite length and numbers (Pérez-Brangulí et al., 2014). Knockout mouse models suggest impairment in autophagic lysosome formation (Varga et al., 2015), lipid accumulation (Branchu et al., 2017) and cholesterol buildup as a result of impaired calcium trafficking (Boutry et al., 2019). Induced pluripotent stem cell (iPSC)-derived motor neurons from *SPG11* knockout models exhibit reduced mitochondrial transport (Güner et al., 2021).

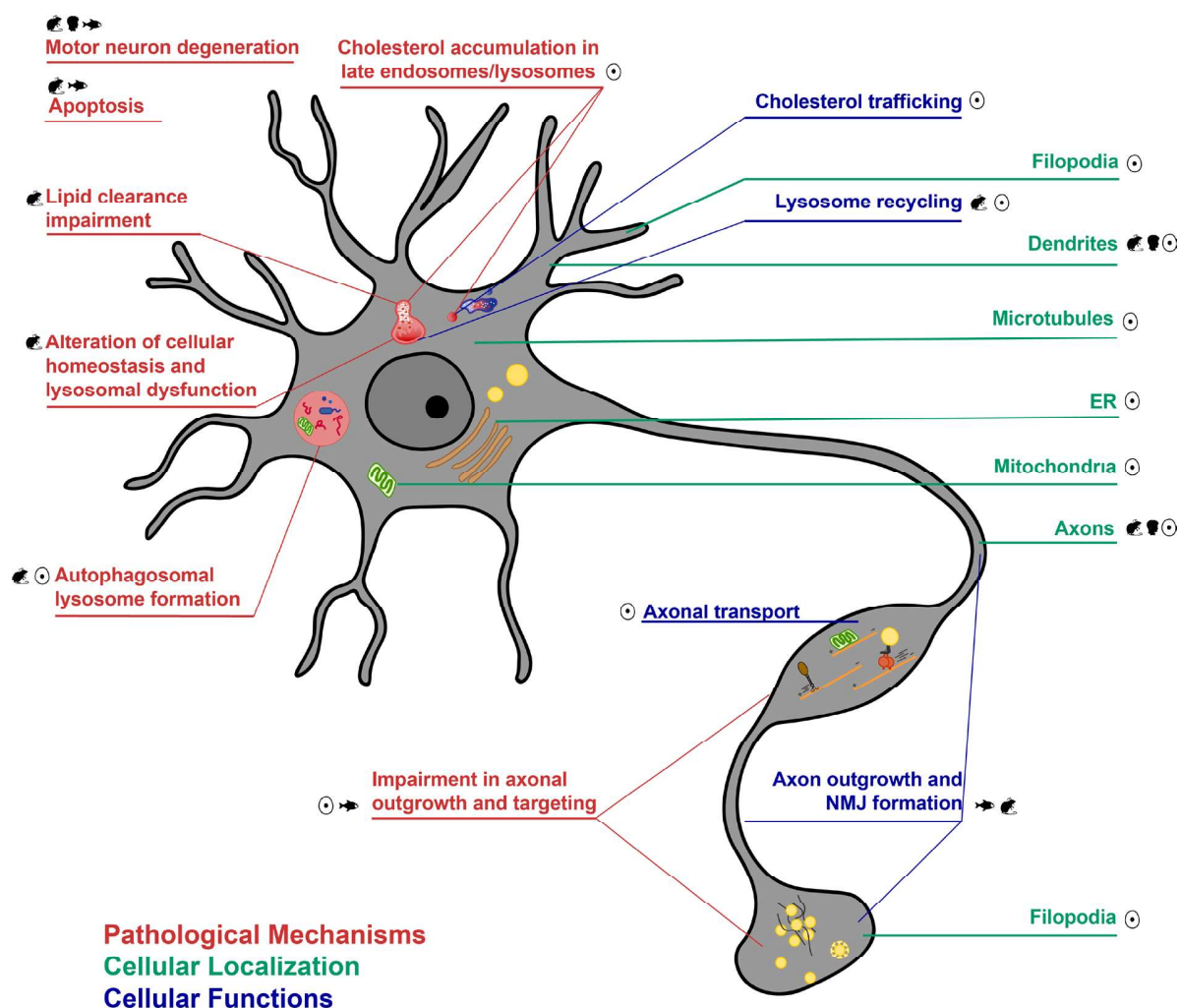


Figure 1.2. Graphical summary of findings on *SPG11*. Pathological mechanisms (red), cellular localization (green), and cellular functions (blue) of spatacsin protein. Icons next to each title represent the organism's (human, mice, zebrafish, cell line) for which these findings were identified. This graphical representation highlights key findings related to *SPG11* and its encoded protein Spatacsin.

These findings collectively highlight the role of *SPG11* in cellular trafficking and lysosomal function. This evidence aligns with localization studies, supporting the hypothesis that spatacsin is integral to maintaining cellular and neuronal homeostasis. The summarized effects of *SPG11* dysfunction are depicted in Figure 1.2.

Mutations in the *SPG11* gene are associated to several neurodegenerative diseases (Figure 1.3), including ALS (Hentati et al., 1998; Orlacchio et al., 2010), HSP (Faber et al., 2018; Hehr

et al., 2007; Winner et al., 2004.), and CMT (Montecchiani et al., 2016). Evidence suggests that these variants often result in a truncated protein, supporting a loss-of-function mechanism as the primary driver of *SPG11* pathogenicity (Lo Giudice et al., 2014).

Patients with *SPG11*-related diseases typically exhibit either homozygous or compound heterozygous mutations. Interestingly, the same mutations in the *SPG11* gene are occasionally reported across different diseases, indicating overlapping or shared pathological mechanisms. This highlights the complexity and variability of *SPG11*-associated disorders and underscores the importance of further research into the gene's role in these diseases.

1.2.2. *SPG11* Association with Motor Neuron Diseases

The correlation between *SPG11* mutations and ALS was first reported by Hentati and colleagues in 1998. Many variants causing autosomal recessive juvenile ALS, also known as ALS5, have been identified since then (Daoud et al., 2012; Iskender et al., 2015a, 2015b; Özoguz et al., 2015). ALS5 is characterized by progressive spasticity in the limb and facial muscles, accompanied by distal amyotrophy. Notably, the onset of ALS5 typically occurs within the first two decades of life, but the disease progresses slowly, and patients tend to have prolonged survival (Orlacchio et al., 2010).

An autosomal recessive form of HSP (ARHSP), initially mapped to the long arm of chromosome 15, can arise from mutations in several genes, including *SPG7*, *SPG20*, *SPG21*, and *SPG11* (Stevanin et al., 2007). In addition to limb spasticity, many ARHSP patients show mental impairment and thinning of corpus callosum (ARHSP-TCC) (Del Bo et al., 2007). Variants in *SPG11* account for 41-77% of all ARHSP-TCC cases, making the TCC a reliable hallmark of *SPG11*-induced autosomal recessive spastic paraplegia 11 (Stevanin et al., 2007). Other clinical features of ARHSP-TCC include axonal neuropathy and cerebellar signs, likely caused by the combined degeneration of central and peripheral axons and neuronal loss within cortical regions and spinal cord (Lo Giudice et al., 2014; Stevanin et al., 2007).

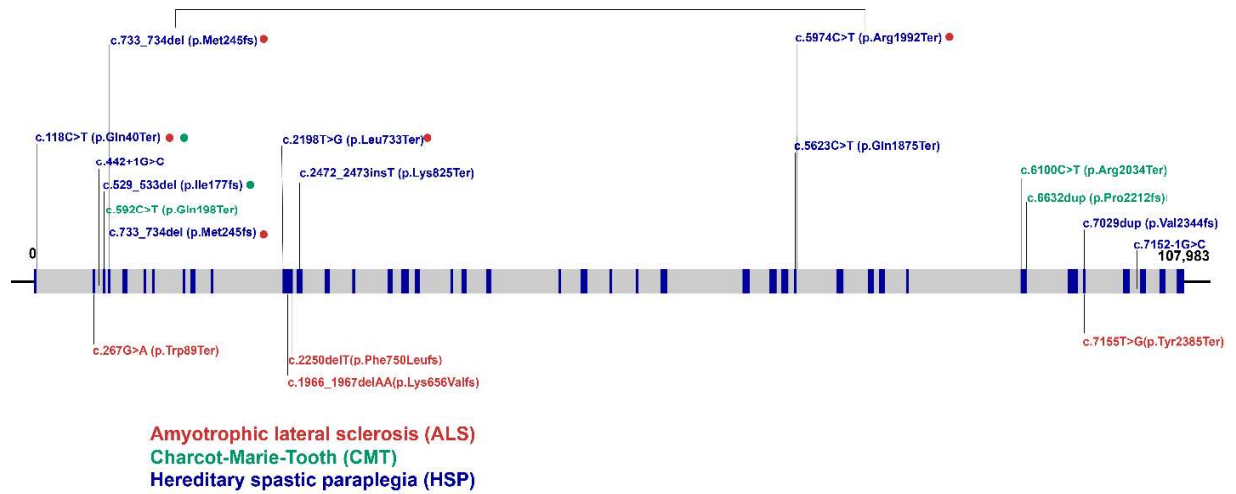


Figure 1.3. Disease-causing mutations in *SPG11* associated with ALS, CMT, and HSP. This schematic highlights mutations in the *SPG11* gene that are linked to various neurodegenerative diseases: Amyotrophic lateral sclerosis (red), Charcot-Marie-Tooth (green), and Hereditary spastic paraplegia (blue). Mutations associated with more than one disease are labeled with the respective disease names.

Charcot-Marie-Tooth axonal type 2X is an autosomal recessive disease caused by pathogenic *SPG11* mutations. CMT2X is characterized by lower limb muscle weakness, distal sensory impairments, and gait difficulties. Patients with CMT2X often display overlapping phenotypes with ALS5 and ARSHP-TCC. Symptoms typically manifest in the first two decades of life, with early-onset patients showing more severe phenotypes. In line with other CMT2 subtypes, NCV values in CMT2X patients remain normal, while motor and sensory axonal neuropathies are observed in the lower limbs (Montecchiani et al., 2016).

1.2.3. *CG13531* Gene, Its Expression, and Orthology to *SPG11*

CG13531 is the only candidate ortholog of the human *SPG11* gene in *Drosophila melanogaster*, sharing 37% similarity and 22% identity with *SPG11* (Stevanin et al., 2008). It is expressed in the adult head of *Drosophila* (Aradska et al., 2015) and is believed to play roles in axonal transport, synaptic vesicle transport, and axon extension.

The *CG13531* gene is 5996 bps long and is located on the second chromosome of *D. melanogaster*. It contains seven exons and encodes a single coding sequence. The protein product of *CG13531* includes the Spatacsin-C domain (Figure 1.4c), which is a defining feature of the Spatacsin protein family. While *CG13531* lacks other components found in human spatacsin, the presence of this domain reinforces the likelihood of being an ortholog of *SPG11* (Blum et al., 2024).

Protein structure modeling further supports this orthology. As shown in Figure 1.5, the AlphaFold-predicted protein structures of the protein encoded from *CG13531* and human spatacsin reveal similar folding patterns, further suggesting functional and evolutionary parallels between these two proteins.

CG13531 protein has predicted functional partners common with Spatacsin as well, according to the String database (Szklarczyk et al., 2023). One of these proteins is zinc finger FYVE domain-containing protein 26 (ZFYVE26 or Spastizin) homolog CG5270 and is coded by the *SPG15* gene. This protein plays a role in HSP disease and functions in lysosome and autophagosome organization (Vantaggiato et al., 2023). Another shared partner is the human Atlastin-1 ortholog, Atlastin, which is involved in the biogenesis of the endoplasmic reticulum tubular network and axonal development (Li, 2022; Zlamalova et al., 2024). The Paraplegin protein, an ATP-dependent zinc metalloprotease, is another partner and contributes to mitochondrial organization and cytokinesis (Ferreirinha et al., 2004). Spastin, an ATP-dependent microtubule-severing enzyme, is critical for cytoskeletal dynamics and proper synapse formation (Costa and Sousa, 2022). Similarly, Spartin regulates axonal growth by modulating endosomal trafficking and regulating microtubule stabilization (Nahm et al., 2013). These shared interaction partners between *CG13531* and Spatacsin support their orthology.

A *CG13531* Gene



B *CG13531* Transcripts



C *CG13531* Protein



Figure 1.4. Structure of the *CG13531* gene, transcripts, and protein. (A) Gene structure: The *CG13531* gene spans 5996 base pairs and consists of six exons (blue). (B) Transcripts: Two alternative transcripts (*CG13531-RB* and *CG13531-RA*) are shown. (C) Protein structure: It contains a Spatacsin C-terminus domain (yellow) span residues 1394–1745 within the full protein length of 1815 residues. (Adapted with permission from Blum et al., 2024).

The *Drosophila* Auxilin protein is another predicted interaction partner of *CG13531* protein. Auxilin acts as a co-factor for heat shock cognate 70 and plays an essential role in neuronal development by regulating clathrin-coating dynamics and Notch signaling (Hagedorn et al., 2006; Cheng et al., 2023). Another partner of *CG13531* is the Parkin protein, which contributes to mitochondrial homeostasis and organization, regulates autophagic turnover (Ziviani et al., 2010). Given that many of these interaction partners have critical roles in neuronal development and degeneration, it is likely that *CG13531* may also function in neurons.

Expression data from the *Drosophila* RNAi Screening Center scRNA-seq database reveal that *CG13531* is expressed most highly in descending neurons (DN) and hemocyte cell types in the adult brain. Although expression levels are lower, *CG13531* is also found in serotonergic, dopaminergic, and peptidergic neurons, as well as in some glial cell types. Additionally, the database indicates that *CG13531* is highly expressed in certain neuronal progenitors and hemocytes in larvae. Lower expression levels are observed in specific glial cells and cholinergic neuron progenitors. Notably, no expression of *CG13531* was detected in muscle tissue according to these datasets (Hu et al., 2024).

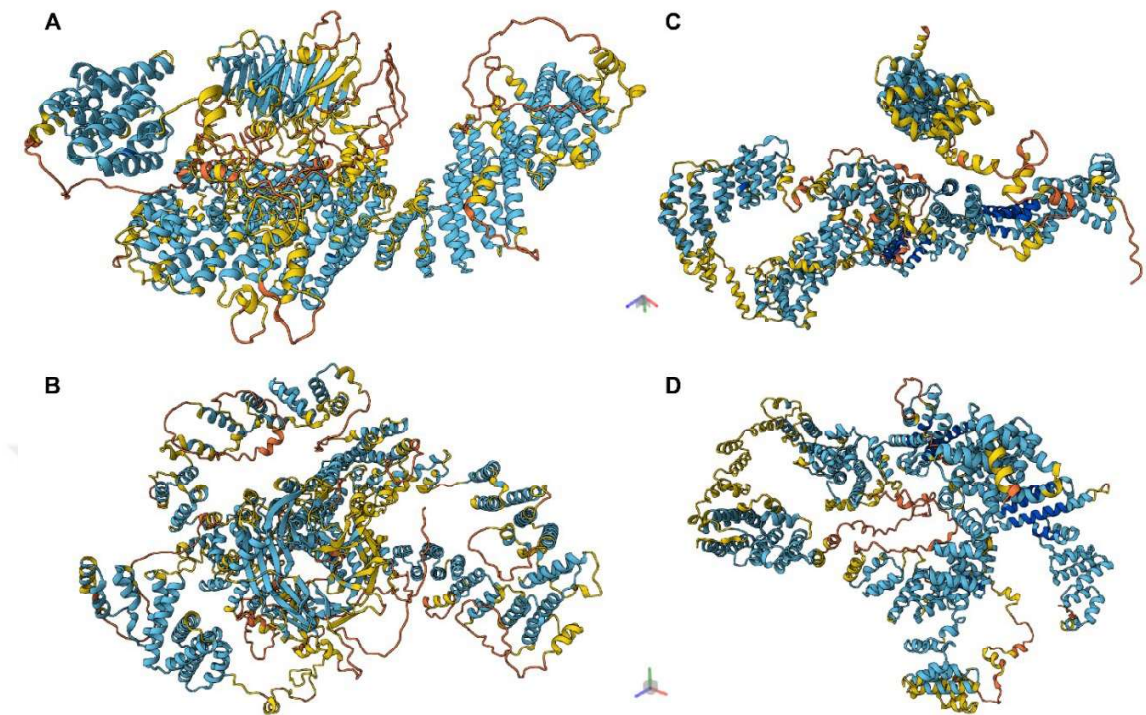


Figure 1.5. Predicted structures of Spatacsin and *CG13531* proteins. (A, B) Predicted 3D structures of Spatacsin, displayed from two different orientations to highlight the protein's secondary and tertiary structures. (C, D) Predicted 3D structures of *CG13531* protein, viewed from alternate orientations. The axes in the center of each panel indicate the orientation for comparison across different views. (Adapted with permission from Jumper et al., 2021).

Taken together, the prominent expression of *CG13531* in descending neurons and neuronal progenitor cells suggests that the gene may have functional roles in motor neurons, supporting its potential involvement in processes like axonal transport and motor neuron function. This expression pattern highlights the importance of further investigation into *CG13531*'s role in neuronal and motor functions.

1.3. Junction Morphology and Development of *Drosophila*

Synaptogenesis is a critical biological process in *Drosophila* that begins during development and continues into adulthood. Neuromuscular junctions (NMJs) are specialized synapses where presynaptic neurons (motor neurons) connect with postsynaptic targets (muscles) through complex cellular interactions (Chou et al., 2020).

The *Drosophila* larval musculature consists of many segments, each housing approximately 30 muscle fibers attached to the inner surface of the epidermis. These segments are broadly categorized as thoracic (T1-3) and abdominal (A1-8), each of which contains symmetrical hemi-segments with visible muscle fibers (Figure 1.6a) (Hooper, 1986). Among these, segments A3 and A4 are commonly investigated due to the larger surface areas, while muscles 6 and 7 are frequently investigated because their associated neurons form multiple contact sites, facilitating detailed study of synaptic events (Fernandes et al., 2023; Menon et al., 2013).

Motor neurons in *Drosophila* arise from individual neuroblast lineages and extend their axons along distinct pathways as they exit the central brain. There are three major nerve roots: the segmental nerve (SN), intersegmental (ISN) nerve roots, and the transverse nerve (TN). Upon reaching the periphery, the SN splits into two branches, the SNa (which innervates lateral muscles) and the SNc (which innervates ventral muscles). Similarly, the ISN splits into three branches, the ISN (which innervates dorsal muscles), the ISNb (which innervates ventrolateral muscles (VLMs)), and the ISNd (which innervates other ventral muscles). Each type of motor neuron has a specific muscle target, as illustrated in Figure 1.6b (Landgraf et al., 1997; Menon et al., 2013).

During embryogenesis, motor neuron axons exit the central brain and extend to their specific muscle fiber targets. Upon reaching the muscle fibers, initial contact is established, and growth cones begin differentiating into synaptic terminals. Cellular adhesion molecules (CAMs) engage with other CAMs or synaptic adhesion molecules (SAMs) at synapses to ensure physical contact between pre- and post-synaptic structures. Neuroxin-1 (Nrx-1) and Neuroligin 1-4

(Nlg1-4) interactions in the extracellular matrix initiate synapse formation first by promoting membrane outgrowth, followed by size expansion. Scaffold proteins such as Synapse-defective (Syd-1) and Liprin- α accumulate in the pre-synaptic area and interact with Nr x -1-Nlg complexes, enabling post-synaptic clustering of scaffold proteins such as Discs large (Dlg) and glutamate receptors (GluRs), which are regulated by Nr x -1. Additionally, synaptic vesicles and active zone marker protein Bruchpilot (Brp) localize to the pre-synaptic site. Proteins downstream of the Bone morphogenetic protein (BMP) pathway and the Wingless-related integration site (Wnt) signaling pathway are subsequently recruited to synapses to facilitate NMJ maturation and maintenance (Chou et al., 2020; Menon et al., 2013).

By the end of embryonic development, a few NMJs with sparse boutons (circular-shaped structures that contain synapses) are established. During larval development, NMJs become fully functional, with specialized boutons, some of which are surrounded by subsynaptic reticulum (SSR), depending on the bouton type. Synaptic refinement events occur in two major stages: Motor neurons initially form contacts with non-target muscles in early embryonic stages, which are later eliminated to ensure specificity. As muscle fibers grow, bouton numbers dramatically increase, but some boutons are selectively pruned during this stage of refinement. Synaptic refinement continues during morphogenesis, optimizing NMJ structure and function (Menon et al., 2013).

Pre-synaptic terminals at the *Drosophila* NMJ are categorized into three types according to their size, morphology, and neurotransmitter type: Type I, type II, and type III, as summarized in Figure 1.6c. Type I boutons are large (Type Ib is larger than type Is), glutamatergic, and possess SSR. Type II boutons are much smaller without SSR; they are primarily peptidergic but may also coexpress glutamate as a neurotransmitter. Type III are small, oval-shaped boutons lacking SSR and exclusively peptidergic. Boutons form on branches in one of three ways: Membrane budding, a process similar to yeast budding; symmetric division, splitting of an existing bouton into two; or de-novo formation, the independent formation of a new bouton (Fernandes et al., 2023; Menon et al., 2013; Pérez-Moreno & O’Kane, 2019).

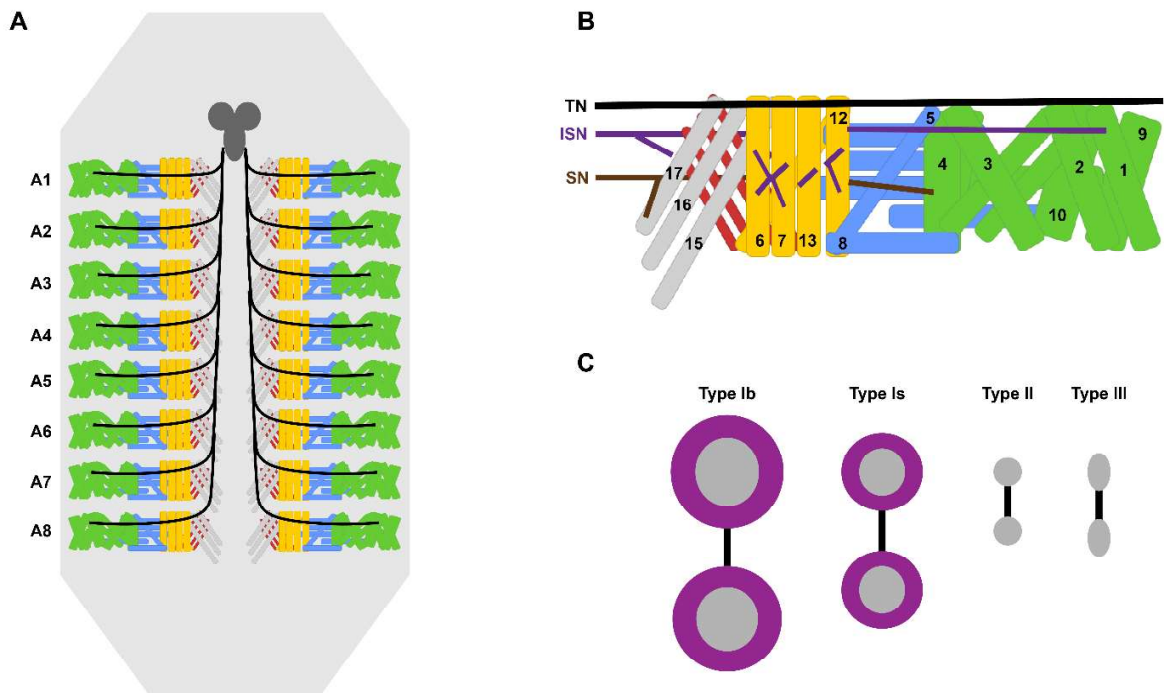


Figure 1.6. Overview of *Drosophila* larval musculature and motor neuron innervation. Color-code is used for ventral (yellow), lateral (blue) and dorsal (green) muscles. (A) Schematic representation of the *Drosophila* larval fillet, illustrating the muscle fibers in A1–A8. (B) Detailed view of muscle fibers and nerve root innervation, TN, ISN and SN are shown. (C) Morphology of boutons, categorized based on their structure and Discs large signal (purple).

Aside from the established bouton types, several transient structures are observed during NMJ formation. These include synaptapods, ghost boutons, synaptic retractions, and satellite boutons, which play roles in the synaptic refinement process. If NMJ development is impaired, these structures may not be eliminated. Synaptapods are filopodial extensions that are highly dynamic structures that are typically only visible through live imaging. Ghost boutons are immature boutons lacking a Dlg signal and an active zone but containing vesicles. They can be visualized by Horseradish peroxidase (HRP) and axonal membrane markers. These boutons are precursors to mature boutons and are not a result of a degeneration process. Synaptic retractions occur when a previously formed bouton disappears, leaving behind a ‘foot-print’ of the former bouton. They are characterized by the loss of pre-synaptic markers such as Synapsin (Syn) and Bruchpilot (Brp) while maintaining post-synaptic clusters marked by Dlg and GluR. Unlike ghost boutons, synaptic retractions can involve multiple boutons. Satellite boutons are small

boutons budding from a normal-sized parent bouton located at the terminal arbor. Different from the above-mentioned boutons these boutons contain functional synaptic markers, including Syn, Brp, Dlg, and GluR, allowing them to serve as functional synapses. In some mutants, satellite boutons are overproduced. This event is characterized by one parent bouton being attached to several small ones, and is usually linked to NMJ overgrowth phenotypes (Menon et al., 2013).

Genes that regulate NMJ growth are categorized as either positive or negative regulators, depending on their role in cytoskeletal organization, microtubule interactions, and bouton formation. Genes that act as positive regulators enhance NMJ growth often alter the cytoskeleton and interact with microtubules. Mutations of these regulators usually result in a reduced number of boutons and branches, highlighting their role in promoting NMJ development. Genes that negatively regulate NMJ growth usually function in cytoskeletal organization or proteasomal degradation. The lack of these negative regulator proteins results in an increased number of boutons, sometimes without a corresponding increase in synaptic area. Genes that play a role in presynaptic activation and calcium regulation can also regulate NMJ growth. Voltage-gated calcium channels and genes regulating neuronal activation are critical for bouton and NMJ formation. Loss-of-function mutants of these genes usually display a reduced number of boutons, indicating their importance as positive regulators in NMJ development (Menon et al., 2013).

1.4. Midline Guidance and Olfactory Receptor Neurons in *Drosophila*

The corpus callosum (CC) is a crucial structure that connects the right and left cerebral hemispheres in humans, facilitating communication between the two hemispheres. It plays an essential role in transferring and integrating cognitive signals, sensory input, and motor output across the hemispheres (Vermeulen et al., 2023). Structurally, the CC consists of heavily myelinated axons and forms the brain's primary commissural region.

A sophisticated cascade of events governs the formation of the CC. Newborn neurons in the subventricular zone extend their axons to the cortical plate, initiating migration to the opposite hemisphere. Axonal projections navigate a corridor whose borders are defined by repellent cues between the ventricular zone and the cortical plate. These neurons then advance further towards the CC, guided by attractant cues and pioneer axon movements. Once the commissural neurons cross to the other hemisphere, continue their migration until they reach their designated targets (Gavrish et al., 2024).

This process is orchestrated by a complex interplay of attractant and repellent cues, ligands, and receptors, and it is evolutionarily conserved in bilaterally symmetrical animals. While *Drosophila melanogaster* has a simpler nervous system with fewer neurons and genes involved in midline crossing compared to humans, the fundamental mechanisms and key molecular players involved in axonal guidance are highly conserved between the two species (Magalhães et al., 2007). This makes *Drosophila* an excellent model organism for studying the genetic and molecular underpinnings of midline-crossing processes.

The majority of neuronal axons in the *Drosophila* embryos are commissural, meaning they cross the midline to connect with contralateral neurons. This process is orchestrated by the secretion of both repulsive and attractive cues from midline glia during early embryogenesis. Two key pathways govern midline crossing: The Netrin-Frazzled (Net-Fra) pathway, where two Netrin ligands act as attractants, signaling to the Frazzled receptor. Frazzled also functions independently as a transcription factor for *Commissureless (comm)*, a critical protein for midline attraction responsiveness. The second evolutionarily conserved pathway of midline crossing is the Slit-Roundabout (Robo) Pathway. Slit, the ligand, interacts with Robo receptors to generate a repulsive signal that prevents ipsilateral neurons (non-commissural) from crossing the midline. Other modulatory genes, such as Amalgam (Ama), Hedgehog (Hh), and cell adhesion molecules such as Down syndrome cell adhesion molecule (Dscam) and Flamingo (Fmi) further regulate midline guidance, among many others (Howard et al., 2019; Magalhães et al., 2007).

Among the commissural neurons in *Drosophila*, olfactory receptor neurons (ORNs) have the largest number of commissural axons in the fly brain. Other commissural neuron types include small ventrolateral neurons (s-LNvs), clock neurons, and certain lateral anterior optic

tubercle neurons (Reinhard et al., 2022; Tai et al., 2021).

ORNs detect environmental odorant signals through dendrites exposed to the external environment and project their axons to glomeruli in the antennal lobe (AL). Here, ORNs form synapses with projection neurons (PNs), which relay information to higher brain centers like the mushroom body (MB) and lateral horn (LH) (Barish and Volkan, 2015).

In *Drosophila*, ORNs are located in two appendages: the maxillary palps and antennae, both of which are covered with hair-like structures called sensilla. Each sensillum houses 1-4 ORNs, which acquire their specific identities during the larval and pupal stages through a sequential expression of different transcription factors (Barish & Volkan, 2015; Jefferis et al., 2007; Tai et al., 2021). Following sensilla formation and identity acquisition, around 1300 ORNs need to establish precise connections with glomeruli in the antennal lobe (AL) and subsequently with their PN targets (Gavrish et al., 2024).

The events leading to the midline crossing of ORN axons in the *Drosophila* brain are depicted schematically in Figure 1.7b, with the ORN axon bundle in the adult brain shown in Figure 1.7a. Antennal nerves enter the AL approximately 20 hours after puparium formation (APF), guided by Dscam and Robo. At this stage, the Semaphorin-2b (Sema-2b)/Plexin-B signaling pathway splits axons to two major bundles surrounding the AL. By around 22 hours APF, antennal nerve axons begin targeting their respective glomeruli. Midline crossing progresses under the influence of key guidance molecules, including Dscam, Robo, Sema-2b, and teneurins. Maxillary palp axons enter the AL around 30 hours APF. At this stage, glomerular compartmentalization begins under the control of N-cadherin (Ncad) and Semaphorin-1a (Sema-1a). Distinct boundaries between glomeruli are established, and projection neuron (PN) connections are formed (Gavrish et al., 2024).

Commissural neurons play a critical role in the proper formation of ORN connections during development. Defects in this process often result in strong phenotypes, demonstrating the importance of precise guidance mechanisms. As the largest commissural neuron bundle in the *Drosophila* brain, ORN commissural axons can serve as an effective model for studying midline crossing processes, such as those observed in the CC of humans. Their well-defined

development and reliance on evolutionarily conserved signaling pathways make ORN commissural axons a valuable model for understanding midline guidance and the genetic and molecular mechanisms underlying commissural connectivity.

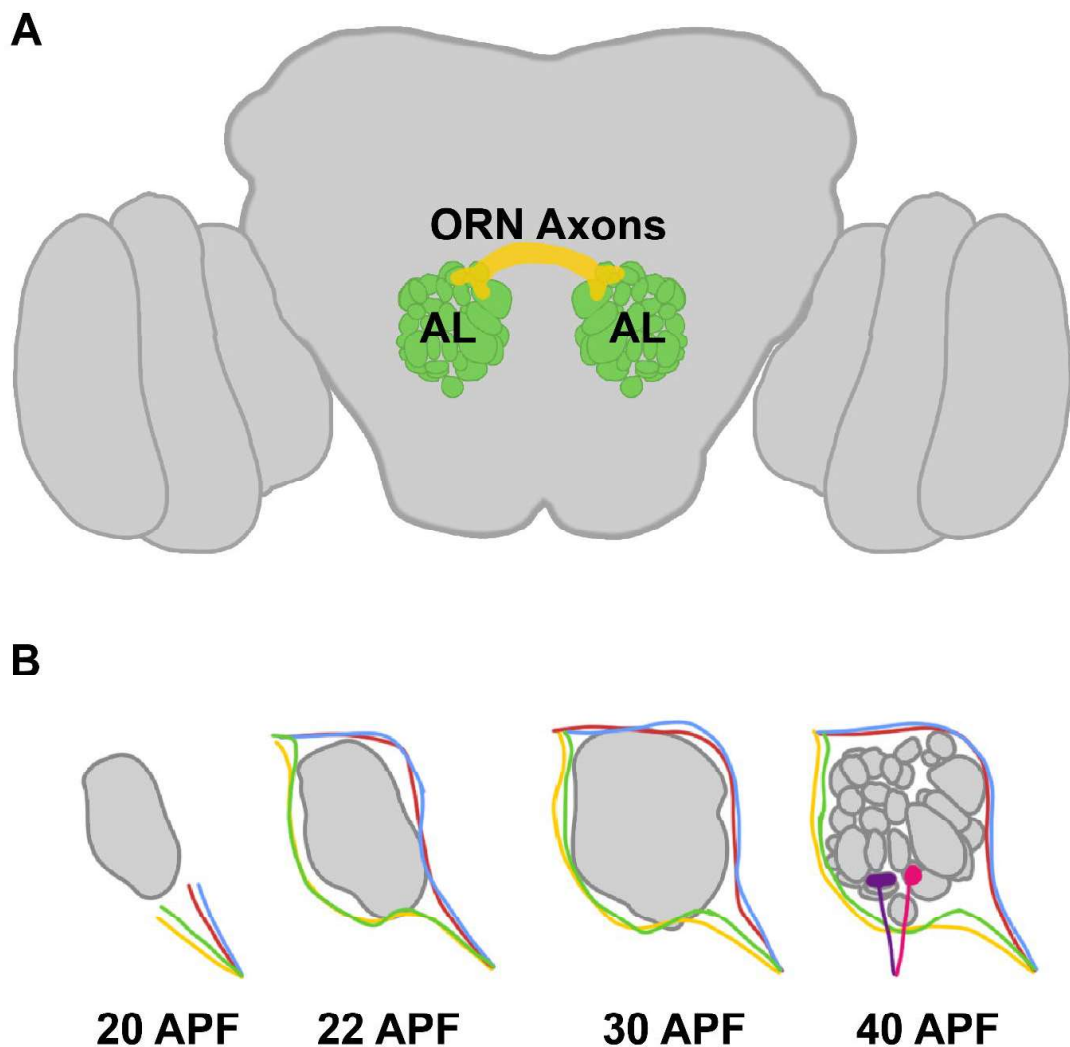


Figure 1.7. Development and morphology of olfactory receptor neuron (ORN) axons in *Drosophila*. (A) Schematic representation of the *Drosophila* brain showing the antennal lobes (AL) and ORN axons. ORN axons (yellow) project to the AL (green). (B) ORN axon guidance during development, visualized at different hours after puparium formation (APF). (Adapted with permission from Barish and Volkan, 2015).

1.5. Mushroom body as Learning and Memory Center of *Drosophila*

The ability to respond appropriately to environmental cues is critical for survival, as organisms must learn and implement responses quickly and efficiently. In *Drosophila melanogaster*, the mushroom body (MB) is the central brain region responsible for integrating sensory input, learning based on this input, and regulating sleep (Li et al., 2021).

The mushroom body forms from unipolar neurons called Kenyon cells, which are activated by sensory input from ORN. Since ORN lineages vary across individual flies, each ORN and its corresponding projection neuron (PN) interact with different Kenyon cells. This variability necessitates that each fly learns how to respond to specific sensory inputs, as the information relayed to each Kenyon cell is not fixed (Aso et al., 2014).

There are around 2000 Kenyon cells in the *Drosophila* brain. Their dendrites form a structure called the calyx, and their axons form the mushroom body itself, giving rise to distinct lobes. The MB arises from four neuroblasts in each hemisphere, which begin dividing during the embryonic stage and continue until the late pupal stage. Through these divisions and axonal projections, distinct morphological structures of the MB are formed. The MB comprises both horizontal (medial) and vertical lobes. The horizontal (medial) lobes include the γ , β , and β' lobes, whereas the vertical lobes include the α and α' lobes. Out of seven types of Kenyon cells, five form the main calyx, which processes sensory input. There are also ventral and dorsal accessory calyces that contribute to the γ dorsal cells and α/β posterior cells; however, these accessory calyces do not receive input from ORNs (Aso et al., 2014; Kunz et al., 2012).

The *Drosophila* MB has long been studied, and it is known to rely on developmentally critical genes and axon-guidance molecules for the proper formation of its complex structure. These molecular components ensure the precise development and organization of the MB, a brain region essential for sensory integration, learning, and memory. Due to its central role in learning and its strictly regulated architecture with high neural plasticity, the MB may serve as a valuable model for understanding the mechanisms underlying intellectual disabilities (Li et al., 2021; Lin, 2023).

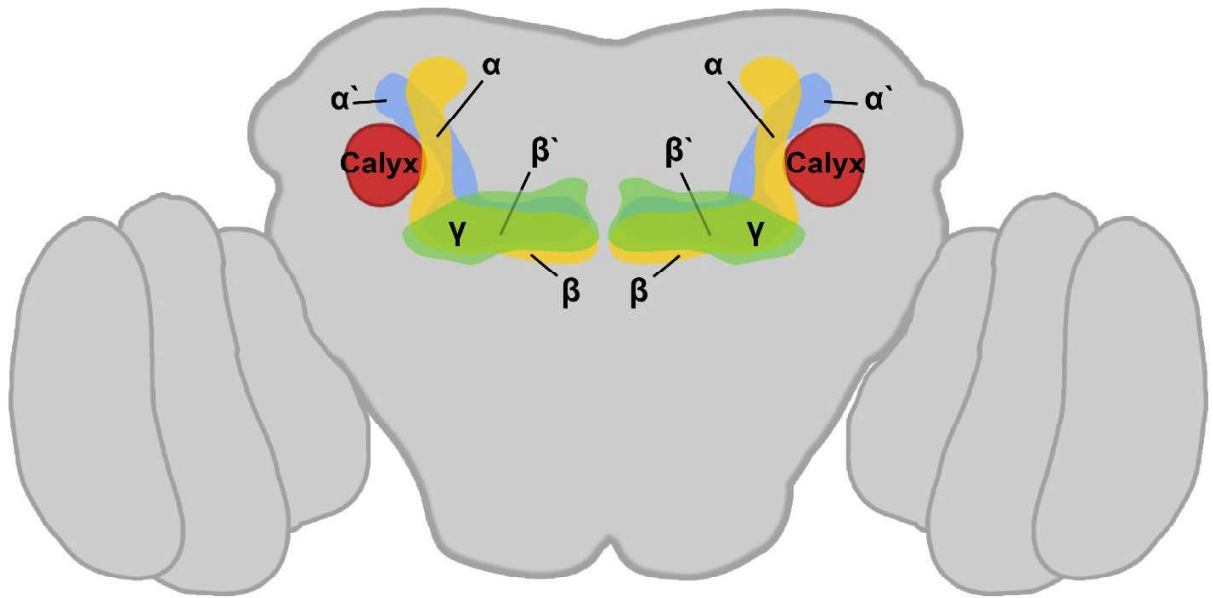


Figure 1.8. Mushroom body structure in the adult *Drosophila* brain. Schematic illustration of the mushroom body, a key center for learning and memory in *Drosophila*. The structure includes: γ lobes (green): Central lobes of the mushroom body. α and β lobes (yellow): Major lobes extending dorsally and medially. α' and β' lobes (blue): Accessory lobes branching from the α and β lobes. Calyces (red): Input regions receiving sensory information.

1.6. Wing Development and Morphology of *Drosophila*

The *Drosophila* wing is a well-established model system for studying various biological processes, including development, cell polarization, and degeneration. Its simple yet highly organized structure makes it an ideal framework for investigating these processes.

The *Drosophila* wing features veins that extend from the proximal body to the distal wing tip. These veins are categorized into: longitudinal veins (LVs) are horizontal, elongated veins named numerically (e.g., LV1, LV2). There are also several crossveins. The humeral crossvein connects longitudinal veins near the wing base. Anterior and posterior crossveins connect longitudinal veins further toward the distal wing.

Studies on cell polarization often utilizes bristle morphology and directionality on the wing surface. The bristles' consistent orientation and patterns provide an excellent model for understanding how cells establish and maintain polarity. Vein axons within the wing are a primary focus in degeneration studies. These studies provide insights into axonal maintenance, degeneration mechanisms, and the molecular pathways underlying neurodegenerative conditions (Carreira et al., 2011).



2. AIM OF THE STUDY

The *spastic paraplegia gene 11* has been associated with various neuropathies, including hereditary spastic paraplegia, amyotrophic lateral sclerosis, and Charcot-Marie-Tooth disease. These motor neuron disorders share overlapping symptoms, such as motor neuron degeneration, movement difficulties, and muscle weakness. *SPG11* encodes spatacsin, a transmembrane protein with potential scaffold functions that plays critical roles in axon extension, axonal transport, and the organization of lysosomes and autophagosomes.

CG13531 is the predicted ortholog of the human *SPG11* gene in *Drosophila melanogaster*. While *CG13531* shares significant structural and functional similarities with *SPG11*, the functional equivalence of these two genes remains to be conclusively demonstrated.

This study aims to characterize the knockout mutants of the *CG13531* gene in *Drosophila* and establish a model that can potentially recapitulate the symptoms associated with *SPG11*-related neuropathies. To achieve this, I examined the morphological and behavioral phenotypes observed in the absence of *CG13531*, providing insights into the cellular and physiological roles of this gene. By developing this model, we aim to create a foundation for further studies exploring the mechanisms of *SPG11*-related neuropathies and potential therapeutic interventions.

3. MATERIALS AND METHODS

3.1. Biological Materials

Biological materials used in this study, including fly lines and their respective markers and balancer chromosome are listed in Table 3.1.

Table 3.1. Fly lines, markers, and balancers used in this study.

Name of line	Chr. No.	Description
Gal4 Drivers		
<i>Act5-Gal4</i>	2	Ubiquitous expression of Gal4 under the control of <i>actin promoter</i>
<i>Act5-Gal4</i>	3	Ubiquitous expression of Gal4 under the control of <i>actin promoter</i>
<i>Elav-Gal4</i>	1	Pan-neuronal expression of Gal4 under the control of <i>elav promoter</i>
<i>D42-Gal4</i>	3	Expression of Gal4 in all motor neurons and interneurons.
<i>TubP-Gal4</i>	3	Ubiquitous expression of Gal4 under the control of <i>tubulin promoter</i>
<i>Dpr-Gal4</i>	2	Expression of Gal4 in a subset of sensory neurons.
UAS Constructs		
UAS- <i>CG13531</i> -RNAi	2	Expresses RNAi for <i>CG13531</i> knock-down
BL36304	2	RNAi background line with attP40 docking site integrated within or upstream of <i>Msp300</i> gene.
UAS- <i>mCD8::RFP</i>	2	Expresses membranous RFP.
UAS- <i>Dicer2</i>	3	Expresses <i>Dicer2</i> under the control of UAS
General Stocks		
<i>yw</i>	1	Mutant alleles of <i>yellow</i> and <i>white</i> genes
<i>w¹¹¹⁸</i>	1	Mutant allele of <i>white</i> gene

Table 3.1. Fly lines, markers, and balancers used in this study (cont.).

<i>yw;CG13531^{CRIMIC}/CyO, Tb</i>		<i>CG13531</i> loss of function line with CRIMIC construct, balanced with CyO,Tb
<i>yw;CG13531^{CRIMIC}/CyO; TM2/TM6B</i>		<i>CG13531</i> loss of function line with CRIMIC construct, balanced with CyO;TM2/TM6B
<i>w; Df(2R)BSC599/CyO, Tb</i>		Deficiency line with deletion on 2 nd chr, including <i>CG13531</i> , balanced with CyO,Tb
<i>w;If/CyO, Tb; Dp(2;3)GV-CH321-21J20/TM6B</i>		Interchromosomal duplication line, partial segment of 2 nd chr inserted on 3 rd chr
Balancers and Markers		
<i>Sp</i>	2	Marker, supernumerary bristles
<i>If</i>	2	Marker, squished eyes
<i>Tb</i>	2	Marker, tubby larvae
<i>Cu</i>	2	Marker, curly wings
<i>Sna[Sco]</i>	2	Marker, misshaped thorax bristles
<i>CyO</i>	2	Balancer chromosome with Curly and/or Tubby markers
<i>Tb</i>	3	Marker, tubby larvae
<i>Hu</i>	3	Marker, supernumerary bristles
<i>Ubx</i>	3	Marker, large haltere with bristles
<i>Sb</i>	3	Marker, stubble bristles
<i>MKRS</i>	3	Balancer chromosome with Stubble marker
<i>TM2</i>	3	Balancer chromosome with Ultrabithorax marker
<i>TM3</i>	3	Balancer chromosome with Stubble marker
<i>TM6B</i>	3	Balancer chromosome with Humeral and Tubby markers
<i>w;Sna[Sco]/CyO, Tb</i>		Flies carrying indicated balancer chromosomes, and markers
<i>yw⁶⁷; ; TM2/TM6B</i>		Flies carrying indicated balancer chromosomes, and markers
<i>w;If/CyO, Tb; MKRS/TM6B</i>		Flies carrying indicated balancer chromosomes, and markers
<i>yw⁶⁷;Sp/CyO; TM2/TM6B</i>		Flies carrying indicated balancer chromosomes, and markers

3.2. Chemicals and Supplies

3.2.1. Chemicals and Kits

Chemicals and kits used in this study are shown in Table 3.2.

Table 3.2. List of the chemicals and kits.

Chemical	Manufacturer
1 kb marker	NEB, USA (N3232L)
100 kb marker	NEB, USA
Agarose	GeneOn, USA (604,001)
Bovine Serum Albumin	Sigma-Aldrich, USA (A9647)
cDNA Synthesis Kit	Nepenthe, Turkey (NP041011610)
Chloroform	
DNase Kit	NEB, USA (M0303L)
EDTA	Sigma-Aldrich, USA (59417C)
Vecta Shield	Vector Laboratories
Ethidium Bromide	Sigma Life Sciences, USA (E1510)
Isopropanol	Sigma-Aldrich (W292907)
KCl	Sigma-Aldrich (29676)
KOAc	Sigma-Aldrich (P1190)
LiCl	Sigma-Aldrich (L9650)
NaCl	Sigma-Aldrich, USA (S7653)
Normal Goat Serum (NGS)	Millipore (S26-100ML)
One-Taq DNA Polymerase	NEB, USA (M0480)
Paraformaldehyde	Sigma-Aldrich, USA (P6148)
Phenol:chloroform:Isoamyl alcohol	Sigma-Aldrich, USA (P2069)

Table 3.2. List of the chemicals and kits (cont.).

Proteinase K	Roche, Germany (139963000)
AccuPower 2X Greenstar qPCR Master Mix	Bioneer, Korea (K6251)
Tris	Sigma-Aldrich, USA (T6066)
Triton-X 100	AppliChem, USA (A4975)

3.2.2. Buffers and Solutions

Buffers and solutions used in experiments are shown in Table 3.3.

Table 3.3. List of the buffers and solutions.

Buffer	Ingredients
HL3	
PBS	137 mM NaCl 2.7 mM KCl 10 mM Na ₂ HPO ₄ 1.8 mM KH ₂ PO ₄
0.3% PBS-T	PBS (1x) 0.3% Triton X-100
0.5% PBS-T	PBS (1x) 0.5% Triton X-100
0.05% PBS-T	PBS (1x) 0.05% Triton X-100
PFA	4% PFA in 1X PBS, pH 7.4

Table 3.3. List of the buffers and solutions (cont.).

PAXD	50 g BSA 3 g Sodium Dexoycholate 0.3% Triton X-100 In PBS
TAE Buffer (1X)	40 mM Tris-Cl 1mM EDTA 0.1% Acetic acid
Squish Buffer	10 mM Tris, pH 8.0 1 mM EDTA 25 mM NaCl
Buffer A	100 mM Tris, pH 7.5 100 mM EDTA 100 mM NaCl 0.5% SDS

3.2.3. Primers

Primers which are used in this study are shown in Table 3.4.

Table 3.4. List of the primers.

Primer Name	Sequence (5'>3')	Tm
<i>CG13531_KG4_F1</i>	GTGCTAGAAAGGGATGGGGC	64.4
<i>CG13531_R1</i>	CCAAGTGTTGATTTC AATTTCAGG	61.1
<i>CG13531_EGFP_F1</i>	CTGGGCATGGATGAGCTGTA	63.7
<i>CRIMIC_ch_for</i>	GTGGTATGGCTGATTATGATCAGAA G	49
<i>CG13531_qPCR_F1</i>	AGCGTCATTGGAGTGCGTT	57.5
<i>CG13531_qPCR_R1</i>	CCCTGAAGCACAACGAATTG	58.4
<i>GAPDH1_Forward</i>	TAAATTCGACTCGACTCACGGT	57

Table 3.4. List of the primers (cont.).

<i>GAPDH1_Reverse</i>	CTCCACCACATACTCGGCTC	57.5
<i>CG3649_qpcr_F9</i>	CCAAGTGGTGTCCCAAGAAG	62.5
<i>CG3649_qpcr_R9</i>	ATGTGCTTTCGTTCCGCCAG	65.5

3.2.4. Antibodies and Dyes

Antibodies which are used in this study are shown in Table 3.5.

Table 3.5. List of the antibodies.

Name	Antigen	Host	Dilution	Source
Primary Antibodies				
Anti-Elav	Elav	Rat	1:50	DSHB
Anti-Repo	Repo	Mouse	1:50	DSHB
Anti-dsRed	GFP	Rabbit	1:1000	DSHB
Anti-FasII	Fasciclin II	Mouse	1:20	DSHB
Anti-B-gal	B-galactosidase	Rabbit	1:100	Cell Signaling
Anti-HRP	Horseradish peroxidase	Rabbit	1:2000	
Anti-Dlg	Discs large	Mouse	1:100	DSHB
Anti-Futsch	Futsch	Mouse	1:50	DSHB
Anti-Fmi	Flamingo	Mouse	1:200	DSHB
Anti-N-Cad	Cadherin, DN	Rat	1:200	DSHB
Secondary Antibodies				
Alexa Fluor 488- conjugated anti-Rabbit	Rabbit IgG H & L	Goat	1:800	Invitrogen
Alexa Fluor 555- conjugated anti-Mouse	Mouse IgG H & L	Goat	1:800	Invitrogen

Table 3.5. List of the antibodies. (cont.)

Alexa Fluor 555-conjugated anti-Rat	Rat IgG H & L	Goat	1:800	Invitrogen
Alexa Fluor 647-conjugated anti-Mouse	Mouse IgG H & L	Goat	1:800	Invitrogen
Alexa Fluor 647-conjugated anti-Rabbit	Rabbit IgG H & L	Goat	1:800	Invitrogen

3.3. Equipment

3.3.1. Disposable Labware

Disposable labware which are used in this study are shown in Table 3.6.

Table 3.6. List of the disposable labware.

Material	Manufacturer
Falcon Tubes (15 - 50) ml	Becton, Dickinson and Company, USA
Microscope cover glass	VWR, USA
Microscope slides	Fisher Scientific, UK
Micropipette Tips (10-200-1000-5000 μ l)	VWR, USA
Microcentrifuge tubes	Citotest Labware Manufacturing, China
PCR tubes (200 μ l)	Bio-Rad, USA
Plastic Pasteur pipette	TPP Techno Plastic Products AG, Switzerland

Table 3.6. List of the disposable labware (cont.).

Syringe (1cc)	Becton, Dickinson and Company, USA
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3.3.2. Equipment

Equipment which are used in study is shown in Table 3.7.

Table 3.7. List of the equipment.

Equipment	Manufacturer
Autoclave	Astell Scientific Ltd., UK
Centrifuges	Eppendorf, Germany (Centrifuge 5424, 5417R)
Cold Room	Birikim Elektrik Soğutma
Confocal Microscope	Leica Microsystems, USA (TCS SP5)
Electrophoresis Equipment	Bio-Rad Labs, USA
Freezers	Arçelik, Turkey
Gel Documentation System	Bio-Rad Labs, USA (Gel Doc XR)
Heating Block	Fisher Scientific, France
Heating Magnetic Stirrer	IKA, China (RCT Basic)
Incubator	Weiss Gallenkamp, USA (Incubator Plus Series)
Laboratory Bottles	Isolab, Germany
Micropipettes	Eppendorf, Germany
Microwave oven	Vestel, Turkey
Nutator	

Table 3.7. List of the equipment (cont.).

pH meter	WTW, Germany (Ph330i)
Refrigerators	Arçelik, Turkey
LightCycler480	Roche
Stereo Microscope	Olympus, USA (SZ61)
Thermal Cycler	LongGene, China
Vortex Mixer	Scientific Industries, USA (Vortex Genie2)

3.4. Fly Husbandry

Flies were maintained at 25°C and 40% humidity in the culture vials with ~10 mL food inside 12-hour light-dark cycle incubators. Fly food consists of dH₂O, 0.5% (w/v) agar, 4% (w/v) corn flour, 2% (w/v) yeast, and 6.25% (w/w) molasses or 4.5% (v/w) sugar. 0.5 g/L Nipagin and 5 mL/L propionic acid were added to prevent any microbial or fungal contamination.

3.5. Histological Techniques

3.5.1. Adult Brain Immunohistochemistry

Adult brains were dissected in cold PBS and fixed in 4% PFA for 20 minutes at room temperature. The samples were washed with 0.3% PBS-T 3 times for 20 minutes and then incubated in PAXD as a blocking solution for 1 hour at room temperature. Antibody dilutions were prepared in PAXD and incubation with primary antibody was done overnight at 4°C on a nutator. Next day the brains were washed 3 times with 0.3% PBS-T for 20 minutes at room

temperature. Secondary antibody dilutions were prepared in PAXD and secondary antibody incubation was performed overnight at 4°C on a nutator. Later, the brains were washed 3 times with 0.3% PBS-T, and once with PBS for 10 minutes. Finally, the brains were mounted in Vectashield on the slides.

3.5.2. Pupal Brain Immunohistochemistry

Pupae were selected according to their stages. Pupal cases were opened, brains were dissected in cold PBS and fixed in 4% PFA for 30 minutes at room temperature. The samples were washed with 0.5% PBS-T 3 times for 20 minutes and then incubated with PAXD for 1 hour at room temperature. The brains were incubated with the primary antibody in PAXD was done overnight at 4°C on a nutator. The following day, washes were done 3 times for 20 minutes at room temperature 0.5% PBS-T. Secondary blocking was performed with 10% NGS for 1 hour at room temperature, and secondary antibody dilutions were prepared in 10% NGS. Then the brains were incubated with the secondary antibody overnight at 4°C. Later, the brains were washed 3 times with 0.5% PBS-T and once with PBS for 10 minutes. Finally, brains were mounted in Vectashield on the slides.

3.5.3. Larval Neuromuscular Junction Immunohistochemistry

Larvae in 3rd instar stage were collected and briefly washed in HL3 buffer. Larval fillets were dissected and fixed with methanol on dissection pad for 5 minutes. After, 3 brief washes were done with HL3 on the dissection pad. Samples were then transferred to 0.05% PBS-T in Eppendorf tubes. After a 5 minutes wash with PBS-T, the fillets were blocked with 5% NGS solution and incubated with the secondary antibody on a nutator overnight at 4°C. Next day, 3 20 minutes washes followed by a 4 hour wash with 0.05% PBS-T were performed at room temperature. Secondary antibody dilutions were prepared in 5% NGS and samples were incubated with the secondary antibody overnight at 4°C. Following day, 3 washes with 0.05% PBS-T were performed at room temperature and an overnight PBS-T wash at 4°C was done.

Next day, the samples were briefly washed with HL3 and mounted in Vectashield on the slides.

3.5.4. Wing Assays

The adults' wings were dissected and washed for 5 minutes at room temperature with 0.3% PBS-T in a cuvette. The wings were then collected from the cuvette and mounted in Vectashield on slides.

3.5.5. Imaging and Visualization

Mounted tissues were visualized with Leica fluorescent confocal microscope and the images were processed using ImageJ Fiji.

3.6. Molecular Biology Techniques

3.6.1. Single Fly DNA Extraction

Adult flies were collected in Eppendorf tubes (one fly per tube) and frozen at -20°C for 40 minutes. 1 μ L of 10 mg/mL Proteinase K was added for each 50 μ L Squish Buffer, and 50 μ L of buffer with Proteinase K was added to each Eppendorf tube. After homogenization for 1 minute, the samples were incubated at 37°C for 1 hour. Then Proteinase K was heat -inactivated by incubation at 95°C for 5 minutes. The samples were briefly centrifuged and stored at -20°C.

3.6.2. RNA Extraction

Adult flies (8-10) were collected in Eppendorf tubes, frozen at -80°C and homogenized with a pestle before they got thawed. 400 µL of TRIZOL was added two times to the tubes, and homogenization was performed each time on ice. After that, homogenization continued using a syringe with a 20-gauge needle on ice for 1 minute. The samples were incubated at room temperature for 5 minutes; then 160 µL chloroform was added, and tubes were vigorously shaken and left for 3 more minutes. Samples are centrifuged at 13200 rpm for 15 minutes at 4°C. The transparent upper aqueous phases were collected from each tube and transferred to the new tubes. Incubation with 400 µL cold isopropanol was performed for 10 minutes at room temperature, followed by a centrifugation at 13200 rpm (15584 g) for 15 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 75% ethanol. Another centrifugation at 13200 rpm for 15 minutes at 4°C was performed. The supernatant was carefully discarded from each tube, and the tubes were left to air dry for approximately 10 minutes at room temperature. The pellets were then dissolved in 20 µL RNase-free water at 55°C for 10 minutes. The samples were stored at -80°C until use.

3.6.3. Polymerase Chain Reaction for Genotyping of *CG13531*

The verification of the CRIMIC cassette presence in *CG13531* gene genomic location was done using PCR. The PCR mixture was prepared in nuclease-free water and consisted of 10µM forward primer, 10µM reverse primer, 10 mM dNTP, 2µL/10 µL reaction of DNA (from single fly DNA extraction), 1X One-Taq standard buffer, and 0.1 U/µL reaction of One-Taq enzyme. Standard PCR cycling conditions were followed, but extension time was adjusted according to the size of the products.

To amplify the 5' end of the cassette, which contained the Kozak-Gal4 sequence and the upstream chromosome region *CG13531_KG4_F1* and *CG13531_R1* primers were used. The reaction conditions were as follows: 3 minutes of initial denaturation step at 95° C followed by 39 cycles of 30 seconds of denaturation at 95° C, 30 seconds of annealing at 60°C, and extension

at 68° C for 1 minute. The final extension was performed for 5 minutes at 68° C, then the reaction was terminated and stored at -20°C.

To amplify the 3' end of the cassette, which contained the 3XP3-EGFP sequence, and the downstream chromosome region *CG13531*_EGFP_F1 and CRIMIC_ch_ for primers were used. The reaction conditions were as follows: 3 minutes of initial denaturation step at 95° C followed by 39 cycles of 30 seconds of denaturation at 95° C, 30 seconds of annealing at 57°C, and extension at 68° C for 1.5 minutes. The final extension was performed for 5 minutes at 68° C, and the reaction was terminated and stored at -20°C.

3.6.4. Gel Electrophoresis

1% Agarose gel was prepared with 1X TAE buffer by boiling, 2 µL of 50 ng/µL EtBr was added for each 10 mL of gel when the temperature of the gel was ~50°C. Then, the gel was cast into the tray with an already-placed comb. DNA Ladder and PCR products mixed with loading dye and loaded on the gel. The electrophoresis was performed for 30 minutes at 120 V, and the gel was imaged using GelDoc.

3.6.5. DNase Treatment and cDNA Synthesis

DNase kit was used according to the manufacturer's instructions. 1 µg RNA was treated with a DNase buffer mixture containing 1U DNase, 1X buffer, and RNase-free water. Incubation is done for 15 minutes at 37°C. Then 1 µL of 0.05M EDTA was added to the solution, and the enzyme was inactivated for 30 minutes at 70°C.

cDNA synthesis is performed according to the manufacturer's instructions. To ensure the maximum amount of RNA template is present, 4 µL of DNase-treated RNA was mixed with 1 µL of enzyme mixture and 5 µL of the buffer provided by the kit.

3.6.6. Relative Gene Expression with RT-qPCR

A reaction master mix was prepared for each primer pair. 1X qPCR mix, 0.2 μ M of forward and 0.2 μ M of reverse primers, and nuclease-free water were present for each reaction. *GAPDH1* was used as a reference gene. *CG13531*_qPCR_F1 and *CG13531*_qPCR_R1 primers were used for *CG13531* and GAPDH1_F1 and GAPDH1_R1 primers for *GAPDH1*. The reaction master mix was aliquoted into individual wells of 96-well qPCR plates. After that, 1 μ L of 100 ng/ μ L cDNA and RNA templates was added to the wall of each well. Plates were centrifuged briefly, and the RT-qPCR program started.

For *CG13531* and *GAPDH1*, 3 minutes of initial denaturation step at 95° C followed by 35 cycles of 30 seconds of denaturation at 95° C, 30 seconds of annealing at 58° C and 30 seconds of extension at 72° C. Fluorescence measurement was performed at the end of each extension step and after all cycles were completed, a gradual increase of temperature from 60° C to 95° C was performed to obtain the melting curve.

3.7. Functional Analysis of *CG13531*

3.7.1. CRIMIC Construct as a Knockout Method

Clustered Regularly Interspaced Palindromic Repeats-Mediated Integration Cassette (CRIMIC) method is a genome editing technique that makes use of CRISPR/Cas9 method. A universal exon trapping technique is integrated with binary expression techniques and fluorescent markers. Basically, the gene of interest is replaced with the CRIMIC construct, and this construct can facilitate the transcription of the *GAL4* gene under the promoter of the gene of interest. At the same time, GFP is expressed in the eyes of the flies as a selection marker (Figure 3.1) (Kanca *et al.*, 2022).

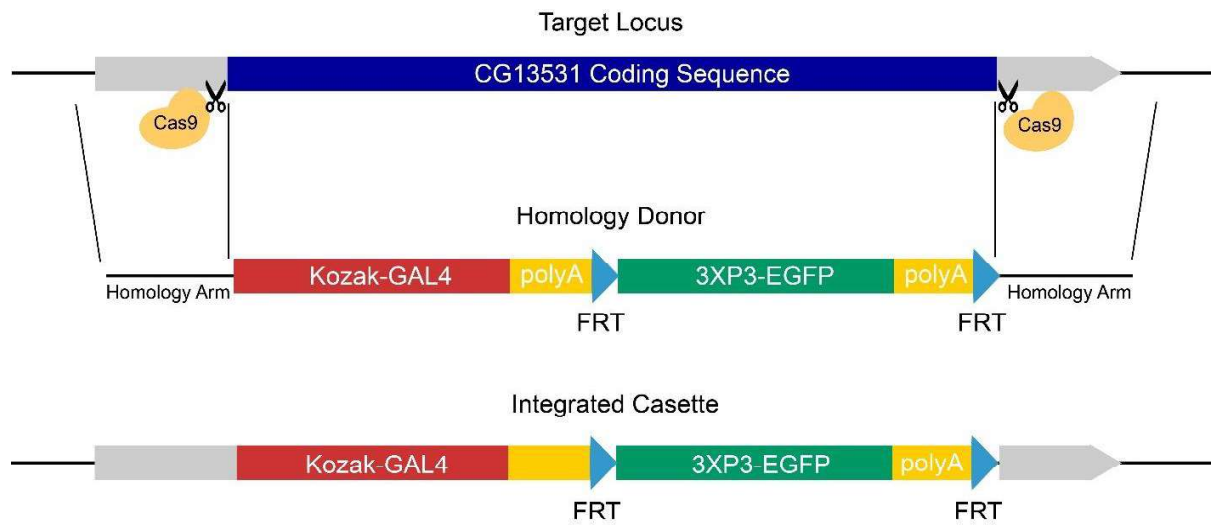


Figure 3.1. CRIMIC construct and knockout mutant generation. Target Locus (*CG13531* coding sequence) shown in blue is flanked by targeted Cas9 cleavage sites. Homology Donor cassette contains: Kozak-GAL4 (red) drive GAL4 expression, 3XP3-EGFP (green) is visible marker for integration. polyA (yellow) ensure transcriptional termination, and FRT sites (blue triangles) enable excision. Integrated Cassette illustrates Cas9-mediated cleavage and homologous recombination of the donor cassette disrupting the *CG13531* coding sequence.

The *CG13531* knockout mutants generated by Bellen Lab with this CRIMIC construct (Figure 3.1) were used in the experiments after being balanced (Figure 3.2). While in the most of experiments the flies with homozygous *CG13531*^{-/-} allele on the 2nd chromosome which didn't carry any balancer on the 3rd chromosome (see F1 in Figure 3.2 B) were used, in the few experiments they did carry TM2 and TM3B 3rd chromosome balancers (See F1 in Figure 3.2. A). As a control group, genetic background *yw* line was used. In cases where the experiment group carried 3rd chromosome balancers, *yw*; ; *TM2/TM6B* line was used as an additional control.

A

P ♂ $yw; \frac{CG13531^{CRIMIC}}{CyO}; \frac{TM2}{TM6B}$ **x** $yw; \frac{CG13531^{CRIMIC}}{CyO}; \frac{TM2}{TM6B}$ ♀

F1 $yw; CG13531^{CRIMIC}; \frac{TM2}{TM6B}$

B

P ♂ $yw; \frac{CG13531^{CRIMIC}}{CyO, Tb};$ **x** $yw; \frac{CG13531^{CRIMIC}}{CyO, Tb};$ ♀

F1 $yw; CG13531^{CRIMIC};$

Figure 3.2. Crosses for generating homozygous knockout mutants.

3.7.2. Tissue-Specific Knockdown Experiments

Tissue-specific knockdown experiments were conducted to understand and determine the role of *CG1531*. In each knockdown experiment, as a control, the same driver line was crossed to the line BL36304, which carried the same attP integration site as *CG1531* RNAi line but does not have any integration to the site.

Act-Gal4-driven and *Tub-Gal4*-driven knockdowns of *CG1531* (Figure 3.3 A and B, respectively) were performed to evaluate the efficiency of the RNAi line.

A

P ♂ ; *UAS-CG13531-RNAi* / CyO ; X ; *act5-Gal4* / CyO ; ♀

F1 ; *UAS-CG13531-RNAi* / *act5-Gal4* ;

B

P ♂ ; *UAS-CG13531-RNAi* / CyO ; X *w* ; *Tub-Gal4* / TM6B ; ♀

F1 *w* / + ; *UAS-CG13531-RNAi* / + ; *Tub-Gal4* / +

Figure 3.3. The schemes of the crosses for the whole body knockdown of *CG13531*.

D42-Gal4- (Figure 3.4 A) and *Elav-Gal4*-driven (Figure 3.5) knockdown animals were subjected to climbing activity assay to characterize the motor neuron-specific or pan-neuronal effects of *CG13531* downregulation. Additionally, NMJ morphology in the animals with *D42-Gal4*-driven knockdown of *CG13531* was investigated. However, homozygous parents are used in that case to ensure larval selection will not fail since parent lines did not carry any larval selection marker (Figure 3.4 B).

A

P ♂ ; $\frac{UAS-CG13531-RNAi}{CyO}$; **X** $w; \frac{Sp}{CyO}; \frac{D42-Gal4}{TM2}$ ♀

F1 $\frac{w}{+}; \frac{UAS-CG13531-RNAi}{Sp}; \frac{D42-Gal4}{+}$

B

P ♂ ; $UAS-CG13531-RNAi$; **X** $w; \frac{Sp}{CyO}; D42-Gal4$ ♀

F1 $\frac{w}{+}; \frac{UAS-CG13531-RNAi}{Sp \text{ or } CyO}; \frac{D42-Gal4}{+}$

Figure 3.4

Figure 3.4. The schemes of the crosses for the motor neuron knockdown of *CG13531*.

Dpr-Gal4-driven knockdown of *CG13531* combined with *UAS-mCD8::RFP* reporter expression was used to evaluate the neurodegeneration in the fly wing (Figure 3.6). While *Dpr-Gal4* ensured knockdown specific to wing neurons, the RFP reporter allowed direct imaging of these neurons in the wings without any further procedures.

P ♂ ; $\frac{UAS-CG13531-RNAi}{CyO}$; **X** $Elav-Gal4$; ♀

F1 $\frac{Elav-Gal4}{+}; \frac{UAS-CG13531-RNAi}{+}$;

Figure 3.5. The scheme of the cross for the pan-neuronal knockdown of *CG13531*.

$$\begin{array}{l}
 \text{P} \quad \text{♂} ; \frac{UAS-CG13531-RNAi}{CyO} ; \quad \times \quad ; \frac{Dpr-Gal4, UAS-mCD8::RFP}{CyO} ; UAS-dicer2 \quad \text{♀} \\
 \\
 \text{F1} \quad ; \frac{Dpr-Gal4, UAS-mCD8::RFP}{UAS-CG13531-RNAi} ; \frac{UAS-dicer2}{+}
 \end{array}$$

Figure 3.6. The scheme of the cross for the wing sensory neuron knockdown of *CG13531*.

3.7.3. Complementation Analysis of *CG13531*- generated by CRIMIC Method Using Deficiency Line Lacking *CG13531*

For the complementation experiments, the deficiency line BSC599 with deletion of 2R:22798321;2R:22854179 region on the 2nd chromosome was crossed to the *CG1351* knockout line generated by the insertion of the CRIMIC construct. Both alleles lack *CG13531* gene. This cross (Figure 3.7) was performed to evaluate whether the deficiency allele could not complement *CG13531*^{CRIMIC} manifestations, thus proving that the phenotypes observed in the *CG13531*^{CRIMIC} line were caused by genetic defects mapped to the same chromosomal region as the one missing in the deficiency line.

$$\begin{array}{l}
 \text{P} \quad \text{♂} \quad yw; \frac{CG13531^{CRIMIC}}{CyO, Tb} ; \quad \times \quad yw; \frac{Df(2R)BSC599}{CyO, Tb} ; \quad \text{♀} \\
 \\
 \text{F1} \quad yw ; \frac{Df(2R)BSC599}{CG13531^{CRIMIC}} ;
 \end{array}$$

Figure 3.7. The scheme of the cross for generating a transheterozygous mutant with *CG13531*^{CRIMIC} and BSC599 lines which lacks *CG13531* on both alleles.

3.8. Behavioral Analysis of the Flies with *CG13531* Knockout and Knockdown

3.8.1. Startle-induced Negative Geotaxis Assay

Assay was performed as described in ref (). For this analysis, 10 males and 10 females were crossed and progeny of the cross is used (Figure N). After eclosion, the progeny was collected and aged either 5-7 or 25-27 days.

A sliding platform (Figure 3.8) positioned on top of a stand was utilized for the measurement. Six 15 mL Falcon tubes were attached to the upper side in an upside-down position. A corresponding number of removable 15 mL Falcon tubes were placed right-side up on a stable part of the platform. This setup allowed flies placed in lower tubes to climb into the corresponding tube of the upper section. By sliding the platform back and forth, flies that ascended to the top were transferred to the next tube on the bottom part.

Each tube used in the experiment was first acclimated with ten *yw* flies, comprising an equal number of males and females, for 1 minute. Following this acclimation period, a maximum of 30 flies of the same genotype were placed in the first lower tube and allowed to acclimate for one minute.

After the one-minute acclimation, the platform was tapped on the surface to startle the flies, causing them to fall to the tube on the bottom part. The flies then had 30 seconds to climb up. After this climbing period, the sliding platform was pushed to the right (Figure 3.8b), and the flies that had climbed to the top tube were startled again, causing them to fall to the bottom of the second tube; immediately after the flies fell, the sliding platform was set back to its original position by pushing it left (Figure 3.8a). This process was repeated five times, where flies without climbing deficiencies would typically reach the sixth and last tube. After the final round, the flies in each section were collected and counted. The room temperature was maintained at 25°C during the experiments, which were always conducted between 4 PM and 6 PM.

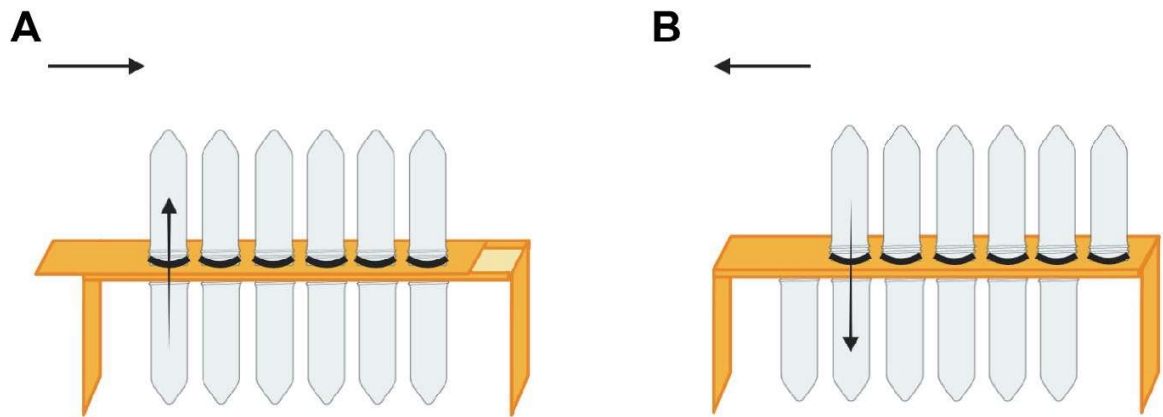


Figure 3.8. Sliding platform depiction used in startle-induced negative geotaxis assay. (A) Starting position: Flies are placed in vertical tubes aligned on a sliding platform. The initial position allows flies to respond to a mechanical stimulus by climbing upwards (indicated by the arrow). (B) Transfer position: The platform slides horizontally, enabling flies that have climbed to the top of the current tube to be transferred to the next tube for subsequent rounds.

A weighted average was employed as a measurement method, where tube one was assigned a score of zero and tube six received a score of one. The index was calculated by dividing the weighted average by the total number of flies in that experimental session.

3.8.2. Eclosion Rate Assay

For this analysis, 10 males and 10 females were used to start the population of each genotype in more than 3 biological replicates. The flies were let to mate for 5 days, and then the parents were transferred to another vial. After the first pupation event, pupae were counted for 4 days. After flies started to eclose, for another 4 days, flies were counted according to their genotype and sex and immediately collected from culture. At the end of eclosion events, the dead pupae number was counted, and their sex was identified and recorded. Dead pupa number in each group was then compared.

3.8.3. Survival Rate Assay

To generate the flies for this analysis 10 males and 20 females of the for each genotype were collected to the vial and let mate. At least 8 biological replicates were used for each genotype. On the first day of eclosion, the first flies that eclosed are discarded and for the next two days, the progeny was collected from the cultures and let mate for two days. On the 3rd day after collection, 30 mated flies of the same sex and genotype were sorted into the vials. After that, flies from each vial were transferred to the new food on Mondays, Wednesdays, and Fridays. The number of dead flies was counted and recorded during transfer.

4. RESULTS

4.1. Verification of CRIMIC Induced Knockout and GAL4-UAS Induced Knockdown of *CG13531*

The genotyping of the *CG13531*^{CRIMIC} allele was performed using PCR to confirm the presence of the *CRIMIC* construct. Primers specifically designed to recognize the 5'UTR and 3' UTR of the *CRIMIC* construct and its surrounding genomic location were used. Single flies of both sexes were carefully selected from different biological replicates of the stock to ensure accurate and reliable results. The protocol was optimized to minimize errors and maximize reproducibility.

For the 5'end of the construct, where the KOZAK-Gal4 sequence is located, successful verification was achieved for all samples except the RT-1 female. This was confirmed by the presence of a distinct 888 bp product band (Figure 4.1a). Similarly, the same DNA samples were genotyped for the 3' end of the construct, where EGFP was inserted, and verified in all samples, producing a 1329 bp product band (Figure 4.1b).

Although some unspecific binding resulted in additional product bands in the samples, including the negative control, these did not interfere with the overall interpretation of the results. The positive results for both the 5' and 3' ends of the construct confirm the successful integration of the *CRIMIC* construct into the *CG13531* locus, validating the effectiveness of the *CRIMIC*-induced knockout and the presence of the desired modifications.

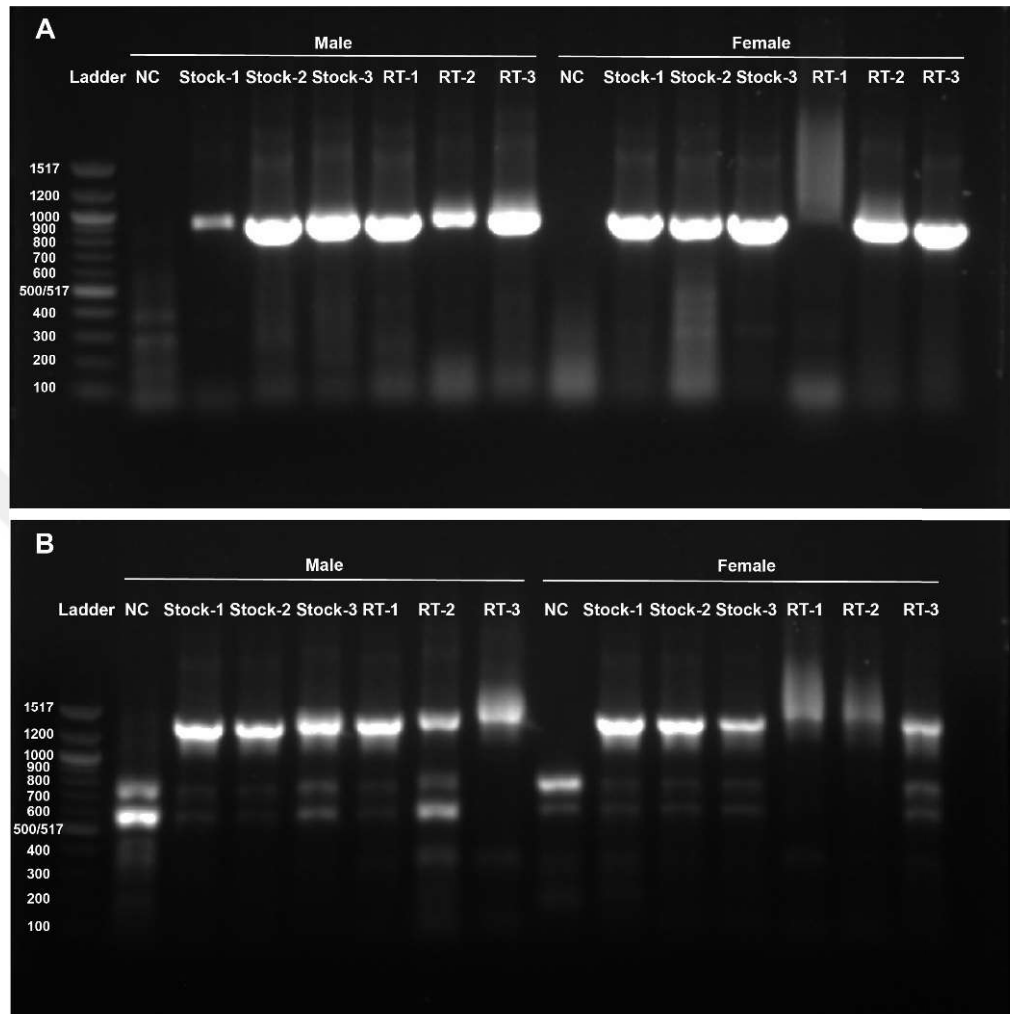


Figure 4.1. PCR products for the verification of the CRIMIC construct. Fly stocks kept at +18°C (Stock labels) and room temperature (RT labels). The *yw* genotype is used as a negative control (NC). (A) 5' end of the construct, where the KOZAK-Gal4 sequence (888 bp) is located. (B) 3' end of the construct where the EGFP sequence is inserted (1329 bp).

The relative expression levels of *CG13531* were analyzed to confirm the efficiency of both the CRIMIC-induced knockout (KO) and RNAi-mediated knockdown (KD) in flies (Figure 4.2). To validate the complete knockout of *CG13531*, homozygous (*yw*; *CG13531CRIMIC/CRIMIC*), heterozygous (*yw*; *CG13531CRIMIC/+*), and balanced heterozygous (*yw*; *CG13531CRIMIC/CRIMIC*; *TM2/TM6B*) flies were analyzed. *yw* flies were used as a control. The results showed almost no detectable *CG13531* expression in homozygous knockout flies compared to the control, confirming that the *CG13531CRIMIC* allele effectively

generates a null mutant. Low expression levels were observed in heterozygous flies, as expected. The balanced heterozygous flies also exhibited reduced expression, consistent with the presence of one wild-type allele. These results confirm the successful knockout of *CG13531* using the CRIMIC strategy.

For RNAi-mediated knockdown of *CG13531*, two different GAL4 drivers were used: *Actin-GAL4* (*act>*) and *Tubulin-GAL4* (*tub>*). The RNAi control line (BL36304) crossed to the same drivers was used as a baseline for comparison. *Actin-GAL4*-driven RNAi showed approximately 50% reduction in *CG13531* expression levels, indicating moderate knockdown efficiency. In contrast, *Tubulin-GAL4*-driven RNAi did not result in a significant reduction in *CG13531* expression, suggesting lower effectiveness of this driver in these experimental conditions. These results highlight the variability in RNAi efficiency depending on the driver used.

The results of the relative expression analysis demonstrate that the CRIMIC-induced knockout of *CG13531* effectively eliminates gene expression, creating a reliable null mutant. For RNAi-mediated knockdown, *Actin-GAL4* was more efficient in reducing *CG13531* expression than *Tubulin-GAL4*, indicating that the choice of driver plays a critical role in achieving effective knockdown. This analysis further confirms the tools used in this study for manipulating *CG13531* expression in *Drosophila melanogaster*.

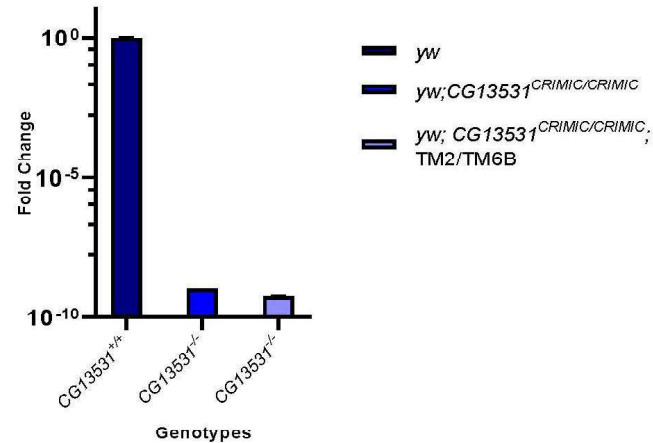
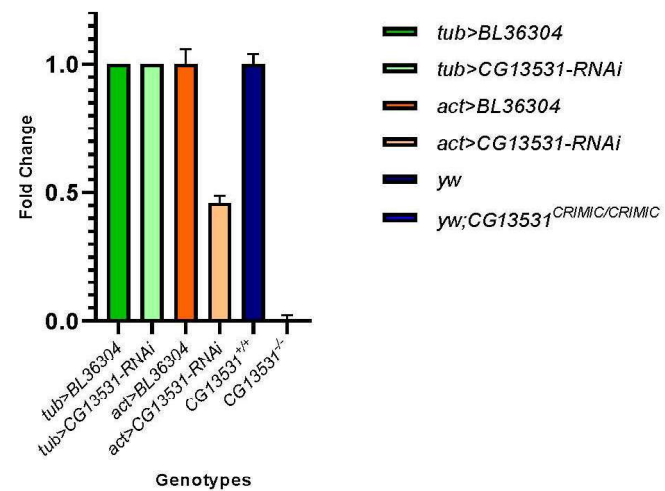
A**Relative Expression Analysis of *CG13531* KO Mutants****B****Relative Expression Analysis of *CG13531* KD and KO Mutants**

Figure 4.2. Relative Expression Levels of *CG13531* Knockout (KO) and Knockdown (KD) Flies. (A) Relative expression analysis of *CG13531* homozygous knockout flies (*yw*; *CG13531*^{CRIMIC/CRIMIC} and *yw*; *CG13531*^{CRIMIC/CRIMIC}; *TM2/TM6B*) and in *yw* flies (control). (B) Relative expression analysis of *CG13531* knockdown flies (RNAi under the control of Actin-GAL4 (*act>*) and tubulin-GAL4 (*tub>*) drivers) and the RNAi control line (BL36304) crossed with the same driver lines serves as the baseline.

4.2. Behavioral Changes in Knockout and Knockdown Flies

4.2.1. The Loss of *CG13531* Causes Locomotor Defects in Adult Flies

Identification of the locomotor defects in flies is crucial for modelling of motor neuron neuropathy. We use a startle-induced negative geotaxis assay to measure the climbing ability of flies across different sexes, ages, and genotypes. The Mann-Whitney-U test was used to identify the mean difference between the control and experiment groups since the samples showed a non-Gaussian distribution. To understand distribution normality the Kolmogorov-Smirnov test was used ($\alpha=0.05$). For females, P-values were calculated as follows: 0.0388 for 5-day-old *yw* control, $P>0.1000$ for 5-day-old *CG13531*^{-/-} mutants, 0.0106 for 25 day-old *yw* control, $P<0.0001$ for 25 day-old *CG13531*^{-/-} mutants. For males, the P-values were 0.0147 for 5-day-old *yw* control, 0.0189 for 5-day-old *CG13531*^{-/-} mutants, 0.3299 for 25-day-old *yw* control, $P<0.0001$ for 25 day-old *CG13531*^{-/-} mutants. Except for 25-day-old control (*yw*) males and 5-day-old mutant (*CG13531*^{-/-}) females, all groups were unable to pass normality test.

Figure 4.3 illustrates the climbing index of *CG13531* knockout flies compared to their control counterparts across two distinct age groups. As anticipated, the climbing ability of each mutant line was significantly reduced relative to its control (P-values of 5-day-old females: 0.0230, 25-day-old females: 0.0002, 5-day-old males: 0.0039, 25-day-old males: 0.0002). Additionally, there was a noticeable decline (comparison between age groups of males $P=0.0003$, and of females $P=0.0065$) in climbing ability with age in the mutant flies, consistent with the characteristics of *SPG11*-related diseases. There is a similar trend of age-related decline between control groups (comparison between age groups of males $P=0.0085$, and of females $P=0.0117$). However, the decline in motor ability due to aging was more pronounced in mutant lines.

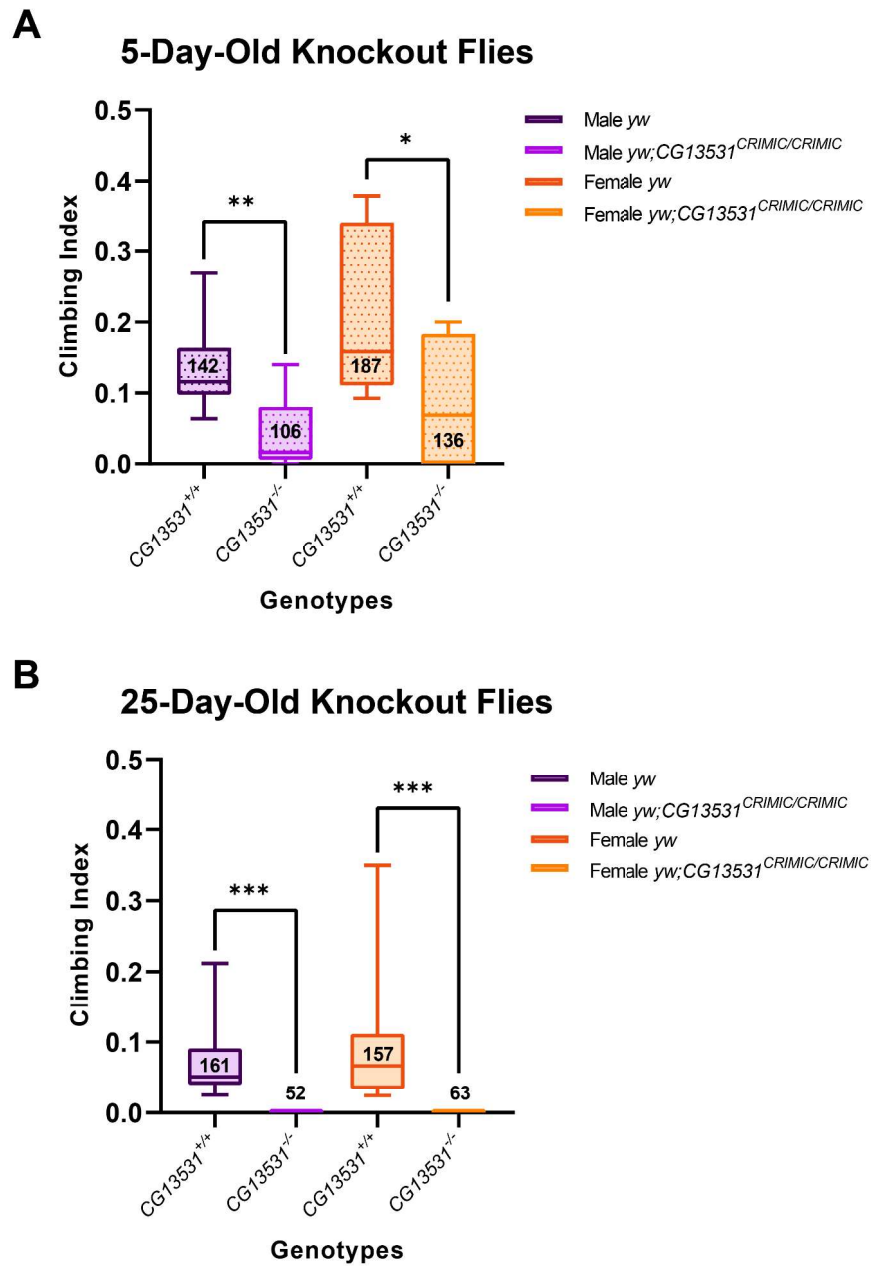


Figure 4.3. Climbing ability analysis of *CG13531* knockout flies. (A) Climbing Index of 5-Day-Old Flies. Climbing index of *CG13531*^{-/-} flies (*yw*; *CG13531*^{CRIMIC/CRIMIC}) are significantly decreased compared to *yw* flies used as control ($P < 0.05$ for females and $P < 0.01$ for males). (B) Climbing Index of 25-Day-Old Flies. The climbing ability further declines *CG13531*^{-/-} flies compared to the *yw* controls ($P < 0.001$ for both sexes).

To understand in which cell type *CG13531* function is crucial for locomotor activity, I further investigated the climbing ability of flies with tissue-specific knockdown of *CG13531* (see Figure 4.4). Specifically, I evaluated the climbing performance of the *Elav-Gal4>UAS-CG13531-RNAi* (pan-neuronal knockdown) and *D42-Gal4>UAS-CG13531-RNAi* (motor neuron-specific knockdown) lines, along with their corresponding controls, to compare the effects of pan-neuronal and motor neuron-specific downregulation of *CG13531*. When normality was tested for these groups using the Kolmogorov-Smirnov test ($\alpha = 0.05$), all groups passed the test ($P > 0.1000$). Therefore, an unpaired t-test was used to assess differences between the groups.

The results showed a slight decline in locomotor ability in all *CG13531* knockdown groups compared to controls. However, no significant differences in climbing ability were observed in most groups (females with pan-neuronal knockdown: $P = 0.1251$, males with pan-neuronal knockdown: $P = 0.1787$, males with motor-neuronal knockdown: $P = 0.085$). The only significant reduction in climbing ability was observed in female flies with motor neuron-specific knockdown ($P = 0.0105$).

This lack of significant differences may be attributed to the strength of the particular *Gal4* drivers or the efficiency of the RNAi line. If it is the latter, this would align with findings from human and mouse models, as *SPG11*-related diseases are recessive genetic disorders requiring loss of both alleles for phenotypic manifestation. Another potential explanation is related to the sample size; a decline in climbing ability may become more apparent if more replicates are added to the analysis.

Overall, these results suggest that *CG13531* plays a role in motor function, but additional studies are required to refine our understanding of its cell-type-specific importance in locomotor activity.

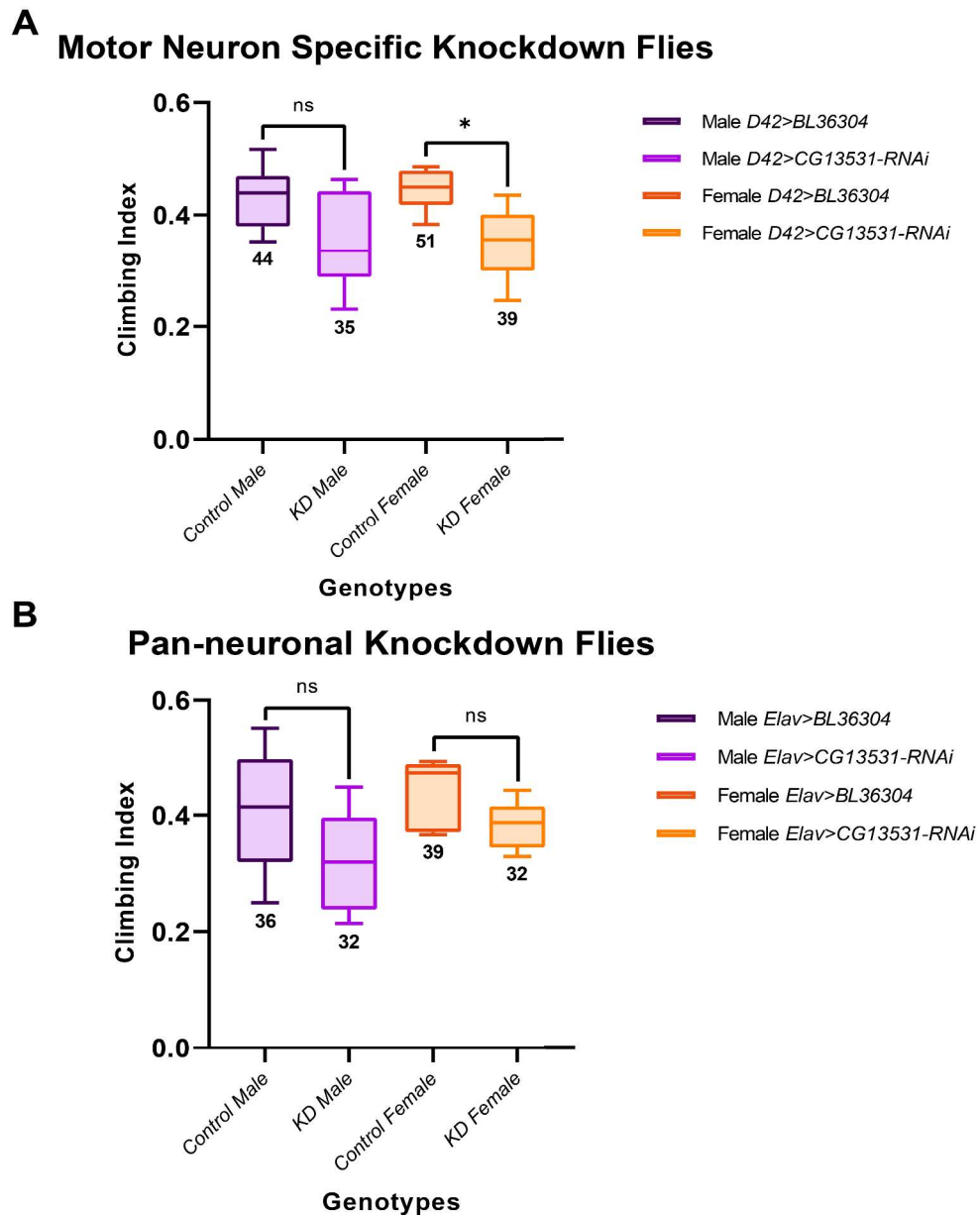


Figure 4.4. Climbing ability analysis of flies with tissue-specific *CG13531* knockdown. (A) Climbing Index of Motor Neuron Knockdown Flies. The knockdown of *CG13531* in motor neurons was driven by *D42-Gal4* (for males $P = ns$, for females $P = 0.0105$). (B) Climbing Index of Pan-Neuronal Knockdown Flies. The pan-neuronal knockdown of *CG13531* was driven by *Elav-Gal4* ($P = ns$ for both sexes).

To confirm that the CRIMIC allele reflects the loss of *CG13531* function specifically, I measured the climbing ability of the *CG13531*^{CRIMIC/BSC599} transheterozygous mutant line. Figure 4.5 illustrates the results of these assays. Due to the small sample size in these (three

experimental repeats), normality could not be measured using the Kolmogorov-Smirnov test. Therefore, comparisons were made using the Mann-Whitney-U test.

The data show a visible decline in the locomotor ability of male progeny for both homozygous and transheterozygous mutants compared to controls. However, these declines were not statistically significant (for *CRIMIC* homozygous mutant: $P=0.1000$, transheterozygous *CRIMIC/BSC599* mutant: $P=0.1000$). Furthermore, when the difference between male *CRIMIC* mutants and transheterozygous *CRIMIC/BSC599* mutant males were compared, the difference was minimal ($P=0.8000$), suggesting that *CRIMIC* homozygous and *CRIMIC/BSC599* transheterozygous mutants behave similarly. Female flies also exhibited a decline in climbing ability for both *CRIMIC* homozygous and *CRIMIC/BSC599* transheterozygous mutants, although the differences were not statistically significant (*CRIMIC* homozygous mutant: $P=0.4000$, *CRIMIC/BSC599* transheterozygous mutant: $P=0.6000$). Similar to the males, when the climbing abilities of *CRIMIC* homozygous and *CRIMIC/BSC599* transheterozygous mutant lines were compared, no significant difference was observed ($P\text{-value} > 0.9999$), indicating identical locomotor ability between the two mutant lines. While only a small number of experimental repeats (three SING assay replicates) were performed with these lines so far, the current data suggest that the *CRIMIC* allele specifically targets *CG13531*, as homozygous and transheterozygous mutants display similar climbing defects, reinforcing the conclusion that observed phenotypes are due to *CG13531* loss of function.

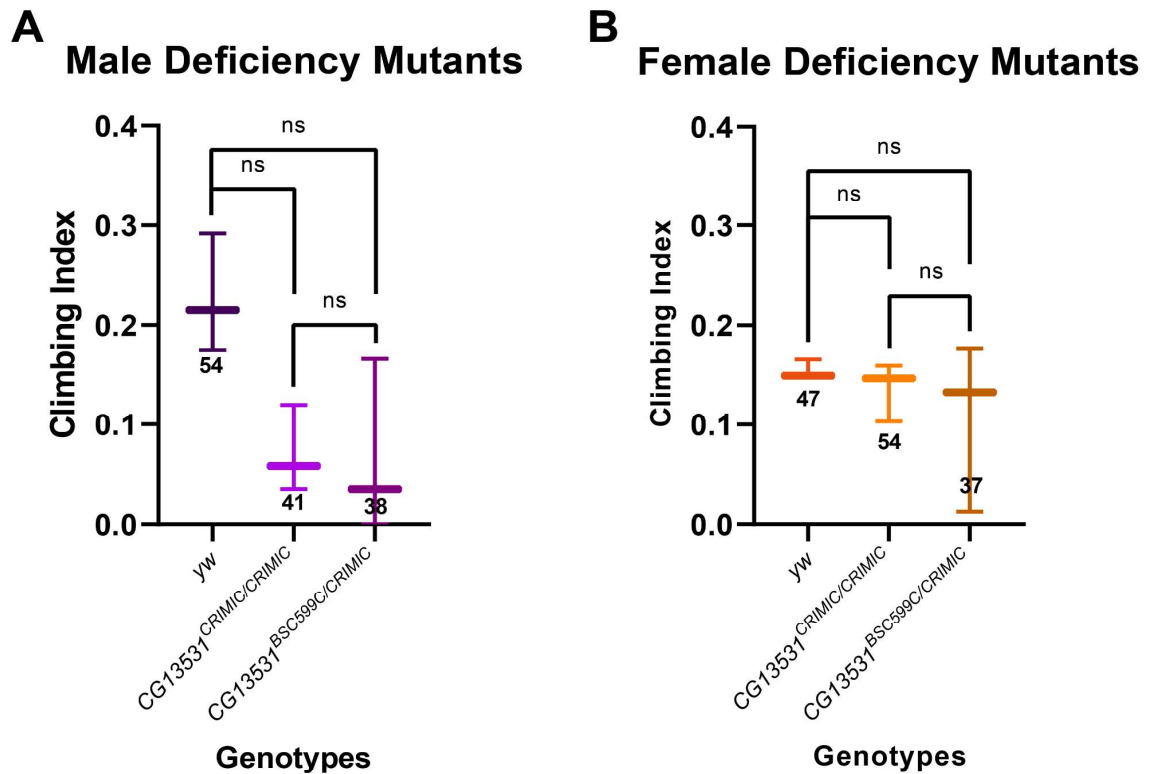


Figure 4.5. Climbing ability analysis of *CRIMIC* homozygous and *CRIMIC/BSC599* transheterozygous mutant flies. Five-day-old male (A) and female flies (B) are evaluated for their climbing ability with SING assay.

4.2.2. Eclosion Rate of Knockout Flies

The eclosion rate is critical for *Drosophila* because it ensures developmental viability and shows reproductive success. A fly must first exert pressure on the pupal case with its ptilinum and then use abdominal muscles to emerge from it (Kimura & Truman, 1990; Kuleesha et al., 2016). If muscles are weakened before this stage, flies may fail to emerge as adults (Caschi and Pandey, 2015). To evaluate this, I investigated the eclosion rate for *CG13531*^{-/-} and control flies (*yw*) and counted the number of dead pupae.

The results showed no significant difference in the number of dead pupae (Figure 4.6), suggesting that defects observed in *CG13531*^{-/-} flies manifest during adulthood rather than

earlier developmental stages. This also indicates that abdominal muscles, critical for eclosion, are likely unaffected at this stage of development.

Additionally, I observed a slight decrease in progeny numbers in *CG13531*^{-/-} flies (~20% for males and ~15% for females) compared to controls. This reduction may be attributed to differences in the number of embryos laid in culture. However, the impact of *CG13531* loss of function on fertility is beyond the scope of this research and could be investigated in future studies.

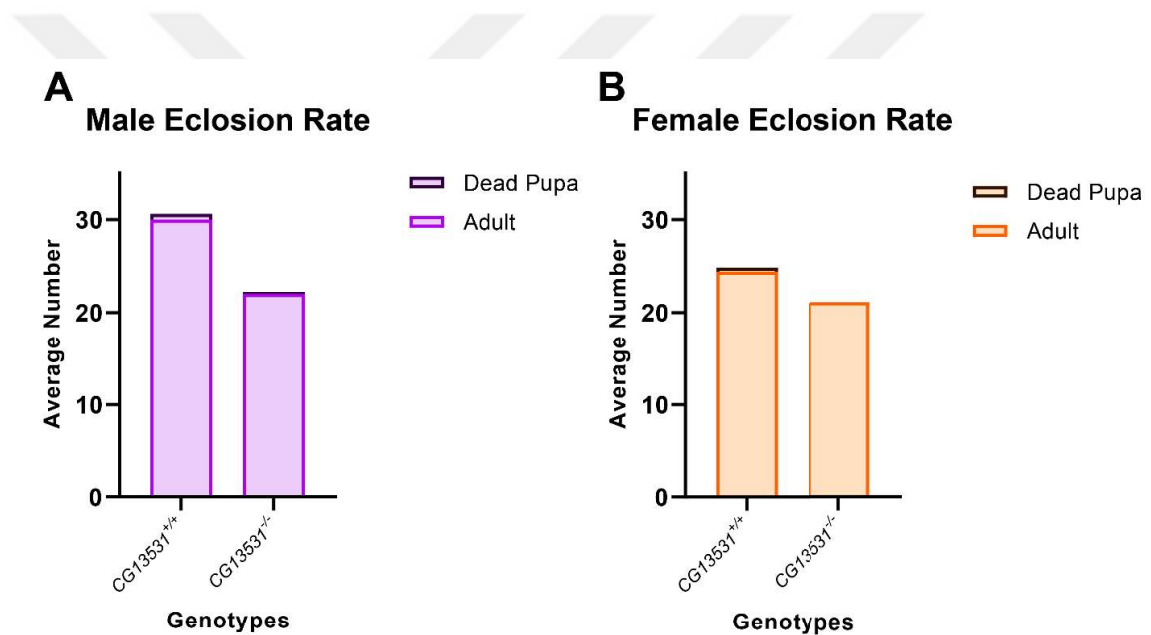


Figure 4.6. Eclosion rates of male and female *Drosophila* with *CG13531* genotypes. (A) Male eclosion rate: Average number of successfully eclosed adult males (light purple) and dead pupae (dark purple) for wild-type (*CG13531*^{+/+}) and mutant (*CG13531*^{-/-}) flies. (B) Female eclosion rate: Average number of successfully eclosed adult females (light orange) and dead pupae (dark orange) for wild-type and mutant flies. ($n \geq 4$ biological replicates per genotype.)

4.2.3. Lifespan of the Knockout Flies

Assessment of survival rate, or lifespan, is crucial in modeling motor neuropathies and understanding the impact of gene mutations on neurodegeneration and longevity (Agudelo et

al., 2020). In this study, I evaluated the survival of flies carrying the *CG13531*^{-/-} mutation alongside two control groups: wild-type (*yw*) and *yw;;TM2/TM6B*.

Since the experimental flies in this assay carried 3rd chromosome balancers, an additional control (*yw;;TM2/TM6B*) was included to account for any potential effects caused by these balancer chromosomes. However, compared to both control groups, the *CG13531* knockout mutants exhibited significantly reduced lifespans in both males (compared to *yw*: $P=0.0001$, and to *yw;;TM2/TM6B*: $P=0.0001$) and females (compared to *yw*: $P=0.0001$, and to *yw;;TM2/TM6B*: $P=0.0001$) (Figure 4.7). The observed reduction in lifespan due to *CG13531* loss-of-function was expected, as *SPG11* patients are known to have shortened life expectancy. Gene mutations associated with locomotor defects often result in decreased activity, paralysis, and respiratory problems, which can further contribute to reduced longevity.

A decreased survival rate is a common feature in *D. melanogaster* models of neurodegenerative diseases. The loss of motor ability, leading to difficulties in walking or feeding, may directly contribute to this reduction in lifespan. Additionally, systemic cellular dysfunctions, such as impaired proteasome activity or defective autophagy could underlie the observed decrease in survival.

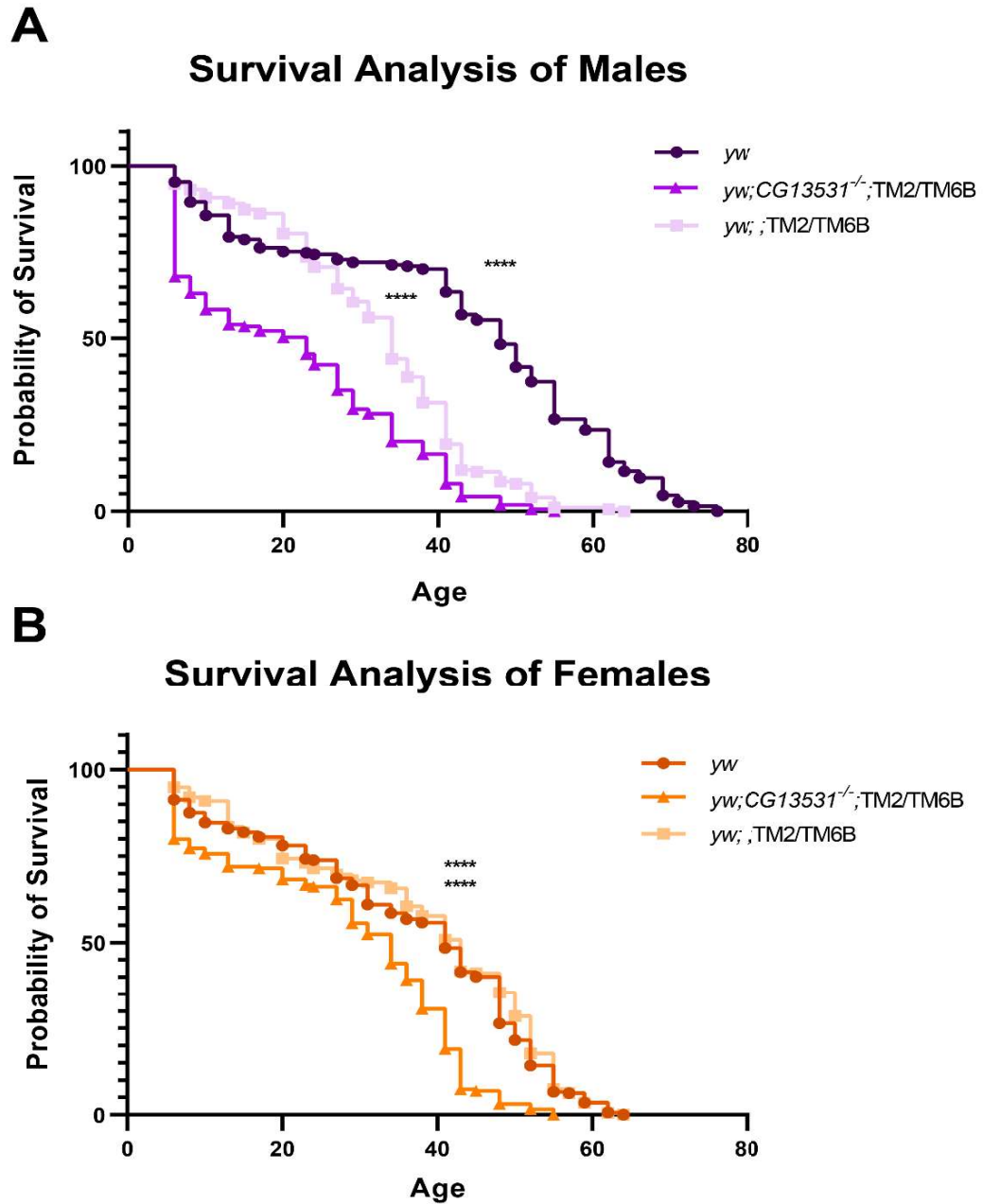


Figure 4.7. Survival analysis of male and female *Drosophila* carrying the $CG13531^{-/-}$ mutation. (A) Male survival: Kaplan-Meier survival curves comparing $CG13531^{-/-}$ mutants ($yw;CG13531^{-/-};TM2/TM6B$) to two control groups (yw and $yw;;TM2/TM6B$), $P = 0.0001$ for both comparisons. (B) Female survival: Kaplan-Meier survival curves comparing $CG13531^{-/-}$ mutants to the same two control groups, $P = 0.0001$ for both comparisons. Sample sizes include $n \geq 200$ flies per sex and genotype. Statistical significance: **** $P < 0.0001$.

4.3. The Loss of *CG13531* Affects the Bouton and Branch Numbers in the Larval NMJ

The neuromuscular junction serves as a key model for studying motor neuron disorders, because it represents the key synaptic connection between motor neurons and muscles. Disruption at this synapse is often a hallmark of motor neuron diseases, leading to impaired signal transmission and muscle function. Examining the NMJ in *Drosophila* larvae provides valuable insights into the mechanisms of motor neuron degeneration, helping to understand when these changes occur and whether synaptic dysfunction contributes to disease progressions. By assessing the structural and functional integrity of the NMJ, such as bouton and branch numbers, one can gain a better understanding of how synaptic defects correlate with motor symptoms and neurodegenerative processes.

To assess the impact of *CG13531* loss on motor neurons, I examined the NMJs in *Drosophila* larvae. Specifically, I focused on the motor neurons innervating muscles 6 and 7 in abdominal segments A3 and A4, selected for their larger surface area, distinct bouton morphology, and ease of accessibility. Homozygous *CG13531*^{-/-} larvae were used as the experimental group, while the *yw* line served as a control. Larval fillets were stained with antibodies against Horseradish peroxidase (HRP) and Discs large (DLG) (see Figure 4.8), which label neuronal membranes and postsynaptic area, respectively. I counted individual boutons to identify potential synaptic changes. The number of branches in each segment was quantified to evaluate alterations in NMJ structure (refer to Figure 4.9).

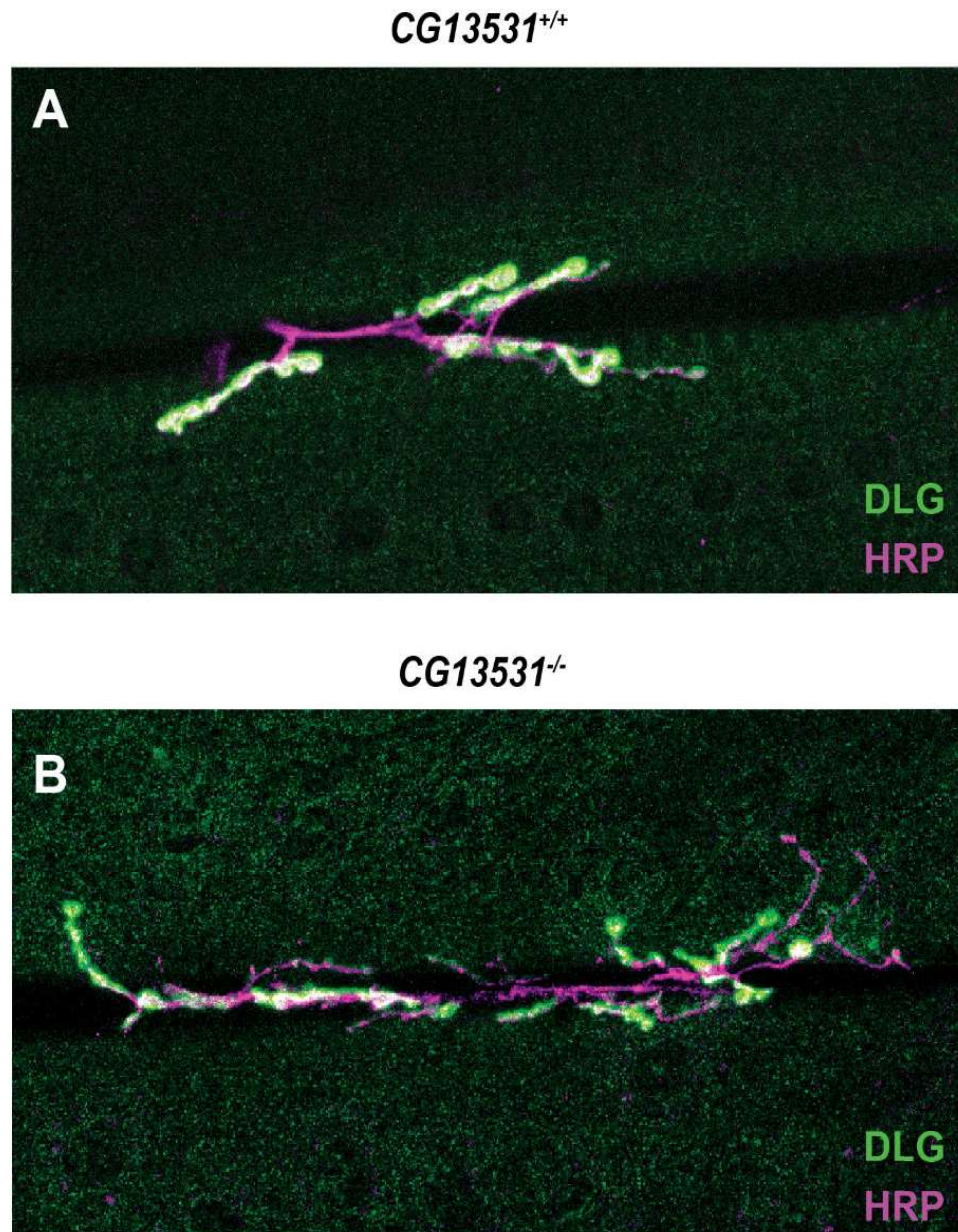


Figure 4.8. Neuromuscular junctions in control and *CG13531^{-/-}* larvae. (A) NMJ from a control (*CG13531^{+/+}*) larva. Antibodies against Discs large (DLG, green) label the postsynaptic area, while Horseradish peroxidase (HRP, magenta) marks the neuronal membranes and axons. (B) NMJ from a *CG13531^{-/-}* knockout larva. Compared to the control, the mutant exhibits an increased number of boutons and branching is also more extensive.

The immunostaining results revealed significant differences between the control and experimental groups in terms of NMJ structure, as shown in Figure 4.8. In the *CG13531*^{-/-} larvae, the number of boutons was increased (for A3 segment: $P < 0.0001$, A4 segment: $P < 0.0001$). Despite the increase in bouton number, many boutons in the *CG13531*^{-/-} larvae were smaller in size compared to those observed in the control group, suggesting altered synaptic morphology. In Figure 4.9, the comparison of bouton and branch numbers (for A3 segment: $P = 0.0063$, A4 segment: $P = 0.0020$) within the different segments revealed a significant increase in *CG13531*^{-/-} larvae for both criteria. The consistent increase in both bouton and branch numbers across segments A3 and A4 confirms the phenotype associated with *CG13531* loss-of-function mutations.

The literature suggests that an elevated number of boutons and branches often reflects suboptimal development of NMJs. This suggests that *CG13531* may play an important role in *Drosophila* NMJ development. The observed phenotype could help to address the behavioral changes that manifest during the adult stage, linking structural abnormalities to functional impairments.

Additionally, numerous boutons in *CG13531*^{-/-} larvae appeared smaller in size and lacked detectable DLG signals. This phenotype could indicate a potential developmental delay. The presence of smaller boutons without post-synaptic markers might indicate incomplete maturation or delayed synapse formation. Alternatively, this phenotype could result from a compensatory mechanism, where the formation of a greater number of new boutons were formed to compensate for reduced synaptic activity. Another possible explanation is the formation of ghost boutons, characterized by incomplete or improper regulation of NMJ development. These boutons might represent immature or nonfunctional synapses. Distinguishing between these possibilities requires further investigation. Future studies could include time-course analyses of NMJ development, electrophysiological assays to evaluate synaptic activity, and molecular analyses to identify underlying regulatory pathways.

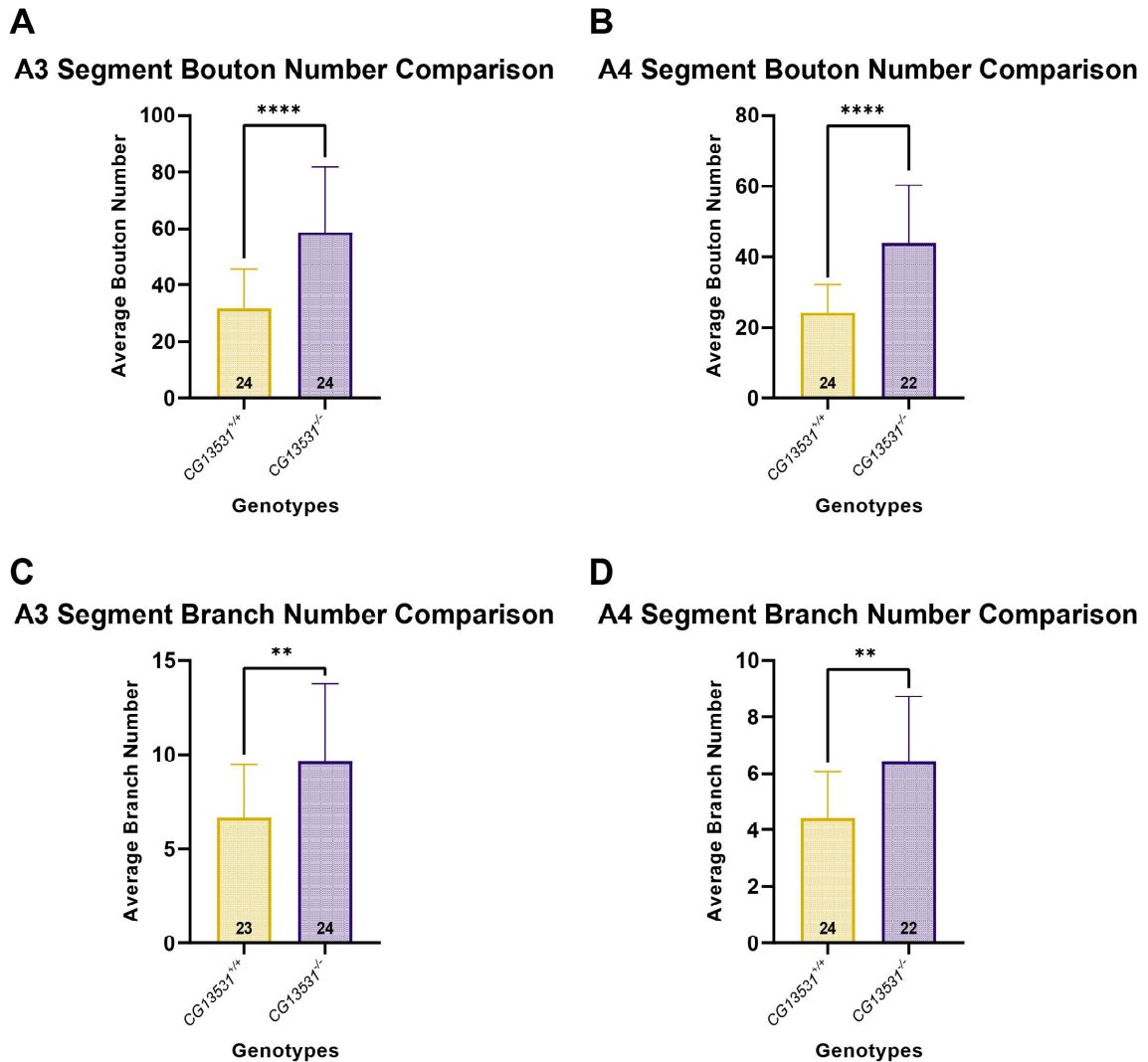


Figure 4.9. Quantification of neuromuscular morphology alterations between knockout and control larvae. (A) A3 segment bouton number comparison of *CG13531*^{-/-} knockout larvae to controls ($P < 0.0001$). (B) A4 segment bouton number comparison between same groups ($P < 0.0001$). (C) A3 segment branch number comparison between same groups ($P < 0.01$). (D) A4 segment branch number comparison between same groups ($P < 0.01$). The data were analyzed using Welch's t-test ($P < 0.05$), with a sample size of $n \geq 22$ for all genotypes.

I performed a simple analysis of these small boutons observed in the experiment and control groups. Figure 4.10 shows an analysis of the number of small boutons and their ratio to the total number of boutons in both groups. These small boutons resembled many of the 'beads-on-a-string' structure similar to the chromosomal organization, making precise measurement of

their area challenging. Consistent with expectations, these small dot-like boutons were indeed increased in both the A3 and A4 segments of *CG13531*^{-/-} larvae compared to controls, further supporting the notion that *CG13531* loss leads to alterations in NMJ morphology.

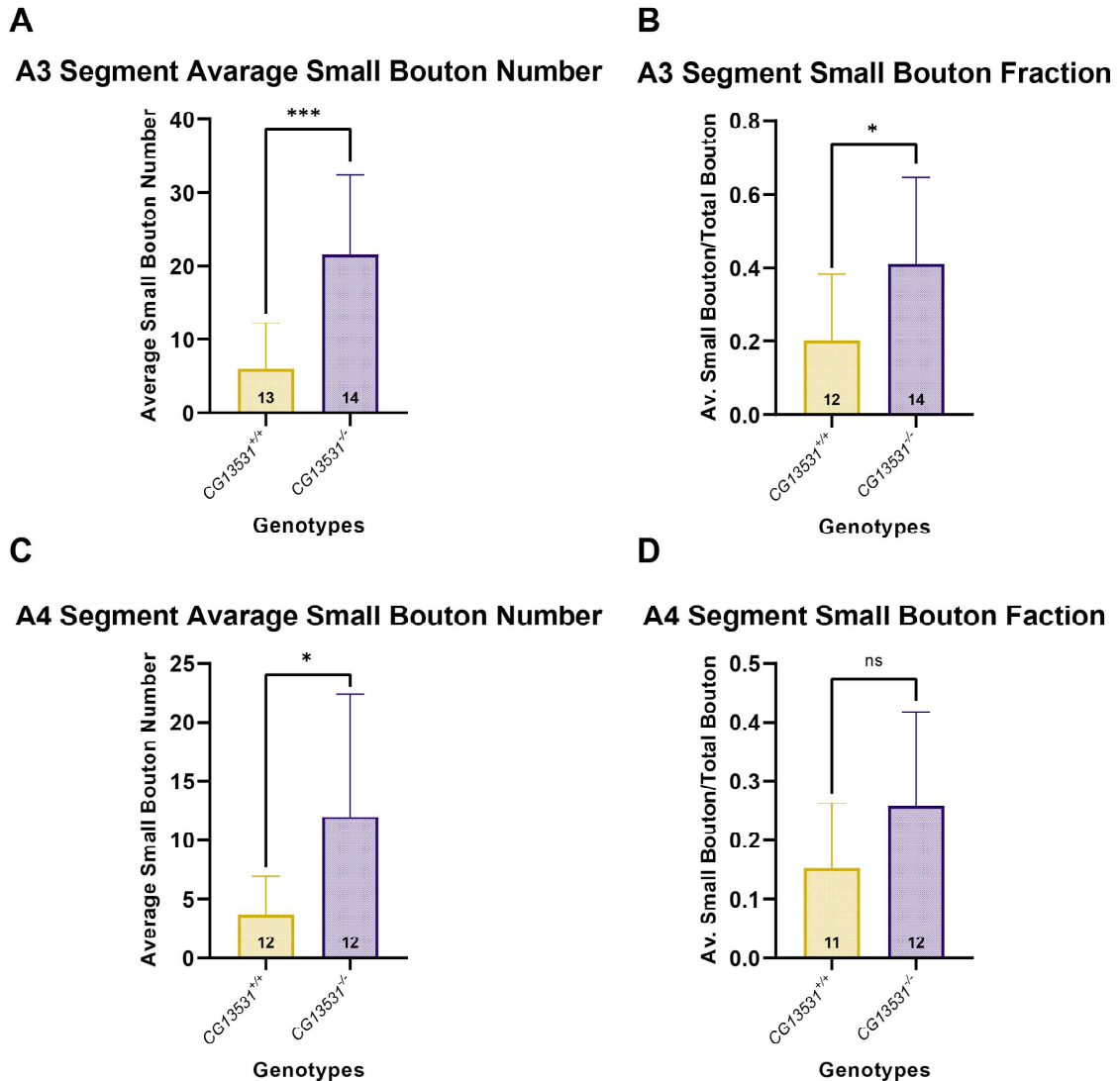


Figure 4.10. Quantification of small boutons. (A) A3 segment average small bouton number comparison between knockout and control groups. ($P < 0.001$). (B) A3 Segment Small Bouton Fraction comparison for same groups ($P < 0.05$). (C) A4 Segment Average Small Bouton Number comparison for same groups ($P < 0.05$). (D) A4 Segment Small Bouton Fraction comparison for same groups ($P = ns$). Welch's t-test ($P < 0.05$) was used to analyze, with a sample size of $n \geq 11$ for all genotypes.

They were subjected to further investigation since their occurrence showed a statistically significant result. Since all groups showed normal distribution ($P\text{-value} > 0.1000$ for all groups according to Kolmogorov-Smirnov test), I used the Paired-t-test with Welch's correction to determine if the differences were significant. The average number of small boutons in the A3 segment ($P=0.0003$) and the fraction of small boutons compared to the total in the A3 segment ($P=0.0222$), as well as the average number of small boutons in the A4 segment ($P=0.0200$), were all significantly different from the control groups. Although the fraction of small boutons in the A4 segment (Figure 4.10A) was not statistically significant ($P=0.0802$), small boutons accounted for nearly one-third of the NMJ.

To ascertain whether the observed phenotype in the NMJ of knockout larvae is a direct consequence of *CG13531*'s absence in motor neurons, I examined and quantified the NMJs of *D42-Gal4 > UAS-CG13531-RNAi* larvae (Figure 4.11 and Figure 4.12). The control group consisted of flies with the *attP40* integration site (BL36304) crossed to *D42-Gal4*.

Although the images showed alterations in NMJ morphology, these changes were observed in both the knockdown and control groups. Quantifying the differences in bouton and branch numbers in flies with *CG13531* downregulated, using Welch's t-test, revealed results that largely followed a reverse trend compared to the knockouts. Specifically, there was a decrease in branch numbers in both segments and a reduction in bouton number in the A4 segment.

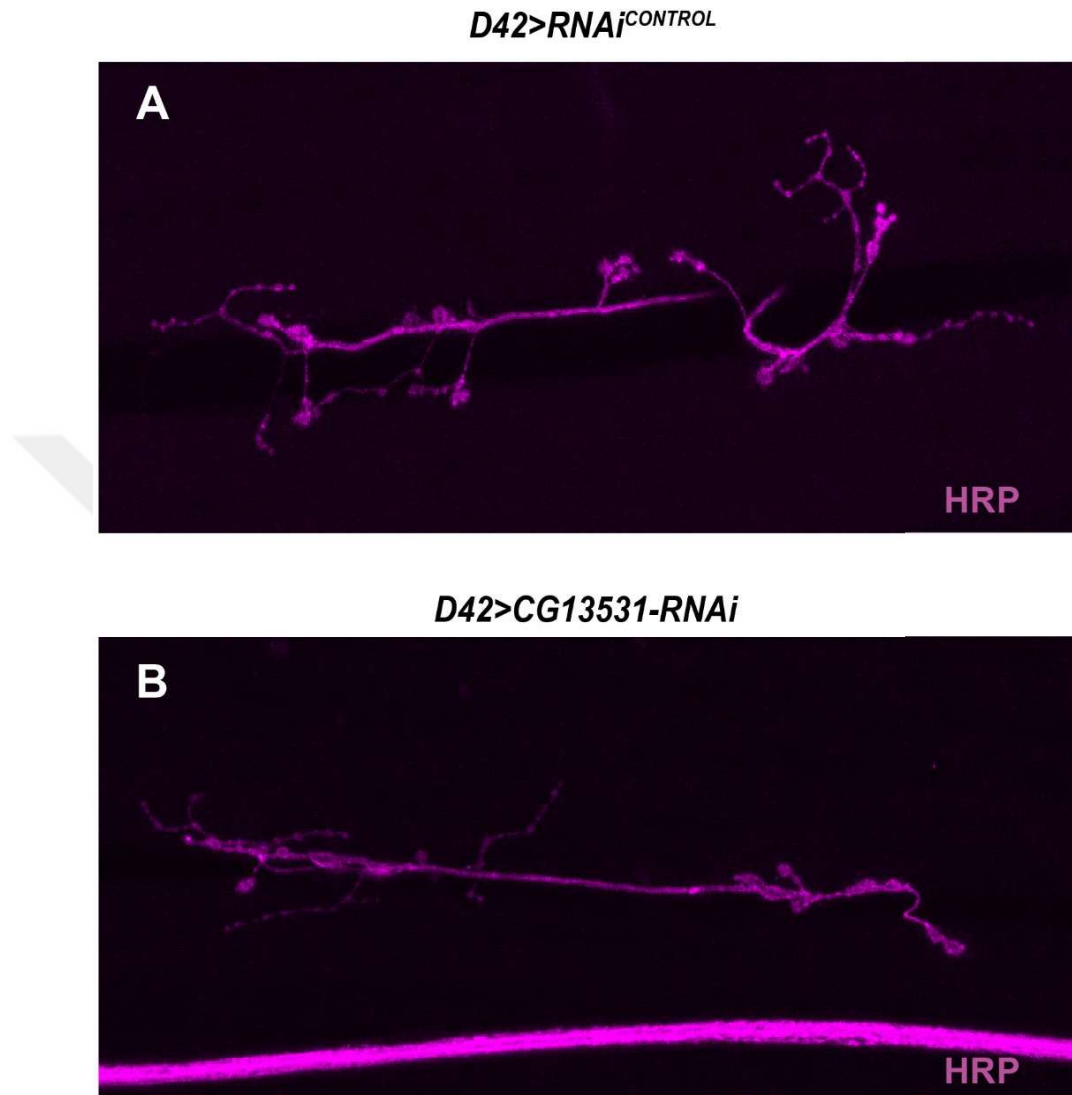


Figure 4.11. Neuromuscular junctions of control (A) and *CG13531-RNAi* knockdown (B) larvae visualized using horseradish peroxidase (HRP) staining (magenta), which marks the axons. The images depict the structural differences in NMJ morphology between the control and knockdown groups, indicating the impact of *CG13531* knockdown on synaptic architecture.

While the changes in bouton ($P=0.6619$) and branch ($P=0.1760$) numbers in the A3 segment were not statistically significant, the decrease in bouton ($P=0.0437$) and branch ($P=0.0128$) numbers in the A4 segment was statistically significant. Regardless of the significance of these results, further investigation should be done. These preliminary results suggest that *CG13531* may not have a direct effect in motor neurons but might exert an indirect

influence instead.

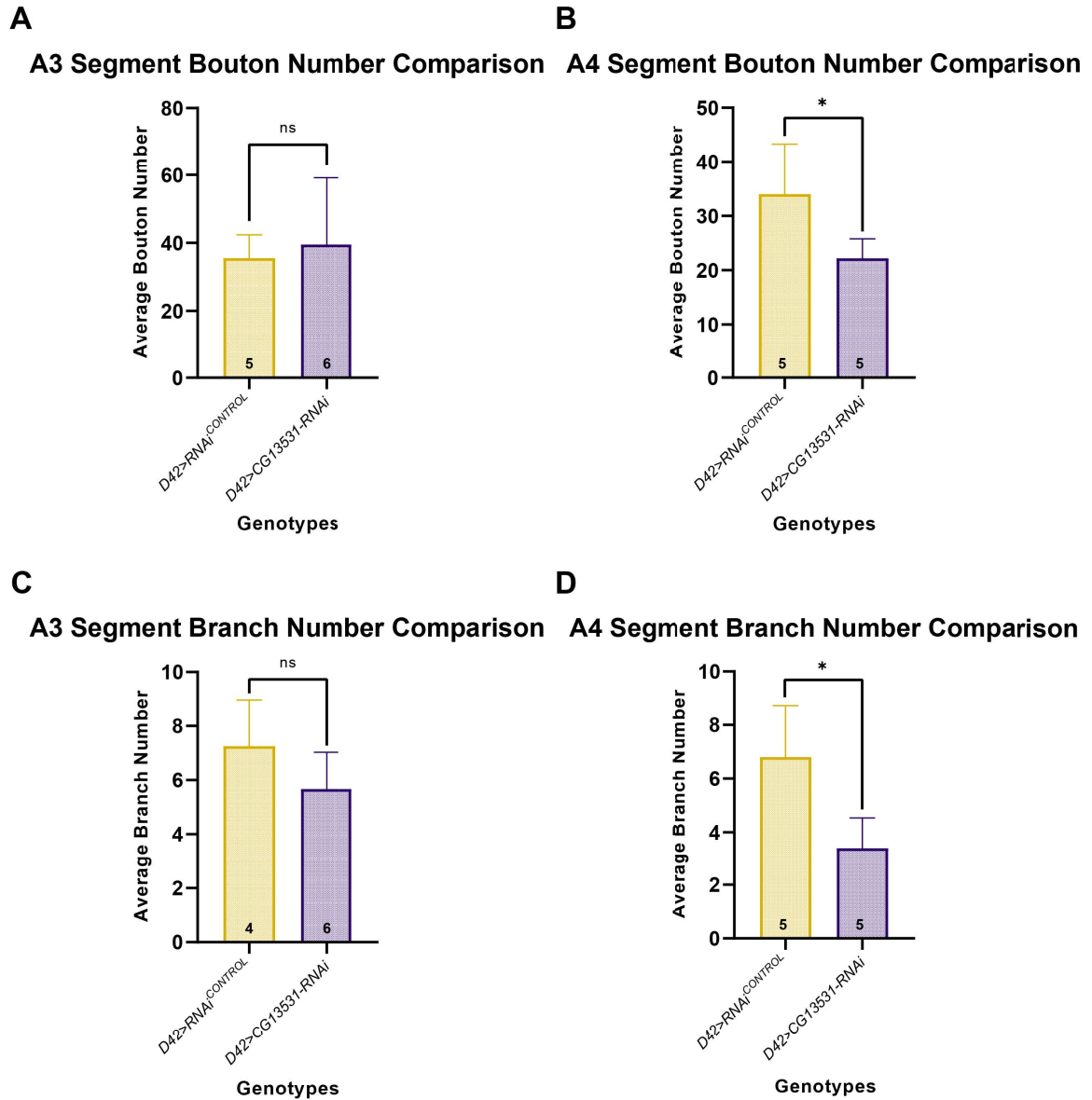


Figure 4.12. Quantification of neuromuscular morphology alterations between motor neuronal knockdown larvae and control. (A) Average branch number in the A3 segment. (B) Average bouton number in A4 segment. (C) Average bouton number in the A3 segment. (D) Average branch number in the A4 segment. Sample size $n \geq 4n$ for all genotypes. Statistical comparisons were made using Welch's t-test, with significance indicated by * for $P < 0.05$.

4.4. The Loss of *CG13531* Leads to Thinning of Olfactory Receptor Neuron Axon Bundle in the Pupal Brain

The corpus callosum is an essential structure for communication between the brain's hemispheres, and its development depends on the proper crossing of axons at the midline. Key molecular mechanisms that guide these axons across the midline include Netrin, Slit, and Robo. These mechanisms are crucial for the proper formation of the corpus callosum and are evolutionarily conserved. A similar mechanism is also responsible for guiding axon projections in the olfactory system of *Drosophila*.

Since *Drosophila melanogaster* does not have a corpus callosum, the midline crossing of neurons is investigated through other commissural neuron structures like the axons of olfactory receptor neurons (ORNs). To determine whether the ORN axon bundle undergoes a similar thinning as reported for the corpus callosum of patients with TCC, the brains of *CG13531*^{-/-} pupae were stained with Flamingo, a cell adhesion molecule essential for axon guidance. Flamingo specifically marks all ORN axons during the pupal stages (P1-P5), allowing visualization of axon bundles (Figure 4.13).

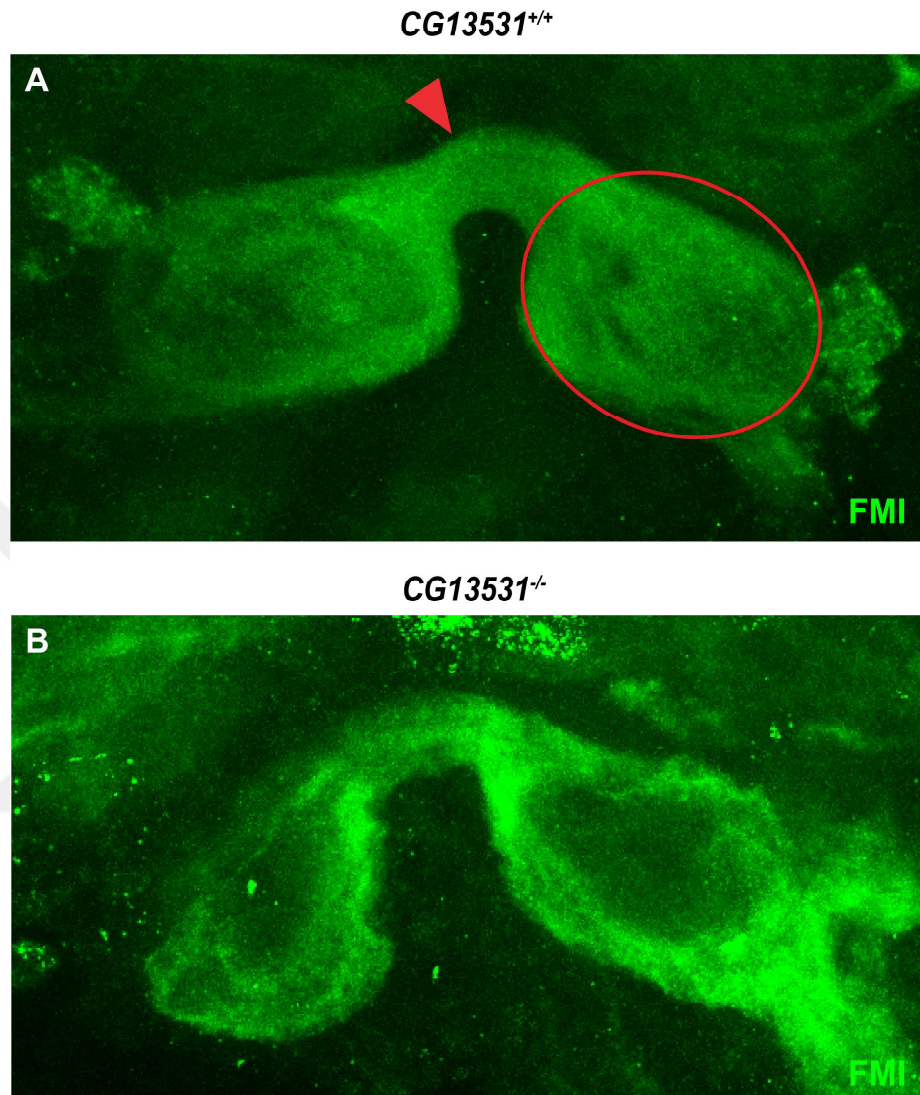


Figure 4.13. Olfactory receptor neuron axon bundle thickness in control (A) and *CG13531*^{-/-} knockout (B) pupae at the P3 pupal stage. ORN axons were stained with Flamingo (FMI, green), a cell adhesion molecule essential for axon guidance. The red arrowhead and circle in (A) highlight the normal thickness of the ORN axon bundle in control pupae, whereas (B) shows the thinning of the ORN axon bundle in knockout pupae.

Pupae were selected around 30 h after pupariation based on body morphology to ensure they were at the same developmental stage. Both antennal lobes and the ORN axon bundle are shown in Figure 4.13; the red arrow indicates the commissural neuron axons, and the red circle indicates the antennal lobe area. The thickness of the commissural olfactory receptor neuron axons was measured on the midline and normalized to the area of the left antennal lobe. The

ratio was then compared between the control and knockout groups (Figure 4.14) using Welch's t-test.

The results indicate a significant decrease in the thickness of the ORN axon bundle ($P=0.0338$), suggesting potential impairment of axon guidance or axonal degeneration, with the latter being the more likely scenario.

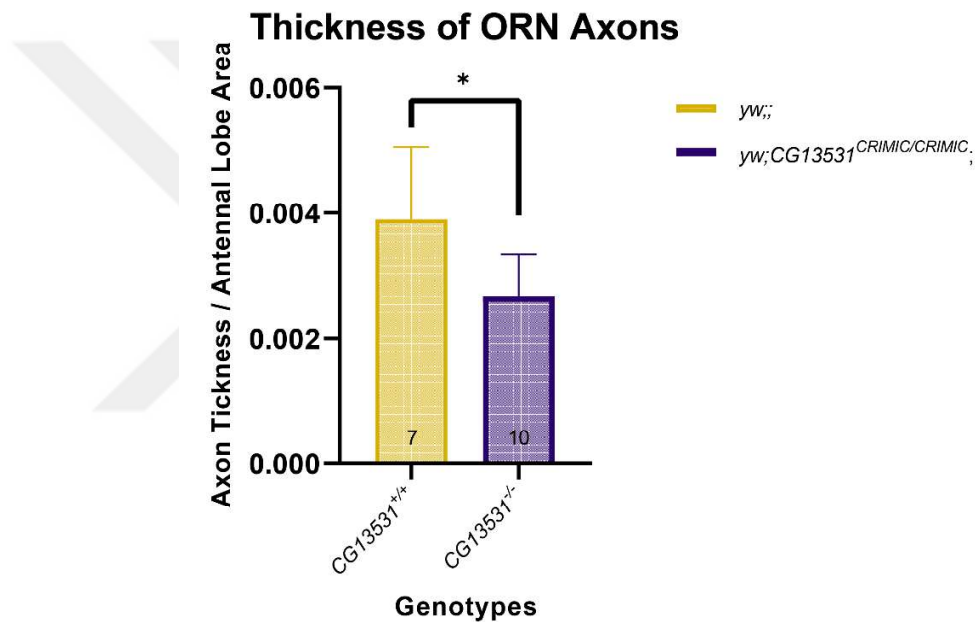


Figure 4.14. Quantification of ORN axon thickness. Axon thickness was normalized to the area of the left antennal lobe and compared between control ($CG13531^{+/+}$) and knockout ($CG13531^{-/-}$) groups. The results show a significant reduction in axon thickness in the knockout group ($n \geq 7$; $P < 0.05$). Data are presented as mean $\pm$ SEM. Statistical significance is indicated by *.

4.5. Assessment of Degeneration of Wing Main Vein

The *Drosophila* eye has long been recognized as a valuable model system for investigating the mechanisms underlying neurodegeneration and related pathological processes. However, no observable phenotype was detected in the retina of the knockout mutants when viewed under a binocular microscope.

In contrast, the wing of *Drosophila* has been less frequently utilized as a model, but it offers a complementary system for studying neurodegeneration. Therefore, in this section, I utilized the wing as a model to assess neurodegeneration.

To examine axons in the wing, the *Dpr-Gal4>UAS-mCD8::RFP, UAS-dicer2, UAS-CG13531-RNAi* line was used, and *Dpr-Gal4>UAS-mCD8::RFP, UAS-dicer2, attP40* (BL36304) was used as the control line. The *Dpr-Gal4* driver ensured knockdown in the sensory neurons of the wing, while *UAS-mCD8::RFP* labeled the axons, enabling analysis of signal loss in axons. Degeneration was evaluated by measuring the frequency of fragmentation in the main wing vein axons.

Experimental and control flies were aged to five days, and their sensory neurons were imaged using confocal microscopy. At this early stage, no significant differences were observed between the two groups (Figure 4.15). To assess whether degeneration occurred at later stages, the same genotypes were aged to 25 days. Even at this advanced age, no evidence of axon fragmentation was observed, as the RFP signal remained intact in the main wing axons (Figure 4.15). These findings are consistent with the absence of neurodegenerative phenotypes observed in the eye.

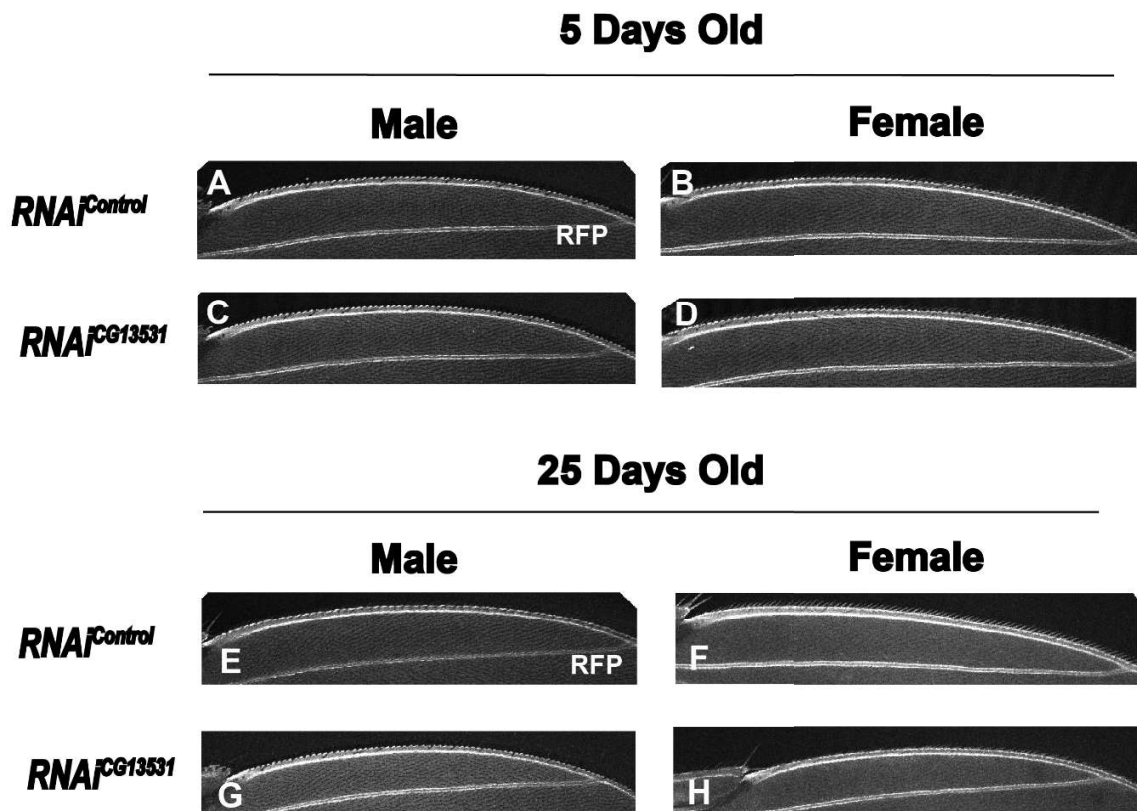


Figure 4.15. Sensory neurons in the wing main vein labeled with RFP in 5-day-old (A-D) and 25-day-old (E-H) groups of the flies with *CG13531* downregulation (C, D, G, H) and controls (A, B, E, F). RFP fluorescence visualizes the sensory neuron axons in the wing. No significant axon degeneration or fragmentation was observed at either 5 days or 25 days in either group.

4.6. Mushroom Body Morphology of Knockout Flies

Given that most patients with *SPG11*-caused hereditary spastic paraplegia are reported to exhibit mild intellectual disabilities, one of our objectives was to investigate whether our model could provide any insights into this condition. The mushroom body, the learning and memory center in the *Drosophila* brain, provided an excellent starting point due to its well-studied structure and its relevance to cognitive functions.

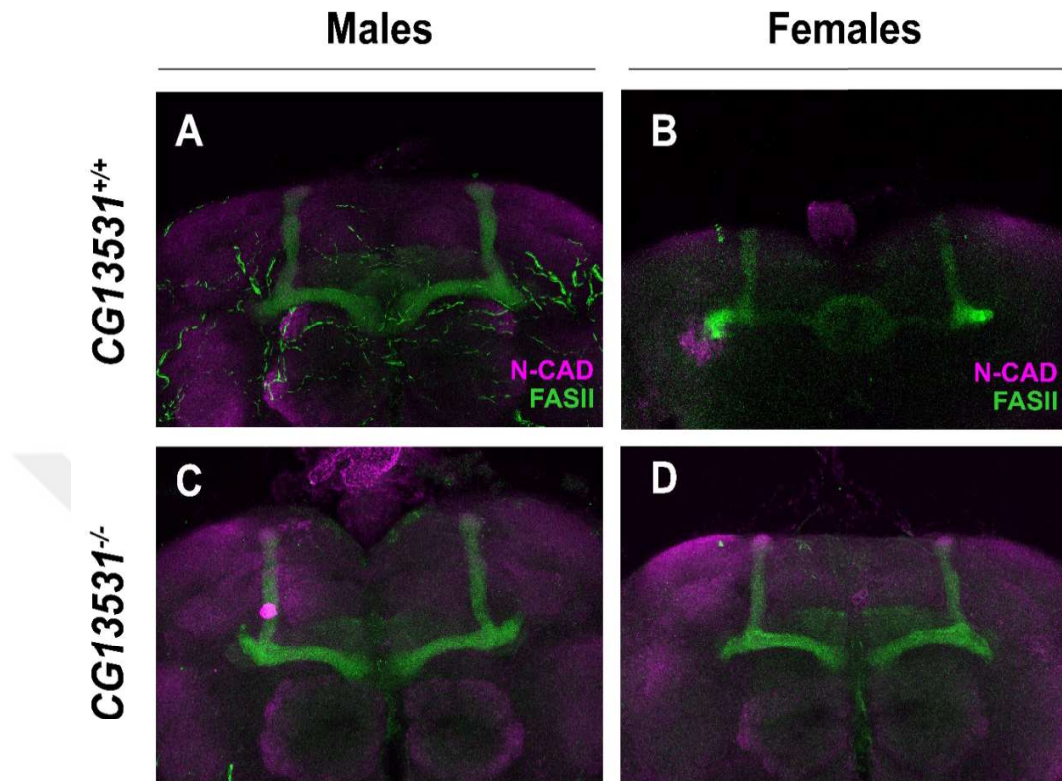


Figure 4.16. The typical mushroom body morphology of *yw* and *CG13531*^{-/-} flies. Male (A) and female (B) control flies, as well as knockout mutant male (C) and female (D) flies, were stained with anti-Fasciclin II (green) and anti-N-Cadherin (magenta) antibodies to visualize the calyx, lobes, and cell body regions of the mushroom body. No morphological differences were observed between the control and knockout flies in either sex.

To examine the mushroom body structure, the brains of five-day-old *CG13531* knockout and control flies were stained with anti-Fasciclin II and anti-N-Cadherin antibodies (Figure 4.16). No observable morphological differences were detected in the calyx, lobes, or cell body regions of the mushroom bodies between the control and *CG13531* knockout flies. Despite the absence of morphological defects, it is possible that behavioral changes may still occur. Further investigation is needed to address the possible changes in cognitive functions in these flies.

5. DISCUSSION

Animal models are essential for uncovering the underlying mechanisms, physiological conditions, and manifestations of diseases, as well as developing and improving treatments. In this study, I aimed to establish a fruit fly model for *SPG11*-related motor neuron neuropathies to gain an in-depth understanding of the disease and, if possible, to distinguish between the different conditions resulting from *SPG11* mutations. The creation of such models is critical for further studies, where these models may be used for drug repurposing efforts.

To begin, I verified the CRIMIC line by genotyping to confirm the presence of the CRIMIC integration in the genome. Subsequently, the expression level of *CG13531* mRNA was assessed by RT-qPCR. Positive genotyping results and the significantly reduced mRNA expression level ensured that the CRIMIC line was indeed a *CG13531* knockout mutant. With this verification, phenotypic evaluations were carried out. The RNAi line was also evaluated by RT-qPCR using two different Gal4 drivers. Actin-GAL4 was effective in partially reducing *CG13531* expression (50% knockdown efficiency), while tub-GAL4 showed limited efficacy (no significant reduction). Unfortunately, no second RNAi line was available, which limited the assessment of RNAi efficiency and phenotypic variability.

Locomotor ability assessment is a key behavioral assessment in modeling motor neuron neuropathies. Negative-geotaxis assays performed with *CG13531*^{-/-} flies and control groups revealed a dramatic decline in the climbing ability of knockout flies, which worsened with age. This critical finding is consistent with clinical reports on patients with *SPG11*-related disorders, highlighting progressive motor deficits.

In addition, tissue-specific knockdown groups, including pan-neuronal and motor neuronal *CG13531* knockdown, were subjected to the same climbing assays. Interestingly, these groups did not display significant impairments in climbing ability. Several explanations can account for this discrepancy. First, the number of experimental repeats or the efficiency of the driver lines may have been insufficient. Second, the RNAi itself may have been ineffective in achieving adequate knockdown. Alternatively, the lack of phenotype could suggest that low

levels of *CG13531* expression are sufficient to maintain its function.

Eclosion and survival rates were measured to determine the effect of *CG13531* absence on development, survival, and lifespan in mutants. The eclosion rate was unaffected between knockout and control flies, suggesting that the disease manifestation likely begins later in life. However, the lifespan of knockout flies was significantly decreased compared to controls. This aligns with expectations, as the loss of motor control and ability could lead to premature death.

After the findings of behavioral changes, I turned my focus to morphological changes, particularly how motor neurons are affected by the absence of *CG13531*. Aligning with our predictions, motor neurons at the NMJs of larvae displayed distinct morphological differences between knockout and control groups. Quantification confirmed that knockout larvae had an increase in number of boutons and branches compared to controls. This observation may be due to a possible overgrowth phenotype, a failure in the negative regulation of NMJ growth, or a general absence in the regulation, causing the formation of more transient structures to compensate for functionally impaired boutons. When the NMJ morphology was investigated in motor neuron-specific downregulation lines, conflicting results were observed. However, the efficiency of this downregulation remains uncertain, which could explain the inconsistencies.

Evaluation of wing main vein axonal degeneration was done to determine if the degeneration process occurs in motor neurons. However, no signs of degeneration were observed in the wing up to 25 days of age. This could be due to the slow progression of the disease, with 25 days being insufficient to detect degeneration, or it may indicate that different motor neurons are affected by the absence of *CG13531*, with wing neurons not being among them.

Analysis of midline-crossing neurons was performed in order to differentiate between three diseases caused by *SPG11* mutations in humans. HSP11 is frequently associated with thin corpus callosum, an important marker of the disease. By modeling the corpus callosum using the ORN axon bundle in flies, I have identified a slight but significant thinning of the axon bundle. This finding is significant and could potentially serve as a distinguishing feature of *SPG11*-related diseases in this model.

Lastly, the mushroom body structures were examined. The mushroom body, as the learning and memory center of the fly brain, could undergo morphological changes that reflect cognitive impairments such as intellectual disabilities. My results showed no significant changes in MB structure, suggesting that intellectual disability might not be associated with the absence of *CG13531*. However, this finding does not fully rule out the possibility of cognitive impairments, which may be present and require further investigation.

5.1. Evaluation of CRIMIC Mutants

Our results regarding the relative expression of *CG13531* CRIMIC mutants confirm the efficiency of the CRIMIC construct, showing a ten-scaffold decrease in *CG13531* expression. The following experiments also revealed deficiencies in these knockout mutants, aligning with our expectations. Thus, it is appropriate to classify *CG13531* CRIMIC mutants as *CG13531* knockout mutants. However, a neighboring gene, *CG3649*, has a 3' UTR that overlaps with the 3'UTR of *CG13531*, and one of the gRNAs used for the CRIMIC construct insertion also targets this overlapping genomic region. This overlap raises the possibility that the expression level of *CG3649* may be affected by the CRIMIC intervention.

To investigate whether the CRIMIC insertion affects the expression of *CG3649*, I planned a relative expression analysis using qRT-PCR and designed a separate set of primers for *CG3649*. Since *CG3649* is expressed in pupal stages and adult females, I tried to amplify cDNA synthesized from adult fly RNA. Despite designing and testing different sets of primers through temperature gradient PCR with qPCR mix, none of the trials successfully amplified the cDNA. Additional trials are necessary to conclusively determine whether the CRIMIC cassette affects only *CG13531* expression.

We also wanted to utilize *Kozak-Gal4* inserted to understand the expression pattern of *CG13531*. To this end, I performed experiments using reporter lines crossed with CRIMIC lines. Adult flies carrying the CRIMIC construct either on one or both alleles, together with reporter

constructs such as the membrane reporter *UAS-mCD8::RFP* and nuclear reporter *UAS-nlacZ*, were dissected to understand the expression pattern of *CG13531*. Samples were stained with antibodies against the reporters, as well as anti-Repo or anti-Elav antibodies to mark glia and neurons, respectively. Surprisingly, the nuclear reporter signal co-localized only with Repo in the brain, indicating glial expression with a limited presence in neurons.

In the larval brain and ventral nerve cord, *Kozak-Gal4*-driven *mCD8::RFP* signals were observed in axons leaving the ventral nerve cord, indicating expression in motor neurons. This finding conflicted with the adult expression pattern. To resolve this, I examined the expression in neuromuscular junctions using the same reporter and co-staining with anti-Futsch to determine axonal co-localization. The results showed strong staining in nerves leaving the ventral nerve cord and motor neuron axons on muscle 6/7, as well as other NMJs on various muscle fibers. Interestingly, signals resembling glial cells were also observed in these NMJ images.

Upon evaluating my expression pattern findings, I noted similarities to patterns observed in other CRIMIC lines used in other studies in our laboratory. We hypothesized that these inconsistencies could arise from the inefficiency of the promoter or transcription factor sequences cloned into the construct, resulting in an incorrect expression pattern that predominantly indicates glial expression. Therefore, I decided to use the CRIMIC line only as a knockout mutant, disregarding the observed expression patterns.

5.2. Expression Pattern of *CG13531*

We investigated the expression pattern of *CG13531* using RNA-seq data from available databases. However, the results were challenging to interpret since cell-specific expression levels and even the types of cells expressing the gene were changing. *CG13531* is not broadly expressed across all cell types and, even within those cells where it is expressed, the levels are relatively low (Hu et al., 2024). Additionally, expression levels in adults changed with age, but

these changes were not consistent across certain cell clusters.

For example, *CG13531* mRNA was detected in the DN1 cell cluster and some clock neurons, where its expression initially increased and later decreased with age. This pattern could be linked to *CG13531*'s predicted role in axonal maintenance, however, it does not entirely explain the observed dynamic changes. If the precise localization of the protein could be determined, it might suggest additional roles, such as involvement in age-related plasticity changes.

Given that *CG13531* expression in wild-type flies is low and is limited to a few cell clusters, developing a construct to investigate the spatiotemporal localization of this protein is critical. Understanding where and when *CG13531* localizes is essential, as it could provide direct evidence for the phenotypes we have observed and help us hypothesize an underlying mechanism of pathogenicity and the gene's role in motor neurons.

5.3. Locomotor Deficiency

CG13531 knockout flies showed a progressive loss in climbing ability, a sign of motor deficiencies. However, additional assays, such as flight tests to evaluate control over wing muscles, should be conducted to fully understand the degree of motor impairment caused by the loss of *CG13531* in fly motor neurons.

Larval crawling assays could also be performed to determine when locomotor deficiencies begin during development. Identifying the onset of these deficiencies is critical for optimizing the timing of future drug trials aimed at alleviating these deficits.

Moreover, during the larval selection process for NMJ morphology investigations, we observed that homozygous knockout larvae were less responsive to light stimuli compared to controls. Although this observation was not quantitatively measured, it was consistently noted. This lack of responsiveness may be due to deficits in light stimulus perception or muscle

contraction, which could slow crawling movement or impair turning behavior. Dedicated assays to measure light stimuli responses, larval crawling speed, and larval bending angles measurements would help clarify these observations.

While the wing experiments did not reveal evidence of axonal degeneration, flight ability could be further assessed by measuring the duration and control of flight. Behavioral phenotypes may not always correlate with immunohistochemical observations, as these techniques allow visualization of only a limited number of markers simultaneously. Impaired axonal transport or synaptic activity, which may not be visible in staining assays, could still underline the observed behavioral deficits.

Overall, the observed locomotor defects and reduced lifespan in *CG13531* knockout flies highlight the potential of this model to represent *SPG11*-related diseases effectively. A mutant model's success in representing *SPG11*-related diseases. Additional experiments are warranted to further validate the relevance of this model in studying the pathology for these conditions.

5.4. Neuromuscular Junction Defects

The morphology of neuromuscular junctions underwent dramatic changes in some *CG13531* knockout larvae. Overall, the mutants showed phenotypes consistent with defects in negative growth regulation, as described in previous studies (Menon et al., 2013). Both bouton and branch numbers increased significantly, suggesting that *CG13531* may play a role in repressing NMJ growth.

Smaller Boutons were observed in almost all *CG13531* knockout samples, and these boutons lacked a postsynaptic area, as shown by the absence of the Dlg signal. This observation was consistent across different sets of replicates, minimizing the likelihood of immunohistochemistry errors as the cause of the Dlg signal loss in specific regions. Yet these boutons do not resemble satellite boutons, as they also lack Dlg signal in surrounding areas. A

possible explanation for this phenotype is the presence of ‘ghost boutons’ which are typically transient structures. However, ghost bouton are generally rare, with only one such bouton appearing at a time.

In *CG13531* knockouts, the prevalence of these small boutons might reflect a compensatory mechanism, where the number of boutons increases regardless of their functionality, likely due to impaired NMJ growth and development. However, to confirm this hypothesis, live imaging of NMJs, or further investigation using Brp (Bruchpilot) as a marker for active zones is required.

In addition to the small boutons, boutons with detectable Dlg signals sometimes showed structure abnormalities. Instead of the typical large, circular shapes, some boutons appeared elongated or oval. This alteration could result from defects in the localization of other synaptic components, potentially indicating a direct role of *CG13531* in proper NMJ development.

CG13531 interacts with proteins such as Auxilin and Spartin. Auxilin plays a role in clathrin dynamics and regulates Notch signaling, and Spartin functions in BMP signaling. Both pathways are essential for the development and maturation of functional synapses, suggesting that the exact nature of these interactions may underlie the phenotypes observed.

Overall, to fully understand the functional capacity of these synapses and the significance of the small boutons, further investigations of active zones and synaptic functionality are necessary.

5.5. Olfactory Receptor Neurons as a Model for Corpus Callosum

To address potential changes in commissural axon structures, I studied their morphology, axon guidance, and structural organization. Two neuronal types were examined: the ORN axon bundle that crosses the midline, and the motor neuron axons that extend to the opposite side of the central brain and ventral nerve cord and exit these structures to target muscles.

I conducted trials with both neuron types. For the ORN axon bundle, Fmi was used as a marker during the pupal stages these axons are still forming. For motor neurons, Fasciclin II (FasII) was used during the third instar larval stage.

Our experiments with motor neurons faced several challenges. FasII staining did not exclusively label motor neurons, making it difficult to analyze them specifically. Additionally, finding a quantification method was challenging. While several axon tracts were visible, their thickness could not be measured reliably. Furthermore, signal strength measurements were compromised due to high background noise.

In contrast, the ORN axon bundle was a more promising target. These axons constitute the densest commissural axon population, stacked closely together. However, a significant limitation was the lack of a general marker specific to ORN axons. While individual ORNs could be labeled and imaged according to their OR type, this approach was insufficient for capturing the overall organization of the axons. To address this, I used Fmi as a marker, which highlights cell adhesion and guidance molecules during midline crossing. Despite its usefulness, the Fmi-based method had limitations, as it could not capture morphological changes in later stages of development. To overcome this, alternative markers such as anti-Tubulin and anti-Dscam could be optimized for staining ORN axons in adult stages, enabling a comprehensive analysis.

Overall, given their evolutionarily conserved midline guidance mechanism and the functional parallels between ORN axons and the human corpus callosum in transmitting sensory information, the ORN axon bundle serves as a suitable model for studying the corpus callosum.

5.6. Degeneration Assessment

I attempted to measure degeneration using wing morphology, as it offers an alternative to the extensively studied eye structure in *Drosophila*. Surprisingly, my results indicated no signs

of degeneration up to 25 days of age. It might be due to the slow progression of *CG13531*-related phenotypes, suggesting that even later ages should be considered for examination in these mutants.

Degeneration may also occur in other parts of the body, such as the legs, which should also be investigated with a focus on motor neurons controlling leg movement. Upstream elements in this circuit, such as motor neurons, may remain unaffected, as no signal loss was observed even the distal parts of motor neurons, ruling out degeneration at this level.

Although no observable phenotypes were noted during expression studies in larvae, I also analyzed the eye-antennal imaginal discs and wing discs. The antibodies used in these experiments may not have been optimal for detecting axonal degeneration, and no morphological abnormalities were observed in the tissues.

Another potential phenotype to consider is the rough-eye phenotype, which covers a large spectrum of manifestations, ranging from severe to mild structural changes. While no morphological abnormalities were observed in the adult eye under a binocular microscope may not provide sufficient resolution to detect subtle changes. Therefore, higher-resolution microscopy should be used to identify any potential changes in the adult eye.

5.7. Orthology Between *SPG11* and *CG13531*

From orthology databases and relevant publications, it is evident that *CG13531* is the only ortholog candidate for *SPG11* in *Drosophila*. Protein sequence similarity, identity, and 3D structural alignment further support their orthology. The presence of the spatacsin-C domain serves as definitive evidence that these proteins belong to the same family, whose only member is the *SPG11* gene and its orthologs. However, the absence of additional domains in *CG13531* that are critical for spatacsin's function in humans-such as transmembrane domains-may affect specific functional roles, such as vesicle trafficking and lysosomal organization.

In higher vertebrates, spatacsin and its orthologs are predicted to have two transmembrane domains, whereas in many insects, including worms, only one is present. This suggests that additional domains, such as the second transmembrane domain, might have been acquired through evolutionary changes. Interestingly, when examining the spatacsin protein of various *Drosophila* species, no transmembrane domains are detected (Blum et al., 2024). This may indicate that the single transmembrane domain present in other insects was lost in *Drosophila*. The functional implications of this loss should be studied further, through investigation of lysosomal function, synaptic activity, and filopodia formation.

RNA sequencing offers a powerful approach to uncover pathways associated with *CG13531*. A transcriptomic analysis could identify differentially expressed genes in the absence of *CG13531*, and subsequent GO-term analysis may provide insights into the biological pathways and processes in which *CG13531* is involved.

5.8. Comparison *CG13531* Knockout Model with Other Animal Models

Mouse models of *SPG11* knockout show locomotor deficits, such as reduced performance on the rotarod (Boutry et al., 2019; Branchu et al., 2017) and increased fall frequency (Varga et al., 2015). Similarly, zebrafish models with *SPG11* downregulation demonstrate motor ability loss and even paralysis (Boutry et al., 2018). In line with these findings, the observed decline in motor ability in our model aligns well with its potential as a motor neuron disease model.

SPG11 knockout mice also display atrophy and motor neuron degeneration (Branchu et al., 2017), while zebrafish models show impaired motor neuron outgrowth (Martin et al., 2012). Additionally, human cell line studies suggest that *SPG11* downregulation negatively affects neurite length (Güner et al., 2021; Pérez-Brangulí et al., 2014). Although motor neuron degeneration was not observed in our study, significant motor neuron deficits further support the utility of this model for studying *SPG11*-related diseases.

Studies on *SPG11* knockout mice highlight lysosomes, autophagosomal, and autolysosomal dysfunction. These include autolysosome accumulation (Varga et al., 2015), autophagosome accumulation (Khundadze et al., 2021), and decreased lysosome numbers (Branchu et al., 2017; Khundadze et al., 2021). Additionally, lipid accumulation (Boutry et al., 2019; Branchu et al., 2017), and reduced ganglioside synthesis result in delayed disease symptoms (Boutry et al., 2018). Furthermore, a decline in axonal mitochondrial transport has been reported in iPSC studies (Güner et al., 2021), suggesting that multiple mechanisms contribute to *SPG11*-related pathogenicity. Considering that *CG13531* interaction partners are involved in mitochondrial and lysosomal organization, defects in lysosomal and autophagosomal functions, as well as changes in mitochondrial transport, should be investigated to further characterize the *CG13531* knockout model.

While we observed progressive locomotor deficits and alterations in NMJ morphology consistent with reduced growth suppression, degenerative events were not detected in this study. Nevertheless, our findings suggest that *CG13531* knockout mutants are a promising model for studying *SPG11*-related diseases.

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