IMPACT OF EXERCISE ON SOD1G93A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS: A BEHAVIOURAL AND METABOLIC ANALYSIS

by

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Dalhousie University is located in Mi'kma'ki, the ancestral and unceded territory of the Mi'kmaq. We are all Treaty people.

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease marked by the progressive loss of upper and lower motor neurons of the spinal cord, motor cortex, and brainstem. There is no cure for the disease, and current treatments are limited. Exercise is a commonly prescribed non-pharmacological treatment; however, studies have shown mixed results regarding its efficacy. This study explored the impact of exercise on the progression of ALS in SOD1G93A mutant mice by investigating how identified biomarkers were affected by exercise at different time points. Behavioural results indicated early changes in hip movement regardless of exercise, and also showed exercise-dependent changes in ankle movement. In a pilot study investigating blood serum biomarkers, 22 metabolites were identified that showed significant differences between exercise and sedentary groups. These results provide a stepping stone for future investigation to better understand the impact of exercise on ALS progression, and whether its prescription to patients is justified.

LIST OF ABBREVIATIONS

μL	Micro Litre
ALS	Amyotrophic Lateral Sclerosis
ANOVA	Analysis of Variance
BDNF	Brain-derived neurotrophic factor
BMI	Body Mass Index
BMMA	β-methylamino-L-alanine
C9ORF72	Chromosome 9 Open Reading Frame 72
DLC	DeepLabCut
fALS	Familial amyotrophic Lateral Sclerosis
FDA	Food and Drug administration
FUS	Fused in Sarcoma
GDNF	Glia-derived neurotrophic factor
GS	Gastrocnemius
HILIC	Hydrophilic Interaction Chromatography
IL-1B	Interleukin-1B
IL-6	Interleukin-6
IL-8	Interleukin-8
IP	Iliopsoas
Me(OH)-ILIS	Methanol - Isotope-Labelled Internal Standard
MN	Motor Neuron

MND	Motor Neuron Disease
^{o}C	Degrees Celsius
OCT	Optimal Cutting Temperature
OS	Oxidative Stress
PBS	Phosphate Buffer Saline
PC	Phosphocholine
PCA	Principal Component Analysis
PFA	Paraformaldehyde
PKC	Protein Kinase C
RPM	Rotation Per Minute
sALS	Sporadic Amyotrophic Lateral Sclerosis
SOD1	Superoxide Dismutase 1
TA	Tibialis Anterior
TARDBP	Transactive Response DNA Binding Protein
TBK1	TANK-Binding Kinase 1
TDP-43	Transactive Response DNA Binding Protein 43
TNF-α	Tumor Necrosis Factor Alpha
WT	Wild Type

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CHAPTER 1: INTRODUCTION

1.1 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease marked by progressive loss of upper and lower motor neurons of the spinal cord, motor cortex and brainstem (Rowland & Shneider, 2001). The loss of the motor neurons leads to muscle weakness and muscle wasting which often starts at a focal onset followed by subsequent spreading throughout the body (Feldman et al., 2022). The disease progresses until the eventual failure of the respiratory system resulting from the improper functioning of the diaphragm. In humans, death due to respiratory failure typically occurs between 3-5 years following diagnosis (Similowski et al., 2000).

The two main categories of ALS are sporadic ALS (sALS) which is the onset of ALS with no clear risk factor or family history of the disease and familial ALS (fALS) for cases where the patient has at least one other family member who has developed the disease. fALS makes up approximately 5% of reported cases, while sporadic (sALS) accounts for the other 95% (Byrne et al., 2011).

ALS is one of the most common motor neurodegenerative diseases worldwide with an estimated incidence of 1 to 2.6 cases per 100,000 people a year and a prevalence of approximately 6 per 100,000 people (Talbott et al., 2016). The age group at the highest risk of developing the disease are those falling between the ages of 45 and 75 with a mean age at onset of 53-63 years for sALS and 40 – 60 years for fALS (Talbott et al., 2016, Nardo et al., 2016). Male sex has also been identified as a risk factor for the development of ALS, with males typically 1 to 2 times more likely to develop the disease compared to females (Longinetti & Fang, 2019, Beghi et al., 2006). However, the sex dependence varies geographically. In Africa, for example, the ratio between males to females was outside this range at 2.9:1 (Longinetti & Fang, 2019). In Europe, the male to female ratio has been showing a regression over time to be closer to 1:1 (Fontana et al., 2021). These differences based on region show evidence for sex to be less of a risk factor than originally thought in some studies.

Although the exact causes of various forms of ALS remain unknown, it is thought to be a complex interplay of genetic factors, environmental and lifestyle factors and age-related dysfunction (Feldman et al., 2022; Masrori & Van Damme, 2020). The relative importance of these factors to fALS and sALS is different.

Over 50 genes have been found to be associated with ALS (Marangi & Traynor, 2015). Among them, the five most studied genes include superoxide dismutase 1 (SOD1), found in 20% of fALS and 1-2% of sALS; hexanucleotide expansions in chromosome 9 open reading frame 72 (C9orf72) found in 30-50% of fALS and 7-10% of sALS; TAR DNA-binding protein 43 (TARDBP) and fused in sarcoma (FUS) both found in 3-5% of fALS and less than 1% of sALS; and lastly TANK-binding kinase 1 (TBK1) found in 1% of patients (Zou et al., 2017). Amongst patients with sALS, their offspring have an increased 30-60% chance of inheriting the disease (Zou et al., 2017).

Non-genetic associated factors implicated in sALS include several environmental, ageing and lifestyle-related risk conditions. Epidemiological research has focused on lifestyle risk factors including blood lipid levels (Zeng & Zhou, 2018), smoking (Zhan & Fang, 2019), body mass index (BMI) (Nakken et al., 2019), physical exercise and physical fitness (Chio, 2005), and diet (Pupillo et al., 2017).

Some of these factors, such as diet, BMI and level of exercise, have been shown to both increase or decrease the risk of developing ALS. Studies investigating BMI have identified a correlation between low BMI and an increased chance of developing ALS and a high BMI to be protective against the progression of the disease (Nakken, 2019). Light physical activity, such as walking for pleasure, has been shown to decrease chances of developing ALS, whereas heavier activity levels of exercise such as a job that requires an increased amount of walking or standing has shown increased risk of developing ALS (Bandres-Ciga et al., 2019).

There are also a number of occupational and environmental risk factors associated with an increased chance of development of ALS (Longinetti & Fang, 2019). Occupations in agriculture, fishing and hunting, and forestry have been associated with higher rates of ALS (Dickerson et al., 2018). Additionally, exposing workers to potentially harmful environmental factors such as chemicals, pesticides, metals, and electromagnetic fields

(Fang et al., 2009) can increase a worker's risk. In addition to metals, such as manganese, well known for their neurotoxicity,a number of other metals have also been identified in higher concentrations in the cerebrospinal fluid of ALS patients. These include copper, aluminum, arsenic, cadmium, cobalt, zinc, vanadium, and uranium (Roos et al., 2012). Thus, additional occupations with increased risk include jobs such as construction and electrical workers, military personnel, medical service workers, and production plant workers where workers may get exposed to metals (Peters et al., 2016).

Environmental factors are not only important considerations in occupational settings but also in areas where people live. β-methylamino-L-alanine (BMAA) for example, is a neurotoxic chemical produced from cyanobacteria and found in greater concentrations in Guam causing an increased incidence of ALS in western Pacific (Plato, 2003) and, more recently, a cluster of ALS in the US (Caller et al., 2009).

Although the pathogenesis of ALS remains largely uncertain, many molecular factors and pathways have been involved in the process such as glutamate excitotoxicity, altered RNA metabolism, defective axonal transport, protein misfolding and aggregation, endoplasmic reticulum stress, disrupted protein trafficking, inflammation, mitochondrial dysfunction and oxidative stress (Cunha-Oliveira et al., 2020).

Oxidative stress (OS) is defined as an imbalance of free radicals and antioxidants in the body. Free radicals are formed as a natural biproduct from several biological processes such as breathing, digestion and metabolism. Strenuous exercise may result in an increased production of free radicals due to the increased energy demands causing mitochondrial superoxide production (He et al., 2016). Under normal circumstances these free radicals would be cleared out and destroyed through the body's antioxidant system. However, in ALS patients, postmortem tissues showed evidence of oxidative stress providing supporting evidence for its role in disease progression (Shaw et al., 1995). More recent studies have shown even more evidence for the role of oxidative stress in the disease progression. These results have sparked a line of research investigating antioxidants as a potential therapeutic to combat against the disease, which led to the approval of the new ALS drug, Endaravone (Barber et al., 2006). Endaravone is a potent

pyrazolone free radical scavenger and antioxidant, helping to reduce oxidative stress in the body.

Another leading hypothesis of disease development is the effect of glutamate excitotoxicity. Glutamate is the predominant excitatory neurotransmitter in the central nervous system. Under normal circumstances, glutamate is released from presynaptic terminals and activates NMDA and AMPA receptors leading to an action potential. Glutamate is then cleared from the synaptic cleft terminating the action of the neurotransmitter. If, however, glutamate were not cleared away adequately, calcium ions would flood into the cell causing an increase in the intracellular concentration of calcium ions leading to excitotoxicity to the point of apoptosis (Foran & Trotti, 2009). The most predominantly prescribed drug for ALS, Riluzole, is a sodium channel blocker and may also directly block the release of glutamate and glutamate receptors, reducing excess glutamate caused excitotoxicity to motor neurons.

Riluzole, however, is an expensive drug which may be able to prolong survival by 2-3 months in ALS patients (Miller et.al., 2012), while Endaravone is even more expensive and only has limited effects on a subgroup of patients with an early diagnosis (Cho & Shukla, 2020). The effectiveness of these drugs is related to when they are administered highlighting the importance for early diagnosis or novel alternative therapeutic strategies for ALS patients.

1.2 Diagnosis of ALS and early behavioral changes in ALS

ALS is defined by the patients' upper and lower motor neuron degeneration. However, apart from this one commonality the disease is incredibly complex and heterogeneous. The variety of genetic, biochemical, and clinical features of the disease make it difficult to diagnose and treat (Swinnen & Robberecht, 2014). The diagnosis of ALS remains a clinical diagnosis and is based on the presence of both upper motor neuron (UMN) and lower motor neuron (LMN) signs, in patients with progressive muscle weakness that has no alternative explanation. As it stands, ALS has no clear-cut diagnostic test (Richards et al., 2020).

To be confidently diagnosed, medical practitioners first eliminate the possibility that the symptoms the patient is experiencing are caused by a more common neuro-muscular

disease like progressive muscular atrophy. Through neurological examination testing reflexes, muscle strength and other responses at regular intervals, practitioners can assess whether the patients are demonstrating progressive spasticity, muscle wasting and fasciculations. This would be evidence of both upper and motor neuron dysfunction and could be used to diagnose the disease more accurately as ALS (Richards et al., 2020). The use of electromyography has been shown to be able to detect subclinical involvement of lower motor neurons as well, although it would be diagnostically more accurate with signs of upper neuronal dysfunction at the same time (Richards et al., 2020).

Disease phenotypes are often classified by a specific site of onset. Approximately 65% of patients present with a spinal onset showing signs in distal upper and lower limb muscle weakness and spreading over time to other parts of the body; 30% of patients present with a bulbar dysfunction beginning with weakness in facial, tongue and pharyngeal muscles eventually producing dysarthria and dysphagia; and the remaining patient present with symptoms elsewhere in the body (Richards et al., 2020).

Due to the heterogeneity and difficulty in accurate early diagnosis, there is an average of approximately one year from symptom onset to the diagnosis (Richards et al., 2020). This can have a huge impact on the ability to prescribe early therapeutic interventions (Richards et al., 2020). By the time muscle wasting is visible, which would be only one of the signs indicating a possibility of ALS based on current diagnostic factors, at least 30% of anterior horn neurons could have degenerated (Masrori & Van Damme, 2020). This delay in treatment could lead to shorter survival times, showing the need for novel and reliable biomarkers to help give accurate earlier diagnosis (Masrori & Van Damme, 2020).

In animal models, the use of gait analysis has been investigated as a possible way to diagnose disease onset due to the frequently observed impact of the disease progression on the locomotor system. Using precise motion capture software, comprehensive locomotor profiles can be created for models of neurodegenerative diseases, such as the SOD1G93A mouse model (Preisig et al., 2016).

1.3 Biomarkers of ALS

A biomarker can be defined as "an objective measurement that acts as an indicator of normal biological processes, pathogenic processes or pharmacological responses to therapeutic interventions" (Verber & Shaw, 2020). These markers therefore must be sensitive and specific for the disease, reliably discriminate between clinical phenotypes, have the ability to change in a predictable way over disease progression, and should be an accessible and practical measurement (Verber & Shaw, 2020).

One of the most common and rich sources of potential biomarkers used when investigating diseases is the blood, where a wide range of symptoms can be investigated including but not limited to neuroinflammation, mitochondrial dysfunction, oxidative stress, excitotoxicity and protein aggregation (Sun et al., 2020). In the case of ALS, blood-based analyses have identified some ALS associated factors. In familial categories, concentrations of specific known targets in the serum of patients, such as TDP-43, are correlated with disease burden over time (Sun et al., 2020). Sporadic cases of ALS have also been investigated by looking at molecular factors, such cytokines, metabolites, stress granules, and neurofilaments. Inflammation, in particular, is a common symptom associated with ALS (Sun et al., 2020). For this reason, cytokines such as TNF-a, IL1B, IL-6, IL-8 TNF receptor 1 and vascular endothelial growth factor can be useful biomarkers of disease found in blood sampling, since they have all shown elevated levels in ALS patients (Sun et al., 2020). It has been reported that cytokine expression in the blood of ALS patients is altered compared to controls, but does not necessarily change over time. Unfortunately, inflammation is not specific to ALS, therefore this is not the ideal biomarker for ALS alone. However, it can be an early indicator compared to waiting for muscle weakness and later stage symptoms.

Blood metabolites being an intermediary product of metabolism offer a promising biomarker for diseases as they can be used to represent ongoing biological processes (Lanznaster et al., 2018). Not only can they help identify early changes, but they can also be useful in providing insight into the underlying mechanisms that lead to the diseased state. Studies have already determined various metabolic pathways related to ALS disease, including lipid and glucose metabolism (Lanznaster et al., 2018). Within these

pathways a number of metabolites of interest have been consistently shown to be related to the disease, such as an elevated circulation of glutamate, which may be leading to glutamate toxicity. Many lines of evidence have identified the role in glutamate toxicity damaging the glutamate transport system, which leads to motor neuron death (Lanznaster et al., 2018).

1.4 Exercise and ALS

Physical activity is often prescribed as a non-pharmacological therapeutic for many diseases. For the general population, exercise can provide a great deal of physiological benefits. Exercise is an important factor in improving cardiovascular health, where research has shown that even moderate exercise such as regular walking was able to play a significant role in reducing the chances of developing cardiovascular diseases (Lin et al., 2015). Physical activity is also important in maintaining neuromuscular function. (Ahtiainen et al., 2003, Tsitkanou et al., 2019). As a therapeutic remedy it has been shown to be beneficial in combating over forty chronic illnesses, as well as improving mental health and cognition, alleviating some of the effects of depression and anxiety, and improving mood and self esteem (Mikkelsen et al., 2017).

Although for many diseases, and for the general population, exercise may show improvements both physically and psychologically, its role as a therapeutic treatment for ALS is considered controversial. Some epidemiological studies have shown an increased incidence of ALS among high performance athletes (Chio, 2005, Lehman et al., 2012). Although not yet understood, theories as to what may drive this correlation include increased oxidative stress, glutamate excitotoxicity, muscle vesicle mediated toxicity, and increased calcium loads (Cunha-Oliveira et al., 2020). Although exercise has numerous benefits, intensive physical activities can cause damage on the body and increased release of potentially harmful by-products, which should be considered for their therapeutic usage.

1.4.1 Positive Effects of Exercises on ALS

Most neurodegenerative diseases start to appear in older populations. With ageing, patients likely experience a reduction in mental and/or physical function often associated

with concomitant diseases exposing them to risks such as decreased body size and composition, reduced cardiovascular and respiratory health and reduced brain function. This period often leads to a lack of autonomy.

As described earlier, these deficits can be improved with physical activity in the general population. However, researchers are actively investigating whether physical activity can specifically be beneficial to patients suffering from progressive neurodegenerative diseases, such as maintaining the psychomotor functions or delaying the progression of the various diseases (Maugeri & D'Agata, 2020).

One mechanism underlying the beneficial effects of the physical activity is the upregulation of several neurotrophic factors in muscle and neural tissue such as brain derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) (Afzalpour et al., 2015). BDNF is a protein that has been associated with cognitive ability and synaptic plasticity. During development, it plays an important role in axonal and dendritic growth and synaptogenesis (Afzalpour et al., 2015). BDNF signalling is reduced in patients with neurodegenerative diseases as reviewed by Zuccato and Cattaneo (2009). BDNF levels have long been shown to be increased in the brain after an increase in physical activity, thus making physical activity a point of interest as a method to circumvent the reduction in BDNF signalling during neurodegenerative diseases, with the hope to utilize its synaptogenic properties (Afzalpour et al., 2015). GDNF on the other hand is a protein produced by skeletal muscle whose increased expression is related to an increase in axonal branching and maintenance, which in turn leads to an increased motor unit size. GDNF may also have anti-inflammatory effects, which is important considering the role of inflammation in the progression of the disease. For these reasons, it is considered one of the most potent survival factors identified for motor neurons (Afzalpour et al., 2015). Potentially, through elevating both GDNF and BDNF, physical activity could increase motor neuron survival rate.

1.4.2 Detrimental Effects of Intensive Exercise on ALS

While physical activity has been shown to have a number of beneficial properties both mentally and physically, epidemiological studies have provided evidence connecting individuals who perform regular intensive exercise with an increased incidence of

developing ALS. However, many of these studies unfortunately suffered from a wide variety of limitations such as heterogeneity of both the classifications of ALS and of the physical activity studied (Maugeri & D'Agata, 2020).

Although the exact mechanisms of ALS are not yet well established, several factors related to intensive physical activity may be implicated in the eventual motor neuron death. The most prominent theories for the mechanisms leading to potential motor neuron disease are from changes in oxidative stress and increased susceptibility to damage through glutamate excitotoxicity (Powers et al., 2011).

It thus remains unclear whether exercise is beneficial or detrimental for ALS patients. While it may improve some aspects of an ALS patient's well being, there is the potential that it accelerates the rate of decline. This leads to inconsistencies in how researchers perceive the effectiveness of exercise as a non-invasive treatment option, as it heavily depends upon which factors are being looking at to identify the effect the treatment may have.

CHAPTER 2: HYPOTHESIS AND THE GOAL OF THE STUDY

Although effects of exercises on ALS have been studied extensively, there have not been consistent and convincing conclusions drawn from these studies due to the fact that they all featured different experimental models and different training protocols. In this study I employed an intensive training program, which has not been widely studied, with the hypothesis that the intensive exercise might negatively impact disease onset and survival in mice. To test this hypothesis, I used the SOD1G93A mouse line, an animal model of ALS, and subjected the animals to different training durations, and employed novel kinematic analysis techniques to analyze the changes of the animal motor behaviors before and after the 'clinical' symptom onset. To further elucidate changes caused by exercise on the mice, we began a pilot study into the metabolomics of the animals in hopes of identifying metabolic pathways and markers that could be used to indicate early changes in metabolism resulting from the progression of the disease and how these change with exercise.

CHAPTER 3: METHODS

3.1 Animals

All experiments were carried out in accordance with the Canadian Council on Animal Care guidelines and approved by the Dalhousie University Committee on Laboratory Animals. Adult B6.Cg-Tg(SOD1-G93A) mice, (Stock#004435, Jackson Laboratories, Bar Harbour , ME) of both sexes and their wild type (WT) litter mates were used in the experiments. Mice were all housed in the Life Science Research Institute Animal Care Facility at Dalhousie University. They underwent a 12-hour light/dark cycle (light from 7:00 19:00, dark from 19:00 to 7:00) and were provided with *ad libitum* access to standard laboratory chow and water. A list of mice used in this experiment can be found in Table 1.

3.2 Clinical Scoring of Murine ALS

SOD1G93A mice were assessed using a rubric scoring system as described by Hatzipetros et al. (2015) to assess disease onset and progression. In brief, mice are suspended by their tails while the observer notes the degree of their hind limb splay. With full extension of hind legs away from the lateral midline, mice are given a score of 0, indicating no evidence of the disease. Collapse of leg extension towards the lateral midline, the first sign of leg muscle weakness, is given a score of 1, and is when we consider the time as the symptom onset. As mice grow weaker, they start showing difficulty in walking and visible tremors. When they walk on a flat surface and exhibit limb dragging, they are assigned a score of 2. At the end stage of ALS, the mouse exhibits extreme weakness in its hind limbs with at least one paralyzed hind limb and an inability to right itself from its side in less than 30 seconds. This is assigned a score of 3.

Table 1 Table of mice used in the experiment for each group and divided by sex.

Group	Total	Males	Females
Sedentary WT	9	4	5
Sedentary Mutant	8	3	5
Young Exercise WT	6	3	3
Young Exercise Mutant	11	4	7
Adult Exercise WT	5	2	3
Adult Exercise Mutant	8	5	3
Post Symptomatic Exercise WT	6	4	2
Post Symptomatic Mutant	8	6	2

3.3 Weighing the Animals

All animals were weighed bi-weekly starting at 40 days of age. After symptom onset (Clinical Score 1) animals were weighed two times per week. When animals reached Clinical Score 2, they were weighed daily until endpoint. Endpoint was marked as either the weight of the animal decreased more than 20% from their maximum weight or if the animals reached Clinical Score 3, marked by hind limb paralysis. Weights were recorded in grams using a standard digital mouse scale.

3.4 Rotarod Test

A Rotarod machine (Ugo Basile, Gemonio, VA, Italy) uses a horizontal rod that rotates around its axis. Mice are placed on the rod and timed for the duration that they remain on it. The animals in this test were subjected to 3 tests of a 2-minute ramp test in which the speed of the rotating cylinder would increase from 10 RPM to 30 RPM over the course of the minute. Following the test, they would rest for 5 minutes before repeating the task for a total of 3 times biweekly, with the average of their performance from the 3 attempts being recorded.

3.5 Grip Strength Test

Grip Strength was calculated using BIO-GS3 (Bioseb) grip strength meter. Holding the mice by the scruff and the tail, mice were brought close to a T bar, and allowed to have

their hind limbs grip the bar. They were then slowly pulled away from the apparatus and the maximum force before they let go was measured. Mice repeated this 5 times each with the average of the 5 experiments being recorded.

3.6 Training Protocol

A treadmill exercise protocol was used to create 3 separate training groups. Using a 5-lane Bioseb mouse treadmill the mice were subjected to 1 hour of running at 15 cm/s with a 15-degree incline, 5 consecutive days a week, with a day of rest followed by either a day of behavioural video recording biweekly or an additional day of rest continuing until mice could no longer consistently run at the set speed on the treadmill. Mice were motivated by either a pulse of air or by tapping their hind with an instrument if they stopped running. 17 Mice were used as an early exercise group, beginning their training at 28 days of age. 13 mice were used as an adult exercise group. Murine adult age is defined as 8 weeks of age, with that group starting with behavioural recordings followed by the start of their exercise program. The final training group of 14 mice was a post-symptomatic training group, where training began after symptom onset/Clinical Score 1. All behavioural video recordings began at 6 weeks of age.

3.7 Treadmill Locomotion Tests

Treadmill locomotion tests were performed on the training mice biweekly from 6 weeks to 16 weeks of age on an in house made treadmill. The treadmill was set at 25cm/s or 40cm/s and either flat or inclined at 22.5-degrees. The belt of the treadmill was made of clear acetate sheet, and a mirror was set at a 45 degrees angle under the belt. Such design allows the camera at the side to capture of the movement of the mouse from underneath as well as from the side. A high-speed camera was used to capture the videos at a capture rate of 200 frames/s. Recordings of 20s in length were taken of the mice running in the various experimental conditions and analysis was done on a consistent set of 15 – 30 steps selected from within this time period. If the mice did not reach the minimum consecutive steps they would rest for one minute, followed by another attempt with a maximum of five attempts.

3.8 Gait Analysis

Mouse gait was tracked using DeepLabCut (DLC) to capture the positions of four calibration points, nose, tail base, tail tip, iliac crest, knee, hind ankle metatarsal pharyngeal joint, hind toe tip, front paw, and the four paws reflected in the mirror. This data was exported and processed in a custom written script in MATLAB (Version 2021b). Output from DLC was converted from pixels to millimeters, using the four calibration points. In order to account for the differences in walking speed between animals, to evaluate differences in phase timing, swing and stance, phases were normalized to 100 frames each, for a total of 200 normalized frames. The parameters extracted from gait and kinematic analyses are illustrated in Figure 10 and described in brief below:

Each step (stride) can be broken down into two phases: swing, and stance. Swing phase was defined as the time between the point when the toe lifted off the track to the point when it touched back down, and stance was defined as the duration between the time of toe touching down until it lifted off the track. The stride timing was calculated from the lateral view of the hind limb as well as the bottom view of four paws taken from the mirror. These values were then used to evaluate the basic step parameters and correlation between four limbs during movement.

Stride length was calculated as the positional change in the toe from the furthest maximum x-value to minimum x-value and corrected by average animal displacement on the track to account for the animal moving forward or back on the treadmill. Reach back distance is the distance the toe reaches back with reference to the base of the animal's tail and toe lift is the maximum to minimum y value of the toe displacement. Posture refers to the height of the base of the tail from the ground level.

Hip angle was calculated between the iliac crest marker, the hip marker, and a calculated knee position. Knee position was based on the measured lengths of the femur and tibia of each animal. Ankle angle was calculated between the metatarsal pharyngeal joint, the ankle, and the calculated knee position.

Phase timing of changes in the hip were divided into flexion and extension phases based on the minimum peak of the joint angle. Phase timing of the ankle was separated into

four phases, extension, yield, propulsion and flexion. Extension was between the joint angle minimum to the first peak. Yielding phase was calculated between that peak and the next trough. Propulsion phase was between this trough and the joint angle maximum. Flexion was between the maximum joint angle to the minimum joint angle.

3.9 Blood Serum Collection

Blood samples were extracted from the left ventricle of the heart from the four groups (sedentary WT, sedentary mutant, adult exercise WT, adult exercise mutant mice), with 3 mice per group at P90. Approximately 1mL of blood was extracted from each mouse and allowed to rest at room temperature for 5 minutes. Samples were then centrifuged at 3000 RPM for 10 minutes until a separation of cells and serum can be seen. Blood serum was then extracted, aliquoted and frozen at -20 ºC for temporary storage or -80 ºC for long term storage.

Preparation for mass spectrometer for analysis: 20µL of each serum sample was used with the addition of 85 µL of cold MeOH-ILIS solution and vortexed for 10s. 30µL of each sample was then mixed with 270µL of dilution buffer (5% HILIC Buffer A/ 95% Acetonitrile) and mixed. This is then transferred to Millipore Ultra Free-MC 0.22µM spin filter capped and spun at max speed for 5 minutes.

Samples were then sent for mass spectrometry analysis.

3.10 Muscle Extraction

Following blood serum extraction, animals were skinned and tibialis anterior (TA), gastrocnemius (GS) and iliopsoas (IP) were removed from the mice. Muscles were immediately weighed, followed by immersing them in 4% PFA for 10 minutes. They were subsequently washed with PBS and covered in a mixture of 1:2 ratio of 20% sucrose and OCT Compound respectively. Samples were then placed on a frozen block of methyl butane until frozen and covered in tinfoil and transferred to a -80 ºC freezer for future use.

3.11 Data Analysis

Statistical analyses were performed, and figures were created using Graph Pad Prism 8 (GraphPad Software, San Diego, Ca) and MATLAB (Version 2021b). All results are

reported as the mean ± standard deviation (SD), unless otherwise indicated. Behavioural parameters were analyzed using a repeated measures 2-way ANOVA with Dunnet's multiple comparisons test to compare the groups and time points to the WT sedentary group. Statistical significance corresponded to $p < 0.05$. Analyses of muscle weights were performed using multiple comparisons ordinary one-way ANOVA with Tukey's multiple comparison test compared against the sedentary WT mice.

CHAPTER 4: RESULTS

4.1 Onset and Survival Changes in Exercised Mutant Mice

The impacts of physical activity/exercises on ALS disease have been inconsistent with patients and in animal models. After comparing many animal studies, I realized that the exercise protocols varied a lot from study to study, and most of them were mild, considering how active mice typically are in their cages. With this in mind, I designed a training protocol, which was closer to what comparable studies referred to as intensive: I trained animals 5 days/week, Monday-Friday. The mice of exercise groups would walk on a treadmill with a 15-degree inclining angle at 15 cm/s for an hour, and then rest during the weekends. Biweekly the mice were recorded on Sunday to track any behavioural changes.

I randomly assigned SOD1G93A mutant mice, and their WT counterparts, into four groups with differing start times:

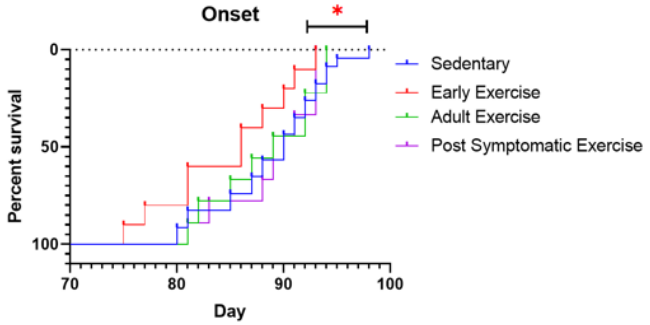
1. Sedentary group, staying in the home cage during training periods;
2. Young exercise group, which began training at four weeks of age until end stage;
3. Adult exercise group, which began the training protocol at 8 weeks of age until end stage;
4. Post symptomatic mice began the exercise protocol after symptom onset till end stage.

SOD1G93A mouse is the most studied animal model for ALS studies and the timelines and biomarkers to indicate animal's symptom onset, progression and end stage have been well established. In my current study, I used the Clinical Scoring System, in which symptom onset, given a score of 1, is assigned when the animals hind limb collapses towards the midline while held by the tail. This is caused by hind limb muscle weakness and is a useful marker to identify early disease progression. I first used these known and frequently studied biomarkers to examine whether intensive exercise could have any impact on the disease onset and progression of SOD1G93A animals.

Most of the SOD1G93A mice in this study had symptom onset days consistent with other studies, Postnatal (P)80 to P100 days (give citations). Early exercise mice were the

exception; in this group symptoms were apparent slightly earlier, from P75 to P90 (Figure 1a). We did not have a sufficient sample size to separate males and females in this study. However, since each group has relatively evenly mixed sexes, I consider the conclusions drawn from this study still reliable. Clinical Score 3 was used as the end stage. Interestingly, there was no significant difference in survival times between the four groups of mutant mice, even though the young exercise group had a trend of a 50% reduced survival rate (Figure 1b).

a)



b)

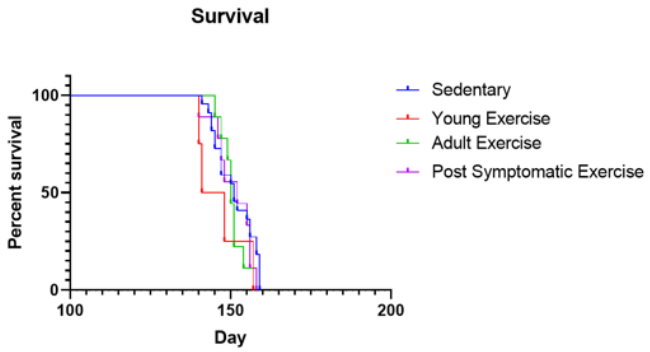


Figure 1: Survival Plot for animal onset and survival

Figure 1a) survival plot of disease onset, which was determined by a Clinical score of 1. The disease onset for the whole group was determined when 50% of the animals in that group were experiencing symptoms in statistical analyses. Significant differences were seen in the early exercise group when compared to the sedentary group. Figure 1b) represents the disease survival time. There were no statistically significant differences found between groups for survival time. Significant differences were investigated using Log-rank (Mantel-Cox) test.

4.2 Behavioural Tests

In addition to the symptom onset and end-stage, I also assessed two commonly used behavior tests, the Rotarod running and hind limb grip strength to examine the symptom progression of the SOD1G93A mutant mice.

4.2.1 Rotarod Tests

The Rotarod behaviour test is a commonly used measure of assessing coordination and motor function in animals. In our experiments, mice were tested and recorded once biweekly, starting at week 6 (Figure 2a). Not surprisingly, all WT mice, regardless of training regimens were consistently able to remain on top of the Rotarod for the entire duration of the experiments (Figure 2a). All mutant mice however, showed progressive decline in performance. Significant differences in the latency of dropping between SOD1 and WT animals started at 14 weeks of age, right after the symptom onset (Figure 2a). Interestingly, although not statistically significant, the early exercise group showed a slight improvement compared to the other mutant groups at week 14 (Figure 2a). These results signify that that the intensive training might influence the balance and global motor control of the SOD1G93A mutant mice differently from hindlimb stretching behavior, which is the determining factor for symptom scoring of 1.

4.2.2 Hind Limb Grip Strength Tests

I also measured hind limb muscle strength of the animals using a BIO-GS3 (Bioseb) force reader. The measure was done by having the animals hold on to a metal bar with their hind limb and the animals were slowly pulled away from the force reader. The maximal force before the animal let go was recorded. This test was done biweekly with the average of 5 measurements used for results. Similar to the Rotarod test, significant decrease in the grip force of mutant mice started at 14 weeks of age compared to the WT mice who showed no change. There were no significant differences seen between the exercised and non-exercised mutant groups (Figure 2b).

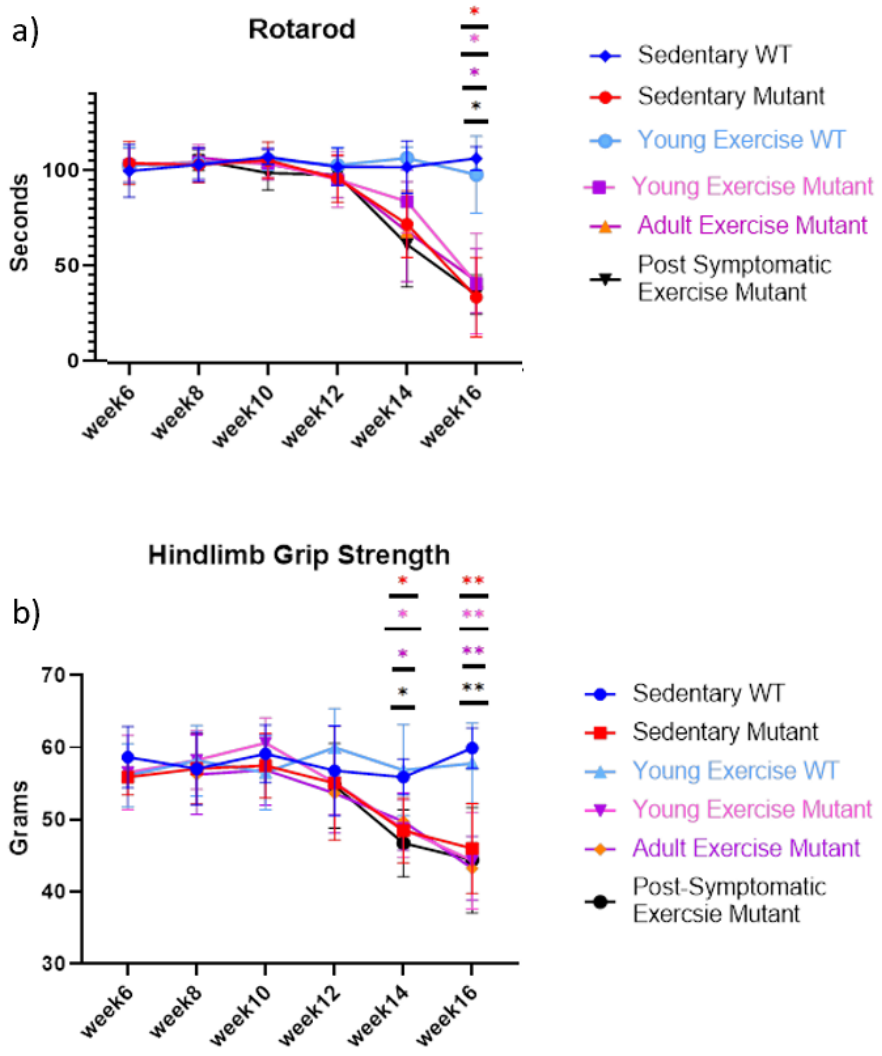


Figure 2: Rotarod and Grip Strength Performance in SOD1G93A mice.

Figure 2a) represents the times in seconds that animals were able to remain on the Rotarod. Mutant mice from all groups started to perform worse than their sedentary counterparts starting at around week 12, showing significantly worse scores among all groups by week 16. Figure 2b) shows the data from the hind limb grip strength test. Mutants progressively showed weaker force scores compared to the WT counterparts following week 12, with significant changes in week 14 and week 16. Repeated measures 2-way ANOVA was used to calculate significant differences. * $p<0.05$ compared to wild type? ** $p<0.01$ colored symbols are corresponding the group represented by the same color.

4.2.3 Gait and Kinematics

At this point, the phenotypic scores and common behaviour tests implied that the long-term exercises might have some impacts on pre-symptomatic and post symptomatic ALS animals. However, current common biomarkers such as Rotarod and grip strength are not sensitive and specific enough to detect subtle behavioural changes during early disease stages and the impacts of exercises. Therefore, I decided to make more detailed analysis of the animals' motor behaviors. To do so, I took videos of the mice while they were running on a treadmill at 25cm/s and 40 cm/s with a flat and a 22.5-degree inclined surface. Recordings were taken biweekly on all mice, and they were analyzed using DeepLabCut, allowing me to capture a wide variety of behavioural features from joint angles to limb position, to gait timing, indicated in the methods. Then, I further employed principal component analysis (PCA) to deduce the variables that changed the most from my raw data of 23 parameters under the four different conditions of speed and incline and between the 8 groups of animals. Figure 3 shows the results from individual PCAs of each of the four running conditions from all experimental animals through the entire training period. Through this analysis, I drew several conclusions. First, four common parameters showed the greatest variability, consistent across the four different groups seen in Figure 3 bar graphs c, f, i and l. These common parameters were animal posture, hip excursion, ankle yielding phase excursion, and ankle flexion excursion (Figure 3). Second, as seen in Figure 3 plots a, d, g, and j, PC1 could clearly separate mutant and WT animals under all running conditions during all recorded periods, which means parameters identifying the greatest variance along PC1 could be used to indicate pre-symptomatic changes in ALS mice. Third, running at 40 cm/s on flat surface, the mutant animals might have shown the most significant separation from the WT animals. While this analysis appears not able to show significant impact of exercises, which might due to the small animal numbers in each group, at 40cm/s flat it did show a trend to pull the exercised groups from the non-exercised groups for both WTs and mutants as seen in Figures 3 a, d, g, and j with the symbol representing the week of animal age. To further dissect how these variables change at different stages of the disease under different conditions, I compared the identified common parameters among the different groups of animals under different running conditions through the whole training period.

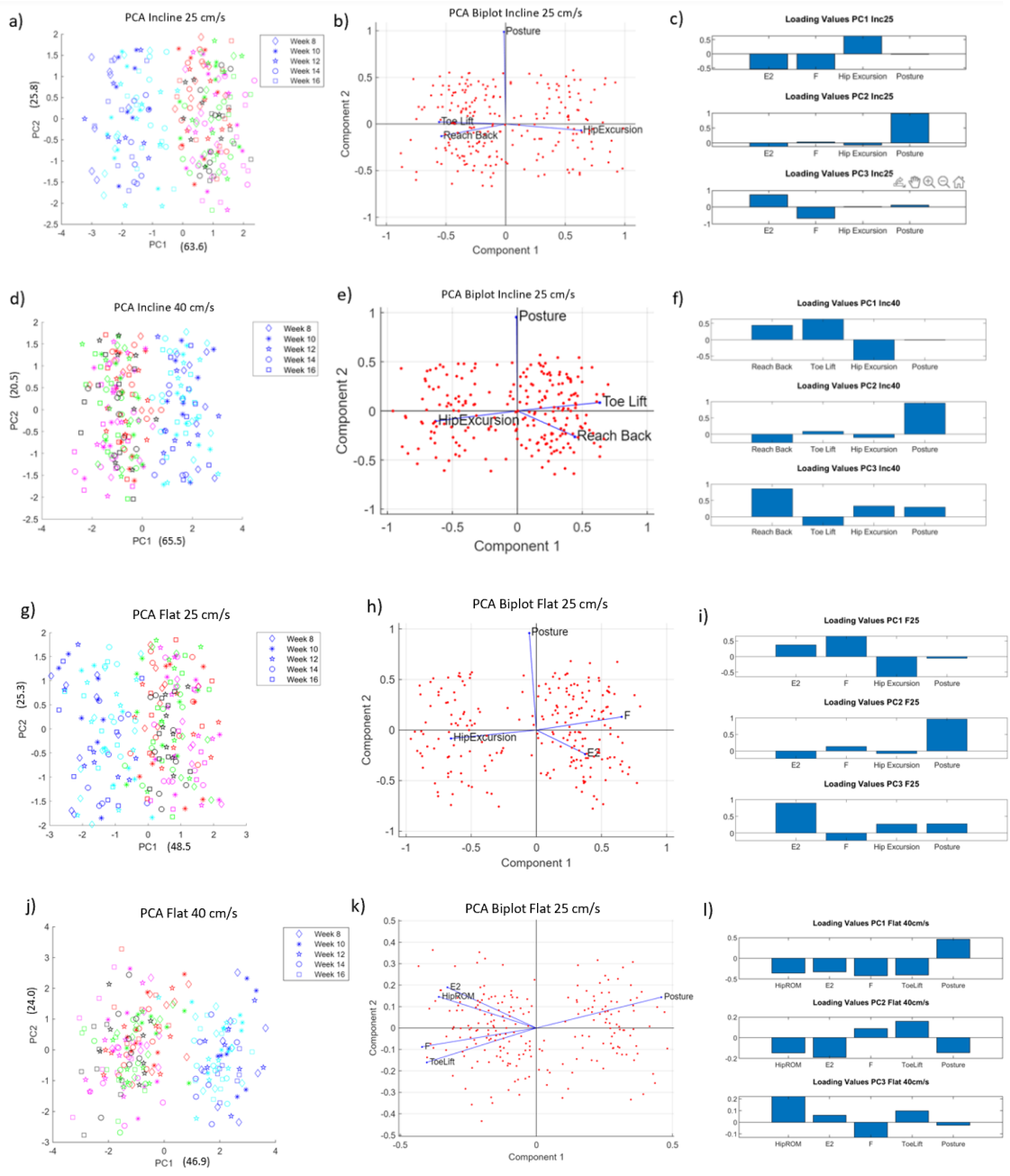

a)
PCA Incline 25 cm/s
Week 8
Week 10
Week 12
Week 14
Week 16
PC2 (25.8)
PC1 (63.6)
b)
PCA Biplot Incline 25 cm/s
Posture
Toe Lift
Reach Back
HipExcursion
Component 2
Component 1
c)
Loading Values PC1 Inc25
Loading Values PC2 Inc25
Loading Values PC3 Inc25
E2
F
Hip Excursion
Posture
d)
PCA Incline 40 cm/s
PC2 (20.5)
PC1 (65.5)
e)
PCA Biplot Incline 25 cm/s
Posture
Toe Lift
HipExcursion
Reach Back
Component 2
Component 1
f)
Loading Values PC1 Inc40
Loading Values PC2 Inc40
Loading Values PC3 Inc40
Reach Back
Toe Lift
Hip Excursion
Posture
g)
PCA Flat 25 cm/s
PC2 (25.3)
PC1 (48.5
h)
PCA Biplot Flat 25 cm/s
Posture
F
HipExcursion
E2
Component 2
Component 1
i)
Loading Values PC1 F25
Loading Values PC2 F25
Loading Values PC3 F25
j)
PCA Flat 40 cm/s
PC2 (24.0)
PC1 (46.9)
k)
PCA Biplot Flat 25 cm/s
E2
HipROM
Posture
F
ToeLift
Component 2
Component 1
l)
Loading Values PC1 Flat 40cm/s
Loading Values PC2 Flat 40cm/s
Loading Values PC3 Flat 40cm/s
HipROM
E2
F
ToeLift
Posture

Figure 3: Principal component analysis outputs

Principal component analysis was run on the parameters extracted from the video recording of the mice. Figures 3 a, d, g, j show the raw PCA output for each group with the different weeks represented by differing symbols (Diamond as week 8, asterisk as week 10, star as week 12, circle as week 14, square as week 16) and the groups represented by the different colours (Dark Blue as sedentary WT, Light Blue as exercise WT, Red as sedentary mutant, pink as young exercise mutant, green as adult exercise mutant and black as post symptomatic exercise mutant. A separation can be seen along the primary principal component in all 4 running modalities between the WT and mutant mice. Figures 3 b, e, h, k show the vector direction that the parameter pulls the plot pattern with the parameters that showed the most variation, which are also shown in bar charts in Figures 3 c, f, i, l as the individual loading values. We can see from these the common parameters that have the greatest impact on the variability in the mouse running performance: hip range of motion, ankle yield excursion, ankle flexion, animal posture.

Animal Posture (Figure 4) refers to the distance between the treadmill and the base of the subject's tail. Starting around symptom onset, at week 12, mutant animals showed a trend with a lower body position. Over the disease progression this position changed into a near crawling position with significant differences seen between all mutant groups when compared to WT counterparts by week 14. Exercises didn't seem affect the posture changes of the SOD1G93A mutant mice.

Hip excursion (Figure 5), refers to the maximal joint angle changes for the hip joint. It was calculated between knee position and the iliac crest (Appendix Figure 10). Interestingly, all mutant animals across groups had a larger hip excursion which could be identified as early as week 8, before the traditional symptom onset point (Figure 5). There was also a trend with both WT and mutant groups to have an increased hip excursion over time.

Ankle flexion excursion (Figure 6) is the difference in the amplitude of the ankle joint angle during the swing phase of the step, where the leg is brought forward. Interestingly, this parameter didn't separate the SOD1G93A mutant animals from WTs, but at the flat surface walking (Figure 6 a, b), it pulled the early exercise groups of both mutant and WT groups away from all sedentary groups. The later exercise groups showed a trend with an increasing flexion excursion as time went on. On the inclined surface, no noticeable differences between all mutant and control mice under all conditions. This result indicates that training on inclined surface could increase the ankle flexion movement for all animals, which could be an adaptive change of the animals to the training.

Ankle yielding phase excursion (Figure 7) is the early phase during stance, when the animal lowers its body position slightly before pulling forward. It can reflect how the animals holds their body weight. At the high speed (40cm/s) on a flat surface, SOD1G93A mutant animals showed an increase in ankle excursion throughout this step phase after symptom onset, except the early exercise mutant group, which had no significant difference from their WT counterparts. Within the 3 groups showing significant increases in angle excursion, in the adult exercise group these changes plateaued at week 16, while post symptomatic and sedentary mutant animal groups kept the up-trending changes, indicating worsening conditions. The post-symptomatic training

group appeared to have the greatest changes in angle excursion even compared to the sedentary mutant group. At 25 cm/s a similar trend was seen with the increase in angle excursion over time, where the same 3 mutant groups have a trend towards a greater yielding phase excursion. However, these differences were not statistically significant. Similar to what seen in the flexion excursion, these same differences were not seen in the incline groups.

These findings did show that detailed kinematic analyses could reveal behavioral changes in SOD1 mutant animals and distinct impacts of intensive trainings before the traditional clinical symptom onsets and more sensitive to the symptom progression.

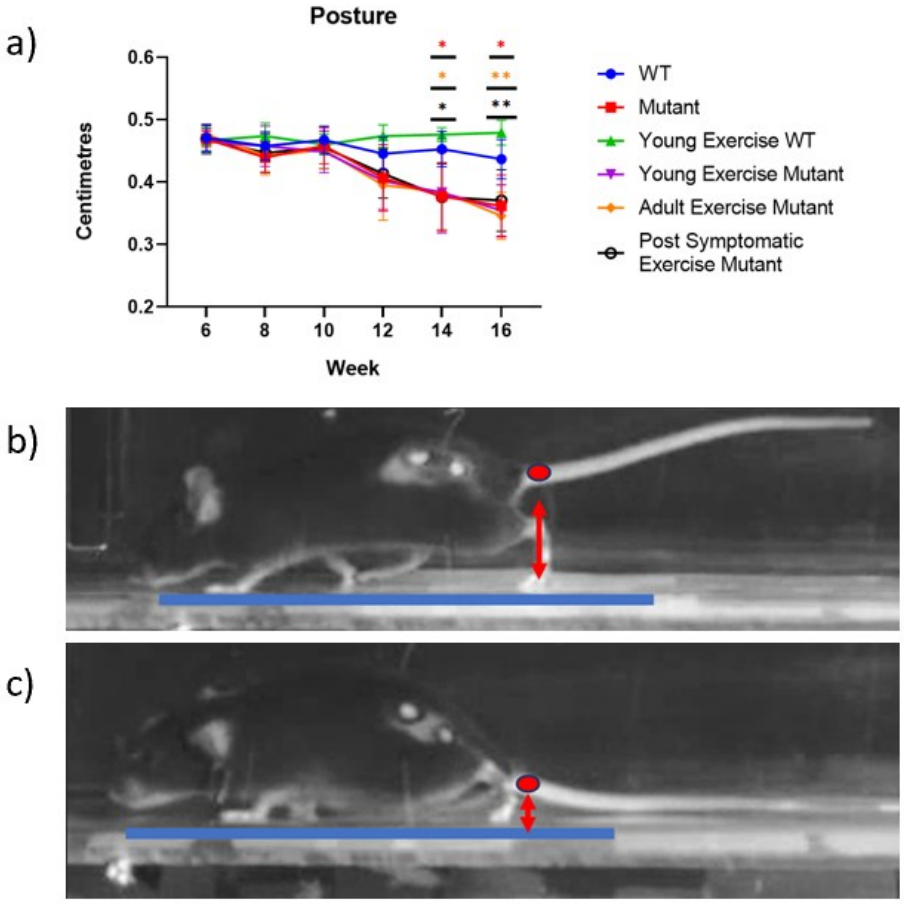


Figure 4: Mouse Posture data and representative images

Figure 4a) represents the results found for mouse posture. Posture was determined as the vertical distance between the base of the tail and the treadmill. Mutant animals experienced a progressively lower posture over time following week 10 in all groups, showing significant differences in weeks 14 and 16. Figures 4b) and 4c) show examples of WT and mutant mice respectively at week 16. Repeated measures 2-way ANOVA was used to calculate significant differences. * $p<0.05$ compared to wild type? ** $p<0.01$ colored symbols are corresponding the group represented by the same color.

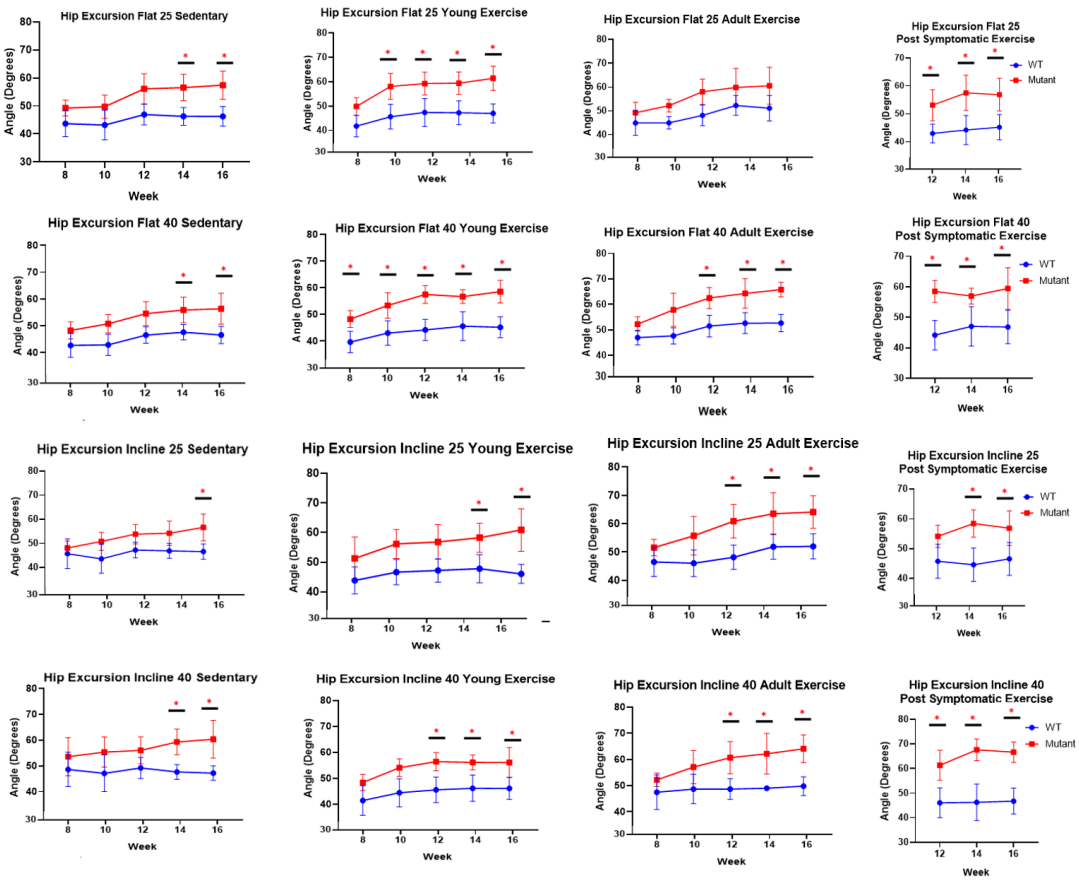


Figure 5: Hip Excursion output

These represent the hip excursion angles calculated as the minimum hip angle through the course of the step subtracted from the maximum hip angle; Column 1: Sedentary, Column 2: Young Exercise, Column 3: Adult Exercise, and Column 4: Post Symptomatic Exercise, with rows as the various running parameters; Row 1: Flat 25cm/s running, Row 2: Flat 40cm/s running. Row 3: Incline 25cm/s running, and row 4: Incline 40cm/s running. Significant differences were seen between the mutant and the WT groups in most conditions and groups after symptom onset with earliest significant differences seen as early as 8 weeks in the young exercise running group at 40 cm/s flat running. There is evidence of progressive difference in hip excursion over the course of time regardless of exercise duration. Repeated measures 2-way ANOVA was used to calculate significant differences. * $p<0.05$ compared to wild type? ** $p<0.01$ colored symbols are corresponding the group represented by the same color.

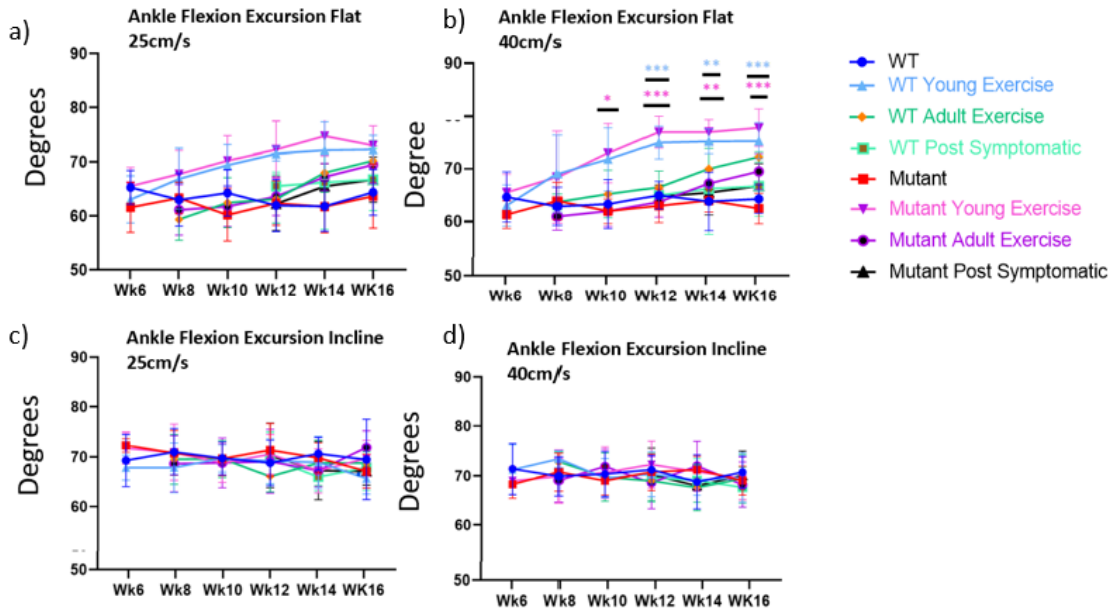


Figure 6: Ankle Flexion outputs for the four recording types

Ankle flexion excursion is calculated as the net difference in the ankle angle in the flexion phase of the stride. Significant differences were found in the ankle flexion in both the Flat 25cm/s (a) and Flat 40cm/s (b) running groups. In Flat 25cm/s, differences were seen at 12 weeks of age in the young exercise groups for both WT and mutant. In the Flat 40cm/s running groups there were differences in the mutant group at 10 weeks of age and for both WT and mutant at 12 weeks and onward. No differences were seen at either speed in the incline recordings. Repeated measures 2-way ANOVA was used to calculate significant differences. * $p<0.05$ compared to wild type? ** $p<0.01$ colored symbols are corresponding the group represented by the same color.

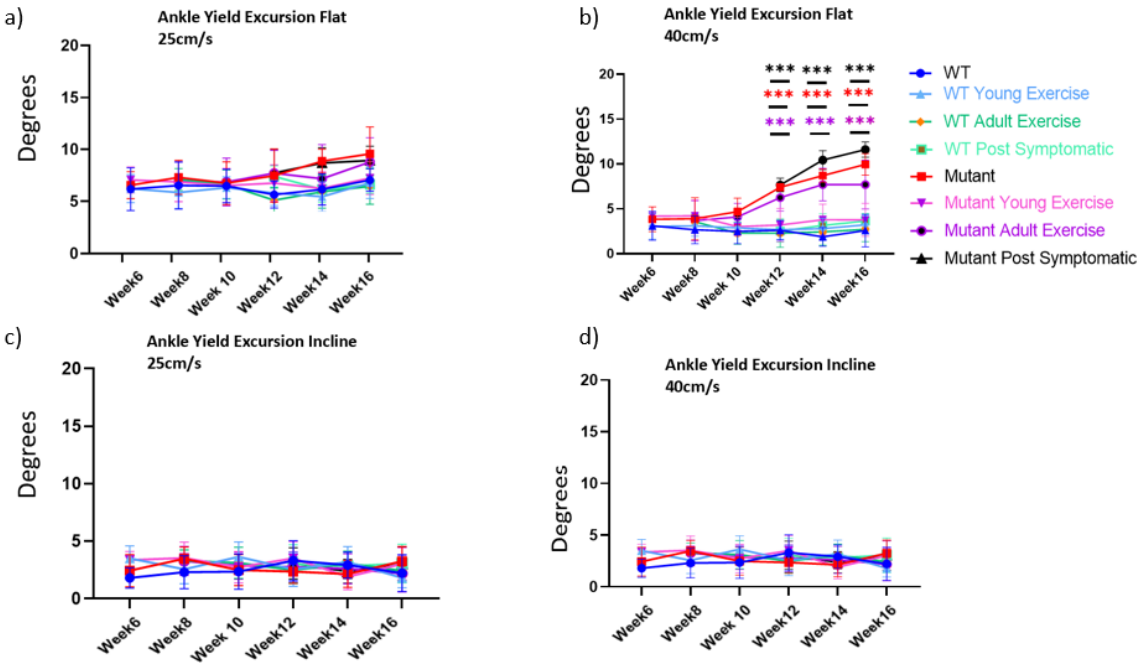


Figure 7: Ankle Yield outputs for the four recording types

Ankle yield excursion was calculated as the net difference in the ankle angle in the yielding phase of the stride. Significant differences were found in the ankle yield in only the 40cm/s flat running group. These changes were seen starting at 12 weeks of age in all mutant groups except for the early exercise mutant group. At the lower speed of 25cm/s in the flat running group, there was a similar trend seen in the same 3 groups. However, it is not a significant difference. Repeated measures 2-way ANOVA was used to calculate significant differences. * $p<0.05$ compared to wild type? ** $p<0.01$ colored symbols are corresponding the group represented by the same color.

4.2.4 Muscle Weight

Animal body weight has also been used as a potential biomarker of disease. It is one of the factors used in defining disease progression whereby a loss of 15% of the animal's body mass is an alternative endpoint stage. Although this is a common diagnostic factor it severely lacks specificity. Causes of weight loss in mice can be attributed to any type of stressful situation, not specific to disease, therefore in a study looking at exercise and ALS, weight loss struggles to remain a reliable biomarker of disease. Muscle weights can however be used to determine changes caused through the exercise regimen. These can then be further used to help elucidate what is happening in the mice in the changes seen in their kinematic analyses.

To investigate these changes, tibialis anterior (TA) and gastrocnemius (GS) muscles were extracted and weighed. The individual animal's total body weight was used to determine how the differences in the individual muscle weights related to their overall changes in body mass. The groups we examined for this experiment included: sedentary WT animals at P90, sedentary mutant animals at symptom onset, exercise WT animals at P90 starting exercise at 8 weeks, exercise mutant animals at symptom onset starting exercise at 8 weeks, mutant mice at end stage with their WT counterparts, and mutant exercise mice at end stage with their WT counterparts.

Although not statistically significant, a trending increase in muscle weight in both the TA and the GS in the exercised mice was observed in all groups. This was coupled with an increase in the percentage of body weight that these mice had, indicating that proportionally these muscles in the exercised mice had a trend of being larger compared to sedentary animals, particularly for TA muscles. These trends indicating potential physiological changes may account for some of the changes seen in the gait kinematics. Significant differences were found only between each of the groups muscle weights when comparing to the end stage muscle weights however.

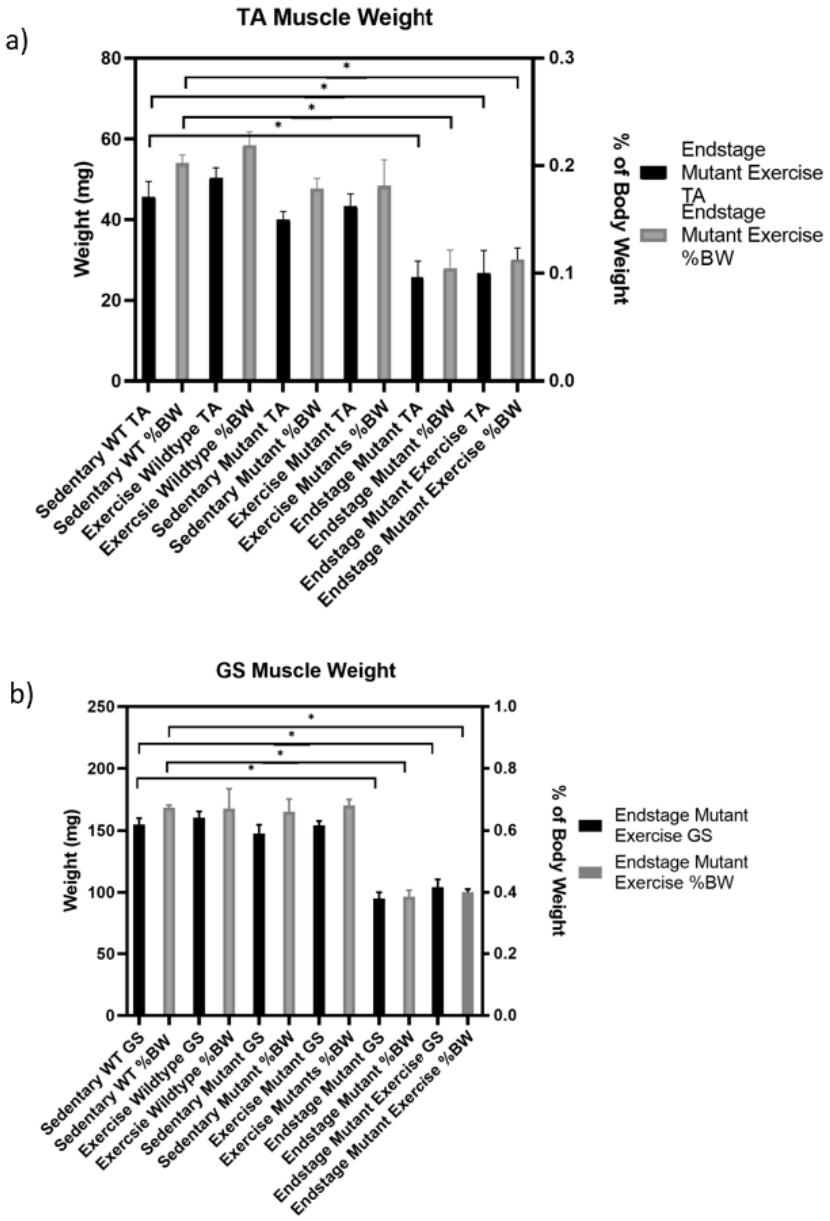


Figure 8: Muscle Weight Data

Figure 8a) represents the muscle weight data for the TA muscle in 3 animals in each of sedentary WT, exercise WT, sedentary mutant, exercise mutant and sedentary end stage groups, which were all at a Clinical Score of 3. Significant differences from the sedentary WT group were only seen in end stage mice in both groups. Figure 8b) shows the data from the GS muscles for the same groups with the same trend of significant differences only seen in the end stage mice. * $p<0.05$ compared to wild type? ** $p<0.01$.

4.3 Blood Metabolites

My behavioral analyses showed that the motor system has significantly changed in SOD1 mutant animal at young age and long-term intensive exercise could make substantial impact on locomotor behaviors in ALS progression. However, such behavioral analyses may not be possible for ALS patients. However, blood samples are much more accessible to most people even when their behavioral symptoms are not obvious. Studies have also shown that blood serum metabolites showed significant changes in ALS patients. I aimed to see if we could find early markers in ALS animals and how they may change after exercise trainings.

We compared metabolomes of 12 mice assigned divided into four groups:

1. Sedentary WT
2. Sedentary mutant
3. WT adult exercised mice who started the exercise training at 8 weeks of age
4. Mutant mice that began exercising at 8 weeks of age

The mass-spectrometry and initial raw data analysis of the metabolites were done by The Biological Mass Spectrometry (BMS) Core Facility in Dalhousie's CORES (Centralized Operation of Research Equipment and Support) program. I then further analyzed the data using Metaboanalyst. Figure 9a shows a heatmap comparing the 25 metabolites that showed the greatest statistical differences between sedentary WT and sedentary mutant animals. Only four of these metabolites showed a significance of $p< 0.05$ changes which were PC(24:0/P18:0), LysoPC(24:1(15Z)),PC(18:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/P-18:0).

Investigating the changes that occurred between all four groups, 22 metabolites were found to have significant differences and 39 metabolites that showed a trending difference. Figure 9b shows the list of the metabolites that had significant differences and shows how the concentration of metabolites relates from one group to the other. As we can see, 15 of the 22 most significant metabolites were found to have an upregulation between the exercise mutants compared to the other 3 groups. Additionally, 6 metabolites showed the greatest difference between the sedentary mutants compared to the other 3

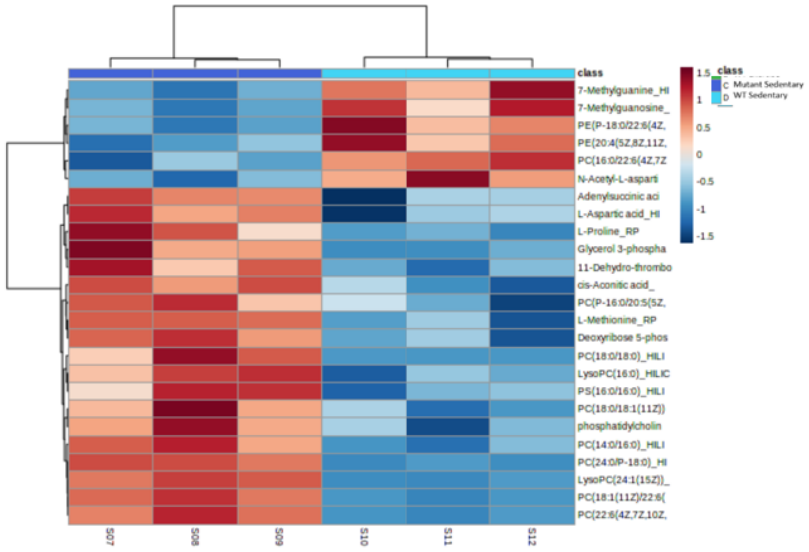
groups. The metabolite showing the most significant difference however, N-acetyl-L aspartine, had an upregulation in both the WT groups compared to the mutant groups.

PCA was used to investigate the differences between the 4 groups along the tested metabolites. Figure 9c shows some clustering of the groups across 3-dimensional component axes. Sedentary mutant animals separate from the other 3 groups along PC-2, whereas there is some separation of the mutant exercise group along PC-1and PC-3.

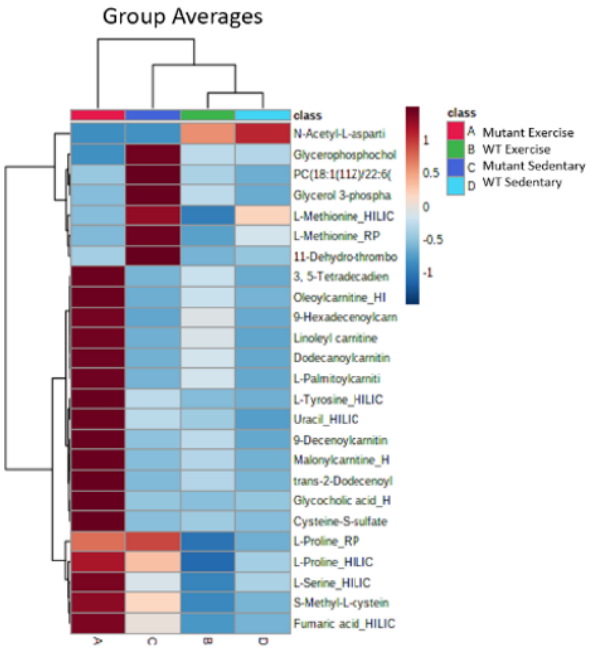
In order to further investigate these differences, pathway analysis was used to further understand the relationship between the significant metabolites. What was found was that the 15 metabolites that showed significant differences between the exercise mutant group and the sedentary WT group highlighted the potential changes in 3 pathways with the highest impact factors as well as the greatest p value. These were the Aminoacyl-tRNA biosynthesis, tyrosine metabolism, and phenylalanine, tyrosine and tryptophan biosynthesis pathways. The remaining 6 significantly different metabolites from the previous analysis, which showed difference amongst the sedentary mutants compared to the rest of the groups, were related to lipid metabolism pathways. In Figure 9d the output of the pathways analysis can be seen showing the impact factor vs the p-value for the highlighted pathways based on the given metabolic changes.

These metabolic differences showing distinct patterns when comparing the groups to one another shows the possible use of this methodology as a biomarker of disease. Future studies with a larger sample size and looking at different time points can further elucidate these differences and may also be used to show stronger indications of what the changes in the metabolites between the groups mean.

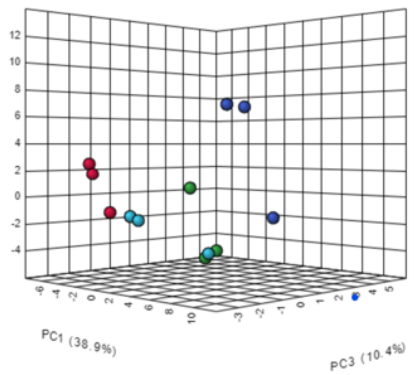
a)


class
7-Methylguanine_HI
7-Methylguanosine_
PE(P-18:0/22:6(4Z,
PE(20:4(5Z,8Z,11Z,
PC(16:0/22:6(4Z,7Z
N-Acetyl-L-asparti
Adenylsuccinic aci
L-Aspartic acid_HI
L-Proline_RP
Glycerol 3-phospha
11-Dehydro-thrombo
cis-Aconitic acid_
PC(P-16:0/20:5(5Z,
L-Methionine_RP
Deoxyribose 5-phos
PC(18:0/18:0)_HILI
LysoPC(16:0)_HILIC
PS(16:0/16:0)_HILI
PC(18:0/18:1(11Z))
phosphatidylcholin
PC(14:0/16:0)_HILI
PC(24:0/P-18:0)_HI
LysoPC(24:1(15Z))_
PC(18:1(11Z)/22:6(
PC(22:6(4Z,7Z,10Z,
S07
S08
S09
S10
S11
S12
class
C Mutant Sedentary
D WT Sedentary

b)


Group Averages
class
N-Acetyl-L-asparti
Glycerophosphochol
PC(18:1(11Z)/22:6(
Glycerol 3-phospha
L-Methionine_HILIC
L-Methionine_RP
11-Dehydro-thrombo
3, 5-Tetradecadien
Oleoylcarnitine_HI
9-Hexadecenoylcarn
Linoleyl carnitine
Dodecanoylcarnitin
L-Palmitoylcarniti
L-Tyrosine_HILIC
Uracil_HILIC
9-Decenoylcarnitin
Malonylcarntine_H
trans-2-Dodecenoyl
Glycocholic acid_H
Cysteine-S-sulfate
L-Proline_RP
L-Proline_HILIC
L-Serine_HILIC
S-Methyl-L-cystein
Fumaric acid_HILIC
A
C
B
D
class
A Mutant Exercise
B WT Exercise
C Mutant Sedentary
D WT Sedentary

c)


PC1 (38.9%)
PC3 (10.4%)

d)

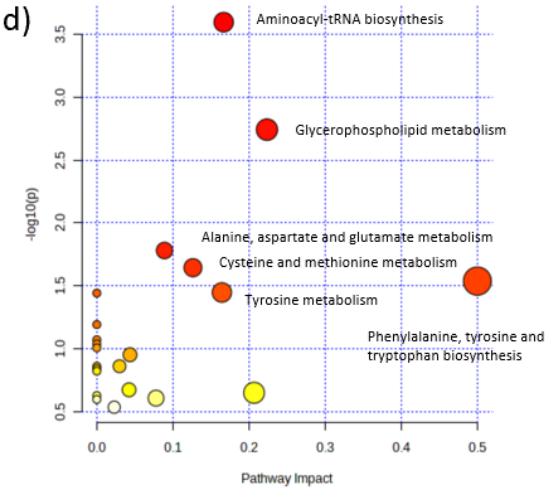

Aminoacyl-tRNA biosynthesis
Glycerophospholipid metabolism
Alanine, aspartate and glutamate metabolism
Cysteine and methionine metabolism
Tyrosine metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
-log10(p)
Pathway Impact

Figure 9: Metabolic Data

Figure 9a) is a heat map of the 25 most significant metabolites comparing the Sedentary WT and the Sedentary mutant animals. Figure 9b) represents the heat map of the four groups, with group averages, for the 25 most significant metabolites. Figure 9c) is a representation of the pathway analysis done on the significant metabolites when comparing all four groups of animals. It is showing the significance of the metabolites compared to the number of metabolites found within that pathway (Pathways Impact). Figure 9d) is a 3-dimensional principal component analysis of the 3 animals in each group.

CHAPTER 5: DISCUSSION

In the current study I systematically recorded and analyzed gait and hindlimb kinematics of SOD1G93A mice at different ages with different exercise training protocols, including sedentary, early exercise, adult exercise and post-symptomatic exercise groups. I found that using the traditional scoring system, only the SOD1 mutant animals that started intensive treadmill training at young age showed earlier signs of disease onset than compared to the sedentary counterparts, while no differences in overall mouse survival times were observed among all the different training groups. However, the gait and limb analyses revealed earlier changes in locomotor behaviors during the disease development and progression of the SOD1 mutants. Interestingly, different exercises training protocols showed different effects on the limb movements at varied disease stages. For example, changes between mutant and WT animals can be identified as early as 8 weeks of age in certain parameters such as in the hip joint angle excursion. Furthermore, the long-term intensive exercises appeared to have triggered adaptative change in leg swings and strengthen the mutant's ability to hold body weight during walking, but had no effects correcting the animals' postures. In addition, in our promising pilot study of blood serum metabolites, we found 22 metabolites that showed significant differences between our four groups (sedentary WT, sedentary mutant, adult exercise WT and adult exercise mutant) many of which differed specifically between the exercise and sedentary mutant mice.

5.1 Physical activities and ALS

5.1.1 Physical activities and symptom onset

ALS can show a variety of symptom onsets, whether it is a bulbar onset or spinal onset of disease, and further where specifically these signs first present themselves. Additional complications arise with the variety of potential causes and the ill-understood interplay between genetic and environmental influences (Richards et al., 2020, Oskarsson et al., 2018).

Exercise is also a broad topic where different exercises can be performed, be it swimming, running, resistance training, etc. and these exercises can be done at a spectrum of intensity levels from an aerobic effort to anerobic effort. Due to the heterogeneity in ALS and in exercise as well as differences in patient lifestyles, it can be difficult to clearly determine whether exercise is a beneficial non-pharmacological therapeutic strategy for individual ALS patients (Hamidou et al., 2014, Lacorte et al., 2016, Harwood et al., 2016). Animal models have been extensively used to understand the pathogenetic mechanisms of ALS and test therapeutic strategies. One beneficial factor using lab animals is to reduce some of the confounding variables, like lifestyles, living conditions and genetic background, in patients. However, even with animal models of ALS, there has been no clear consensus on the impacts that exercises may have on ALS as seen in the review by Tistkanou et al, 2019. One obvious variable in these studies was their training protocols. The duration of training times, speeds of running, incline vs flat treadmill, treadmill vs running wheel access, running vs swimming varied from study to study. Not only were the types of exercise different, but the timeline throughout which the animals were trained was also inconsistent (Liebetanz et al., 2004, Kirkinezos et al., 2003, Carreras et al., 2010). Some evidence has shown that mild-moderate endurance training may have positive effects on certain assessments (Kirkinezos et al., 2003) whereas vigorous endurance training had null to harmful effects (Mahoney et al., 2004). These inconsistencies may account for the wide variability in conclusions, which may also further indicate exercise/physical activities may not all beneficial to ALS, and much more vigorous studies are needed in both human and animal models.

To overcome some of the confounding issues in these studies, I used one of most studied mouse models, SOD1G93A mouse, which expresses human SOD1G93A mutant gene. We frequently checked the copy number of the mutant genes, which ensured our animals showed consistent landmarks of ALS-like symptom onset and progression process. In addition, I used treadmill running, which would also be one of the most used and least variable training procedures. At the same time, however, I used an inclined surface, which added the intensity of the exercise. More importantly, I started the training protocol in animals at different ages, which is one of the least known, but crucial impact factors that cannot easily be studied in humans. In my study, the youngest mouse group

started training at 4 weeks old at which point they are big enough to run on the treadmill without falling through the openings. I then included a group starting at 8 weeks, an adult age for mice, and another at P 90 days, the symptom onset stage. There was also a sedentary group as positive control. Among all these 4 mutant groups, only young training group showed significant earlier symptom onset, compared to other groups, but all groups had similar survival time. Studies on the impact of exercise are highly variable in their investigations, some studies such as that by (Mahoney et al., 2004) who did look at both survival times and onset in mice doing intensive exercise found trends for earlier clinical onset in males but not in females and found in males a reduced survival time in the exercise mice. These results differ from our investigations where we saw no change in survival time but changes in onset in our earlier trained group. Other studies (Carreras et al., 2010, Mahoney et al., 2004) that looked at different parameters such as motor neuron density, did not show a change in clinical symptom onset times but did observe a hastened onset of motor performance deficits in their intensive exercise groups, with both studies investigating moderate and intensive levels of treadmill running. Although the cause of the observed early onset in some studies has yet to be elucidated, the predominant theory is that exercise causes increased levels of oxidative stress in the diseased animals leading to faster motor neuron loss (Powers et al., 2011). The possibility that intensive physical activities at young age could potentially lead to earlier ALS symptom onset of ALS was implied in several human studies, particularly with patients carrying C9ORF72 mutations (Julian et al., 2021). All these studies and our studies have further supported the importance of identifying the populations that carry ALS related gene mutations to get suitable care.

5.1.2 Complex impacts of treadmill training on walking behavioural changes of SOD1G93A

The fact that the young training SOD1 group had a similar survival period in spite of earlier onset may indicate that the long-term exercises might have certain beneficial effects to slow disease progression after the symptom onset.

To investigate detailed effects of exercises on ALS, I first did some of the classical measurements, such as the grip strength and Rotarod tests. Our results were consistent with other studies (Carreras et al., 2010). Over time, mutants lose grip strength progressively, starting around 12 weeks when symptom onset occurs. Rotarod also showed a similar parallel progressive decline. However, exercises did not seem to aid the mice on either of these parameters. We then wondered whether both tests might be too rough to detect subtle behavioral changes at early stage of the disease.

I then chronically recorded the mouse running on the treadmill at different speeds and different angled surfaces to search for biomarkers that more accurately reflect the behavioral changes of the disease. We used DeepLabCut, a markerless pose estimation program that allowed us to investigate a wide variety of parameters related to mouse movement. To find the most representative parameters, I utilized principal component analysis, and revealed the factors having the most variability. The top variables between the wildtype and SOD1 mutant animals across different ages and training groups were posture, ankle flexion excursion, ankle yield excursion, and hip excursion.

More interestingly, these four parameters showed significant different between mutant and wildtype and among different training groups with different temporal trajectories. Among them, the posture, indicating the ability that the mice hold up the hip, started declining in all mutant groups regardless of exercise, around the symptom onset. Over time the mutants showed progressive decrease in their hind amplitude. By the end stages the mice appeared to be almost dragging their tails along the ground. Similarly to the grasping and rotarod tests, this parameter should directly reflect the weakened limb and trunk muscle force holding the rear part of the body up due to significant loss of MNs at symptom on set. One notable muscle that may be related to this change is the iliopsoas muscle. This muscle attaches between the vertebral column and the femur of the animal and plays an important role in hip movement and postural control. Weakening control of this muscle would also help explain the differences seen in the hip angle excursion seen where differences between mutant and WT mice were seen as early as 8 weeks old. Mutants showed a consistently greater excursion of the hip which could possibly be

related to less control over the movement of the leg along the hip muscles such as the iliopsoas. This reduced control and strength in hip muscles could also be associated with the mouse's ability to bear weight causing detriments in its posture.

Similar to muscles of the hip associated with weight bearing, the weight bearing muscles in the ankle also showed weakness over time, which was revealed in an increase of yielding excursion of the ankle during the treadmill walking at a medium speed, 40 cm/min, in most mutant groups starting at approximately week 12, slightly before the symptom onset. One exception here, however, is the young exercise mutant group which remained at approximately the same level of excursion as the WT group. Long-term treadmill training starting at young ages might have greater impact on stabilizing ankle muscle force through protecting the MNs or/and muscles from fast deterioration. It should be noted that while adult exercise mice also showed an increase in excursion, these changes plateaued at 16 weeks. In contrast, the post symptomatic exercise group performance worsened along time even compared to the sedentary mutant group. This worsening may indicate that intensive exercise following symptom onset could cause further damage to these weight bearing MN and muscles by over taxing them. For example, it may possibly be related to an increase in reactive oxygen species on the already weakened muscles without the benefits of earlier exercise in strengthening the muscles and having the body adapt to this insult.

Furthermore, another ankle related parameters, ankle flexion, was also affected by treadmill training. In this case, however, the treadmill exercise caused changes regardless of disease state. All exercised groups, mutant and their WT counterparts showed some increase in flexion movement at flat running. The young exercise group showed a higher degree of ankle flexion by 10 weeks compared to the other groups, whereas the adult exercise group increased the degree of ankle flexion compared to the sedentary groups after 14 weeks. At 16 weeks the adult exercise group of both mutant and WT showed a degree of ankle flexion comparable to the young exercise at 10 weeks of age. The post symptomatic group also showed a change in week 16 but not levelling out to the same extent as the groups that underwent a longer exercise protocol. Notably, there were no changes in any group of animals at inclined walking. Since our treadmill training was on

an inclined surface, this result could indicate adaptive changes by training. The animals have to reinforce the movement habit of lifting their toes higher by flexing their ankle in order to compensate for the incline surface. This is not uncommon among professional athletes. Depending on their sport, their gait behaviour may be changed to best suit their professional performance. An example of this can be seen in a study investigating the kinematic differences between football and futsal players (Daneshjoo et al., 2021) where football players showed less extension in landing and jumping maneuvers as their legs were more adapted to these actions while futsal players had an increased flexion in their knees and hips relating to cutting maneuvers due to the tighter turns and movement that the players regularly perform.

Nevertheless, our results indicated that the treadmill training may have impacts on ankle movement in both mutant and wildtype animals compared to any other differences observed between the exercised and sedentary mice. It is known that the exercise can increase muscle mass. When I compared the cross sections of the GS and TA muscles at different stages, exercise groups showed a trend of larger muscle mass, particularly the TA muscle, at symptom onset, but the exercise couldn't prevent the muscle loss at end stage. This may partially account for the changes of a few gait parameters. For example, exercise induced change in ankle flexion would be controlled by the TA muscle.

In summary, our detailed kinematic analysis revealed a complex picture of changes of locomotor behavior of SOD1G93A animals at different disease stages and even more complex impacts of exercises on these changes. We have shown that long intensive exercise may cause earlier onset of symptoms such as inducing shakiness, but some beneficial factors, such as increased muscle mass and potential MN protection after the symptom onset. These beneficial factors may help maintain the mobility of the mutant animals. In addition, changes of hip excursion started at very young age in SOD1 mutant animals, which could be an early sign of the disease.

5.2 Blood Serum Metabolites

Behavioural biomarkers are very useful as a method to indicate the presence of disease in the place when there would be no obvious other reason to suspect the presence of disease, however, as an early detector of disease onset it is often a parameter that is the

consequence of an established internal dysregulation. For this reason, it can be beneficial in less common diseases such as ALS, where physicians would not regularly test their patients for the disease until they show early behavioural changes. This study tries to establish that behaviour can be used earlier than it has been in animal models, however, other biomarkers of disease such as blood metabolites can possibly show even earlier signs of disease onset. One such biomarker is blood serum metabolites.

Recently, blood metabolomics have been studied extensively in human patients and animal models in an effort to find specific biomarkers for diagnosis and prognosis of ALS disease. Although there haven't been any conclusive indicators revealed through these studies, some common pathways have been identified. For example, many studies have shown that dysfunctions in lipid metabolism may be potential drivers of ALS pathogenesis, causing denervation of neuromuscular junctions, mitochondrial dysfunction, excitotoxicity, impaired neuronal transport, and inflammation (Agrawal et al., 2022). In our study phosphatidylcholine metabolites showed a significant upregulation in the sedentary mutant mice compared to the sedentary WT mice indicating dysfunction in lipid metabolism similar to other studies. When the analysis included the exercised mice we saw that these same four phosphatidylcholines that showed significant differences remained showed a similar expression between the sedentary WT and exercise mutant group. This indicates that exercise could help in the restore of lipid metabolism in ALS patients.

Other molecules which showed trends of differences between the two Glycerol-3-phosphate is an important molecule in the synthesis of triglycerides. Although inconsistent, some studies have also shown increased levels of triglycerides in ALS patients, and those with greater relative levels of triglycerides showed an increased survival time (Dorst et al., 2010). This could be an indication of the body responding to the increased energy needs that have been associated with ALS where upregulation of the building blocks for lipids can help combat against the disease.

Guanosine diphosphate plays a role in many different biological pathways including but not limited to PKC activation, aspartate metabolism, citric acid cycle, purine, and pyruvate metabolism. One pathway in particular that may be relevant to ALS is its role in

gluconeogenesis. Glucose metabolism has been shown to be dysregulated in ALS (Lee et al., 2022). Specifically, in response to low blood glucose concentrations, glucagon is released from a- pancreatic islet cells which promote gluconeogenesis and glycogenolysis. SOD1G93A mice were shown to have elevated glucagon levels following insulin induced hypoglycemia with minimal changes shown in overall blood glucose levels. This suggests perturbations with regards to downstream glucagon signalling pathways and glucagon insensitivity contributing to aberrant glucose homeostasis as reviewed in (Lee et al., 2022).

When the two exercise groups were included, 22 metabolites showed significant differences between the groups. Surprisingly, the exercised mutant mice showed the most drastic differences from other groups. Among the 22 metabolites, 15 of them were increased compared to those in the control groups and sedentary mutant group. Looking at a pathway analysis for these 15 metabolites, we find that the top three pathways that show both the highest impact factor as well as the greatest p value were aminoacyl-tRNA biosynthesis, tyrosine metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis. The common metabolite shared between these three pathways is L-tyrosine. Tyrosine is a non-essential amino acid and is an essential component for the production of several neurotransmitters such as dopamine, epinephrine, and norepinephrine. Looking at the graph of the metabolite concentration we can see that L-tyrosine was elevated in both mutant groups however much more in the exercise groups. In exercise science L-tyrosine has been extensively studied with a variety of focuses. For example, researchers have been identifying the use of L-tyrosine supplementation in improving animals' endurance in increased air temperatures (Tumilty et al., 2011). By contrast, other studies have shown that dysregulation in tyrosine metabolism may play a role in several neurodegenerative diseases, and specific to ALS, may be implicated in motor neuron apoptosis (Peluffo et al., 2004). When the body is experiencing inflammation or oxidative stress, a reactive nitrogen species can react with tyrosine to form nitrotyrosine. It has been hypothesized that increased nitrotyrosine may not directly cause motor neuron death, but in elevated concentrations it may increase the production of free radicals, further increasing oxidative stress and damage to the motor neurons (Peluffo et al., 2004). With respect to our results, the increase in L-tyrosine in the exercised mutants suggests

that intensive exercise may leave the motor neurons more vulnerable to degeneration for ALS patients.

As mentioned previously, of the pathways identified through our pathway analysis, the three pathways with the highest impact factor as well as the greatest p value were aminoacyl-tRNA biosynthesis, tyrosine metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis. Looking at studies of blood metabolites in ALS patients, phenylalanine, tyrosine and tryptophan biosynthesis and tyrosine metabolism were also identified (Chang et al., 2021). These metabolites are essential components in the production of several neurotransmitters including epinephrine and norepinephrine, dopamine, and serotonin. (Chang et al., 2021) Although common pathways have been identified among blood serum metabolite analyses, the exact role and of these metabolites in ALS pathogenesis is still unclear.

Our pilot study identified some highly promising metabolic differences in exercised and sedentary mutant mice compared to their WT counterparts. The trends seen in this study indicate differences in the metabolic pathways may be activity dependent. We showed that with exercise, metabolic differences such as differing expression of PC could be restored to the levels of WT mice through exercise. This however was not the only change occurring. Mutant exercise mice showed many metabolic differences from both WT groups as well as from the sedentary mutant group. These results are in agreement with the results seen in behavioural studies, where by certain metrics exercise can show beneficial changes, while in other ways it can show its own set of potentially harmful effects.

CHAPTER 6: LIMITATIONS AND FUTURE DIRECTIONS

The biggest limitation in these studies was sample size. Although the mice in each group were genetically the same and trained with the same exercise protocol, there was still a large variability in behavioural measurements between mice within each group. With so much individual variability in behaviour, a larger sample size is required. With this as well, this study combined results from males and females to increase overall sample size, however many studies have shown differences in disease response to therapies depending on sex. Therefore, future studies could benefit from separating the two sexes and determining whether there are sex related differences. This is especially true with regards to blood serum metabolites, which in many cases show a lot of individual variability. Increasing the sample size may help uncover other relevant metabolites. We identified 22 statistically significant metabolites with our pilot study numbers yet there were over 50 metabolites that showed a promising trend.

Future studies can also look for additional biomarkers, such as such as identification of stress granules and stress granule related factors. Stress granules play an important role in RNA metabolism, however many recent studies have begun investigating them in relation to ALS (Dudman & Qi, 2020). Chronic stress can cause these granules to build up in prion like fashion which can lead to apoptosis and have been identified in ALS patients. Using tools such as RT-QPCR could be utilized to investigate the presence of these biomarkers and their factors in various tissues to see how it relates to the behavioural outcomes.

Future directions can also investigate what the effect of the reduction in interneurons has. Given that the animals maintained their gait coordination it would be worth investigating whether this is due to a re-circuitry that overcomes the reduction in interneurons or if the reduction does not fall below the minimum required input to maintain stable gait.

CHAPTER 7 : CONCLUSIONS

This study sought to identify early biomarkers of disease in SOD1G93A mice, and see what influence exercise had on those biomarkers. We were able to identify a number of early behavioural changes between four groups of mice (Sedentary mutant and WT, early exercise mutant and WT, adult exercise mutant and WT mice, and post symptomatic exercise mutant and WT mice) through behavioural analysis. Differences found in this analysis included changes in animal posture, ankle flexion excursion, ankle yielding phase excursion, and hip excursion. A key biomarker that showed early differences was the hip excursion, showing changes as early as 6 weeks of age however it was not influenced by the exercise protocol. We were able to identify other parameters that showed differences amongst exercise groups compared to the sedentary group such as worsening ability to bear weight in the mutant groups that started exercise regime after symptom onset.

A pilot study investigating the use of blood serum biomarkers shed interesting light on a number of potential metabolites that may be related to the disease progression, and changes within these biomarkers between the exercise and sedentary groups. Future work will look to increase the sample size of these experiments and determine if there is any correlation between these metabolomic changes and the changes seen in the animal behaviour.

While my study was not able to answer the question of whether intensive exercise is beneficial or harmful for SOD1 mice, it indicated that behavioural measurements alone may not be able to answer the question. Promisingly, using detailed metabolic information, the complex biochemical pathways impacted by both ALS and exercise may be untangled.

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APPENDIX A: GAIT ANALYSIS

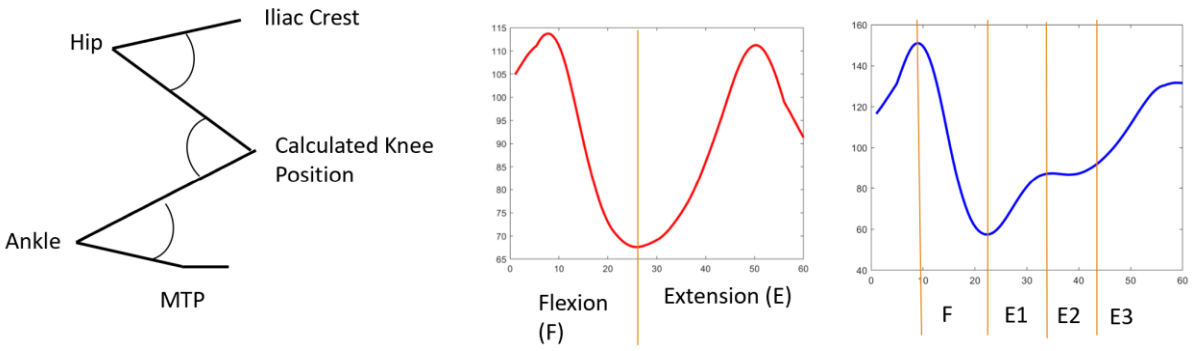

Figure 10: Gait Analysis

Diagram of mouse leg and points of interest for gait analysis. B) Hip angle graph showing the separation between flexion and extension. C) Ankle angle graph showing the separation between the four phases.

APPENDIX B: SPINAL CIRCUITRY IN ALS

As discussed, ALS is marked by progressive motoneuron degeneration, however the pathophysiology of the disease is not restricted solely to this one facet of the disease and evidence of degeneration has also been found in the brainstem and spinal circuits in which motor neurons are embedded. In the SOD1G93A mouse model, studies of cultured motor neurons and interneurons, showed that mutant mice expressed smaller glycine currents compared to their WT counterparts. This loss is specific to large motor neurons and is not observed in in presumed gamma and small fatigue resistant motor neurons that innervate type I muscle fibres (Chang and Martin, 2011).

On the other hand, it has also been proposed that enhanced motoneuron excitability is often proposed as a mechanism contributing to the pathophysiology of motoneurons. Motoneurons receive monosynaptic glutamatergic input from other motoneurons as well as recurrent excitation from V3 interneurons (Falgairolle & O'Donovan, 2020). Ideally, the Renshaw pathway exhibits a strong inhibitory effect on motoneurons balancing out motoneuron excitability, however if the motor neural inputs to inhibitory Renshaw cells are lost before those to motoneurons, this can lead to a powerful, recurrent glutamatergic excitation without the inhibition leading hypothetically to glutamate toxicity (Falgairolle & O'Donovan, 2020).

Studies investigating interneuron health over disease progression have revealed a significant loss in both inhibitory and excitatory interneurons including a 40% reduction in V1 inhibitory interneurons, and a 60% loss of V2a excitatory interneurons (Salamatina et al., 2020). Previous work in our lab has indicated differences in the counts of V3 interneurons as well in the SOD1G93A population, however the effect exercise may play on this noted decrease in spinal circuitry has yet to be extensively researched.

Many researchers have investigated the relation between interneurons and disease state. V3 interneurons, in particular, are associated with a robust rhythmic output allowing for coordinated gait movement. In the behavioural analysis there was no significant difference found in coordinated gait movement between the four limbs (supplementary

figures). However, studies have shown improved coordination in individuals who undergo regular exercise. We wanted to therefore investigate whether exercise could potentially curtail the loss of V3 interneurons, regardless of the lack of noticeable difference in coordination.

This experiment used of SOD1G93A;Sim1CreTdTom mice to allow us to visualize the sim1cre expressing V3 interneurons with Td tomato. Staining for the expression of C- fos we are able to identify the cells that were activated after a bout of activity. We used four groups of mice consisting of sedentary WT, sedentary mutant, exercise WT and exercise mutant with three animals per group. The exercise groups began exercising at 8 weeks of age and all mice were sacked for analysis at 90 days with the mutants all showing symptom onset as described in the rubric scoring system in the methods section.

Counting the number of V3 cells in each Lumbar section, a consistent significant decrease in the number of V3 cells in both the exercise mutant and the sedentary mutant groups as compared to both the exercise and sedentary WT groups was found. Looking at the V3 cells which also express C-fos, the number of activated V3 cells maintains a similar ratio between the mutant and the WT groups where the proportion of activated to non activated V3 cells remains the same.

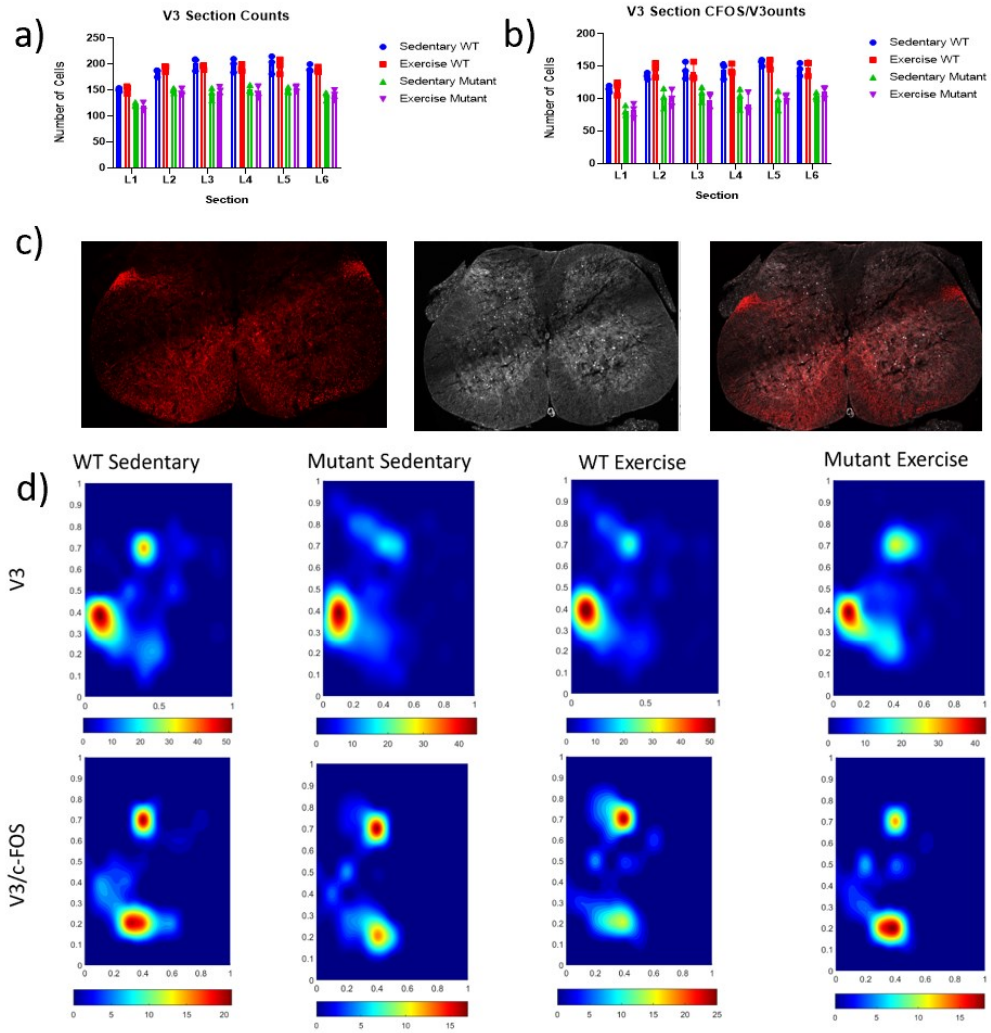

Figure 11: V3 interneuron count and V3/C-fos count in the mouse spinal cord

Figure a) represents the V3 count in the mouse spinal cord through each lumbar section. Figure b) shows the V3 and C- fos count in the mouse spinal cord through each lumbar section. Figure c) shows representative images of the spinal cord with V3 highlighted on the left, C- fos images in the middlem and the combination on the right. Figure d) shows the heat map distribution of the cells found.