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Uncovering Early Synaptic Defects in Amyotrophic Lateral Sclerosis

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS), also known as Lou Gerhrig's disease, is a progressive neurodegenerative disorder that currently holds the worst prognosis of this class of neurological disorders. The name may sound familiar due to the famous Ice Bucket Challenge that began four years ago with the goal to raise money and awareness for this devastating disease. Because of the rapid nature of this progression, the average survival time from the onset of symptoms is only 3-5 years, giving it the worst prognosis of the neurodegenerative disorders. Stephen Hawking is a famous anomaly, having survived over fifty years with the disease. However, this is sadly not the case for most patients. Only about 5% of cases survive over 20 years with ALS. Although other disorders such as Alzheimer's Disease or Parkinson's Disease are more prevalent in our society, it is important to also draw attention to ALS which currently has no cure and few effective treatments.

The disease is primarily characterized by degeneration of the upper and lower motor neurons which innervate muscles controlling voluntary movements (Chio et al., 2009). When these motor neurons degenerate, nerve cells can no longer provide nourishment to the muscle cells and the muscles begin to atrophy or become weaker and smaller. This disease targets the lateral regions of the spinal cord and results in sclerosis or hardening of those regions as the disease progresses. Voluntary muscles are those which are under conscious control. This means that muscles in the arms or legs can be affected, but the heart and digestive system will not be. However, swallowing and breathing is controlled by voluntary muscles and will become progressively more difficult as the disease advances. Weakening of the diaphragm muscle will leave the patient unable to breathe, making respiratory failure the most common cause of death in ALS patients (Wolf et al., 2017).

Nearly all cases of ALS occur sporadically, making this fatal disease even more difficult to treat. About 5-10% of cases have been found to be familial, with links to heritable mutations in a diverse range of proteins (Machamer et al., 2014). Therapies related to its pathogenesis remain elusive, with the greatest advances being made in provision of care and supportive care, such as nutrition and respiratory support (Morgan et al, 2016). Disease-modifying treatment is limited to Riluzole, a glutamatergic antagonist used to block the release of glutamate from nerve cells, ultimately reducing the glutamate-induced deterioration of neurons. This drug is the only known treatment which extends the life of a patient, with about a 36% reduction in mortality (Fang et al., 2018). Although it has been effective at slowing the disease progression in some cases, it only focuses on treating the symptoms, rather than the causes. This highlights the need for identifying the underlying mechanisms responsible for maintenance of the synapses of these affected motor neurons. This knowledge is crucial for understanding the basis of early neuronal defects in aging and neurodegenerative diseases.

Abnormal intracellular protein aggregates are commonly found in neurodegenerative diseases. A few key proteins have been identified as being involved in the pathogenesis of ALS, including TAR DNA-binding protein 43 (TDP-43) and fused in sarcoma (FUS) (Mackenzie et al, 2010). These disease-related proteins share both functional and structural similarities and are both DNA/RNA binding proteins. Their cytoplasmic accumulation has been linked to neurodegeneration, although the mechanism behind this accumulation is still unknown. There are currently more than 35 ALS-linked mutations identified in TDP-43, and 30 ALS-linked mutations reported in FUS. This indicates that these proteins are promising candidates for developing biomarker assays and targeted therapies for patients with ALS (Mackenzie et al., 2010). Acquiring these biomarkers could be used to more effectively screen and diagnose patients, evaluate their risk for developing the disorder, and to eventually create drug therapies targeted specifically for these individuals (Hottstein et al., 2017).

In instances where ALS is found to be genetically inherited, mutations in the FUS-encoding gene have been found responsible for approximately 3% to 5% of cases (Sama et al., 2014). Normally, the wildtype FUS protein is a highly conserved nucleic-acid binding protein found predominantly in the cell nucleus. It is involved in a variety of cellular functions including DNA damage repair, RNA processing and cellular stress response, among others (Sama et al., 2014). All of these functions make this protein critical to the transcriptional and translational processes of the cell. FUS mutants, on the other hand, can cause irreversible protein aggregates associated with the pathogenesis of ALS.

In terms of its role in the pathology of ALS, it is unclear whether disease-related mutations cause the loss of normal FUS function or instead induce the protein to acquire a gain of toxic function (Sama et al., 2014). Similar to TDP-43, FUS-related ALS pathology is characterized by mislocalization of FUS to the cytoplasm, resulting in a loss of its nuclear expression (Ishigashi et al., 2018). Previous studies on *Drosophila* and zebrafish have found that the functional loss of FUS results in neuronal cell death (Wang et al., 2011). On the other hand, accumulation of FUS in the cytoplasm has been strongly associated with stress granules, which are typically induced by various cellular stresses, such as oxidative stress or mitochondrial dysfunction that inhibit the initiation of protein translation. The presence of stress granules supports the gain of toxicity hypothesis of FUS-related pathology (Ishigashi et al., 2018). Based on the current body of knowledge, there are two possible mechanisms for which FUS could contribute to ALS pathology.

With these hypotheses in mind, we turn to model organism *Drosophila* to help us better understand the synaptic defects underlying ALS pathogenesis. The fundamental mechanisms and morphology of neuromuscular junctions (NMJs), where motor neurons synapse onto muscle fibers, in *Drosophila* are well-conserved from fly to man. Additionally, studies have found that flies contain functional orthologs for nearly 75% of known human disease genes (LLoyd & Taylor, 2017). This makes *Drosophila* an ideal model organism to study neuromuscular development and degeneration. These flies contain a human homolog of the FUS protein, known as the *Cabeza* (*caz*) gene. *Caz* is a member of the RNA binding proteins that are conserved from flies to humans. We hypothesize that since loss of FUS function may contribute to neuronal death, selectively knocking down *caz* in flies may tell us more about the pathogenesis of ALS.

Most current research on ALS using a *Drosophila* model is performed during the larval stage of development. However, *Drosophila* are holometabolous, undergoing a remodeling of their muscle system when transitioning from the larval to adult stage during metamorphosis (Veverytsa et al., 2013). During this time, some neurons are either removed or restructured, and new neurons are integrated. The result is the generation of an adult specific neural circuit, which is responsible for behaviors such as copulation and flight (Veverytsa et al., 2013). Developing ALS early in life is extremely rare, with the average age of onset being 60 years, and most patients being diagnosed between ages 50 and 74 (Traxinger et al., 2013). A study found that altering electrical activity and cell adhesion molecule FasciclinII levels affected development at the adult abdominal NMJ in ways that are distinct from their larval and adult thoracic counterparts (Hebbar et al., 2006). Therefore, it is unclear if the results found in larval flies will mimic those found in the adult stage. We aim to fill this knowledge gap by assessing adult NMJs in transgenic flies containing ALS-related genes.

At this time, most research studying muscular development in *Drosophila* has targeted the indirect flight muscles (IFMs). These muscles share many features with vertebrate somatic muscle cells, which has made them excellent models for examining the genes involved in myogenesis, the development of muscle tissue (Gunage et al., 2017). Although the IFMs have a behavioral flight assay to assess their integrity, we chose to focus on the NMJs found in abdominal muscles, which are very similar in structure to larval NMJs. Many presynaptic and postsynaptic markers can be used in this tissue, making them a great model to analyze the synaptic integrity of the NMJs in transgenic flies. The NMJs are very visible in the abdominal tissue, with terminal boutons that form in a beads-on-a-string arrangement that is easy to visualize and assess under a microscope (Pons et al., 2017). We hope that this abdominal model will help us answer the questions that remain unanswered about the pathogenesis of neurodegeneration in ALS.

Given the existing body of knowledge on this disease, our goal is to detect the genes responsible for maintaining synaptic structure and function and clarify their roles in neuroprotection in vivo. This field currently lacks a genetically traceable model for assessing how synaptic maintenance occurs in aging and in neurodegenerative diseases such as ALS. There is a limited amount of knowledge on how the structure of the synapse becomes altered with age. Using the UAS-Gal4 system, the FUS gene can be driven in *Drosophila* for tissue-specific expression. We hypothesize that abnormalities in synaptic maintenance underlie the mechanism behind neuronal loss in neurodegenerative diseases. We propose that targeting these early defects could prevent neuronal death, thereby addressing the root of the disease rather than simply treating its symptoms. Using wildtype flies as a baseline, synaptic markers can be expressed in the FUS gene model at various time points to determine early synaptic defects that relate to neurodegeneration in ALS. We hope that these results will help develop biomarkers critical to early detection and treatment for this disease.

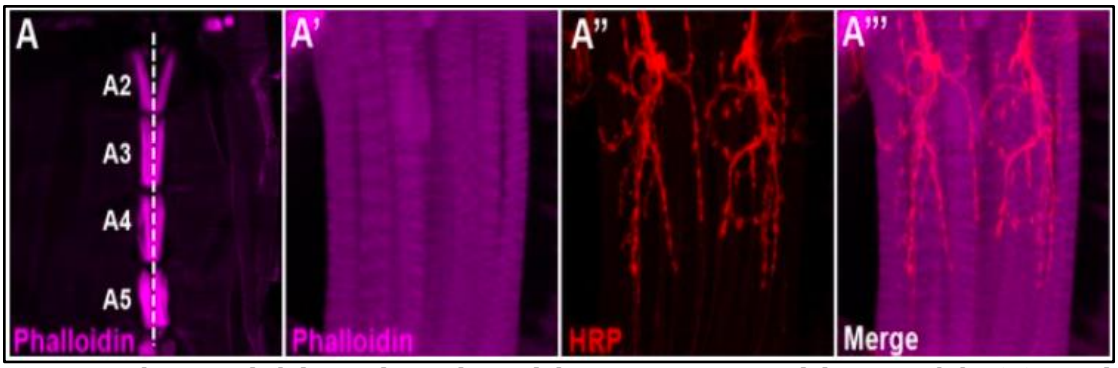


Figure 1: The ventral abdominal muscles and their innervation in adult *Drosophila*. **(A)** In wild type flies, the VAMs are organized in a stereotyped pattern on the abdomen as observed at low magnification. Muscles are labeled using a phalloidin staining. The white dotted line represents the ventral midline. **(A'–A''')** At higher magnification, motor neurons innervate each muscle fiber and synaptic boutons are visible. Adapted from Pons et al., 2017.

Previous research on the ventral abdominal motor neurons was important to consider before beginning our experiments. The abdominal muscles in wild type flies have a stereotyped pattern containing 4 muscles labeled A2-A5, with each segment containing two hemisegments, as seen in Figure 1. The A2 segment is noted by the chevron pattern due to the presence of two chordotonal organs, which separate the two hemisegments (Pons et al., 2017). This was an important structural feature which helped us navigate the orientation of our tissue when observing it under a microscope.

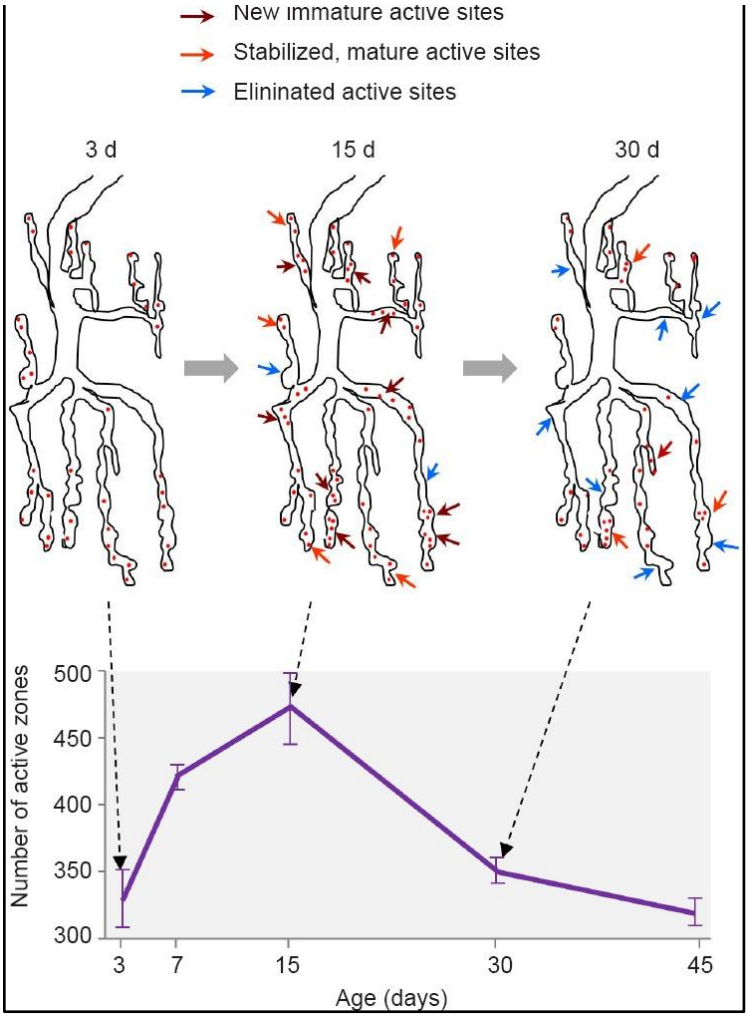


Figure 2: Age-dependent changes in synaptic active zones at the fly abdominal neuromuscular junction (NMJ). Adapted from López-Arias et al., 2017.

The larval NMJ has been successfully used to study the molecular bases of synaptic formation and physiology in *Drosophila*. Figure 2 illustrates the age-dependent changes in the number of active zones present at synapses. In the NMJ of wild type adult flies, there is a progressive increase in the number of active zones during the first two weeks of life. Afterwards, the active zones decrease. This transition from synapse addition to synapse elimination corresponds with the onset of fly behavioral and synaptic senescence (López-Arias et al., 2017).

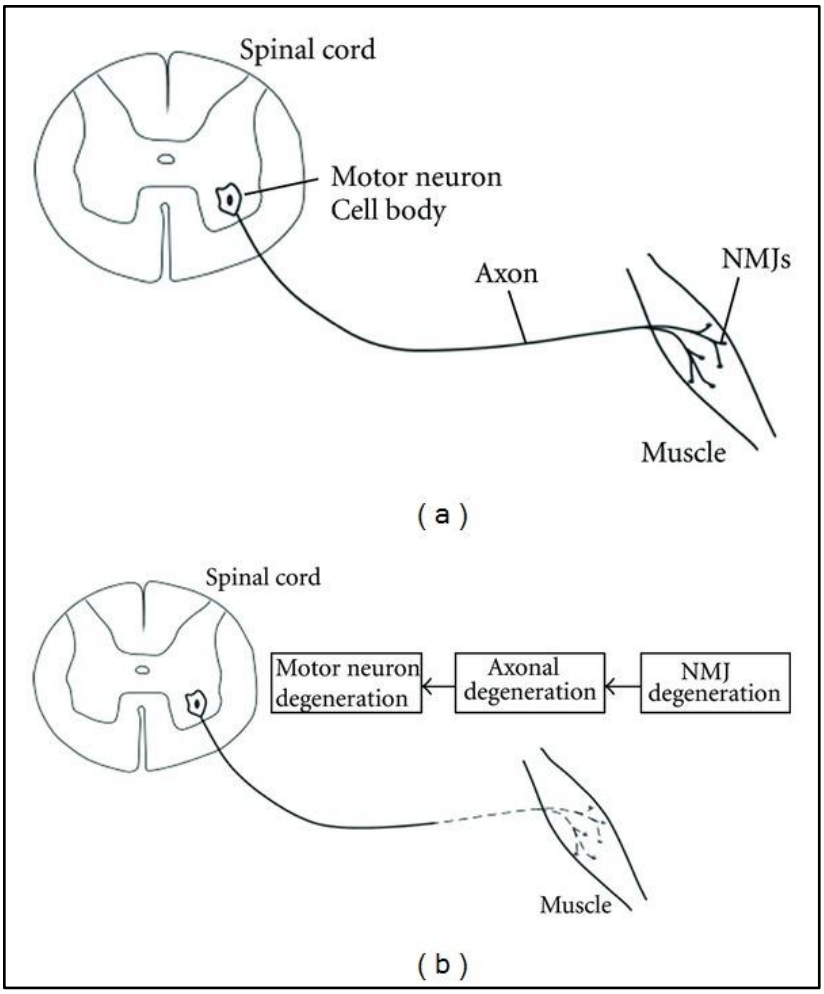


Figure 3: "Dying back" model of motor neuron degeneration in ALS. Adapted from Krakora et al., 2012.

Previous studies have suggested a "dying back" mechanism to explain the nature of the degeneration of motor neurons in ALS, as seen in Figure 3. Dying back involves the slow evolving distal to proximal axonal degeneration commonly observed in a wide variety of degenerative and toxic conditions of the central and peripheral nervous system (Dadon-Nachum et al., 2010) (Fischer et al. 2003). As the NMJs degenerate, the axons begin to slowly pull away as the motor neuron eventually degenerates, resulting in atrophy of the muscle fiber.

RESULTS

RNAi knockdown of *Caz* demonstrates age-dependent motor neuron degeneration in VAMNs.

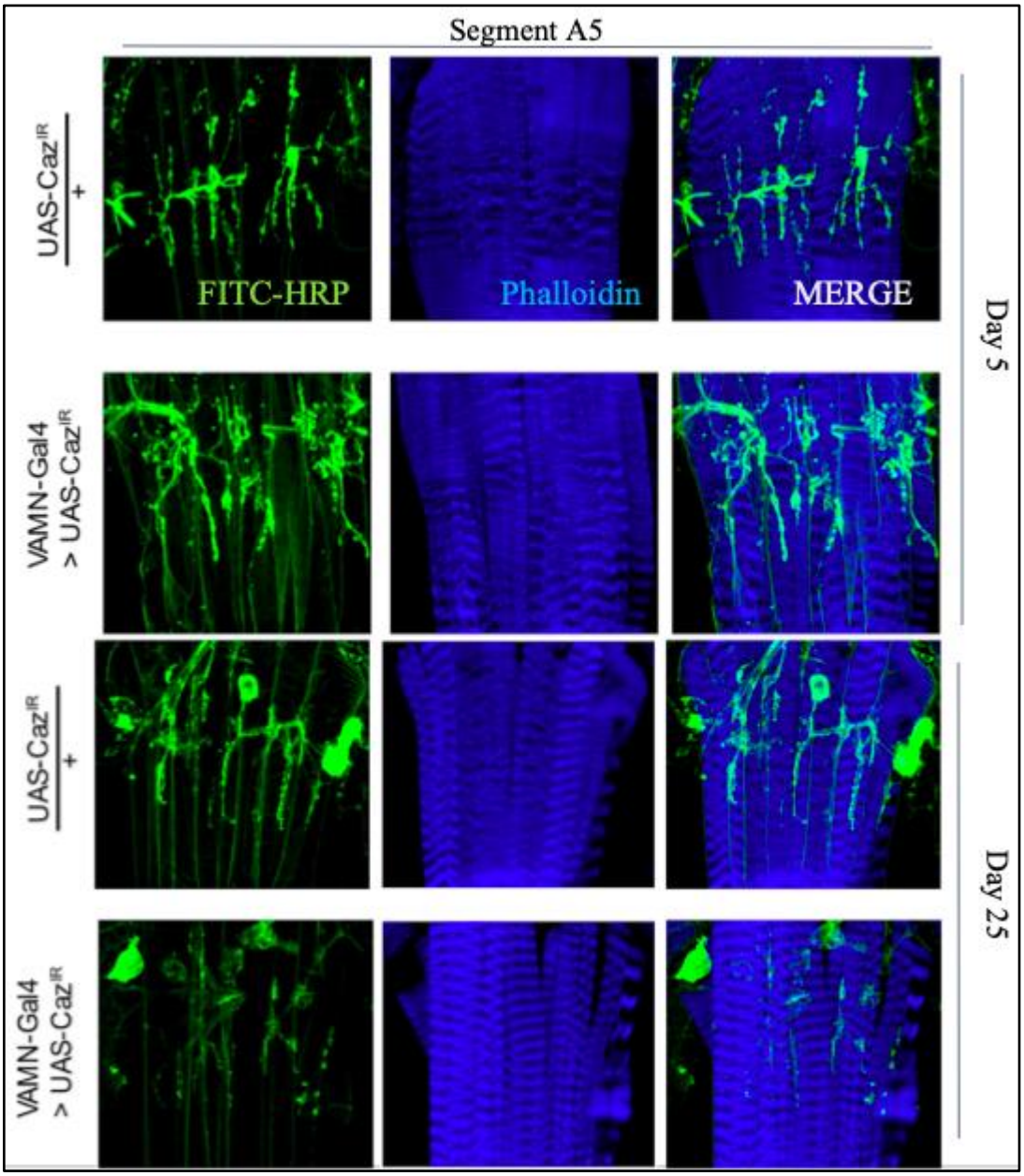


Figure 4: Age-dependent motor neuron degeneration in RNAi knockdown of *Caz* using a VAMN-specific driver.

Our goal was to determine if knocking down *Caz* using RNAi and expressing the tissue-specific knockdown would lead to motor neuron degeneration. This would help us better understand the effects of the loss of FUS in humans, to determine if it is involved in the early pathology of ALS. We examined the phenotypic differences in the motor neurons using a VAMN-specific driver when knocking down *Caz.* When examining the HRP staining, we observed that the motor neurons in which *Caz* was knocked down seemed to contain a reduced number of visible boutons. The Phalloidin staining of the muscle tissue displayed no significant damage, indicating

that the cell death was exclusive to the motor neurons. This indicates that the loss of FUS function in humans may lead to enhanced degeneration of the motor neurons at the synaptic level.

Abdominal motor neurons show decreased expression of postsynaptic DLG when overexpressing wild type and mutant FUS.

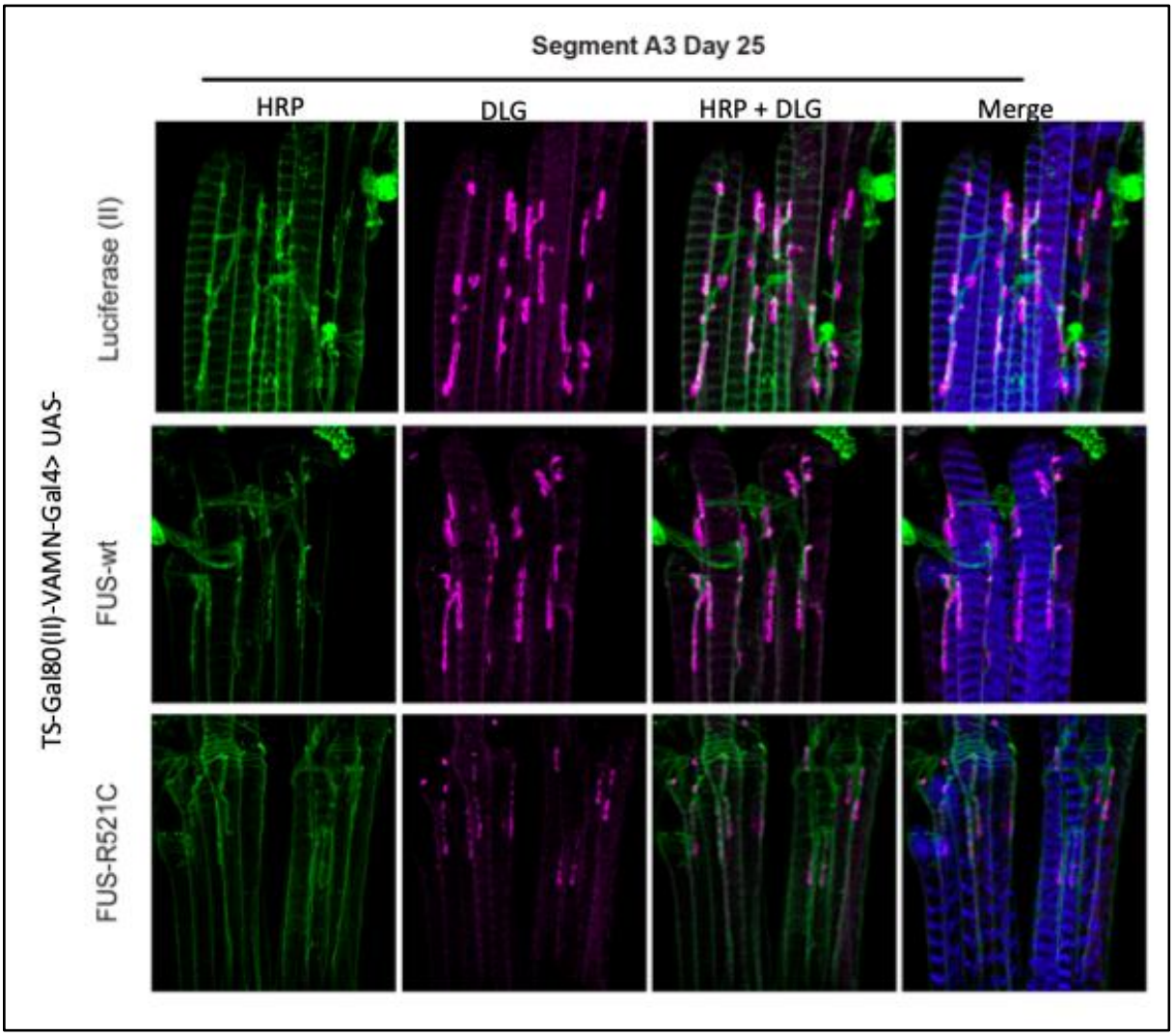


Figure 5: Abdominal motor neurons show decreased number and size of boutons when overexpressing mutant FUS-R521C, compared to control expressing gene reporter Luciferase (II).

We next used the TSGal80 system to drive the expression of FUS wild type and mutant R521C, as well as our gene reporter control on the 2nd chromosome. We collected and aged females at 29°C, the temperature at which the FUS gene is turned on and can be expressed within the ventral abdominal motor neurons. We then compared the structural differences between the wild type and mutant FUS to assess their effects on the structural integrity of the synapse. DLG is a postsynaptic marker found at the subsynaptic reticulum in both larval and adult *Drosophila*. Previous studies have found that mutations in DLG alter the expression of DLG and cause significant changes in the structure of the subsynaptic reticulum at those synapses. The results

indicate that DLG is required for normal synaptic structure and offer insights into the role of its homologs in maintaining vertebrate synapses (Lahey et al., 2015).

The goal of staining for DLG was to better assess the integrity of the synapse in transgenic flies after aging. We observed decreased DLG expression in both wild type and mutant FUS compared to our control. However, the DLG was most visibly reduced in the mutant, suggesting that FUS overexpression could contribute to the loss of this vital protein. Expressing wild type FUS may have its own toxic effects since it is not endogenous to the flies.

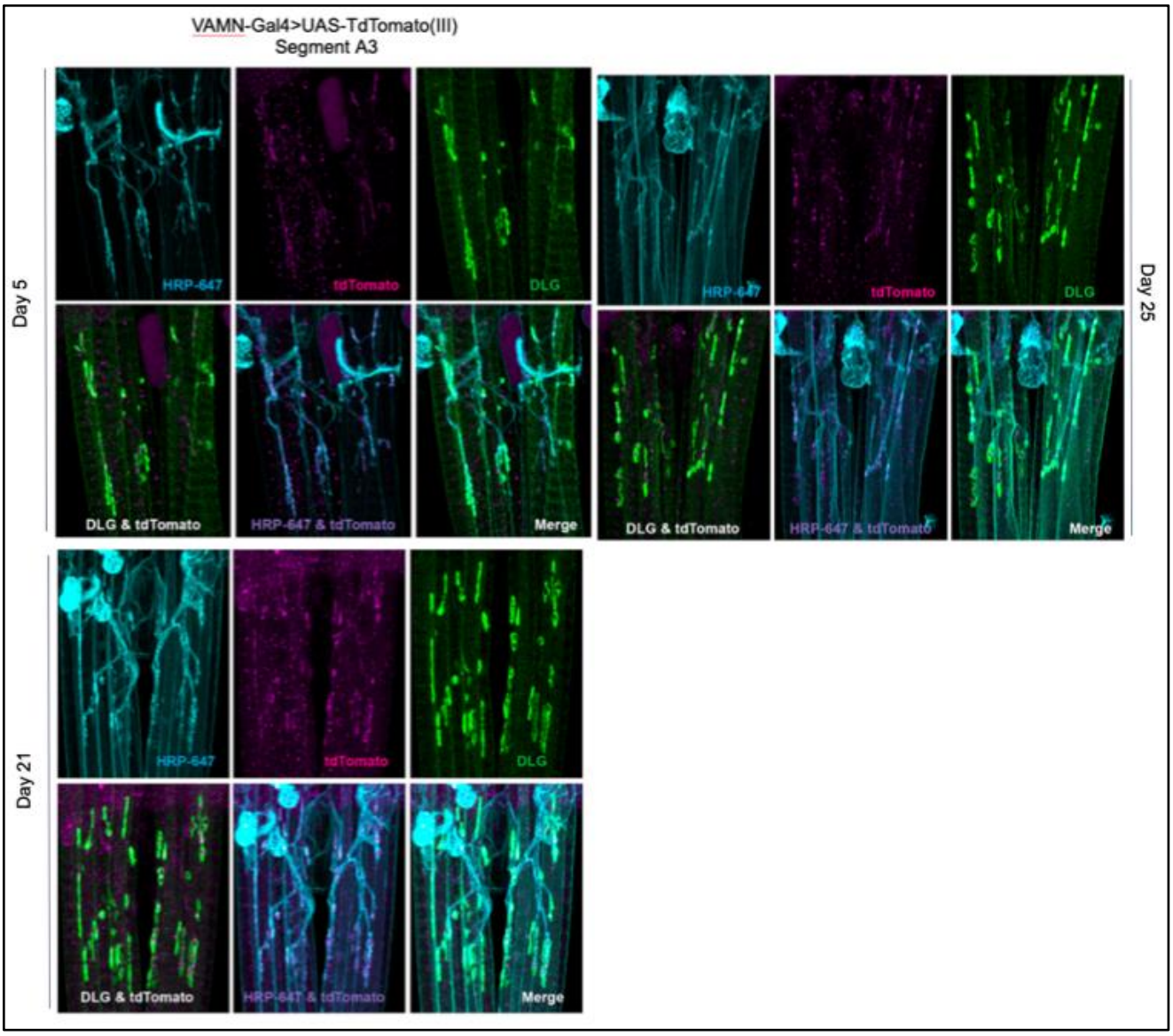


Figure 6: Characterizing our control using gene reporter TdTomato (III) in an age-dependent manner.

We decided upon this gene reporter as a control for our experiments because it maintained synaptic integrity similar to wildtype flies. For this experiment, we did not utilize the TSGal80 system. Instead, they were collected and raised in a 28.7°C. The goal was to examine the flies at several time points to assess any morphological changes that occur. Although we imaged segments A3-A5, we decided to narrow our focus to segment A3. We stained for DLG protein again to assess the synaptic integrity in aged flies. We noticed an apparent increase in DLG expression at age 21 compared to age 5, and then another decrease at age 25. This is consistent with the expression pattern of the TdTomato gene reporter used and the HRP staining of the motor neurons. The phalloidin staining looks consistent between tissues, indicating that there was no muscular tissue damage to the preps.

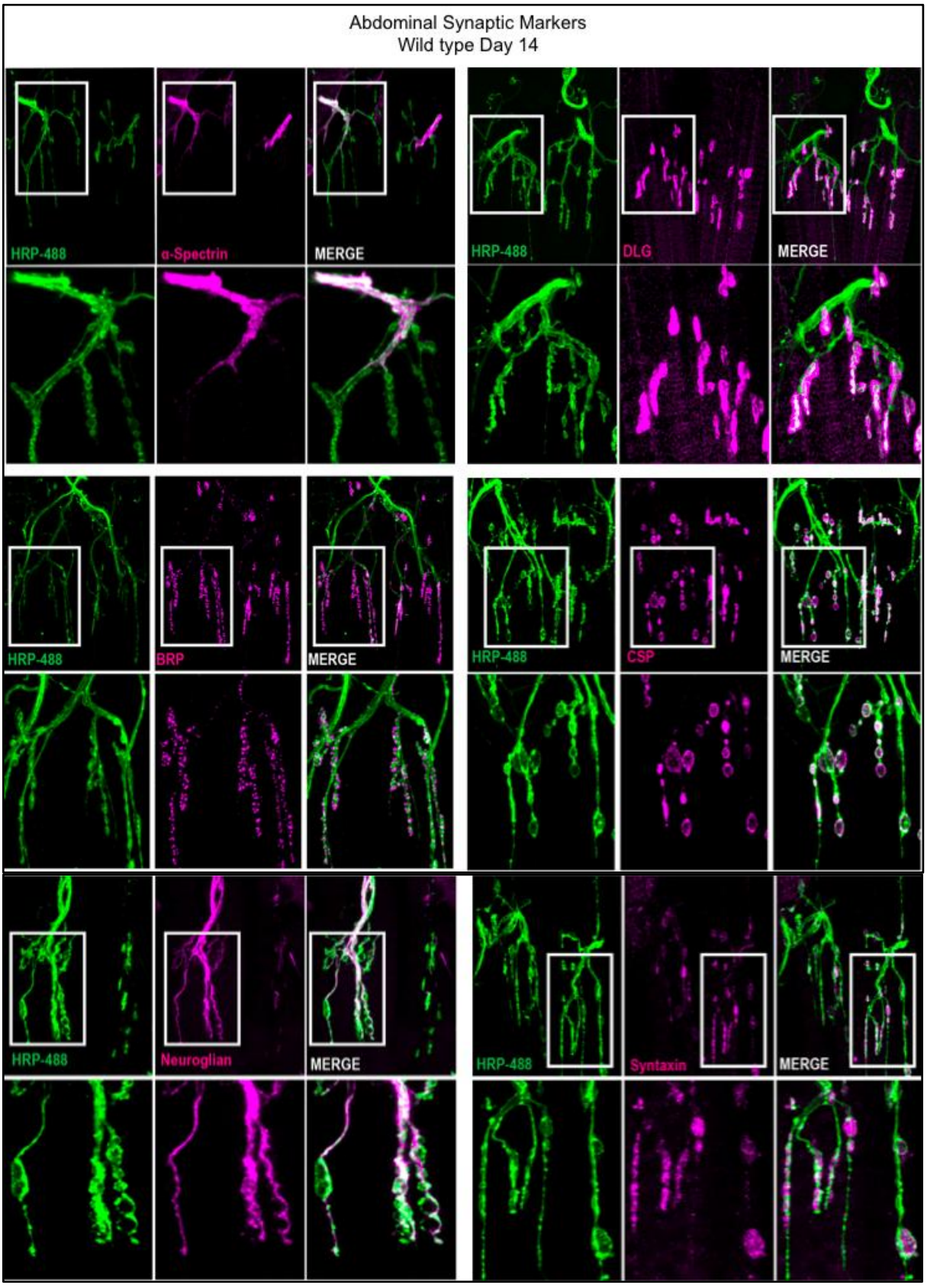
A previous study examined how aging affects the synaptic ultrastructure in the adult *Drosophila* NMJ using fluorescence and electron microscopy. The morphological changes they observed included increased bouton sizes with proportionally rising number of active zones (AZs) and increased synaptic vesicle density at AZs is increased in aged flies. Their findings suggest that aging is possibly accompanied by impaired synaptic vesicle release and recycling and a potentially compensatory expansion of AZs and postsynaptic densities (Wagner, 2015). These observations were important to consider when comparing our results. It is possible that the increased expression of our reporter in aged flies is due to an expansion of AZs at the synaptic level. To further characterize these age-dependent synaptic changes in the VAMNs, we stained for a series of synaptic markers in wild type flies to get an understanding of the baseline levels of their expression.

Staining for presence/absence of synaptic markers to analyze their baseline expression levels in wild type flies.

Table 1: Antibodies to assess synaptic structure

Antibody	Synaptic Component
Bruchpilot	T-Bar, Active Zones
Syntaxin	SNARE proteins
Synaptotagmin	Synaptic Vesicles
Synapsin	Synaptic Vesicles
Cystein String Protein-2	Synaptic Vesicles
Cystein String Protein-3	Synaptic Vesicles
Fasciclin II	Cell-Adhesion Molecules
Neuroglian	Cell Adhesion Molecule
22C10 "futsch"	Synaptic Microtubules
Spectrin	Cytoskeletal Proteins
Disc Large	Subsynaptic Reticulum
Glutamate Receptor Type IIc	Postsynaptic Receptors
Glutamate Receptor Type IIb	Postsynaptic Receptors

Table 1: Common antibodies used to assess synaptic structure at the NMJ. Adapted from Dr. Daniel Babcock and Jess Sidisky.


Abdominal Synaptic Markers
Wild type Day 14
HRP-488
α-Spectrin
MERGE
HRP-488
DLG
MERGE
HRP-488
BRP
MERGE
HRP-488
CSP
MERGE
HRP-488
Neuroglian
MERGE
HRP-488
Syntaxin
MERGE

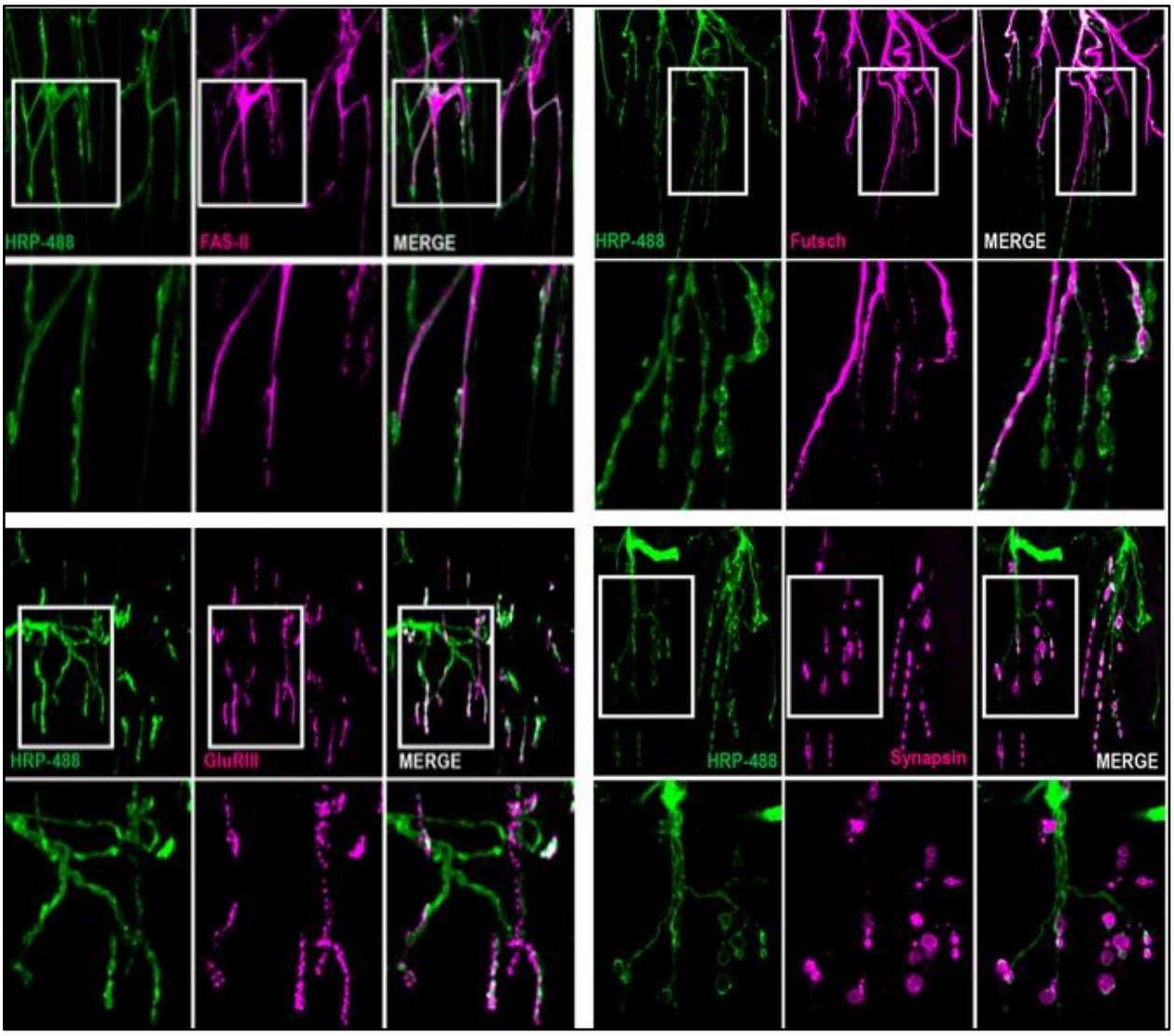


Figure 7: Staining for presence/absence of synaptic markers to analyze integrity of aging synapse. Alpha Spectrin -1:200, DLG-1:50, BRP-1:50, CSP-1:50, Neuroglian 1:25, Syntaxin 1:50, FAS II-1:100, Futsch-1:200, GluRIII-1:2500, Synapsin 1:50, as seen in Table 1.

Prior to analyzing these synaptic markers in the FUS/ *caz* model of ALS, we examined them in wild type flies. The synaptic markers chosen are commonly used to assess the structure of NMJ's. The goal of this experiment was to characterize the synaptic density of several synaptic markers on the abdominal NMJ's in wild type flies. These flies were collected and aged at 28.7°C for 14 days.

Alpha spectrin is typically associated with the plasma membrane and functions in a lipoprotein pathway that delivers dietary fat to the larval fat body for storage (FlyBase). Our imaging displays alpha spectrin localization primarily on the axon of the motor neurons that does not extend towards the boutons. Boutons are defined as oval-shaped structures that contain multiple AZs where neurotransmitters are released, and each of these is apposed to a glutamate receptor (GluR) cluster (Menon et al., 2013). We can see in our images that the glutamate receptor

IIc (GluRIII) is localized at the postsynaptic boutons of the motor neurons, as expected. The presynaptic bouton is surrounded by the subsynaptic reticulum (SSR) that contains neurotransmitter receptors, scaffolding proteins, and postsynaptic signaling complexes. DLG is a postsynaptic marker found in the subsynaptic reticulum. It is seen wrapping around the boutons, which is consistent with its known function. Bruchpilot (BRP) molecules form an elongated conformation that stretches from the AZ membrane towards the boutons, forming the well-characterized T-bar structure at the NMJs (Ackerman et al., 2015). BRP is essential for enabling Ca^{2+} channels to cluster beneath the T-bar at the center of the AZ, which brings the Ca^{+2} source close and allows Ca^{+2} to fuse into the presynaptic membrane (Ackerman et al., 2015). BRP's localization allows us to visualize the active zones in each bouton, which could be used in future experiments to characterize the levels of AZs in ALS transgenes.

Cysteine string protein (CSP) and synapsin are both components of synaptic vesicles and are seen to localize around the boutons in a similar fashion. CSP is critical for neurotransmitter release at the NMJ of *Drosophila*, with previous studies showing it is also required for nerve terminal growth and the prevention of neurodegeneration in *Drosophila* and mice (Dawson-Scully et al., 2007). Synapsin is important for vesicle clustering, neurodevelopment, and plasticity (Vasin et al., 2014). Syntaxin is a T-SNARE transmembrane protein that drives membrane fusion during neurotransmitter release (Harris et al., 2016). It is also seen localized near the boutons, as expected.

Neuroglian is a cell adhesion molecule that has been shown to be required for synaptic stability (Enneking et al., 2013). It is imaged along the entire length of the axon. We find Fascicillin II localized in a similar way in our images. FasII is another cell adhesion molecule which has been found to play a role in the stabilization of motor neuron branches during pruning in *Drosophila* flight muscles (Hebbar et al., 2005). Futsch is a component in synaptic microtubules that has been found to control synaptic growth at the *Drosophila* NMJ by regulating the synaptic microtubule cytoskeleton (Roos et al, 2000).

Each of these postsynaptic markers could lead to deficits in neuronal integrity, making them a crucial component of our study. Once we characterized what these proteins look like in the adult *Drosophila* NMJ, we were able to begin characterizing them in flies expressing gene reporter Luciferase (III) or the wild type or mutant FUS gene. At the NMJ, studies have found that overexpressing either wild-type or mutant FUS reduced the number of active zones at the presynaptic terminal. Additionally, the postsynaptic terminal's glutamate receptor subunit composition was altered, ultimately reducing the amount of neurotransmitters released (Machamer et al., 2014). They also found that motor neurons expressing mutant FUS-R521C resulted in decreased size of postsynaptic density and decreased number of synapses per unit area in mice (Qui et al., 2014). We expanded on these results by staining for several synaptic markers and examining their density at ages 5, 14, 21, 25 and 28 in both wild type and mutant FUS. Although these results have not been imaged, they are a vital step forward towards discovering the synaptic defects underlying motor neuron degeneration in ALS.

Discussion

Overall, we've gained a greater understanding of the development of adult *Drosophila* NMJs, which are still not well characterized in this field. By aging them to different time points,

we can better understand the characteristics of an aging motor neuron. Our transgenic crosses have given us a better understanding of the role that FUS protein overexpression and knockout of its fly homolog *Cabeza* plays in neuronal development. Although we were not able to image the synaptic markers in these transgenic flies, our characterization of their localization in wild type flies has broadened our knowledge on the abdominal NMJs as a whole.

To further our experiments, we planned to begin to quantify the AZs in the boutons using synaptic marker BRP. We were able to stain for these markers in our control, wild type and mutant FUS flies. Quantifying the AZs would allow us to determine if there are structural differences that we cannot observe through simply looking at the confocal images. Perhaps there is a significant decrease in the localization of a vital synaptic marker that is initiating cell death in these motor neurons. As mentioned in our results, each synaptic marker analyzed has been found to play a crucial role to the integrity of the synapse and aberrations in their functions could lead to neurodegeneration. If we observe abnormalities in one or more synaptic markers, this will allow us to move forward and discover the genetic defect underlying these changes.

We could expand on our results using the fly homolog *Cabeza* using the FLP/FRT system to knockdown the *caz* gene more specifically, rather than using the RNAi method that has known limitations. Cell-type-specific knock-down by transgenic RNAi typically displays a significant knockdown of only ~50-80%, which increases the risk of off-target effects in our flies. The FLP/FRT system will increase the efficacy of the knockdown, yielding more precise results.

Despite our study, we are still left with many questions regarding the pathogenesis of ALS at the synaptic level. Moving forward, we could analyze the quantal content and size of the vesicles using electrophysiological methods. This will allow us to understand if ALS mutations are affecting the amount of neurotransmitter produced or released, giving us insight into how the motor neurons are starting to deteriorate. We could continue to explore the role of the loss of function or gain-of-toxic function of FUS in early synaptic defects to determine a genetic marker of the disease. Overall, our results help the scientific community gain more insight into detect the genes responsible for maintaining synaptic structure and function. The goal is to one day clarify their neuroprotective roles in hopes of developing a therapeutic treatment that will address the neuronal defects before the motor neurons even begin to die. Early intervention could potentially save many lives of those who have the potential to develop the symptoms of this fatal disease.

MATERIALS AND METHODS

Fly rearing and genetics

GAL4 protein activates transcription through binding to upstream activation sequence (UAS), whereas GAL80 protein prevents GAL4-dependent transcription. The temperature-sensitive GAL80[ts] provides a temporal regulation of UAS transgene (Duffy, 2002).

Flies were raised on standard *Drosophila* medium at 25°C. For aging experiments, adult flies were stored at 18°C and collected each day and raised at 29°C. The following fly stocks were obtained from the Bloomington Drosophila Stock Center: *Oregon-R, UAS-FUS-HA, UAS-FUS-R521C, UAS-CAZ- RNAi, UAS-Luciferase (II), UAS-Luciferase (III), UAS-TdTomato (II), UAS-TdTomato (III), UAS- Luciferase-RNAi (II).*

Ventral Abdominal Dissection Procedure (adapted from Dr. Daniel Babcock)

Flies were anesthetized with CO2 and dunked in 70 % ethanol, to reduce waxy coating. 4% Paraformaldehyde Fix was prepared in PBS. A 6cm sylgard dish was used. Tools used: Dissection scissors, 1 non-sharp pin forceps, 2 fine tipped forceps. In each dish we prepared 3 preps as follows: a) Added PBS (room temperature) to the dish and removed wings. b) Next, used the scissors to cut at the distal end of the halters, removing the thorax, legs and head. Removed the genitalia next. Bisected the tissue along the dorsal midline of the abdominal segment. Next, pinned each corner of the tissue into the siligard dish. Used fine forceps to remove viscera (organs). Repeated for additional preps. Placed in Fix solution for 30 minutes at room temp. After 30 minutes, removed Fix and washed dish 3X with PBS. Discarded waste in the waste container under fume hood.

Immunohistochemistry

Following dissection, tissue was placed in 1.5 mL (1500µL) Blocking Buffer (BB) solution in the fridge (4º) for 1 hour. Primary antibodies (Fig.7) were diluted in BB and placed in 4º fridge overnight. The tissue was then washed 4x with 1.5mL PBS with 0.3% Triton solution (PBST). Secondary antibody staining was applied and spun for 2 hours in a dark box at room temperature. The following secondary antibodies were used: 568 goat-α-mouse (1:200), 568 goat-α-rabbit (1:200), 488 HRP (1:200), 647 phalloidin (1:1000). Once secondary staining was complete, tissue was washed 4x with 1.5mL PBST (5 mins each). The tissue was then mounted onto a slide using Vectashield.

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