2017

# Investigating the Role of an SK Channel Activator on Survival and Motor Function in the SOD1-G93A, ALS Mouse Model

Matthew Thomas Dancy
*Wright State University*

# INVESTIGATING THE ROLE OF AN SK CHANNEL ACTIVATOR ON SURVIVAL AND MOTOR FUNCTION IN THE SOD1-G93A, ALS MOUSE MODEL

A thesis submitted in partial fulfillment
of the requirement for the degree of
Master of Science

By

MATTHEW THOMAS DANCY
B.S., Kent State University, 2013

2017
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

DATE OF DEFENSE
01/18/2017

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Matthew Thomas Dancy ENTITLED Investigating the role of an SK Channel Activator on Survival and Motor Function in the SOD1-G93A, ALS Mouse Model BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF Master of Science.

Sherif Elbasiouny, Ph.D., P.E.
Thesis Director

Eric S. Bennett, Ph.D.
Department Chair of
Neuroscience, Cell Biology and Physiology

Committee on
Final Examination

Sherif Elbasiouny, Ph.D., P.E.

Mark Rich, M.D., Ph.D.

Keiichiro Susuki, M.D., Ph.D.

Robert Fyffe, Ph.D.
Vice President for Research and
Dean of Graduate School

ABSTRACT

Dancy, Matthew Thomas. M.S., Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2017. Investigating the role of an SK Channel Activator on Survival and Motor Function of the SOD1-G93A, ALS Mouse Model.

Amyotrophic Lateral Sclerosis (ALS) is a fatal, adult-onset progressive degenerative motor neuron disease that is characterized by muscle atrophy and weakness due to the loss of upper and lower motor neurons. Average survival time for individuals diagnosed with the disease is three to five years; currently there is no cure and only one drug approved by the Food and Administration (FDA). Scientists have proposed various theories in order to solve the mystery which surrounds ALS. One of these theories hypothesizes how hyperexcitability and excitotoxicity leads to the death of motor neurons. In this study, we will address ways of combatting the effects of hyperexcitability as well as excitotoxicity by targeting a specific channel type. The channels in question are small conductance calcium activated potassium channels (SK channels). We chose to target these channels because they directly affect the medium after-hyperpolarization (mAHP) of the cell which controls firing rate. We postulate that SK channels are being altered in such a way that cell firing rate has been increased, leading to phenotypes associated with the disease such as abnormal excitability, mitochondrial dysfunction, axonal loss motor impairment, muscle atrophy as well as excitotoxicity, thus leading to the spread of motor neuron death. Upon administration with a specific SK channel activator in the form of CyPPA; improvements in motor function and survival were found. These improvements

suggest that SK channels are indeed viable drug targets and specific SK channel activators may be treatment options for individuals suffering from ALS.

TABLE OF CONTENTS

Page

INTRODUCTION ..... 1
Amyotrophic Lateral Sclerosis ..... 2
Hyperexcitability/Excitotoxicity and Motor Neuron Death ..... 4
Small Conductance Calcium Activated Potassium Channels (SK Channels) ..... 10
Transgenic Mouse Line SOD Mutation ..... 14
SK Channels in the SOD1 Model ..... 16
Drug Treatments ..... 19
Rationale ..... 22
Hypothesis ..... 22
MATERIALS AND METHODS ..... 23
Animals ..... 24
Drugs ..... 24
Behavioral Studies: Protocol and Criteria ..... 26
Survival Analysis ..... 28
Statistics ..... 31
RESULTS ..... 32
NEONATAL DRUG TREATMENTS ..... 33
Study 1 - 16 Day Treatment of CyPPA starting at P5 – P20 ..... 34
Study 2 - 16 Day Treatment of Riluzole starting at P5 – P20 ..... 43
CLINICALLY RELEVANT STUDIES ..... 51
Study 3 - Daily Treatment of CyPPA Starting at P90 – Death ..... 52
Study 4 - 7 Day Treatment of CyPPA starting at P90 – P96 ..... 60
DISCUSSION ..... 70
REFERENCES ..... 79

TABLE OF FIGURES

PAGE

Figure 1: Excitotoxicity and Motor Neuron Death ..... 8
Figure 2: Mechanism of the Mitochondrial Apoptotic Pathway ..... 9
Figure 3: SK Channel Mechanism ..... 13
Figure 4: Rotarod ..... 28
Figure 5: Assessment Criteria ..... 29
Figure 6: Motor Function – 16 Day Treatment of CyPPA Starting at P5 – P20 ..... 37
Figure 7: Rate of Motor Function Loss – 16 Day Treatment of CyPPA starting at P5 – P20 ..... 38
Figure 8: Final Failure – 16 Day Treatment of CyPPA starting at P5 – P20 ..... 39
Figure 9: Final Failure (Kaplan Meier) – 16 Day Treatment of CyPPA starting at P5 – P20 ..... 40
Figure 10: Survival – 16 Day Treatment of CyPPA starting at P5 – P20 ..... 41
Figure 11: Survival (Kaplan-Meier) – 16 Day Treatment of CyPPA starting at P5 – P20 ..... 42
Figure 12: Motor Function – 16 Day Treatment of Riluzole starting at P5 – P20 ..... 45
Figure 13: Rate of Motor Function Loss – 16 Day Treatment of Riluzole starting at P5 – P20 ..... 46
Figure 14: Final Failure – 16 Day Treatment of Riluzole starting at P5 – P20 ..... 47
Figure 15: Final Failure (Kaplan-Meier) – 16 Day Treatment of Riluzole starting at P5 – P20 ..... 48
Figure 16: Survival: 16 Day Treatment of Riluzole starting at P5 – P20 ..... 49
Figure 17: Survival (Kaplan-Meier) 16 Day Treatment of Riluzole starting at P5 – P20 ..... 50
Figure 18: Motor Function – Daily Treatment of CyPPA starting at P90 - Death ..... 54
Figure 19: Rate of Motor Function Loss – Daily Treatment of CyPPA starting at P90 - Death ..... 55
Figure 20: Final Failure – Daily Treatment of CyPPA starting at P90 – Death ..... 56
Figure 21: Final Failure (Kaplan-Meier) – 16 Day Treatment of CyPPA starting at P90 – Death ..... 57
Figure 22: Survival – Daily Treatment of CyPPA starting at P90 – Death ..... 58
Figure 23: Survival (Kaplan-Meier) - Daily Treatment of CyPPA tarting at P90 – Death ..... 59
Figure 24: Motor Function – 7 Day Treatment of CyPPA starting at P90 – P96 ..... 63
Figure 25: Rate of Motor Function Loss – 7 Day Treatment of CyPPA starting at P90 – P96 ..... 64
Figure 26: Final Failure – 7 Day Treatment of CyPPA starting at P90 – P96 ..... 65
Figure 27: Final Failure –7 Day Treatment of CyPPA starting at P90 – P96 ..... 66
Figure 28: Survival – 7 Day treatment of CyPPA starting at P90 – P96 ..... 67
Figure 29: Survival (Kaplan-Meier) – 7 Day Treatment of CyPPA starting at P90 - P96 ..... 68

# LIST OF TABLES

PAGE

Table 1: Drugs used during testing, along with their structure and interactions. ..... 25

Table 2: Rotarod Protocol ..... 28

Table 3: Comparison of All Drug Studies ..... 69

LIST OF ABREVIATIONS

AHP – After Hyperpolarization

ALS – Amyotrophic Lateral Sclerosis

α-MN – Alpha Motor Neuron

AP – Action Potential

BK – Large Conductance Calcium Activated Potassium Channel

CAM – Calmodulin

CMBD – Calmodulin Binding Domain

EATT2 - Excitatory Amino Acid Transporter 2

fALS – Familial ALS

FDA – Food and Drug Administration

FP – Fasciculation Potential

IK – Intermediate Conductance Calcium Activated Potassium Channel

LMN – Lower Motor Neuron

mAHP – Median After Hyperpolarization

MT - Mutant

MN – Motor Neuron

nAChrs– Nicotinic Acetylcholine Receptors

NMDA – N-Methyl-D-aspartic acid

sALS – Sporadic ALS

SK Channel – Small Conductance Calcium Activated Potassium Channel

SK1, SK2, SK3 – Isoforms of SK channels

SMA – Spinal Muscular Atrophy

SOD1 – Cu/Zn Superoxide Dismutase Enzyme 1

UMN – Upper Motor Neuron

ACKNOWLEDGEMENTS

I would like to begin by thanking my advisor, Dr. Sherif Elbasiouny, for his support, expertise and knowledge. He gladly welcomed me into his lab, sharing his experiences along the way and revealing to myself along with my other lab mates the amount of patience, hard work and dedication it takes to be a researcher. Furthermore, I would also like to thank Teresa Garrett. I can't imagine how difficult it must be managing a lab of this size but somehow she pulls it off with ease. If you are struggling with anything, she is always there to lend a hand and keep you motivated and her work ethic is out of this world.

I would also like to thank all the members of the NERD Lab, both past and present. In one way or another, each one of you has helped me in more ways than you can ever imagine. A special thanks goes out to all of the undergraduate students who have helped me complete these studies. Your help was tremendous and I probably could not have finished these tasks without you.

Thank you to my committee members, Dr. Mark Rich and Keiichiro Susuki, for their guidance, insight and patience. I would also like to thank Dr. Larry Ream, for giving me a chance and allowing me entrance into this program. The time spent at Wright State has been quite rewarding and I have learned a tremendous amount of knowledge regarding myself and research as a whole. I would like to thank the Department of Neuroscience, Cell Biology & Physiology for the breadth of knowledge and for the opportunity to expand my research experience. Finally, I would like to thank my parents, family, and friends for their continuous support. Bow Tie Friday's will live on…

# INTRODUCTION

## Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a fatal, adult-onset neurodegenerative disease that affects upper and lower motor neurons causing weakness, paralysis, and eventual death (Bruijn, Miller, & Cleveland, 2004). In ALS, cortical and spinal motor neurons (MNs) degenerate progressively (Rowland & Shneider, 2001). Survival time for ALS patients is 3-5 years and currently there is no cure (Hirtz et al., 2007). Patients diagnosed with the illness initially suffer from muscle weakness and tremors that later progress to paralysis (Byrne et al., 2011). Roughly 90% of all ALS cases are of an unknown etiology and are classified as sporadic (SALS) (Simpson & Al-Chalabi, 2006). While the remaining cases are considered familial (FALS) (Gros-Louis, Gaspar, & Rouleau, 2006). FALS is inherited in a dominant manner and approximately 20% of these are linked to mutations of Cu/Zn superoxide dismutase type-1 enzyme (SOD1) (Rosen et al., 1993). The clinical similarity between familial and sporadic cases of ALS indicates that although etiology may be multifactorial, different disease-triggering factors converge on a common pathway of MN death.

To combat MN death drugs have been administered as potential treatments to stave off disease related symptoms. Riluzole is currently the only drug FDA approved to treat ALS (Worldwide ALS, 2015). It is known for having many actions which include, targeting TTX sensitive $Na^+$ channels, inhibiting kainate and N-Methyl-D-aspartic acid (NMDA) receptors and activating SK channels (Dimitriadi et al., 2013; Gurney et al., 1996). This non-specific drug, only produces modest increases in survival of up to 2 – 3 months in ALS patients (Bensimon, Lacomblez,

Meininger, & Group, 1994; R. G. Miller et al., 1996; R. Miller, Mitchell, Lyon, & Moore, 2002) while also producing slight extensions (~10 days) of survival in SOD1 mice (Bellingham, 2011; Gurney et al., 1996). Interestingly little to no changes in motor function have been reported with treatments of Riluzole (Gurney et al., 1996).

The lack of available treatments for individuals suffering from ALS makes it important for scientists to test various therapeutic avenues with the hopes of ameliorating the phenotypes ALS presents. Data compiled from computational modeling in our lab predicted a downregulation of SK channel conductance. Immunohistochemistry experiments in our lab confirmed this prediction by showing a decrease in SK channel cluster area and density in mutant MNs as compared to WT. This data suggested to us that SK channel could be a novel drug target in ALS that if activated may suppress disease related symptoms. Pharmaceutical activation with an SK channel could allow for the reduction of glutamate-induced excitotoxicity as well as cell hyperexcitability, allowing the preservation of neurons; thereby leading to enhanced motor function and prolonged survival in ALS.

**Hyperexcitability/Excitotoxicity and Motor Neuron Death**

Glutamate induced excitotoxicity is the concept of neural degeneration caused by the over-stimulation of glutamate receptors (Olney, Mcgeer, & Mcgeer, 1978). Glutamate excitotoxicity and subsequent MN death has been increasingly recognized as an important pathophysiological factor in ALS. In addition, reduced expression of the glutamate transporter, excitatory amino acid transporter 2 (EAAT2), has been reported in the SOD1 mouse model and in the spinal cord of ALS patients (Philips & Robberecht, 2011; Rothstein, 1996). Increased expression of glutamate receptors permeable to influx of $Na^+$ and $Ca^{2+}$ ions has been reported on MNs in ALS, thus increasing susceptibility to glutamate toxicity (Williams et al., 1997). Ultimately, an influx of $Ca^{2+}$ ions through NMDA receptors occurs, resulting in increased intracellular $Ca^{2+}$ concentration and activation of $Ca^{2+}$-dependent pathways that mediate neuronal death (Choi, 1987; Meldrum & Garthwaite, 1990) (Figure 1, A & B). It has been suggested that neuronal cell death by excitotoxicity can be attributed to either apoptosis or necrosis (Ientile et al., 2001).

Calcium plays a vital role in the cell signaling pathway which leads to the apoptotic cell death of MNs (Figure 2). Calcium is usually free and unbound in the synaptic cleft after being released from presynaptic vesicles. Once glutamate has bound to the NMDA receptors on the postsynaptic neuron, $Ca^{2+}$ is free to enter the cell (Figure 2A). Once inside the cell $Ca^{2+}$ enters the endoplasmic reticulum (ER) via the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) pump (Joseph & Hajnóczky, 2007; Mattson & Chan, 2003). Enhanced translocation of $Ca^{2+}$ from the ER to the

mitochondria results in mitochondrial $Ca^{2+}$ overload. Initiating the release of numerous apoptotic proteins into the cytoplasm, one of which is known as cytochrome C (CYT C) (Joseph & Hajnóczky, 2007) (Figure 2B). Coincidently, enhanced $Ca^{2+}$ efflux from the ER can also be promoted by CYT C, by directly activating IP3 receptors which allow increased efflux of $Ca^{2+}$ from the ER (Mattson & Chan, 2003). In time mitochondrial $Ca^{2+}$ overload will induce swelling of the mitochondria leading to disturbances and eventual mitochondrial rupture. Once ruptured, the release of apoptotic factors enters the cytosol. CYT C together with apoptosis protease activating factor 1 (APAF-1), forms "apoptosome" which results in the activation of procaspase-9, and in turn activation of effector caspases (caspases-3, -6, and -7) ultimately leading to eventual cell death (Pinton, Giorgi, Siviero, Zecchini, & Rizzuto, 2008) (Figure 2C). Execution of mitochondria-related apoptosis can be connected with mitochondrial dysfunction, increased reactive oxygen species (ROS) production, and alterations to $Ca^{2+}$ homeostasis; all of these pathologies are observed in the ALS phenotype which eventually leads to MN degeneration (Mattiazzi et al., 2002; Pinton et al., 2008).

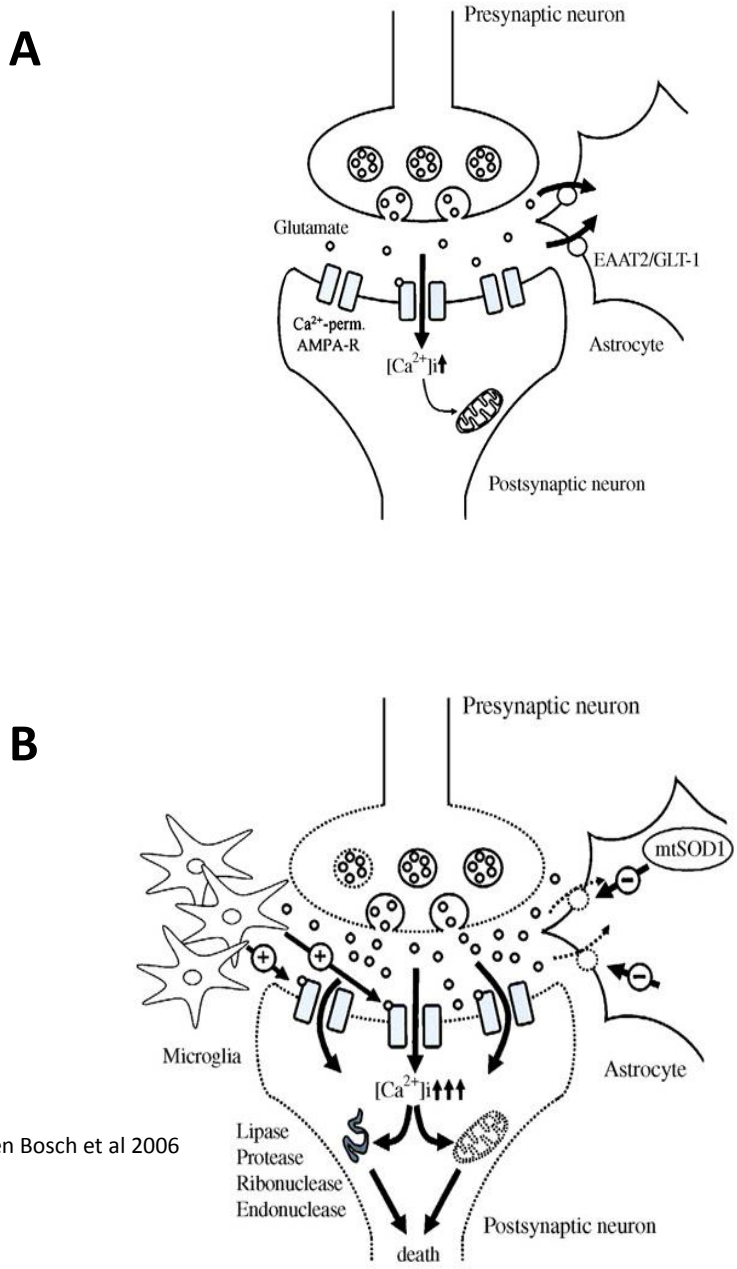
All voluntary movement relies on lower motor neurons (LMNs), which innervate skeletal muscle fibers and act as a link between upper motor neurons (UMNs) and muscles (Bear, Connors, & Paradiso, 2007). UMNs originate in the motor region of the cerebral cortex; they give rise to descending tracts that terminate on interneurons or MNs in cranial nerve motor nuclei or in spinal cord gray matter (Fletcher, 2012; Saladin, Sullivan, & Gan, 2015). LMNs are located in either the anterior grey column and anterior nerve roots (spinal MNs) (Fletcher,

2012). Eisen et al 1992 (Eisen, Kim, & Pant, 1992) suggested that increased excitability might be a mechanism causing both UMN and LMN degeneration in ALS (Plaitakis & Caroscio, 1987).

Excitability may be defined as the "capacity to be activated by or react to a stimulus" ("Excitable.," n.d.). Thus, hyperexcitability means an increased or exaggerated response to a stimulus. An example of hyperexcitability in denervated skeletal muscle and ALS occurs in the form of fasciculations (Mamede de Carvalho & Swash, 2016; Neelands, Herson, Jacobson, Adelman, & Maylie, 2001). Fasciculations are brief, spontaneous contractions affecting a small number of muscle fibers, often causing a flicker of movement under the skin (Oxford Dictionaries, n.d.). Fasciculation and cramp are commonly observed clinical features in ALS and are considered to be representative features of LMN hyperexcitability (Mamede De Carvalho & Swash, 2013). A hallmark characteristic of the disease, fasciculation potentials (FPs) have been shown to precede the disease by many months and the presence of FPs is commonly used as a clinical diagnostic feature (M. De Carvalho & Swash, 2004; T. M. Li, Alberman, & Swash, 1988). With disease progression, FPs become complex and unstable (Mamede de Carvalho & Swash, 2016), and in 2014 and 2016 Shimizu et al and Bokuda et al concluded that a high frequency of FPs correlated with increased disease severity (Bokuda et al., 2016; Shimizu et al., 2014). It seems that early identification of a hyperexcitable motor system might help in the predication of the development of ALS and, may provide a chance to make some early "neuroprotective" intervention prior to disease onset (Mamede de Carvalho, 2011). Interestingly, Shibuya et al 2011 suggested that fasciculations in

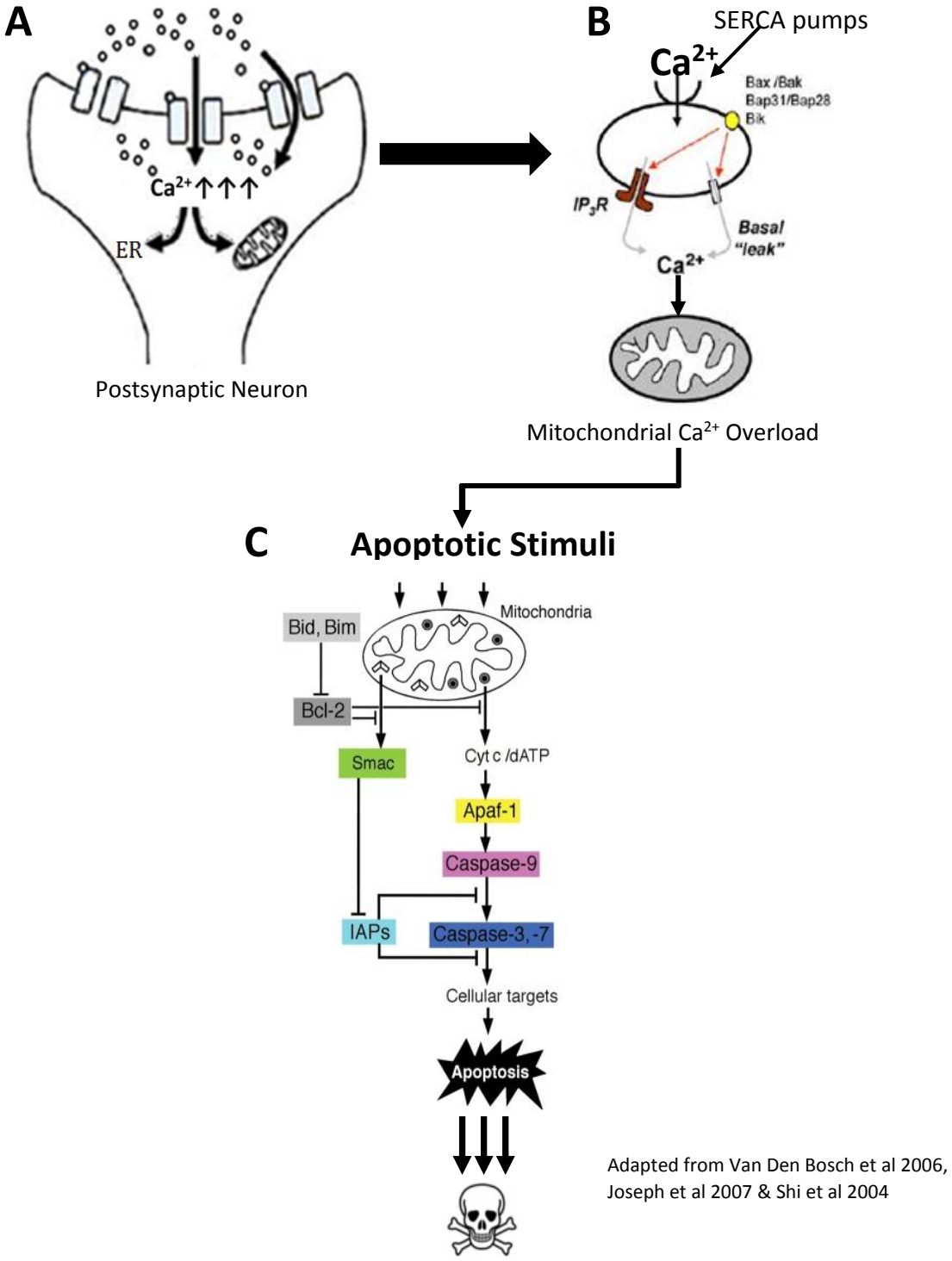
ALS are caused by reduced potassium channel clusters (Shibuya et al., 2011). A reduction of these clusters would cause decrease $K^+$ conductance increasing excitability. However, increasing $K^+$ conductance by activating channels that directly affect the AHP could also allow for the reduction of a hyperexcitable state.

There is evidence that hyperexcitability and excitotoxicity play key roles in the disease mechanism and Riluzole is used to suppress the effects of both of these pathologies (Van Den Bosch, Van Damme, Bogaert, & Robberecht, 2006). However, a number of concerns regarding therapeutic effect still remains regarding Riluzole. Patients treated with Riluzole only see slight extensions of survival, usually only by a few months (2 – 3) and previous studies have shown no improvements in motor function in SOD1 mice. (Gurney et al., 1996; Lacomblez, L., Bensimon, G., Meininger, V., Leigh, P., & Guillet, 1996; R. Miller et al., 2002). Riluzole has numerous targets, most of which are ion channels. This suggests that the disease may be caused by a type of channelopathy. If this is the case, discovering affected channel(s) would improve our understanding of the disease while also leading to potential therapeutic targets. Targeting a channel type which directly affects cell excitability could allow for potential MN protection leading to a novel treatment.




Adapted from Van Den Bosch et al 2006

Figure 1: Excitotoxicity and Motor Neuron Death
A. Under normal conditions, the glutamate released by the presynaptic neuron stimulates the $Ca^{2+}$-permeable AMPA receptors present in the postsynaptic motor neuron. This leads to a moderate, non-toxic increase of the cytoplasmic $Ca^{2+}$ concentration (Van Den Bosch et al 2006). B. Damage to the EAAT2 transporter can be caused due to the presence of mutant (mt) SOD1. Moreover, activated microglial cells can secrete substances that increase the glutamatergic stimulation of the postsynaptic neuron. A further increase in these factors leads to an increase in the cytoplasmic $Ca^{2+}$ concentration in the postsynaptic neuron. This leads to the generation of toxic reactive oxygen species in the mitochondria. Once the buffering capacity of the mitochondria is exceeded, intracellular $Ca^{2+}$ over-activates $Ca^{2+}$ dependent enzymes. This interferes with normal neuronal functionality leading to neuronal death and ultimately the spread of neurodegeneration (Van Den Bosch et al 2006).




Adapted from Van Den Bosch et al 2006, Joseph et al 2007 & Shi et al 2004

Figure 2: Mechanism of the Mitochondrial Apoptotic Pathway
A. Binding of glutamate to the NMDA receptors and free $Ca^{2+}$ entering the postsynaptic neuron. B. $Ca^{2+}$ enters the ER and mitochondria. C. Apoptotic pathway and death.

**Small Conductance Calcium Activated Potassium Channels (SK Channels)**

Small conductance calcium activated potassium channels (SK channels) are widely expressed in the central nervous system as well as α-MNs (Adelman, Maylie, & Sah, 2012; Deardorff et al., 2013). SK channels mainly contribute to the after hyperpolarization potential (AHP) following action potentials (APs) and suppress firing rate (spike frequency) of neurons (Mendelowitz, 1996). This effect allows for a regular firing pattern and a reduction in spontaneous activity preventing hyperexcitability. SK channels are so named because they have a small conductance of approximately 10–14 pS (Blatz, A. L., & Magleby, 1986). SK channels share the same tetrameric architecture with voltage-dependent potassium channels. Each subunit is composed of six transmembrane α-helical domains that are denoted S1-S6. A loop between the S5 and S6 transmembrane domains forms the potassium ion selectivity filter (Cui, Qin, Yu, Bowers, & Zhang, 2014). Most potassium selective channels are voltage gated, however SK channels become modified due to their response to elevated $Ca^{2+}$ levels. They are highly sensitive to calcium, being activated in the sub-micromolar range (Kohler et al., 1996) The $Ca^{2+}$ binding protein calmodulin (CaM) is constitutively associated with SK channels at the CaM-binding domain (CaMBD). The CaMBD is located in the proximal C-terminus of the channel subunit (Wissmann et al., 2002). $Ca^{2+}$ binding to CaM induces conformational changes in both CaM and CaMBD that subsequently trigger an opening of the channel pore, (Schumacher, Rivard, Ba, & Adelman, 2001) allowing potassium efflux (Figure 3). SK channels are activated by elevations in cytosolic $Ca^{2+}$ from several sources which include; voltage dependent N-type calcium (Cav1.3) channels or

through permeable agonist-gated ion channels, such as NMDARs and nicotinic acetylcholine receptors (nAChrs) (Schutter & Smolen, 1998).

In many neurons a single AP or bursts of APs are followed by a prolonged AHP. (Barrett & Barret, 1967). The AHP has several overlapping components, which include the fast, medium and slow AHPs. SK channels have been found to be present in most types of neurons, including MNs, and are responsible for the medium-duration postspike after hyperpolarization (mAHP) (McLarnon, 1995). Potassium efflux through SK channels hyperpolarizes the cell, modulating cell excitability (Lam, Coleman, Garing, & Wulff, 2013; Wulff & Köhler, 2013). The mAHP activates rapidly and decays over several hundred milliseconds. In many cases the mAHP is blocked by apamin, which increases excitability and identified that SK channels indeed alter cell firing rate. These apamin sensitive SK channels can be found in a wide variety of neurons including MNs. The contribution of SK channels to the AHP is due largely to their activation by $Ca^{2+}$ influx via Cav1.3 channels (Edgerton & Reinhart, 2003). In LMNs there are two functionally distinct SK channel populations. Specifically, ones that mediate the mAHP are activated by $Ca^{2+}$ influx through N- and P/Q-type $Ca^{2+}$channels (X. Li & Bennett, 2007). It has been suggested that activation of SK channels may elicit protection against glutamate-induced excitotoxicity because of the hyperpolarizing signal SK channels produce (Benítez et al., 2011; Dolga et al., 2008).

While SK channels have not been directly implicated in ALS, there has been evidence that they may be a target for study. By modifying the AHP conductance SK channels play an important role in regulating MN excitability. Elimination of SK

conductance abolishes the mAHP, increasing repetitive firing (X. Li & Bennett, 2007). Therefore, increasing SK conductance could decrease firing frequency in hyperexcitable cells. This signal would allow MNs to have a normalized firing rate, reducing spontaneous activity, alleviating metabolic demands and oxidative stress. In ALS, protection against these pathologies would likely lead to the survival of MNs, thus mitigating the symptoms of MN death which include paralysis and reduced motor output.

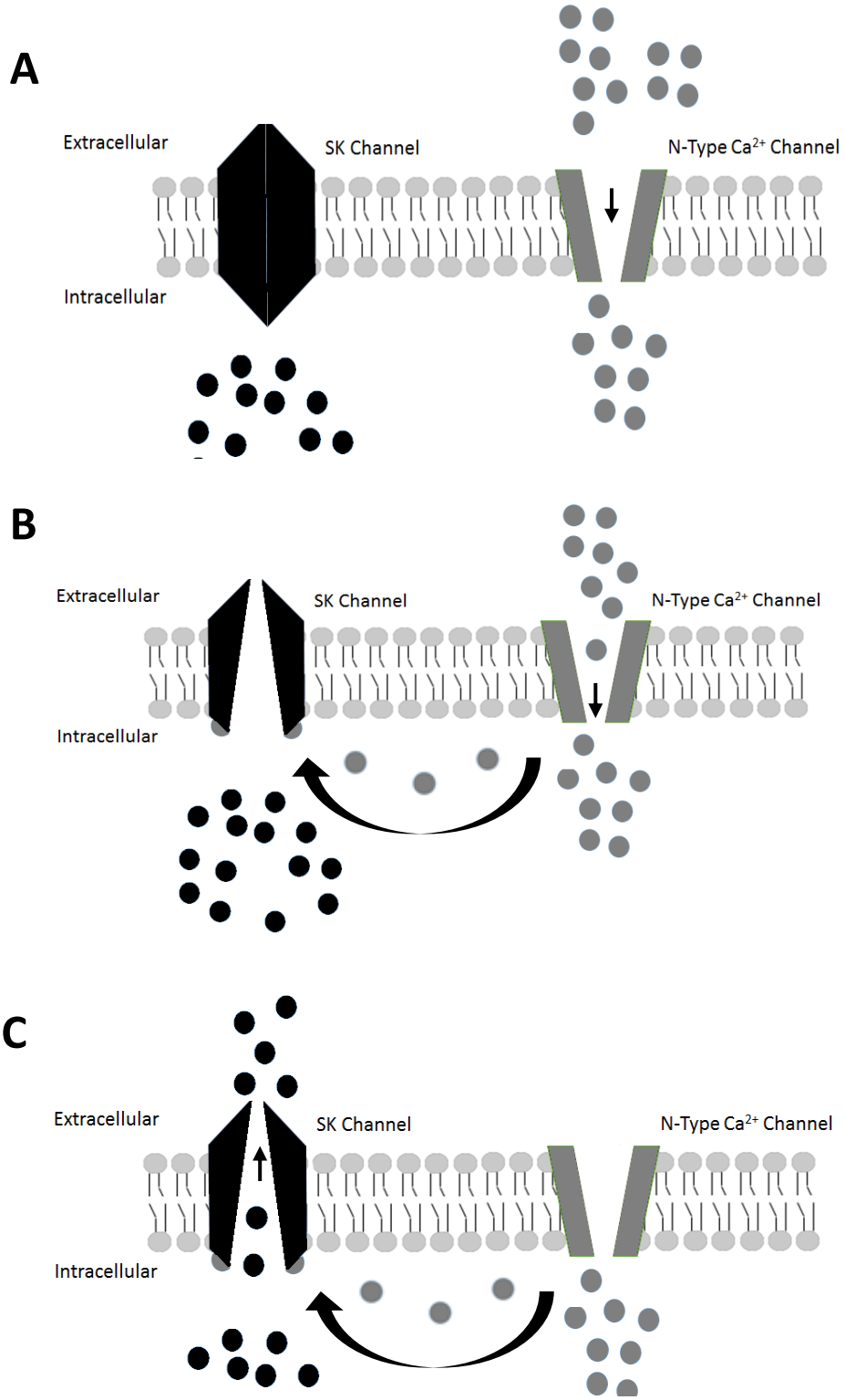



Figure 3: SK Channel Mechanism
A. N-Type $Ca^{2+}$ channel opens allowing an influx of $Ca^{2+}$ into the cell. B. $Ca^{2+}$ binding to CaM induces conformational changes in both CaM and CaMBD. C. This induces conformational changes in both CaM and CaMBD that triggers an opening of the channel pore allowing $K^{+}$ efflux. Activation of SK channels regulate cell firing and excitability. Black circles indicate potassium, gray circles indicate calcium

**Transgenic Mouse Line SOD Mutation**

To study ALS in the lab, it is common for animal transgenic lines to be utilized. The FUS, TDP-43, C9orf72 and the SOD1 mutations are the four main genes which are most commonly tested and each represents a form of FALS (Vajda et al., 2017). In our behavioral drug studies we will be using one of the SOD1 mutations, which are the most studied animal transgenic lines in the field.

SOD1 mutant lines exhibit a point mutation in the superoxidase dismutase 1 gene, which were generated by constitutively expressing mutant human SOD1 mini-genes in mice (Gurney et al., 1994). Mutations in the SOD1 gene are the most studied form of inherited ALS, accounting for ~20% of all the FALS forms (Rosen et al., 1993). Of all of the SOD1 mutant models, the G1H (high-copy line with 25 copies) line of the SOD1-G93A mutant murine strain is one of the most widely characterized and studied models of ALS currently available (Turner & Talbot, 2008). Mice overexpressing this form of mutant SOD1 develop a phenotype similar to that of ALS patients including motor impairment, axonal loss, MN death, muscle atrophy and limb weakness (Fischer et al., 2004). This transgenic line is the most aggressive form of the disease presenting a phenotype of early disease onset and rapid disease progression. Disease onset occurs in 3 months (90 days) and has a duration of only 1-2 months (125 days); (Chiu et al., 1995). The G93A-H transgenic line, contrasts with the low-copy G93A strain (G93A-L) which has a disease onset of 6-8 months (170 days), and duration of 2-3 months (225 days); (Gurney, 1997; Quinlan, Schuster, Fu, Siddique, & Heckman, 2011).

Early observations of abnormal glutamate metabolism and decreased glutamate transport in the brain and spinal cord of ALS patients and SOD1 mice led to the hypothesis that the excitatory amino acid neurotransmitter glutamate may be involved in the ALS pathogenesis (Plaitakis, 1990; Rothstein, Martin, & Kuncl, 1992). Therefore, drugs affecting the glutamatergic system were suggested as therapeutic agents to reduce cell firing. The reduction of firing rate of α-MN seems to be imperative for sustaining a healthy motor system. This can be accomplished by targeting specific channel types which alter the AHP in along with setting cell firing rate.

**SK Channels in the SOD1 Model**

Quinlan and colleagues reported a decrease in the AHP half-duration although no changes were found in the AHP amplitude of their neonatal; postnatal day 10 (P10) SOD1 mutant MNs using a slice preparation (Quinlan, 2011). Inhibition of SK channels has been shown as a source of neuromodulation of the AHP and has been shown to increase motor neuron firing rate(Herrik et al., 2012). AHP modulation has been linked to disease-related changes in the G93A model (Casas et al., 2013; Herron & Miles, 2012; Saxena et al., 2013). There is also computational evidence that reduction of the AHP may lead to persistent inward current facilitation, leading to further increases in cell excitability (Elbasiouny, Bennett, & Mushahwar, 2006). A reduction in AHP can cause enhanced firing rates, leading to the development of oxidative stress (Lancelot et al 1998) and an increased load on mitochondrial metabolism. Increased firing rates also contribute to disturbances of the cells ion metabolism (Heath & Shaw, 2002). Measures of the AHP (which are mediated partially by the SK) have been demonstrated to be significantly decreased in human patients with ALS (Piotrkiewicz & Hausmanowa-Petrusewicz, 2011). Piotrkiewicz et al 2011 monitored single motor unit potentials and found that patients with ALS showed increased accurances of motor unit firing and decreased AHP when compared to healthy individuals, thus suggesting an increase in excitability. Furthermore, a down regulation of SK (a decrease in SK conductance) was predicted in simulations of neonatal mutant SOD1 MNs (Elbasiouny laboratory, unpublished data). As a means of confirming this prediction, a later study which used immunohistochemistry to stain MNs and SK channels was

completed (Dukkipati, 2016). It was concluded that area and density of SK channel clusters were also reduced in SOD1-G93A mice. The decrease in these cluster properties may have altered channel functionality leading to changes in MN excitability.

Considering that computational modeling predicted a downregulation of SK conductance and this prediction was confirmed using immunohistochemistry. It was decided that CyPPA, a specific SK channel activator and Riluzole, a non-specific drug which also activates SK channels would be administered in our behavioral drug studies (Cao, Dreixler, Couey, & Houamed, 2002; Grunnet et al., 2001). We are hypothesizing that treatment with an SK channel activator would extend survival and improve motor function in the SOD1- G93A mouse model. The significance of these studies is that we can investigate whether SK channels are potential drug targets. We can also investigate whether the SK channel plays a vital role in the ALS disease mechanism and test if drug specificity is importatnt for therapeutic efectiveness.

With a channel type identified it was imperative to determine a proper time point for drug treatment. Many labs administer treatments with hopes of improving disease progression and survival at late time points, typically during symptom onset. Koschnitzky et al 2014 completed similar behavioral testing with Fluoxetine (Koschnitzky et al., 2014). Fluoxetine increases synaptic serotonin levels which also increases spinal MN excitability. It was reported that chronic treatments with Fluoxetine, administered from P70-end stage and P-30-endstage presented no

impact on disease progression (Koschnitzky et al., 2014). After finding no changes on disease progression a new 7 day dosing paradigm was then chosen at a neonatal treatment time point (P5 – P11). This time point was chosen because Amendola and Durand et al 2008 showed dendritic changes at this same age in SOD1 mice (Amendola & Durand, 2008). Koschnitzky and colleagues used this information and administered temporary neonatal drug treatments with Fluoxetine from P5-P11. Interestingly, this treatment paradigm produced significant impairments in motor behavior and a severe reduction in survival. They concluded that short term neonatal interventions can affect ALS disease progression, which supports the idea that developmental processes may contribute later in the disease (Koschnitzky et al., 2014).

**Drug Treatments**

In these studies we used two drugs which target SK channels. The first drug chosen was (N-cyclohexyl-2-(3,5-dimethylpyrazol-1-yl)-6-methylpyrimidin-4-amine) (CyPPA) a specific SK channel activator. CyPPA, is a small (MW: 285), trisubstituted pyrimidine that belongs to a completely different compound class from that of the prototype IK/SK channel activators 1-EBIO and NS309 (Hougaard et al., 2007). CyPPA is a positive modulator of SK but not intermediate conductance (IK) channels. It is thought that CyPPA act as an allosteric modulator of SK2/SK3 channels, which increases their sensitivity to activation by cytosolic $Ca^{2+}$ (Hougaard et al., 2007).

The use of CyPPA during *in vivo* preparations have produced pronounced increases in the duration of the mAHP (Herrik et al., 2012). These increases reduced neuron firing when compared to controls (Herrik et al., 2012). Moreover, inhibition of SK channels by apamin, an SK channel blocker leads to faster firing rates and increased instantaneous firing rates (Lorenzon & Foehring, 1992; Schwindt, Spain, Foehring, Chubb, & Crill, 1988). Apamin has also been shown to block the postspike AHP and abolishes the SK-mediated component of the AHP (Herrik et al., 2012; X. Li & Bennett, 2007). In other studies, oral administration of CyPPA has improved motor performance in the neurodegenerative disorder, Spinocerebellar ataxia type 2 (SCA2) and in a model of spinal muscular atrophy (SMA). (Dimitriadi et al., 2013; Kasumu et al., 2012). These findings suggest that CyPPA may be a possible treatment option for other neurodegenerative disorders such as ALS.

The second drug used in these experiments was Riluzole (2-amino-6-triflurомethoxy-benzothiazole) which is currently the only FDA approved drug to treat ALS (Gurney, Fleck, Himes, & Hall, 1998; Worldwide ALS, 2015). Riluzole was initially identified as a paralytic agent (Domino, Unna, & Kerwin, 1952) and was found to significantly improve muscle strength and disease progression in ALS patients (Bensimon et al., 1994; Lacomblez, L., Bensimon, G., Meininger, V., Leigh, P., & Guillet, 1996). Riluzole has a variety of pharmacologic actions, including inhibition of glutamate release from presynaptic nerve terminals (Martin, Thompson, & Nadler, 1993), modulation of the NMDA ionotropic receptor (Debono, Le Guern, Canton, Doble, & Pradier, 1993), stabilization of the voltage-dependent sodium channels, (Pratt et al., 1992) and inhibition of the high affinity uptake of λ-aminobutyric acid (GABA), a major inhibitory neurotransmitter (Mantz, Laudenbach, Lecharny, Henzel, & Desmonts, 1994).

In 1998 Gurney et al showed that treatment with Riluzole increases survival of adult G93A-H mice (Gurney et al., 1998). Gurney et al treated adult animals with Riluzole from P42 until death at an optimal dosage of 44mg/kg and saw a 10 day increase in survival (Gurney et al., 1998), interestingly no changes in motor function were shown in their behavioral drug studies (Gurney et al., 1996). Given that Riluzole has numerous targets which also includes SK channels (Cao, Dreixler, Couey, & Houamed, 2002; Grunnet et al., 2001), the mechanism of Riluzole protection remains unclear (Bellingham, 2011; Kuo, Siddique, Fu, & Heckman, 2005; Schuster et al., 2012). However, in 2013 Dimitriadi et al suggested that Riluzole was beneficial in two models of SMA and works via SK channels by improving

neuromuscular function (Dimitriadi et al., 2013). Considering that Riluzole acts via SK channels and has been shown to have some therapeutic effect by reducing excitability (Dimitriadi et al., 2013; Kuo et al., 2005). Perhaps treatment with CyPPA, a more potent and specific channel activator which modifies excitability by increasing the AHP could lead to increased motor output and prolonged survival in ALS mice.

## Rationale

A shortening of the α-MN mAHP has been noted directly in a mouse *in vitro* electrophysiological study (Quinlan et al., 2011) and indirectly in a study of human muscular electrical activity in ALS patients (Piotrkiewicz & Hausmanowa-Petrusewicz, 2011). A modeling prediction from Elbasiouny et al suggested that a downregulation of SK conductance is present in mutant neonatal SOD1 MNs (Elbasiouny lab, unpublished results) and Dukkipati et al confirmed this prediction using immunohistochemistry (Dukkipati, 2016).

## Specific Aims

1. Investigate whether the SK Channels is an effective drug target in an ALS mouse model.
2. Investigate whether SK channels play a major role in ALS pathogenesis.
3. Investigate whether drug specificity can affect therapeutic outcomes.

## Hypothesis

Treatment with a specific SK channel activator would extend survival and improve motor function of the SOD1-G93A ALS mouse model. Thus, it is hypothesized that treatment with a specific SK channel activator would extend survival and improve motor function of the SOD1-G93A ALS mouse model.

# MATERIALS AND METHODS

**Animals**

SOD1-G93A-H male mice were grouped housed in barrier facilities on a 12:12-h light-dark cycle. Food and water were provided ad libitum. All mice tested had a background of B6SJL-Tg (Jackson Laboratories, stock #002726) and were age matched littermates. All procedures were approved by the Wright State University LACUC. In these types of studies a minimum of twelve animals per group is recommended to ensure minimal variability (Scott et al., 2008). We will adhere to these guidelines and have at least twelve animals in each treatment group.

**Drugs**

CyPPA (Sigma-Aldrich, CAT No.5493) was administered via three separate dosing paradigms. Treatment of CyPPA was given to neonatal mice from P5 to P20 (CyPPA P5-P20), adult mice from P90 – P96 (CyPPA P90 7 Day) and a daily dosing paradigm starting a P90 and ending at death (P90 Daily study). During the neonatal dosing paradigm a 16 day treatment period was chosen because SK channels are coupled with N-type $Ca^{2+}$. Since N-type $Ca^{2+}$ channels mature around P18 (Inagaki & Lee, 2013), we thought overlapping drug administration with channel maturation during development would be key for therapeutic effects to become present. For all studies using CyPPA drug was administered through once daily injections (14mg/kg body weight, Intraperitoneal (IP)) or vehicle control (11% DMSO and saline – 16 Day treatment of CyPPA at P5 and 7 Day treatment of CyPPA at P90; 11% DMSO, 5% Kolliphore and Saline – Daily CyPPA treatment at P90) injections. During preliminary testing it was found that CyPPA resulted in healthy animals not completing the rotarod protocol 30 minutes after dosing. Animals receiving the drug

became lethargic and motor function was significantly reduced as they fell off the rotarod prematurely. These changes were present up to an hour after injection. However, during experimentation drug was administered after behavioral testing to avoid any motor performance changes caused by the drug.

Riluzole (MP Biomedicals, LLC, CAT No. 193713) was administered to neonatal mice from P5 – P20 (Riluzole P5-P20), Drug was administered through once daily injections (22mg/kg body weight IP) or vehicle control (0.1 Normal HCl, diluted in water (DDI)) injections. Riluzole concentration was determined by halving the optimal dosage reported in literature since higher dosages of 44mg/kg and 33mg/kg was too strong and would kill the neonatal mice.

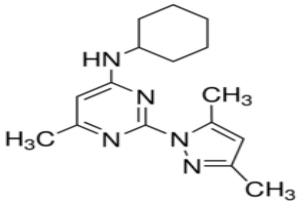
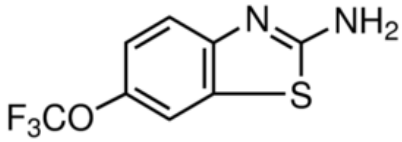
| Drug | Structure | Interactions |
|---|---|---|
| CyPPA<br>(N-cyclohexyl-2-(3,5-dimethylpyrazol-1-yl)-6-methylpyrimidin-4-amine) |  | EC50 = 6μM<br>Target: Activates KCA2.3, Inhibits $Na_V$ Channels<br>Half Life: Unknown<br><br>-Hougaard et al 2007 |
| Riluzole<br>(2-amino-6-trifluromethoxy-benzothiazole) |  | EC50 = 12μM<br>Targets: KCA2.3, TTX Sensitive Na+ channels, Inhibits kainate and NMDA receptors, BK Channels<br>Half Life: 12 Hours<br>FDA Approved<br><br>- Grant et al 2010 |

Table 1: Drugs used during testing, along with their structure and interactions.

**Behavioral Studies: Protocol and Criteria**

Behavior studies were performed every other day by individuals blinded to genotype and drug condition. Rotarod testing and data collection started at P85 for each drug study. Rotarod testing did not start at an earlier age because SOD and WT mice show no differences in motor ability until symptoms become present, typically around P90. Weight, motor function, end stage, first failure and final failure were monitored for all mice.

Animals received a period of 4 consecutive days of training on rotarod testing paradigm. The training period started at P72. During testing and training animals would run on the rotarod for a total of 240seconds. The rotarod was set at 5rpm for the first 30s, then increased to 10rpm to complete the first minute. The rotarod then increased to 15rpm for another 30s and increased to 20rpm for a full minute. To finish testing, the rotarod was set at its maximum rotation speed of 25 rpm for 90 seconds (Table 2). The fall time was recorded via photo beam break on the rotarod device (AccuRotor Multi-Animal Rotarod. Omnitech Electronics, Inc (Figure 4)). Mice ran three times with at least a five minute break between runs. The maximum time from each of the three trials was recorded to determine motor function. Maximum time spent on the rotarod was used to prevent daily motor performance variance. The age at which mice failed to complete the entire protocol (240s) for each of the three trials was recorded; this was considered First failure. Final failure was recorded when the animals failed to stay on the rotarod for <12s for each of the three trials. Final failure was representative of the animal completing <5% of the rotarod protocol and severe motor deficits being present (Figure 5).

Rate of Motor Function Loss was used to determine slope of rotarod decline. A ten percent drop from the baseline was used to ensure motor decline was present. The lower limit of the regression test was determined when animals reached an average of ten percent completion (Figure 5). This point was chosen due to the rapid disease progression seen in the model. We wanted to ensure our slope was a good representation of overall motor function.

Survival was determined when animals failed the righting reflex test. If they could not right themselves 30s after being placed on their back the animals were humanely euthanized.

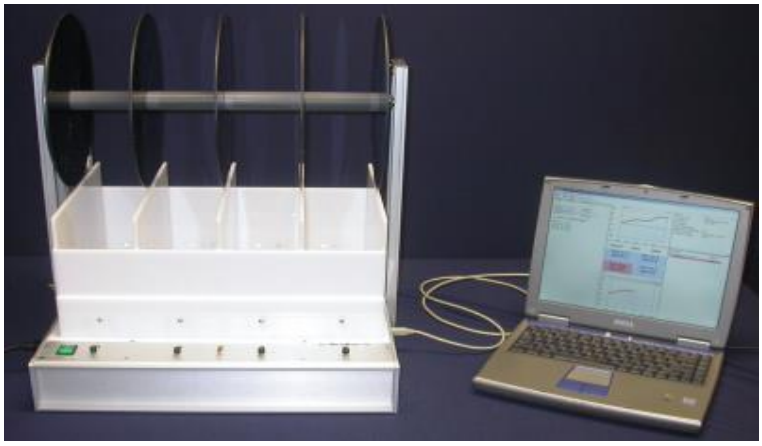


Figure 4: Rotarod
AccuRotor – Multi Animal Rotarod, Omnitech Electronics, Inc

| Rotarod Protocol | |
|---|---|
| Time (s) | Speed (rpm) |
| 0-30 | 5 |
| 30-60 | 10 |
| 60-90 | 15 |
| 90-150 | 20 |
| 150-240 | 25 |

Table 2: Rotarod Protocol

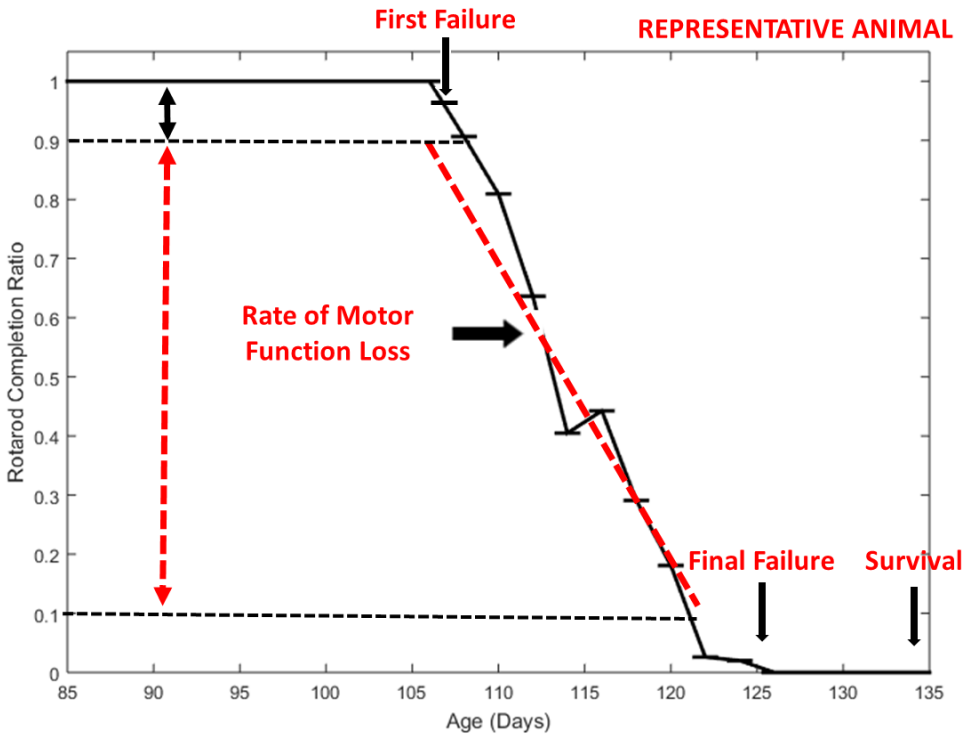



Figure 5: Assessment Criteria

First failure, time point when animals no longer fully complete the rotarod protocol. Rate of Motor Function Loss, Slope and linear fit of rotatod line. To determine slope, a baseline for motor function was determined. A ten percent drop from the baseline was instituted to ensure motor function decline was present. The lower limit of the regression test was determined when animals being tested reached an average of ten percent completion. Ten percent completion is equivalent to 24 seconds of time spent on the rotarod. This point was chosen due to the fact that many animals drop out of the study because of rapid disease progression. Also, we wanted to ensure our slope was a good representation of overall motor function. Final failure was recorded when animals failed to stay on the rotarod for <12s for each of the three trials. Final failure was representative of the animal completing <5% of the rotarod protocol and severe motor deficits being present. Survival was determined when animals failed the righting reflex test. At this point animals were humanely euthanized.

**Survival Analysis**

The Kaplan Meier Log Rank survival test was used to analyze final failure and overall Survival of the animals. The final failure time point is indicative of severe gross motor deficits and a lack of motor control. Survival represents the age when animals fail the righting reflex test. At this time point animals are humanely euthanized.

## Statistics

Values are represented as mean ± standard error of the mean. Data, including Kaplan Meier survival analysis was analyzed using Statistica 12. Student's t-test were used to analyze differences between first failure, final failure, survival and every day the animals ran. All data had normal distributions and equal variance. Kaplan Meier survival analysis was used to determine probability of survival for motor function and overall survival. The Mann-Whitney U test a nonparametric analysis was also used to analyze motor function data. Figures were made with Matlab R2015A. P-values less than 0.05 were considered statistically significant.

# RESULTS

# NEONATAL DRUG TREATMENTS

**Study 1 - 16 Day Treatment of CyPPA starting at P5 – P20**

Analysis of motor function was completed as described (Figure 6), n for the animals receiving CyPPA was 12 and n for the animals receiving the vehicle control animals was 13. It was found that animals receiving CyPPA presented no differences when testing first failure using a student's t-test ($p = 0.99$). First failure for both the treatment and the vehicle control groups occurred at P111. However, significant differences were found when testing motor function. Analysis of motor function was completed using a student's t-test by testing each day the animals ran. Animals receiving CyPPA showed sustained motor function as the disease progressed. Starting at P123 – P128 significant differences were found using a student's t-test (P123 - P128: $p \leq 0.0264$). Analysis of the Mann-Whitney U test also showed significant improvements in motor function between the treatment and control vehicle groups from P125 – P128 ($p \leq 0.019$). Overall, animals receiving CyPPA had significant improvements in motor function (Figure 6).

To assess motor function, a regression test was utilized to measure the slope of motor function decline (Figure 7). Comparison of the SOD-treated vs. the SOD-Control of the Regression test showed significant differences between the slopes for CyPPA and the Vehicle Control. Slope for animals receiving CyPPA was -0.0211, $R^2 = 0.85$, while the slope for the Vehicle Control animals was -0.0349, $R^2 = 0.74$ ($p = 0.007$).

As the study progressed, animal performance continued to drop. The next analysis completed tested final failure (Figure 8). Final failure is the age at which the animals stopped running on the rotarod and the presence of gross motor deficits

and a lack of motor control is very evident. Animals receiving CyPPA showed a significant 7% increase in final failure using a student's t-test ($p = 0.03$). Average final failure for animals receiving CyPPA occurred at P128, while animals receiving the vehicle control occurred at P120.

Final failure tracks "survival" of motor function and indicates when animals lose their ability to balance and coordinate themselves on the rotarod (Figure 9). Some labs use a functional time point like final failure as their "survival" criteria because they believe it may be a more sensitive gauge (Lutz et al 2009). This analysis in the form of a Kaplan-Meier curve shows the percentage of animals running over time. Significant differences were found using the Cox's proportional F-Test ($p = 0.03$), indicating that animals receiving CyPPA retain motor function longer as the disease advances.

In the latter stages of the study, disease progression is quite rapid and many animals succumb to the illness (Figure 10). The survival analysis is then completed on the animals. Survival is determined when animals fail the righting reflex test within a 30 second period. On average animals receiving CyPPA showed a significant 8% increase in survival using a student's t-test ($p = 0.0063$). On average animals receiving CyPPA lived 136 days while animals receiving the vehicle control lived 126 days (a 10 day extension in survival).

A Kaplan-Meier curve was generated using the survival data and showed significant differences for survival between the two groups (Figure 11). Significant differences were found using a Cox's Proportional F-Test ($p = 0.007$). The oldest

animal receiving CyPPA survived for 152 days which was nearly 20 days longer than the oldest Vehicle Control animal.

**Study 1 Conclusions**

Significant improvements in motor function and survival were found, suggesting a slower disease progression. Animals receiving the 16 day treatment of CyPPA presented extended motor function in the latter stages of the disease and an increased likelihood that motor function would persist based on the final failure analysis using the Kaplan Meier curve. Survival was also improved in the animals receiving CyPPA, this 10 day increase equated to nearly an 8% change overall.

These results show that SK channels could be a potential drug target and CyPPA may have therapeutic properties. These results also suggest that CyPPA is a potential treatment for SOD1 mutant mice. To further assess its effects, we compared CyPPA to Riluzole's effects on survival and motor function of SOD mice.

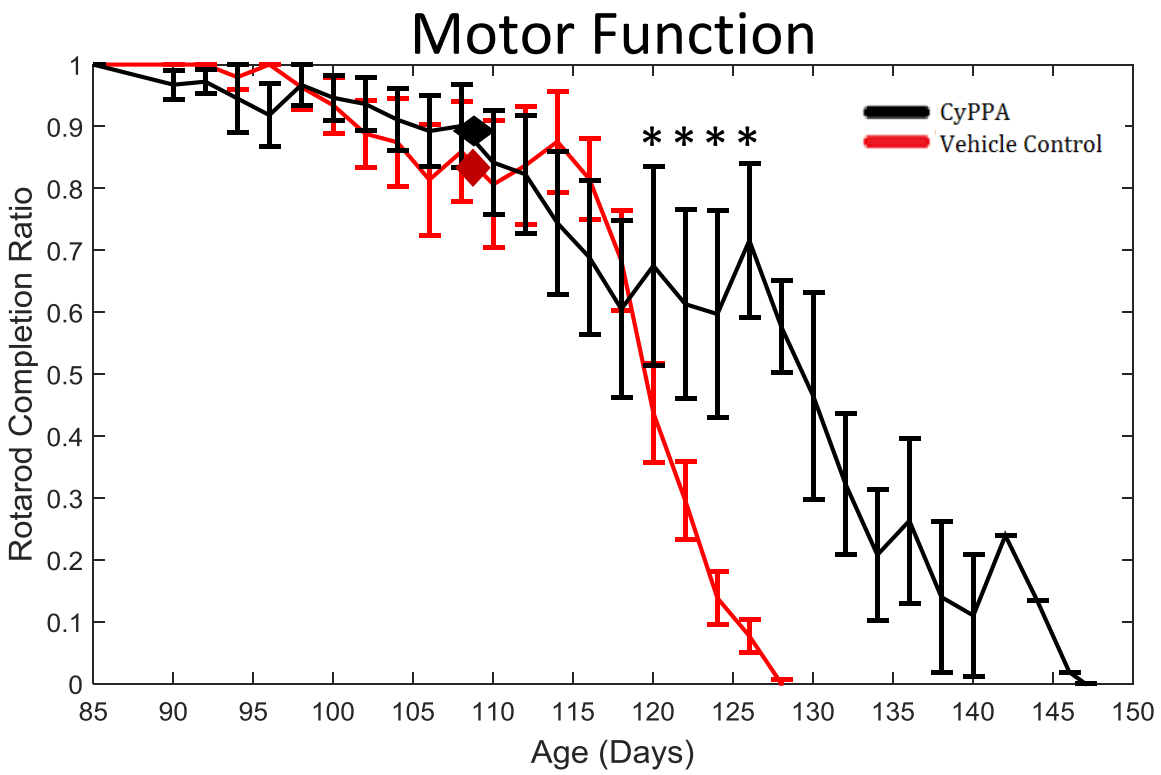



Figure 6: Motor Function – 16 Day Treatment of CyPPA Starting at P5 – P20
Graph showing average rotarod completion for animals in both treatment groups. Diamonds represent average first failure for each group being dosed. Average first failure for the animals receiving the vehicle control and CyPPA occurred at P111. No significant differences were present testing first failure. Motor function for animals receiving CyPPA was significantly different from P123 – P128 using the student's t-test and from P125 – 128 using the Mann-Whitney U test. Vehicle Control n = 13, CyPPA n =12; Data is Mean ± S.E.

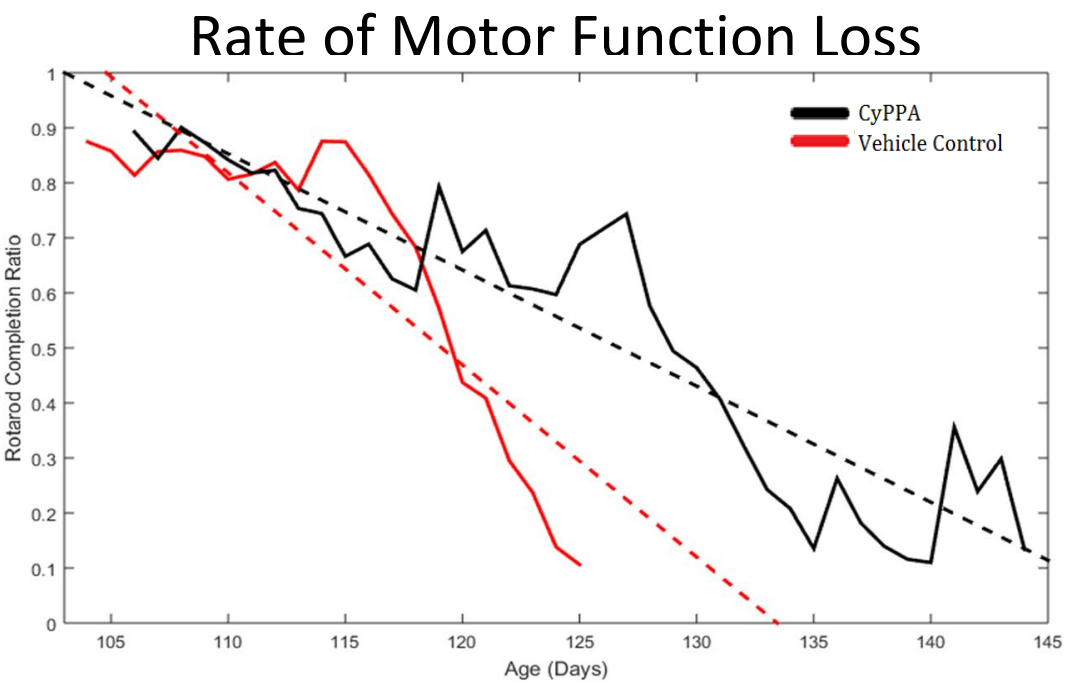



Figure 7: Rate of Motor Function Loss – 16 Day Treatment of CyPPA starting at P5 – P20
Slope for animals receiving CyPPA was -0.0211 while animals receiving the vehicle control was -0.0349. Using a regression test we determined that motor function decline was significantly different between the two groups, p-value 0.007. Vehicle Control n = 13, CyPPA n =12; Data is Mean ± S.E.

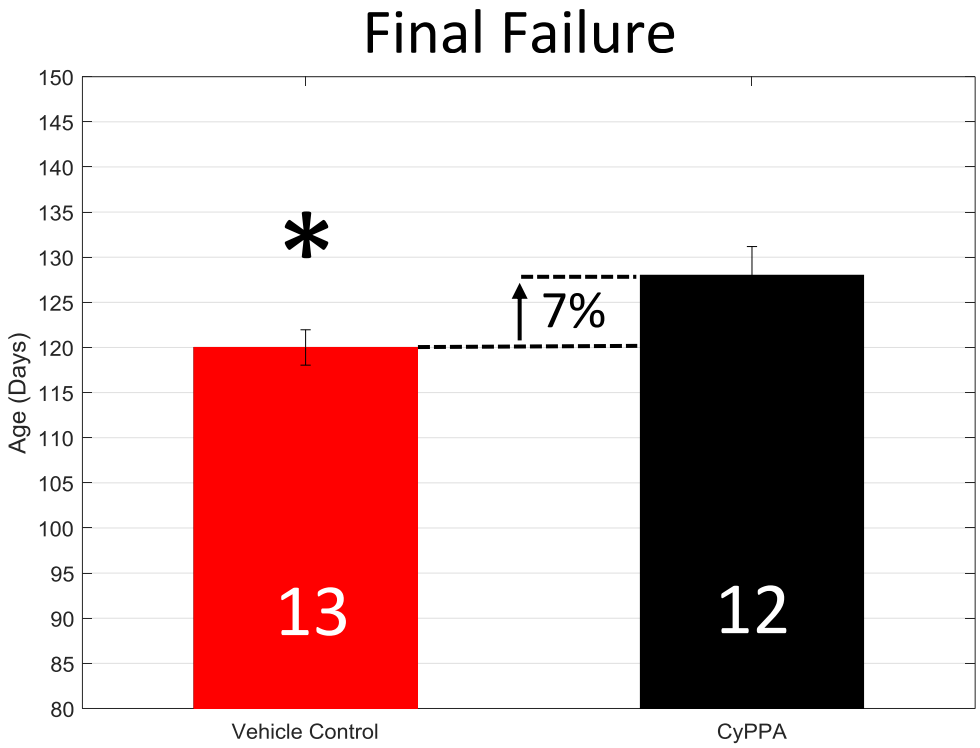



Figure 8: Final Failure – 16 Day Treatment of CyPPA starting at P5 – P20
Animals receiving CyPPA increased average final failure by 7%. Final Failure was significant when analyzed using a student's t-test ($p = 0.03$). Vehicle Control n = 13, CyPPA n =12; Data is Mean ± S.E.

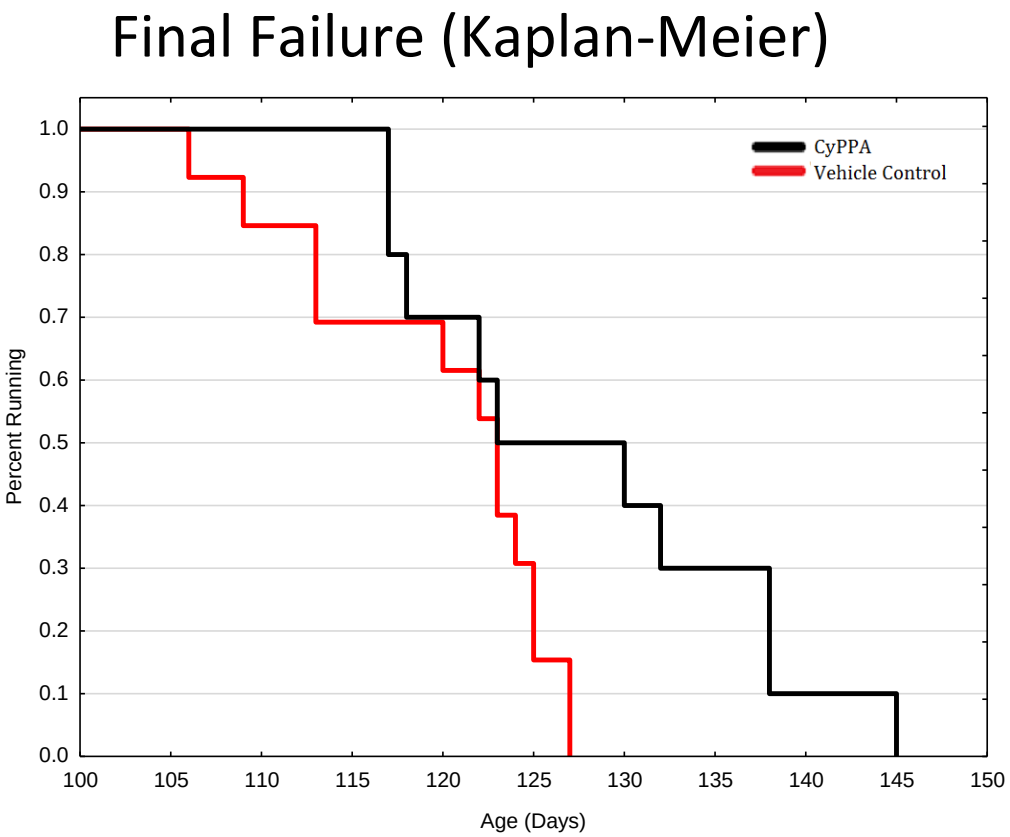



Figure 9: Final Failure (Kaplan Meier) – 16 Day Treatment of CyPPA starting at P5 – P20
Animals receiving CyPPA showed significant survival improvements testing motor function using Cox's proportional F-Test (p = 0.03). Vehicle Control n = 13, CyPPA n =12;

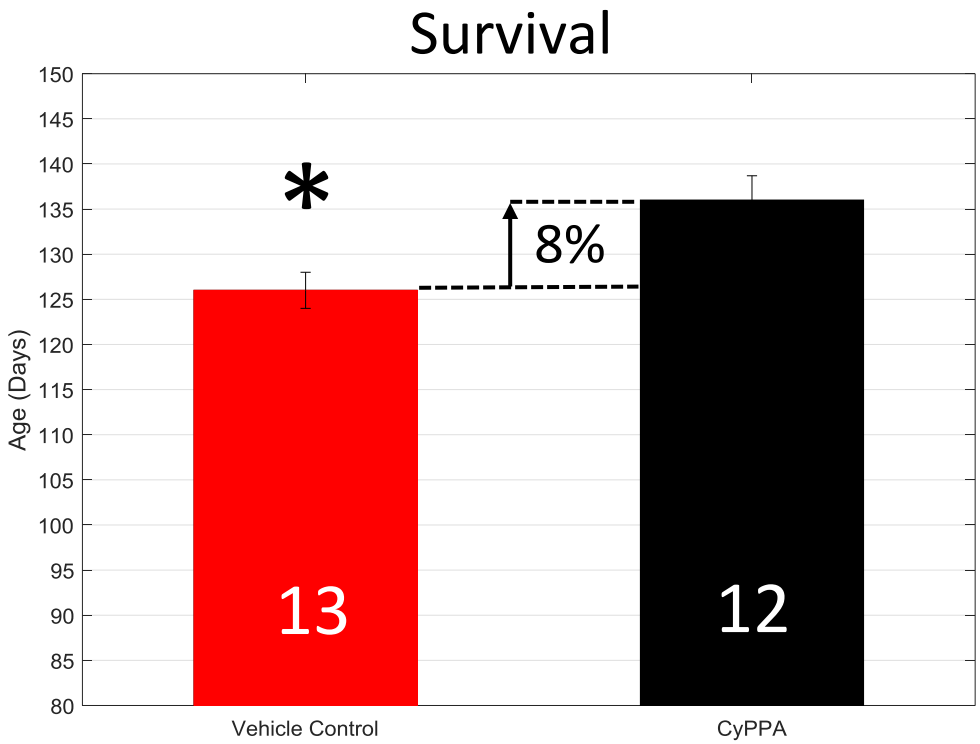



Figure 10: Survival – 16 Day Treatment of CyPPA starting at P5 – P20
Animals receiving treatment of CyPPA lived 10 days longer on average. Which equated to a significant 8% increase ($p = 0.006$). Animal's receiving CyPPA lived 136 days while animals receiving the vehicle control lived 126 days on average. Vehicle Control n = 13, CyPPA n =12; Data is Mean ± S.E.

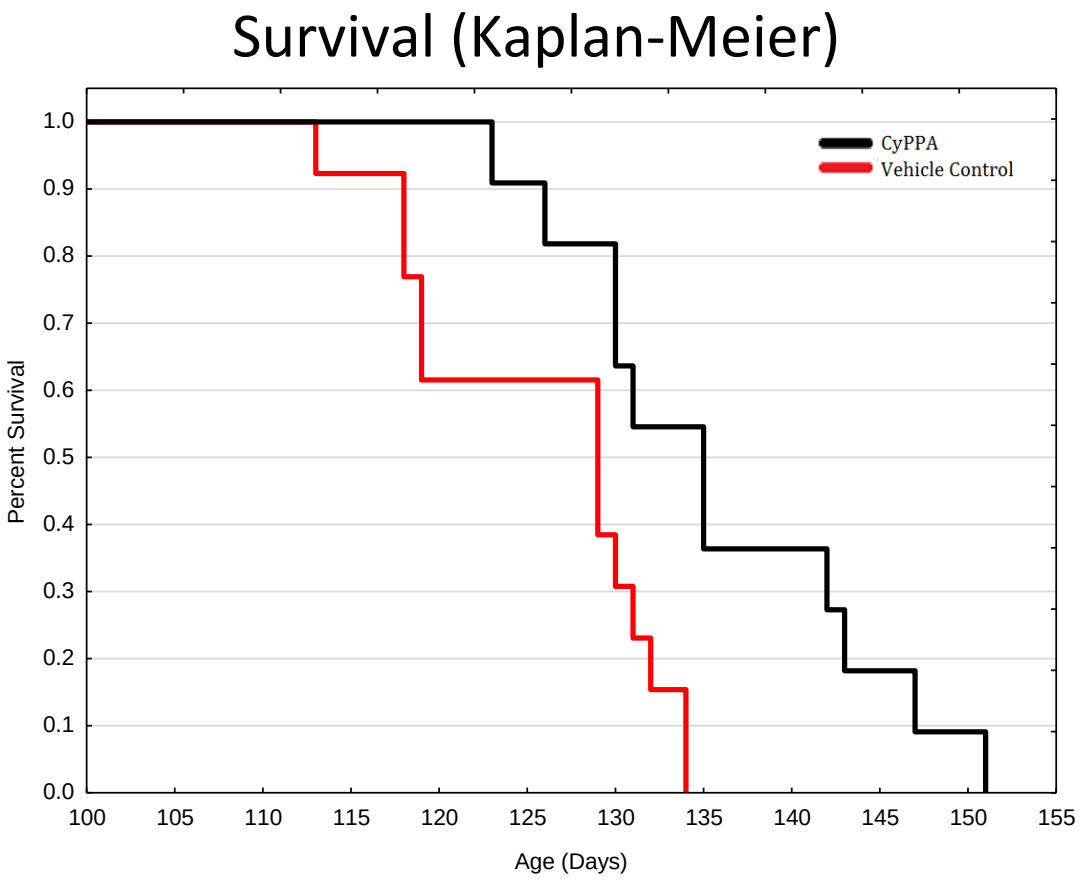



Figure 11: Survival (Kaplan-Meier) – 16 Day Treatment of CyPPA starting at P5 – P20
Animals receiving CyPPA showed significant extension in survival using Cox's proportional F-Test, p-value 0.007. The oldest CyPPA animal lived 152 days, nearly 20 days longer than the oldest Vehicle Control animal

**Study 2 - 16 Day Treatment of Riluzole starting at P5 – P20**

Riluzole was administered via once daily intraperitoneal injections at concentration of 22 mg/kg. A concentration of 22 mg/kg was preferred over the optimal dosage (44 mg/kg) reported in literature because when tested higher concentrations lead to the death of the neonatal mice. The n for animals receiving Riluzole and the vehicle control was 12.

Animals receiving Riluzole showed no significant differences in the first failure analysis which was completed using a student's t-test ($p = 0.19$). Average first failure for animals receiving Riluzole occurred at P93 while animals receiving the vehicle control occurred at P88 (Figure 12). Analysis of motor function was completed by using a student's t-test. Unfortunately, no differences were found testing motor function.

A regression test was used to analyze the slope of motor function (Figure 13). Analysis of the regression test showed no significant differences between the two groups ($p = 0.29$). Slope for animals receiving Riluzole was -0.0144, $R^2 = 0.82$, whereas the slope for the animals receiving the vehicle control was -0.0183, $R^2 = 0.89$.

The next analysis completed tested final failure (Figure 14). Again, no significant differences were found between the two groups ($p = 0.45$). Average final failure for the animals receiving Riluzole occurred at P121 while final failure for animals receiving the vehicle control occurred at P119. Upon completion of the final failure analysis, Kaplan-Meier curves were also analyzed for the final failure criteria

(Figure 15). No significant differences were found when analyzing the Cox's proportional F-Test ($p = 0.34$).

Animals receiving Riluzole showed no changes in survival when assessing means using a student's t-test ($p = 0.99$). Survival for both the treatment and the vehicle control groups occurred at P131 (Figure 16). A Kaplan-Meier curve was generated and showed no significant differences for survival between the two groups by using the Cox's proportional F-Test ($p = 0.37$) (Figure 17).

**Study 2 Conclusions**

Riluzole administration showed no changes in either motor function or survival. These results as well as the results from the preceding CyPPA study suggest that drugs with specific SK-channel activation properties may have greater therapeutic effects than non-specific drugs.

These short term neonatal studies were very beneficial for studying disease mechanisms and pathogenesis. Interestingly, the major takeaways from these neonatal studies is that SK channels seem to play a role in the disease mechanism and that activation of SK channels may produce therapeutic effects. Given that clinical drug treatment starts after ALS diagnosis, we then tested administration of CyPPA at symptom onset.

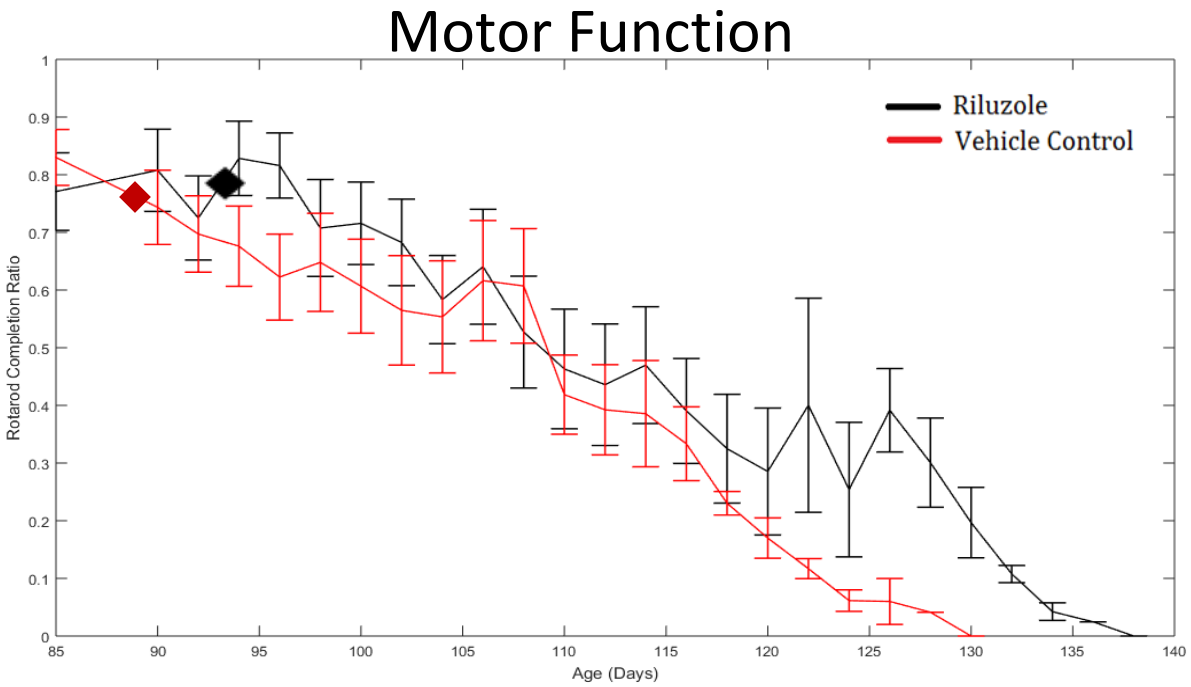



Figure 12: Motor Function – 16 Day Treatment of Riluzole starting at P5 – P20
Diamonds represent average first failure for each group being dosed. Average first failure for the animals receiving the vehicle control occurred at P88 and Riluzole occurred at P93. No significant differences were present testing first failure or motor function. Vehicle Control n = 12, Riluzole n =12; Data is Mean ± S.E.

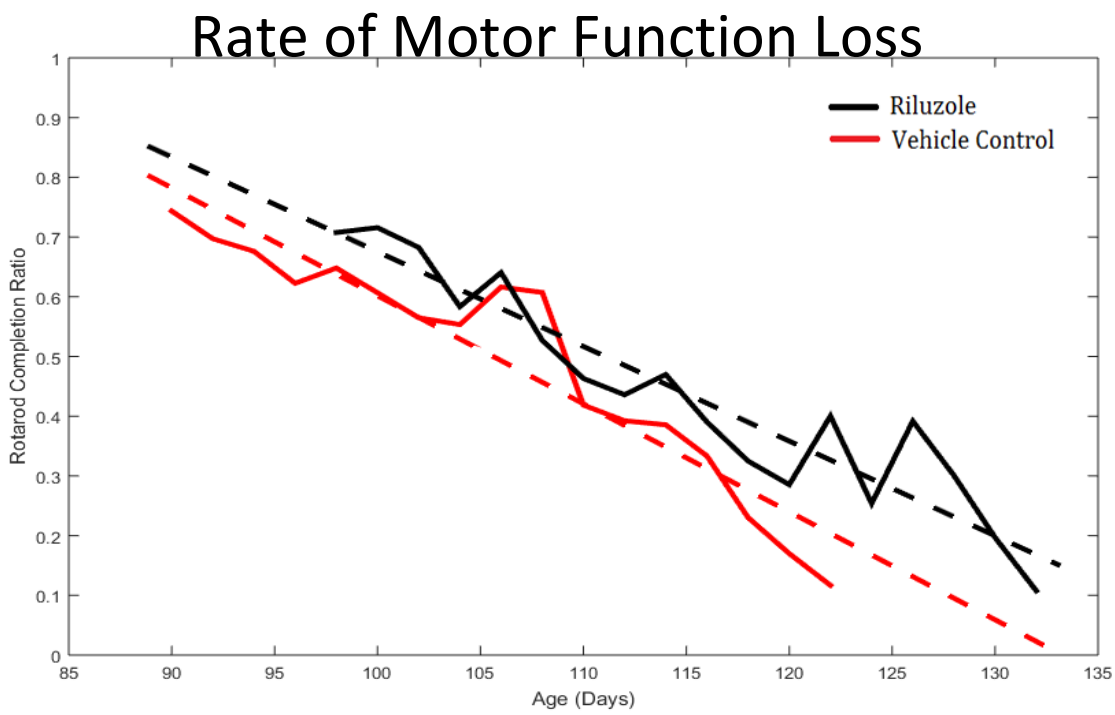



Figure 13: Rate of Motor Function Loss – 16 Day Treatment of Riluzole starting at P5 – P20
Slope for animals receiving Riluzole was -0.0144 while animals receiving the vehicle control was -0.0186. Using a regression test we determined that motor function decline was not significantly different between the two groups. Vehicle Control n = 12, Riluzole n =12; Data is Mean ± S.E.

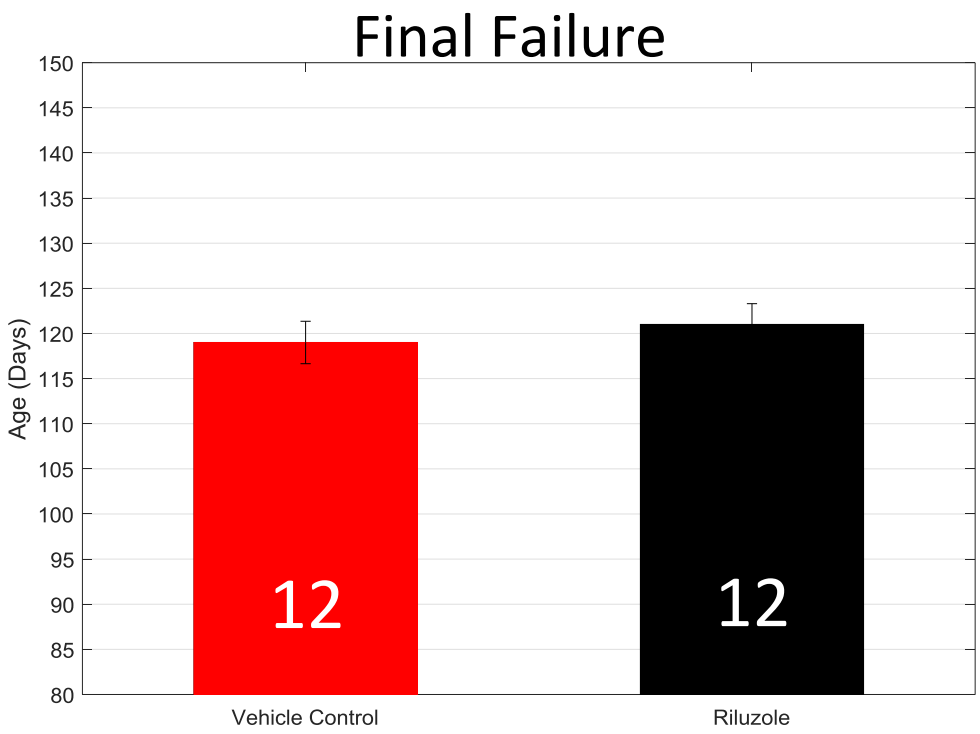



Figure 14: Final Failure – 16 Day Treatment of Riluzole starting at P5 – P20
Animals receiving Riluzole treatment had no change in average final failure. Vehicle Control n = 12, Riluzole n =12; Data is Mean ± S.E.

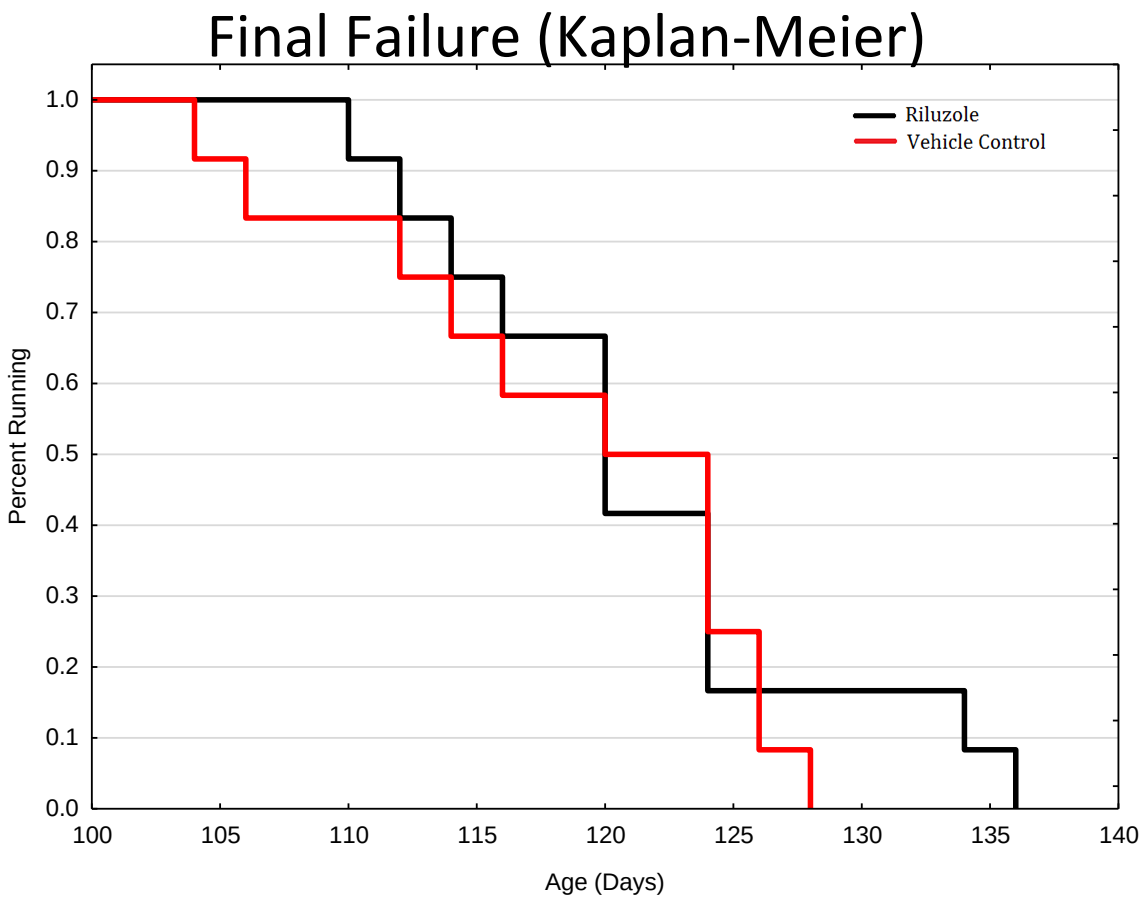



Figure 15: Final Failure (Kaplan-Meier) – 16 Day Treatment of Riluzole starting at P5 – P20
Animals receiving Riluzole showed no changes in motor function using Cox's proportional F-Test.
Vehicle Control n = 12, Riluzole n =12; Data is Mean ± S.E.

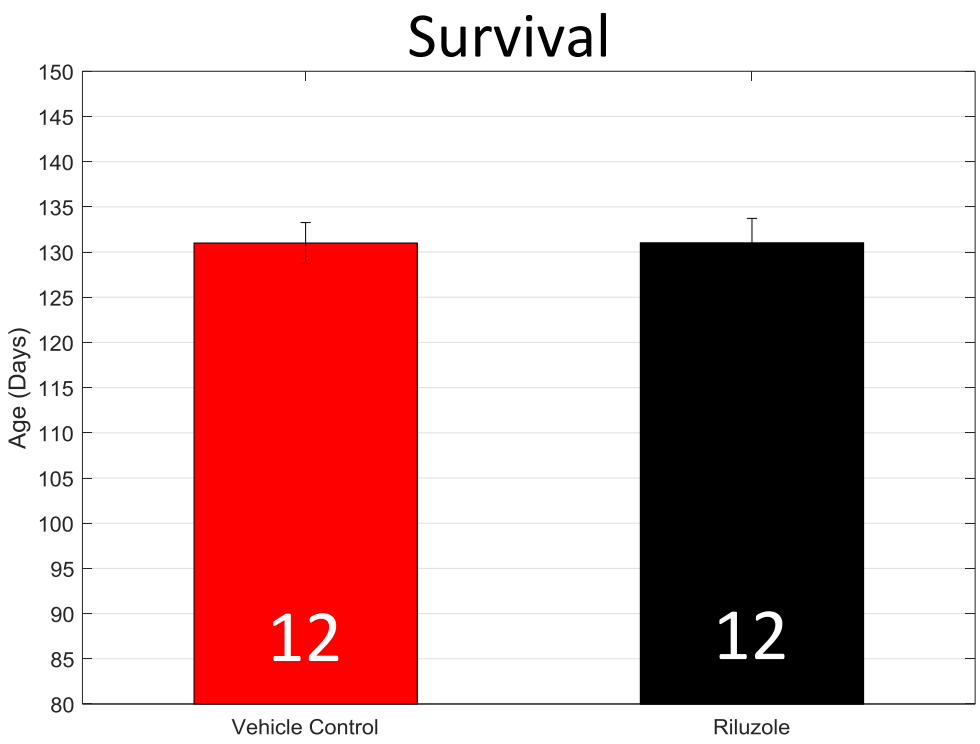



Figure 16: Survival: 16 Day Treatment of Riluzole starting at P5 – P20
Animals receiving Riluzole and the vehicle control presented no changes, Riluzole and Vehicle Control animals on average lived 131 days. Vehicle Control n = 12, Riluzole n =12; Data is Mean ± S.E.

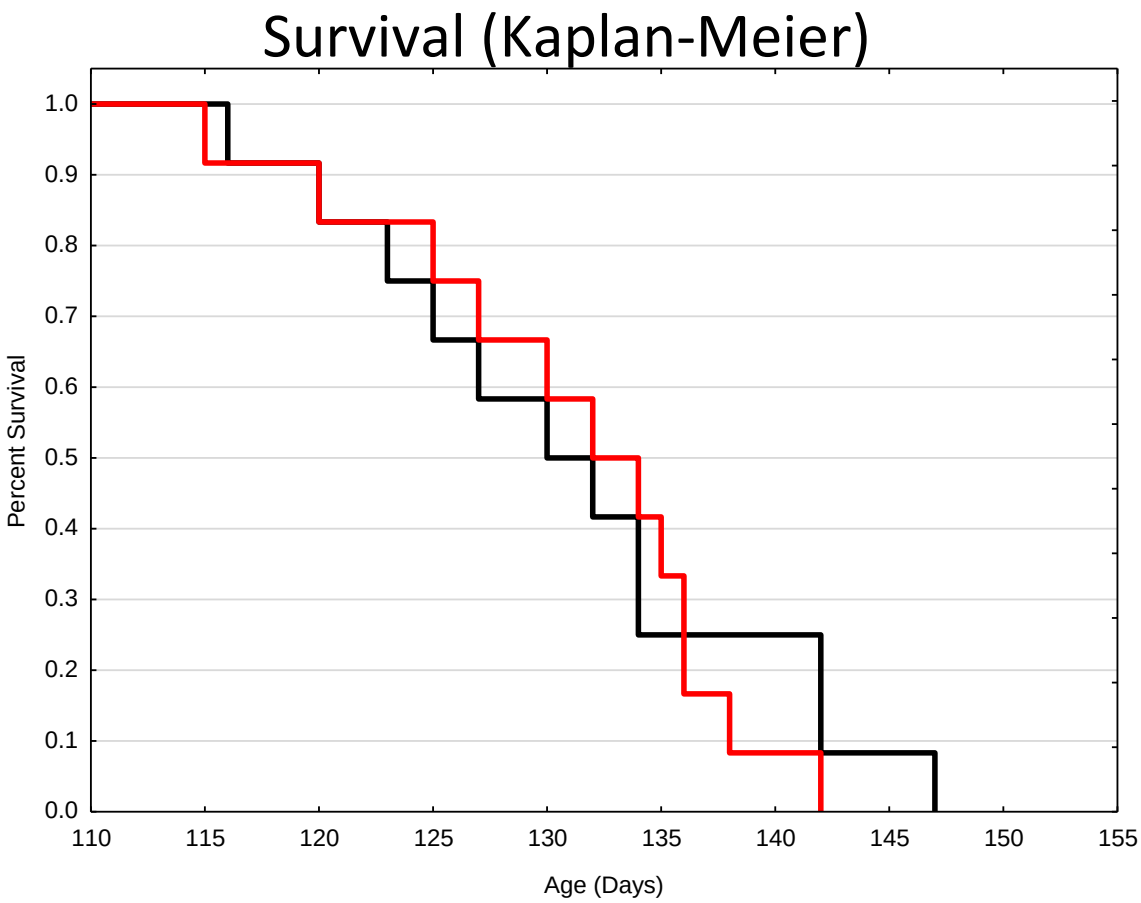



Figure 17: Survival (Kaplan-Meier) 16 Day Treatment of Riluzole starting at P5 – P20
Animals receiving Riluzole showed no changes in survival using Cox's proportional F-Test. Vehicle Control n = 12, Riluzole n =12; Data is Mean ± S.E.

# CLINICALLY RELEVANT STUDIES

**Study 3 - Daily Treatment of CyPPA Starting at P90 – Death**

CyPPA was administered via once daily intraperitoneal injections at concentration of 14 mg/kg. We decided on this concentration because of the therapeutic effects observed from the previous study (16 Day Treatment of CyPPA at P5 – P20) which used the same concentration and we wanted our drug concentration to remain consistent throughout the studies. The n for animals receiving CyPPA was 13 and n for animals receiving the vehicle control was 14.

Animals receiving daily treatments of CyPPA presented no significant differences in the first failure analysis which was completed using a student's T-test ($p = 0.09$). Average first failure for animals receiving daily treatments of CyPPA occurred at P91 while animals receiving the vehicle control occurred at P87. Analysis of motor function was completed by using a student's T-test (Figure 18). No differences were found testing motor function.

Slope for animals receiving daily treatments of CyPPA was -0.0211, $R^2 = 0.92$. Slope for animals receiving the vehicle control was -0.0172, $R^2 = 0.87$. Analysis of the regression test again showed no significant changes ($p = 0.158$) (Figure 19).

The next criteria analyzed tested final failure or the time in which of gross motor deficits and a lack of motor control become present (Figure 20). Final failure was not significant between the two groups using a student's t-test ($p = 0.19$). Average final failure for animals receiving daily treatments of CyPPA occurred at P123 while animals receiving the vehicle control occurred at P127. To complement

the final failure t-test, Kaplan-Meier survival curves were created (Figure 21). No significant differences were found using the Cox's proportional F-Test ($p = 0.9$).

In the later stages of the study, disease progression is quite rapid and many animals succumb to the disease (Figure 22). Survival analysis using the student's t-test ($p = 0.23$) and the Kaplan-Meier ($p = 0.23$) analysis showed no differences between the two groups (Figure 23).

**Study 3 Conclusions**

Results from the third study did not show beneficial effects that were present in the 16 day CyPPA neonatal study. We hypothesized that treatment with CyPPA during symptom onset would produce some therapeutic effect. Given that daily administration of SK-channel activators reduce MN firing frequency, daily administration could mask the potential beneficial effects of motor function. We therefore decided to test short-term administration of CyPPA at symptom onset which occurs around P90.

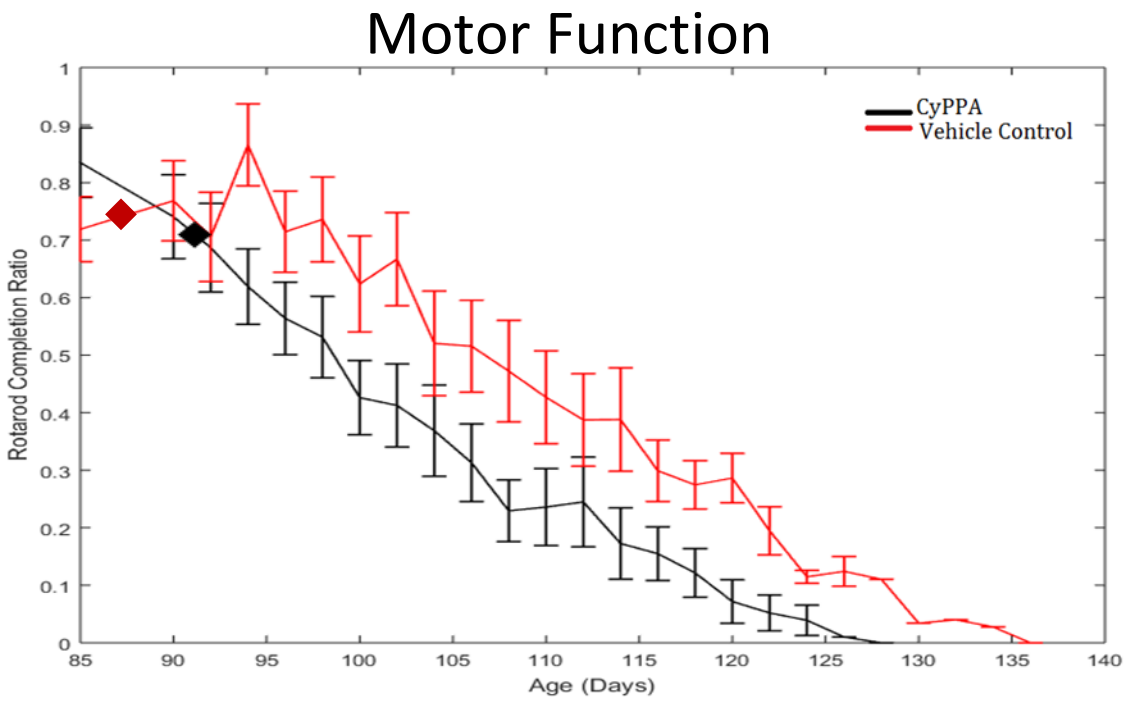



Figure 18: Motor Function – Daily Treatment of CyPPA starting at P90 - Death
Diamonds represent average first failure for each group being tested. Average first failure for the animals receiving the vehicle control occurred at P87 and CyPPA occurred at P91. No significant differences were present testing first failure or motor function. Vehicle Control n = 14, CyPPA n =13; Data is Mean ± S.E.

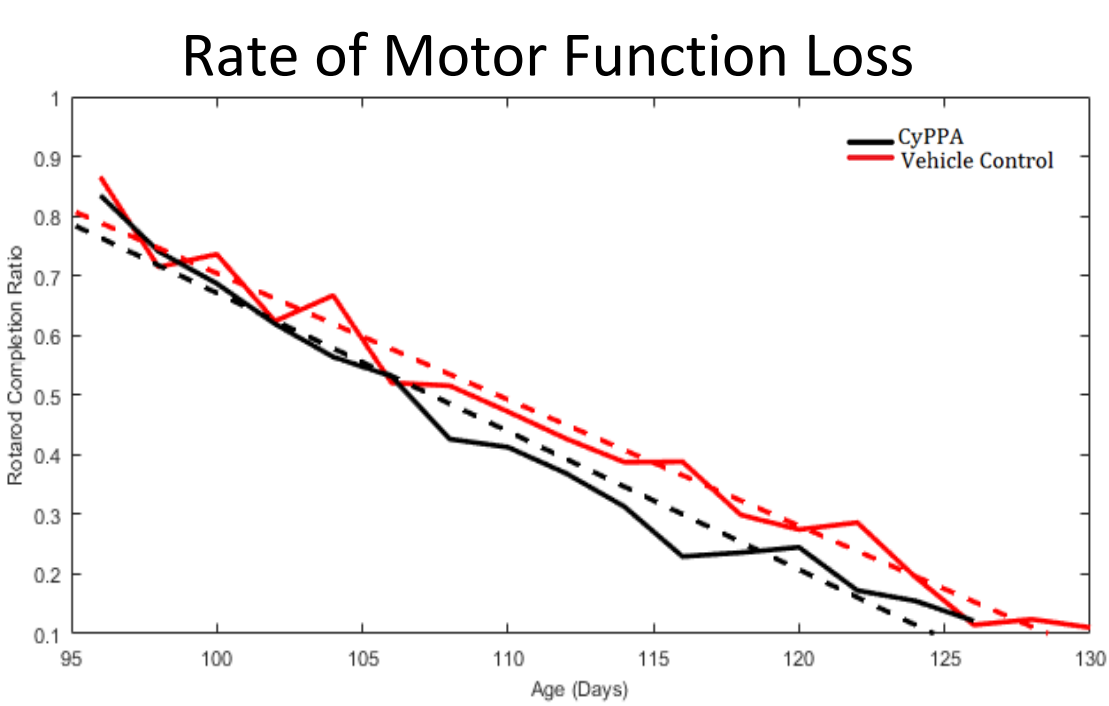



Figure 19: Rate of Motor Function Loss – Daily Treatment of CyPPA starting at P90 - Death Slope for animals receiving CyPPA was -0.0211 while animals receiving the vehicle control was -0.0172. Using a regression test we determined that motor function decline was not different. Vehicle Control n = 14, CyPPA n =13; Data is Mean ± S.E.

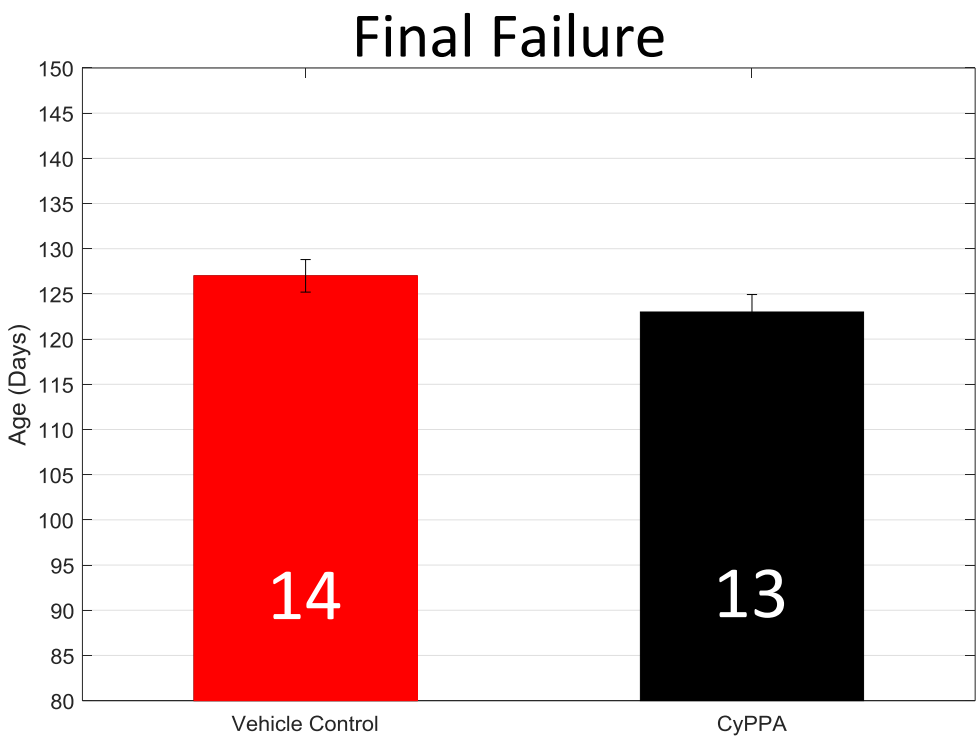



Figure 20: Final Failure – Daily Treatment of CyPPA starting at P90 – Death
Animals receiving CyPPA had no change in average final failure. Vehicle Control n = 14, CyPPA n =13; Data is Mean ± S.E.

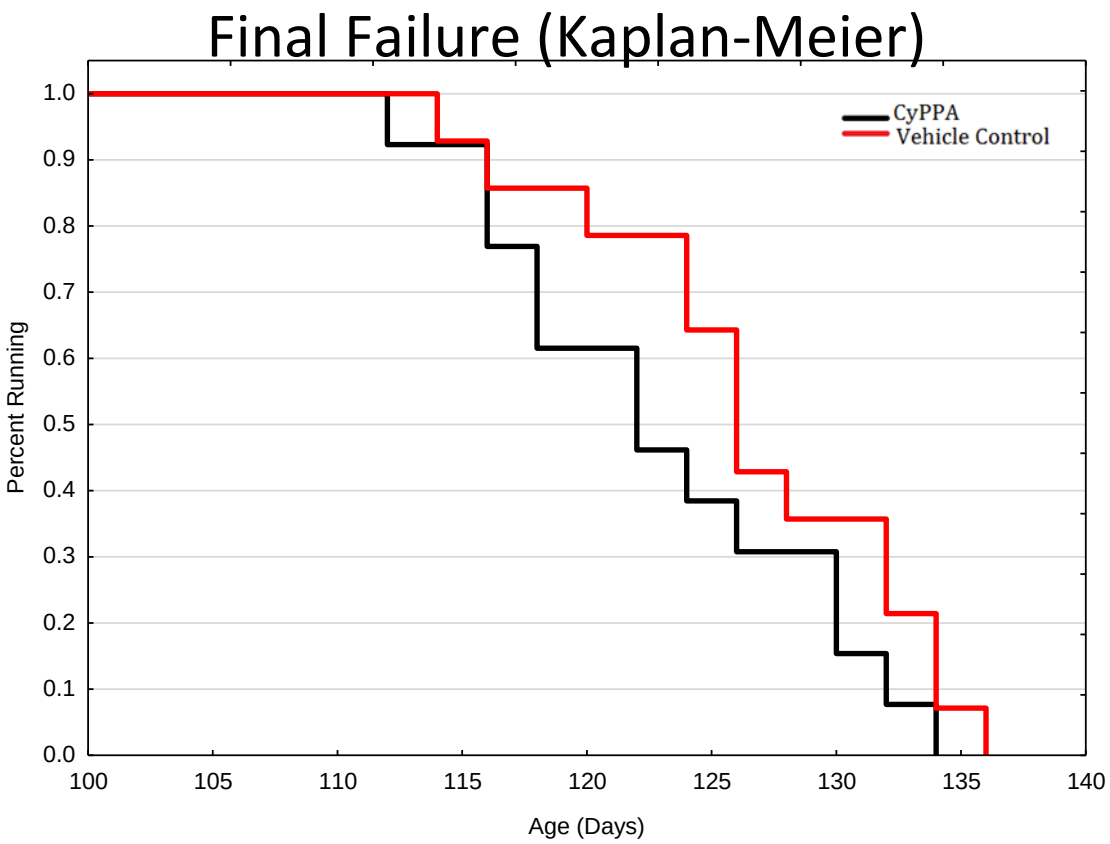



Figure 21: Final Failure (Kaplan-Meier) – 16 Day Treatment of CyPPA starting at P90 – Death Kaplan-Meier curve showing percent of animals still running on the rotarod. Animals receiving CyPPA showed no changes in motor function using Cox's proportional F-Test. Vehicle Control n = 14, CyPPA n =13; Data is Mean ± S.E.

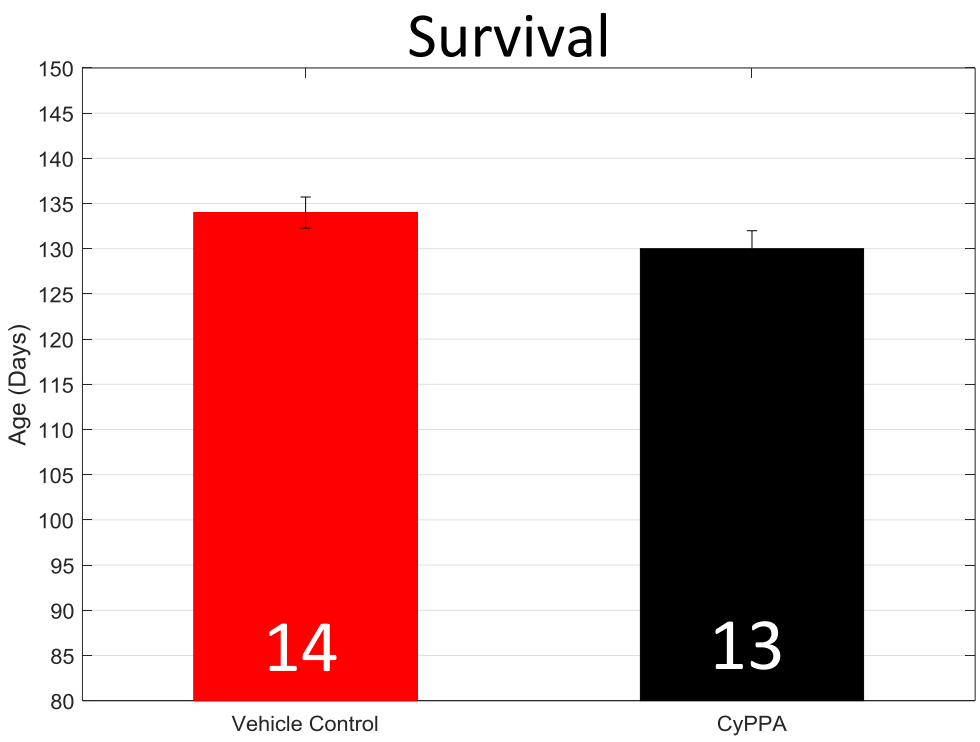



Figure 22: Survival – Daily Treatment of CyPPA starting at P90 – Death
Animals receiving CyPPA and the vehicle control presented no changes in Survival. On average animals receiving CyPPA lived for 130 days and Vehicle Control animals lived 134 days. Vehicle Control n = 14, CyPPA n =13; Data is Mean ± S.E.

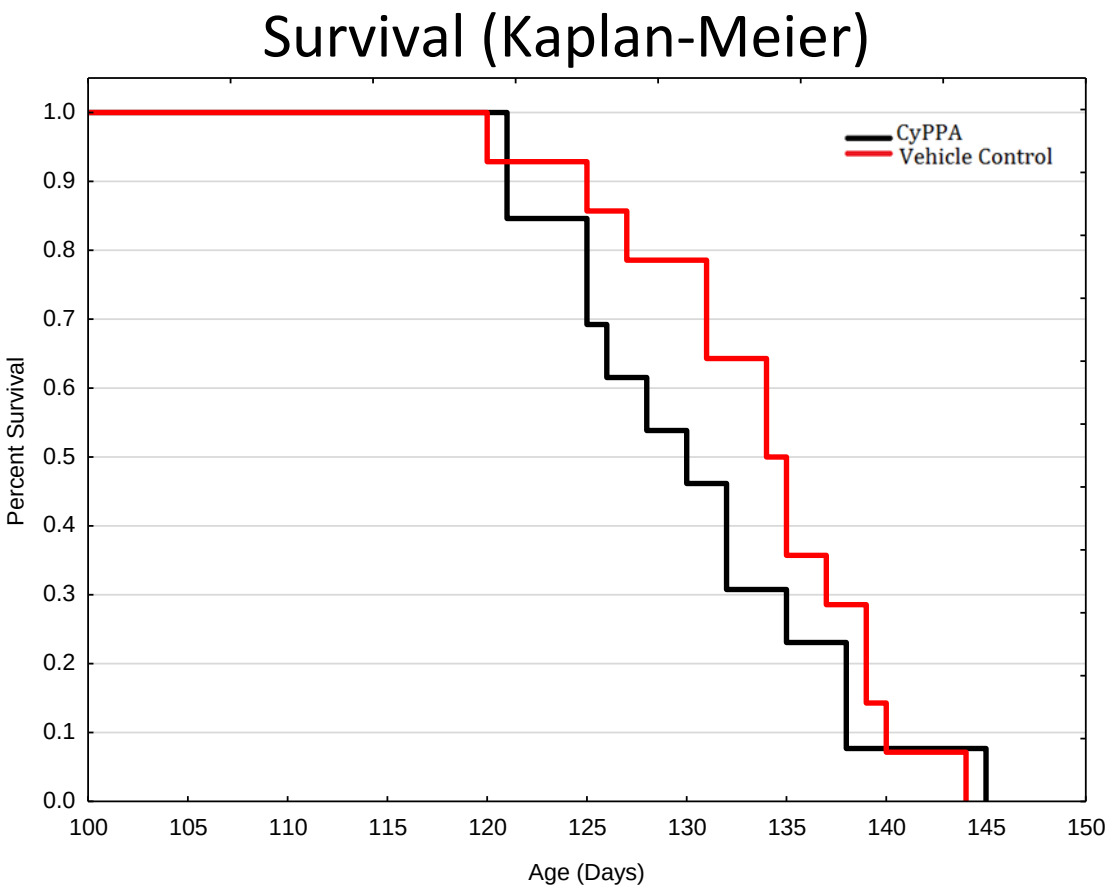



Figure 23: Survival (Kaplan-Meier) - Daily Treatment of CyPPA  tarting at P90 – Death
Kaplan-Meier curve showing percent of animal's surviving. Animals receiving CyPPA showed no changes in survival using Cox's proportional F-Test. Vehicle Control n = 14, CyPPA n =13; Data is Mean ± S.E.

**Study 4 - 7 Day Treatment of CyPPA starting at P90 – P96**

CyPPA was administered via once daily intraperitoneal injections at concentration of 14 mg/kg. We decided on this concentration because of the therapeutic effects observed from a previous study (16 Day Treatment of CyPPA at P5 – P20) which used the same concentration and we wanted our drug concentration to remain consistent throughout each of the studies. The n for animals receiving CyPPA was 14 and n for animals receiving the vehicle control was 14.

Animals receiving short term treatments of CyPPA showed no significant differences in first failure ($p = 0.06$). Average first failure for animals receiving the temporary treatment of CyPPA occurred at P101 (Figure 24). Average first failure for animal's receiving the vehicle control occurred at P93. To our excitement, we observed several time points along the motor function graph with significant improvements. Animals receiving CyPPA showed sustained motor function as the disease progressed. Significant differences were found using a student's t-test and occurred from P92 – P105 and P112 – P119 (P92 – P105, $p \leq 0.05$; P112 – P119, $p \leq 0.03$). Analysis of the Mann-Whitney U test also showed significant improvements in motor function between the treatment and control vehicle groups from P94 ($p \leq 0.032$), P97 - P100 ($p \leq 0.027$), P102 - P103 ($p \leq 0.014$), and P114 - P118 ($p \leq 0.022$).

After finding differences between each day the animals received CyPPA. A regression test was used to analyze slope of motor function (Figure 25). Analysis of

the regression test showed significant differences between the slopes for the temporary treatments of CyPPA and the vehicle control. Slope for the animals receiving CyPPA was -0.0207, $R^2 = 0.92$, while vehicle control animals had a slope of -0.0263, $R^2 = 0.92$ ($p = 0.029$).

The next analysis completed tested final failure (Figure 26). The improvements in motor function and rate of motor function loss suggested that the final failure criteria may also be significant. Final failure for the animals receiving CyPPA occurred at P126 while animals receiving the vehicle control occurred at P119. Unfortunately, this 5% increase did not to reach significance ($p = 0.06$). However, generation of the Kaplan-Meier curve did show significant differences amongst the percentages of animals running over time (Figure 27). Significant differences were found using the Cox's proportional F-Test ($p = 0.03$). This suggested that animals receiving temporary CyPPA treatments maintain motor function as the disease progresses and have a higher probability of motor function enduring during the latter stages of the disease.

Unlike the first study, this final study using CyPPA did not extend survival in the SOD1 mutants (Figure 28). On average animals treated with CyPPA lived 135 days while vehicle control animals lived 129 days ($p = 0.25$). The Kaplan-Meier curve also showed no significant differences for survival between the two groups ($p = 0.06$) (Figure 29). It is interesting to note that in this study we had an animal who survived the longest out of any study. This animal lived for 162 days which was nearly 20 days longer than the oldest vehicle control animal.

**Clinically Relevant Conclusions**

Chronic treatments of CyPPA had no therapeutic effect. This lack of beneficial effects might have occurred because CyPPA does present some acute motor effects after administration. In a preliminary study, it was found that thirty minutes after drug administration animals injected with CyPPA would become lethargic and motor function was significantly reduced. For all of the clinically relevant studies, animals were given drug after behavioral testing to avoid any changes to motor output and prevent variances in the data. It is likely that the drug did not directly affect motor performance during testing. However, continuous dosing with CyPPA during the daily treatment study, potentially kept the animals in a poor state, thus producing no therapeutic effects.

In contrast, short term treatments with the same drug produced significant improvements in motor function though survival was unchanged. In the latter stages of the disease, motor function was sustained in the animals receiving the treatment and they had a higher probability of retaining their motor function later in the disease. This suggests that CyPPA is having some therapeutic effect on the animals, possibly preserving MNs.

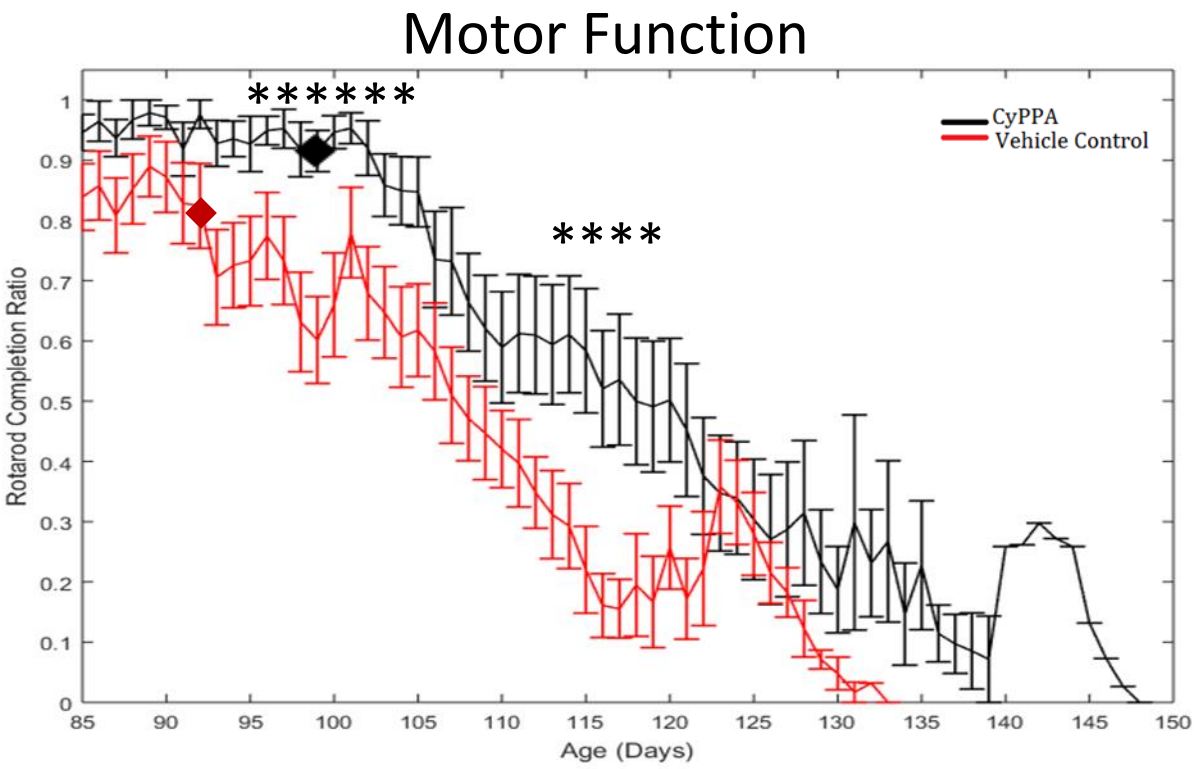



Figure 24: Motor Function – 7 Day Treatment of CyPPA starting at P90 – P96
Diamonds represent average first failure for each group being dosed. Average first failure for the animals receiving the vehicle control was 93 days while CyPPA occurred at P101. No significant differences were present testing first failure, though the p-value was 0.06. Motor function for animals receiving CyPPA was significantly different from P92 – P105 and P112 – P119usign the student's t-test.The -Whitney U test also showed significant improvements in motor function between the treatment and control vehicle groups from P94 ($p \leq 0.032$), P97 - P100 ($p \leq 0.027$), P102 - P103 ($p \leq 0.014$), and P114 - P118 ($p \leq 0.022$). Significance was determined when p-value was <0.05. Vehicle Control n = 14, CyPPA n =14; Data is Mean ± S.E.

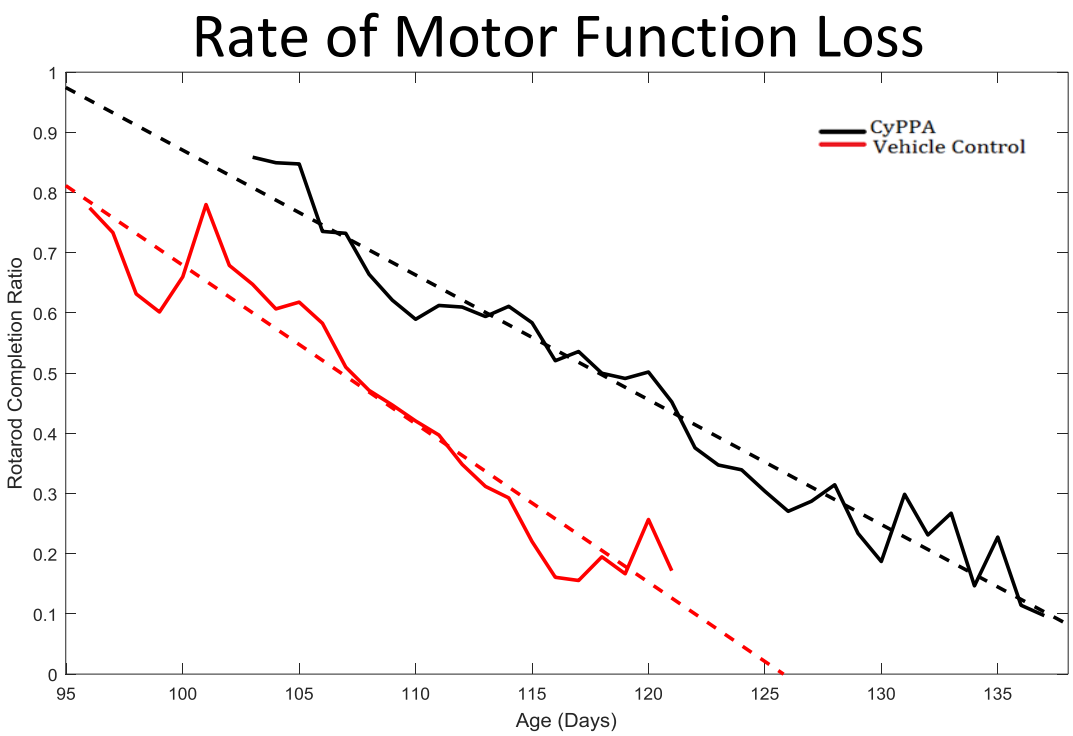



Figure 25: Rate of Motor Function Loss – 7 Day Treatment of CyPPA starting at P90 – P96
Slope for animals receiving CyPPA was -0.0207 while animals receiving the vehicle control was -0.0263. Using a regression test we determined that motor function decline was significantly different between the two groups, p-value was 0.029. Vehicle Control n = 14, CyPPA n =14; Data is Mean ± S.E.

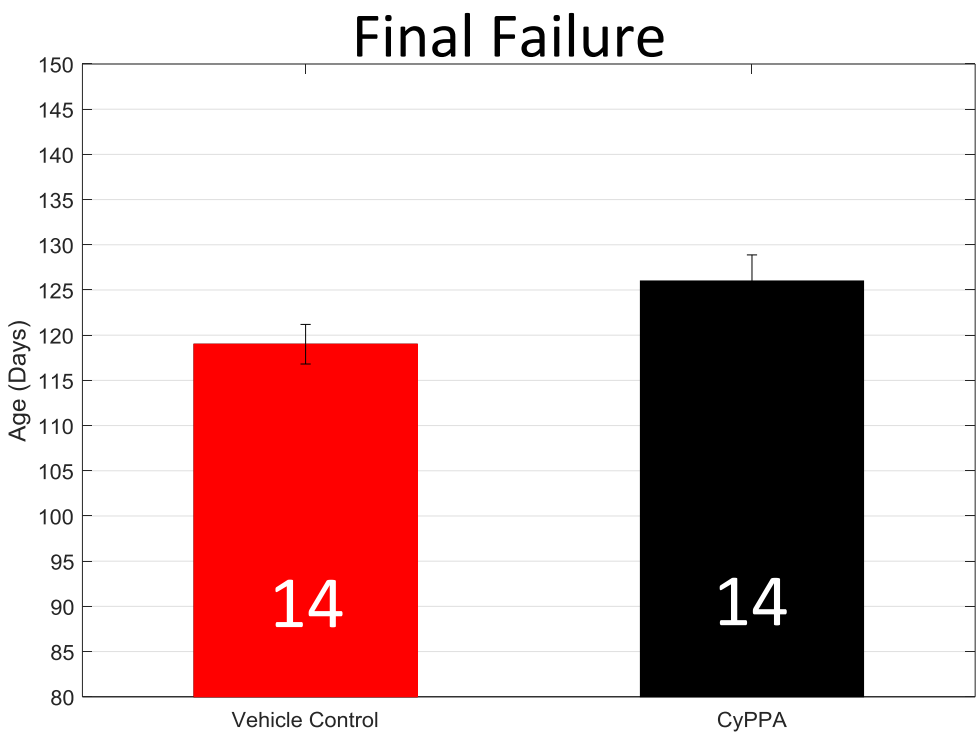



Figure 26: Final Failure – 7 Day Treatment of CyPPA starting at P90 – P96
Animals receiving CyPPA treatment increased average final failure by 5%. Average final failure for the animals receiving the vehicle control was 119 days while CyPPA occurred at P126. No significant differences were present testing final failure. Vehicle Control n = 14, CyPPA n =14; Data is Mean ± S.E.

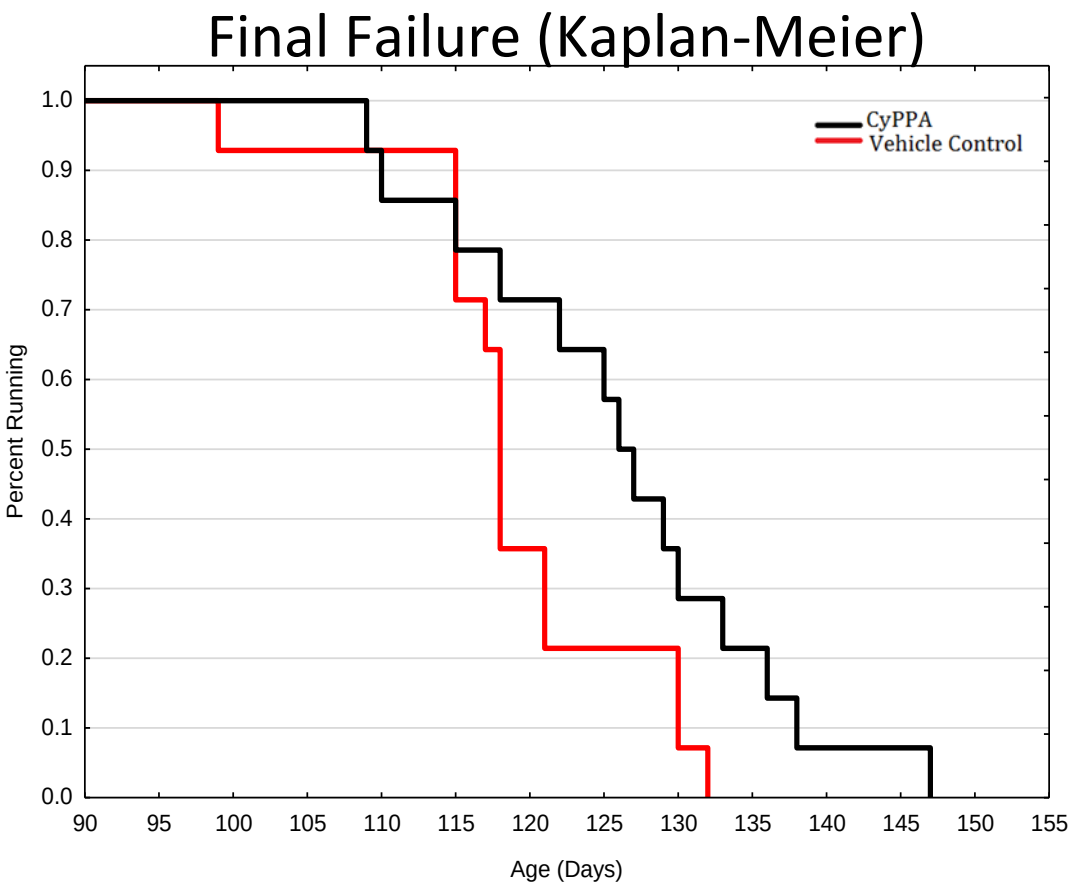



Figure 27: Final Failure –7 Day Treatment of CyPPA starting at P90 – P96
Animals receiving CyPPA showed significant improvements in motor function by staying on the rotarod for an extended number of days. Significant differences were found using Cox's proportional F-Test, p-value 0.0321. Vehicle Control n = 14, CyPPA n =14; Data is Mean ± S.E.

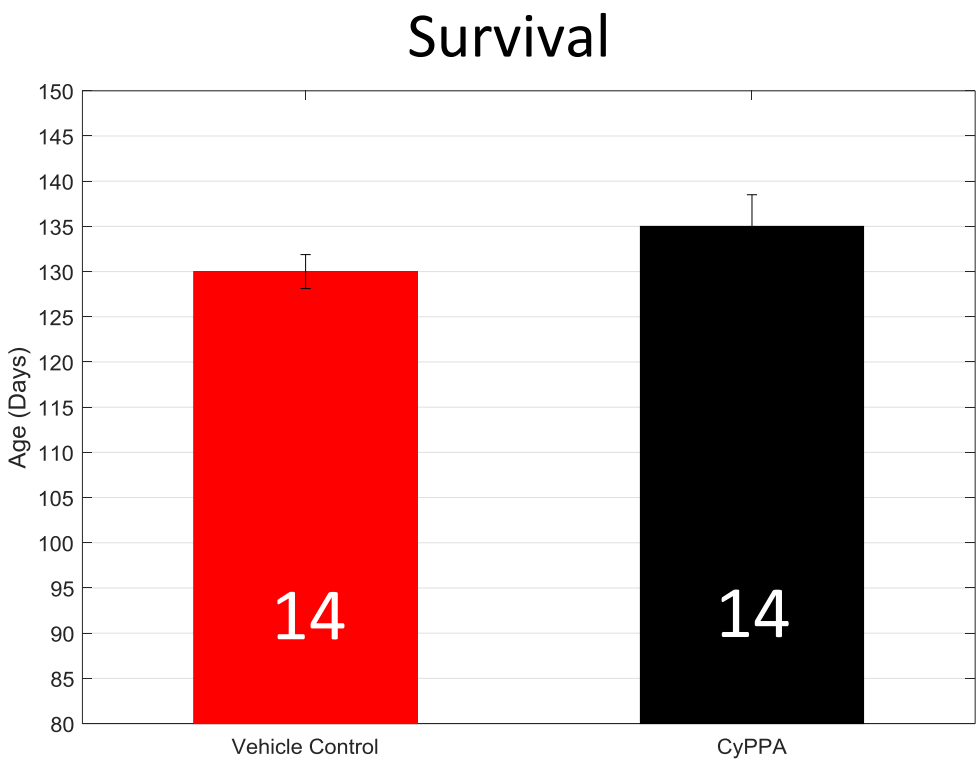



Figure 28: Survival – 7 Day treatment of CyPPA starting at P90 – P96
Animals receiving CyPPA treatment increased survival by 4%. Average survival for the animals receiving the vehicle control was 130 days while CyPPA occurred at P134. No significant differences were found. Vehicle Control n = 14, CyPPA n =14;  Data is Mean ± S.E.

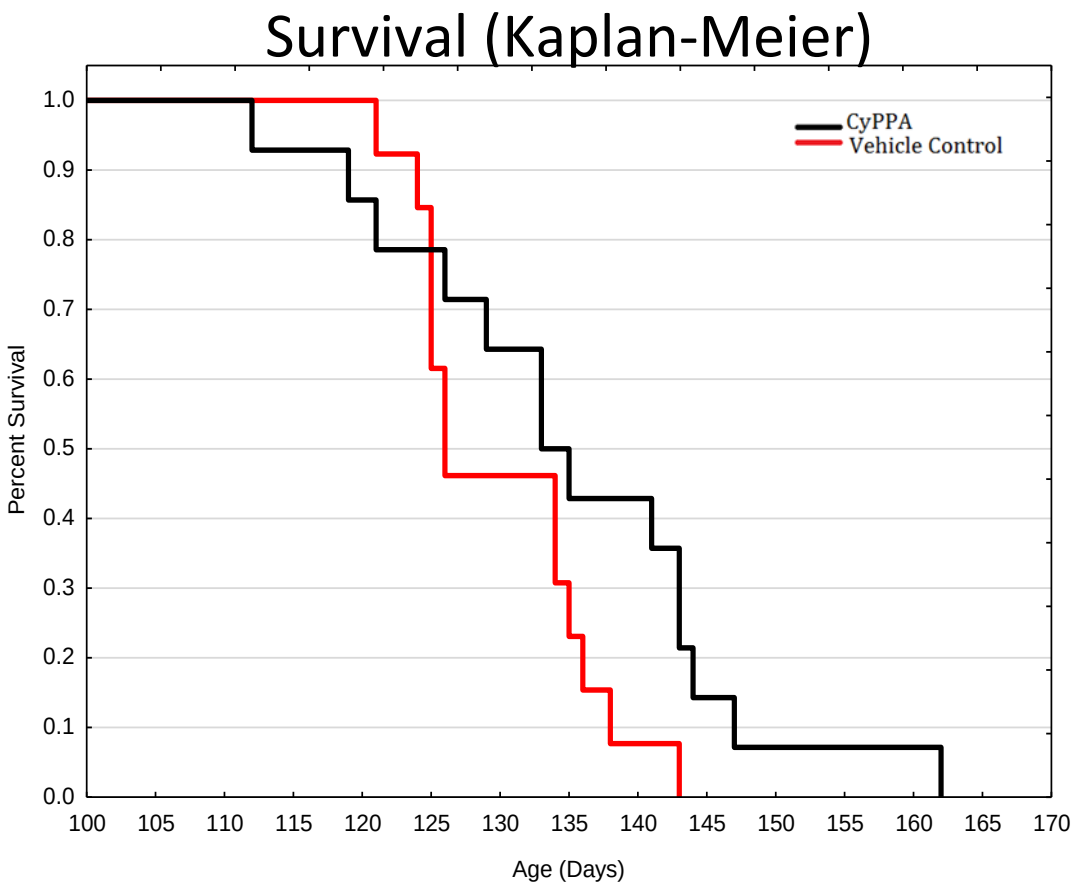



Figure 29: Survival (Kaplan-Meier) – 7 Day Treatment of CyPPA starting at P90 - P96
Animals receiving CyPPA showed no significant improvements in survival using Cox's proportional F-Test ($p = 0.06$). The oldest CyPPA animal lived 162 days (best of all studies), 20 days longer than the oldest surviving vehicle control animal. Vehicle Control n = 14, CyPPA n =14; Data is Mean ± S.E.

| Table | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Drug Studies | | Drug | First Failure | | Final Failure | | Survival | |
| | | | Mean | ± S.E. | Mean | ± S.E. | Mean | ± S.E. |
| Drug Studies | 16 Day CyPPA P5 | Vehicle Control | 111 | 2.53 | 120 | 1.96 | 127 | 1.98 |
| | | CyPPA | 111 | 4.36 | 128* | 3.18 | 136* | 2.98 |
| | 16 Day Riluzole P5 | Vehicle Control | 88 | 1.2 | 119 | 2.35 | 131 | 2.26 |
| | | Riluzole | 93 | 3.46 | 121 | 2.30 | 131 | 2.72 |
| | Daily CyPPA P90 | Vehicle Control | 87 | 0.85 | 127 | 1.79 | 134 | 1.72 |
| | | CyPPA | 91 | 2.08 | 123 | 1.92 | 130 | 1.99 |
| | 7 Day CyPPA P90 | Vehicle Control | 93 | 3.23 | 119 | 2.19 | 130 | 1.88 |
| | | CyPPA | 101 | 3.49 | 126 | 2.88 | 135 | 3.50 |

Table 3: Comparison of All Drug Studies
Table depicting all motor and survival criteria for each study completed. * denotes significance and p-values < 0.05. Data is Mean ± S.E.

# DISCUSSION

To date the underlying mechanism which initiates MN death in ALS is still unknown. However, there is evidence that hyperexcitability and excitotoxicity play a key role in the disease mechanism. Previous studies from our lab used computational modeling as well as immunohistochemistry to test specific channel types. Our modeling predicted that SK channels become downregulated and immunohistochemistry showed a decrease in SK cluster density and area in the SOD1-G93A mutant (Elbasiouny et al, unpublished and Dukkipati, 2016). It is well known that SK channels mediate the mAHP regulating cell excitability (Lam et al., 2013). In the disease, it could be possible that a decrease in these channel types may lead to increased cell firing, hyperexcitability, $Ca^{2+}$ overload and eventual cell death due to excitotoxicity. In our studies, drug administration with short term 16 day neonatal (P5 – P20) drug interventions with CyPPA produced significant improvements in both motor function and survival. While, short term 7 day treatments with CyPPA during symptom onset (P90 – P96) also produced significant improvements in motor function.

Our first study which administered CyPPA at neonatal time point (P5-P20) showed significant improvements in motor function and survival. Animals receiving the treatment of CyPPA had 5 days where motor function was significantly different (Figure 6,Table 3). CyPPA also produced a lower slope compared to its vehicle control, suggesting a reduced rate of motor function loss (Figure 7). Both analyses of final failure and the survival criteria produced significant differences in the favor of CyPPA (Figure 8,Figure *9*,Figure *10*,Figure *11*). In support of our findings, a study from 2008 suggested that SK activation by either NS309 or CyPPA may have

neuroprotective function as they provided protection against glutamate-induced excitotoxicity in primary cortical neurons (Dolga et al., 2008). Our data and this information suggests that activation of SK channels by CyPPA may reduce the effects of excitotoxicity, alleviating symptoms associated with the disease, thus leading to the improvements we have reported.

CyPPA also produced significant motor function improvements during the clinically relevant time point, though these results were limited to the short term (7-Day) treatment. Overall, chronic long term treatments showed no changes in motor function or survival. We think this phenomenon was observed because CyPPA has acute motor effects, which significantly impairs motor output after injection. Since CyPPA has been shown to increase mAHPs which result in a reduction of cell firing rate. Continuous administration of CyPPA and the subsequent reduction of MN firing likely left animals in a weak state. Animals kept in this state might have become more vulnerable to disease changes, thus hampering any therapeutic effect of the drug. On the other hand, short term treatments showed significant improvements. Motor function and rate of motor function loss were all improved compared to the vehicle control and the daily studies (Figure 18Figure 19Figure 24 ,Figure *25* and Table 3). Our 7 day treatments also produced significant improvements in the final failure Kaplan-Meier analysis (Figure 9). Unfortunately, most of the other analysis criteria from the 7 day study produced no significant differences. First failure, final failure, and the survival (Kaplan-Meier) analysis all presented p-values of 0.06. It is interesting that motor function was improved in the short term treatment even though the long term treatment presented no changes. The improvement from the

short term treatments suggests that CyPPA may induce functional plasticity. The short term 7 day treatments of CyPPA may have allowed for a metabolic break, giving the MNs a chance to recover from excitotoxicity, $Ca^{2+}$ overload, increased excitability, causing them rejuvenate. These changes could have harbored a protective therapeutic effect on MNs as wells SK channels, preserving motor function. Protection of these channels might have led to the normalization of APs to a state which is more reminiscent of the pre-symptomatic state. A return to this state would have allowed surviving MNs to function normally, staving off disease related symptoms, thus producing the significant improvements in motor function. This plasticity may have allowed MNs to adapt to the drug effects created by CyPPA, producing the therapeutic effects we observed. Conversely, it is also possible that continuous drug administration with CyPPA from the daily study caused numerous health issues to these mice. It is unknown what actions CyPPA has on the liver, respiratory system and cardiovascular systems and so daily interventions with this drug might have caused the lack of change in motor function or survival

The most significant results from this project were the 16 day treatment of CyPPA at P5 and the 7 day treatment with CyPPA at P90. When we compared our results from these studies we did find some similarities. Motor function and rate of motor function loss were both improved (Figure 6,Figure *7*,Figure *8*Figure 24,Figure *25*, Figure *27* and Table 3) again suggesting that SK channels and treatment with CyPPA are suited for protecting against excitotoxic injury (Benítez et al., 2011). Unlike the neonatal study, no improvements in survival were observed during the clinically relevant time point. During development there are a plethora of MN's. If

CyPPA has therapeutic properties it is likely that these MNs become more robust with treatment during development and end up less affected by disease changes. Conversely, treatment with CyPPA during symptom onset starts long after MN death has begun. With a limited number of MNs left during the clinically relevant time point, the beneficial effects of CyPPA might have been diminished leading to improvements in motor function but no changes in survival.

When we compated CyPPA with Riluzole we saw that drug administration with Riluzole presented no effects on motor function or survival in our neonatal studies. Interestingly, previous literature has shown significant increases in survival when adult G93A-H mutants are treated with Riluzole (Gurney et al., 1998). There are vast differences in the Gurney study compared to our study. Gurney et al treated adult animals with Riluzole from P42 until death at a concentration of 44mg/kg where as our study treated neonatal animals from P5-P20 at a concentration of 22mg/kg. Neonatal animals given a dosage of 44mg/kg and 33mg/kg ended dying from these high concentrations and so a lower concentration of Riluzole was used in our studies. The lack of improvements in motor function and survival demonstrates that neonatal interventions with Riluzole are not therapeutic and that drug treatment with a specific channel activator may be more beneficial than treatment with a non-specific drug. Riluzole targets numerous channel types along with SK channels (Cao et al., 2002). However, Pedarzani et al 2005 showed that CyPPA is far more potent than Riluzole when activating SK channels (Pedarzani et al., 2005). This information along with our data, which shows improvements in both motor function

and survival by CyPPA, suggests that activation by specific drug therapies can be more beneficial than activation by non-specific drugs.

Completion of these studies suggests that CyPPA and SK channels may have therapeutic properties. These changes could have lead to the improvements in motor function and survival. Beneficial effects produced by CyPPA may have also occurred due to an increased number of SK channels, increased SK expression or conductance through the channels. These increases could reduce MN firing rate and mAHP, reducing the effects of hyperexcitability and excitotoxicity. The reduction of these pathologies in G93A- H mutants may have led to the protection of MNs thereby improving motor function as well as survival in some of our studies. In support of our findings, previous studies have shown that SK channel activation by CyPPA could be considered as a therapy for glutamate-induced excitotoxicity (Benítez et al., 2011; Dolga et al., 2008) which would also ameliorate disease related symptoms. Given that behavioral testing was completed for these studies determining the preservation of MNs is difficult. It is possible that treatment with CyPPA during development could strengthen the respiratory system as well as producing stronger skeletal muscle. If this is the case, these changes might have also produced the improvements in motor function and survival.

There are several limitations in our studies. First, the half-life of CyPPA is unknown. Pharmacokinetic data as well as a dose response curve would have ensured proper dosing of the drug and potentially more favorable outcomes from these experiments. Without this pharmacokinetic data, choosing a concentration

which could produce therapeutic effects was just based on concentrations in literature. A concentration of 14mg/kg was settled upon and we did see significant effects. It is possible that far greater benefits could have been produced if an optimal dosage was obtained. Improvements in motor function were found at this concentration in the 16 day treatment of CyPPA commencing at P5 and the 7 day treatments of CyPPA starting at P90. Moreover, the 16 day neonatal treatments of CyPPA also prolonged survival in our SOD1 mutants. The presence of pharmacokinetic data would have guided our efforts in selecting an optimal dosing concentration. Even though CyPPA produced therapeutic effects we are unsure whether these effects are mediated via SK channels on MNs . SK channels are found in numerous cell types. Our lab currently has ongoing experiments to test whether CyPPA effects are mediated through MNs. Published data support the idea of CyPPA as a therapy for glutamate-induced excitotoxicity (Dolga et al 2008) and there is evidence showing that treatment with CyPPA could improve motor function in other neurodegenerative diseases which share similarities to ALS (Benítez et al 2011).

Another limitation arose during the Riluzole study. Gurney et al 1998 suggested the optimal dosage for Riluzole in adults SOD1 mutants should be 44mg/kg. Initially we started with this concentration for our neonatal injections but we were met with complications. 44mg/kg was too high of a concentration for pups and several of the animals did not survive the treatment. The concentration was then dropped to 33mg/kg and pups were still being lost. Finally, at a concentration of 22mg/kg, half of the optimal dosage in adults, pups were able to survive the full 16 day dosing period.

For future studies it would be imperative to test other SK channel activators to ensure the beneficial effects are not limited to one drug. Conversely, instead of testing SK channel activators it would be interesting to test an SK channel blocker as well. Apamin, a neurotoxin found in bee venom selectively blocks SK channels, eliminating the mAHP and increasing cell firing rate (Castle, Haylett, & Jenkinson, 1989; Herrik et al., 2012). It would be interesting to see if ALS symptoms become exacerbated when SK channels become blocked and if our SOD1 mutants become symptomatic earlier.

There have been some criticisms about the G93A-H transgenic line. Using a transgenic line with that has longer disease progression such as the G93A-L or the G85R lines might increase the understanding of disease mechanism and could potentially provide insight into other pathologies which might become altered. Finally, the search for early diagnosis has been an ongoing struggle in this field. Using various techniques, such as nerve conduction studies, electrical impedance myography (EIM), Dual-energy X-ray absorptiometry (DEXA) ( which measures lean body mass), ultrasound, histology or electrophysiology may uncover novel biomarkers. Enabling early diagnosis and allowing patients to receive treatments in a timely manner, before symptoms become too severe.

In conclusion drug administration with CyPPA produced significant improvements in both motor function and survival. Short teom neonatal drug administration and short term drug administration during symptom onset proved to be most beneficial suggesting that activation of SK channels via CyPPA can

produce therapeutic effects and that SK channels are indeed viable drug targets. Another interesting outcome from this study was that neonatal treatment with Riluzole did not have an impact on survival or motor function while CyPPA did. This suggests that neonatal interventions with Riluzole are not beneficial but more importantly that channel specificity could be required for therapeutic effectiveness. Perhaps the most encouraging fact about this drug is that it has been previously shown to improve motor function in other neurodegenerative diseases such as SCA2 and SMA (Dimitriadi et al., 2013; Kasumu et al., 2012). While CyPPA is not yet FDA approved, positive results like these suggest that in time CyPPA may become a potential therapy for patients suffering from ALS.

# REFERENCES


Adelman, J. P., Maylie, J., & Sah, P. (2012). Channels: Form and Function. *Annu. Rev. Physiol*, *74*, 245–69. https://doi.org/10.1146/annurev-physiol-020911-153336

Amendola, J., & Durand, J. (2008). Morphological differences between wild-type and transgenic superoxide dismutase 1 lumbar motoneurons in postnatal mice. *Journal of Comparative Neurology*. https://doi.org/10.1002/cne.21818

Barrett, E. F., & Barret, J. N. (1967). Separation of two voltage-sensitive potassium currents, and demonstration of a tetrodotoxin-resistant calcium current in frog motoneurones. *The Journal of Neuroscience*, *255*(3), 737–774. https://doi.org/10.1113

Bear, M., Connors, B., & Paradiso, M. (2007). *Neuroscience: Exploring the brain* (3rd ed.). Philadelphia, PA: Lippincott Williams & Wilkins.

Bellingham, M. C. (2011). A Review of the Neural Mechanisms of Action and Clinical Efficiency of Riluzole in Treating Amyotrophic Lateral Sclerosis: What have we Learned in the Last Decade? *CNS Neuroscience and Therapeutics*. https://doi.org/10.1111/j.1755-5949.2009.00116.x

Benítez, B. A., Belálcazar, H. M., Anastasía, A., Mamah, D. T., Zorumski, C. F., Mascó, D. H., … De Erausquin, G. A. (2011). Functional reduction of SK3-mediated currents precedes AMPA-receptor- mediated excitotoxicity in dopaminergic neurons. *Neuropharmacology*, *60*(7–8), 1176–1186. https://doi.org/10.1016/j.neuropharm.2010.10.024

Bensimon, G., Lacomblez, L., Meininger, V., & Group, and the A. S. (1994). A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *The New England Journal of Medicine*, *330*(3), 585–591. https://doi.org/10.1056/NEJM199403033300901

Blatz, A. L., & Magleby, K. L. (1986). Single apamin-blocked Ca-activated K+ channels of small conductance in cultured rat skeletal muscle. *Nature*, *323*, 718–720. https://doi.org/10.1038/323718a0

Bokuda, K., Toshio Shimizu, B., Hideki Kimura, B., Toshihiro Yamazaki, B., Kamiyama, T., Kazuhiko Watabe, B., … Eiji Isozaki, B. (2016). Quantitative analysis of the features of fasciculation potentials and their relation with muscle strength in amyotrophic lateral sclerosis. *Neurological Sciences*, *37*(12), 1939–1945. https://doi.org/10.1007/s10072-016-2692-9

Bruijn, L. I., Miller, T. M., & Cleveland, D. W. (2004). Unraveling the Mechanisms Involved in Motor Neuron Degeneration in Als. *Annual Review of Neuroscience*, *27*(1), 723–749. https://doi.org/10.1146/annurev.neuro.27.070203.144244

Byrne, S., Walsh, C., Lynch, C., Bede, P., Elamin, M., Kenna, K., … Hardiman, O. (2011). Rate of familial amyotrophic lateral sclerosis: a systematic review and meta-analysis. *Journal of Neurology, Neurosurgery, and Psychiatry*, *82*(6), 623–627. https://doi.org/10.1136/jnnp.2010.224501

Cao, Y. J., Dreixler, J. C., Couey, J. J., & Houamed, K. M. (2002). Modulation of recombinant and native neuronal SK channels by the neuroprotective drug riluzole. *European Journal of Pharmacology*, *449*(1–2), 47–54. https://doi.org/10.1016/S0014-2999(02)01987-8

Casas, C., Herrando-Grabulosa, M., Manzano, R., Mancuso, R., Osta, R., & Navarro, X. (2013). Early presymptomatic cholinergic dysfunction in a murine model of amyotrophic lateral sclerosis. *Brain and Behavior*. https://doi.org/10.1002/brb3.104

Castle, N. A., Haylett, D. G., & Jenkinson, D. H. (1989). Toxins in the charaderization of potassium channels. *Trends in Neuroscience*, *12*(2), 59–65.

Chiu, A. Y., Zhai, P., Dal Canto, M. C., Peters, T. M., Kwon, Y. W., Prattis, S. M., & Gurney, M. E. (1995). Age-Dependent Penetrance of Disease in a Transgenic Mouse Model of Familial Amyotrophic Lateral Sclerosis. *Molecular and Cellular Neuroscience*. https://doi.org/10.1016/j.cub.2012.07.004

Choi, D. W. (1987). Ionic dependence of glutamate neurotoxicity. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *7*(2), 369–379.

Cui, M., Qin, G., Yu, K., Bowers, M. S., & Zhang, M. (2014). Targeting the Small-and Intermediate-Conductance Ca 2+ -Activated Potassium Channels: The Drug-Binding Pocket at the Channel/Calmodulin Interface. *Neurosignals*, *22*, 65–78. https://doi.org/10.1159/000367896

de Carvalho, M. (2011). Why is ALS so excited? *Clinical Neurophysiology*, *122*(122), 1689–1690. https://doi.org/10.1016/j.clinph.2011.02.004

de Carvalho, M., & Swash, M. (2016). Lower motor neuron dysfunction in ALS. *Clinical Neurophysiology*. https://doi.org/10.1016/j.clinph.2016.03.024

De Carvalho, M., & Swash, M. (2004). Cramps, muscle pain, and fasciculations: Not always benign? *Neurology*, *63*(4), 721–723. https://doi.org/10.1212/01.wnl.0000134609.56166.15

De Carvalho, M., & Swash, M. (2013). Fasciculation potentials and earliest changes in motor unit physiology in ALS. *J Neurol Neurosurg Psychiatry*, *84*, 963–968. https://doi.org/10.1136/jnnp-2012-304545

Deardorff, A. S., Romer, S. H., Deng, Z., Bullinger, K. L., Nardelli, P., Cope, T. C., & Fyffe, R. E. W. (2013). Expression of postsynaptic Ca2+-activated K+ (SK) channels at C-bouton synapses in mammalian lumbar -motoneurons. *J Physiol*, *591*(Pt 4), 875–97.

https://doi.org/10.1113/jphysiol.2012.240879

Debono, M. W., Le Guern, J., Canton, T., Doble, a, & Pradier, L. (1993). Inhibition by riluzole of electrophysiological responses mediated by rat kainate and NMDA receptors expressed in Xenopus oocytes. *European Journal of Pharmacology*, *235*(2–3), 283–289.

Dimitriadi, M., Kye, M. J., Kalloo, G., Yersak, J. M., Sahin, M., & Hart, A. C. (2013). The Neuroprotective Drug Riluzole Acts via Small Conductance Ca 2+ -Activated K Channels to Ameliorate Defects in Spinal Muscular Atrophy Models. *Journal of Neuroscience*, *33*(15), 6557–6562. https://doi.org/10.1523/JNEUROSCI.1536-12.2013

Dolga, A. M., Blank, T., Knaus, H., Spiess, J., Luiten, P. G. M., Eisel, U. M., … Pharmacology, C. (2008). TNF- α mediates neuroprotection against glutamate-induced excitotoxicity via NF-κ B-dependent up-regulation of SK2 channels. *Journal of Neurochemistry*. https://doi.org/doi:10.1111/j.1471-4159.2008.05701.x

Domino, E. F., Unna, K. R., & Kerwin, J. (1952). Pharmacological Properties of Benzazoles I. Relationship Between Structure and Paralyzing Action. *Journal of Pharmacology and Experimental Therapeutics*, *105*(4), 486 LP-497. Retrieved from http://jpet.aspetjournals.org/content/105/4/486.abstract

Dukkipati, S. (2016). *SK Channel Clustering in SOD1-G93A Motoneurons*.

Edgerton, J. R., & Reinhart, P. H. (2003). Distinct contributions of small and large conductance Ca2+-activated K+ channels to rat Purkinje neuron function. *The Journal of Physiology*, *548*(Pt 1), 53–69. https://doi.org/10.1113/jphysiol.2002.027854

Eisen, A., Kim, S., & Pant, B. (1992). Amyotrophic lateral sclerosis (ALS): A phylogenetic disease of the corticomotoneuron? *Muscle & Nerve*, *15*(2), 219–224.

https://doi.org/10.1002/mus.880150215

Elbasiouny, S. M., Bennett, D. J., & Mushahwar, V. K. (2006). Simulation of Ca2+ persistent inward currents in spinal motoneurones: mode of activation and integration of synaptic inputs. *J Physiol*, *570*(Pt 2), 355–374. https://doi.org/10.1113/jphysiol.2005.099119

Excitable. (n.d.). Retrieved October 28, 2016, from https://www.merriam-webster.com/dictionary/excitable

Fischer, L. R., Culver, D. G., Tennant, P., Davis, A. A., Wang, M., Castellano-Sanchez, A., … Glass, J. D. (2004). Amyotrophic lateral sclerosis is a distal axonopathy: Evidence in mice and man. *Experimental Neurology*, *185*(2), 232–240. https://doi.org/10.1016/j.expneurol.2003.10.004

Fletcher, T. F. (2012). Clinical Neuroanatomy Guide. Retrieved November 8, 2016, from http://vanat.cvm.umn.edu/mLocLesGui/clinAnat.html

Gros-Louis, F., Gaspar, C., & Rouleau, G. A. (2006). Genetics of familial and sporadic amyotrophic lateral sclerosis. *Biochimica et Biophysica Acta*, *1762*(11–12), 956–72. https://doi.org/10.1016/j.bbadis.2006.01.004

Grunnet, M., Jespersen, T., Angelo, K., Frøkjær-Jensen, C., Klaerke, D. A., Olesen, S. P., & Jensen, B. S. (2001). Pharmacological modulation of SK3 channels. *Neuropharmacology*, *40*(7), 879–887. https://doi.org/10.1016/S0028-3908(01)00028-4

Gurney, M. E. (1997). The use of transgenic mouse models of amyotrophic lateral sclerosis in preclinical drug studies. *Journal of the Neurological Sciences*, *152*(SUPPL. 1), 67–73. https://doi.org/10.1016/S0022-510X(97)00247-5

Gurney, M. E., Cutting, F. B., Zhai, P., Doble, A., Taylor, C. P., Andrus, P. K., & Hall, E. D. (1996).

Benefit of vitamin E, riluzole, and gabapentin in a transgenic model of familial amyotrophic lateral sclerosis. *Annals of Neurology*, *39*(2), 147–157. https://doi.org/10.1002/ana.410390203

Gurney, M. E., Fleck, T. J., Himes, C. S., & Hall, E. D. (1998). Riluzole preserves motor function in a transgenic model of familial amyotrophic lateral sclerosis. *Neurology*, *50*(1), 62–66. https://doi.org/http://dx.doi.org/10.1212/WNL.50.1.62

Gurney, M. E., H Pu, A. C., Canto, M. D., Polchow, C., Alexander, D., Caliend, J., … H.Deng. (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science*, *264*(5166), 1772–1775. https://doi.org/10.1126/science.8209258

Heath, P. R., & Shaw, P. J. (2002). Update on the glutamatergic neurotransmitter system and the role of excitotoxicity in amyotrophic lateral sclerosis. *Muscle and Nerve*, *26*(4), 438–458. https://doi.org/10.1002/mus.10186

Herrik, K. F., Redrobe, J. P., Holst, D., Hougaard, C., Sandager-Nielsen, K., Nielsen, A. N., … Christophersen, P. (2012). CyPPA, a positive SK3/SK2 modulator, reduces activity of dopaminergic neurons, inhibits dopamine release, and counteracts hyperdopaminergic behaviors induced by methylphenidate. *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2012.00011

Herron, L. R., & Miles, G. B. (2012). Gender-specific perturbations in modulatory inputs to motoneurons in a mouse model of amyotrophic lateral sclerosis. *Neuroscience*, *226*, 313–323. https://doi.org/10.1016/j.neuroscience.2012.09.031

Hirtz, D., Thurman, D. J., Gwinn-Hardy, K., Mohamed, M., Chaudhuri, A. R., & Zalutsky, R. (2007). How common are the “common” neurologic disorders? *Neurology*, *68*(5), 326–337.

https://doi.org/10.1212/01.wnl.0000252807.38124.a3

Hougaard, C., Eriksen, B., Jørgensen, S., Johansen, T., Dyhring, T., Madsen, L., … Christophersen, P. (2007). Selective positive modulation of the SK3 and SK2 subtypes of small conductance Ca. *British Journal of Pharmacology*, *151*, 655–665. https://doi.org/10.1038/sj.bjp.0707281

Ientile, R., Macaione, V., Teletta, M., Pedale, S., Torre, V., & Macaione, S. (2001). Apoptosis and necrosis occurring in excitotoxic cell death in isolated chick embryo retina. *Journal of Neurochemistry*, *79*(1), 71–78. https://doi.org/10.1046/j.1471-4159.2001.00532.x

Inagaki, A., & Lee, A. (2013). Developmental alterations in the biophysical properties of CaV 1.3 Ca2+ channels in mouse inner hair cells. *Channels (Austin)*, *7*(3), 171–181. https://doi.org/10.4161/chan.24104

Joseph, S. K., & Hajnóczky, G. (2007). IP3 receptors in cell survival and apoptosis: Ca2+ release and beyond. *Apoptosis*, *12*(5), 951–968. https://doi.org/10.1007/s10495-007-0719-7

Kasumu, A., Hougaard, C., Rode, F., Jacobsen, T., Sabatier, J., Eriksen, B., … Bezprozvanny, I. (2012). Novel selective positive modulator of calcium-activated potassium channels exerts beneficial effects in a mouse model of spinocerebellar ataxia type 2. *Chemistry & Biology*, *19*(10), 1340–1353. https://doi.org/10.1016/j.chembiol.2012.07.013

Kohler, M., Hirschberg, B., Bond, C. T., Kinzie, J. M., Marrion, N. V., Maylie, J., & Adelman, J. P. (1996). Small-Conductance, Calcium-Activated Potassium Channels from Mammalian Brain. *Science*, *273*(5282), 1709–1714.

Koschnitzky, J. E., Quinlan, K. A., Lukas, T. J., Kajtaz, E., Kocevar, E. J., Mayers, W. F., … Ej, K. (2014). Effect of fluoxetine on disease progression in a mouse model of ALS. *Journal of Neurophysiology*, *111*(11), 2164–2176. https://doi.org/10.1152/jn.00425.2013

Kuo, J. J., Siddique, T., Fu, R., & Heckman, C. J. (2005). Increased persistent Na+current and its effect on excitability in motoneurones cultured from mutant SOD1 mice. *The Journal of Physiology*, *563*(3), 843–854. https://doi.org/10.1113/jphysiol.2004.074138

Lacomblez, L., Bensimon, G., Meininger, V., Leigh, P., & Guillet, P. (1996). Dose-ranging study of riluzole in amyotrophic lateral sclerosis. *The Lancet*, *347*(9013), 1425–1431. https://doi.org/10.1016/s0140-6736(96)91680-3

Lam, J., Coleman, N., Garing, A. L., & Wulff, H. (2013). The therapeutic potential of small-conductance KCa2 channels in neurodegenerative and psychiatric diseases. *Expert Opinion on Therapeutic Targets*, *17*(10), 1203–1220. https://doi.org/10.1517/14728222.2013.823161

Li, T. M., Alberman, E., & Swash, M. (1988). Comparison of sporadic and familial disease amongst 580 cases of motor neuron disease. *Journal of Neurology, Neurosurgery & Psychiatry*, *51*(6), 778–784. https://doi.org/10.1136/jnnp.51.6.778

Li, X., & Bennett, D. J. (2007). Apamin-Sensitive Calcium-Activated Potassium Currents (SK) Are Activated by Persistent Calcium Currents in Rat Motoneurons. *J Neurophysiol*, *97*, 3314–3330. https://doi.org/10.1152/jn.01068.2006

Lorenzon, N. M., & Foehring, R. C. (1992). Relationship between repetitive firing and afterhyperpolarizations in human neocortical neurons. *Journal of Neurophysiology*, *67*(2), 350–363. Retrieved from http://jn.physiology.org/content/jn/67/2/350.full.pdf

Mantz, J., Laudenbach, V., Lecharny, J., Henzel, D., & Desmonts, J. (1994). Riluzole, a novel antiglutamate, blocks GABA uptake by striatal synaptosomes. *European Journal of Pharmacology*, *257*(1–2), R7–R8. https://doi.org/10.1016/0014-2999(94)90716-1

Martin, D., Thompson, M. A., & Nadler, J. (1993). The neuroprotective agent riluzole inhibits release of glutamate and aspartate from slices of hippocampal area CA1. *European Journal of Pharmacology*, *250*(3), 473–476. https://doi.org/10.1016/0014-2999(93)90037-i

Mattiazzi, M., D'Aurelio, M., Gajewski, C. D., Martushova, K., Kiaei, M., Beal, M. F., & Manfredi, G. (2002). Mutated Human SOD1 Causes Dysfunction of Oxidative Phosphorylation in Mitochondria of Transgenic Mice. *Journal of Biological Chemistry*, *277*(33), 29626–29633. https://doi.org/10.1074/jbc.m203065200

Mattson, M. P., & Chan, S. L. (2003). Calcium orchestrates apoptosis. *Nature Cell Biology*, *5*(12), 1041–1043. https://doi.org/10.1038/ncb1203-1041

McLarnon, J. G. (1995). Potassium currents in motoneurones. *Progress in Neurobiology*, *47*(6), 513–531. https://doi.org/10.1016/0301-0082(95)00032-1

Meldrum, B., & Garthwaite, J. (1990). Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends in Pharmacological Sciences*, *11*(9), 379–387.

Mendelowitz, D. (1996). Firing properties of identified parasympathetic cardiac neurons in nucleus ambiguus. *The American Journal of Physiology*, *271*(125 mM), H2609–H2614.

Miller, R. G., Bouchard, J. P., Duquette, P., Eisen, A., Gelinas, D., Y, H., … Sufit, R. L. (1996). Clinical trials of riluzole in patients with ALS. *Neurology*, *47*(4 Supplement 2), 86S–92S. https://doi.org/10.1212/wnl.47.4_suppl_2.86s

Miller, R., Mitchell, J., Lyon, M., & Moore, D. (2002). iluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *Cochrane Database of Systematic Reviews*. https://doi.org/10.1002/14651858.cd001447

Neelands, T. R., Herson, P. S., Jacobson, D., Adelman, J. P., & Maylie, J. (2001). Small-

conductance calcium-activated potassium currents in mouse hyperexcitable denervated skeletal muscle. *Journal of Physiology*, *536*(2), 397–407. https://doi.org/10.1111/j.1469-7793.2001.0397c.xd

Olney, J., Mcgeer, P., & Mcgeer, E. (1978). *Kainic acid as a tool in neurobiology*. New York: Raven Press.

Oxford Dictionaries. (n.d.). fasciculation - definition of fasciculation in English. Retrieved from https://en.oxforddictionaries.com/definition/us/fasciculation

Pedarzani, P., McCutcheon, J. E., Rogge, G., Jensen, B. S., Christophersen, P., Hougaard, C., … Stocker, M. (2005). Specific Enhancement of SK Channel Activity Selectively Potentiates the Afterhyperpolarizing Current IAHP and Modulates the Firing Properties of Hippocampal Pyramidal Neurons. *Journal of Biological Chemistry*, *280*(50), 41404–41411. https://doi.org/10.1074/jbc.m509610200

Philips, T., & Robberecht, W. (2011). Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease. *The Lancet Neurology*, *10*(3), 253–263. https://doi.org/10.1016/s1474-4422(11)70015-1

Pinton, P., Giorgi, C., Siviero, R., Zecchini, E., & Rizzuto, R. (2008). Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis. *Oncogene*, *27*(50), 6407–18. https://doi.org/10.1038/onc.2008.308

Piotrkiewicz, M., & Hausmanowa-Petrusewicz, I. (2011). Motoneuron afterhyperpolarisation duration in amyotrophic lateral sclerosis. *J Physiol Piotrkiewicz and I. Hausmanowa-Petrusewicz J Physiol*, *58911*(58911), 2745–2754. https://doi.org/10.1113/jphysiol.2011.204891

Plaitakis, A. (1990). Glutamate dysfunction and selective motor neuron degeneration in amyotrophic lateral sclerosis: a hypothesis. *Annals of Neurology*, *28*(1), 3–8. https://doi.org/10.1002/ana.410280103

Plaitakis, A., & Caroscio, J. T. (1987). Abnormal glutamate metabolism in amyotrophic lateral sclerosis. *Annals of Neurology*, *22*(5), 575–579. https://doi.org/10.1002/ana.410220503

Pratt, J., Rataud, J., Bardot, F., Roux, M., Blanchard, J. C., Laduron, P. M., & Stutzmann, J. M. (1992). Neuroprotective actions of riluzole in rodent models of global and focal cerebral ischaemia. *Neuroscience Letters*. https://doi.org/10.1016/0304-3940(92)90108-J

Quinlan, K. A. (2011). Links between electrophysiological and molecular pathology of amyotrophic lateral sclerosis. In *Integrative and Comparative Biology*. https://doi.org/10.1093/icb/icr116

Quinlan, K. A., Schuster, J. E., Fu, R., Siddique, T., & Heckman, C. J. (2011). Altered postnatal maturation of electrical properties in spinal motoneurons in a mouse model of amyotrophic lateral sclerosis. *The Journal of Physiology*, *589*(Pt 9), 2245–60. https://doi.org/10.1113/jphysiol.2010.200659

Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., … Brown, R. H. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, *362*(6415), 59–62. Retrieved from http://dx.doi.org/10.1038/362059a0

Rothstein, J. D. (1996). Excitotoxicity hypothesis. *Neurology*, *47*(4 Supplement 2), 19S–26S. https://doi.org/10.1212/wnl.47.4_suppl_2.19s

Rothstein, J. D., Martin, L. J., & Kuncl, R. W. (1992). Decreased Glutamate Transport by the Brain

and Spinal Cord in Amyotrophic Lateral Sclerosis. *New England Journal of Medicine*, *326*(22), 1464–1468. https://doi.org/10.1056/nejm199205283262204

Rowland, L., & Shneider, N. (2001). The clinical diagnosis of ALS is probably correct in more than 95 percent of cases. 1 However, because. *New England Journal of Medicine*, *344*(22), 1688–1700. https://doi.org/10.1056/NEJM200105313442207

Saladin, K. S., Sullivan, S. J., & Gan, C. A. (2015). *Anatomy & physiology: The unity of form and function* (Seventh ed). New York: McGraw-Hill Education.

Saxena, S., Roselli, F., Singh, K., Leptien, K., Julien, J., Gros-Louis, F., & Caroni, P. (2013). Neuroprotection through Excitability and mTOR Required in ALS Motoneurons to Delay Disease and Extend Survival.No Title. *Neuron*, *80*(1), 80–96. https://doi.org/10.1016/j.neuron.2013.07.027

Schumacher, M. A., Rivard, A. F., Ba, H. P., & Adelman, J. P. (2001). Structure of the gating domain of a Ca2+-activated K+ channel complexed with Ca2+/calmodulin. *Nature*, *410*(6832), 1120–1124. https://doi.org/10.1038/35074145

Schuster, J. E., Fu, R., Siddique, T., Heckman, C. J., Amendola, J., Durand, J., … Harris-Warrick, R. (2012). Effect of prolonged riluzole exposure on cultured motoneurons in a mouse model of ALS. *Journal of Neurophysiology*, *107*(1), 484–92. https://doi.org/10.1152/jn.00714.2011

Schutter, E. D. E., & Smolen, P. (1998). Calcium Dynamics in Large Neuronal Models. *Methods in Neuronal Modeling: From Ions to Networks*, (1996), 211–250. https://doi.org/10.1017/CBO9781107415324.004

Schwindt, P. C., Spain, W. J., Foehring, R. C., Chubb, M. C., & Crill, W. E. (1988). Slow conductances in neurons from cat sensorimotor cortex in vitro and their role in slow

excitability changes. *Journal of Neurophysiology*, *59*(2), 450–467.

Scott, S., Kranz, J. E., Cole, J., Lincecum, J. M., Thompson, K., Kelly, N., … Heywood, J. A. (2008). Design, power, and interpretation of studies in the standard murine model of ALS. *Amyotrophic Lateral Sclerosis*, *9*(1), 4–15. https://doi.org/10.1080/17482960701856300

Shibuya, K., Misawa, S., Arai, K., Nakata, M., Kanai, K., Yoshiyama, Y., … Kuwabara, S. (2011). Markedly reduced axonal potassium channel expression in human sporadic amyotrophic lateral sclerosis: An immunohistochemical study. *Experimental Neurology*, *232*(2), 149–153. https://doi.org/10.1016/j.expneurol.2011.08.015

Shimizu, T., Fujimaki, Y., Nakatani-Enomoto, S., Matsubara, S., Watabe, K., Rossini, P. M., & Ugawa, Y. (2014). Complex fasciculation potentials and survival in amyotrophic lateral sclerosis. *Clinical Neurophysiology*, *125*(5), 1059–1064. https://doi.org/10.1016/j.clinph.2013.10.052

Simpson, C. L., & Al-Chalabi, A. (2006). Amyotrophic lateral sclerosis as a complex genetic disease. *Biochimica et Biophysica Acta*, *1762*(11–12), 973–985. https://doi.org/10.1016/j.bbadis.2006.08.001

Vajda, A., McLaughlin, R. L., Heverin, M., Thorpe, O., Abrahams, S., Al-Chalabi, A., & Hardiman, O. (2017). Genetic testing in ALS: A survey of current practices. *Neurology*, *88*(10), 991–999. https://doi.org/10.1212/WNL.0000000000003686

Van Den Bosch, L., Van Damme, P., Bogaert, E., & Robberecht, W. (2006). The role of excitotoxicity in the pathogenesis of amyotrophic lateral sclerosis. *Biochimica et Biophysica Acta - Molecular Basis of Disease*. https://doi.org/10.1016/j.bbadis.2006.05.002

Williams, T. L., Day, N. C., Ince, P. G., Kamboj, K., R., & Shaw, P. J. (1997). a-amino-3-hydroxy-5-

methyl-4-isoxazole propionic acid receptors: A molecular determinant of selective vulnerability in amyotrophic lateral sclerosis. *Annals of Neurology*, *42*(2), 200–207. Retrieved from 10.1002/ana.410420211

Wissmann, R., Bildl, W., Neumann, H., Rivard, A. F., Klöcker, N., Weitz, D., … Fakler, B. (2002). A helical region in the C terminus of small-conductance Ca2+-activated K+ channels controls assembly with Apo-calmodulin. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M109240200

Worldwide ALS. (2015). Rilutek (riluzole). Retrieved from http://alsworldwide.org/research-and-trials/article/rilutek-riluzole

Wulff, H., & Köhler, R. (2013). Endothelial small- and intermediate-conductance KCa Channels: An Update on Their Pharmacology and Usefulness as Cardiovascular Targets. *Journal of Cardiovascular Pharmacology*, *61*(2), 102–112. https://doi.org/10.1097/FJC.0b013e318279ba20.Endothelial