A Sex-dependent Response to *dl*-PHPB in an Amyotrophic Lateral Sclerosis Disease

Mouse Model

by

Uduak Ime MacKenzie

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To my parents, especially my Mother, thank you for giving me the wings to fly; quite literally- across the globe to Canada. Without your vision and sacrifice, pursuit for excellence and value for education, I wouldn't be here today. Though this may sound cliché, but words cannot begin to express how lucky I am to have such a strong mother. I love you mom and thank you for believing in your girl-child!

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To my little one Caleb, mama will place you on her shoulder, so you see farther and reach farther.
In this world, you can be whatever you want to be. I love you son.

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Abstract

Amyotrophic Lateral Sclerosis is a fatal neurodegenerative disease affecting upper and lower motor neurons resulting in their death. There are currently only two FDA approved drugs for its treatment, and both offer mild effects on the disease. Hence the need for a continued search for a better and more effective treatment. dl-Potassium 2-(1-Hydroxypentyl)-benzoate (dl-PHPB) is a novel drug for the treatment of cerebral ischemia and has shown potential neuroprotective, anti-oxidative and anti-apoptotic benefits in several neurodegenerative diseases. In this study, the effects of dl-PHPB treatment are investigated in SOD1^{G93A} mice, an animal model for ALS disease. Our results show that dl-PHPB has neuroprotective effects that are able to delay disease onset and progression, and also improve locomotor function particularly in female animals. Further investigation will be needed to confirm dl-PHPB as a drug candidate for the treatment of ALS and its effects on different gender groups.

List of Abbreviations and Symbols used

%	Percent
°C	Degrees Celsius
±	Plus or Minus
<	Lesser than
AAV	Adeno-Associated Virus
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
ALSAQ-40	ALS Assessment Questionnaire
ALSFRS-R	ALS Functional Rating Scale- Revised
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	Amyloid Precursor Protein
ASOs	Antisense Oligonucleotides
ATP	Adenosphine Triphosphate
Aβ	Amyloid beta
BDNF	Brain Derived Neurotrophic Factor
C9ORF72	Chromosome 9 Open Reading Frame 72
cDNA	Complementary Deoxyribonucleic acid
ChAT	Choline Acetyltransferase
CNS	Central Nervous System
CSF	Cerebrospinal fluid
***dl*-NBP**	3-n-butylphthalide
***dl*-PHPB**	*dl*-Potassium 2-(1-Hydroxypentyl)-benzoate

EAAT	Excitatory Amino Acid Transporter
EAAT2	Excitatory Amino Acid Transporter 2
ER	Endoplasmic Reticulum
fALS	Familial Amyotrophic Lateral Sclerosis
FDA	Food and Drug Administration
FF	Fast Fatigable
FR	Fast fatigue-resistant
FUS	Fused in Sarcoma
FUS/TLS	Fused in sarcoma/Translocated in liposarcoma
FVC	Forced Vital Capacity
g	grams
GPNMB	Glycoprotein Non-Metastatic Melanoma Protein B
GRP	Glial- restricted Precursors
H_2O_2	Hydrogen Peroxide
HCN	High Copy Number
HCN SOD1^{G93A}	High Copy Number SOD1^{G93A}
HRP	Horseradish peroxidase
IL-1B	Interleukin-1B
IL-4	Interleukin 4
IL-6	Interleukin 6
IV	Intravenous
l-NBP	L-3-n-Butylphalide
L5	Lumbar Spinal Cord segment 5

LCN	Low Copy Number
LCN SOD1^{G93A}	Low Copy Number SOD1^{G93A}
LMN	Lower Motor Neuron
mg/kg	milligram/kilogram
MHC	Myosine Heavy Chain
MIF	Macrophage migration inhibitory factor
ml	Milliliter
MND	Motor Neuron Diseases
mSOD1	mutated SOD1
Na$^+$	Sodium
NMDA	N-Methyl-d-aspartic acid
NMDARs	N-Methyl-d-aspartate receptors
NMJ	Neuromuscular Junction
NOX2	NADPH Oxidase 2
NSC	Neural Progenitor Stem Cells
O$_2^-$	Superoxide Radical Anion
·OH	Hydroxyl radical
ONOO−	Peroxynitrite oxidant
PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline – Triton X-100
PFA	Paraformaldehyde
PLA	Plantaris
PLMN	Pure Lower Motor Neuron

PMSF	Phenylmethane Sulfonyl Fluoride
PS1	Presenilin 1
PUMN	Pure Upper Motor Neuron
QOL	Quality of Life
qPCR	Quantitative Polymerase Chain reaction
rhEPO	recombinant human Erythropoietin
RNA	Ribonucleic acid
ROS	Radical Oxygen Species
ROS	Reactive Oxygen Species
RPM	Rotations Per Minute
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
S	Slow muscles
sALS	Sporadic Amyotrophic Lateral Sclerosis
S.D	Standard Deviation
SEM	Standard Error of the Mean
SFDA	State Food and Drug Administration of China
SOD1	Super Oxide Dismutase 1
SOL	Soleus
TARDBP	Transactive response DNA binding protein
TBST	Tri-buffer Saline Tween-20
TNF-α	Tumor Necrosis Factor – alpha
TUDCA	Tauroursodeoxycholic acid
UBQLN2	Ubiquilin 2

UCN SOD1$^{\text{G93A}}$	Unknown Copy Number SOD1$^{\text{G93A}}$
UDCA	Ursodeoxycholic acid
UMN	Upper Motor Neuron
USFDA	United States Food and Drug Administrations
VAMP	Vesicle Associated Membrane Protein
VAPB	Vesicle Associated Protein B
VCP	Valosin-containing Protein
VEGF	Vascular Endothelial Growth Factor
WT	Wild type
µl	Microliter

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CHAPTER 1: INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a relentless progressive fatal neurodegenerative disease branded by the loss of both upper and lower motor neurons of the spinal cord, motor cortex and brainstem (Al-Chalabi & Hardiman, 2013; Fischer et al., 2004; Geloso et al., 2017; T. Philips & Rothstein, 2014). This systemic death of motor neurons leads to weakness, spasticity, and atrophy of the muscles. Eventually in 3-5 years, following diagnosis, death of the individual is imminent as a consequence of respiratory failure and paralysis (Cantor et al., 2018; Haverkamp et al., 1995). Since its discovery in 1869 by French Neurologist Jean Martin Charcot, ALS has remained the most common motor neurodegenerative disease affecting middle aged adults aged 45-60 years (Boillée et al., 2006; Byrne et al., 2011; Nardo et al., 2016).

Environmental exposures to fertilizers and pesticides have been shown to adversely worsen the prognosis of the disease (Yu et al., 2014). Other adverse prognostic indicators include an increased age at symptom onset, shortness in time from first symptom observed to clinical examination, bulbar onset of disease, and rate of deterioration of the respiratory system (Haverkamp et al., 1995). Correlational links have also been suggested between military personnel and a higher risk of dying from ALS, more than members of the general population (G. Weisskopf M. et al., 2005). Potential link between ALS and smokers remains to be elucidated, despite its discussed associations as a risk factor (M. G. Weisskopf et al., 2004).

ALS falls under two broad umbrellas- the sporadic form accounts for 95% of cases reported, while the familial form makes up approximately 5% of reported cases (Byrne et al., 2011). Sporadic ALS (sALS) is termed such, as there is no indication of inheritance of the disease, while familial ALS (fALS) show typical signs of the Mendelian inheritance pattern (Casci & Pandey, 2015). Over the years, mutations in various genes have been linked to both the

sporadic and familial variant of the disease. Some of these genes include Fused in Sarcoma (FUS), TAR DNA Binding Protein (TARDBP), Chromosome 9 Open Reading Frame 72 (C9ORF72), Superoxide Dismutase (SOD1), Vesicle associated membrane protein (VAMP)-associated protein B (VAPB) gene, to mention a few (Arai et al., 2006; Belzil et al., 2009; Connolly et al., 2020; DeJesus Hernandez et al., 2011; Mathis et al., 2019; Rosen et al., 1993; Zou et al., 2017).

There are currently no known cures for ALS due to the genetic, molecular, and cellular complexity of the disease and its rapid progression. There are however two drugs marketed for treatment purposes. The first drug – Riluzole, works by hindering the presynaptic release of glutamate thus reducing chronic glutamate excitotoxicity. It is a rather expensive drug, with mediocre effectiveness- prolonging median survival by approximately 2-3 months in ALS patients (R. G. Miller et al., 2012). The second approved drug- Edaravone is an antioxidant- a free radical scavenger and its effect is seen in the removal of both lipid peroxides and hydroxyl radicals (T. Watanabe et al., 1994). In the SOD^{G93A} mouse model, Ito et al., (2008) reported no increase in survival following treatment with Edaravone. However, motor function decline is ameliorated, spinal cord motor neurons are protected, and the aggregation of SOD1 protein is inhibited (Ito et al., 2008). It is still too soon to determine how effective this is in the treatment of ALS in patients. The cost of this drug is estimated to be around $145,500 per year, and in addition to this is the extra burden of committing to frequent intravenous (IV) infusions and the accompanying health complications that may arise for a patient having an IV port constantly (Chang & Ross, 2017).

1.1   Cellular Mechanisms & Pathologies of Amyotrophic Lateral Sclerosis

At clinical diagnosis of disease, approximately two thirds of patients present with a classical 'Charcot ALS', which is recognized as Limb onset (Wijesekera & Leigh, 2009). Typical symptoms are related to focal muscle weakness that may begin in the upper or lower limbs, with a gradual but certain onset of spasticity developing in the already atrophied muscles that eventually affects gait and dexterity (Wijesekera & Leigh, 2009). One third of patients present with Bulbar onset, marked by dysarthria of speech and dysphagia for solids or liquids (Jawdat et al., 2015). Eventually almost all patients with bulbar onset will develop excessive drooling, medically termed sialorrhoea due to the difficulty in swallowing (Wijesekera & Leigh, 2009). It is important to note that both limb symptoms and bulbar symptoms can occur simultaneously(Wijesekera & Leigh, 2009). However, bulbar onset progresses more rapidly than limb onset (Jawdat et al., 2015) and people diagnosed with this typically have a shorter survival time of approximately 2 years..

Even though the exact mechanisms underlying ALS diseases are still unclear, several cellular and molecular factors and processes, including mitochondrial dysfunction, oxidative stress, axonal transport dysregulation, glutamate excitotoxicity, have been shown to contribute to the pathogenesis and progression of ALS disease

1.1.1   Reactive Oxygen Species, oxidative stress, and SOD 1 mutations.

Oxidative stress occurs as a result of an imbalance in the production and elimination of Reactive Oxygen Species (ROS) and the system's inability to remove or repair the damage. (Barber & Shaw, 2010; Gall et al., 2020). Cellular ROS occurs due to aerobic metabolism and are formed as by-products of this process; which is mainly incurred due to electron leakage in the mitochondrial respiratory chain, leading to a production of superoxide radical anion (O_2^-) and

hydrogen peroxide (H_2O_2) (Barber & Shaw, 2010). Neither of these oxides are highly reactive, however they do undergo further reactions that lead to a production of the more potent peroxynitrite oxidant ($ONOO^-$) (Beckman & Koppenol, 1996), and the highly reactive radical - hydroxyl radical ($\cdot OH$) (Barber & Shaw, 2010). These oxidizing agents are very powerful, and capable of causing irreparable damage to cells; modifying proteins conformation, destroying lipids and altering DNA and RNA species (Barber & Shaw, 2010). Increased oxidative stress has been shown to affect mitochondrial function leading to irreversible damage of mitochondrial DNA(Kowaltowski & Vercesi, 1999), worsening or heightening of endoplasmic reticulum stress (Malhotra & Kaufman, 2007), and negatively affecting protein homeostasis pathways and mechanisms (Reichmann et al., 2018) eventually leading to apoptosis, cellular damage and neuronal loss or death (Gall et al., 2020).

Superoxide dismutase (SOD1), a small protein of 153 residues, is both abundant and ubiquitously expressed both in prokaryotes and eukaryotes (Fridovich, 1995). Its main function is to catalyze the transformation of superoxide anion – a reactive oxygen species (ROS) to hydrogen peroxide (H_2O_2) and by so doing, play an essential and dynamic role in cell functions. (F. Johnson & Giulivi, 2005).

In 1993, SOD1 gene mutations were the first missense mutations to be identified in a causal relationship with ALS (Rosen et al., 1993). A gain of toxic function, but not loss of dismutase activity in SOD 1 mutants contributes heavily to motor neuron death in ALS. Mutations in this gene account for about 3% of all sALS cases and 20% of fALS (Acevedo Arozena et al., 2011; Pasinelli & Brown, 2006). The mechanism of toxicity for the mutated SOD (mSOD1) gene is not fully elucidated; however, two potential theories have been proposed. The first proposal describes a toxic gain of function of the mutated SOD 1 gene, where the zinc

binding is attenuated and thus converts the protein into a toxic pre-oxidant (Sharp et al., 2018). The second proposal postulates a tendency for a modicum fraction of mSOD1 proteins to misfold, aggregate and form clusters which in turn saturate chaperone proteins, impede proteasomes and/or coalesce with mitochondrial enzymes disrupting their function (Boillée et al., 2006; Julien & Kriz, 2006; Liu et al., 2012; Rakhit et al., 2007).

The over-expression of mutated human SOD1 genes in different animal models, including mouse and zebrafish, is one of the most popular systems used to study ALS disease. The different mutations of the SOD1 gene have varying effects on the phenotype, survival rate, severity, and progression of ALS disease. SOD1^{G93A} mutant is among one of the few that recapitulated a quick disease progression and short survival period in patients (Mejzini et al., 2019). The SOD1^{G93A} mouse model has been the most studied and instrumental in providing ALS researchers with invaluable knowledge on ALS and its mechanism of action and potential treatments (Hwee et al., 2014; J. Knippenberg et al., 2013; Weerasekera et al., 2018).

1.1.2   *Mitochondrial and Endoplasmic Reticulum dysfunction in ALS*

Mitochondria are a vital organelle for energy metabolism, generating Adenosine Triphosphate (ATP), are a main source of ROS, act as gatekeepers to the intrinsic apoptotic pathways and help in the stabilization of free calcium ions concentration in a cell(Gall et al., 2020; Kaus & Sareen, 2015). Several mechanisms such as defective axonal transport, flawed glutamate uptake process, increase in oxidative stress, increase in protein aggregates accumulation, to mention a few can trigger mitochondrial dysfunction in ALS (Gall et al., 2020) in both the nervous system and other regions of the body such as the liver and blood lymphocytes (Cozzolino & Carrì, 2012; S. Sasaki et al., 2007).

Post-mortem human tissues as well as tissues from animal models of ALS, that exhibit abnormalities in the mitochondria have equally been shown to possess some Endoplasmic Reticulum (ER) dysfunction (Kaus & Sareen, 2015). Due to the intrinsic structures and functions of ER, such as its involvement in the synthesis and folding of both secreted and membrane properties; stress conditions such as those caused by mitochondrial dysfunction can interfere with ER abilities as well. Thus, leading to misfolds and aggregation of accumulated proteins as observed in TDP-43 and FUS/TLS mutations (Andersen & Al-Chalabi, 2011; Turner et al., 2013), and therefore, aggravated ER stress (Matus et al., 2013). Taken together, aberrant mitochondria morphology and its consequent dysfunction in ALS, can in part be attributed to insoluble mutated protein aggregates as observed in the case of SOD1-causing ALS (Higgins et al., 2003), resulting in a cascade of events that ultimately leads to increase in oxidative stress (Kausar et al., 2018).

1.1.3   Glutamate-mediated excitotoxicity

Glutamate is the major excitatory neurotransmitter or chemical messenger in the CNS (Gall et al., 2020; Zhou & Danbolt, 2014). When released from the presynaptic neuron into the synaptic cleft, it activates glutamatergic receptors, including the ionotropic α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-d-aspartic acid (NMDA) receptors, which in turn facilitates the influx of calcium and sodium ions into the post synaptic neurons (Ferraiuolo et al., 2011; Gall et al., 2020; Van Den Bosch et al., 2006).

Excessive production and release of glutamates can result in extreme neuronal firing and influx of calcium ions into postsynaptic neuron leading to excitotoxicity (Gall et al., 2020). The first reported observations of glutamate-mediated excitotoxicity in ALS, dates to 1987 when Plaitakis and Caroscio showed or reported elevated levels of glutamate in blood plasma. Even

more so important was a study by Rothstein and colleagues in 1990 showing elevated levels of glutamate in the CSF of ALS patients (Plaitakis & Caroscio, 1987; Rothstein et al., 1990).

Glutamate excitotoxicity in ALS was considered as one of the major causes for the death of motoneurons (Rattray & Bendotti, 2006). This excitotoxicity can be explained by faulty glutamate uptake and transporters. For instance, an error in the function of excitatory amino acid transporter (EAAT2), leads to the presence of excessive calcium influx in the neurons, which ultimately triggers aberrant calcium homeostasis in the neurons, downstream mitochondria dysfunction resulting in energy failure and elevated levels of ROS production (Netzahualcoyotzi & Tapia, 2015; Y. Wang & Qin, 2010). Glutamate excitotoxicity has been implicated in both sporadic and familial forms of ALS, which is evident by the occurrence of heightened glutamate release and activity, motor neuron and interneuron hyperexcitability and cortical hyperexcitability in ALS animal models and patients (Milanese et al., 2011; Shi et al., 2019; van Zundert et al., 2008; Vucic et al., 2008). Though there is overwhelming evidence of glutamate excitotoxicity and its role in neurodegeneration in ALS, it is still not completely understood why the anti-glutamatergic and anti-excitotoxic drug -Riluzole is able to prolong life for roughly only 2-3 months following an ALS diagnosis. (Petrov et al., 2017).

1.1.4   *Axonal Transport dysregulation*

Axonal transport impairment is another key pathological feature of ALS and has been observed in several ALS animal models as well as in other neurodegenerative diseases (De Vos & Hafezparast, 2017; Ferraiuolo et al., 2011; Sleigh et al., 2020). Axonal transport is an important procedure whereby essential elements such as RNA, proteins, lipids and organelles are delivered to and from the synapses (Ferraiuolo et al., 2011; Gall et al., 2020). Motor neurons are highly polarized cells that possess exceptionally long axons, sometimes reaching 1meter in

length, and as such place heavy demands on cellular and physiological functions needed to transport and deliver those necessary essential components. (Ferraiuolo et al., 2011; Gall et al., 2020).

Characteristically, motor neurons employ microtubule-dependent kinesin and cytoplasmic dynein to either transport molecules anterogradely towards the NMJ or retrogradely towards the soma, respectively (Ferraiuolo et al., 2011). The function of axonal transport, maintenance of the axon structure, as well as electrical impulse conductance, intrinsically relies on the structure of the neurofilament (Yuan et al., 2017). Neurofilaments are stretch-resistant intermediate filaments, major constituents of cytoskeleton proteins, and are specific to neurons (Prokop, 2020). Neurofilaments form cross bridges with themselves as well as with actin filaments, actin rings and microtubules, that form a protein network crucial to the axonal structure (Dubey et al., 2020; Prokop, 2020). These microtubules together with motor protein complexes, like the dynein-dynactin complex, and kinesin are involved in the long- distance transportation of cellular cargo such as mRNAs, proteins, and organelles (Gall et al., 2020; Heisler et al., 2014; Tempes et al., 2020)

Due to the heavy involvement of protein complexes like dynein-dynactin and kinesin in axonal transport, alterations and mutations in their structure and function have led to impairment and axonal transport. Studies have shown that mutations in dynactin subunit 1 (DCTN1) affected the tertiary structure of the dynactin protein, disrupting its ability to bind to microtubules, which eventually led to ALS (Puls et al., 2003). Furthermore, interruptions in the dynein-dynactin complex have also evidently led to late onset motor pathology in ALS mice that recapitulate what is observed in late onset ALS patients (LaMonte et al., 2002). Finally, mutations in one of

the kinesin proteins, particularly the KIF5A isoform have also been shown to be associated with ALS (Nicolas et al., 2018).

Based on the evidences presented, it can be concluded that different mutations concomitant with ALS have the ability to directly alter the structure and functional dynamics of the cytoskeletons, impairing the axonal transport mechanism (Gall et al., 2020). This impairment furthermore contributes to mitochondrial dysfunction at distal sites (Pasinelli et al., 2004), reducing energy or ATP production, interfering with calcium homeostasis at the NMJ and consequently leading to distal axonopathy (Gall et al., 2020) observed in the SOD1^{G93A} mice (Fischer et al., 2004; Tallon et al., 2016) as well as in SOD1 patients (Boillée et al., 2006; De Vos et al., 2007).

1.1.5 Protein aggregates

Protein aggregates identified as ubiquitinated inclusions are a well-defined pathological hallmark of ALS (Ferraiuolo et al., 2011; Gall et al., 2020; Piao et al., 2003), present in TDP-43, FUS, and SOD1 ALS patients (Gill et al., 2019; Sreedharan et al., 2008; Vance et al., 2009). Among them, aggregates of TDP-43 positive proteins are most commonly seen, occurring in 98% of both sporadic and familial ALS cases (Feneberg et al., 2018). In 2008, mutations were discovered in TARDP, the gene that encodes TDP 43, in ALS patients (Sreedharan et al., 2008), consolidating the evidence for TDP 43 dysfunction in ALS etiology and also making it a vital key to our understanding of protein aggregation and its pathogenic effect in both sporadic and familial forms of ALS (Ferraiuolo et al., 2011).

In addition to TDP-43, other proteins, such as SOD1 and FUS inclusions were identified (Ferraiuolo et al., 2011) in ALS. SOD1 inclusions were mainly found in motoneurons of familial ALS patients, as well as in mouse models and cellular models, expressing SOD1 mutations

(Shibata et al., 1994). Cytoplasmic inclusions that contain mutant FUS proteins were identified in patients with FUS- associated familial ALS (Groen et al., 2010; Hewitt et al., 2010). Hyaline conglomerate inclusions, rich in accumulated neurofilaments, were also identified in the proximal dendrites of motoneurons, particularly in patients with SOD1 mutations (Ince et al., 1998). Discoveries of other genetic variants like the missense mutations in Valosin-containing protein (VCP) gene and mutations in Ubiquilin 2 (UBQLN2) (Deng et al., 2011; J. O. Johnson et al., 2010) have further strengthened the evidence that protein deregulation contributes to the pathophysiology of ALS.

Even though I am only summarizing a small portion of the current studies in ALS, it is quite clear such complicated cellular and molecular mechanisms underlying ALS disease makes it extremely difficult to develop any effective treatments.

1.2 Gender-related differences in Amyotrophic Lateral Sclerosis

There have been reported cases of gender differences in ALS disease progression and response to drug treatment. The prevalence and incidence rate of ALS in men versus women is approximately 1.5:1 to 2.0:1 respectively (Cervetto et al., 2013a). The authors also reported that in vehicle treated animals, female mice outperformed males on the rotarod and grip strength tests. They also reported that motor deficit onset was first observed in males prior to females (Cervetto et al., 2013a).

Studies have shown that there is a complex interplay between gender and the pathology and progression of ALS disease that result in differences in the manifested clinical phenotype of the disease (McCombe & Henderson, 2010; Palmieri et al., 2015). Though few possible reasons such as differences in biological response to exogenous toxins; differences in male and female

nervous system, that thereby results in the immune system's ability to handle this damage; and differences in exposure to environmental toxins exist, there are still no known definitive causes on why these differences are present. (McCombe & Henderson, 2010)

1.2.1 Effect of Gender on Incidence and Prevalence ratio in ALS

The worldwide incident rate of occurrence in ALS is estimated to affect 1.5 -2 per 100,000 persons per year with a prevalence approximated at 7 per 100,000 , and a 1.6:1 ratio existing for the occurrence of the disease in males versus females respectively (J. D. Mitchell & Borasio, 2007; Talbot, 2009). Incidence being understood to be the number of new cases per year and prevalence the number of people living with the disease. Studies have shown that the incidence and prevalence rate of ALS is higher in males than in females, and this is true for both fALS and sALS cases (Abhinav et al., 2007; A. Chio et al., 2009; Nalini et al., 2008; Vázquez et al., 2008; Werneck et al., 2007). Both incidence and prevalence ratios are age specific. Incidence ratio steadily increases with age until approximately 75 years of age before it declines, while that of prevalence consistently declines as the age of the individual increases (Beghi et al., 2007; Manjaly et al., 2010; Murphy et al., 2008; Nalini et al., 2008).

1.2.2 Effect of Gender on Survival

Due to strides made in research and advances gained in the studies of ALS, it is now understood that survival in ALS is in part dependent on factors such as rate of disease progression, clinical phenotypes, early onset of respiratory failure and importantly the nutritional status of the patient (Kiernan et al., 2011). Certain factors such as older age at symptom onset, bulbar onset of disease, and respiratory problems beginning earlier in disease progression are linked to a worse prognosis for survival; while limb-onset of disease, younger age at presentation and longer diagnostic delay allow for a prolonged survival in the patient (Talbot, 2009). In

regards to gender differences however, there appears to be no particular correlation or overall effect as an individual predictor of survival in ALS (McCombe & Henderson, 2010; Murphy et al., 2008; Sajjadi et al., 2010; Talman et al., 2009).

1.2.3   *Effect of Gender on Age at Disease Onset*

Age of onset is a common and rather important factor in determining progression and survival in ALS patients, as well as deterioration in activities of daily living and quality of life; with an older age decreasing survival time, as well as increasing functional decline in patients suffering from sporadic ALS (H. Watanabe et al., 2015). In an Indian cohort of ALS patients, mean age of ALS disease onset was 46 years old, and a mean age of death was 53 years old (Nalini et al., 2008). Age at disease onset being defined to be the age at which patients first notice or become aware of muscle weakness (Atsuta et al., 2009) or difficulty or impairment in other activities such as swallowing, speech, or respiration (H. Watanabe et al., 2015) . In several studies around the world, the mean age of onset is reported to be younger in males than it is in females (Atsuta et al., 2009; A. Chio et al., 2009; Fang et al., 2009; Vázquez et al., 2008). This is not unexpected considering the fact mentioned earlier that incidence ratios increase with age because they are age-specific, and that these incidence ratios are greater for males than it is for females. Therefore, when ALS patients are subdivided into groups based on gender, then the observation is that average age at onset is younger in males than in females.

1.2.4   *Effect of Gender on Site of Onset & Disease Phenotypes*

ALS has varying forms of clinical manifestations or phenotypes. These clinical presentations are based on two main factors which are the site of onset or weakness (Bulbar, upper limb or lower limb), and the type of weakness observed, that is whether it's affecting upper motor neurons or lower motor neurons (McCombe & Henderson, 2010). Based on any of

the above combinations, there are at least four major clinical presentations of ALS. According to Kiernan and colleagues (2011), they include: (1) Limb Onset ALS which is a combination of both Upper and Lower motor neurons; (2)Bulbar onset ALS which mainly present with swallowing and speech impairment and later on in its progression, features of limb atrophy; (3) Primary Lateral Sclerosis affecting purely upper motor neurons and which is a rarer form of ALS accounting for only about 1-3 % of ALS cases (Singer et al., 2005; Statland et al., 2015) ; and finally (4) Progressive Muscle Atrophy which only involves lower motor neurons (Gordon et al., 2006; Kiernan et al., 2011). In addition to the four major ones noted, there are other manifestations such as pseudobulbar palsy (Abrahams et al., 1997; Ganesalingam et al., 2009) flail leg and flail arm presentation (Talman et al., 2009).

Gender affects onsite of disease, as seen for instance in bulbar onset where there is a higher percentage of women than men that are affected (McCombe & Henderson, 2010). In an Italian ALS epidemiological study, 46% of women presented with bulbar onset while it was 31% for men (A. Chio et al., 2009) and in a similar study in India, it was 40% for women and 23 % for men (Nalini et al., 2008). The rest of the proportion of patients fell under limb onset. In another population study that focused on patients with bulbar onset phenotype, of the 49 bulbar onset case reported, women accounted for 63% of that number (Manjaly et al., 2010). However, in limb onset, the reverse is the case, as there are more reports of male incidence than there are females, like in a study in Ireland, where the proportion is 62% of men in comparison to 48% of women (Traynor et al., 1999). Another phenotypical variant of ALS, the flail-arm syndrome also shows a preponderance of males to female clinical manifestation, with males being three to nine times likely to have this than females (Czaplinski et al., 2004; M. T. M. Hu et al., 1998). Taken

together, these suggests that there might be a genetic link to gender predisposing one to a certain ALS phenotypical variant

1.3   Clinical signs and symptomology of ALS

1.3.1   *Neuromuscular Denervation & Motor neuron loss*

The Neuromuscular junction is ternary in nature consisting of three elements which include the presynaptic motor neuron, the post synaptic muscle and the synapse-associated glial cells- Terminal Schwann Cells (TSC) (Campanari et al., 2016).   It is a specialized cholinergic synapse that allows for the transmission of action potentials from the motoneurons to skeletal muscles, and a disruption to this function results in muscle weakness and or paralysis (Campanari et al., 2016). In ALS, Neuromuscular denervation precedes motor neuron loss, and as such clinical symptom or manifestation of the disease (Dadon-Nachum et al., 2011). This distal axonopathy is known as the "die-back" phenomenon, where retrograde degeneration of motoneurons, starts distally at the nerve terminals or at the neuromuscular junction and then continues towards the cell body also known as the soma (Campanari et al., 2019). Pathological denervation of NMJ in SOD1^{G93A} mice was observed as early as P47, followed by loss of motor axons in the ventral root between P47 and P80, then the loss of α- motor neuron somas in the lumbar spinal cord after P80, when clinical ALS like symptoms started (Fischer et al., 2004).

There are different kinds of muscle fiber types and several documented ways to categorize them, based on the abundance of the following factors: Myosin Heavy Chain (MHC), mitochondria, proteins, capillary density, fatigue resistance, strength and metabolic strategies (Schiaffino & Reggiani, 2011). Of all these, the most helpful way to define muscle fiber types are based especially on the MHC isoform profile; where which some muscle fibers contain a

single MHC isoform otherwise known as pure fiber types, and others have a combination of two or more MHC isoform-known as hybrid muscle types (Pette & Staron, 2000). When they are defined based on their myosin composition, there are four types of muscle fibers (Schiaffino & Reggiani, 2011).

This classification is the most current consensus in muscle fiber type research, and categorizes them as such: Slow Oxidative (Type 1) fibers, Fast-twitch oxidative glycolytic (Type 2A) fibers that are also fatigue resistant, Fast-twitch glycolytic fibers (Type 2B) that are fast fatigable (Peter et al., 1972); and finally an additional fast fiber type known as Type 2X which has a different myosin composition from 2A and 2B (Schiaffino et al., 1989). Motor units which consist of motor neurons and the muscle fibers they innervate are intrinsically divided into three major functional types; slow (S), fast fatigue-resistant (FR), and fast fatigable (FF) (Frey et al., 2000). Motor units with 2X muscle fiber types show fatigue resistance that is in-between that of 2A and 2B and their twitch type is comparable to those of 2A and 2B units (Larsson et al., 1991). In ALS, the fast-twitch glycolytic fibers otherwise known as Type 2B fast fatigable fibers, are the first fiber type to deteriorate. Degeneration occurs first in distal motor axons that innervate Type 2B fast fatigable fibers, while those fibers that are innervated by Slow Type 1 motor axons are last to experience deterioration and death (Tallon et al., 2016).

Muscle fibers are also innervated by different types (and sizes) of motor neurons, and as can be imagined, this would present with varying susceptibilities and vulnerability to SOD1 toxicity (Boillée et al., 2006). Due to the extensive work done in ALS research, it has long been shown that motoneurons that produce myelinated axons that are greater than 5μm in diameter are the most susceptible to loss or degeneration in ALS mice model (Bruijn et al., 1998) . In spite of the significant motor neuron loss, especially that of lower motor neurons in the ventral horn of

the spinal cord and brainstem; that is estimated to about 50% by the time of autopsy (Wijesekera & Leigh, 2009), some motor neurons, particularly those of the Onuf's nucleus in the sacral spinal cord and the cranial nerve oculomotor nuclei are spared degeneration and loss (Brockington et al., 2012)

1.3.2   *Non-cell autonomous pathology and the role of glia cells in ALS*

Amongst the multifactorial hallmark characteristics that define ALS pathology and its progression, the role and importance of glial activity or dysregulation cannot be overemphasized. Despite the deep involvement of neuromuscular junctions and motoneuron loss in ALS disease the role of neuroinflammation played both by microglia and astrocytes is of equal importance, as many studies have investigated the impact of these factors in novel treatment approaches and interventions for ALS (Geloso et al., 2017).

Using ALS animal models, studies have shown that ALS is a non-cell autonomous multifactorial disease, and that the well-being of motoneurons and interneurons is in part, heavily dependent on the role that glial cells play in the disease progression (T. Philips & Rothstein, 2014). Glial cells include Astrocytes, Microglia, NG2- oligodendroglial progenitor cells, Oligodendrocytes, Pericytes, and Ependymal glia (T. Philips & Rothstein, 2014). Of all these mentioned, microglia are the major macrophage-like cells in the Central nervous System (CNS). They release various anti and pro-inflammatory cytokines and chemokines under different conditions (Cherry et al., 2014; T. Philips & Rothstein, 2014). In very broadly loose terms, microglia can be defined in either a "resting" state or an "activated" state (Cherry et al., 2014). However, we do know that this overly simplistic way of defining microglia mode is not a true reflection of the complexities of microglia function. Two photon microscopy recordings done by Nimmerjahn and colleagues in 2005 showed that in a healthy CNS, microglia in the so-called

"resting" state is carrying out surveillance work, constantly scanning the CNS environment, like vigilant housekeepers for any potential danger in a strong effort to maintain homeostasis (Nimmerjahn et al., 2005).

M1 or "classically activated" and M2 or "alternatively activated" microglial phenotypes represent the continuum in which microglia activated state swings from pro-inflammatory to anti-inflammatory respectively, with the M1 state designed to phagocytize the hazards or threats, and the M2 phase to induce repair and restoration to a healthy system (T. Philips & Rothstein, 2014). Some examples of pro-inflammatory cytokines and factors include Interleukin 6 (IL-6), Tumor Necrosis Factor – alpha (TNF-α), Nitric Oxide Synthase (iNOS), Cyclooxygenase 2 (COX-2) etc., while the anti-inflammatory ones include interleukin 4 (IL-4), Interleukin (IL-10), Insulin growth factor 1 (IGF-1), Vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor, to mention a few (Mishra et al., 2017; T. Philips & Rothstein, 2014).

ALS studies in mutant SOD mice models have shown that glial cells can play both a pathogenic and a protective role in ALS progression (Puentes et al., 2016). Following onset and subsequent progression of ALS, microglia have been shown to release TNF-α, IL-6 and IL-1β, which are pro-inflammatory mediators; thus prompting a pathogenic response that contributes to neuronal dysfunction and cell death (Heneka et al., 2014). Yet, at the same time beneficial anti-inflammatory factors such as IL-4 and IL-10, as well as growth factors are secreted augmenting repair and protection, delaying disease progression and extending survival (Beers, Henkel, et al., 2011; Lewis et al., 2012) suppressing release of reactive oxygen species (ROS) while enhancing insulin-like growth factor secretion 1 (Weihua Zhao et al., 2006). Taken together, it is evident that microglia activation in ALS progression is a complex

neuroinflammatory response that can play the dual roles of pro-inflammation and anti-inflammation.

1.4   Drugs and Therapeutic approaches in Amyotrophic Lateral Sclerosis

Several decades of persistent research has gone into finding a cure for ALS, however despite the relentless efforts, there are no known cures. Clinical diagnosis for ALS is typically reached using the El Escorial criteria that comprises a combination of clinical assessment, electrophysiological tests, and neuropathologic examination (Jakobsson Larsson et al., 2016; Lu et al., 2012). The evidence of the disease must be proven in both upper and lower motoneurons, using a combination of earlier mentioned assessment tools, signs that there are progressive spread to other parts of the body, and in addition to these, a clear absence of neurologic, electrophysiologic and pathological proof of other diseases that could be deemed to be motor neuron degeneration observed in ALS patients (J. J. Chen, 2020).

A diagnosis of ALS is bleak due to its lethality and fact that there are currently no known cures. Although there are two approved FDA (Food and Drug Administration) drugs for treatment, both have very limited modest effects. Currently, many drugs and therapeutic approaches are investigated. Some show promises towards better treatments and maybe a cure for ALS. The ALS Ice bucket challenge, a social media fundraising campaign that went viral in 2014 was prodigious in that it raised $115 million dollars in a span of 8 weeks for ALS research and charities. This campaign brought much needed hope to a bleak situation, a global awareness of the disease, and fueled progress in its treatment (Kuehn, 2018). I will briefly expatiate on current treatments for ALS, milestones achieved as well as future prospective towards the race for finding more promising treatments for ALS.

1.4.1   Riluzole as a drug treatment for ALS

Riluzole (2-amino-6-trifuromethoxybenzothiazole), was the first and, for a long time,

only approved treatment for ALS (Gaber et al., 2016). A benzothiazole derivative, it is an anti-

excitotoxic drug with anti-convulsant (Sarro et al., 2000) and neuroprotective properties. The

exact mechanisms underlying Riluzole's cellular effects are not completely clear, but it has been

shown that Riluzole can inhibit glutamatergic neurotransmission, block the voltage-dependent

and persistent Na+ currents (Benoit & Escande, 1991; Bensimon et al., 2002; Lacomblez et al.,

1996; Urbani & Belluzzi, 2000; Vucic et al., 2013; Y.-J. Wang et al., 2008).

Despite what is known of Riluzole and its cellular activity, since its clinical availability in

the mid 1990's, much is left to be fully understood and elucidated on its mechanism of action

towards neuroprotection (Cheah et al., 2010). Riluzole is currently the only drug treatment that

has been clinically shown, based on statistics, to improve survival in ALS patients(R. G. Miller

et al., 2012). Taken at 100mg per day, it prolongs median survival in patients by about 2-3

months for approximately 50% of patients, or a 9% gain in survival probability of up to 1 year

(Kiernan et al., 2011; R. G. Miller et al., 2012). As with any drug , there are potential side effects,

and in the case of Riluzole it can cause nausea and lethargy, gastrointestinal problems, and

elevated liver enzyme levels (Jaiswal, 2019). But these effects- especially those of nausea and

lethargy are reversible on discontinuing the drug (R. G. Miller et al., 2012).

1.4.2   Edaravone as a drug treatment for ALS

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent anti-oxidant agent,

meaning it scavenges free Radical Oxygen Species (ROS) including peroxynitrites, and

purportedly eliminates lipid peroxides and radical hydroxyls (Jaiswal, 2019; Nowicka et al.,

2019; K. Watanabe et al., 2018). It was originally developed by a Japanese company and was

approved for use in treatment of acute cerebral infarction in 2001 (Sawada, 2017; K. Watanabe et al., 2018). In 2015, it was then approved for ALS treatment in Japan, and then in 2017 by the FDA in the United States of America (K. Watanabe et al., 2018); in Canada, this approval came in 2018 by Health Canada ("Access to Therapies," 2020). It is marketed under the trade name Radicava, and is the first treatment to have been approved for ALS in over 20 years (since Riluzole), therefore has gained a lot of public attention as it brings hope to ALS patients, their families and healthcare providers (Yeo & Simmons, 2018).

Since ROS and free radicals play a crucial role in the pathogenesis of certain diseases including ALS, by dramatically increasing in quantity, attacking cell membranes and promoting inflammation, then drugs that are able to scavenge these free radicals are desirable therapeutic approaches (K. Watanabe et al., 2018). Though Edaravone fits this bill as an excellent radical scavenger, by markedly reducing 3 -nitrotyrosine levels in the cerebrospinal fluid, which is the laboratory marker of oxidative stress, its exact mechanism of action is still not fully understood (M. T. Mitchell & Chamberlin, 2017).

Currently Edaravone is considered safe for use hence the approvals, though not yet approved for use in Europe (Yeo & Simmons, 2018). However, the cost of the drug is high while the benefits are considered to be modest (Yeo & Simmons, 2018). The initial phase III clinical trial of Edaravone for ALS patients showed that there was no significant difference in efficacy of Edaravone for ALS treatment between patients who were treated with Edaravone and those who received placebo specifically in regards to rate of decline score as measured by the ALS Functional Rating Scale- Revised (ALSFRS-R) (Abe et al., 2014). It was not until a *post-hoc* analysis was performed that another subgroup in the study was identified that had noticeably

better outcome, as it was detected that this subgroup had a slower rate of decline score in the ALSFRS-R.

This Efficacy-Expected Subpopulation of Definite or Probable diagnosed ALS patients, otherwise referred to as dpEESP2y scored a 2 or higher on all levels of the ALSFRS-R, had a vital force capacity equal to or greater than 80%, and only had symptoms for 2 years or under (THE EDARAVONE (MCI-186) ALS 16 STUDY GROUP, 2017). Following this, a randomized double-blind placebo-controlled clinical Phase III trial that selected for patients that met the above criteria was carried out and the results reported in 2017 by The Writing Group, & Edaravone (MCI-186) ALS 19 Study Group (Abe et al., 2017). They reported smaller decline in the ALSFRS-R, the ALS Assessment Questionnaire (ALSAQ-40), the Modified Norris Scale and a health-related Quality of Life (QOL) measure, in those treated with edaravone versus those that received a placebo treatment. However, there was no significant difference in Forced Vital Capacity (FVC), grip strength and pinch strength (Abe et al., 2017).

Despite the attention the announcement of edaravone for ALS treatment garnered, its result is modest at best, and there are lots of unclarity surrounding its effect. Some of the areas of concerns not fully understood or known include 1) Its effect on respiratory function and muscle strength, 2) its effect on survival, 3) are there any benefit beyond 6 months of use? 4) the benefits appear to be for only ALS patients who meet the criteria of the special sub-population as mentioned in the prior paragraph, 5) finally, the benefits for non-Asians are not fully understood as broad clinical trials inclusive of other ethnic diversities are lacking (Yeo & Simmons, 2018). This unclarity in effects are not fully understood by the public, as the layman may in error perceive that because a drug is approved for use by all, then it must be effective for all.

1.4.3   Emerging Therapies & Approaches in ALS

As the long and tedious research in understanding ALS, its pathology and hopeful cure ensues, several therapies have emerged as promising for the treatment of this disease. These therapies and approaches can be categorized into 7 distinct groups based mainly on the pathophysiological representations of the disease, and secondarily on whether they are pharmacological or non-pharmacological interventions (Nowicka et al., 2019). Those groups with pharmacological intervention include: 1) Anti-apoptotic, 2) Anti-inflammatory, 3) Anti-excitotoxicitory, 4) Anti-oxidant, 5) Anti-aggregation 6) Neurotropic growth factors & Neuroprotective approaches, and 7) Stem cell therapies which is non-pharmacological (Nowicka et al., 2019).

1.4.3.1 Anti-apoptotic therapies

Anti-apoptotic approaches for ALS therapy examines issues such as mitochondrial dysfunction of damaged motoneurons and abnormal calcium handling that leads to the start of the apoptotic cascades. Outcomes in this area of therapies steered large studies like that of Dexpramipexole; which in a randomized, double-blind, placebo-controlled phase III clinical trial, aimed to assess the effects of the drug on patients with both fALS and sALS that had been experiencing symptoms for 2 years or less (Cudkowicz et al., 2013). The results from this large study failed to prove an effectiveness of this drug in two different models of ALS; which were 1) High-copy B6-SJL-SOD1.sup.G93A /Gur1 mice, and 2) Wild-type human and mutant human TDP-43 transfected primary rat cortical neurons (Vieira et al., 2014). The results showed that in the SOD1.sup.G93A mice, there were no observable effects in slowing down motoneuron degeneration or in increasing survival, while in the transfected rat cortical neurons, undergoing a

dosage range of 5-50 µM only a small margin of improvement was identified in only one

indicator of neuronal survival, and this was at 10 µM of the drug (Vieira et al., 2014)

Other preliminary clinical work includes that which was done more recently on

Ursodeoxycholic acid (UDCA) and Tauroursodeoxycholic acid (TUDCA), which show a modest

positive outlook on their effectiveness and safety in treating ALS patients (Elia et al., 2016; Gj et

al., 2016). Both of these acids are potent inhibitors of apoptosis; which they do by interfering

with the upstream mitochondrial pathway of cell death, reducing endoplasmic reticulum stress,

preventing radical oxygen formation, and steadying the unfolded protein response (Vang et al.,

2014), all important factors in ameliorating ALS symptoms.

1.4.3.2 Anti-inflammatory therapies

This group of research work is focused on neuroinflammation activities that affect and

are affected by microglia, reactive astrocytes, infiltrating T-lymphocytes and microphages which

play vital roles in the neurodegenerative disease progression of ALS (Beers, Henkel, et al., 2011;

Chiu et al., 2008; Henkel et al., 2009; Puentes et al., 2016). Studies that have shown potential

treatment opportunities in this area include recombinant human erythropoietin (rhEPO), NP001

immune regulator, and Masitinib (Nowicka et al., 2019).

The rhEPO study was a a multicentre, randomized, double-blind placebo controlled

phase III study of 208 patients carried out in Italy, patients were aged 18-75, had a slow vital

capacity of equal or greater than 70%, and their onset of weakness was set at 18months or less

(Lauria et al., 2015). The results from this study showed that the dosage of 40,000 IU of rhEPO

every 2 weeks did not change the outcome on the rate or course of ALS progression (Lauria et al.,

2015).

That of NP001 immune regulator was a phase II randomized double-blind placebo-controlled trial in 136 ALS patients , that ran for 6 months and of which the inclusion criteria was that patients must have had the disease for less than 3 years and an FVC of less than or equal to 70% (G. Miller et al., 2015). In general, results for this study show a slowing of disease progression in the group of patients that had received a high dose of the drug, but yet showed no overall conclusive evidence on its effectiveness as a drug treatment for ALS (G. Miller et al., 2015).

The last one in this group, and probably with better potential than the rest is the study on Masitinib. It has undergone a randomized double-blind placebo-controlled phase 2/3 clinical trial, where the patients were given Masitinib together with Riluzole. The results are yet to be published, but preliminary analysis shows a slowing of disease progression by 27% in "normal progressor" ALS patients (ALSFRS-R < 1.1 drops per month), but not in "fast progressor" ALS patients (ALSFRS-R > 1.1 drops per month) (AB Science, 2018; Khairoalsindi & Abuzinadah, 2018)

1.4.3.3 Anti- Excitotoxicitory therapies

Excitotoxicity in ALS is mainly a product of excessive production and release of glutamate and also a combination of modifications in postsynaptic glutamate receptors and glutamate transport (Nowicka et al., 2019). Excitatory amino acid transporter (EAAT) with the main duty of glutamate clearance loses its receptors in ALS, and therefore a direct impact of this is elevated levels of glutamate in the Cerebrospinal fluid (CSF) (Rosenblum & Trotti, 2017). The main drug that targets excitotoxicity and has shown a modest degree of effectiveness and an increase in survival is Riluzole, and this drug has already been discussed in reasonable details. Another drug compound worthy of mention in this category is the FDA–approved beta-lactam

antibiotic. This drug significantly increases EAAT2 activity in rat brain, elevates EAAT2 promoter activity and protects motoneurons from excitotoxicity in human astrocyte cultures, and delays disease progression while subsequently prolonging survival in ALS mice (Jeffrey D. Rothstein et al., 2005; Lipski et al., 2007; Melzer et al., 2008; Thöne-Reineke et al., 2008). Notwithstanding, regardless of the promising results shown in animal and in-vitro models, it failed to translate into clinical trials involving individuals afflicted with ALS (Cudkowicz et al., 2014).

1.4.3.4 Anti-Oxidant therapies

Oxidative stress plays a prominent role in the pathogenesis of ALS and appears to be a common denominator in multiple risk factors that make one susceptible to the disease (Niedzielska et al., 2016). As mentioned earlier in one of the previous sections, Edaravone targets this pathophysiological pathway. Apart from Edaravone, another drug molecule which has shown promising results in this area is the drug called AEOL 10150, a small molecule that catalytically consumes ROS and reactive nitrogen species (Nowicka et al., 2019). Although the drug has limited data on human studies of its effect on ALS, a meta-data analysis of pre-clinical trials done on the SOD1^{G93A} model shows that this molecule may be the most hopeful compound for assessment of treatment in a clinical trial (Benatar, 2007).

1.4.3.5 Anti- Aggregation therapies

Misfolded cellular proteins aggregation is a hallmark of ALS pathogenesis, and of the over 200 mutations present in ALS, roughly 90% of them are missense and encode to varying degrees, damaged SOD1 variants (Keskin et al., 2019; Nowicka et al., 2019). The other protein that experiences a similar fate in ALS is the TAR-DNA binding protein TDP-43 that can equally form aggregates in motoneurons (Benkler et al., 2018; Nowicka et al., 2019). Potential

candidates for the treatment of ALS, targeting aggregates includes the macrophage migration inhibiting factor (MIF) for SOD 1 amyleoid aggregates, and the acridine derivative, 4,5-bis{(N-carboxy methyl imidazolium)methyl}acridine] dibromide that targets TDP-43 aggregates (Nowicka et al., 2019). MIF in *in vitro* experiments, has been shown to modify the usual SOD1 amyloid aggregation pathway, and stimulating the formation of disordered aggregates (Shvil et al., 2018). While the acridine derivative on the other hand, works by disbanding the connected low-complexity domains of consecutive TDP-43 monomers, hence interrupting the establishment of toxic pathological aggregates (Afroz et al., 2017).

1.4.3.6 Neurotropic growth factors & Neuroprotective approaches

Since degeneration of neuronal tissue is central to ALS pathogenesis, some research investigate how damaged neurons can be stimulated for repair or better yet, how to generate new ones. Neurotrophic and neuroprotective approaches investigate drugs that can do this instead of interfering with the pathological activity path (Nowicka et al., 2019). Substances such as the flavonoid 7,8-Dihydroxyflavone which is a tyrosine kinase receptor B agonist which imitates the effect of Brain Derived Neurotrophic Factor (BDNF), have been shown to improve survival of lower motor neurons, improve motor deficits and preserve dendritic spines and spinal motor neuron in ALS mice models (Korkmaz et al., 2014). Another study by Nagahara and colleagues (2017), showed that glycoprotein non-metastatic melanoma protein B (GPNMB) is also effective in being a potential neuroprotective treatment. They showed that recombinant GPNMB was able to alleviate motor neuron cell degeneration and death that were caused by the presence of transfected mutant TDP-43 plasmids (Nagahara et al., 2017).

1.4.3.7 Cell based Therapies

Stem cells are cells that have multi- or pluripotent features with renewal or regenerative capacities (Forostyak & Sykova, 2017). Pluripotency can be defined as the ability of a cell to differentiate into any mature cell type in the organism deriving from all three germ layers - ectoderm, endoderm and mesoderm (Lee et al., 2007; Nistor et al., 2005). Cell based therapies are a viable treatment for several neurodegenerative diseases including Parkinsonism, Huntington's disease, ALS, Alzheimers etc, (Y. Chen et al., 2014; de Munter et al., 2013; Forostyak & Sykova, 2017; Hsu et al., 2013), and they have been investigated and proposed as an eventual source for motor neuron regeneration (Kim et al., 2014). There are two main categories of study under stem cell research for ALS. They include Glial- restricted Precursors (GRP) and Neural Progenitor Stem Cells (NSC)

Given the multifactorial nature of ALS disease and progression, it has made fully understanding its mechanism a daunting task. However our current knowledge on ALS, shows that neuroinflammation underlies motor neuron damage; of which astrocytes plays a key function in this neuroinflammatory reaction, and as such makes them a possible target for the treatment of ALS (Kruminis-Kaszkiel et al., 2014). Using various stem cell therapies to restore or replace damaged motor neurons can be a difficult and challenging task. Therefore, turning our focus instead towards utilizing enriched healthy donor-derived astrocytes may be more beneficial and a likely better course of treatment action (Kruminis-Kaszkiel et al., 2014). Glial- restricted Precursors (GRP) that are derived from astrocytes are lineage restricted precursors or progenitors and have been shown to have the ability to differentiate into astrocytes after transplantation. To this end, studies on transplantation of astrocytes and GRP-derived astrocytes have been shown to stimulate the neural regeneration process and that the therapeutic benefit is probably due to the

astrocyte-derived function (Angelo C Lepore et al., 2008; Lepore et al., 2011; Nowicka et al., 2019)

Neural stem cells (NSC) are self -renewing multi-potent progenitor cells that are located both in the developing and mature CNS. Characterized by their ability and capacity not only for self-renewal but also to differentiate into neurons, astrocytes and oligodendrocytes through asymmetrical fate-committed division (Reekmans et al., 2012). Due to these features, they are identifiable as a potential therapy target for ALS disease (Nowicka et al., 2019). Studies using animal models have shown that following motor neuron degeneration, endogenous NSCs resident in the CNS are triggered to multiply, migrate, and stimulate neurogenesis in the spinal cord, the body's natural reaction in an attempt to defend itself (Nowicka et al., 2019). However, this reaction does not provide enough defense in the face of debilitating neurodegenerative diseases like ALS (Gincberg et al., 2012). Regardless, NSCs are able to support motor neurons that were earlier damaged during the course of ALS progression through its neurotrophic and anti-inflammatory properties (Mazzini et al., 2016). Stem cells also produce immunomodulatory molecules that lead to the process of neurogenesis and angiogenesis through their ability to regulate cell migration, growth and differentiation (Mazzini et al., 2016). Given all that has been stated of NSCs, their characteristics and work in the CNS, there is much hope that they will lead to beneficial and positive results in the movement towards better therapy for ALS.

1.4.3.8 Gene Therapy Strategy

As the search for better treatment for ALS continues, new alternative therapy continues to emerge. One of such therapies is gene therapy which includes utilizing antisense oligonucleotides (ASOs) and RNA interference approaches. In a study by Smith and his colleagues, they showed that by continuous intraventricular infusions of modified ASO, in rodent

models of ALS they were able to reduce both SOD1 protein and mRNA levels throughout the brain and spinal cord, and this significantly delayed disease progression, while extending survival in this rodent model (Smith et al., 2006). Another helpful approach has been through the use of viral vector AAV serotype 9 (AAVs9) to deliver glial cell line derived neurotrophic factor (GDNF) intravenously to adult motor neurons (Sandra Duque et al., 2009). By targeted delivery via systemic injections of AAV9-SOD1-shRNA, Thomsen and colleagues showed a significant delay of disease onset, expansion of lifespan, enhanced survival of spinal motor neurons, and maintenance of NMJs in rodent models of ALS (Thomsen et al., 2014).

In a more recent study by Martier and colleagues (2019), they tried to reduce the gain of toxicity function caused by hexanucleotide repeat expansion as observed in the more occurring (in comparison to SOD 1) C9orf72 mutation of ALS. To accomplish this task, they used AAV (adeno-associated virus) 5-miC to reduce the repeat containing transcripts; (miC is artificial engineered anti-C9orf72-targeting miRNAs). Post-mortem analysis following their experiments of the ALS mice treated with this method, showed a reduction in nuclear RNA foci, and this is important as the accumulation of RNA foci in the cytoplasm and nucleus of affected cells leads to a gain of toxic function of those cells (Martier et al., 2019). Scientists are in a constant pursuit and search for better treatment options for people suffering from neurodegenerative diseases like ALS, and gene therapy poses a promising avenue to reach this goal. It can be used to replace mutated genes with the normal copies of the proper or healthy genes; can be used to target RNA of the disease causative gene, thereby reducing the expression of that gene (gene- knockout); can be used for the purpose of gene editing by its ability to modify the genome, and finally, gene therapy is of pertinent importance in its ability to introduce a beneficial or protective factor

through the technique of gene addition in very complex and or infectious diseases (Cappella et al., 2019; D. Wang & Gao, 2014)

1.5   *dl*-NBP and *dl*-PHPB in Neurodegenerative diseases

dl-PHPB 2-(1-hydroxypentyl)-benzoate is a derivative of *dl*-NBP and has shown beneficial and positive results as a treatment for certain neurodegenerative disease. In the subsequent subheadings, both drugs, their mechanism of action and benefits will be succinctly explained.

1.5.1   dl-NBP and Neurodegenerative diseases

dl-NBP (dl-3-n-butylphalide) is a synthesized racemic form of L-3-n-Butylphalide (l-NBP), a compound that was isolated from Apium graveolens Linn (Chinese Celery) in the 1980's by the Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China (Xiaoliang, 2012). *dl*-NBP has similar pharmacological effects as l-NBP, and has been shown to possess neuroprotective benefits in animal models of cerebral ischemia, prevent blood platelets aggregations, protect mitochondrial structure and function during neuronal apoptosis cascade, improve brain microcirculation, inhibit oxidative damage, and modulate the inflammatory response (Dong & Feng, 2002; Xiaoliang, 2012; Xu et al., 2012; Yang et al., 2015).

In addition to this, *dl*-NBP was shown to assuage the symptoms of vascular dementia in rats brought upon by chronic cerebral hypoperfusion. These animals performed better on tests of spatial memory and learning, and showed lessened cerebral pathology including less neuronal damage, glial activation and white matter density diminution (Peng et al., 2007). In a more recent study by Sun and colleagues (2017), *dl*-NBP was shown to promote neuroplasticity and motor function in rats with cerebral ischemia. They investigated the role of this drug compound on

axiogenesis, neurogenesis and behavioral performance in rats following cerebral ischemia, and found that *dl*-NBP promoted behavioral performance in rats, and significantly increased axonal growth and neurogenesis (Sun et al., 2017).

In Alzheimer's disease (AD), formation of neuritic and senile plaques, as a result of accumulation of amyloid beta (Aβ), is pertinent to the hallmark of the disease, and this ultimately leads to oxidative stress, mitochondrial dysfunction, inflammatory cascade responses and eventually neuronal cell death (Bhatt et al., 2017). Zhang and colleagues in 2016 showed that l-NBP; which *dl*-NBP is derived from, showed promising beneficial results in ameliorating symptoms of AD in transgenic mice models of AD by reducing Aβ induced damage in the synapse, and enriching synaptic plasticity; which inherently leads to a modulation of cognitive function (Yu Zhang et al., 2016). *dl*-NBP was approved in China in 2005, as a drug for the treatment of acute cerebral ischemia (Xiaoliang, 2012). It is hydrophobic in nature, making it difficult to be used intravenously as a mode of administration; thereby subjecting it to oral administration only. This limiting property of the drug would prove to be a challenging difficulty in administering the drug to patients who have suffered a more severe stroke and have had their swallowing abilities impaired; therefore the need for the synthesis of a hydrophilic drug – *dl*-PHPB was essential (Xiaoliang, 2012).

1.5.2   dl-PHPB and Neurodegenerative diseases

dl-PHPB 2-(1-hydroxypentyl)-benzoate as earlier noted is a derivative of *dl*-NBP. It is a new drug candidate for the treatment of cerebral ischemia, and was approved in 2009 by the State Food and Drug Administration (SFDA) of China for a clinical phase I trial as an anti-stroke and neuroprotective drug (Y. Hu et al., 2012). In 2013 the phase I trials were concluded and it was then approved for clinical phase II and III trials (J. Li et al., 2017; P. Li et al., 2014). In 2006,

Zhang and colleagues showed that *dl*-PHPB was able to reduce infarct volume and improve neurobehavioral deficits in the cerebral ischemic rat model (Yi Zhang et al., 2006). In another study of *dl*-PHPB neuroprotective effects on neuroblastoma cells, it was shown to attenuate hydrogen peroxide induced cell apoptosis (Y. Hu et al., 2012).

dl-PHPB is hydrophilic in nature, thereby making it easily administered orally or intravenously (Y. Hu et al., 2012; Xiaoliang, 2012). It also converts easily, rapidly, and completely *in vitro* or *in vivo* to *dl*-NBP (J. Li et al., 2018; Yi Zhang et al., 2004, 2006). The 2004 study by Yi Zhang and colleagues showed that *dl*-PHPB administered both orally and intravenously led to an inhibition of thrombus formation in a rat Arteriovenous (A-V) Shunt thrombosis model, and as such it has potential to be an anti-platelet and anti-thrombotic treatment agent for cerebral ischemia (Yi Zhang et al., 2004). In a 2013 study, Zhao et al investigated the effects of *dl*-PHPB on the cognitive deficits brought on by chronic cerebral hypoperfusion in rats, and found that administering *dl*-PHPB significantly improved spatial learning and memory, and in addition to this, the drug reduced SOD activity, lipid peroxide and astrocyte activation in the cortex of these rats (Wanhong Zhao et al., 2013). Li and colleagues in 2014 showed that chronic administration of *dl*-PHPB in transgenic mice models of AD reversed memory deficits, improved learning and memory and enhanced long term potentiation possibly via an increase in p-GluN2B expression in the hippocampus (P. Li et al., 2014). GluN2B subunits containing N-Methyl-d-aspartate receptors (NMDARs) are important for synapse plasticity (Shipton & Paulsen, 2014), and are associated with neurodegenerative process observed in patients with AD, Huntington's and Parkinson's disease (Parsons & Raymond, 2014). Therefore modulation of NMDARs containing GluN2B subunits are potentially promising targets for treatment of these neurodegenerative diseases (Schreiber et al., 2019). Peng and

colleagues also show that *dl-* PHPB improved memory deficits and reduced amyloid and tau

pathologies in amyloid precursor protein (APP) and presenilin 1 (PS1) double-transgenic AD

mouse models (APP/PS1) (Peng et al., 2014)

Pharmacokinetic bioavailability of the drug following oral administration in rats and dogs

show that *dl*-PHPB is 50 to 100% higher than *dl*-NBP (Xiaoliang, 2012). There were no side

effects or toxicity observed in rat models of cerebral ischemia when compared to *dl*-NBP

administration (Xiaoliang, 2012), and similar findings were observed in a study with beagle dogs

(J. Li et al., 2017). Taken together, *dl*-PHPB appears to have not only neuroprotective effects,

but also anti-apoptotic and anti-oxidative benefits in the different studies that have been carried

out, including what was observed in a neurodegenerative disease like AD. This makes it an ideal

candidate to investigate its effect on a multifactorial, fatal neurodegenerative disease such as

ALS.

1.6   Hypothesis

Based on the aforementioned studies, and many more, highlighting the benefits of *dl-*

PHPB, my hypothesis is that *dl*-PHPB will have similar comparable effects on the SOD1^{G93A}

ALS mice model used in this work by attenuating neurodegeneration, reducing inflammatory

response, rescuing motor neuron loss and improving motor behavior.

There is already evidence that neuronal dysfunction precedes the clinical symptom

manifestation of the disease (Dadon-Nachum et al., 2011; Frey et al., 2000). Of the neuronal

dysfunction that occurs, neuromuscular junction (NMJ) or synapse loss are one of the earliest

symptoms that occur, which then leads to axon retraction- denervation of the muscle fibers and

eventually motor neuron death (Frey et al., 2000). Therefore, my objectives to test the hypothesis for this present study are to:

1.) Determine if NMJ loss can be attenuated and or rescued in the SOD1^{G93A} mice following treatment with *dl*-PHPB,

2.) Ascertain if Motor Neuron (MN) loss can be diminished in the spinal cord of the SOD1^{G93A} mice, thus improving motor coordination and balance in the SOD1^{G93A} mice, and finally

3.) Determine if inflammation response can be reduced by observing the expression levels of microglia in the treated SOD1^{G93A} mice.

If these objectives are achieved, then a pivotal groundwork would have been established for a better innovative treatment for ALS sufferers, thereby providing a better quality of mobility and life. This is the crux of my research with the aim and intention to shed more light on ALS disease and treatment knowledge.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Experiments were all carried out in accordance with the Canadian Council on Animal Care guidelines and were approved by the Dalhousie University Committee on Laboratory Animals. Adult B6.Cg -Tg(SOD1*G93A) mice, (Stock # 004435, Jackson Laboratories, Bar Harbor, ME) of both sexes and their wildtype litter mates were used in the experiments. According to Jackson Lab, SOD1^{G93A} (Stock # 004435), mice have approximately 16-31 copies of human SOD1^{G93A} mutated gene. After many generations of in-house breeding, however, we noticed that the symptoms of our SOD1^{G93A} mice were getting milder. After Quantitative Polymerase Chain Reaction (qPCR) testing, we discovered that our in-house mice had only 9-12 copies of the human transgenes. We then referred to these mice as low copy number SOD1^{G93A} (LCN SOD1^{G93A}) mice. In addition to this, there were a group of mice which were already sacrificed, and their tissues harvested for experiments, before we noticed the change in the symptoms of the mice. This group of mice we referred to as unknown copy number SOD1^{G93A} (UCN SOD1^{G93A}) mice. We then purchased again Adult B6.Cg -Tg(SOD1*G93A) mice (stock # 004435, Jackson Laboratories, Bar Harbor, ME) and re-established the original SOD1^{G93A} mouse line, which we refer to as high copy number SOD1^{G93A} (HCN SOD1^{G93A}) mice. All the breeders were then checked for their copy number range using qPCR.

All mice were housed in the Life Science Research Institute Animal Care Facility located here at Dalhousie University. They were placed on a 12- hour light/dark cycle (light from 07:00-19:00, dark from 19:00-7:00+1day) with *ad libitum* access to standard laboratory chow and water.

2.2   *In-vivo* procedures

2.2.1   *Weighing of animals*

All animals were weighed every weekday to document their progress and or the progress of the disease. Weights were recorded in the gram measuring unit, using a standard digital mouse scale. Typically, weight was recorded before the animals were given the drug via oral gavage. The animals were determined as terminal and euthanized if their weight decreased more than 20% of their initial weight at start of experiment.

2.2.2   *Oral gavage*

The drug *dl*-PHPB 2-(1-hydroxypentyl)-benzoate (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China), was prepared in water in three different dosages in correspondence to their weight: 30mg/kg, 60mg/kg and 100mg/kg. All SOD1^{G93A} animals were randomly placed to either the *dl*-PHPB treatment group or placebo (water) group. The *dl*-PHPB treated LCN SOD1^{G93A} animals' group were then randomly selected and placed in either the 60mg/kg and 100mg/kg dosage groups. Those that fell under the group UCN SOD1^{G93A} received 30mg/kg dosage only. Finally, the HCN SOD1^{G93A} animals only received the 60mg/kg, as this dosage was found to be the most cost effective and efficient dosage following the LCN SOD1^{G93A} experiments. All Wild type (WT) animals received only the placebo treatment of water. Animals were oral gavaged every weekday for a pre-determined period. LCN mice category were gavaged for 16 weeks, HCN for 9 weeks and UCN for 14 weeks.

Enteric administration of the drug or placebo is done by the oral gavage method, where a lubricated stainless-steel bulb-tipped gavage needle, attached to a syringe is used to administer the precise amount of drug directly into the stomach of the mouse. For this method, proper training and handling of the mice is necessary to ensure minimal stress as possible to the animal

when administering the drug or placebo. In this method, the animal is restrained by grasping the loose skin of the neck and back of the mouse, enough to immobilize the head and body of the animal but not to cause discomfort or stress to the animal, which are typically indicated by body movements or audible vocalizations from the animal. The lubricated needle is then gently passed through the esophagus into the stomach. In the case where visible stress or discomfort is detected, then the needle position is adjusted; as this may indicate that the needle is in the wrong path; that is, entering the trachea towards the lungs instead of esophagus towards the stomach.

2.2.3   *Phenotypic Neurological Scoring (Rubric scoring)*

The Rubric scoring system for the $SOD1^{G93A}$ mice is designed to quickly assess the neuromuscular junction function of the mice as ALS disease progresses. By using this method to observe the hindlimb splay and general gait pattern, one can estimate how far along the disease has progressed, and when the mice have finally reached endpoint. In this method, all mice were suspended by their tail for a few seconds to observe their degree of splay. This scoring system was done every weekday for the length of the project. The subsequent paragraphs detail the Rubric scoring scale used. The scoring method used is extracted from work done by Hatzipetros and colleagues (Hatzipetros et al., 2015) and is summarized below

0 = Full extension of hind legs away from lateral midline when mouse is suspended by its tail, and mouse can hold this for two seconds. No evidence of disease.

1= Collapse of leg extension towards lateral midline (weakness) during tail suspension.

2= Change in gait e.g. Weakness in one hindlimb or lowering of hinder parts during locomotion, or visible tremors

3 = Extreme weakness in both hindlimbs, usually toes curl under at least twice during walking of 12 inches, or any part of foot is dragging along cage bottom/table.

4 = Functional paralysis in both hindlimbs and cannot right themselves to sternum within 20secs after being placed on their sides, that is absence of righting reflex (end point)

2.3   Behavioral Tests

2.3.1   Rotarod test

The rotarod test is the conventional scientific method for measuring and assessing motor coordination, balance, and strength in rodents. The mice are placed on a horizontal rod that rotates around its axis, demarcated into sections by an opaque piece of plastic, so that up to five mice can be placed at a time and their performance observed and recorded, without them being distracted by their litter mate. The Rotarod machine for mouse (Ugo Basile, Gemonio, VA, Italy) can be programmed at a set speed or at a gradual accelerating speed over a defined time limit. Animals belonging to LCN and HCN SOD1^{G93A} groups were trained on the machine for three days before the experiments began. For the training, the animals were allowed to run freely at a ramped speed from 10 to 40RPM for 300 seconds, 3 times a day, with 10 minutes intervals for breaks. That of UCN SOD1^{G93A} were trained at a constant speed of 20 RPM for 180 seconds, 3 times a day.

For the experiments itself, in the LCN and HCN SOD1^{G93A} groups, the rotarod machine was set at an accelerating speed from 10 rotations per minute (RPM) to 40 RPM over a 300 second time frame, and the latency to fall of the mouse was recorded. If mice fell before the 300 second time limit, they were given a minimum of 10-minute rest period before they were placed back on the rotarod and the experiment repeated. Each mouse would have up to 3 chances in any given experiment but ended at any trial when they could reach 300 second time limit without falling off. That of the UCN SOD1^{G93A} group were different, as they ran at a constant speed of

20RPM for a maximum time of 180 seconds. They were however subjected to three chances if needed, and their latency to fall was also recorded. All mice were subject to this experiment once a week, and their performance recorded.

2.3.2 Hanging Grid experiment

The hanging grid test was used to assess the limb strength of the mice. Fundamental to this, is the basic evaluation of neuromuscular weakening or decline and consequentially, motor ability and function. In this experiment, mice were placed on a 1 centimeter by 1centimeter metallic grid, and then the grid was turned upside down, so that the mice were suspended from the grid atop a plastic bin, 60 centimeters from a soft bedding to cushion or break their fall and prevent injury. Each mouse was given up to 300 seconds to hang, and a maximum of 3 trials if they fell before the time limit of 300 seconds. The height of 60 centimeters from the metallic wire grid to the bottom of the bin was necessary to deter the mice from simply falling willingly and therefore negatively impacting the observed results. All mice were subjected to this experiment once a week, and their performance recorded.

2.4   Molecular & Cellular Tests

2.4.1 Harvesting Tissues

Tissues used for this project were Plantaris (PLA) and Soleus (SOL) muscles for the analysis of Neuromuscular Junction (NMJ) and the Spinal cord for the analysis of both motoneurons and microglia. To harvest the tissue, mice were anesthetized with a mixture of Ketamine and Xylene and then transcardially perfused with cold phosphate buffer saline (PBS, 25-30ml) solution, which was then immediately followed by cold 4% paraformaldehyde (PFA, 15-20ml). The spinal cords were harvested first, then followed by the muscles.

2.4.1.1 Spinal cord Harvesting

To briefly explain, the internal organs were removed after perfusion, and the head decapitated to allow for easier access to harvest the spinal cord. Using a pair of forceps and surgical scissors, the vertebrae was cut open longitudinally, being careful to not damage the spinal cord. The spinal cord was then harvested, by carefully snipping off the nerve rootlets. The harvested spinal cord was then pinned to a sylgard plate containing cold 4% PFA and allowed to fix for 2.5 hours. This short fixation time is necessary, so the anti-body staining is optimized, and images of microglia and motoneurons can be clearly seen. Fixation time longer than 3 hours will result in poorer quality images in addition to background staining of non-target cells. Once fixation time has elapsed, the spinal cord was rinsed in 1X PBS 3 times at 30 minutes intervals. Following these rinses, the spinal cord was transferred into a falcon tube filled with cold 1X PBS and left overnight in 4ºC fridge to remove all traces of PFA completely. The next day, it was transferred into a falcon tube containing 30% sucrose solution to cryo-protect the spinal cord and left in the fridge in the solution for 2-4 days. After the cryoprotection period had elapsed, spinal cord was removed from 30% sucrose solution, and cut into half at the T13 region. By doing this, the spinal cord is divided into two, where one part consists of the cervical and thoracic region, and another consists of the lumbar and sacral region. Cords were then frozen in individual small molds filled with Optimum Cutting Temperature compound (Tissue-Tek, Sakura Finetek, USA), and utilizing 100% ethanol and dry ice, wrapped in parafilm and then tinfoil, labels affixed, and then transported to -80 ºC freezer for storage until they are ready to be sectioned.

2.4.1.2 Muscle tissue Harvesting

To evaluate and analyze NMJ terminals, Plantaris (PLA) and Soleus (SOL) muscles were examined for this project. Mouse hindlimbs muscles were exposed by creating a small skin-deep

incision near the ankle of the mouse and then carefully peeling off the skin until reaching the hip area. The identified Soleus and Plantaris muscles were removed from the mouse, by snipping the tendons on both ends of the muscles and transferred to a PBS-filled Eppendorf tube, until all muscles needed were harvested. Then the muscles were straightened and pinned to a thin flat piece of cork and immersed in 4%PFA, post-fixed for 15 mins at room temperature in 4%PFA on a Microplate Shaker (VWR International, Radnor, PA), followed by 3 PBS rinses at intervals of 5 minutes each. Following this, muscles were permeabilized (for better staining) in cold Methanol for 5 minutes, and then rinsed again once in PBS for 5 minutes, before the muscle fibers were ready to be teased apart. With the muscles pinned by the tendons to a sylgard plate, using a No. 5 forceps, the muscle fibers were gently and carefully teased apart, taking care not to rip them, and fanned out like the bristles of a paint brush; then they were transferred into a 4 well plate for immunohistochemical staining. Special care was taken not to damage the fibers during the whole process.

2.4.2   *Immunohistochemistry for Neuromuscular Junction Innervation*

To visualize the NMJ structures, antibody Purified anti-Tubulin beta3 (also known as b3Tuj1) (BioLegend, San Diego, CA) was used to label motor neuron axons (1:500 dilution with 10% Goat serum/ 0.3%PBS-T), and those against-Synaptophysin (Invitrogen, Frederick, MD) for the synaptic vesicles located throughout the pre-synaptic terminals (1:500 dilution with 10% Goat serum/ 0.3%PBS-T). Primary antibodies were incubated overnight at room temperature on a micro plate shaker.

The following day, primary antibodies were vacuumed out of the wells, and the muscles were rinsed with PBS 3 times at 5-minute intervals each. Then they were ready for secondary antibody staining as well as the staining for the nicotinic acetylcholine receptors on the muscles.

They were incubated with goat anti-mouse 488 (1:1500 dilution in 0.3%PBS-T) (A11029, Invitrogen, MD) to visualize motor axons, and goat anti-rabbit 647 (1:750 dilution in 0.3%PBS-T) (Jackson Immuno Research lab, West Grove, PA) to visualize the pre-synaptic terminals. To label the motor endplates which bind to nicotinic acetylcholine muscle receptors, tetramethyl rhodamine-conjugated α-bungarotoxin (T1175, Life Technologies, CA) was used (1:500 dilution in 0.3%PBS-T). The incubation for this part of the procedure is done in the dark for a period of 1-2 hours max; then followed by three PBS rinses at 5 minutes intervals. At this conjecture, muscles were then transferred to a slide, the attached tendon is snipped off, covered moderately in few drops of fluorescent mounting medium (Dako Inc.,Carpinteria, CA) to aid better fluorescent images and prevent muscle drying from air exposure. The muscle fibers were arranged neatly in rows of 5-10 muscles fibers per portion of the slide, covered with a cover slip, transferred to a slide holder and then stored in a -20° freezer until they were ready to be viewed and imaged.

2.4.3 Analysis and Quantification of Neuromuscular Junction innervation

To analyze the NMJ innervation patterns, co-localization of pre-synaptic terminals and muscle fiber motor endplates were assessed. They were visualized and manually counted using a Leica DM LFS fluorescent microscope. The classification of NMJ innervation were based on the assessed extent of which pre-synaptic terminals (Synaptophysin, 647, far-red) was co-localized with the motor end plates (α-bungarotoxin, rhodamine-conjugated). This assessment resulted in 3 categories of NMJ innervation patterns.

They were considered fully innervated when the pre-synaptic terminals (Synaptophysin, 647, far-red) was completely co-localized with the motor end plates (α-bungarotoxin, rhodamine-conjugated); partially innervated if the pre-synaptic terminals labelling

(Synaptophysin, 647, far-red) was not completely co-localized with motor end plates (α-bungarotoxin, rhodamine-conjugated); and finally denervated when only the motor end plates (α-bungarotoxin, rhodamine-conjugated) labeling were observed with the complete absence of pre-synaptic terminals (Synaptophysin, 647, far-red) labelling. NMJs for each category was manually counted (to a minimum total of at least 250 NMJs per muscle) and recorded for each mouse. This experiment was done on both Plantaris and Soleus muscles of both *dl*-PHPB treated and water treated SOD1^{G93A} mutant mice. The experimenter was blinded for the entire process to prevent experimenter-bias.

2.4.4   Immunohistochemistry for Motor neurons & Microglia

2.4.4.1 Sectioning and Staining the spinal cords

Using a cryostat, spinal cord tissue was cross sectioned at a thickness of 30μm and transferred to slides. After sectioning, slides were allowed to dry for 2 hours at room temperature, or overnight in 4ºC fridge. Once dried, slides were transferred to a -20ºC freezer for storage until they are ready to be stained. Once they are ready to be stained, they are then air-dried for 45mins to 1 hour before staining procedure can begin.

Using an *ImmEdge* Hydrophobic barrier pen (Vector Laboratories, San Francisco), a barrier is created around the edge of the slides preventing the antibody solutions from spilling out when applied to the slides. Slides were rinsed 3 times at 5 minutes intervals with 0.003%PBS-T. To label and identify motoneurons, polyclonal goat anti- Choline acetyltransferase (also known as ChAT) antibody (AB144P, Millipore, MA) (1:100 dilution with 2% Horse serum/ 0.003%PBS-T) was used. To label and identify microglia, monoclonal rabbit anti- IBA1 antibody (1:1000 dilution with 2% Horse serum/ 0.003% PBS-T) (AB178847, Abcam,

Cambridge, UK) was used. They are left to incubate for 12 hours at room temperature in a dark covered slide box container.

After the primary antibodies' incubation time had elapsed, the slides were rinsed 3 times at 5 minutes interval with 0.003% PBS-T, and then incubated in secondary antibodies for 1 hour at room temperature. Donkey anti-goat (A11055, Invitrogen, MD) (Alexa 488; 1:500 dilution with 2% Horse serum/ 0.003% PBS-T) to visualize motoneurons and donkey anti-rabbit (Jackson Immuno Research lab, West Grove, PA) (Alexa 594; 1:250 dilution with 2% Horse serum/ 0.003% PBS-T) to visualize microglia. Following the end of incubation time, they were rinsed with 0.003% PBS-T 3 times at 5 minutes intervals. 2-3 drops of fluorescent mounting medium were added, covered with a cover slip, sealed with nail polish around the edges, and transferred into 4ºC fridge for storage until time to take picture images of the cells.

2.4.4.2 Imaging & Quantification of Motor neurons

Motor neurons were manually counted using a Leica DM LFS fluorescent microscope and were only counted from the L5 region of the spinal cord - the area of the lumbar enlargement. Motoneurons counted were from the three experimental treatment categories which were Wildtype control, the *dl*-PHPB treated, and the water treated HCN SOD1^{G93A} mice groups only. The images were half of the cross-sectioned spinal cord encompassing the entirety from the dorsal to the ventral regions. 5 of these half-sections per mouse were imaged and used for analysis and quantification

2.4.4.3 Microglia analysis & imaging

Microglia analysis and image capture were carried out using a Leica DM LFS fluorescent microscope and were also taken from the L5 region of the mouse spinal cord under the three treatment categories which were Wildtype control, *dl*-PHPB treated, and water treated HCN

SOD1^{G93A} groups only. Unilateral 10X images (1860 X 2208 pixels) of spinal cord cross-sections were captured. The images were half of the cross-sectioned spinal cord encompassing the entirety from the dorsal to the ventral regions. 5 of these half-sections per mouse were imaged and used for microglia analysis and quantification. Identical exposure and illumination were set for all images captured.

2.4.4.4 Densitometry and microglia cell count

The density of IBA-1 immunoreactivity as well as IBA 1 immunoreactive cells were captured in the images taken, and were analyzed using ImageJ (NIH, Bethesda, MD). ImageJ is an open source Java processing program with thousands of macros and plugins useful in life and biomedical sciences for data imaging and visualization, advanced image processing and statistical analysis (Schindelin et al., 2015; Schneider, 2012).

As earlier stated, to analyze and quantify microglia, the densitometry of IBA-1 reactive cells was measured from 10X fluorescent images of the hemi- spinal cord cross sections, a set scale of measurement in inputted globally for all the images, images were then cropped to a set pre-determined width and height measurement. XY coordinates were created for the image using the cell counter plug-in function on ImageJ, and then the images were turned into an 8-bit grayscale image. Following this, the image threshold was adjusted based on the calculated percentage that best reflects IBA-1 immunoreactivity of the microglia cells. Specific measurements were then set to obtain and record areas of interest for the analysis. For this project, those specific measurements functions include: Area, Minimum & Maximum gray value, Limit to Threshold, Mean gray value and Centroid. Centroid is especially important for providing the individual microglia cells XY coordinates and needful if heat maps showing areas of microglia cell concentration were to be generated.

The image was then transformed to a binary image using the Binary plug-in, and then the

"watershed tool" function was used to mark out each aligned cell, such that cells that were larger

than a set size was broken and counted into 2 or more microglia cells that happen to be lying

adjacent in close proximity. Following these sequences of steps, the cells were then analyzed and

quantified by the ImageJ program software; with both the summary and full results presented.

These results were saved to be used at a later time during statistical analysis

2.5   Statistics

Statistical analysis was performed on all the behavioral tests using the Student's T-test

function from Microsoft Excel for Mac Version 16.47 (Microsoft Corporation, Redmond, WA),

as well as GraphPad Prism 9.0.0 software (GraphPad Software, San Diego, CA).

Molecular/cellular experiment done using GraphPad Prism 9.0.0 software. This software was

also used for the in-vitro experiments that involved analyzing the weights of the animals during

the course of disease progression. Different statistical analytical methods were employed

depending on the parameters being analyzed. Used Log-rank (Mantel-Cox) Test to determine

significant differences in disease onset curve between the treatment groups. Used Kaplan Meier

Survival curve to analyze survival differences among the groups. Used Student's T-test and

Repeated Measures 2- Way ANOVA test to determine significance in behavioral experiments.

Also used Mixed Effects Model analysis whenever the requirements of Repeated Measures 2-

Way ANOVA test were not met.

All cellular/ molecular experiments statistics were carried out using One-way ANOVA,

followed by Tukey's Post hoc test and, in some cases, when the assumptions of One-Way

ANOVA are not met, Kruskal-Wallis test was used followed by Dunn's multiple comparison

Post-hoc test. Statistical significance was considered achieved if $p<0.05$.

CHAPTER 3: RESULTS

As the most used ALS animal model, SOD1^{G93A} mice possess and display typical ALS phenotypical traits especially limb paralysis that is as a result of the loss of spinal motor neurons. The goal of my project is to determine the effectiveness of the drug *dl*-PHPB in mitigating the disease symptoms and progression of ALS disease.

I have indicated in the introduction that many factors, such as genetic backgrounds, transgene copy numbers and gender identity, may affect and modify the ALS-like phenotypes of SOD1^{G93A} mice. In my current study, the standard strain purchased from the Jackson lab, which has approximately 16-31 copies of human mutated SOD1-G93A gene, will be referred as high copy number (HCN) SOD1^{G93A}. The colony that we found to have a lower copy number of the transgene, estimated at 9-12 copies, will be referred as low copy number (LCN) SOD1^{G93A}. These LCN mice also possess a more mixed genetic background than the original strain. In addition, all my analyses will also consider gender differences and dosage-dependent effects of *dl*-PHPB.

Our pilot study was done with mice whose transgene numbers were not checked, and they will be referred to as Unknown Copy Number SOD1^{G93A} or UCN SOD1^{G93A}. In this early study, we found that disease onset and early progression were delayed in mice treated with drug when compared to the control group (See Appendix A, Fig. 12 & 13). In addition, the outcomes for drug treated females were better than the drug treated males in some of the experimental tests performed. Therefore, my studies have mainly focused on the early stages of the disease, not venturing into late end stages and survival of the animals.

3.1   Disease onset in the SOD1^{G93A} mice following treatment with *dl*-PHPB.

There are different standards to determine the disease on-set point for human and animal models. In our preliminary work leading to this current study, we utilized weight changes of the mice to determine disease onset. However, we found there were a couple of caveats. First, the weight of SOD1^{G93A} mice fluctuated from day to day due to various treatments and behavior tests during the week. More problematically, since we didn't realize that our animals lost copies of the SOD1^{G93A} mutant genes (i.e. the LCN group), during the whole experimental period till our limitation for drug gavage (16 weeks), most animals' weight kept increasing (Table 1), indicating that the animals' symptoms were relatively delayed or slower to reach than those with HCN SOD1^{G93A} mutant genes. Therefore, we decided to use the Phenotypic Neurological Scoring colloquially called Rubric scoring; and this manner of determining disease onset has been explained in the Materials and Methods section.

Average weight (g) at	LCN SOD1^{G93A} - 100mg/kg			LCN SOD1^{G93A} - 60mg/kg			HCN SOD1^{G93A} - 60mg/kg		
	Start of experiment (P 75)	Middle of experiment (P 138)	End of experiment (P184)	Start of experiment (P 80)	Middle of experiment (P 146)	End of experiment (P189)	Start of experiment (P 58)	Middle of experiment (P 85)	End of experiment (P115)
Drug treated	26.58±1.1g	29.09±2.0g	30.37±1.5g	26.22±1.1g	26.81±1.6g	28.71±1.9g	20.51±0.6g	20.91±0.6g	21.68±0.6g
Water treated	27.55±1.3g	30.16±1.7g	31.62±1.8g	25.64±1.5g	28.08±2.0g	30.43±2.4g	21.02±0.8g	21.33±0.8g	22.02±0.8g

Table 1: Average weight gain in LCN SOD1G93A & HCN SOD1G93A mice groups.
This table represents a snapshot data from both 60mg/kg & 100mg/kg LCN SOD1^{G93A} mice group, and 60mg/kg HCN SOD1^{G93A} mice group. Analysis done using One-Way Analysis of Variance (ANOVA) followed by a post hoc Tukey's test. No significant differences between water treated and drug treated mice in all 3 groups. ± indicates S.E.M.

3.1.1 Rubric scoring-based disease onset in the SOD1^{G93A} mice following treatment with dl-PHPB.

According to the rubric scoring system that we are using, a rubric score of 1 is described as the collapse of leg extension towards the lateral midline, signifying weakness during tail suspension, which was used as the commencement of disease onset for any given mouse in this project.

As I mentioned earlier, we had 2 groups of mutants with different genetic back grounds; the HCN SOD1^{G93A} mice that have approximately 16-31ccopies of mutant genes, and the LCN SOD1^{G93A} mice with only about 9-12 copies. As expected, the HCN SOD1^{G93A} mice have a much earlier onset of disease when compared to the LCN counterparts with or without *dl*-PHPB treatment (Fig 1). Treatment with 60 mg/kg *dl*-PHPB slightly, but significantly delayed the symptom onset time of HCN SOD1^{G93A} mice, but not that of the LCN SOD1^{G93A} mice.

Comparison of Disease Onset curve in LCN & HCN SOD1^{G93A} mice group
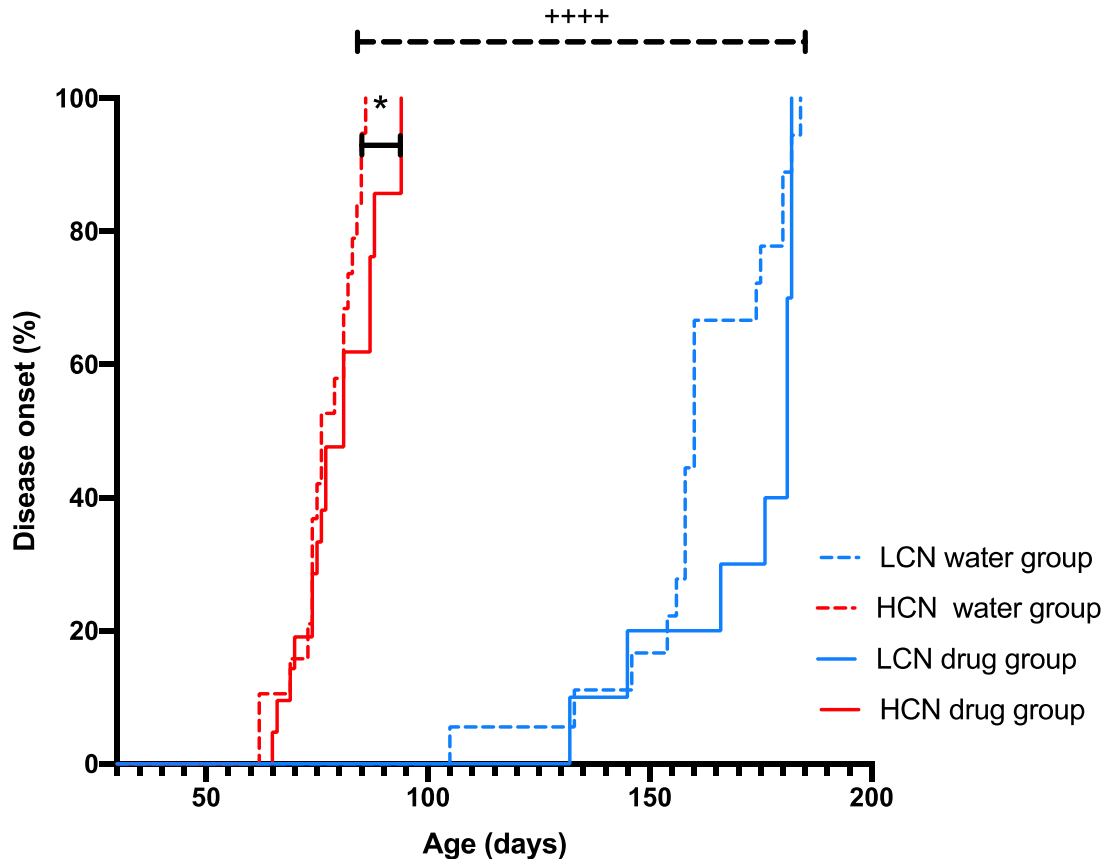
Figure 1: Comparison of Disease Onset in LCN & HCN SOD1^{G93A} mice.
Disease onset for mice were determined using a rubric score of 1. Used Log-rank (Mantel-Cox) Test to determine significant differences in onset curve between the groups. Significant difference observed in onset of disease between HCN 60mg/kg drug treated group and their water treated counterparts. No significant difference between treatment groups of LCN mice. However, significant difference observed between LCN and HCN SOD1^{G93A} mice groups. n= 19 HCN water group, 22 HCN drug group, 18 LCN water group and 10 LCN drug group; p<0.05 (*); p<0.0001 (++++).

Since it has been reported that males and females have different susceptibility in ALS, we then investigated the details of *dl*-PHPB effects on HCN SOD1^{G93A} mice (Fig. 2). Similar to previous reports, male HCN SOD1^{G93A} mice had a slightly early onset of disease symptoms compared to the females, with 50% (5 of 10) of the water treated males at P75, then followed closely at P76 by the water treated females. Interestingly, *dl*-PHPB had no significant effect on HCN SOD1^{G93A} males, but it significantly delayed the symptoms of disease onset of the female HCN SOD1^{G93A} mice group by a week, which was also significantly different from that of *dl*-PHPB treated SOD1^{G93A} male mice.

Disease Onset curve in HCN SOD1^{G93A} mice group differentiated by gender
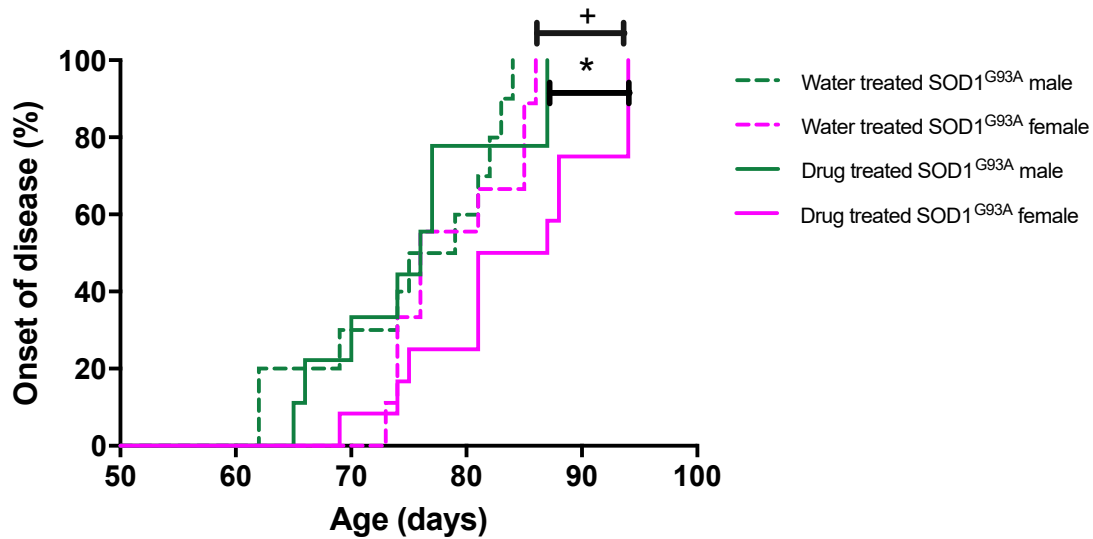
Figure 2: Disease Onset based on rubric score in HCN SOD1G93A mice category
Disease onset for this group were determined using a rubric score of 1. Used Log-rank (Mantel-Cox) Test to determine significant differences in onset curve between the groups. Significant differences observed between the water treated and drug treated females **(+),** also between the drug treated males and drug treated females **(*).** n= 12 drug treated females; 9 drug treated males and water treated females each and 10 water treated males. $p<0.05$ **(*, +).**

3.2   The influence of genetic background and gender on the effectiveness of *dl*-PHPB in behavioral tests

3.2.1   Rotarod performance outcome in the SOD1^{G93A} mice

The Rotarod performance test, documenting animal's ability or lack thereof to run on a rotating cylinder, is the most common and frequently used test for monitoring and assessing motor function and coordination in animals (Barnéoud et al., 1997; S. Knippenberg et al., 2010). All mice in this project were subjected to rotarod tests, in which the rotating frequency of the rod was ramped up from 5 RPM to 40 RPM in 5 mins. Most adult wild type mice can follow this speed well and keep running throughout the 5 mins period (data not shown). However, SOD1 mutant mice would gradually lose their motor function, and the severity of the disease is reflected at the latency with which the animals drop off from the rotating rod (Figures 3, 4).

As I described earlier, the LCN SOD1^{G93A} mutant mice had very low rubric score during the whole testing period, indicating little decay of their motor function. Therefore, even till approximately P120, on average, water treated LCN mutant mouse could still run close to the time span of 5 mins in the rotarod test, and only some of them started dropping off at high frequency, as the disease progressed (Fig. 3A &B). However, the LCN SOD1^{G93A} mutant mice treated with either 100 mg/kg (Fig 3A) or 60 mg/Kg (Fig 3B) of *dl*-PHPB could do even better and could run well on the rotarod up to the age of P140. This result indicated that *dl*-PHPB delayed the progression of the disease for approximately 3 weeks.

A. Rotarod performance in LCN SOD1^{G93A} mice group at drug dosage of 100mg/kg.
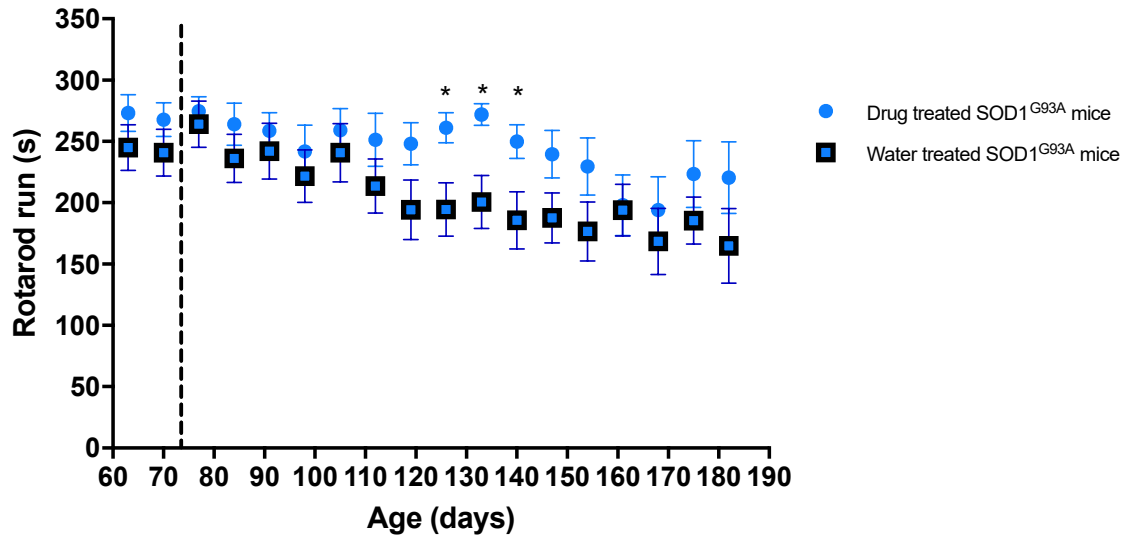
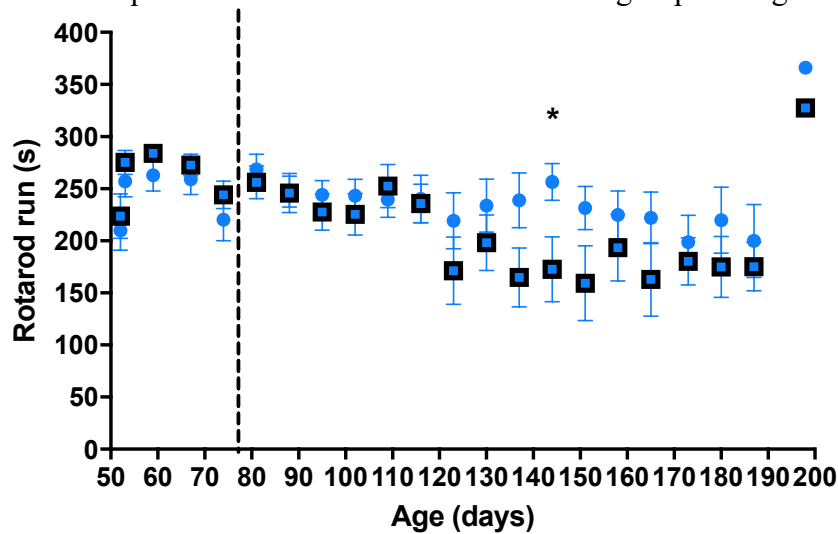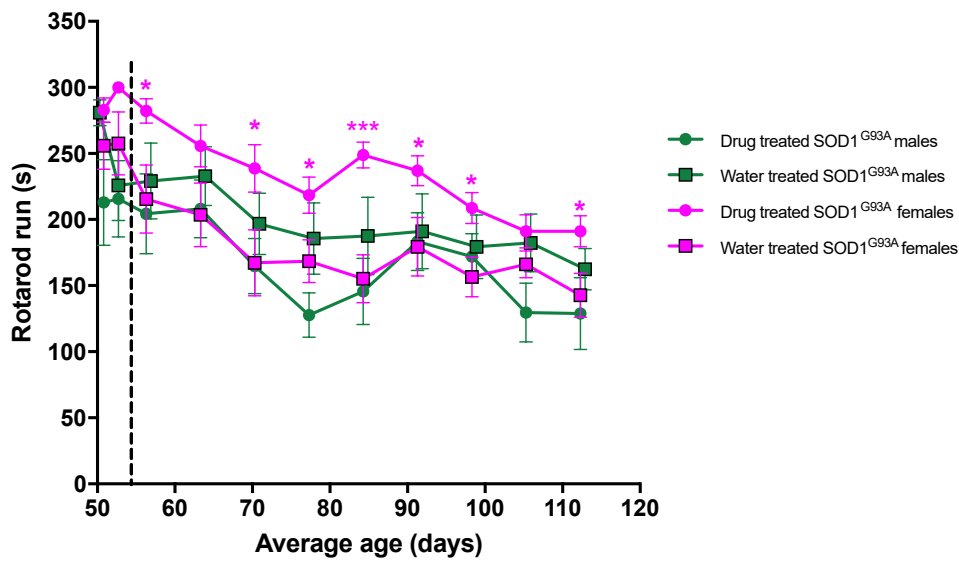B. Rotarod performance in LCN SOD1^{G93A} mice group at drug dosage of 60mg/kg

Figure 3:Rotarod performance in both categories of LCN SOD1G93A mice
Student's T-tests used to calculate significance for each data point. Significant differences observed in 100mg/kg dl-PHPB treated group **(A),** and also in 60mg/kg dl-PHPB treated group **(B).** Vertical dashed lines indicate baseline/pre-treatment. n= 16 per treatment group for 100mg/kg LCN category, and 14 per treatment group for 60mg/kg LCN category. $p<0.05$ (*); error bars indicate SEM.

Not surprisingly, the mice with HCN of SOD1^{G93A} began displaying motor disability much earlier. Some of the HCN mice couldn't run through the 5 min course when the rotarod tests began at around P50, which was earlier than we expected. In addition, the mobility of the SOD1 mutant animals had a considerable amount of variation, right from the start of the Rotarod tests (Fig 4). Even with the gradual decline of the HCN SOD1^{G93A} mice ability to run, drug-treated females still significantly outperformed their water-treated litter mates, and more interestingly, they performed significantly better than the drug-treated males in their rotarod tasks. Drug treatment didn't help in improving motor performances of SOD1^{G93A} males.

These results show that *dl*-PHPB treated HCN SOD1^{G93A} mice are able to better retain their motor coordination and balance than those who do not receive treatment, and that these observations are even more prominent in the drug-treated female mice.

A. Rotarod performance in HCN SOD1^{G93A} mice group at drug dosage of 60mg/kg

B. Rotarod perfomance between females and males of 60mg/kg drug treated HCN SOD1^{G93A} mice group.

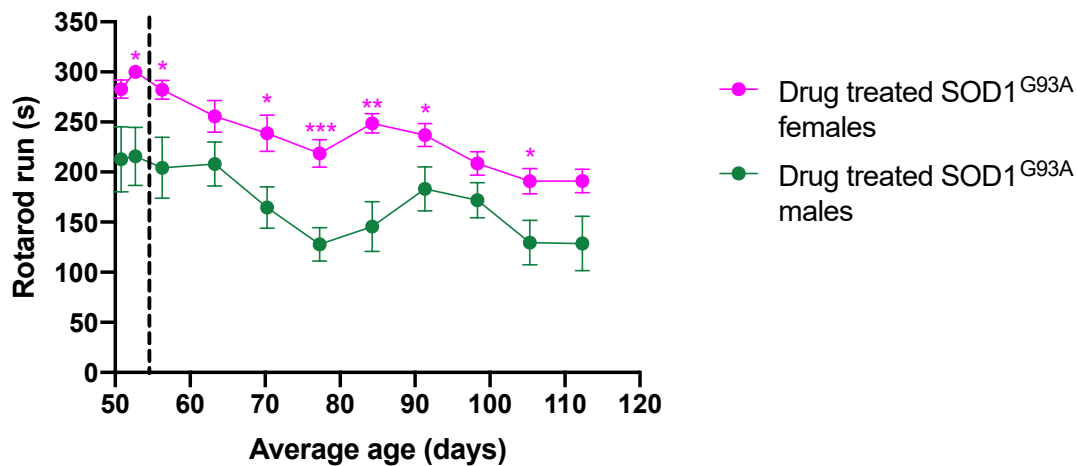**Figure 4: Rotarod performance in HCN SOD1G93A mice based on gender.**
Student's T-tests used to calculate significance for each data point. Significant difference observed between drug treated females and water treated females **(A)**, and between drug treated females and drug treated males **(B)** of the HCN SOD1^{G93A} mice category..Vertical dashed lines indicate baseline/pre-treatment. n=12 drug-treated females, 9 drug-treated males and water-treated females each, 10 water-treated males; $p<0.05$ (*), $p<0.01$ (**), $p<0.001$(***); error bars indicate SEM.

3.2.2 *Hanging performance in the SOD1*^{G93A} *mice*

Inverted hanging in mice is used to examine both muscle strength and motor function of the animals (Alves et al., 2011). The inverted hanging test also known as the Hanging wire test is designed to detect muscle wasting or atrophy. Muscle wasting is a major symptom of neuromuscular and neurodegenerative diseases like ALS (Pasetto et al., 2018).

In our experiments, we used a metal grid on the top of a barrel to make the mice hang on their two or four limbs before jumping or falling into a container that had its bottom lined and padded with paper mesh. It was a simple experimental design, but turned out not very easy to execute, as the mice tried to avoid and evade hanging in that inverted position. Particularly, when the mice were healthy enough to move around more freely, they dropped off from the grid, which might not necessarily be due to a decline or weakness of their grabbing force. Therefore, as is observed in Figs. 5A and B, that the latencies of dropping for LCN SOD1^{G93A} mice fluctuated in a wide range from animal to animal or week to week. However, as seen during the entire testing period, the basic trend for latency to fall didn't vary drastically for water-treated and drug-treated animals until the very end when the male animals started showing some decline (Fig. 5B). These results may indicate that the grabbing force and strength of LCN SOD1^{G93A} mice didn't decline significantly during our experimental period.

However, hanging time for HCN SOD1 mice began decreasing from approximately age P58. By the end of our experiment at P114, most HCN animals could only hold on hanging for a very short period of time. Females and their male littermates showed significant differences on their performance in hanging. Females could always maintain their hanging posture longer than males at all time points. In *dl*-PHPB treated animals, the females still out-performed their male littermates, but there was no difference between drug- and water-treated animals (Fig 6C).

A. Hanging performance in 100mg/kg drug treated LCN SOD1^{G93A} mice group
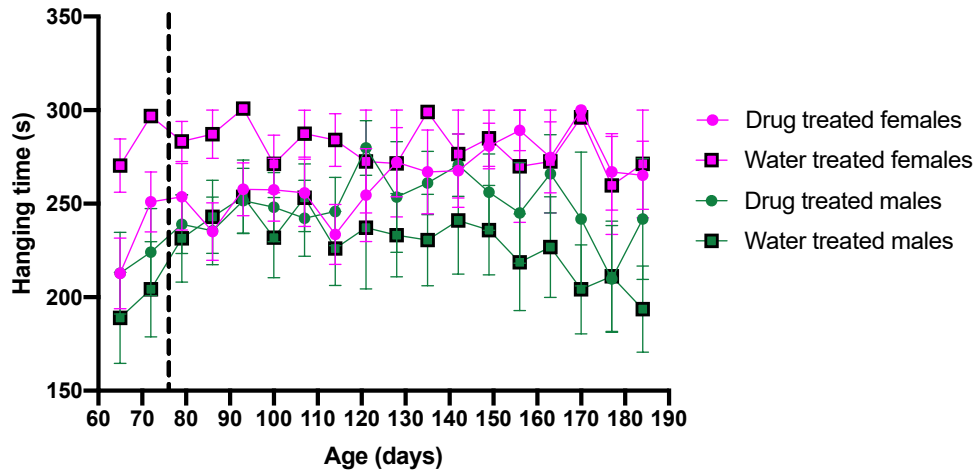
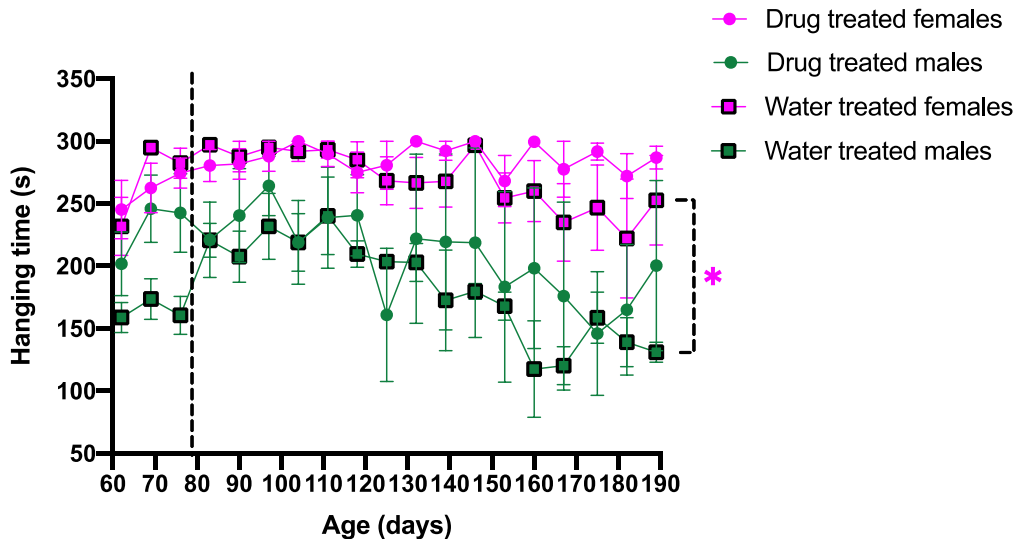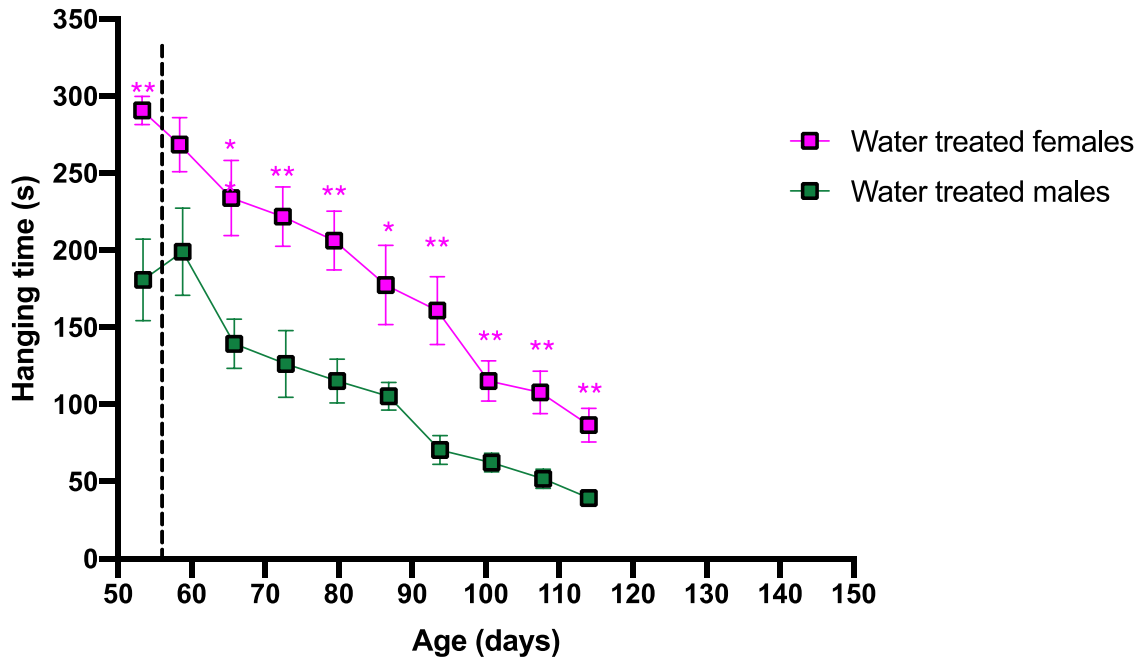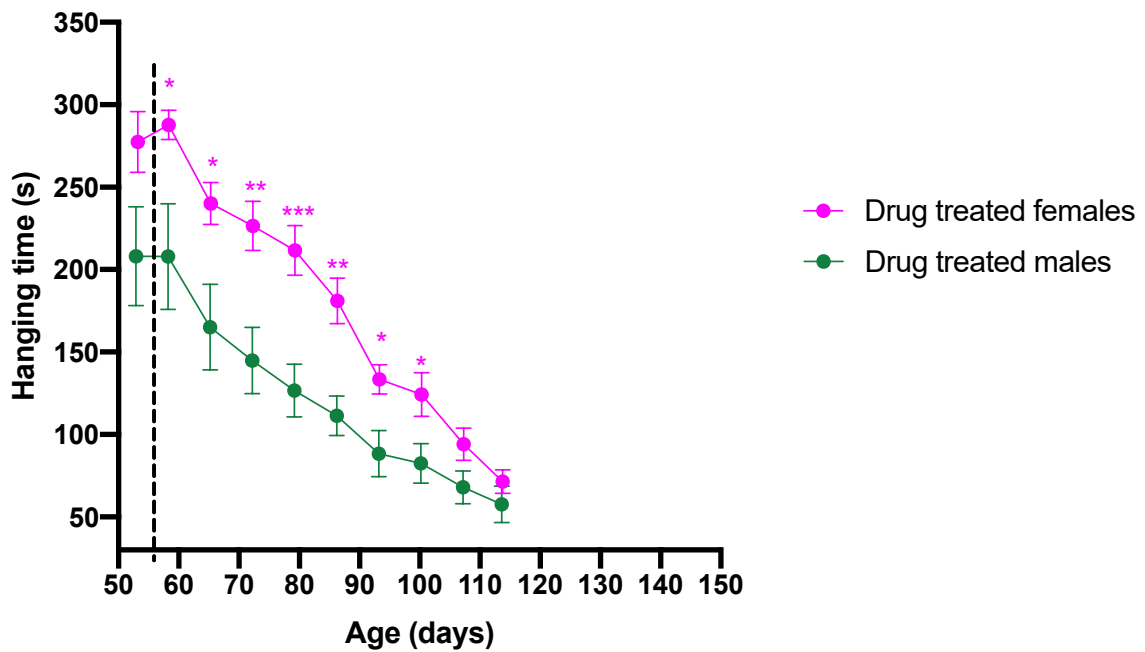B. Hanging performance in 60mg/kg drug treated LCN SOD1^{G93A} mice group

Figure 5: Inverted Hanging performance in the LCN SOD1G93A mice.
Repeated measures 2-way ANOVA used to calculate significant differences between treatment groups. No significant differences observed in the hanging tests of the 100mg/kg LCN SOD1^{G93A} mice group **(A),** significant differences (*) observed between the water treated females and water treated males of the 60mg/kg LCN SOD1^{G93A} mice group **(B).**Vertical dashed lines indicate baseline/pre-treatment. n=5 drug treated females and males respectively, 6 water treated females and 4 water treated males **(A)**; n= 5 water treated females and 3 water treated males, 5 drug treated females and 3 drug treated males **(B)**; $p<0.05$ (*); error bars indicate SEM.

A. Hanging performance in water treated HCN SOD1^{G93A} mice category

B. Hanging performance in drug treated HCN SOD1^{G93A} mice group at dosage of 60mg/kg

C. Hanging performance in HCN SOD1^{G93A} mice group at drug dosage of 60mg/kg across gender and treatment groups
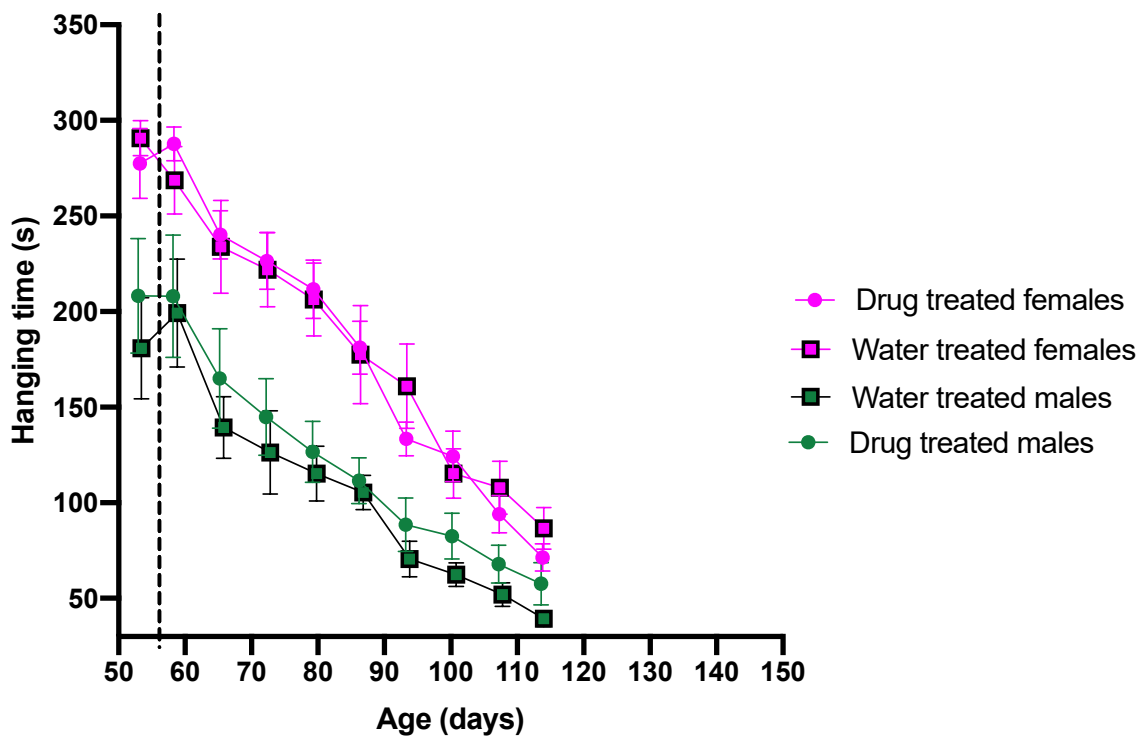

Figure 6: Inverted Hanging performance in the HCN SOD1G93A mice.
Student's T-tests used to calculate significance for each data point. Significant differences observed in the water-treated females and males of the HCN SOD1^{G93A} mice category **(A),** and also in the drug-treated females and males litter mates **(B)**. Graph showing overall inverted hanging performance of mice over time as age progressed **(C).** Vertical dashed lines indicate baseline/pre-treatment. n=12 drug-treated females, 9 drug-treated males and water-treated females each, 10 water-treated males; p<0.05 (*), p<0.01 (**), p<0.001(***); error bars indicate SEM.

3.3 **The influence of genetic background and gender on the effectiveness of *dl*-PHPB in cellular assessments**

3.3.1 Loss of innervation and function in Neuromuscular Junction (NMJ) in the SOD1^{G93A} mice

Denervation or loss of neuromuscular junctions is a significant pathological event that occurs both in ALS patients and in animal models of the disease (Clark et al., 2016; Fischer et al., 2004; Frey et al., 2000), and it typically precedes motor neuron loss as well as clinical symptoms or manifestations of the disease (Clark et al., 2016; San Pun et al., 2006; Vinsant et al., 2013). Using immunohistochemistry, one can visualize the neuromuscular junctions and their innervation patterns. For this project, we employed the use of this method to visualize neuromuscular junctions from 2 different muscles of both the SOD1^{G93A} mice and wild type control litter mates. The muscles stained were the Plantaris (PLA) muscle which is fast fatigable muscle with a mix of Type IIB, Type IIB /IIX hybrid and IIX fibers; and Soleus (SOL) muscle which is a slow- twitch muscle with a mixture of Type I and Type IIA fibers (DeNies et al., 2014).

Figure 7 is a representative image of the innervation patterns of the Neuromuscular Junction, in which the postsynaptic muscle Ach receptors were stained by rhodamine-conjugated α-bungarotoxin, the synaptic vesicles of the presynaptic terminals were stained with synaptophysin and the motor axon was labeled by β3Tuj1. When NMJs were fully innervated, rhodamine -conjugated α-bungarotoxin and synaptophysin signals would be fully overlapped, as shown in the column labelled 'Innervation' of Fig 7. At partial innervation, the receptors on the muscle are still present, but no longer fully overlapped by the corresponding presynaptic terminals, as axonopathy is becoming more evident, with the withdrawal of some of the axon terminals (Fig 7, right; 'P. innervation'). This is even more so during the stages of full denervation, all the axon terminals have disappeared, and have retracted from the receptor on the

muscle (Fig 7; middle 'Denervation'). The three patterns for NMJ status were observed and quantified for both LCN SOD1^{G93A} and the HCN SOD1^{G93A} mice. As I described earlier, at the end of the experiments, when we collected the muscles, most LCN mice had mild symptoms, and as such still had relatively strong muscle force. Correspondingly, we found that most NMJs from either PLAs or SOLs of the LCN group were fully innervated, and there was only a small number of partially innervated NMJs. For instance, drug treated fully innervated PLAs in the LCN group treated with 60mg/kg mice (P189), reduced to 92(SEM±3%) from 98(SEM±0.2%) of the same muscle type in the wildtype control mice NMJs (Fig. 8A, i.). And in the fully innervated SOL muscles of the drug treated mice, the numbers were comparable with a reduction to 90(SEM ±1%) from 98(SEM ±1%) of the same muscle type in the wild type controls (Fig. 8A, ii). The partially innervated NMJs of the PLA muscles for this drug treated LCN group were at 8(SEM ±2%,) while those of the SOL muscles were 10(SEM ±1%) (Fig. 8A iii & iv).

Contrary to the LCN SOD1^{G93A} group of mice, the muscle strength of HCN SOD1^{G93A} mice at age P115, decreased dramatically by the end of the experiments, which was also reflected in the deterioration of NMJs. Particularly, on average, drug treated fully innervated NMJs from PLAs muscles decreased to 57(SEM ±4%), while the partially and completely denervated NMJs increased to 21(SEM ±2%), and 22(SEM ±3%), respectively, compared to the wild type control mice with 99(SEM ±0.2%) at full innervation (Fig. 8B i, iii, v). However, under all conditions in the different experimental categories, the treatment with *dl*-PHPB didn't significantly affect the NMJ structures in all muscles (data not shown).

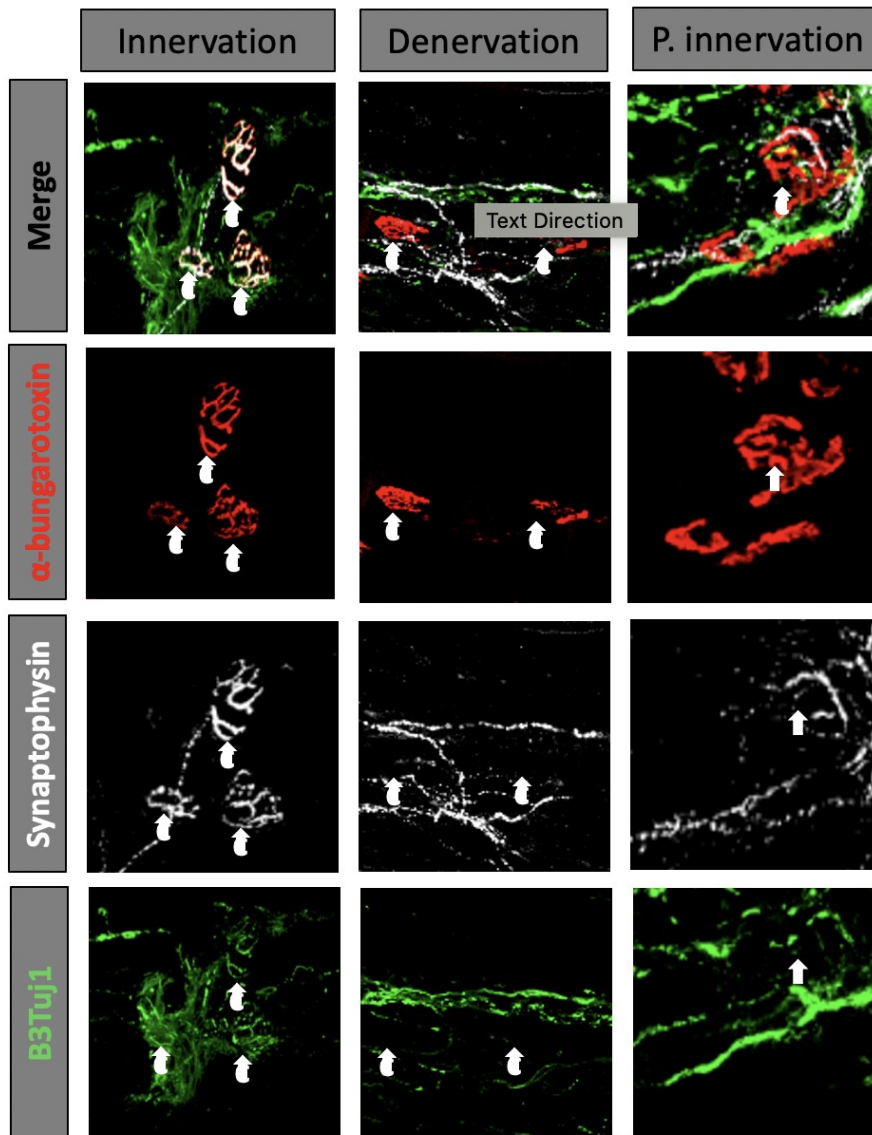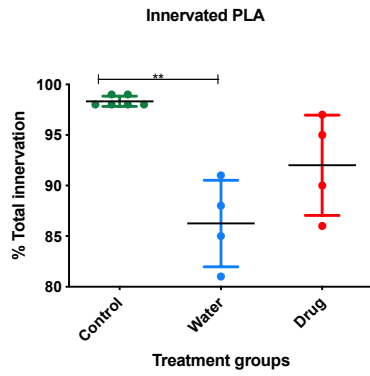Neuromuscular innervation pattern representation

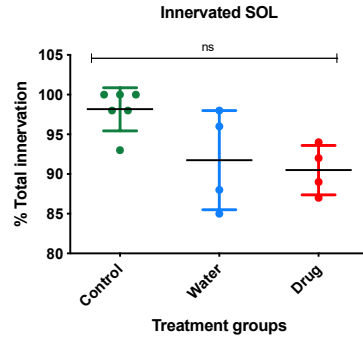**Figure 7: Innervation pattern of the Neuromuscular junction (NMJ).**
Representative images of NMJ staining for both Plantaris (PLA) and Soleus (SOL) muscle.
Immunohistochemistry staining reveals 3 innervation patterns- full innervation, partial
innervation and denervation. α-bungarotoxin stains the receptors on the muscles, Synaptophysin
stains the synaptic vesicles on the presynaptic axon terminals and B3Tuj1 stains the motor axons.
These representative NMJs were all obtained from the UCN SOD1^{G93A} mice category. NMJs
showing 'innervation' were obtained from a wild-type control mouse aged P180, that of
'denervation' was from an UCN SOD1^{G93A} mouse aged P149, and that of 'partial (p) innervation'
was from an UCN SOD1^{G93A} mouse aged P160.
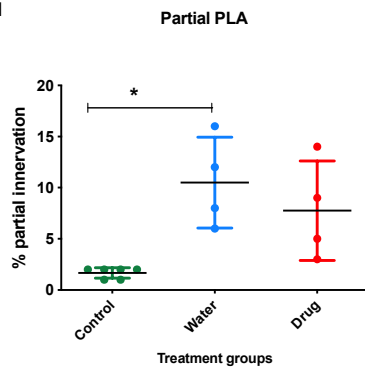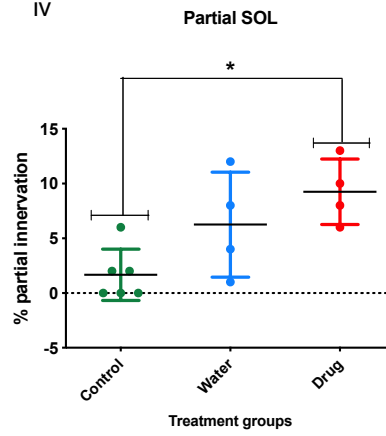
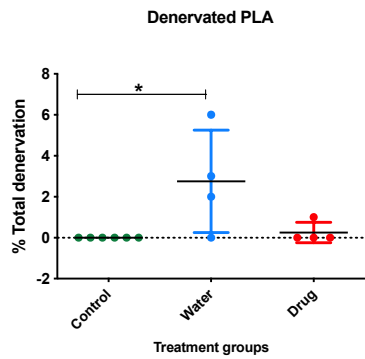## A. NMJ innervation in LCN SOD1^{G93A} group

i

Innervated PLA

ii

Innervated SOL

iii

Partial PLA

iv

Partial SOL

v

Denervated PLA
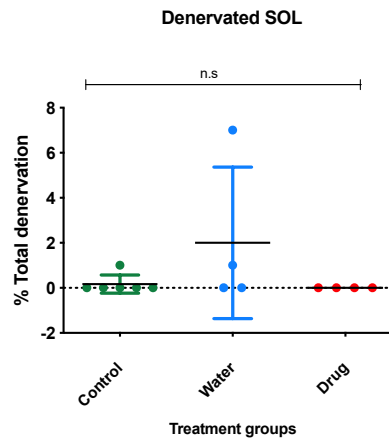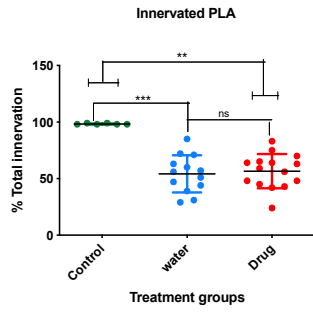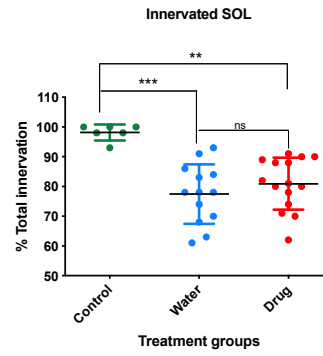

vi

Denervated SOL

B. NMJ innervation in HCN SOD1^{G93A} group

B. NMJ innervation in HCN SOD1^{G93A} group

i

Innervated PLA

ii

Innervated SOL

iii

Partial PLA

iv

Partial SOL

v

Denervated PLA

vi

Denervated SOL
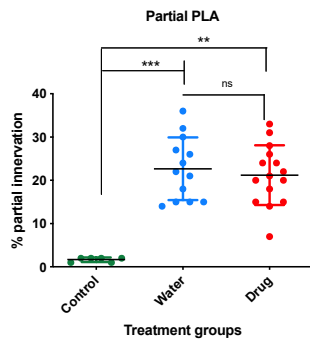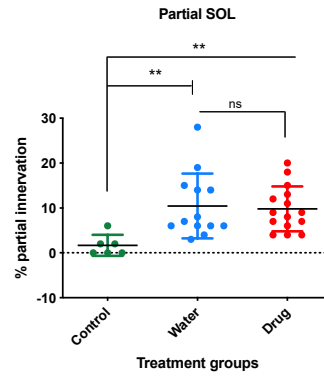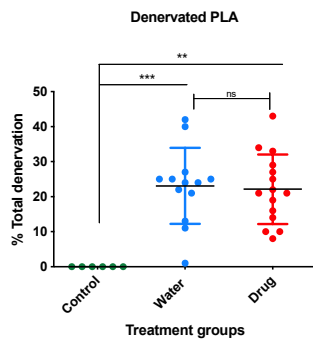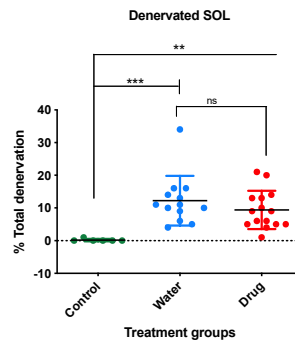
Figure 8: NMJ innervation results for LCN & HCN SOD1G93A mice group
Used Kruskal-Wallis test to calculate significant differences between groups, followed by Dunn's multiple comparison post-hoc analysis. Significant differences present between the wild type control and treatment groups, however no significant differences between the drug treated and water treated groups of both the PLA and SOL. n= 6 control, 4 water and 4 drug treated mice **(A)**. n=6 control, 13 water and 15 drug treated mice **(B)**; $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$(***).

NMJ innervation in LCN SOD1^{G93A} and HCN water treated SOD1^{G93A} groups

Figure 9: NMJ innervation results for water treated LCN & HCN SOD1G93A mice group. One-way ANOVA test, followed by Tukey's post-hoc analysis for calculating significant differences between treatment groups. Significant differences present in both PLA and SOL. n= 15 Wild-type control, 10 water treated LCN mice, and 13 water treated HCN mice. p <0.05 (*), p<0.01 (**), p<0.001(***), p<0.0001(****).

Figure 9 above shows the results between both water-treated groups of LCN and HCN SOD1^{G93A} mice. Though these results do not reflect *dl*-PHPB 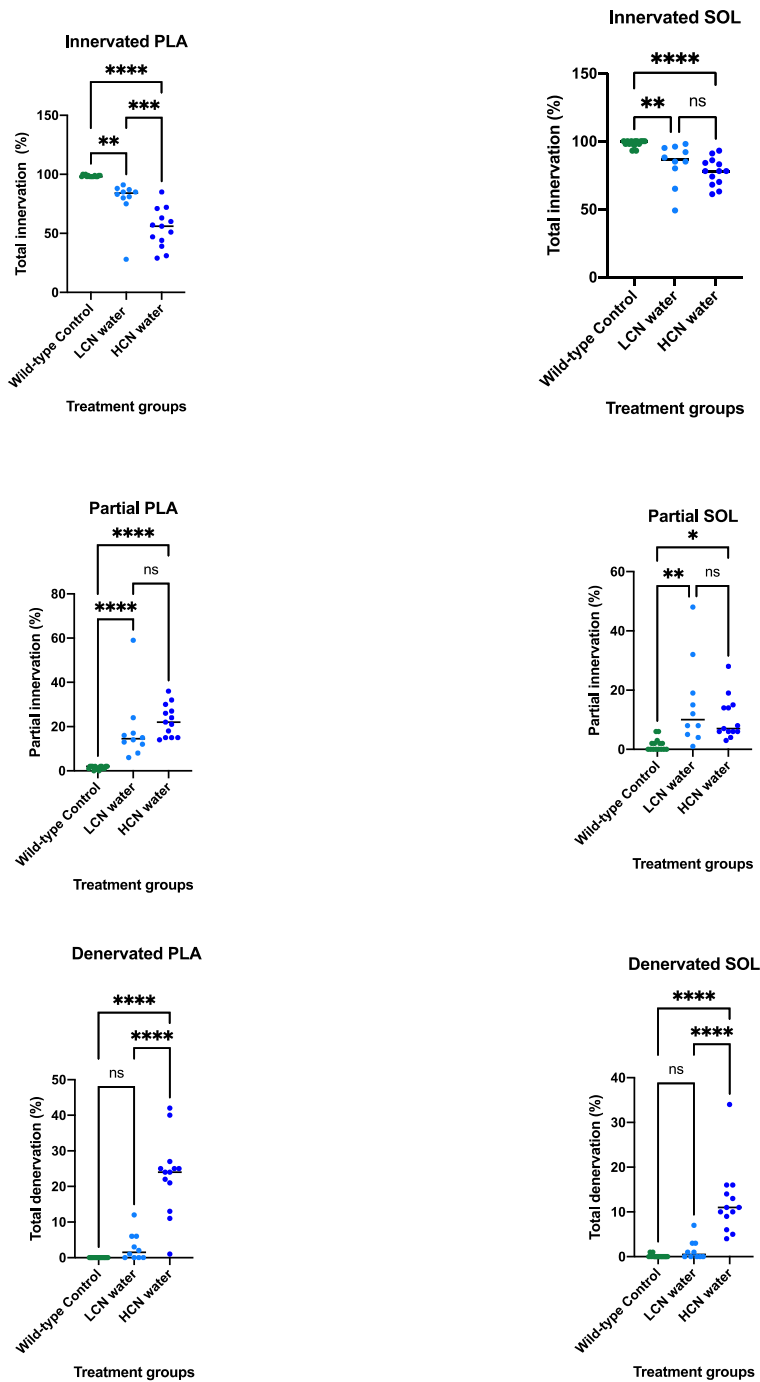effect on NMJ loss during ALS, it does however show that the genetic copy number of the two groups directly affect the extent and impact of NMJ loss during disease progression. Overall, wild-type control animals maintained their NMJs significantly more than the 2 mutant groups, but the LCN SOD1^{G93A} mice did significantly better in maintaining innervation; especially in th PLA muscles. There are no significant differences in full innervation between the LCN and HCN SOL muscle groups, but in the denervated muscles, the LCN group have significantly less denervation than those in the HCN group, and this was consistent in both the PLA and SOL muscles.

3.3.2 *Motoneuron count analysis in the HCN SOD1^{G93A} mice*

Loss of motoneurons is at the core of ALS disease , and is what eventually leads to muscle weakness, paralysis and eventually death of the individuals (Benkler et al., 2018; Boillée et al., 2006; Kim et al., 2014; Martineau et al., 2018). Therefore, given the important role motoneurons play in ALS pathology, we investigated if *dl*-PHPB drug treatment can rescue motor neuron loss and ameliorate disease outcome. Motoneuron quantification analysis was only done in the HCN SOD1^{G93A} experimental group and was extracted only from the L5 lumbar region of the spinal cord (Fig. 10).

At the end of the experiment when we collected the spinal cord, in addition to the weak muscle strength, rubric scores of SOD1 mutant ranged between 2-3: with 2 meaning a change in gait e.g weakness in one hindlimb or lowering of hinder parts during locomotion, and or visible tremors, and 3 being extreme weakness in both hindlimbs, usually toes curl under at least twice during walking approximately 12 inches; or any part of the foot is dragging along cage bottom/table. Therefore, it is not surprising that the averaged MNs in L5 of the mutants

significantly decreased from 31(SEM ±2) per half section (30 μm) in the wild type animals to

19(SEM ±1) per half section in the water treated mutants (Fig. 10). Even though the *dl*-PHPB

treated mutants showed a trend of increased MN number, with an average count of 22 (SEM±1)

per half section, the difference was not statistically significant compared to the water treated

animals, which might, at least, be partially due to a small sample size (Fig. 10).

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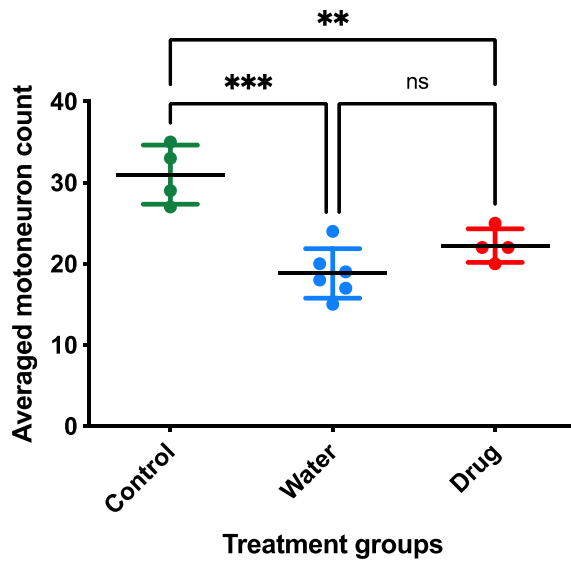**Figure 10: Averaged Motoneuron count in the HCN SOD1G93A mice groups.**
One-Way ANOVA followed by Tukey's post-hoc analysis. There were no significant differences in motoneurons count between the treatment groups. Though differences were present between the Wildtype control and the drug treated or water treated SOD1^{G93A} mice. n= 4 control, 6 water treated mice and 4 drug treated mice. p <0.01 (**), p<0.001(***).

### 3.3.3 *Microglial analysis in the HCN SOD1^{G93A} mice*

Microglial activation in ALS disease is a common pathological neuroinflammatory feature of the disease; and genetic mutation like what is observed in SOD1, TARDP and C9orf72 forms of ALS, enhance this neuroinflammatory response, providing convincing evidence of immune dysfunction and dysregulation in ALS (Beers & Appel, 2019). The appearance of reactive microglia following "injury", which in the case of ALS is neurodegeneration, leads to neuroinflammation and contributes to the death of motor neurons (Thomas Philips & Robberecht, 2011).

Our studies investigated how it behaved in the SOD1 mutant animals using IBA-1 antibody staining to visualize the microglia in the spinal cord (Fig 11A) with water or *dl*-PHPB treatment. We found that there is significant increase in IBA-1 positive cells in HCN SOD1^{G93A} *mice* groups (Figure 11B), compared to wildtype Control mice. Interestingly, the *dl*-PHPB drug treated SOD1^{G93A} mice had more microglia than water treated animals. I further compared the IBA-1 staining in the Dorsal, Intermediate and Ventral regions of the spinal cord. Interestingly, more microglia in the drug treated mice were shown in both the dorsal, and especially in the ventral regions of the spinal cord, compared to water treated mice, but intermediate region appeared to have no significant difference between the water treated mice and the drug treated mice.

Microglia quantification in this study is based on IBA-1 antibody staining to visualize the microglia in the spinal cord. This method does not determine if the quantified microglia are of the M1 phase (pro-inflammatory) or M2 phase (anti-inflammatory). In the Appendix B section (Figs. 14-16), we show results from western blots and q-PCR that were carried out to specifically check the levels of IBA-1 expression and gene expression of some pro-inflammatory markers

from the spinal cord of the SOD1^{G93A} mouse treated with either *dl*-PHPB or water. The results showed that there were no significant differences between the drug treated and water treated animals in either the expression levels of IBA-1 or gene expression levels of the different pro-inflammatory markers investigated. Though the levels of IBA-1 expression and proinflammatory cytokines are significantly heightened in mutants in comparison to the wild-type control animals, these differences are not translatable between the drug and water treatment groups (Appendix B, Figs.14-16).

A. Representative image of fluorescent microglia and motor neuron staining in spinal cord
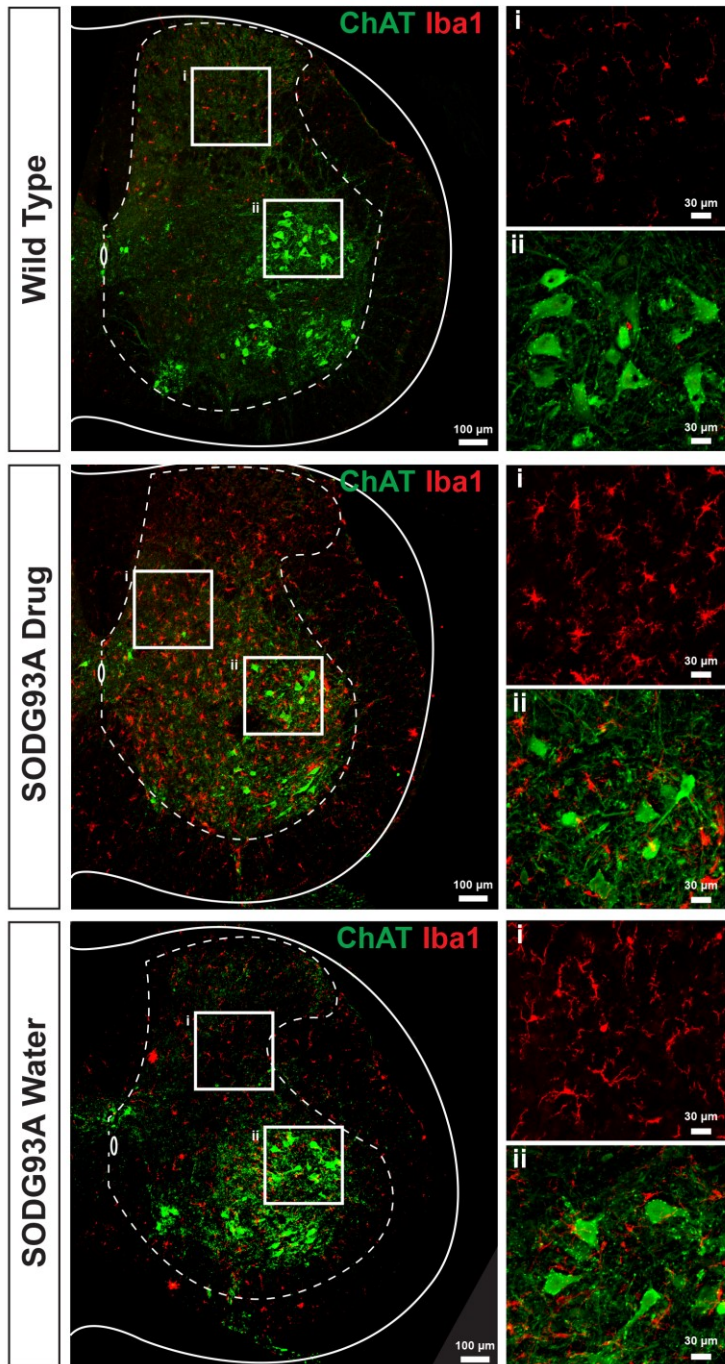
B. Microglia analysis of the HCN SOD1^{G93A} experimental group
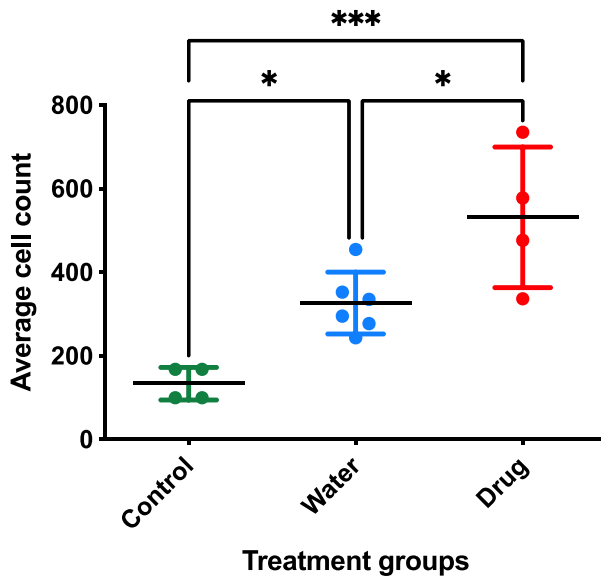
C. Microglia analysis of HCN SOD1^{G93A} experimental group separated into the 3 spinal cord regions
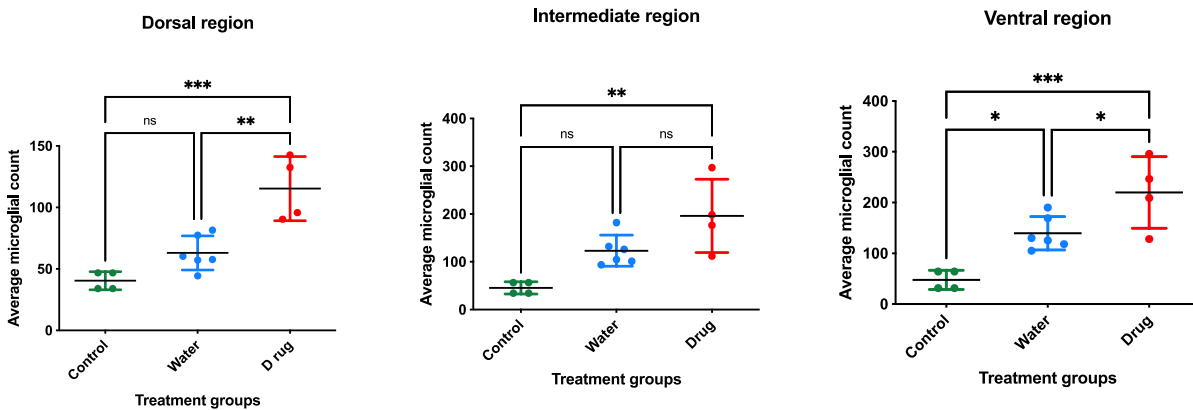
Figure 11: Microglia analysis for HCN SOD1G93A experimental group.
Representative image of microglia and motor neuron staining in a cross-sectioned spinal cord **(A),** Averaged microglia count from L5 region of spinal cord. Total combined averaged microglia cell count across treatment groups disregarding distinct spinal cord regions **(B),** Averaged Microglia cell count differentiated by Dorsal, Intermediate and Ventral regions of the spinal cord **(C)**. One-Way ANOVA, followed by Tukey's post-hoc analysis; n= 4 wild-type control, 6 water treated mice and 4 drug treated mice. p <0.05 (*), p<0.01 (**), p<0.001(***)

CHAPTER 4: DISCUSSION

In our current study, we assessed the therapeutical potential of *dl*-PHPB, a new chemical, on ALS disease, using an animal model, SOD1^{G93A} mouse. Our results indicate that *dl*-PHPB has moderate, but significant benefits on delaying symptom onset of disease and disease progression. More interestingly, these beneficial effects of *dl*-PHPB were more evident in the female SOD1^{G93A} mice than in their male counterparts. Our preliminary analysis also implies that *dl*-PHPB may generate the neuroprotective effects through affecting the microglial activity in the spinal cord at the early stages of the disease development.

4.1   Genetic background and Copy numbers

4.1.1   Disease onset as influenced by copy numbers

As earlier stated, the copy numbers of the animals used for this project varied. Some of the mice were only 9-12 copies termed LCN SOD1^{G93A}, while some were 16- 31 copies termed HCN SOD1^{G93A}, and yet there were some of which we did not know the copy number -referred to as UCN SOD1^{G93A}. As a result of these variations, the effect of *dl*-PHPB on the mice were also affected. We have shown *dl*-PHPB significantly delayed the symptom onset of HCN SOD1^{G93A} mutant mice with Mantel-Cox Test, but not in the LCN group, even though we could see a trend of a delay with LCN group at certain stages during the treatment.

Several studies have shown that the phenotype, severity of the disease as well as its progression is heavily reliant on the copy numbers of the mutated gene (Acevedo Arozena et al., 2011; Alexander et al., 2004; Deitch et al., 2014; Heiman-Patterson et al., 2005), and that progression is variable even in patients with the same genetic mutation of the disease (Valbuena et al., 2019). In a foremost study by Gurney and colleagues (1997), they discovered that the

B6SJL-TgN(SOD1-G93A)^{dl}1Gur transgenic mouse line with a low copy number of approximately 8 copies demonstrated delayed disease onset and increased survival span when compared to the transgenic line B6SJL-TgN(SOD1-G93A)1Gur that possesses 25 copies of the mutated gene (Gurney, 1997) .

Our current project also reported similar results in our LCN transgenic mouse line, bringing about a delay in disease onset. And though survival analysis were not formally carried out in the LCN group for the purpose of this current study, out of curiosity we did however keep some of the animals (without *dl*-PHPB treatment) to monitor when they would reach endpoint. We found out that they reached this stage at about 11-12 months of age. An advantage to such a slowly progressing ALS mouse model is that it mimics more the phenotypical progression of ALS in humans, and as such will be an excellent model for longitudinal studies, research into early-stage pathological processes, as well as early-stage treatments. The effect of *dl*-PHPB may depend on the state of the disease development influenced by the copy number of mutated genes,

Independent of the copy numbers, there is a possibility that the genetic background of our mouse line may have affected the results reported. Genetic background of the LCN group mice were also not the same as that used for HCN. The HCN mice breeders acquired from Jackson Lab were the B6.Cg-Tg(SOD1*G93A)1Gur/J (Stock #004435, Jackson Lab, Bar Harbor, ME). Their genetic background according to the Jackson lab website information shows that their strain of origin is (C57BL/6 x SJL)F1. Our initial breeders for the LCN group were also from the same stock as the HCN with the same parent strain. However, following several generations of breeding in the lab, they were inadvertently bred against other backgrounds that were not (C57BL/6 x SJL)F1. We assume that some of them would have also been bred against a CD-1 background, as the mice fur began appearing in different colors, sometimes white or brown

instead of the standard black. Therefore, the LCN mice, in addition to having low copy numbers of genetic mutation also had a mixed genetic background, which greatly influenced the results we observed from the LCN with or without the drug interaction.

In addition, we had a relatively small sample size for LCN animals and didn't have enough animals to separate the males and females when conducting onset assessment, which may have also affected the final results.

4.2   Gender and its influences on *dl*-PHPB efficacy.

One of the most interesting results in our study is that gender greatly affected both ALS symptoms and *dl*-PHPB effects. As is already known in ALS, females have a better prognosis of the disease than males, with a lower incidence and prevalence rate (Cervetto et al., 2013b), as well as a longer period of mobility maintenance when compared to males (Turner et al., 2010). Our studies showed that females outperformed males in all behavioral tasks during the tested period. In most cases, the *dl*-PHPB treatment showed more significant effects on females than males. As a matter of fact, this made the differences between males and females even more amplified, with disease onset being delayed, motor ability longer preserved, muscle strength conserved, and overall neuroprotective benefits that are advantageous to the motor neuronal circuit.

These beneficial prognosis for females versus males in ALS disease have already been reported in several studies (Abhinav et al., 2007; A. Chio et al., 2009; Nalini et al., 2008; Vázquez et al., 2008), posing the question of why are these differences present and striking? Some studies have investigated the role that endogenous female hormones play in ALS progression and survival, and found that certain female reproductive hormones such as estrogen

have a neuroprotective effect on ALS pathology (de Jong et al., 2013). In an *in vitro* study by Nakamizo and colleagues (2000), they showed that estradiol – an estrogen steroid hormone naturally produced by the body, hindered nitric oxide and glutamate- induced spinal motor neuron death in primary cultures of rat spinal cord; hence providing neuroprotective benefit from excitotoxicity (Nakamizo et al., 2000). Additionally, a 2004 study showed that ovariectomized SOD1^{G93A} mice accelerated ALS disease progression. However, when these mice were treated with 17β-estradiol, the disease progression was then significantly delayed (Groeneveld et al., 2004). Furthermore, in a large population-based study, the relationship between lifetime estrogen exposure in women and ALS was investigated, using their reproductive history. They found out that a longer reproductive life span which is a stand-in for a longer life-time exposure to estrogen, was associated with a lower risk of ALS, and also a prolonged survival of women with ALS (de Jong et al., 2013).

The results from our current study also show a better overall performance in behavioral tests and a delayed onset of disease in the female SOD1^{G93A} mice; regardless of their copy numbers and genetic backgrounds, and the effects of the drugs, in some cases were more amplified at higher doses, suggesting a potential interaction with the drug and female hormones that bring about neuroprotective benefits. The better outcome of *dl*-PHPB treatment could be due to a potential interaction with the drug and female hormones that bring about neuroprotective benefits.

4.3   *dl*-PHPB and its neuroprotective properties in ALS

The neuroprotective benefits of dl-PHPB have been shown in several studies. In Alzheimer's disease, it was shown to attenuate tau and amyloid pathologies, therefore

simultaneously improving memory deficits (Peng et al., 2014); reduced hydrogen peroxide-induced apoptosis in neuroblastomas (Y. Hu et al., 2012); decreased superoxide dismutase activity and lipid peroxide in the cortex of hypoperfused rats (Wanhong Zhao et al., 2013), to mention a few. Though most of the disease models the drug was tested on were neurodegenerative disease, this is the first time it's been tested on ALS- a disease that specifically leads to motor neuron death.

4.3.1   Treatment times and dosing impact on dl-PHPB benefits

Our results show that *dl*-PHPB provides mild benefits in the SOD1^{G93A} ALS mice model improving their rotarod performance. However, it doesn't impact the muscle strength of the animals, as all the animals declined with similar pace on the inverted hanging tests. Correspondingly, we didn't detect significant differences in the NMJ deterioration rate and motor neuron loss between the drug-treated and water treated groups. We could speculate several possibilities that led to such discrepancies in different motor performances.

One is that *dl*-PHPB has different protective effects on different neurons and regions in the CNS. Even though all the ALS pathogenetic factors, such as SOD1 or other mutated genes, exists in all cells of affected individuals, most studies have focused on LMNs. Recent studies, however, have shown that some interneurons in the spinal cords may be affected before MNs in SOD1 mutant animal (McGown et al., 2013; Salamatina et al., 2020). Moreover, studies also show that changes in UMNs begin early on in the progression of the disease with signs like cortical hyperexcitability, therefore making UMN degeneration an early event in ALS and possible early detection marker (Geevasinga et al., 2016; Vucic et al., 2008). Therefore, it is possible that *dl*-PHPB has more protective impacts on neurons in the brain or spinal interneurons during early stages of the disease development. As a result, we could see an improvement in the

coordinated locomotor performance during the early treatment, but not the motor neuron in the group tested (HCN SOD1^{G93A}). Possibly, earlier administration of *dl*-PHPB could generate even stronger effects.

In addition, although we did utilize varying dosages, the only group that actually received 2 different doses was the LCN group. We found that in rotarod performance at least, those LCN SOD1^{G93A} that received the higher dose of 100mg/kg (instead of 60mg/kg), had a longer window of significantly better performance on the rotarod test. This dose was not tested with the HCN group, so it's impossible to state exactly what the results would have been. Nevertheless, based on the result observed from the rotarod behavior in the LCN group, it could be postulated that at higher doses of 100mg/kg and above, the results could have been more beneficial, and possibly conserved muscle strength.

The effects of dosing do not however appear to function as an independent factor but are contingent to some degree on the mutant copy numbers present in the animal. For instance, when the results of the Rotarod tests were examined, we found that 60mg/kg appeared to have a more striking effect on the HCN than on the LCN group of animals. To briefly elucidate, while there was a short window of 1 week and 3 weeks of efficacy in the 60mg/kg and 100mg/kg drug treated LCN group respectively, the HCN group drug efficacy is seen throughout the entire 12 weeks of testing. Disease onset was also more strikingly delayed in the HCN group receiving 60mg/kg, but no differences were observed in the LCN group. These results suggest an intricate interplay with dosage and mutant copy numbers of the animals.

4.3.2   *dl-PHPB and its effect on microglia activity*

Our microglia cells quantification showed that there was a slight, yet significant increase in the amount of microglia present in the drug treated mice versus those in the water treated mice.

Further investigation with the use of western blots to check the levels of IBA-1 expression, however showed no significant differences between the drug treated mice and those that received water (Fig 14, Appendix B). Moreover, q-PCR testing of various pro-inflammatory cytokines also showed no significant differences in levels of gene expressions (Figures 15 & 16; Appendix B).

Microglia are divided into M1 (pro-inflammatory) phenotype and M2 (anti-inflammatory) phenotypes. Even though all pro-inflammatory markers showed no significant differences in their levels of expression between treatment groups, it is possible that the M2 phenotype microglia could have made up the increase in microglia presence observed in the *dl*-PHPB treated mice.

Proinflammatory cytokines or molecules such as Interleukin 6 (IL-6), Interleukin-1B (IL-1B), NADPH Oxidase 2 (NOX2), to mention a few, have been shown to play a critical role in microglial activation with ALS disease, thereby negatively impacting the progression of ALS pathology (Beers, Zhao, et al., 2011; Martínez et al., 2017; Weihua Zhao et al., 2013). For instance, inactivation of NOX2 has been shown to reduce microglial-derived Reactive Oxygen Species (ROS) production in an ALS SOD1 mouse model, delay neurodegeneration, and thereby increase survival of the mice (Marden et al., 2007; Wu et al., 2006). In a study by Mishra and colleagues (2017), they show that exposure of microglial cells to Cerebrospinal fluid (CSF) from ALS patients, resulted in potent microglia activation that was skewed primarily towards pro-inflammation, as seen in the upregulation of proinflammatory cytokines such as IL-6, TNF-α, while simultaneously a downregulation of anti-inflammatory cytokines and beneficial neurotrophic factors such as Vascular Endothelial Growth Factor (VEGF) were observed (Mishra et al., 2017).

As stated earlier, our investigations showed no difference in pro-inflammatory markers between the treatment groups across the various mutant copy number categories. A possible explanation for this, is that the drug effect is probably diminished by the time of animal sacrifice and tissue harvesting. A study by Zhao and colleagues, explained that with the progression of ALS disease, mutant SOD1 microglia take on the M1 phenotype which promotes inflammation, and simultaneously motor neuron degeneration, and as a result could lead to an increase the in the rate of ALS progression (Weihua Zhao et al., 2013).

4.4   Limitations

As with most studies, there are several limitations that can arise during its course; and our study was no exception. These limitations will be summarized in this section. Some evident limitation includes our sample sizes and experiment run times. In some of our molecular and behavioral tests, the sample sizes were insufficient, and so therefore partially impacted the results observed. For instance, our motor neuron and microglia count, were done on a limited number of mice, and also was restricted to only the HCN SOD1^{G93A} mutant groups. This in addition to the small size, makes comparing and drawing more clear conclusions difficult. The experiment run time also added to the complexity of things, as it appeared by not starting treatment earlier, and in some cases sacrificing the animals earlier than later, led to missing some observations that could have shown a possibly more potent effect of the drug.

Another limiting factor was the fact that the different copy number categories of mice didn't always go through the same experiments. For instance, our UCN mice in the preliminary run were kept to endpoint before they were sacrificed. By doing so, we were able to obtain the effects of the drug on survival, even though the results showed no significant differences in

prolonging survival among treatment groups. However, this wasn't done for the LCN and HCN groups, because of the need to investigate and capture the effect of the drug on a different parameter such as rotarod tests that would test motor coordination and function in the mice.

Finally, we cannot underestimate the impact varying copy numbers and genetic background had on the results observed. In some cases, we were unable to definitively state what the copy numbers and genetic background were, as we were not aware of the changes until the experiments were completed and animals sacrificed. At which time it was too late to remedy the situation, therefore making it difficult to draw concise and confident conclusions on our results or make inference to what may have been already been observed in the field.

4.5   Future directions

Future directions should address the issues that have already been outlined, which includes sample size, experiment run times, mutant copy number variations and genetic backgrounds. In addition to this, future work should investigate the gene expression levels of anti-inflammatory cytokines too, and not only pro-inflammatory markers. A study by Benito and colleagues that investigated inflammation in blood samples of sporadic ALS patients at early clinical stages, showed that the levels of anti-inflammatory cytokines like IL10, that codes for interleukin 10, and TGFB2, coding for transforming growth factor beta 2 were significantly reduced (Andrés-Benito et al., 2017).

Another potential area of study would be to investigate the changes of microglia morphology as they have been shown to play an important role in the pathophysiology of ALS. Studies have shown that microglia may take on a neuroprotective role for MNs at the early stages of the disease, but then switch to secreting neurotoxic factors towards later stages of the disease,

quickening neurodegeneration and MN death (Brites & Vaz, 2014; Gerber et al., 2012; Weydt et al., 2004). In another study by Dibaj and colleagues, using two-photon imaging they show *in vivo* evidence in the SOD1^{G93A} mice, that at preclinical stages(at about P60), microglia takes on the highly reactive morphology and function and then in later clinical stages shift to microglia morphology that have lost their ability of tissue surveillance and injury-directed response (Dibaj et al., 2011). This area of study would be important and interesting to investigate in our SOD1^{G93A} mice in relation to how it is affected by the effects of dl-PHPB treatment.

4.6   Conclusion

ALS is a fatal neurodegenerative disease that is both multifactorial in nature and that affects multi-systems and cellular pathways in the body. There are currently only 2 approved FDA drugs – Riluzole and Edaravone available to ALS suffers, which are generally of mild effect. *dl*-PHPB has neuro protective, anti-apoptotic and anti-oxidative beneficial properties, making it a promising candidate for studies in ALS disease. The results presented in this study provide evidence that *dl*-PHPB has some neuroprotective benefits that can preserve motor function and coordination, while delaying progression in some of our mice categories; and these benefits were amplified in female mice. The effects of the drug, based at least on this study, appear to not have strong effects on the motor neuron circuity and thus do not translate to parameters that test attenuation of NMJ and MN loss, as well as the conservation of muscle strength in the animals. Microglial morphology and function present an area of great interest that could further test the effectiveness of the drug and provide insight on a cellular mechanism that is of great pathological importance in the progression of ALS. The field of treatment and drug discovery for ALS is vast and complex, and one more study in this area adds to the knowledge

and hope of developing more effectual therapies that will lead to an improvement of the mobility

and quality of life for ALS sufferers.

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APPENDIX A UCN SOD1^{G93A} BEHAVIORAL TESTS RESULTS

1.1   Disease Onset & Weight loss in UCN SOD1^{G93A} mice group differentiated by gender

To determine disease onset for the UCN SOD1^{G93A}, we utilized weight drop as the defining parameter. During the course of the experiments, individual mice weight displayed fluctuations. However, there was a point in time where their weight dropped below the initial weight at the beginning of the experiment and did not return to said weight or exceeded it, instead steadily declined. This point in time was recorded as the start of disease onset for the individual mouse.

In the UCN SOD1^{G93A} mice group, as represented in Figure 12, disease onset is first observed within the water treated male mice contingent, where roughly 66% (4 of 6 mice) of them demonstrated significant weight loss by P99, then followed by 57% (4 of 7) of the drug treated counterparts at P117, 75% (6 of 8) of the water treated female mice at P119 and the final group of treatment mice that experienced onset was the drug treated female mice; with approximately 57% (4 of 7) at P143.

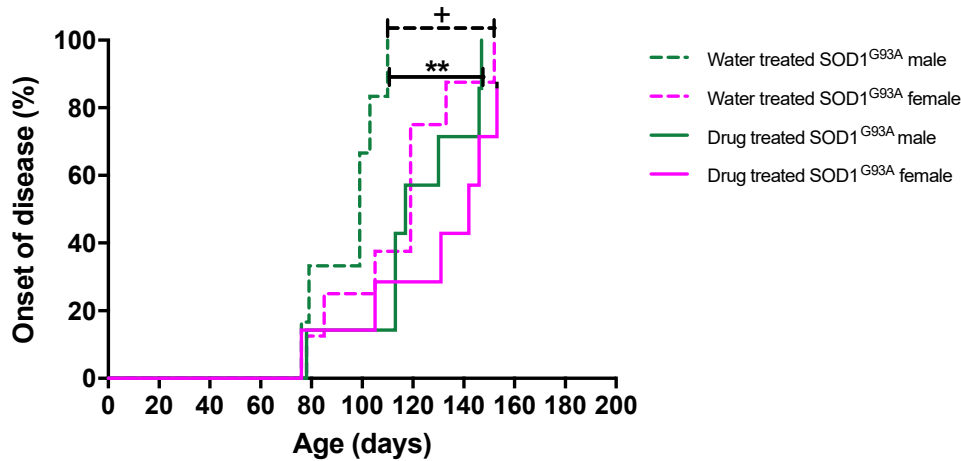*Disease Onset in UCN SOD1^{G93A} mice group*

Figure 12: Disease onset based on weight in 30mg/kg drug treated UCN SOD1G93A mice. Disease onset for mice in this group were determined using their weight loss. The point in time where a mouse weight drops and stays lower than its initial weight at beginning of experiment, without superseding said initial weight is determined as disease onset for that mouse. Used Log-rank (Mantel-Cox) Test to determine significant differences in onset curve between the groups. Significant differences observed in water treated males versus their drug treated littermates (**) and also between water treated males and water treated females (+). n=6 water treated males, 8 water treated females, 7 drug treated males and females respectively. p<0.01 (**), p<0.05 (+).

Disease onset was significantly delayed in male mice treated with the drug in comparison to male counterparts that receive water. Also, when examining only the water treated group, it was observed that water treated mutant males experienced disease onset at a significantly earlier time, and in more of their mice than the water treated mutant females. For example, by P110, 100% of the water treated mutant males have reached disease onset, while this was not true for water treated mutant females until P152. This observation could be interpreted to correlate with what was observed in the progression and prognosis of ALS. Females tend to have a better prognosis of the disease than males do, with a lower incidence and prevalence rate in females than what is observed in males (Nakamizo et al., 2000), and even maintaining their ability to remain mobile for several months despite the progression of anarthria, which is a severe loss in the power to articulate speech (Turner et al., 2010).
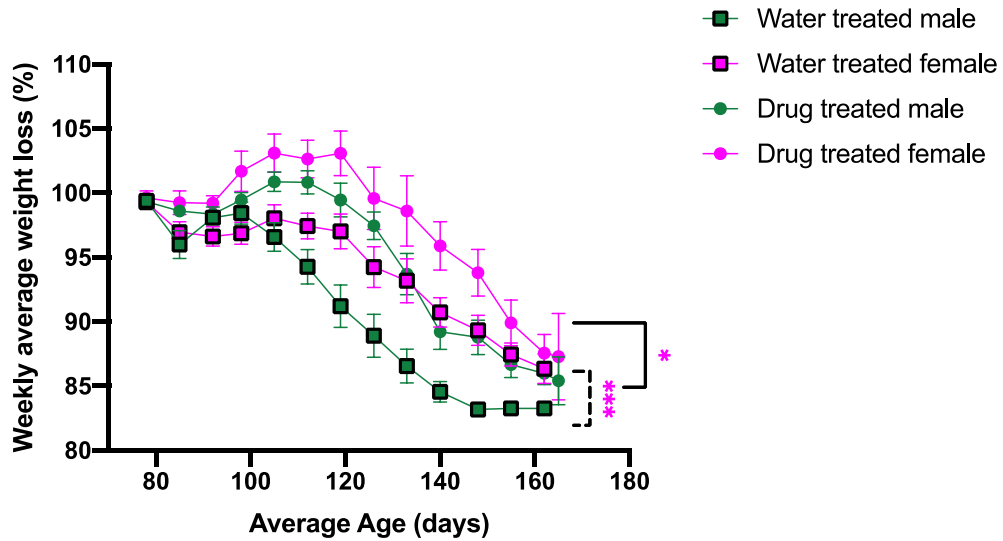
Weight change in 30mg/kg UCN SOD1^{G93A} mice

Figure 13: Percentage Weight loss in 30mg/kg UCN SOD1G93A mice group.
Mice in all treatment groups lost weight as disease and time progressed. Statistics done using
Mixed effects model analysis. Significant differences observed between Water treated females
and males (***) and also between Drug treated females and Drug treated males (*).. n=6 water
treated males, 8 water treated females, 7 drug treated males and females respectively. p<0.001
(***); p<0.05 (*).

Weight loss was a noticeable trend, with all mice regardless of treatment group (Fig 13). A steady decline in their weights were observed with the passing of time and progression of disease, until they reached endpoint of disease and were sacrificed. Endpoint of disease was determined when the animal had lost at least 20% of their initial weight at beginning of experiemt; at which point they were sacrificed. Significant difference observed between the Water treated females versus Water treated males, with the former outperforming the latter by preserving more of their body weight as disease and time progressed. Significant differences were also observed between the Drug treated females and the Drug treated males. Drug treated females were able to preserve significantly more of their weight than their drug treated male littermates. In other words, female mice treated with 30mg/kg of *dl*-PHPB were better able to preserve their muscle strength and maintain a healthier weight througout the length of the experiment (Fig 13).

APPENDIX B MOLECULAR TESTS RESULTS

1.1 The influence of genetic background and gender on the effectiveness of *dl*-PHPB in molecular tests

1.1.1 Levels of IBA-1 expression as detected by Western blotting across treatment groups in the different experimental categories.

Western blotting was used to check for the levels of IBA-1 in the SOD1^{G93A} mutants, either treated with *dl*-PHPB or water as a placebo, and for the wild-type controls as well. It is a molecular and cell biological technique used in identifying and separating specific protein molecules from a complex mixture of proteins, based on their molecular weight via gel electrophoresis (Mahmood & Yang, 2012). Ionized calcium-binding adaptor protein-1 (IBA-1) is a 17-kDa actin-binding protein that is explicitly expressed in macrophages and in both ramified and activated microglia; therefore it is used as an immunohistochemical marker for microglia (Hovens et al., 2014; Imai et al., 1996; Y. Sasaki et al., 2001). Our experiment investigated the levels of microglia through its IBA-1 expression in the LCN SOD1^{G93A} group and HCN SOD1^{G93A} groups. The results of each group are reported next.

A. Levels of IBA-1 expression as detected by Western blots across treatment groups in the LCN SOD1^{G93A} group treated with 100mg/kg dl-PHPB.
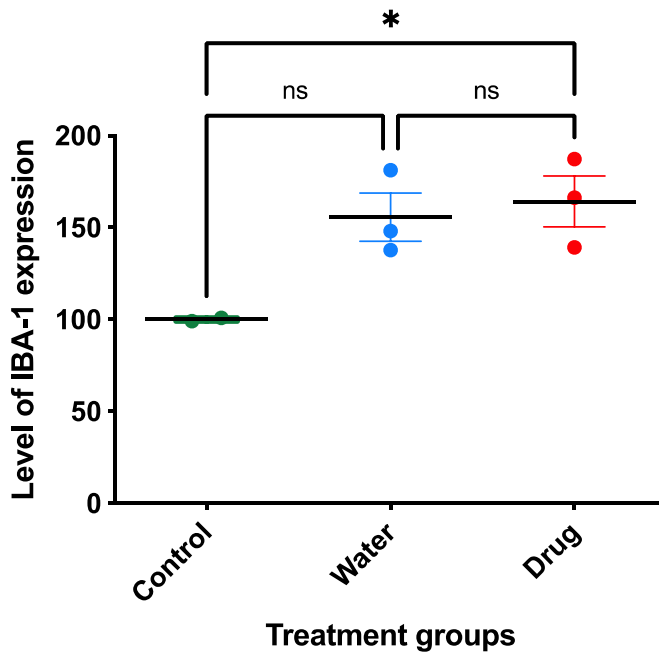
B. Levels of IBA-1 expression as detected by Western blots across treatment groups in the HCN SOD1^{G93A} group treated with 60mg/kg dl-PHPB.
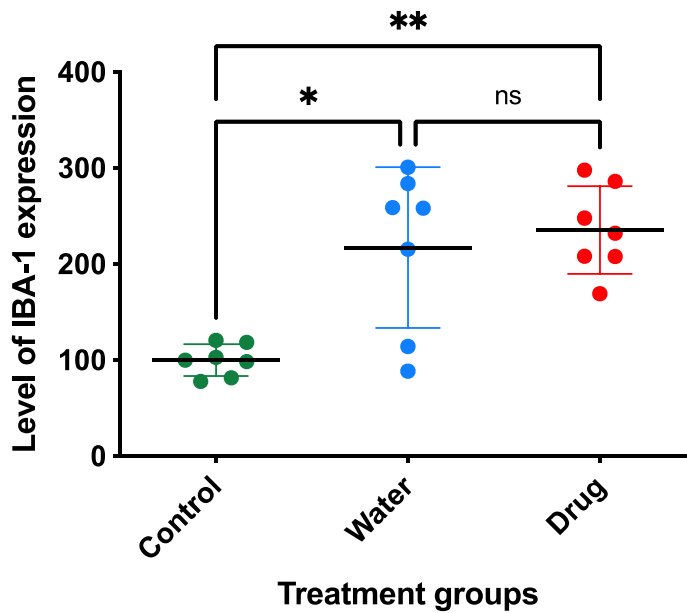
Figure 14: Levels of IBA-1 expression as determined by Western Blot analysis in the mice categories.

IBA-1 expression levels indicative of microglia presence. IBA-1 expression in LCN SOD1^{G93A} mice group treated with 100mg/kg dl-PHPB **(A),** and HCN SOD1^{G93A} mice group treated with 60mg/kg dl-PHPB **(B).** One-Way ANOVA analysis followed by Tukey's post-hoc to test for significant difference. Sample size (n) for LCN SOD1^{G93A} groups are set at 2 control, 3 water and drug treated mice respectively; for HCN SOD1^{G93A} group, 7 mice each in control, water and drug treated groups respectively. $p < 0.05$ (*), $p < 0.01$ (**).

In Figure 14(A) there is a significant difference between the expression levels of IBA-1 within the Wild-type control mice and the 100mg/kg dl-PHPPB drug treated LCN SOD1^{G93A} mice; with the expressions level being greater in the latter than the former. However, there remains no significant differences between SOD1^{G93A} mutant group treated with water versus those treated with dl-PHPB. Figure 14(B) depicts the results of IBA-1 levels of expression in the HCN SOD1^{G93A} mice. The drug treated mice in this group received a dose of 60mg/kg dl-PHPB. Significant differences were evident between the drug treated group and wild-type control, and again between the water treated group and wild-type control. There were however no significant differences in IBA-1 expression between the SOD1 mutant group treated with dl-PHPB versus those that received only water.

The results reported could be attributed to the small sample size, especially in the LCN SOD1^{G93A} groups. It is likely that the low copy number plays a factor in the lack of significant differences observed between the water and dl-PHPB drug treated group. Because the genetic mutation is expressed in a low amount of copy number, the animals are slow to reach diseased state, and as such there is a correlating low IBA -1 expression when compared for instance to HCN SOD1^{G93A} group. Increase in levels of IBA-1 expression is directly correlated to an increase in activated microglia state (Hovens et al., 2014; Imai et al., 1996; Y. Sasaki et al., 2001). In Figure 14(A), which depicts LCN SOD1^{G93A} group, on average the level of IBA-1 expression is slightly above 160 in the SOD1 mutant mice, while in Figure 14(B), which is the HCN SOD1^{G93A} group, the IBA levels in the mutant mice is averaged at approximately 235. Therefore, with low copy number of SOD1 mutation that leads to a delayed ALS -diseased state, one can infer and expect that IBA-1 levels would also be low, and consequently there will be

similar expression levels in those SOD1 mutants treated with water versus those treated with *dl*-PHPB.

1.1.2 Levels of gene expression as detected by qPCR across treatment groups in the different experimental categories.

Quantitative Real-time Polymerase Chain reaction (qPCR) is a powerful tool and standard method used for measuring levels of gene expression (Boulter et al., 2016). Since significant differences in gene expression between diseased states and wild type controls, or across treatment groups can be heavily influential in navigating the course of a research study, therefore it is of optimal care that the methods for quantifying mRNA is standardized and accurate (Boulter et al., 2016).

Using qPCR technique of quantifying mRNA, my research sought to investigate and quantify the level of gene expression of different pro-inflammatory cytokines or molecules to test for the presence of microglial activation in both the diseased SOD1^{G93A} mice groups treated with the *dl*-PHPB drug and those treated with water as a placebo. Considering that *dl*-PHPB has anti-oxidative effects and neuroprotective properties (Y. Hu et al., 2012; Yi Zhang et al., 2004, 2006; Wanhong Zhao et al., 2013), it is imperative to investigate how this drug affects neuroinflammation, and by relation microglia which are the resident immune cells of the Central Nervous System(CNS). The results below show the level of gene expression for different pro-inflammatory molecules in the 3 major animal experimental categories: UCN, LCN and HCN SOD1^{G93A} mice.

A. Level of IL-1B expression in LCN SOD1^{G93A} mice
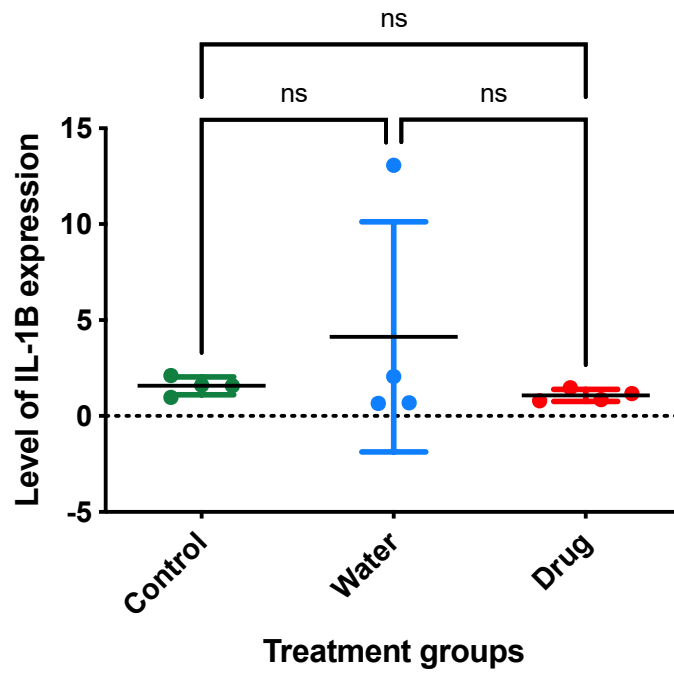
B. Level of IL-6 expression in LCN SOD1^{G93A} mice
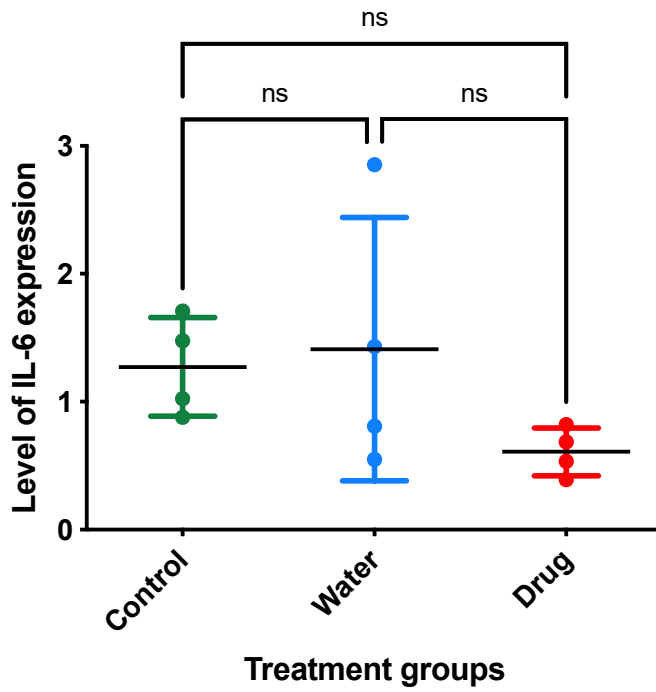
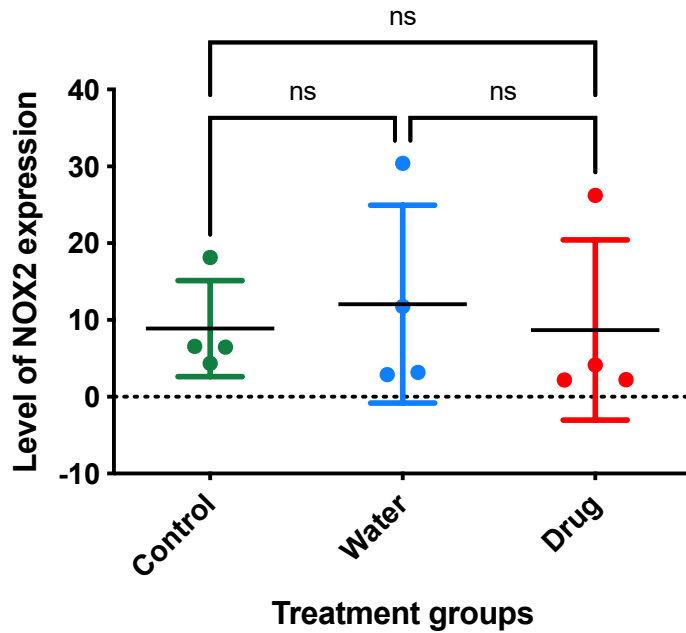C. Level of NOX2 expression in LCN SOD1^{G93A} mice

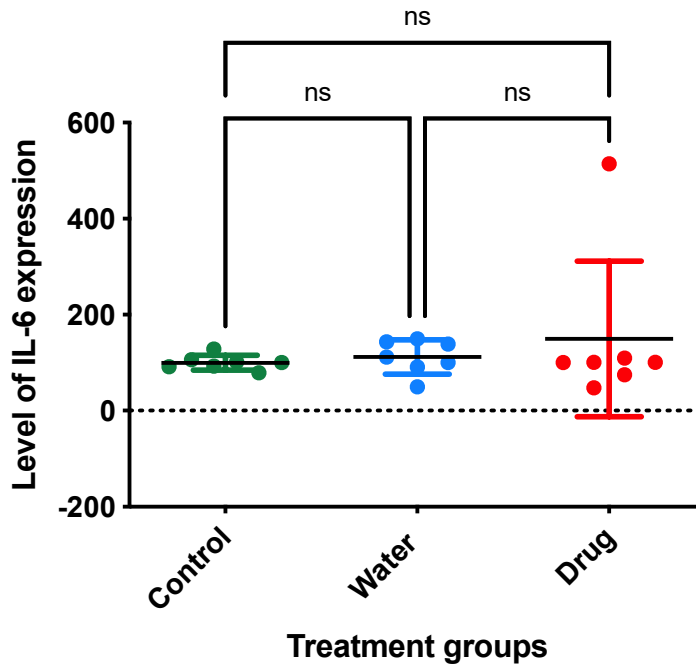**Figure 15: Levels of different gene expression in the LCN SOD1G93A mice groups treated with 60mg/kg dl-PHPB.**
Using qPCR technique, the level of gene expression for the genes mentioned were investigated across the three experimental groups. These are markers for pro-inflammation. Statistical significance calculated using One-Way ANOVA, followed by Tukey's Post-hoc test. There are no significant differences between the drug and water treated groups for all 3 genes checked. Level of IL-1B expression **(A),** Level of IL-6 expression **(B),** Level of NOX2 expression **(C)** in LCN SOD1^{G93A} mice. n= 4 for control, drug-treated and water-treated mice each.

There were no significant differences in pro-inflammation levels between water treated and 60mg/kg *dl*-PHPB drug treated LCN SOD1^{G93A} mice category (Fig. 15). These are similar results to what is reported in the IBA-1 expression levels of the same animal experimental category (Fig 14A), which likewise can be attributed to the low copy number and small sample size.

Figure 16 below investigates again, pro-inflammation markers, with the exception being that IL-1B is now replaced by TNF-α. The experimental category represented here are for those mice with HCN SOD1^{G93A} treated with 60mg/kg of *dl*-PHPB drug. There were no significant differences between treatment groups. However, significant differences were observed between the wild-type control and the mutant groups. In Figure 16 (B), there are significant differences between the wild-type control and the drug treated mutant group, and again between the wild-type control and the water treated mutant group. In Figure 16 (C), similar observations are made, with significant differences evident between the Wild-type control and the water treated mutant group. In both these figures, levels of TNF-α and NOX2 are significantly elevated in the mutant groups, whether treated with water or drug, compared to the wild-type control group.

These results show that levels of pro-inflammation, of the cytokines investigated are elevated in the diseased animal. However, it appears the drug may not have the desired effect of reducing the expression levels of the pro-inflammatory cytokines tested.

A. Level of IL-6 expression in HCN SOD1^{G93A} mice

B. Level of TNF-α expression in HCN SOD1^{G93A} mice
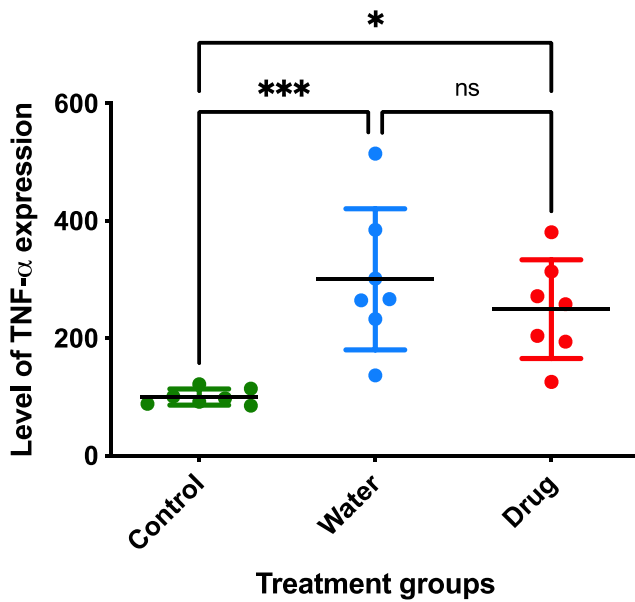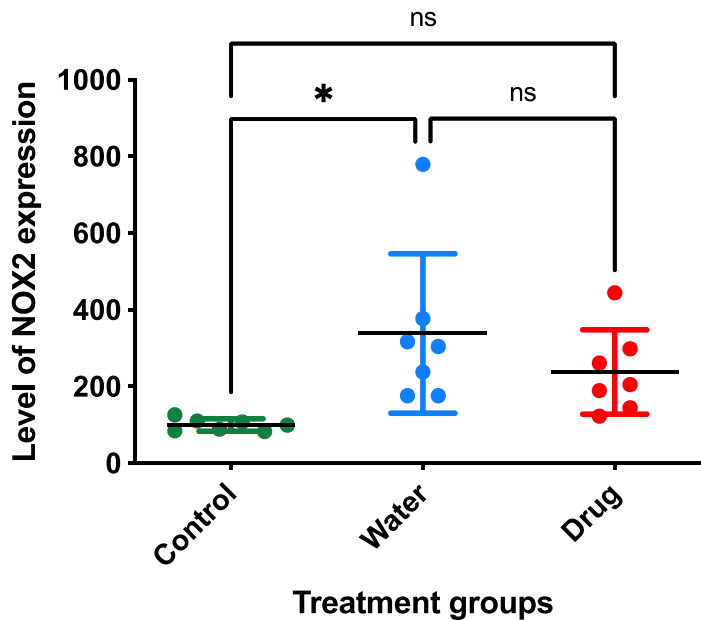
C. Level of NOX2 expression in HCN SOD1^{G93A} mice

Figure 16: Levels of different gene expression in the HCN SOD1G93A mice groups treated with 60mg/kg dl-PHPB
Using qPCR technique, the level of gene expression for the genes mentioned were investigated across the three treatment groups. These are markers for pro-inflammation. Statistical significance calculated using One-Way ANOVA, followed by Tukey's Post-hoc test. They were no significant differences between the drug and water treated groups for all 3 genes checked, though there was significant difference in the control group versus the treated mutant groups of images **(B)** and **(C)** only. Level of IL-6 expression **(A),** Level of TNF-α expression **(B),** Level of NOX2 expression **(C)** in HCN SOD1^{G93A} mice treated with 60mg/kg dl-PHPB. n= 7 for control, drug-treated and water-treated mice each. p <0.05 (*), p<0.001(***).