Towards an Animal Model to Study Sporadic ALS

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Abstract

Amyotrophic lateral sclerosis (ALS) is a complex and presently incurable disease that causes progressive degeneration of motor neurons. About ten percent of cases are attributed to genetic factors while ninety percent are sporadic and have unknown causes. Thus, animal models created by genetic means cannot be studied to learn about sporadic ALS, so another method for inducing ALS must be discovered. The incorporation of *D. melanogaster* models of neurodegeneration holds tremendous promise for discovery of therapeutic targets. In order to induce ALS-like motor impairment, the light-sensitive protein Mito-Killer Red was expressed in the mitochondria of fly motor neurons. Mito-Killer Red photoactivation is known to release hydrogen peroxide; herein the neuromuscular junctions of instar 3 larvae. The motor function was examined before and after photoactivation. Larvae were then dissected and processed for immunostaining of the neuromuscular junction and fluorescence microscopy imaging. Photoactivation caused a slight but not statistically significant effect on motor function (P=.10) and the structure of the neuromuscular junction (P=.13). On average, the Mito-Killer larvae performed 2.4 times worse than the controls at the 90 minute mark and 2.5 times worse at the 120 minute mark. The structural integrity of the neuromuscular junction decreased by 11.7%. To confirm this relationship, future studies should use larger sample sizes and find ways to increase the intensity of ALS-like motor impairment in larval and adult stages. This study is an important step towards a model for sporadic ALS, which has the potential to provide insight into the underlying causes of sporadic ALS and allow for the development of drug screening protocols.

Introduction

Amyotrophic lateral sclerosis (ALS) is a complex and presently incurable disease that causes progressive degeneration of motor neurons. Although it affects approximately 220,000 people across the world [1], there is currently no known cure or treatment. About ten percent of ALS cases are attributed to genetic factors while the remaining ninety percent are sporadic. In these cases, the cause is unknown [2]. This distinction means animal models of study created by purely genetic means cannot be relied on to learn about sporadic ALS. In order to assay drugs or treatments for sporadic ALS, an animal model must be created without using the genetic inheritance of ALS.

One potential contributor to ALS is the neuromuscular junction: a chemical synapse that connects a motor neuron to a muscle fiber. This junction is responsible for controlling communication between the nervous system and the body's muscles [3]. Since ALS symptoms include loss of muscle function, it follows to reason that an appropriate site of study is where the nervous system meets the muscles.

Optogenetics is the combination of optics, the use of light, and genetic manipulation to cause specific changes to occur on a cellular level. This technique involves genetic modification to express a protein that can be activated with a light treatment [4]. This combination allows researchers to coordinate certain molecular changes at a given time during experimentation. One such protein is Mito-Killer Red, which releases hydrogen peroxide (H_2O_2) from mitochondria upon exposure to green light [5]. Mitochondria are the power plants of all eukaryotic cells, and in addition to supplying energy they release toxic waste just like real power plants. One waste product in particular is hydrogen peroxide, here referred to as reactive oxygen species (ROS).

ROS are known to elicit cellular damage mainly through oxidation of proteins [6]. This oxidative damage has been linked to sporadic ALS [7] because it can lead to the degeneration of motor neuron synapses. Therefore, by exposing the Mito-killer transgenic *Drosophila melanogaster* to green light and producing free-radicals, oxidative damage may induce ALS-like symptoms, creating an effective and reproducible sporadic ALS model.

Objective

To determine the effect of Mito-killer activation on motor behavior and the structure of the neuromuscular junctions.

Methodology

We resorted to crosses of fly lines that where either previously generated in the laboratory or purchased from the Bloomington Stock Center (BDSC, Indiana), taking advantage of the UAS/Gal4 system, which allows targeted expression of the Mito-killer proteins. To accomplish Mito-Killer expression in motor neuron mitochondria, we crossed the Mito-killer fly line (w+; UAS-Hsp70-Mito-KillerRed; +) with a D42-Gal4 driver line (D42 fly) and confirmed expression by sorting wandering L3 larvae that with red fluorescent motor neurons in the ventral nerve cord. Green light exposure is anticipated to release hydrogen peroxide (H_2O_2) in motor-neuron mitochondria.

The overall procedure involved monitoring the motor function of the *Drosophila melanogaster* larvae before and after a green light treatment. Motor impairment was determined using flipping assays during which the instar 3 larva is flipped into a "belly up" position and the time until it flips back over is recorded. After the final flipping assay, the larvae were dissected into larval fillets and immunohistochemistry was utilized to prepare the fillets for fluorescence microscopy to image the neuromuscular junctions of both the Mito-killer and D42 lines. This process allowed for the analysis of motor impairment and changes in morphology of the neuromuscular junction in an effort to induce ALS symptoms and create a disease model for sporadic ALS.

Motor function flipping assaying

Materials

1) Fly Lines (Stage 3 larvae)
    a) Mito-killer Red: UAS-Hsp70-Mito-KillerRed / D42-Gal4 (Mito-killer)

b) Wildtype / D42-Gal4 (D42)

2) Stopwatch
3) Paintbrush
4) Stereomicroscope
5) Apple juice agar plates

Methods

Four transgenic (UAS-Hsp70-Mito-KillerRed / D42-Gal4) and four wildtype (D42-Gal4/D42-Gal4) stage 3 larvae were collected and individually separated into apple juice agar plates. Their phenotypes were confirmed using brief exposure to green light to test for red fluorescence in the motor neurons. The larvae were then allowed to acclimate to the agar plates for a minimum of 30 seconds.

The flipping assay procedure was then carried out on each larva and repeated for a total of four assays: while in a "belly-down" position, the larva was rotated to a "belly-up" position and a stopwatch was started. Once the larva flipped back the timer was stopped.

At this point in experimentation, the larvae were subjected to green light treatment (see below). At the 90-minute mark, the flipping assay was repeated four times again for each larva. The assay was repeated another four times at the 120-minute mark.

Dissection and larval fillet imaging

Materials

1) Fly Lines (Stage 3 larvae)
   a) Mito-killer Red: UAS-Hsp70-Mito-KillerRed / D42-Gal4 (Mito-killer)
   b) Wildtype / D42-Gal4 (D42)
2) Fly dissection tools (2mm tweezers, scissors, 1mm dissection pins)
3) Paintbrush
4) Stereomicroscope
5) NIKON TE2000 automated fluorescence microscope

6) Reagents:
   a) PBS - phosphate buffer solution
   b) PBT - as above, plus 0.1 % triton X-100
   c) FITC-HRP (FITC Anti-horseradish peroxidase) antibody to label the presynaptic membrane
   d) Anti-discs large antibody to label the postsynaptic membrane

Methods

Following the final flipping assay, the larvae were dissected along abdominal walls using a stereomicroscope, fine tweezers, scissors, and dissection pins. They were immediately treated in 4% paraformaldehyde in PBS for 15 minutes to prevent decay. The larval fillets were then washed three times in 100 μL PBT.

Each fillet was then incubated with 100 μL (1:500 FITC to PBT), refrigerated overnight. The next day, the fillets were washed and treated with 100 μL of a 1:500 dilution of anti-horseradish peroxidase in PBT and a 1:500 dilution of anti-discs large in PBT at room temperature for 2 hours, after which they were washed again.

The fillets were then mounted on slides using 50 μL mounting solution to be imaged under 40X immersion oil lens of the NIKON TE2000 automated fluorescence microscope. Images of 1398p x 1040p were taken of neuromuscular junctions in the 3rd muscle segment (if possible). Both red and green fluorescence were captured. ImageJ was used to measure the area of both green and red fluorescence.

Green Light Treatment

Materials

1) Fly Lines (Stage 3 larvae)
   a) Mito-killer Red: UAS-Hsp70-Mito-KillerRed / D42-Gal4 (Mito-killer)
   b) Wildtype / D42-Gal4 (D42)
2) Green LED

3) Computer with pulse simulator software
4) Power source
5) Transparent 96-well plate
6) HL3.1 buffer (70 mM NaCl, 5 mM KCl, 1.5mM CaCl2, 4mM MgCl2, 10mM NaHCO3, 5mM Trehalose, 115mM Sucrose, 5 mM Hepes)
7) Paintbrush

Methods

The larvae were individually placed into wells of a 96-well plate with 100 μL HL3.1 buffer and positioned under the green LED such that all larvae would be directly underneath the strongest light. The LED was then activated in pulses of 100 ms at 5Hz at an intensity of 10 V. This light activation was administered for 5 minutes, after which the larvae rested for 15 minutes in the same well plate but with no green light exposure. This process was repeated for a total of five exposures each lasting 5 minutes. After the last exposure, the larvae rested for 90 minutes in apple agar plates before assaying continued.

Results

In order to determine whether ALS symptoms were induced in the larvae, fluorescence images of the neuromuscular junction following photoactivation were analyzed to detect morphological changes. The ratio of red fluorescence (muscle) to green fluorescence (neuron) was measured in both Mito-Killer and D42 *Drosophila* larvae. Additionally, flipping assays were carried out to measure the motor function of *Drosophila* larvae before, 90 minutes after, and 120 minutes after photoactivation. The key data taken from this assay was the ratio of flipping times from before photoactivation to 90 minutes and 120 minutes following photoactivation.

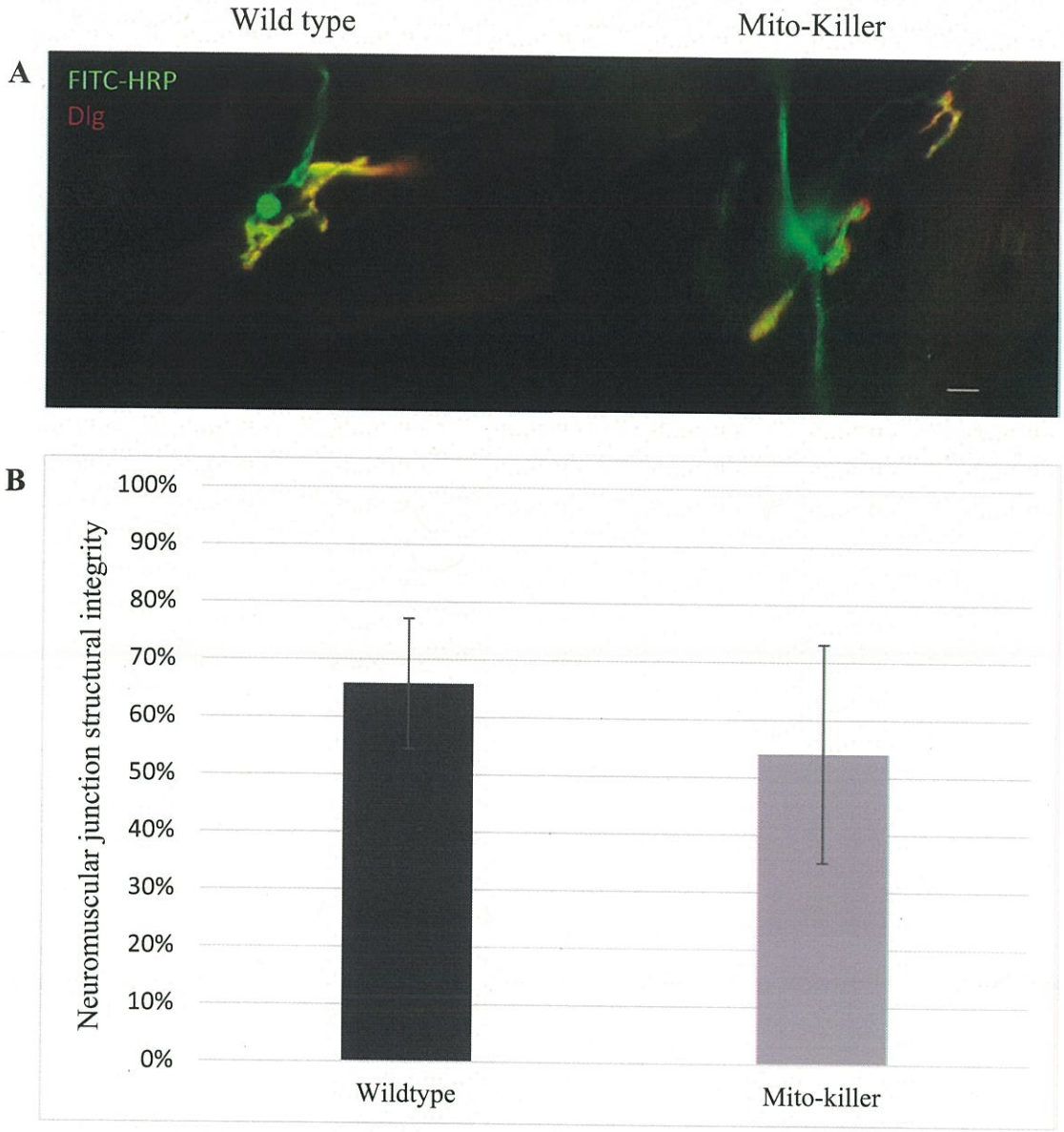

Figure 1: Effect of Mito-killer photoactivation on structural integrity of the neuromuscular junction. A) The ratio of Anti-discs large fluorescence (muscle) to FITC-HRP fluorescence (neuron) was measured to determine the structural integrity of the neuromuscular junction after photoactivation in both D42 (left) and Mito-killer (right) Drosophila larvae. The scale bar is 100 μm. **B)** The ratio of presynaptic membrane area to postsynaptic membrane area.

The ratio of the presynaptic membrane area to postsynaptic membrane area was measured in fluorescence images using the software ImageJ to determine the structural integrity of the

neuromuscular junction. The average ratio was 0.541 in Mito-killer larvae (N=5, STD=0.11) and 0.658 in D42 larvae (N=5, STD=0.19), showing an average decrease in neuromuscular junction structural integrity by 11.7% in Mito-killer larvae compared to wildtype. However, the data were not statistically significant (*P*=.13).

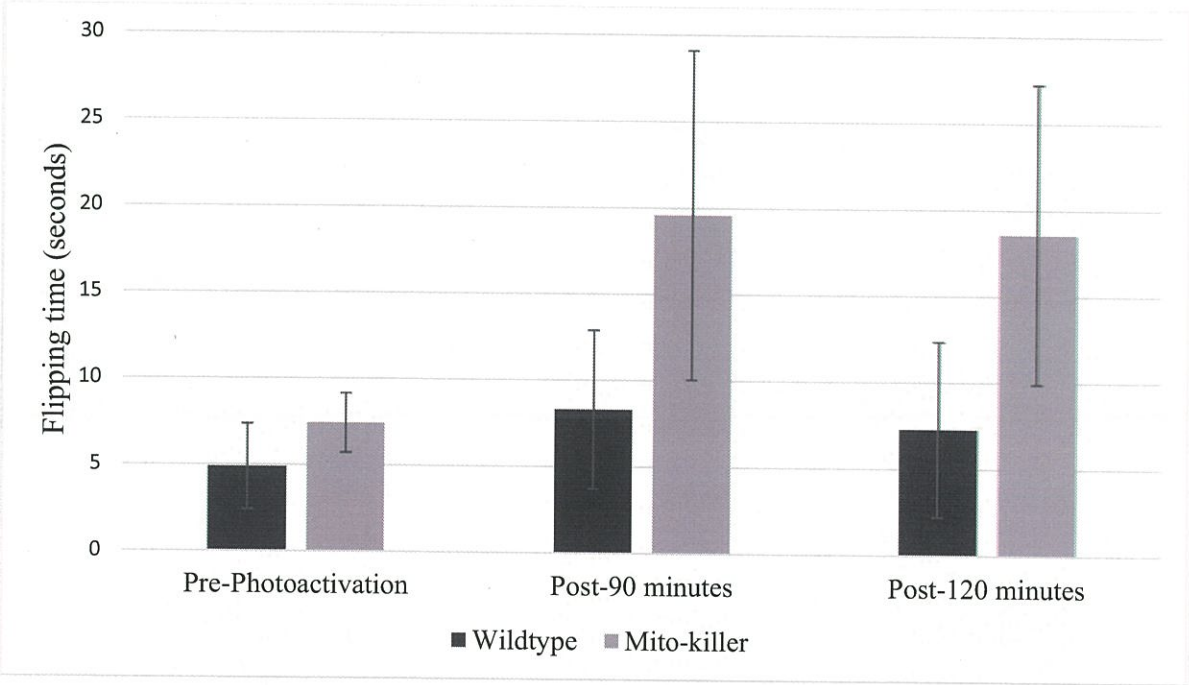

Figure 2: Motor function in response to Mito-killer photoactivation. The bar histograms show the average flipping times of D42 (wildtype) and Mito-killer (Mito-killer) larvae before, 90 minutes following, and 120 minutes after photoactivation gathered from flipping assays.

The flipping assays indicate that motor function decreased in both lines after photoactivation, and although Mito-killer experienced a stronger decrease, the difference between the Mito-killer and D42 is not strong enough to be statistically significant from before photoactivation to 90 minutes (*P*=.13) and to 120 minutes (*P*=.10) after photoactivation. At 90 minutes following photoactivation, Mito-killer larvae performed an average of 2.6 times worse compared to initial performance (N=5, STD=1.0), and D42 larvae performed an average of 1.9 times worse (N=6, STD=0.9). At 120 minutes following photoactivation, Mito-killer larvae performed an average of 2.7 times worse compared to initial performance (N=6, STD=1.4), and D42 larvae performed an average of 1.7 times worse (N=6, STD=1.1).

Discussion

Previously, researchers have applied optogenetics to motor behavior [8], but this project seeks to utilize this technique in disease research. To create a disease model for sporadic ALS, disease symptoms must be induced in *Drosophila melanogaster*. If successful, this would be the first model of its kind and would allow future researchers to screen for drugs to treat ALS. The incorporation of *D. melanogaster* models of neurodegeneration holds tremendous promise for discovery of therapeutic targets compared to screenings based on *in vitro* cell culture, enzymatic assays, or receptor binding assays [9]. *Drosophila* models of human diseases provide several unique features such as powerful genetics, highly conserved disease pathways, and very low comparative costs. With over 75% of the human disease-associated genes having similar genes in *Drosophila* [9], human diseases are now being studied in an animal that is more cost and time effective and is easier to maintain than conventional vertebrate models. Combined with the relative ease to induce tissue specific expression of mutant genes, it is not surprising that a large collection of neurodegeneration models is available in *Drosophila* lines. The repertoire includes SOD1, TDP43 (tbph), VAPB, FUS, Alsin, TAF15, EWSR1, C9orf72, and hnRNPA2 mutants [10]. Of critical importance to this proposal, most of the features described in mice have been confirmed in fly models, i.e., the accumulation of damaged presynaptic mitochondria, impairment of synaptic plasticity, and degeneration of the synapses [11, 9].

The ratio of the areas of the muscle to the neuron of the neuromuscular junction (structural integrity of the neuromuscular junction) between the Mito-killer and D42 *Drosophila* larvae was not statistically significant (P=.13). The ratio of flipping assay times from before photoactivation to 90 minutes following photoactivation (how much photoactivation changed flipping time) between Mito-killer and D42 *Drosophila* larvae was not statistically significant (P=.13), but interestingly the ratio of times from before photoactivation to 120 minutes following photoactivation was slightly more significant (P=.10). Because there is no statistical significance, it cannot be said for sure that a relationship is present. Yet, the purpose of this project was to detect the possibility of a link, and it seems like there is one. In order to validate this possible

relationship, it is imperative to repeat experimentation, collect more data, and increase the sample size.

The green light treatment shows a slight but apparent relationship which may also indicate that in order to produce stronger symptoms of ALS, the intensity or duration of the treatment may need to be increased. This may prove difficult because increasing the intensity may kill the larvae and subjecting them to a longer treatment gives them more of a chance to pupate. Notably, the statistical significance of the data becomes slightly stronger when more time elapsed following photoactivation. This may indicate that after initial photoactivation, ROS linger in the neuromuscular junction and cause further oxidative damage; therefore the dissection may have taken place too early to allow for the full effect to happen.

If successful, this endeavor will result in a sporadic animal model for ALS to be created. This would be a great boon in the development of a cure for ALS, as an animal model would allow researchers to more easily test potential cures and treatments.

Conclusion

Green light treatment and the production of ROS in *Drosophila* larvae seem to induce ALS symptoms, but further experiments are needed to troubleshoot variability in the results. Future studies should explore ways to cause a greater disparity between the control and experimental groups, as well as increasing the sample size. Researchers should especially focus on increasing the duration of treatment. It is worth continuing research down this path because a sporadic model for ALS will lead to drug screening protocols in a model that makes up about 90% of cases and has no known cure.

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