Molecular and neurological effects of fenretinide on Amyotrophic Lateral Sclerosis

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Dedicated to my sister Kate,
for saving my life.

ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is the most common adult motor neuron disease. Currently there is only one modestly beneficial pharmacological treatment, Riluzole, approved by the FDA. It has been documented that polyunsaturated fatty acid (PUFA) concentrations can affect the progression of neurodegenerative conditions however most interventions rely on nutritional supplementation and have limited long-term effectiveness. This thesis describes experiments using fenretinide, a synthetic retinoid capable of altering membrane PUFA concentrations, in a mouse model of ALS (SOD1(G93A)mice). Our treatment resulted in delayed onset, improved motor coordination, and increased life expectancy. Fenretinide also increased plasma levels of the ω-3 PUFA docosahexaenoic acid (DHA) while decreasing ω-6 PUFA arachidonic acid (AA) and products of lipid peroxidation malonyldialdehyde (MDA) and nitrotyrosine (NT). Spinal cord immunohistochemistry revealed a significant reduction in inflammation as assessed by the quantity of activated microglia and astrocytes. These results indicate that fenretinide represents a promising treatment strategy for ALS.

RÉSUMÉ

La Sclérose latérale amyotrophique (SLA) est la maladie affectant les neurones moteurs adultes la plus commune. Il n'existe qu'un seul traitement pharmacologique approuvé par la FDA ayant certains effets bénéfiques, soit le Riluzole. Il est par ailleurs documenté que des concentrations d'acides gras polyinsaturés (PUFA) peuvent affecter la progression d'un état neurodégénératif. Cependant, la plupart des interventions s'appuient sur des suppléments nutritifs et ont une efficacité à long terme plutôt limitée. Cette thèse décrit une série de traitements utilisant le fenretinide, un rétinoïde synthétique capable d'altérer la concentration de PUFA dans les membranes, dans un modèle de souris de SLA (souris SOD1(G93A)). Les traitements ont entrainé un retardement du déclenchement de la maladie avec une meilleure coordination motrice ainsi qu'une espérance de vie améliorée. Le fenretinide a également accrue les niveaux plasmatiques de l'acide docosahexaenoique tout en diminuant les niveaux d'arachidonate ainsi que les produits de peroxydation lipidiques tel que malonyldialdehye et nitrotyrosine. L'analyse immunohistochimique de la moelle épinière a révélé une réduction significative de l'inflammation déterminé par la quantité d'astrocytes et de microglies activés présentes. Ces résultats indiquent que le fenretinide représente un traitement prometteur contre la SLA.

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PRÉFACE

Contribution of Authors:

The author of this thesis has chosen to present it in the format of "manuscript-based thesis"; the following section indicates the "Contribution of Authors".

The entirety of **Chapter One** and **Chapter Three** were written by the thesis candidate including all literature review. Danuta Radzioch contributed to the editing. **Chapter Two** is based on a manuscript entitled "Fenretinide improves functional recovery and extends survival in a transgenic mouse model of Amyotrophic Lateral Sclerosis" by Thomas A. Skinner, Juan B. De Sanctis, Rubèn López-Vales, Marie-Christine Guiot, Jennifer Henri, Samuel David, Danuta Radzioch. TAS was in charge of experimental design, planning of all experiments and performing experimental procedures including: breeding, genotyping, preparing and delivering drug treatments, Rota-rod testing, blood extraction, organ harvests, spinal cord preparations, histological staining, and data and statistical analysis. TAS was also responsible for writing the manuscript and for all preliminary investigations. JBS performed lipid analysis, RLV provided instruction on proper removal of lumbar spinal cord segments and immunohistochemistry techniques, MCG performed histological sectioning, and JH provided technical assistance with animals. SD and DR co-supervised this project.

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KEYWORDS

Amyotrophic Lateral Sclerosis

Arachidonic acid

Ceramide

Docosahexaenoic acid

Fenretinide

Inflammation

Lipid Metabolism

Malonyldialdehyde

Neurological disorders

Neuron survival

Nitrotyrosine

Novel treatments

Reactive gliosis

Reactive oxygen species

Retinoids

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ABBREVIATIONS AND DEFINITIONS

4-HNE- 4-hydroxy-2-nonenal
4-HPR- N-(4-hydroxyphenyl) retinamide, or fenretinide
AA- Arachidonic acid
AD- Alzheimer's disease
ALS- Amyotrophic Lateral Sclerosis
B6- C57BL/6 mouse strain
BBB- Blood brain barrier
BHA- Butylated hydroxy anisole
CAT- Catalase
CF- Cystic Fibrosis
CNS- Central nervous system
COX2- Cyclooxygenase-2
$cPLA_2$- Cytosolic phospholipase A_2
CSF- Cerebrospinal fluid
DHA- Docosahexaenoic acid
EDTA- ethylenediaminetetraacetic acid (calcium chelator)
EFA- Essential fatty acid
ELISA- Enzyme-Linked ImmunoSorbent Assay
EPOX- Epoxygenase
ERK1/2- Extracellular signal-regulated kinase 1/2
fALS- Familial Amyotrophic Lateral Sclerosis
FEN- Fenretinide
GBS- Guillain-Barré syndrome
GFAP- Glial fibrillary acidic protein
GPx- Glutathione peroxidase
GSH- Reduced glutathione
HPLC- High Performance Liquid Chromatography
IL-1ß- Interleukin-1 beta
LOX- Lipoxygenase
Mac-2- Macrophage surface antigen
MAG- Myelin-associated glycoprotein
MAPK- Mitogen-activated protein kinase
MDA- Malonyldialdehyde
MS- Multiple Sclerosis
NADPH- Nicotinamide adenine dinucleotide phosphate
NF-κB- Nuclear factor-kappa B
NIH- National Institutes of Health
NO- Nitric oxide

NOS- Nitric oxide synthase
NSAIDs- Non-steroidal anti-inflammatory drugs
NT- Nitrotyrosine
NYU- New York University impactor
OSU- Ohio State University impactor
PBS- Phosphate buffered saline
PCR- Polymerase Chain Reaction
PD- Parkinson's disease
PGE_2- Prostaglandin E_2
PLA_2- Phospholipase A_2
PUFA- Polyunsaturated fatty acid
qPCR- Quantitative Polymerase Chain Reaction
RNS- Reactive nitrogen species
ROS- Reactive oxygen species
S1P- Spingosine1-phosphate
sALS- Sporadic Amyotrophic Lateral Sclerosis
SCI- Spinal cord injury
SEM- Standard error of the mean
SK1- Sphingosine kinase-1
SM- Sphingomyelinase
SOD- Superoxide dismutase
$sPLA_2$- Secreted phospholipase A_2
Tg- Transgenic
TLC- Thin Layer Chromatography
TNF-α- Tumor necrosis factor-alpha
UV- Ultraviolet
Veh- Vehicle-treated
WD- Wallerian degeneration
WT- Wild-type

Chapter One

1.1 Introduction:

1.1.1 Neurodegenerative Disorders

Neurodegenerative disorders have long provided researchers and clinicians with treatment challenges. Due to the delicate nature of the central nervous system (CNS) many promising pharmaceuticals possess risks that outweigh their therapeutic potential. In addition, mechanisms of homeostatic and inflammatory control in the CNS are relatively poorly understood and appear to have conflicting functions that prove challenging for pharmacological regulation. While neurological disorders such as Amyotrophic lateral sclerosis (ALS, or Lou Gehrig's disease), spinal cord injury (SCI), and multiple sclerosis (MS) have quite different causes and phenotypic expression, they also share many pathological features. In order to proceed with the development of novel treatment options, CNS processes need to be better understood. As results generated from research on the CNS accumulate it is becoming increasingly apparent that many neurodegenerative diseases share certain metabolic aberrations. Although it is frequently difficult to establish causal relationships between the metabolic change observed and the pathological outcome, the inflammatory process seems to represent one of the common denominators. With this in mind it is conceivable that when effective therapies for one disease are found, they may be applied to related pathologies.

Several studies including some recent reports have documented an important role for membrane lipids in a number of pathological conditions [1-4]. Sphingolipids such as ceramides and polyunsaturated fatty acids (PUFAs) are

found in high concentrations in the membranes of neural cells and especially in myelin sheath; which surrounds nerve cell axons. Membrane lipids were once thought to play only a structural role but have now been found to provide other important roles such as signaling. Ceramides, for example, have second messenger signaling capabilities when hydrolyzed from sphingomyelin by sphingomyelinase (SM) and a number of these pathways will be discussed in a later section [5]. Therapies targeted to membrane lipids may lead to development of novel and effective strategies allowing successful treatment of certain neuropathies.

The following introduction will provide a review of the current literature on a number of related neurodegenerative diseases. It also includes a discussion of membrane lipids, their biological activity and their role in neurodegenerative processes. In addition, a summary of some promising new pharmaceutical approaches for neurological disorders will be discussed.

1.1.2 Spinal Cord Injury

SCI is a traumatic neurological injury that leads to permanent functional deficits as a result of axonal loss in the spinal cord. SCI occurs with an incidence of approximately 40 new cases per million people each year and there are currently more than 250,000 people living with SCI in North America (The University of Alabama National Spinal Cord Injury Statistical Center - March 2002). SCI can lead to partial or complete loss of sensation and motor function often rendering victims dependent on the care of others. The damage in SCI is mediated by two factors: the primary injury resulting from the initial physical

trauma and the secondary injury caused by a multitude of factors occurring later on and results in the loss of neurons. Wallerian degeneration (WD) represents one of the processes responsible for secondary damage whereby the axon distal to the site of injury degenerates, removing the necessary trophic support for oligodendrocytes [6]. This eventually leads to apoptosis of oligodendrocytes and subsequent demyelination of neurons and it is through this pathway that axons which have survived the initial impact may suffer impaired conduction after a period of time [6]. The loss of myelin during WD seen in animal models follows a much shorter time course then it does in humans; which can continue for several years, however it has many parallels to the human condition so it represents an excellent surrogate for research purposes [7]. Historically it was believed that CNS neurons could not regenerate following damage however it has been shown that descending axons continue to show sprouting for a prolonged period of time after injury [8]. Nevertheless, myelin associated molecules such as NOGO-A and myelin-associated glycoproteins (MAGs) have been shown to persist in the site of injury and contribute to the environment that is unreceptive to neuron regeneration [7]. Another important mechanism of secondary injury is the inflammatory response, however its role in secondary damage is a source of some conflict because it both promotes the regeneration of neurons but at the same time it is associated with production of factors which impair axon regeneration and survival [9]. The leading theories suggest, however, that inflammation likely perpetuates damage more than it assists in healing especially in traumatic SCI where often, during the course of inflammation, mediators are produced at

excessive levels [9, 10]. As a result, dissecting the inflammatory response in SCI represents an important area of investigation and efforts to control this inflammation may reveal new and effective therapies.

1.1.3 Inflammation and Oxidative Stress in SCI

Astrocytes and microglia infiltrate the site of injury following trauma and cause damage by releasing cytokines, free radicals and reactive oxygen species (ROS) [11]. Another mechanism induced by inflammation is the nitric oxide synthase (NOS) pathway which converts L-arginine into nitric oxide (NO), an important mediator of secondary injury after SCI [12, 13]. NO reacts with superoxide to form peroxynitrite which has a role in cell destruction, causing lipid peroxidation to membranes and oxidative damage to proteins and nucleic acids [13]. The persistence of ROS after injury may also provide possible targets for effective treatment using antioxidant drugs however there is insufficient scientific evidence yet to conclude that such therapies are sufficiently effective. Following the secondary phase of response to SCI mentioned above, the third phase of SCI takes over, which often takes place months to years post initial SCI and is referred to as the chronic phase. This period extends for years after the trauma and includes the formation of a cavity and the accumulation of scar tissue [14]. At this stage very little can be done to improve the neuronal damage or the paralytic condition so treatment efforts must be targeted to minimize secondary injury processes.

1.1.4 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is the most prevalent motor neuron disease occurring in adults. It is a neurodegenerative disorder characterized by the loss of motor neurons, primarily in the spinal cord but also to a lesser extent in the brainstem and cerebral cortex [15]. The progressive loss of neurons leads to muscle weakness, atrophy, paralysis and eventually death caused by respiratory failure usually 3-5 years after diagnosis [16, 17]. Approximately 10% of all cases are caused by an inherited dominant mutation and these forms are collectively known as familial ALS (fALS) [18]. In 1993, Rosen *et al*. determined that roughly 20-25% of fALS cases (2% of all ALS types) are caused by mutations in the Cu/Zn superoxide dismutase gene 1 (SOD1) [18]. In most cases, fALS is clinically similar to sporadic ALS (sALS) and transgenic mice expressing the mutant human Cu/Zn-SOD1 are phenotypically representative of human ALS patients [19, 20]. As a result, transgenic mouse models (Cu/Zn-SOD1 mutant mice) are often used to investigate the causes, pathology and treatments of ALS and the results can be applied to both fALS and sALS in humans [20, 21].

1.1.5 Superoxide Dismutase in ALS

The discovery of SOD1 mutations in fALS and the knowledge of SOD's antioxidant activity lead many researchers to postulate that fALS, and possibly sALS are caused by oxidative damage from ROS and free radicals [18, 20]. Superoxide dismutases are a family of enzymes that catalyze reactions converting superoxide anions ($O_2^{\cdot -}$) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) which is thought to protect from radical induced cellular damage [13]. Initially, it was thought that the mutations in SOD1 would decrease or increase its activity

but recent work has shown that there may be a novel gain of function rather than a change in activity that is responsible for the associated neuronal death [18, 22]. Although some uncertainty of the pathway persists, high levels of oxidative damage have been documented by several research groups as the causative agent of the motor neuron damage in ALS [22]. The novel cytotoxic property has yet to be determined but Liu and colleagues found elevated levels of hydrogen peroxide and hydroxyl radical and diminished levels of superoxide anion in ALS mice suggesting impairment of H_2O_2 detoxification and potentially affected interactions between SOD1 and H_2O_2 detoxification enzymes [23]. This is relevant because mice over expressing normal human SOD1 did not have elevated levels of H_2O_2 but those expressing mutant SOD1 did, supporting the theory that the SOD1 mutations affect more than just the activity of the enzyme [19]. Recently several other hypotheses have been postulated to account for the damage seen in ALS neurons including transport and mitochondrial dysfunctions, glutamate excitotoxicity, insufficient growth factors, protein aggregation and inflammation [24, 25].

1.1.6 Oxidative Stress and Inflammation in ALS

It has been well documented in numerous neurodegenerative conditions that oxidative stress-induced damage targets membranes, proteins, genetic material and mitochondria [26]. This process can cause irreversible damage and eventual cell death [26]. Inflammation is also an important contributing factor in ALS as both microglial and astrocytic activation have been demonstrated to precede disease onset [27]. In 2003, Clement and colleagues generated chimeric

mice that possessed both normal SOD1 and mutant SOD1-expressing cells in a breakthrough experiment which demonstrated that non-neuronal cells from a wild-type background could prolong survival of motor neurons expressing mutant SOD1 [27]. Other groups have since confirmed these results and explained that the disease progresses in a "non-cell autonomous" mechanism meaning that non-neuronal cells (such as microglia and astrocytes) play an important role in motor neuron death [25, 28, 29]. Oxidative damage and inflammation are certainly not mutually exclusive processes because upon activation, microglia upregulate NADPH oxidase which generates oxygen radicals and other ROS [30]. Astrocytes also have a role in motor neuron death in ALS which is, in part, caused by impaired glutamate uptake at synapses due to loss of glutamate transporters in patients with ALS; this leads to glutamate excitotoxicity [31]. It is for these reasons that controlling both inflammation and oxidative stress in neurodegenerative processes has become a topic of intense investigation and this introduction will focus on many of the proposed outcomes of these mechanisms.

1.1.7 Related Pathologies

In addition to SCI and ALS a number of other neurodegenerative disorders possess similar pathological features. MS, Guillain-Barré syndrome (GBS), Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's chorea are examples of diseases that share several phenotypes. Most notably inflammation, imbalances in redox homeostasis and excessive ROS have been documented in all of these disorders and contribute to the environment which is hostile to neurons [32]. Oxidative stress-induced damage is indeed one of the hallmarks of many

neuroinflammatory or neurodegenerative diseases and can disrupt the lipid profile of important cell species in the CNS including glial cells and neurons themselves [26, 33]. It is well known that membrane sphingolipids such as ceramides are released in response to oxidative and environmental stress and can trigger a number of downstream reactions [34-37]. Altered lipid metabolism is involved in many abnormal conditions but may be of considerable importance in neurological disorders because the lipid content in the CNS is one of the highest among the physiological systems in the body. In fact, the CNS contains the second highest level of lipids after adipose tissue [33]. Also, neurons are particularly vulnerable to oxidative damage due to their poor antioxidant defense, high demand for oxygen and elevated proportion of membrane PUFAs, which are disposed to lipid peroxidation [33]. For these reasons a new trend of targeting therapies to antioxidant control and maintaining lipid homeostasis is increasing in popularity for neurodegenerative diseases and may provide a vital link for targeting therapies to multiple disorders.

1.2 Membrane Lipids

Historically, membrane lipids were thought to have few biological functions aside from structural support. Today, bioactive lipid molecules are becoming increasingly recognized for their numerous signaling functions. These endogenous molecules are cleaved from cell membranes and evidence is accumulating regarding their mitogenic activity, cell activation potential, and their ability to induce apoptosis by mediating cell signaling [5]. Through various

intermediate steps and enzymatic reactions, lipid molecules within plasma membranes can be cleaved to release a variety of lipid second messenger species. The activity of sphingomyelinases release sphingolipids such as ceramides whereas phospholipases act on phosphatidyl choline to generate free PUFAs including docosahexaenoic acid (DHA) and arachidonic acid (AA) [5, 38-40]. These molecules are recognized as having additional significance in the CNS due to their high proportion in glia and neurons and their interaction with neurotransmitters [40]. Lipid signaling is extremely important in inflammatory pathways and is thus implicated in many neurodegenerative disorders. The biolipid signaling molecules implicated in these disorders and their associated pathways and effects are explained in further detail below.

1.2.1 Sphingolipids and Ceramides

Sphingomyelin and glycosphingolipids are complex sphingolipids which form an integral part of cellular membranes in eukaryotic cells. These molecules provide structural support, organize membrane composition and mediate cell signaling. The metabolites of complex sphingolipids; simple sphingolipids, have other crucial roles in cell signaling which involve both extra- and intracellular targets [41]. Simple sphingolipids include ceramides, sphingosine and sphingosine 1-phosphate, which are important modulators of the cell cycle. Ceramides, second messengers which play a central role in regulating the cell cycle, are composed of sphingosine and fatty acids and can be degraded into these components by ceramidases [41]. Subsequently sphingosine can be phosphorylated to sphingosine 1-phosphate (S1P) by sphingosine kinase-1 (SK1).

The degradation of sphingomyelins to ceramides requires the enzymatic action of sphingomyelinases; however ceramides can also be synthesized de novo with the help of palmitoyltransferase and ceramide synthase enzymes. The metabolic pathways of ceramides are illustrated in Fig 1.1.

1.2.2 Ceramides and the Cell Cycle

The cell cycle processes that are mediated by ceramides (and other sphingolipids) include growth, differentiation, stress response, and apoptosis [42]. Ceramides have been shown to induce apoptosis whereas S1P is anti-apoptotic [43]. As a result, when SK1 levels are increased there is a shift from ceramides to increased S1P leading to cell proliferation, conversely if SK1 levels are reduced, as they are during the stress response, this induces a rise in ceramide levels resulting in apoptotic cell death [43]. Ceramides may also act as second messengers in response to several signals including oxidative stress, ultraviolet (UV) radiation, X-rays and tumor-necrosis factor (TNF)-α [44]. Because of their apoptosis inducing properties, ceramides have become the target of many therapeutic drugs including cancer chemotherapy regimens. Evidence has begun to accumulate on the involvement of ceramides in apoptosis of neurodegenerative disorders [45]. Although apoptotic cell death is a vital component of CNS development it has also been implicated in the neuronal death seen in ALS, SCI and other neurodegenerative diseases [45]. Based on the known involvement of ceramides in the apoptotic pathway it is a putative target for therapies in these

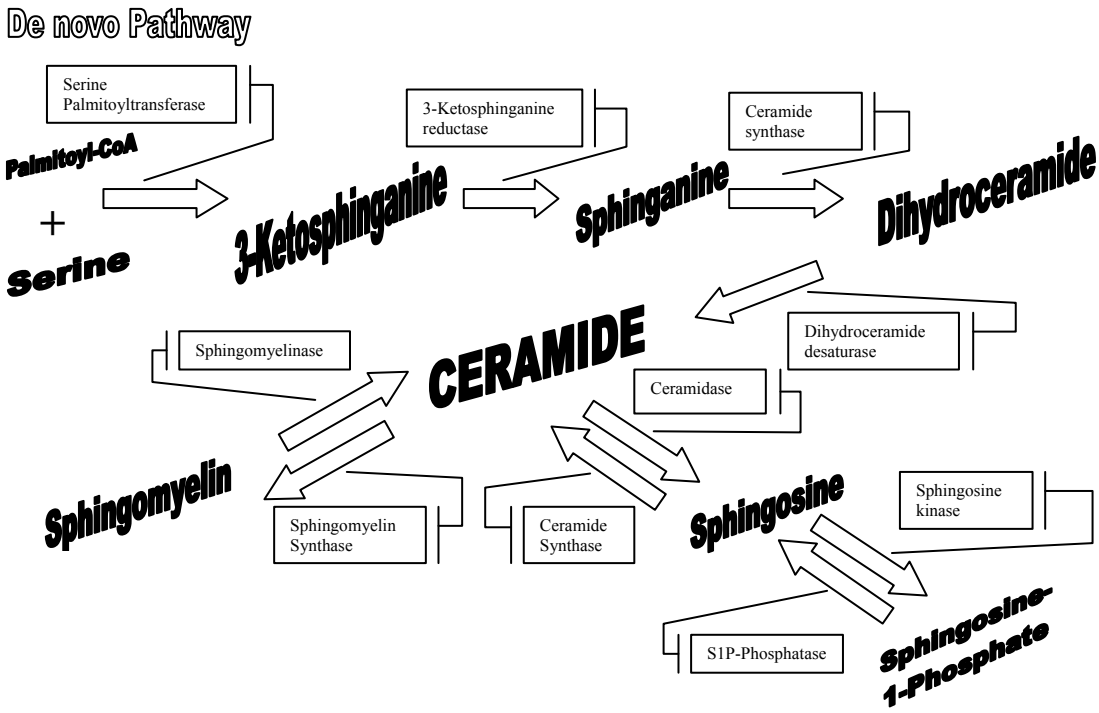


Fig 1.1 Metabolic pathways of ceramides. This figure provides a summary of both anabolic and catabolic pathways of ceramides. The upper row of the figure depicts de novo synthesis of ceramides while the lower portion indicates pathways of ceramide catabolism. Necessary enzymes are depicted in boxes between the products of the reactions they catalyze.

disorders as well. Ceramides on the other hand are also implicated in disrupting stress or immunological responses through the inhibition of nuclear factor-kappa B (NF-κB) and therefore may play a dual role in neurological diseases by also protecting from excessive inflammation [46].

There is currently a debate on the role of ceramides and this is perhaps attributable to their pleiotropic effects. It is important to remember however that this molecule is involved in many vital functions and an imbalance of either excess or insufficiency is likely to have pathological consequences. Our own studies have in fact shown that increasing ceramides correlates with neuron sparing and improved recovery after spinal cord injury perhaps through ceramides' ability to mitigate inflammation (manuscript in preparation). Clearly these molecules have several roles and must be studied further to adequately understand the complexity of their actions.

1.2.3 Polyunsaturated Fatty Acids

PUFAs are an essential component in cellular membranes and most commonly occur as either omega-3 or omega-6 fatty acids. The nomenclature indicates the position at which they share a common inter carbon double bond: at n-3 or n-6 respectively. PUFAs are also known as essential fatty acids (EFA) because humans must ingest them in their diet due to an inability to generate them *de novo*. Diets rich in omega-3 fatty acids are recognized for a wide variety of health benefits ranging from reducing the risk of cardiovascular disease to promoting brain development and memory [47-49]. Omega-6 fatty acids on the other hand, while also considered essential, can contribute to a number of

pathologies when ingested in excessive amounts affecting the relative proportion of omega-6 to omega-3 fatty acids systemically [50]. Current Western diets consist of a dietary omega-6 to omega-3 ratio of over 15:1 while it has been proposed that humans evolved on a diet of equal proportions [51]. Elevated levels of omega-6 EFA have been implicated in the pathogenesis of diseases ranging from cardiovascular disease to inflammatory conditions and recent research has demonstrated that a dietary ratio of 4:1 or lower may reduce the risk of many diseases [51]. *Alpha*-linolenic acid is the short-chain omega-3 fatty acid precursor to all long-chain n-3 EFAs while linoleic acid is the omega-6 short-chain equivalent. These molecules give rise to a number of long-chain products including DHA and AA respectively, both of which play important roles in neurological pathologies and can affect gene expression [52].

1.2.4 Docosahexaenoic Acid

DHA is a long-chain omega-3 EFA that is abundant in the CNS and is an extremely important regulator of neural function. It is important not only in cell signaling but has a role in neurotransmission processes as well, which is supported by the high concentration of this PUFA found at neurological synapses [52, 53]. DHA has essential anti-inflammatory and anti-oxidant properties and provides many neuroprotective effects [54-56]. Such effects have been documented in many well-known neurological conditions and a number of pathways have been proposed. For example, regulation of NOS expression and the production of NO is the subject of intensive study and it has been demonstrated that NO production is inhibited by DHA *in vitro* [57-59]. NO is a

lipid soluble ROS that has important regulatory functions in the CNS affecting signaling and neurotransmission and it is generated from neurons and glia alike [58, 60]. It is also well known for its role in inflammation and oxidative damage and reactions involving this molecule promote the genesis of more ROS. When endogenous production of NO in the CNS leads to elevated levels, the molecule can be neurotoxic [60]. The CNS's intrinsic ability to generate ROS and free radicals matched with the high proportion of membrane PUFAs, which are susceptible to lipid peroxidation, and relative lack of antioxidant systems render it prone to oxidative insult [61]. It has been demonstrated however that dietary DHA provides anti-oxidative defense in the brain by enhancing the activity of important anti-oxidant enzymes including catalase (CAT), glutathione peroxidase (GPx), SOD and reduced glutathione (GSH) [61, 62].

The primary outcome of ROS damage in the CNS is lipid peroxidation. The products of lipid peroxidation, notably 4-hydroxy-2-nonenal (4-HNE), have been implicated in neurodegenerative processes by blocking glutamate transport and causing a buildup of extracellular glutamate [63]. This is accomplished by 4-HNE-induced covalent modification of glutamate transporters that render them less active [63]. Glutamate is the most abundant excitatory neurotransmitter in the mammalian nervous system however when present at high levels this amino acid can damage neurons in a process termed excitotoxicity [64, 65]. This mechanism can have devastating consequences and might significantly contribute to both the etiology and progression of a number of neurodegenerative disorders [64, 65]. The pathway responsible for excitotoxicity has been well documented and studies

indicate that aberrant regulation of calcium levels may significantly contribute to the neuronal destruction [64, 66]. Extracellular glutamate causes a massive influx of Ca^{2+}, the results of which are pluripotent. Elevated intracellular Ca^{2+} inhibits mitochondrial functions dependant on ion gradients which is detrimental to the cells energy metabolism [64]. Ca^{2+} also activates the previously mentioned NOS which generates excessive levels of neurotoxic NO [56]. In addition to these enzymes, Ca^{2+} dependent proteases become activated leading to physical destruction of the neural cytoskeleton while activated phospholipases break down cell membranes to release AA yielding further ROS which contribute to lipid peroxidation [64]. This process is self-perpetuating through a positive feedback loop whereby Ca^{2+} influx triggers the release of more glutamate [64]. Not only can DHA reduce the lipid peroxidation that precedes these events, but it has also been demonstrated to reduce neurodegenerative glutamate cytotoxicity by inhibiting NO production and Ca^{2+} influx while increasing the activity of antioxidant enzymes such as GPx and glutathione reductase [56].

While maintaining sufficient or elevated levels of DHA have been reported to treat many of the above-mentioned conditions other research groups have demonstrated the consequences of the opposite: DHA deficiency. Recently, Chalon used a rat model with chronic α-linolenic acid-deficiency to demonstrate that both dopamine and serotonin neurotransmission were impaired in the absence of dietary n-3 PUFA and that these effects were reversible with an n-3 balanced diet [52, 53]. These effects can be explained by the effect on membrane fluidity of disrupted PUFA concentrations which would impair vesicle formation [52].

Interestingly, low DHA is also linked to cognitive decline and memory deficits in Alzheimer's disease and some psychiatric disorders [67]. While this may also be explained by impaired neurotransmission others have postulated that deficiencies in DHA may in fact be apoptotic to neurons in a process that involves downregulating the expression of cellular phosphatidylserine [68]. What might be most remarkable is the finding by Calderon and colleagues that demonstrates DHA has pro-regenerative properties [69]. In fact, this group was able to demonstrate that DHA supplementation in primary hippocampal cell cultures was sufficient to increase the length and branching of neurites [69]. These findings have generated speculations as to whether DHA or other n-3 PUFAs have therapeutic potential in neurological disorders. Recently King and colleagues were able to show that administration of DHA contributes to recovery after traumatic SCI. These findings offer promise for future development of novel lipid-based treatments [70].

1.2.5 Arachidonic Acid

AA is a long-chain omega-6 free fatty acid found in the plasma membrane of cells including neurons and immune cells. It is cleaved from the phospholipid bilayer of cell membranes by the enzyme phospholipase A_2 (PLA_2). PLA_2 occurs in a number of different isoforms and can be found in the extracellular (secreted PLA_2 or $sPLA_2$) or intracellular space (cytosolic or $cPLA_2$). This enzyme is largely calcium dependant and provides substrate (AA) for cyclooxygenase (COX), lypoxygenase (LOX), and epoxygenase (EPOX) enzymes, which in turn generate eicosanoids that are harmful to cells [71, 72]. Interestingly, these same

enzymes also metabolize DHA, generating a class of molecules collectively referred to as docosanoids. This group includes resolvins and neuroprotectins, which as their name indicates, protect the nervous system from eicosanoids by both anti-inflammatory and immune regulatory actions [72, 73]. Eicosanoids derived from AA include pro-inflammatory prostaglandins such as prostaglandin E2 (PGE_2), thromboxanes and leukotrienes all of which play important roles in inflammation and oxidative damage [72]. The action of these molecules has also been linked to many neuroinflammatory conditions and elevated levels of PGE_2 have been found in brain tissue, cerebrospinal fluid and serum of ALS patients [74, 75]. The actions of AA affect many biological processes in a contrasting manner to DHA, as the release of AA perpetuates the inflammatory response with detrimental consequences in the CNS. The conversion of AA to prostaglandins requires the oxidation of AA by COX-2 enzymes; which subsequently are the target of non-steroidal anti-inflammatory drugs (NSAIDs) [76]. It has been demonstrated by several groups that inhibiting COX-2 in animal models of ALS is able to ameliorate symptoms and retard disease progression [77-79]. In addition, Kiaei and colleagues have shown that $cPLA_2$ plays a pivotal role in ALS by providing AA to be metabolized in the pro-inflammatory COX-2 pathway [71]. AA is an intermediate in numerous important pathways and imbalances in this fatty acid have been implicated in several neurodegenerative disorders by causing abnormalities in inflammation and neurotransmission [80]. Chang and colleagues demonstrated the treatment with lithium (an effective treatment for some neurological disorders) could decrease both the level of AA and the activity

of PLA_2 in the CNS indicating that aberrant AA levels could be responsible for neurological conditions [81, 82]. Furthermore, studies by Song and colleagues demonstrated that AA enriched diets increased anxiety in rats similar to that seen with interleuking-1β (IL-1β) treatment supporting the pro-inflammatory pathway of this lipid [83]. While the metabolic pathway of AA may be well known, many of the outreaching effects are still poorly understood. What is clear however is that AA levels must be carefully regulated and that finding efficient means of pharmacological intervention in the cases of excessive AA accumulation may bring therapeutically relevant benefit to patients suffering from various neuropathies. A summary of ω-3 and ω-6 PUFA pathways and their effects on neuroinflammatory conditions is depicted in Fig 1.2.

1.3 Oxidative stress

1.3.1 Reactive oxygen species

The presence of ROS and some of its effects have been mentioned throughout this introduction. The following section will provide a brief summary of the key pathways involved in ROS generation and its downstream effects in the CNS. Particular emphasis will be placed on the role of oxidative stress in ALS as this pertains to the topics of this thesis.

The majority of intracellular ROS are generated by the mitochondria during oxygen metabolism. Other sources in the CNS include NOS, xanthine oxidase and cytochrome P_{450} oxidase; enzymes responsible for generating reactive nitrogen species (RNS), superoxide and oxygen radicals respectively [84]. Motor

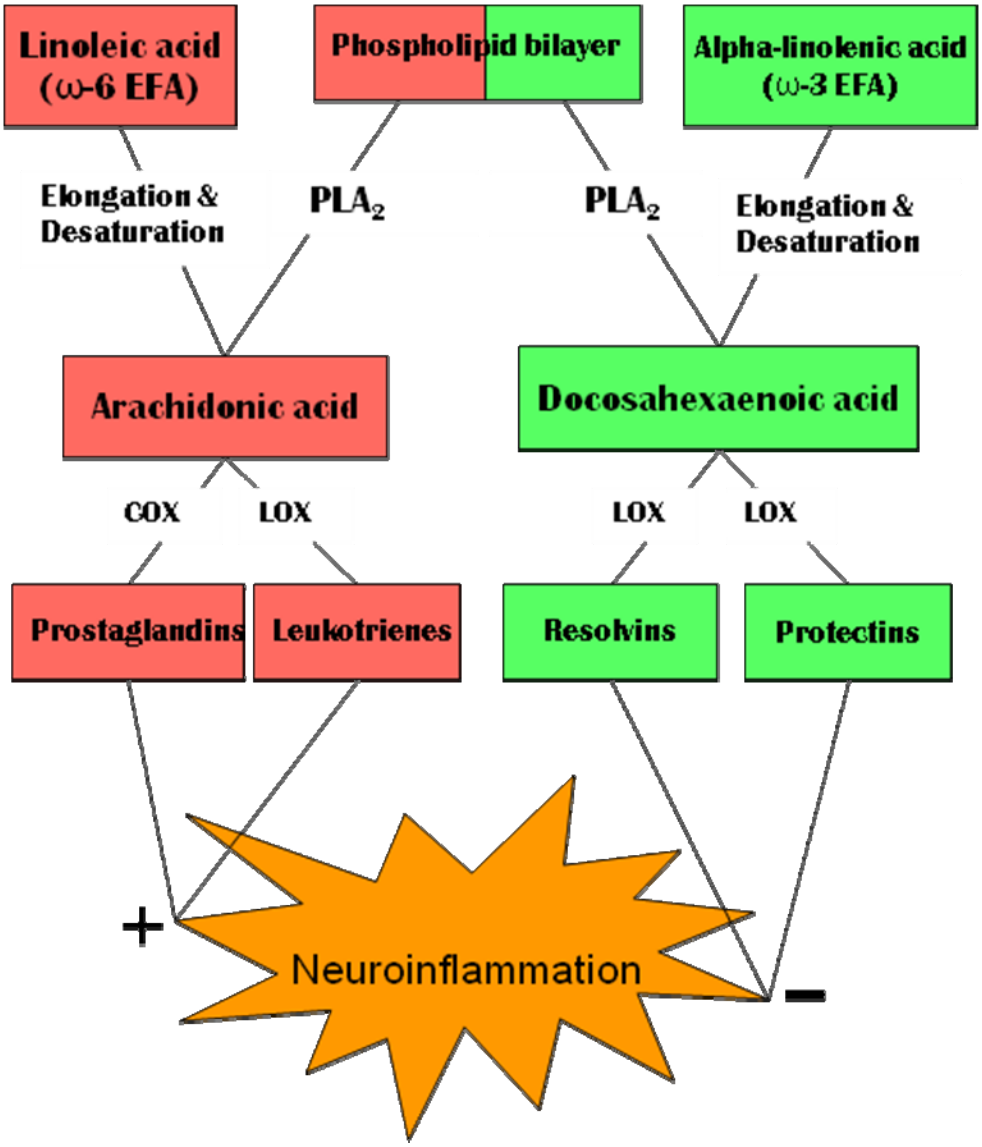


Fig 1.2 Metabolic pathways of PUFAs and their effects on neuroinflammatory status. The pathways of ω-6 and ω-3 PUFA generation and degradation are depicted on the left and right portions respectively. The effect each PUFA species has on neuroinflammation is represented by either a '+' or '-' sign to denote proinflammatory or anti-inflammatory actions respectively.

neurons, which receive a high level of excitatory inputs, can also suffer oxidative damage from the generation of ROS, which accumulates as a result of the glutamate excitotoxicity pathways discussed previously [84, 85].

Lastly, phagocytic cells, notably microglia, are capable of generating high levels of both ROS and RNS in order to destroy foreign targets and timely down regulation of activated microglia can protect from CNS damage [84, 86]. The resulting outcome of such oxygen and nitrogen species involves damage to protein, nucleic acid and of particular importance to the CNS, lipids (PUFA). Lipid peroxidation is a self-perpetuating phenomenon that generates many products including malonyldialdehyde (MDA), 4-HNE and nitrotyrosine (NT) [63]. MDA is the predominant aldehyde product produced from lipid peroxidation and its intra- and extracellular reactivity and long half-life make it an excellent marker of oxidative damage [87, 88]. 4-HNE is another aldehyde responsible for many adverse effects. It is formed by the peroxidation of n-6 PUFA and has been found in elevated levels in the cerebrospinal fluid (CSF) of ALS patients [87, 89]. It has also been demonstrated that treatment with 4-HNE leads to a decrease in the number of motor neurons in the spinal cord and a potentially pathological reduction of intracellular calcium in surviving neurons [89]. NT is also used as a marker of oxidative stress caused by peroxynitrite, which has recently been shown to contribute to neurotoxicity and secondary damage processes after acute traumatic spinal cord injury and other neurological insults [90, 91]. By simply measuring the levels of these oxidation by-products one can ascertain a reasonable estimate of the presence of ROS and oxidative damage.

1.3.2 Glutamate

Glutamate is the major excitatory neurotransmitter in the CNS. The role of glutamate in neuropathy has been briefly described above however it is interesting to note that it is also linked to both oxidative stress and antioxidant pathways. In fact, Rao and colleagues showed that spinal motor neurons cultured in the presence of glutamate had significantly higher levels of ROS and that when these ROS were transferred to an animal model of ALS they were able to affect the ability of astrocytes to take up glutamate and caused oxidation of proteins [92]. It was also demonstrated recently that mouse models of ALS are associated with elevated levels of this neurotransmitter [93]. In addition to contributing to excitotoxicity and oxidative stress pathways, glutamate is also a building block of one of the primary antioxidant molecules: glutathione. Reduced glutathione, known as GSH, along with SOD plays a crucial role in protecting the CNS from both ROS and RNS as well as against glutamate excitotoxicity [94, 95]. It is, in fact, the first line of non-enzymatic defense against the hydroxyl radical, which no enzymes can quench [95, 96]. It has also been established that glutathione is necessary for astrocytes to provide neuroprotective effects and that its presence can enhance neuron survival in pathological conditions such as ALS [97, 98]. Others have found that depletion of GSH can induce motor neuron death through impaired mitochondrial function and low levels of GSH have been well documented in neurodegenerative disorders such as ALS and SCI [94, 95]. These results support the conclusion that enhancing antioxidant defense systems in the CNS is an appropriate target for improved therapies in neurodegenerative conditions.

1.4 Therapeutic strategies for neurological disorders

Unfortunately there are currently very few approved pharmacological agents that can effectively treat neurodegenerative disorders such as ALS and SCI. However hope is certainly on the horizon as many promising therapies have reached or are approaching clinical trials. This section will serve to briefly highlight those therapies currently in use and will discuss some promising new agents and the potential future direction for therapeutics.

1.4.1 Therapeutic strategies for SCI

At the present time there is only one approved pharmacological option for victims of acute SCI in Canada but it has not gained approval by the FDA or many other international governing bodies [99, 100]. Methylprednisolone, a steroid drug, was discovered in the early 1990's to help minimize secondary damage when given promptly (within 8 hours) after the traumatic event [101]. It is generally recognized that Methylprednisolone, like other steroids exerts its effects by inhibiting the inflammatory response and reducing oxidative damage [100]. This treatment however has come under scrutiny as a number of controlled trials have failed to show significant improvements after treatment with methylprednisolone [102, 103]. Further investigation and approaches aimed at reducing inflammation, decreasing oxidative stress-induced lipid peroxidation and enhancing neuroprotection are likely to provide the best therapeutic outcomes.

1.4.2 Therapeutic strategies for ALS

ALS presents many of the same pharmaceutical hurdles as SCI and much like with SCI there exist few treatments with proven efficacy. Currently only one drug, Riluzole, is approved by the FDA for the treatment of ALS yet it provides

only a modest survival improvement [104-106]. This agent exerts its effect by inhibiting glutamate and Na channel activation [104, 105]. While the prevention of glutamate excitotoxicity is an important pathway to target it is reasonable to assume that therapies directed at controlling oxidative stress, inflammation and enhancing neuroprotection could provide additional therapeutic benefit to ALS patients. There are currently a number of promising pharmaceuticals undergoing trials, which target these neuropathic precursor pathways. At the moment the most promising agents are those which provide antioxidant, anti-inflammatory, immune regulatory or anti-apoptotic functions:

Thalidomide

Thalidomide has shown efficacy in delaying functional motor deficits and extending survival in a mouse model of ALS [107]. It can be safe for human use under controlled conditions and can effectively cross the blood brain barrier (BBB) [104, 108]. Thalidomide, well known for its antiangiogenic effects also provides immune regulatory effects by inhibiting TNF-α expression, which likely explains its success in ALS studies [107, 108].

AEOL 10150

AEOL 10150 is an antioxidant of the metalloporphyrin class. This agent is of interest for the treatment of ALS because it is a powerful antioxidant able to scavenge a variety of ROS identified in ALS and inhibit lipid peroxidation [109]. This manganese porphyrin has also been tested in a $SOD1^{G93A}$ transgenic mouse

model of ALS and when administered by IP injection at the onset of symptoms, treated mice showed significant improvements in survival [110]. As expected from the antioxidant properties of this drug, mice treated with AEOL 10150 showed lower levels of lipid peroxidation including MDA and NT [110]. In addition, treated mice had less activation of glia and increased motor neuron sparing within the ventral horn of the lumbar spinal cord [110].

Minocycline

Minocycline is a second-generation tetracycline that possesses pleiotropic effects in addition to its antimicrobial action. Yrjänheikki and colleagues have demonstrated in a brain ischemia model that minocycline is anti-inflammatory, can inhibit microglial activation and is neuroprotective [111, 112]. These results suggest that minocycline therapy could be beneficial in ALS and this has been confirmed in a mouse model of the disease [113]. Dietary supplementation of the tetracycline derivative at the late pre-symptomatic stage in $SOD1^{G37R}$ transgenic mice improved muscle strength, delayed disease onset and significantly enhanced survival [113].

These drugs represent only a sample of those which show promise for use in ALS. Further research is certainly necessary and controlled clinical trials should be pursued where possible as such studies have often revealed weaknesses in hopeful therapies such as with Vitamin E, which demonstrated excellent results in animal models but failed to show effects in humans [114]. Management of the

oxidative phenotype and reducing inflammation are likely to provide the greatest therapeutic benefit in the treatment of this aggressive disease.

1.5 Animal models of neurological disorders

Animal models have had an important impact on medical research and represent a great surrogate for studying complex human diseases. Often disease phenotypes can be accurately replicated in animals and allow researchers to pinpoint mechanistic pathways or genetic involvements that can be extrapolated and applied to the related human condition. Animal models also provide an added level of complexity over *in vitro* methods, which cannot account for interactions between cells in organs or organ systems. It is important to note however that animal models also have limitations and results from studies using these models should be "taken with a grain of salt". When studying neurological disorders with a genetic component transgenic animal models are popular. Such models allow specific genetic targeting but also have certain drawbacks including the abnormal expression of exogenous genes, which may lead to gene interactions that do not occur in nature. It is also important to recognize that most animal models are derived from inbred strains which by definition cannot mimic the genetic diversity of the human population. For the same reasons, studies involving inbred mice allow one to study the influence of recessive genes, which otherwise would be masked by the genetic heterogeneity observed in humans or outbred mice. While certain models offer fewer limitations than others, it is important to continue utilizing a variety of models to generate a more complete and useful picture of physiological processes.

1.5.1 SCI Models

There exists a long history of modeling acute spinal cord injury in animals. Early models utilized large animals such as dogs and cats because inducing injury in these animals was technically more feasible [115]. Currently rat and mouse models are favored likely due to both availability and economy, however primate models offer essential information when analyzing the outcomes of SCI on hand and digit function [116]. Essentially each model is designed to replicate a different type of primary injury with acute, mechanical injuries (such as shearing, compression, contusion or transection) being the most common [115]. While complete transection can provide a paradigm for axon regeneration most human SCI do not completely transect the spinal cord. Therefore models of acute compression are favorable and have evolved from the original weight-dropping and inflated balloon compression techniques [115, 117]. Clip compression is used to provide a simple and reproducible compression force on the spinal cord but requires complete laminectomy and is not measurable [118, 119]. The advantage of this system is that compression can be applied for a longer duration than that provided by impactor devices [117, 119]. Impactor devices are very popular and are of two primary types: the New York University (NYU) impactor or the Ohio State University (OSU) impactor [117]. The NYU impactor drops a weighted rod from a predetermined height onto the exposed spinal cord [117]. The OSU impactor is a more sophisticated version that electronically controls the impact force and tissue displacement and has been shown to generate a highly reproducible injury [120, 121]. The variety of models available for SCI provides researchers with the opportunity to select those appropriate to their needs. Using

these models in conjunction with different transgenic mouse strains provides diverse opportunities to study SCI under different conditions.

1.5.2 ALS Models

Unlike other diseases that are often modeled in large animals such as dogs, sheep and primates, ALS models have been limited almost exclusively to rodents. Although rat models exist, murine models have become the most popular and are available commercially [122]. However, the first Drosophila model of ALS was very recently developed and could provide new insight into the disease as this model gains popularity and use within the scientific community [123]. Perhaps the overwhelming use of rodents is due to the relative complexity of these models; most of which are transgenic and would be difficult to generate in other species. As mentioned in a previous section, a proportion of genetically inherited cases of ALS have been linked to a mutation in the gene encoding the antioxidant enzyme SOD1 [18]. As a result, mutant forms of this gene are the most commonly used transgene in the generation of ALS mice [20]. While several mutant forms of this gene have been used in mice the two most popular murine models of ALS are $SOD1^{G93A}$ and $SOD1^{G37R}$. $SOD1^{G93A}$ transgenic mice possess a mutant form of human SOD1 with a glycine to alanine substitution at position 93 [20]. $SOD1^{G37R}$ is also a mutant form of human SOD1 that has a glycine to arginine substitution at position 37 [124]. Mice expressing these transgenes are very similar in their phenotypic expression however the time course of disease progression is quite different.

1.5.3 SOD1^{G93A}

The SOD1^{G93A} strain is known for its extremely fast disease progression. Although many of the pathological features mirror human ALS, these animals display the initial onset of impaired motor function at approximately 90 days of age [20]. The initial symptoms present as impaired hind limb function and rapidly progress to complete hind limb paralysis and eventual death typically by 4 months of age [124-126]. While this model allows for a reasonable duration of studies, the extremely aggressive phenotype may result in limited effectiveness of some treatments which could otherwise be therapeutically useful.

1.5.4 SOD1^{G37R}

The SOD1^{G37R} strain presents with many of the same disease hallmarks as the G93A strain, however initial onset is not detectable until 9 months of age and the clinical endpoint is usually reached around 11 months to one year after birth [113]. While working with this strain does lead to extremely long experimental protocols, it is perhaps a more representative model of human ALS, which also progresses slowly.

1.6 Fenretinide

Retinoids, molecular species derived from vitamin A, are a family of molecules that contain the retinyl group. These compounds are considered essential human nutrients and have a role in numerous cellular functions including growth, differentiation, gene transcription and antioxidant activity [127-130]. Fenretinide [N-(4-hydroxyphenyl) retinamide, 4-HPR] is a semi-synthetic retinoid that has demonstrated promise for the use as an anti-tumor and chemotherapeutic

treatment in many cancers including breast cancer, Kaposi sarcoma, glioma and neuroblastoma for which is it currently undergoing clinical trials [131-137]. Fenretinide is particularly appealing because of its minimal toxicity when compared to other retinoids [138]. Interest for its application to neurodegenerative diseases have arisen because when used in neuroblastoma and gliomas it seems to have apoptotic effects on malignant cells while protecting surrounding neurons [133, 139].

Previous findings demonstrated that treatment with fenretinide induces ceramides *in vitro* [140, 141]. Fenretinide was also previously shown to have anti-inflammatory actions, possibly by inducing ceramides; which as mentioned previously inhibits pro-inflammatory transcription factor NF-κB [46, 142]. In addition to treating particular symptoms associated with Cystic Fibrosis (CF) such as increasing bone density, the drug was found to correct ceramide deficiency in a mouse model of CF [143, 144]. In addition, fenretinide treatment was able to correct imbalances in AA and DHA and decrease markers of oxidative stress [143]. Based on these findings demonstrating the powerful effect of fenretinide against chronic inflammation associated with CF, our group recently proposed a novel use of this drug to control neuroinflammation associated with SCI and other neural diseases. Our initial studies targeted spinal cord injury and were followed by application of fenretinide therapy to the $SOD1^{G93A}$ transgenic mouse model of ALS. These experiments will be discussed in the following section of this thesis. For an overview of disease mechanisms of ALS and the proposed targets of fenretinide therapy refer to Fig 1.3.

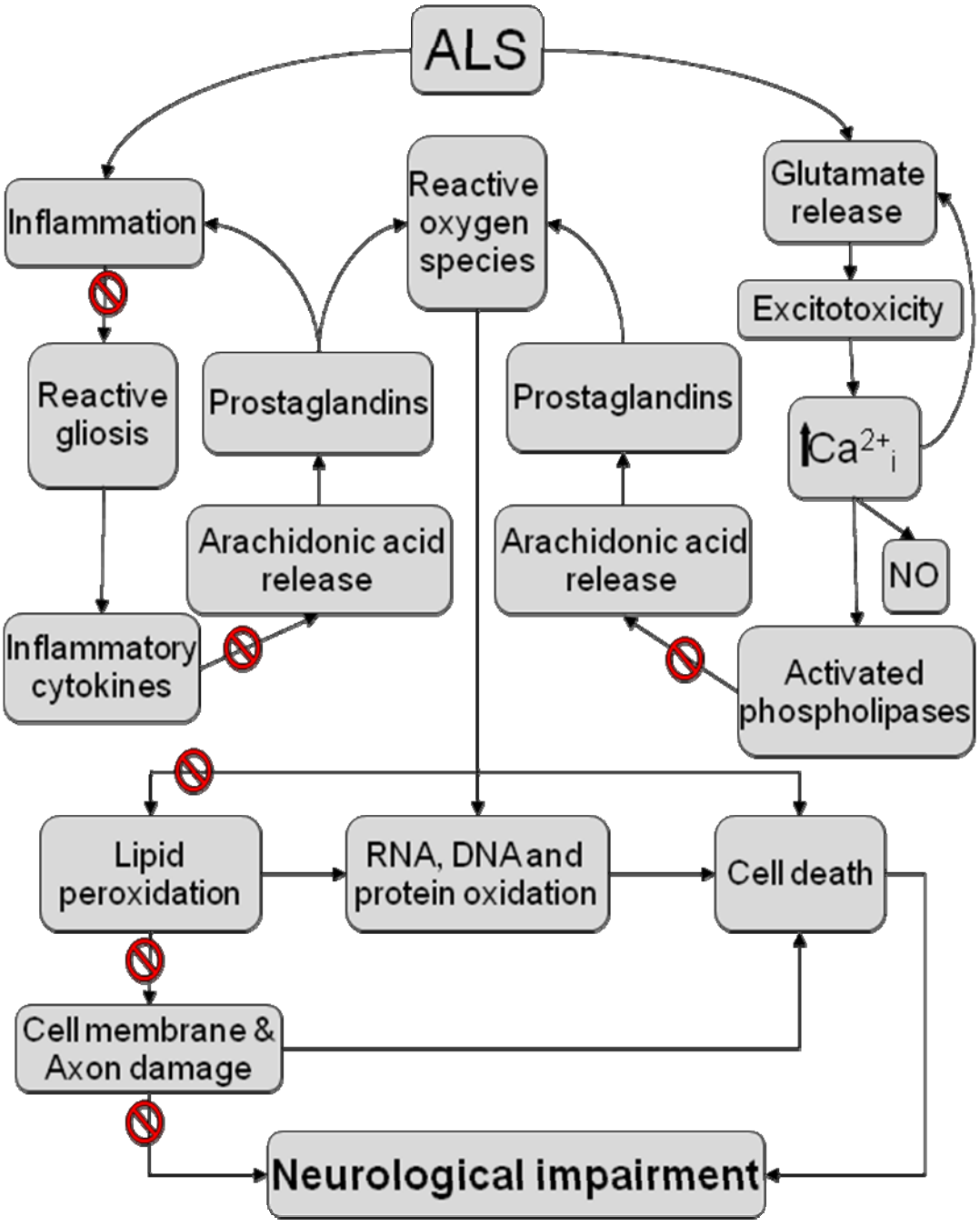


Fig 1.3 Mechanisms of ALS progression. This schematic represents a simplified view of the various factors that contribute to ALS. The circular symbol with a diagonal line through the center (Ø) is used to highlight particular portions of the pathway which we believe are targeted or inhibited by fenretinide treatment.

1.7 Conclusion

Many advancements in understanding and treating CNS disorders have been developed through the meticulous research of several laboratories. This introduction provides a review of many of these accomplishments which offers much greater hope for those affected today than in the past. While many neurological diseases seem quite different they often share some commonalities and it is interesting to note the important role that membrane lipids play. While treatments for neurodegenerative disorders are currently scarce, it is conceivable that pharmacological targeting of lipid imbalances may provide a novel therapeutic option that could be applied to diverse neurological disorders. The study in this thesis will demonstrate that fenretinide; a drug, which effectively corrects phospholipid imbalances, can treat many of the phenotypes of ALS.

Preface to Chapter Two

Rationale: The introduction provided an up-to-date description of the pathophysiology of ALS and a number of related neurodegenerative disorders. As well, the molecular mechanisms and role of membrane lipids involved in disease progression, and pharmaceuticals with therapeutic promise were discussed. Nevertheless, ALS remains a difficult disease to untangle and our understanding is limited. While a cure may never be discovered and treatment options only improve slowly, further developments are urgently needed.

Our group has shown previously that fenretinide is capable of correcting membrane lipid imbalances in an animal model of CF as well as down-regulating particular pro-inflammatory cytokines [143, 144]. We also demonstrated that treatment with fenretinide had a similar effect on lipid concentrations in an animal model of spinal cord injury and was sufficient to improve motor function and neurological impairment in these animals (manuscript in preparation). Analysis of plasma and spinal cords from these animals also showed a decrease in markers of oxidative stress and improved neuron sparing respectively (manuscript in preparation). *In vitro* experiments, which we conducted on macrophages, demonstrated a significant ablation of TNF-α release when stimulated with various toll ligands (Lachance *et al.*, 2008 *submitted*). Before embarking on the present experiments we tested two animal models of ALS ($SOD1^{G93A}$ and $SOD1^{G37R}$) and were able to show that, when compared to their wild-type counterparts, transgenic mice had an imbalance in two lipids which fenretinide

has been shown to correct, AA and DHA. Transgenic mice also displayed significantly higher levels of oxidative stress, almost two-fold the level seen in wild-type littermates for some markers. Taken together these results provided compelling evidence that fenretinide could have therapeutic effects in transgenic mouse models of ALS by correcting the lipid imbalance observed in these animals as well as decreasing their oxidative burden and improving neuron sparing.

Chapter Two

Chapter Two

Based on manuscript in preparation:

Fenretinide improves functional recovery and extends survival in a transgenic mouse model of Amyotrophic Lateral Sclerosis

Thomas A.A. Skinner, Juan B. De Sanctis, Ruben López-Vales, Marie-Christine Guiot, Jennifer Henri, Samuel David, Danuta Radzioch

Abstract :

Amyotrophic Lateral Sclerosis is a late-onset neurodegenerative condition affecting the motor neurons of the spinal cord, brainstem and primary motor cortex. The disease usually presents with mild paralysis around age 40 and rapidly progresses to complete paralysis and respiratory failure in 3-5 years. At the present time Riluzole is the only drug approved by the FDA and treatment effects are not substantial. In this study we demonstrate that fenretinide, a drug with proven efficacy in regulating phospholipid imbalances, mitigating inflammation and reducing oxidative stress may be a suitable candidate for treating ALS. Using a $SOD1^{G93A}$ transgenic mouse model of ALS we demonstrate for the first time that low-dose fenretinide therapy is sufficient to delay onset, improve motor function and extend survival in affected animals. Our results also reveal that fenretinide treatment improves ω-3:ω-6 poly unsaturated fatty acid (PUFA) ratios without a change in diet, reduces lipid peroxidation and mitigates reactive gliosis in the spinal cord. These findings warrant further investigation as they suggest excellent potential for fenretinide as a therapeutic treatment for ALS.

2.1 Introduction

Amyotrophic lateral sclerosis is a multicausal neurodegenerative disease of the motor neurons characterized by progressive muscle weakness, paralysis and eventual death usually occurring 3-5 years after diagnosis [17, 145, 146]. It is a relentlessly devastating disease that targets both upper motor neurons of the cerebral cortex and lower motor neurons of the spinal cord and brainstem culminating in respiratory failure [147]. It is the most common adult-onset neurodegenerative disorder and while the great majority of ALS cases are of a sporadic nature with no known cause, approximately 5-10% of cases are inherited with various genetic targets identified [145-147]. While sporadic and familial forms are often discussed separately it is important to note that their clinical presentation is nearly identical. The most prominent gene identified in familial ALS (fALS) cases is the gene encoding Cu,Zn-superoxide dismutase (SOD1) which is thought to be responsible for approximately 25% of fALS cases [18, 147-150]. The disease is inherited in an autosomal dominant manner and a variety of missense mutations responsible for the disease have been identified in the SOD1 gene [18, 19, 148, 149, 151]. Many theories on the etiology of sporadic ALS (sALS) have been proposed ranging from auto antibodies to impaired amino acid transport, however discovery of SOD1's involvement in fALS has provided important information for both fALS and sALS [152-154].

Transgenic mouse models expressing mutant forms of human SOD1 have become widely used as a surrogate to study human ALS. Over expression of mutant SOD1 variants in these animals produces a progressive motor neuron disease with similar pathological features to both the familial and sporadic forms

of human ALS although the disease progresses with a much faster time course [155, 156]. The discovery of SOD1 involvement lead to the natural assumption that oxidative stress could play a role in disease progression and elevated levels of reactive oxygen species (ROS) and impaired redox mechanisms have been confirmed in a number of studies [157-161]. In addition to the ROS generated by impaired SOD1 function, ALS is also associated with inflammation and activated glia, which are known to release both ROS and reactive nitrogen species (RNS) [162-164]. Uncontrolled ROS and RNS can cause cellular damage in a variety of ways but of particular interest is the effect on the integrity of lipid membranes.

It is well known that membrane lipids can be altered by oxidative damage and this can impair proper cell function, especially in the CNS, which possesses the highest lipid concentration after adipose tissue [33]. Membrane integrity is essential for many functions including transport, physical support and signal transduction, which is especially important for neurons, and it has been documented that polyunsaturated fatty acids (PUFAs), in particular, are extensively damaged when exposed to oxidative stress [165]. Of central importance in inflammatory conditions is maintaining a balance between ω-3 and ω-6 PUFAs as each can have immune regulatory functions. Elevated levels of the ω-6 PUFA arachidonic acid (AA) is associated with an increase in inflammation by promoting the genesis of pro-inflammatory prostaglandins, thromboxanes and leukotrienes, collectively termed eicosanoids [166]. When these mediators are produced in high quantities it can result in neuron damage [166]. Conversely, the ω-3 PUFA docosahexaenoic acid (DHA), which is found in high concentrations in

neural and synaptic membranes, has been shown to help alleviate inflammation and even protect against neurodegeneration [167]. In fact the metabolites of DHA, dubbed neuroprotectins and resolvins, inhibit the production of all three eicosanoids mentioned above [167]. These lipids play an important part in neurological defenses but are commonly overlooked. It is our belief that targeting these PUFAs with pharmacological agents could provide new potential for therapy in disorders of the nervous system.

Fenretinide [N-(4-hydroxyphenyl) retinamide, 4-HRP] is a semi-synthetic retinoid currently in clinical trials for the treatment of several cancers [132-138, 168]. It is a particularly promising drug for neurological cancers and has the advantage of possessing very low toxicity [133, 136-138]. Our lab and others have demonstrated that treatment with fenretinide increases ceramides [140, 143, 144]. This pathway has been linked to activation of 12-lipoxygenase (LOX-12), which catalyzes AA oxidation thereby decreasing its concentration [169, 170]. We reported recently that treatment with fenretinide decreases AA and increases DHA both *in vitro* and *in vivo* [143], (Lachance *et al.*, 2008 *submitted*). We have also found that fenretinide treatment reduces production of pro-inflammatory cytokines by inhibiting levels of phospho-ERK-1/2 in a macrophage cell line (Lachance *et al.*, 2008 *submitted*). In addition, we recently discovered that fenretinide treatment reduced oxidative stress, improved neuron sparing and enhanced motor function in a mouse model of spinal cord injury (manuscript in preparation).

Currently there is only one drug approved for human use in ALS, Riluzole, and it provides only a modest survival improvement of 2-3 months [171]. Evidently new therapies are desperately needed. While many of the effects of oxidative imbalance have been explored in mouse models of ALS, investigation into lipid involvement has been largely untouched. Here we use a $SOD1^{G93A}$ transgenic mouse model of ALS to examine the efficacy of fenretinide therapy in this neurodegenerative condition. We show for the first time that fenretinide, an agent capable of altering PUFA concentrations, is able to extend survival and improve motor function in $SOD1^{G93A}$ mice. We also demonstrate that inflammation in the spinal cord is ameliorated and propose a pathway for fenretinide's actions in a transgenic mouse model of ALS.

2.2 Materials and Methods

2.2.1 Transgenic mice

SOD1^{G93A} [B6SJL-TgN(SOD1-G93A)1Gur/J; 002726] transgenic mice were generously donated by Dr. Jean-Pierre Julien from Laval University (Quebec, PQ, Canada). These animals were derived from a colony maintained on a C57BL/6 background. All animals were housed and bred at the McGill University Health Centre Research Institute Animal Facility. Mice were maintained in cages with sterile wood-chip bedding and kept in ventilated racks. All animal housing, breeding and experimentation were performed under specific pathogen-free conditions in a barrier facility. All mice were derived from matings of wild-type C57BL/6 females with SOD1^{G93A} transgenic male mice. Pups were genotyped between 21 and 28 days of age using real-time quantitative polymerase chain reaction (qPCR) in accordance with The Jackson Laboratory protocols (see below). SOD1 transgenic animals selected for experimentation were separated (1 animal/cage) for the duration of all studies. All mice used for experiments were female and were derived from the offspring of original breeding pairs donated by Dr. Julien. Mice were supplied with NIH-31–modified irradiated mouse diet (Harlan Teklad, Indianapolis, IN) *ad libitum* at all times. Beginning at day 30, mice used for experimentation were also given 12.5 mL of liquid diet (Peptamen liquid diet; Nestle Canada, Brampton, ON, Canada) 5 days per week containing either 5mg/kg of fenretinide or an equivalent volume of vehicle (95% ethanol). After randomly assigning mice to either the fenretinide or control (vehicle) group, each mouse was then assigned randomly to one of three experimental groups to analyze behavior, plasma lipid concentration or histology. All procedures

performed followed Canadian Council of Animal Care guidelines and were approved by the McGill University Animal Care Committee.

2.2.2 Fenretinide preparation and treatment

Fenretinide was generously donated by Dr. Robert Smith of the National Institutes of Health (NIH) (Bethesda, MD, USA). Fenretinide powder was resuspended under sterile conditions to make a 2 μg/μL stock solution in 95% ethanol. This preparation was incorporated into Peptamen liquid diet at a dose of 5mg/kg body weight (approximately 45 μL) per mouse as previously explained [144]. 12.5 mL of Peptamen was selected for the daily liquid diet to ensure that all Fenretinide was consumed as this represents approximately 4/5 of daily mouse food consumption and the mice ate Peptamen preferentially. Mice were treated 5 days per week beginning at 30 days of age until they reached the clinical endpoint. Diet preparation for sham-treated animals was identical except that the equivalent volume of 95% ethanol was used instead of ethanol containing fenretinide. After preparation, the diets were kept protected from light at all times and at 4ºC until their administration. Prepared fenretinide in 95% ethanol was kept protected from light at -20ºC for a maximum of 1 month.

2.2.3 Histological evaluation

Mice were deeply anaesthetized with a cocktail of ketamine (7.5mg/ml) and xylazine (0.5mg/ml) administered via intraperitoneal injection at a dose of 20ml/kg of body weight. Animals were then sacrificed by transcardial perfusion

with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS). The fourth lumbar spinal cord segment, identified by its contribution to the sciatic nerve was carefully removed, post-fixed for 1 hour in 4% paraformaldehyde solution and cryoprotected overnight in 30% sucrose in 0.1 M PBS. 10μm serial sections were cut on a Leica cryostat (Leica Microsystems GmbH, Wetzlar, Germany) and immunostained with rat polyclonal antibodies against GFAP (1:400; Zymed Labs) or rat polyclonal antibodies against Mac-2 (1:4; kindly provided by Dr. Samuel David) to detect the presence and activation of glia. Digital images of both ventral horns were captured every 300 μm. Images were imported into SigmaScan Pro Image Measurement Software Version 5.0.0 (SPSS Inc., Chicago, IL) and activated astrocytes and microglia were quantified by exceeding an intensity threshold. Values were then normalized for the total area examined. Serial sections from a separate set were stained for Nissl body detection using cresyl violet staining and the number of motor neurons surviving in the ventral horn were quantified. Outliers were identified as data falling outside ± 2 standard deviations from the group mean when the point in question was removed.

2.2.4 Tissue collection for fatty acid analysis

Mice were euthanized by inhaling CO_2 followed by cardiac puncture exsanguination. Blood collected was processed as described in the next section. The lumbar spinal cord segments, identified using the ribs and vertebrae as a guide, were transected and all spinal cord tissue was removed and homogenized before storing in 1mM butylated hydroxy anisole (BHA) in chloroform/methanol

(2:1 vol) at -80°C until analysis was performed to maintain sample integrity. Cerebral cortex and brainstem samples were collected, homogenized and stored separately in the same BHA solution. Blood samples collected were treated under the same protocol as the samples extracted at different time points.

2.2.5 Blood collection

Blood samples were collected at day 60, 90 and 120 in addition to the time at the clinical endpoint mentioned above. Mice were placed under a heating lamp for 5 minutes before sampling. Mice were then placed in a holding device and one hind limb was immobilized and shaved. The saphenous vein was pierced with a 25G needle and 100 µL of blood was collected and mixed with 10 µL of 0.5M EDTA to prevent coagulation. Samples were then centrifuged at 350x*g* for 7 minutes at 4°C and 40 µL of plasma was removed and stored in 400 µL of the BHA solution described above. All samples were stored at -80°C until analysis was performed.

2.2.6 Lipid analysis

Plasma, spinal cord, cerbral cortex and brainstem samples were all analyzed to determine the lipid concentration of each. Ceramide, phospholipid-bound docosahexaenoic acid (DHA), phospholipid-bound arachidonic acid (AA), malonyldialdehyde (MDA) and nitrotyrosine levels were assessed in all samples. To determine the lipid concentration of tissue and plasma samples, analysis was performed using an enzyme-linked immunosorbent assay (ELISA) method.

The extracted lipid fractions were dried under nitrogen and resuspended in heptane. Separation of phospholipids was performed by thin-layer chromatography (TLC), detected by iodine. The separated lipid samples were then subjected to ELISA to ascertain the concentrations of each lipid species. The phospholipids from the dry silica, once resuspended in ethanol, were used to coat Nunc plates specific for lipid binding. The plates were then washed and incubated with blocking buffer for 1 hr at 37° C (PBS, 0.1% Tween 20, and 1% bovine serum albumin). Following the blocking step, the plates were incubated with murine IgM (Sigma-Aldrich) antibody (Ab) specific for the particular lipid species desired for 1 hr at 37°C. Following another wash, the plates were incubated with anti-mouse IgM Ab conjugated with peroxidase for 1 hr at 37°C. The final step involved incubating the plates with the peroxidase substrate (TMB; Roche, Laval, QC). The intensity of the colorimetric reaction was determined by spectrophotometry at 405 nm and the level of each lipid species was calculated using a standard curve as a reference. Outliers were identified as data falling outside ± 2 standard deviations from the group mean when the point in question was removed.

2.2.7 Motor function analysis (Rota-rod)

Motor function was assessed by Rota-rod (Med Associates Inc., St. Albans, VT) two times per week beginning at 70 days of age. An acclimatization period of 3 days was implemented before beginning measurements to allow animals to become familiar with the apparatus. Animals were placed on the rod

with a constant rotation of 16 rpm and the time latency to fall was used as a measurement of motor function. Animals remaining on the apparatus after 300 seconds were given a perfect score and the trial was ended. Three consecutive trials were performed with a one minute rest period between each trial and the best result of the three trials was recorded. Mice used for plasma analysis were also included in the Rota-rod experiments as it was determined after evaluating their performance that it was not impaired. In the rare event that an animal's performance improved, the lower "pre-improvement" time point was dropped as it was not considered representative of their true ability given the progressive nature of paralysis in $SOD1^{G93A}$ mice.

2.2.8 Survival

$SOD1^{G93A}$ mice typically develop the first signs of motor impairment around 90 days of age. The initial stages present with a resting tremor and slight gait impairment which progress to complete hind limb paralysis at the end stage. The clinical end point was determined to occur when a mouse was unable to right itself in less than 30 seconds after being turned on its side or when greater than 20% weight loss had occurred. While both methods were used to assess each animal, in the vast majority of cases animals reached the weight loss endpoint prior to losing the ability to right themselves within the allotted time.

2.2.9 Analysis of genotype by real-time RT-QPCR

Amplification of DNA obtained from tail tissue was performed on the Stratagene MX-4000 sequence detector (Stratagene, La Jolla, CA). PCR was performed using the SYBR Green Quantitative RT-PCR kit (Sigma, St. Louis, MO). The amplification program for $SOD1^{G93A}$ DNA consisted of an enzyme activation step for 3 min at 95°C, followed by 40 cycles of a denaturing step for 30 s at 95°C, an annealing step for 30 s at 60°C and an extension step for 45 s at 72°C. A melting-curve analysis was performed after amplification to determine specificity of the PCR products (which were also confirmed with gel electrophoresis). Two sets of primers were used, in separate reaction flasks, to amplify both the WT and transgenic SOD1 genes. Both primer sets (Tg forward: 5'-CAT CAG CCC TAA TCC ATC TGA-3', Tg reverse: 5'-CGC GAC TAA CAA TCA AAG TGA-3', WT forward: 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3', and WT reverse: 5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3') were diluted to a final concentration of 250nM and tested to optimize conditions.

2.2.10 Statistical analyses:

Data was analyzed and statistics were calculated with GraphPad Prism Version 4.03 software (GraphPad Software, San Diego, CA). Analysis of Rota-rod performance was performed by two-way analysis of variance (ANOVA) with Bonferroni post-tests at each time point. Survival was analyzed by log rank test of Kaplan-Meier cumulative survival plots and an un-paired, non-parametric t-test of mean survival time. Comparisons of lipid concentrations across the duration of

the study were also analyzed by two-way ANOVA with Bonferroni postests while comparisons at day 120 were made with unpaired, non-parametric t-tests. Motor neuron, microglia and astrocyte comparisons were also made with non-parametric t-test analysis. Significance for all analyses was set at a two-tailed P value of $\leq$ 0.05. Data are displayed as mean $\pm$ SEM.

2.3 RESULTS

2.3.1 Fenretinide improves motor function in SOD1^{G93A} mouse model of ALS

Impairment of motor function was measured by twice-weekly Rota-rod testing. SOD1^{G93A} transgenic mice treated with fenretinide performed significantly better on the Rota-rod then sham treated control animals suggesting that the disease phenotype can be modulated by fenretinide treatment. Sham-treated animals showed a decline in motor function 2 weeks before any impairment was detected in the fenretinide-treated group indicating that treatment with Fenretinide was sufficient to delay disease onset in this model. At all time points after day 91, the fenretinide-treated group performed better than control animals and this difference was significant ($p \leq 0.05$) independently at day 112, 116, and 119, as shown in Fig 2.1. Performance at day 112 was increased from 162 ± 23 sec (n=26) for vehicle-treated animals to 216 ± 25 sec (n=19) in 5mg/kg fenretinide treated animals. Performance at day 116 was improved from 98 ± 18 sec (n=26) for vehicle treated animals to 154 ± 27 sec (n=20) observed in fenretinide treated animals. On day 119, fenretinide treatment enhanced performance from 46 ± 13 (n=25) in vehicle treated animals to 109 ± 27 (n=18) in fenretinide treated animals as shown in Fig 2.1. In addition to these specific time points at which performance was significantly improved by fenretinide, overall Rota-rod performance across the entire duration of the study was found to be significantly enhanced in fenretinide treated animals compared to control animals ($p \leq 0.0001$), depicted in Fig 2.1. The treatment was only able to delay the

symptoms of ALS but was not able to cure the animals since the performance score eventually also declined in the fenretinide treated animals.

2.3.2 Fenretinide improves survival

To establish whether fenretinide could enhance survival of $SOD1^{G93A}$ mice, the day at which animals reached the objective clinical endpoint was recorded as their duration of survival. Kaplan-Meier curves were used to calculate survival differences as well as a comparison of mean survival between drug treated and control groups. The mean survival of $SOD1^{G93A}$ mice treated with fenretinide was significantly improved from 143 ± 1.4 d (n=17) for control animals to 148 ± 1.4 d (n=12) for drug treated animals ($p \leq 0.05$), as shown in Fig 2.2A. This difference constitutes an increase in survival of almost 10% from the onset of disease. The median survival of treated animals was also significantly ($p \leq 0.05$) higher than untreated as evident from the Kaplan-Meier cumulative survival plot shown in Fig 2.2B. Early mortality was also more common in control animals. More than 22% of sham-treated mice died before a single mouse treated with fenretinide reached the clinical endpoint and 17% of drug treated animals remained alive after all control mice had reached the endpoint, depicted in Fig 2.2B.

2.3.3 Plasma EFA profiles are altered with fenretinide treatment

In order to determine the effect of treatment on systemic lipid profiles, plasma samples were collected at day 60, 90, 120 and at the clinical endpoint for

both fenretinide treated and control groups. Each sample was analyzed for the contribution of DHA, AA, MDA, NT and ceramides. As expected from previous work, phospholipid bound DHA was found to be significantly elevated in the plasma of fenretinide treated animals compared to control mice. Significantly elevated DHA concentrations were detected for the duration of the experiment ($p \leq 0.0001$), shown in Fig 2.3. In addition, significant increases in DHA were found independently at day 60 and 120 in fenretinide treated mice. Treatment with fenretinide increased phospholipid bound DHA at day 60 from 2.22 ± 0.15 (n=9) from control animals to 2.88 ± 0.18 (n=8) an increase of 30% ($p \leq 0.05$). A similar increase was seen on day 120 from 2.63 ± 0.07 (n=8) for sham treated mice to 3.26 ± 0.17 (n=8) ($p \leq 0.01$) for the drug treated group equating to an increase of approximately 25% (Fig 2.3 & 2.4A). Interestingly phospholipid bound AA, while slightly lower at 60 days of age in fenretinide treated mice, only reached a statistically significant decline in samples taken on day 120, falling 12% from 39.50 ± 0.94 (n=8) for control mice to 34.87 ± 0.91 (n=7) ($p \leq 0.01$) for the fenretinide treated group, as shown in Fig 2.4B. Interestingly, differences in ceramide concentrations failed to reach a statistically significant threshold at any of the time points, which is in contrast to our previous studies. Additionally none of the lipids tested displayed significant differences between vehicle treated and fenretinide treated groups when measured at the clinical endpoint (data not shown). Taken together these results are quite consistent with our previous findings from both CF and SCI models suggesting that fenretinide treatment

contributes to maintaining an environment of decreased ω-6 fatty acids while increasing ω-3 fatty acids.

2.3.4 Plasma lipid peroxidation

The samples tested for EFAs were also analyzed for the presence of two markers of oxidative stress: NT and MDA. At 60 days of age fenretinide treated mice had ameliorated levels of NT, however the difference became statistically significant at day 120 at which point control animals possessed 45% higher levels of NT than mice treated with fenretinide ($p \leq 0.001$), as shown in Fig 2.5A. MDA showed a similar profile becoming significantly ($p=0.001$) reduced at 120 days of age in drug treated mice, as shown in Fig 2.5B. The difference in MDA between groups was 38%. These results provide compelling evidence that treatment with fenretinide is associated with large decreases in lipid peroxidation and may provide protection from reactive oxygen and nitrogen species and oxidative stress, which are present in high quantities in $SOD1^{G93A}$ mice.

2.3.5 Organ essential fatty acid profiles and lipid peroxidation

To establish whether the difference in plasma lipid profiles could be seen in the organs directly affected by ALS, samples of lumbar spinal cord, brain stem and cerebral cortex were analyzed for the presence of the same lipid species and markers of lipid peroxidation. Samples were harvested when each animal reached the clinical endpoint and similar to what was seen in plasma samples taken at the same time, few results were statistically significant. Neither AA nor DHA reached a significant difference between vehicle or fenretinide treated groups in the brain stem samples (data not shown). AA in spinal cord samples and DHA in cortex

samples were also not significantly affected by fenretinide treatment, as shown in Fig 2.6C and D respectively, however DHA did show an increasing trend in the fenretinide treated group. There was however a statistically significant 28% increase in phospholipid-bound DHA found in the lumbar spinal cord of fenretinide treated mice ($p \leq 0.05$), as shown in Fig 2.6A. In addition, phospholipid-bound arachidonic acid was roughly 10% lower in the cerebral cortex of treated animals ($p \leq 0.05$), as depicted in Fig 2.6B.

2.3.6 Nissl–stained neuronal cell count

All histological preparations were derived from L4 spinal cord segments taken at 130 days of age. In order to quantify the number of surviving motor neurons, histological sections were stained with cresyl violet and the motor neurons of each ventral horn were counted by an individual blinded to the animal number and treatment conditions.

Representative images depicting motor neuron staining are shown in Fig 2.7A. Counts were performed from both ventral horns on sections every 300μm and an average of 4 sections (8 ventral horns) was taken for each animal. A total of 4 animals (n=4) was used for each group. Mice treated with fenretinide displayed approximately 20% more motor neurons than control mice (Fig 2.7B) however with only the small sample size available results failed to reach a statistically significant threshold.

2.3.7 Glial activation

Immunohistochemistry was also performed on different serial sections from the same animals. Antibodies against both glial fibrillary acidic protein (GFAP), shown in Fig 2.8A, and the Mac-2 antigen (Mac-2), shown in Fig 2.8B, were used to identify activated astrocytes and microglia respectively. Images of both ventral horns were captured every 300μm and imported into SigmaScan Pro image quantification software. The area occupied by darkly stained cells was quantified and normalized for the total area. As illustrated in Fig 2.8A, analysis of GFAP stained sections revealed that sham treated mice had more than 50% greater staining of the ventral horn then mice treated with fenretinide and this difference was found to be statistically significant ($p \leq 0.05$). Mac-2 positive stained area was also significantly ($p \leq 0.05$) higher in control animals with 60% more area stained, as shown in Fig 2.8B. It can be seen from the representative images in Fig 2.8C and D that vehicle treated mice not only possessed a greater number of glial cells but these cells displayed greater hypertrophy. These findings suggest that treatment with fenretinide diminishes activation of glia, the inflammatory cells of the CNS.

2.4 Discussion

There is currently very little information on the role of lipids in ALS and despite extensive research there remains only one approved drug for treating this devastating disease. It has however, been well documented that oxidative stress-induced lipid peroxidation is present in both humans and animals models and it is proposed to be one of the most important factors precipitating the neurodegenerative phenotype [172-178]. Lipid peroxidation has far reaching consequences by generating diffusible reactive products, which can spread further damage and by irreversibly altering the composition of plasma membranes. The loss of PUFA due to lipid peroxidation from neural membranes may be of particular relevance in ALS because these lipid species contribute to the essential membrane fluidity in neurons [179]. In addition, the concentrations of ω-3 and ω-6 PUFAs have important relevance on cellular function and can affect an organism's inflammatory status. No studies have ever tested the ability of fenretinide to alter lipid profiles and correct the disease phenotype in human ALS or animal models of the disease. In previous work, we demonstrated that treatment with fenretinide improved survival in a mouse model of SCI by correcting the PUFA imbalance, reducing inflammation and improving neuron survival (manuscript in preparation). In the present study we applied a similar treatment strategy to $SOD1^{G93A}$ transgenic mice to investigate the therapeutic potential of fenretinide in ALS.

Here we show for the first time that fenretinide therapy is able to improve the disease phenotype in a transgenic mouse model of ALS. One of the most

prominent features of ALS is paralysis and our results demonstrate that motor function and coordinated movements were improved in animals treated with 5 weekly doses of 5 mg/kg fenretinide compared to vehicle treated control animals. Motor performance was significantly improved for the duration of the study but also showed independently significant improvements at three consecutive time points at the midpoint of disease progression. The greatest improvement occurred at 119 days of age when the treated group outperformed sham treated animals by nearly 2.5 fold. Our results also demonstrate a clear survival advantage for treated mice with mean survival from disease onset almost 10% higher in treated mice. The elevation of these two parameters provides considerable promise for fenretinide's potential in treating ALS. Another promising aspect of fenretinide therapy is that it has been extensively tested in humans and is associated with only minor and reversible side effects. Studies performed with long-term low, medium, and high-dose fenretinide in humans have reported few or no side effects. The only consistently reported side effect is Nyctalopia, (impaired low-light vision) which is reversible when treatment is discontinued and does not appear to be cumulative [180-186]. Given that the present expected survival for humans after disease onset is 36-60 months, if fenretinide could provide an equivalent advantage in human disease as it appears to have in mice, it may have the potential to extend survival by several months [17]. One can also presume that with enhanced motor function the quality of life experienced would be improved.

In order to determine the mechanism of fenretinide's action we then analyzed the level of certain lipid species affected by fenretinide in our previous

studies, which also have important functions in neurological systems; principally AA, DHA and ceramides. Measuring the level of phospholipid bound PUFAs is a reliable method of determining their contribution to plasma membranes and provides an accurate quantification of their relative abundance. This method is more accurate than measuring the free forms because when cleaved from the plasma membrane they are quickly converted into downstream products or oxidized and thus cannot be accurately measured in their free form [187, 188]. By measuring at multiple time points we were able to establish kinetics of the therapy and observe changes over time. The use of plasma samples provided insight into the systemic effects of the treatment whereas analysis of specific organs was also used to pinpoint local effects in the lumbar spinal cord, motor cortex and brainstem; the regions most affected by ALS [189]. While most of the data obtained from organs was not significantly different between groups we did find elevated levels of DHA in lumbar spinal cord segments of mice treated with fenretinide. In addition, samples taken from the motor cortex of drug treated mice displayed lower levels of phospholipid bound AA. These results are in agreement with fenretinide's known effects on PUFA concentrations and support the hypothesis that it has both systemic and local effects. The failure to find statistically significant differences in the other analytes, both organs and plasma, collected at the experimental endpoint is not entirely surprising. In order to measure survival, it was necessary to keep animals until they reached an objective clinical endpoint. As a result, all organ and plasma samples collected at this time were harvested at different time points and when the mice had reached an

equivalent and severe phenotypic expression of the disease. Given the data obtained from both behavioral and plasma analysis at day 120 it is conceivable that a greater difference between groups would have been observed in organ samples taken at an earlier stages of disease.

Our plasma lipid analysis offers considerable insight into the mechanism of fenretinide's action. The most striking results were the consistently elevated levels of phospholipid bound DHA for the duration of the study (excluding the samples extracted at the end point). DHA has been linked to improved oxidative status and anti-inflammatory effects in diseases ranging from diabetes to cardiovascular disease [54, 190]. It is generally recognized that increasing dietary consumption of ω-3 PUFAs contributes to better health and reduced inflammation, however our results demonstrate that fenretinide is capable of elevating membrane ω-3 levels without a change in diet. This is likely a result of reduced oxidation from lipid membranes by ROS. DHA's high level of unsaturation provides a greater number of sites for radical species to target than other PUFAs such as AA rendering it more susceptible to lipid peroxidation [188]. The peroxidation of DHA in lipid membranes creates a feed-forward loop whereby increased ROS lowers DHA removing an important regulator of inflammation and oxidative damage. Interestingly our results also show that phospholipid bound AA is lower in the plasma of treated mice measured at day 120. AA and other ω-6 fatty acids are closely linked to inflammation by acting as precursors for pro-inflammatory molecules such as prostaglandins. Fenretinide has been proposed to act directly on membranes by increasing AA cleavage by

Phospholipase-A_2 and its subsequent degradation by activating 12-Lox and our results support this theory [191, 192]. The results of our plasma and organ lipid analysis confirm that both inflammatory and antioxidant mechanisms are compromised in $SOD1^{G93A}$ mice and suggest that fenretinide therapy may be acting on these pathways.

In order to assess the oxidative status we measured two markers of lipid peroxidation; MDA and NT. Both markers were significantly reduced in mice treated with fenretinide by day 120. These markers have been assessed by other research groups to be elevated in inflammatory conditions including SCI and ALS and it was recently demonstrated by Tokuda and colleagues that decreased lipid peroxidation correlated with improved disease outcome in the same mouse model of ALS that we used [193]. Most commonly ROS and RNS are released by immune cells and we have already discussed the effect of high ω-6: ω-3 ratios on induction of inflammation so we next looked at the number of glia infiltrating the most affected area of the spinal cord to see if these cell types could be leading to the oxidative stress observed.

Astrocytes and microglia represent the primary immune cells of the CNS. They are recruited and activated in high numbers in many neurodegenerative conditions and contribute to the loss of motor neurons in ALS [194-197]. Interestingly it has been demonstrated that astrocytes both release NO and are activated by peroxynitrite creating a dangerous cycle that accelerates disease progression [194]. Recently Yamanaka and colleagues demonstrated the importance of regulating reactive gliosis to moderate ALS severity showing that

disease progression is directly related to the inflammatory response of microglia and astrocytes [198]. Our findings indicate that fenretinide therapy reduces the inflammatory response in the lumbar spinal cord of $SOD1^{G93A}$ mice. Both GFAP-positive astrocytes and Mac-2-positive microglia were significantly diminished in fenretinide treated spinal cords. In addition, qualitative observation also revealed a greater level of hypertrophy in control mice reflecting the greater overall immune response in these animals. The lower glial burden in mice treated with fenretinide is likely at least partially responsible for the decrease in oxidative stress as both cells possess inducible NOS and produce inflammatory cytokines and ROS [198, 199]. Based on these findings, the mechanistic pathway of fenretinide's actions began to emerge and we proposed that these differences might account for a difference in neuron survival.

Perhaps our most surprising result was the lack of a significant difference in motor neuron number between groups. There was however certainly a trend and we suggest a number of theories that may explain why this trend was not statistically significant. Given the length of such a project we were not able to obtain extensive preliminary results and consequently ran many experiments simultaneously. From our behavior and plasma analysis it is evident that fenretinide's effects were most prominent between 15-16 weeks and fell sharply after that. Presumably histological samples obtained at 120 days, when behavior showed the greatest disparity, would provide better insight into fenretinide's effects on motor neuron populations. We chose to collect the samples for histological evaluation at 130 days of age to ensure sufficient neurodegeneration

however the disease phenotype of both groups had just begun to converge at this time point. We also noted that remaining neurons appeared more robust in fenretinide treated animals which could explain their performance and survival advantage despite the small difference in neuron number. With the number of samples available the power to detect a true difference was less than 0.25 so it is likely that a greater number of samples would also have provided more statistically relevant results.

In conclusion, this study shows for the first time that fenretinide therapy possesses exceptional promise for treating ALS. Our survival results indicated a 10% increase in survival, which is almost equivalent to the results seen for Riluzole in the same strain of mice [200]. It is of course premature to extend our conclusions to predict benefits for human applications based on these studies alone, however fenretinide has the benefit of being proven safe as an oral drug which is an important component for the consideration for clinical trials [104, 201]. It is our belief that fenretinide is an excellent candidate for further exploration for the therapeutic treatment of ALS.

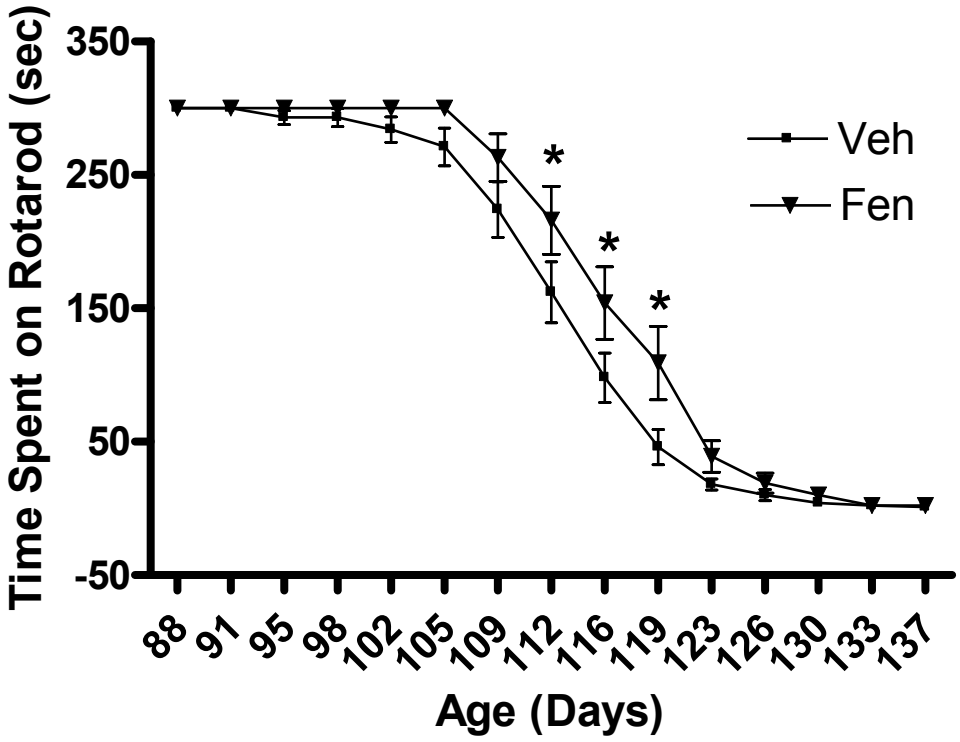
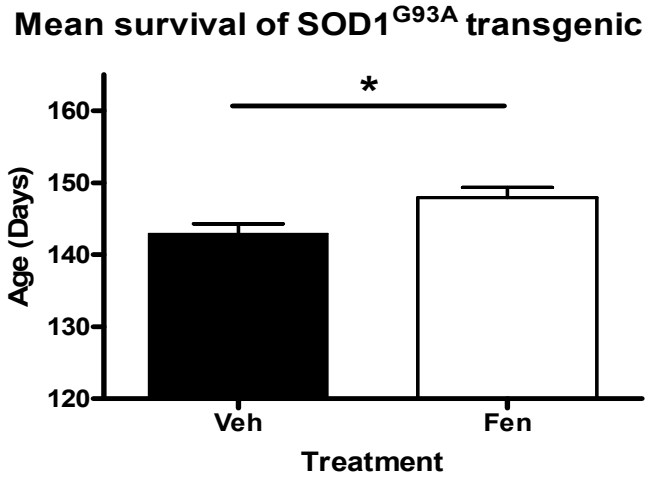


Fig 2.1 Effects of fenretinide on Rota-rod performance. The effect of fenretinide treatment on motor performance in SOD1^{G93A} transgenic mice from 88 to 137 d of age. Mice treated with 5 mg/kg fenretinide (Fen) exhibited significantly improved motor performance compared to vehicle treated controls (Veh) by two-way ANOVA ($p \leq 0.0001$). This test was followed by Bonferroni posttests which additionally revealed significant improvements in the fenretinide-treated group at day 112, 116 and 119 ($p \leq 0.05$). Values are mean ± SEM. ▼, fenretinide; ■, vehicle treated SOD1^{G93A}. * signifies $p \leq 0.05$. n≈20 for each group.

A.



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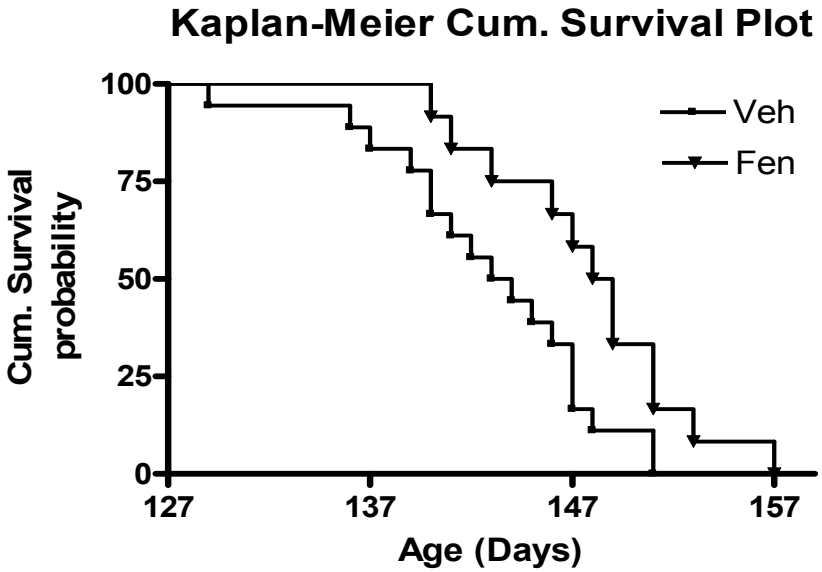


Fig 2.2 The effect of fenretinide treatment on survival in SOD1^{G93A} transgenic mice. A. Figure A depicts the mean survival of fenretinide (Fen) and vehicle treated control (Veh) mice. The mean survival of mice treated with 5 mg/kg fenretinide was significantly improved from 142.9 ± 1.3d to 147.9 ± 1.4d compared to controls ($p \leq 0.02$) by non parametric t-test. Values are mean ± SEM. * signifies p≤0.05. **B.** Figure B depicts the cumulative (Cum.) probability of survival for mice beginning treatment at 30 d of age with vehicle (Veh) or 5 mg/kg fenretinide (Fen). There is a significant increase in survival in treated SOD1^{G93A} mice ($p \leq 0.05$). Median survival values are 148.5d for Fenretinide and 143.5d for vehicle-treated mice. ▼, fenretinide; ■, vehicle treated SOD1^{G93A}. n=18 for Veh, n=12 for Fen.

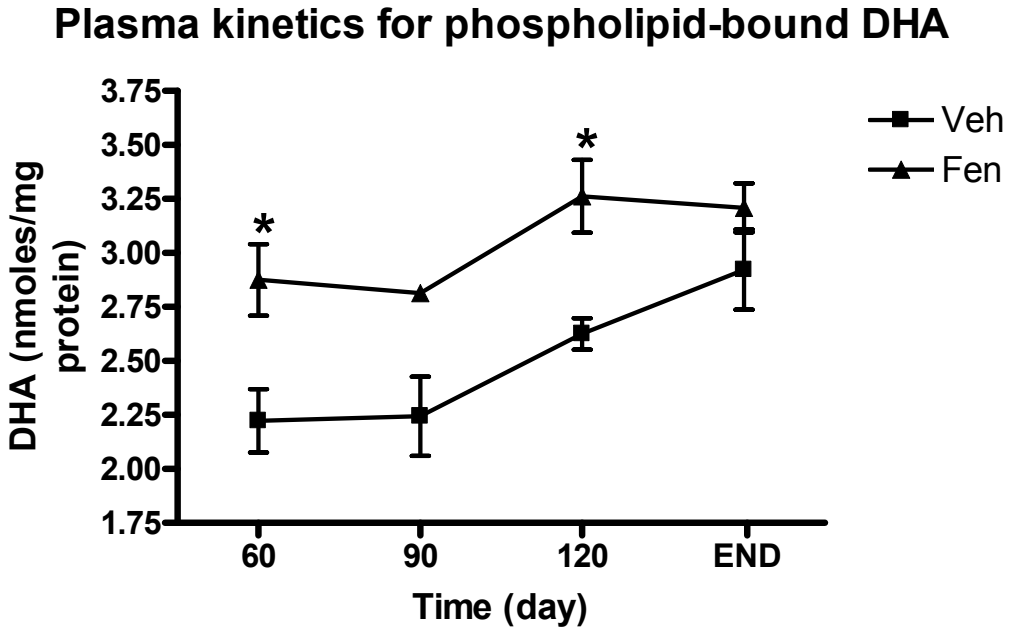
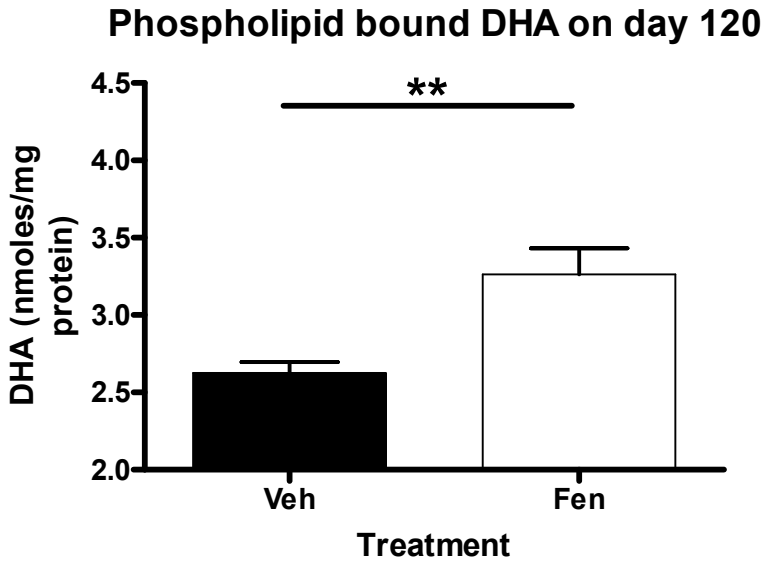


Fig 2.3 Plasma kinetics for phospholipid-bound DHA. This graph displays the phospholipid-bound DHA concentration in mice treated with fenretinide (Fen) or vehicle (Veh). Two-way ANOVA revealed a very significant elevation in DHA in animals treated with fenretinide compared to vehicle treated controls for the duration of the study ($p \leq 0.0001$). Bonferroni posttests also revealed significantly higher DHA at both 60 and 120 days in fenretinide treated mice. * signifies $p \leq 0.05$. $n \approx 10$ for each group.

A.



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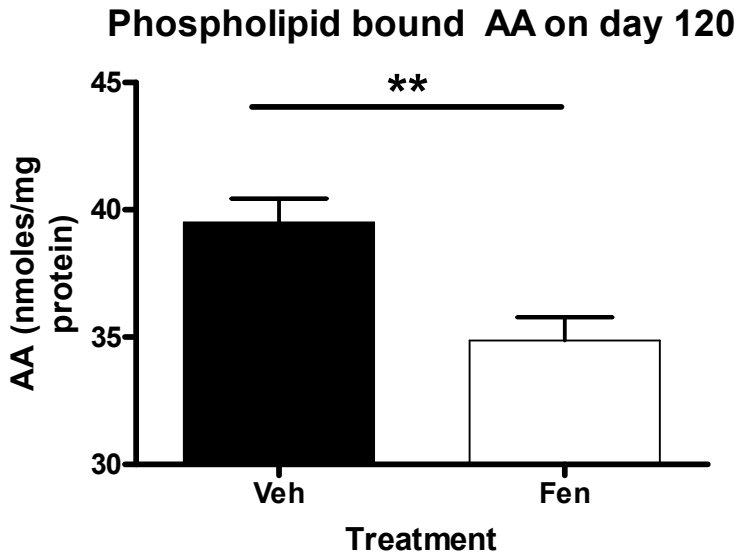
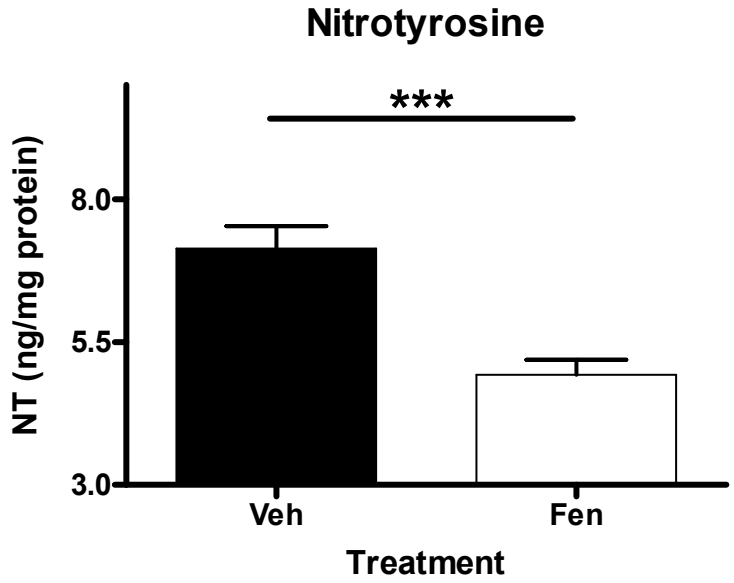


Fig 2.4 Plasma PUFA concentrations. PUFA levels as measured from plasma samples obtained 120 days after birth. Mice treated with 5 weekly doses of fenretinide (Fen) displayed significantly elevated levels of phospholipid bound ω-3 PUFA DHA (**Fig 2.4A**) compared to vehicle treated (Veh) controls. The same mice exhibited significantly reduced levels of phospholipid bound ω-6 PUFA AA (**Fig 2.4B**) compared to control animals. n=7 or 8 for all groups. ** signifies $p \leq 0.01$.

A.



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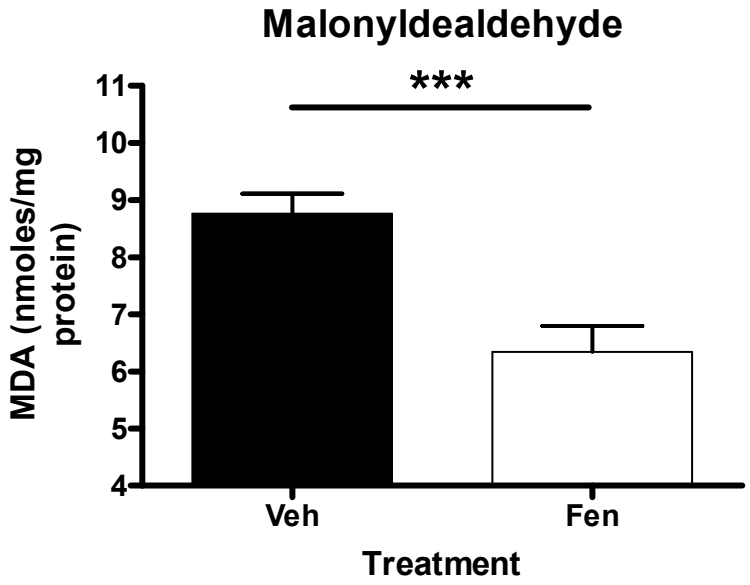


Fig 2.5 The effect of fenretinide therapy on plasma lipid peroxidation. Two markers of lipid peroxidation, NT (**Fig 2.5A**) and MDA (**Fig 2.5B**) were significantly decreased on day 120 in fenretinide treated mice (Fen) compared to vehicle treated (Veh) control animals in plasma samples measured at 120 days of age. *** signifies $p \leq 0.001$. n=7 or 8 for all groups.

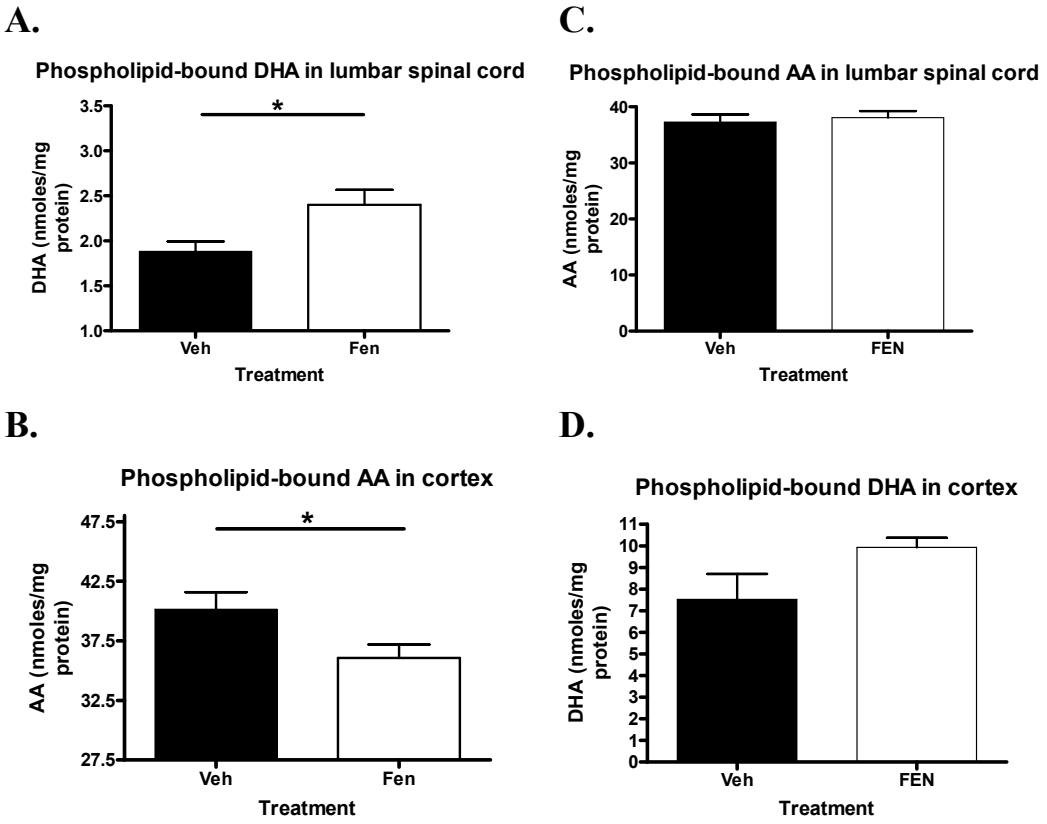
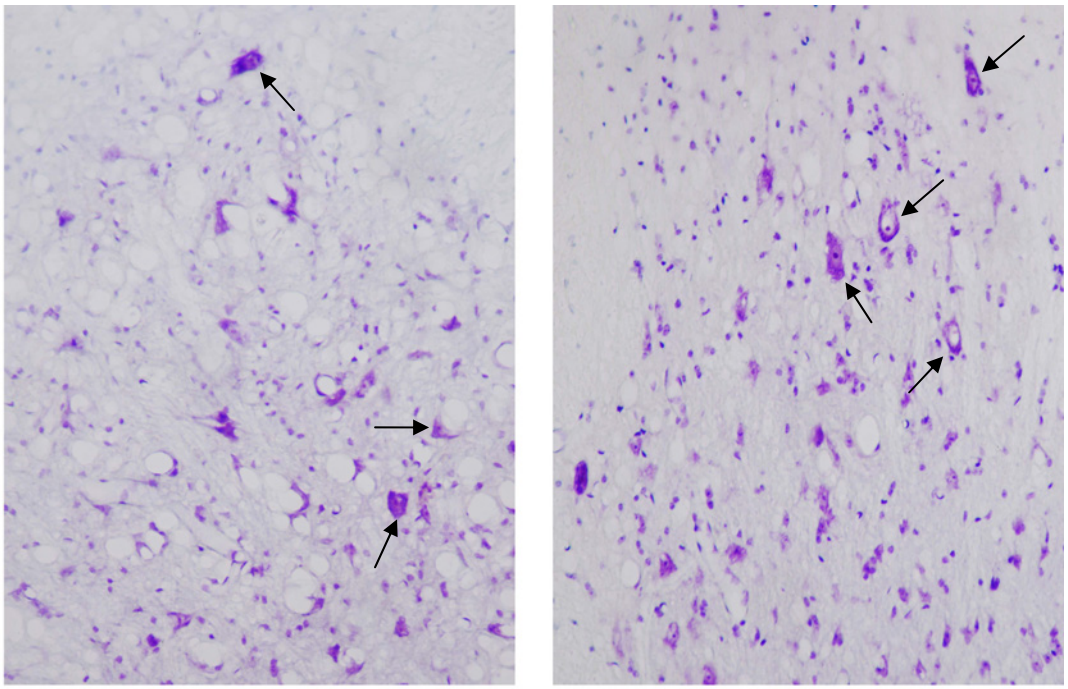


Fig 2.6 PUFA concentrations in organs affected by ALS at 120 days of age. Phospholipid-bound DHA was significantly increased in the lumbar spinal cord of mice treated with fenretinide (Fen) when compared to vehicle treated (Veh) controls (**Fig 2.6A**). A difference in AA from the same spinal cord samples was not detected (**Fig 2.6C**). Motor cortex samples revealed significantly lower levels of phospholipid-bound AA in fenretinide treated mice (**Fig 2.6B**). DHA concentrations were slightly higher in cortex samples derived from fenretinide treated mice measured at 120 days of age, however this difference was not statistically significant (**Fig2.6D**). * signifies $p \leq 0.05$. n=11 or 12 for all groups.

A.



Veh **Fen**

B.

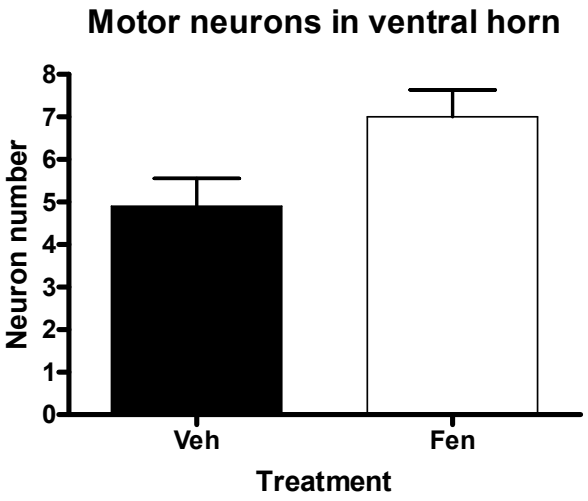


Fig 2.7 Quantification of surviving motor neurons at 130 days of age. A. Images in figure A depict motor neuron staining. Qualitative observations indicate that samples from fenretinide treated (Fen) mice possessed more robust motor neurons then their vehicle treated (Veh) counterparts. Arrows indicate alpha motor neurons identified in the ventral horn. **B.** Motor neurons counted in the ventral horn of the 4th lumbar spinal cord segment appeared to be more numerous in mice treated with fenretinide however this difference failed to reach statistical significance. n=4 for both groups.

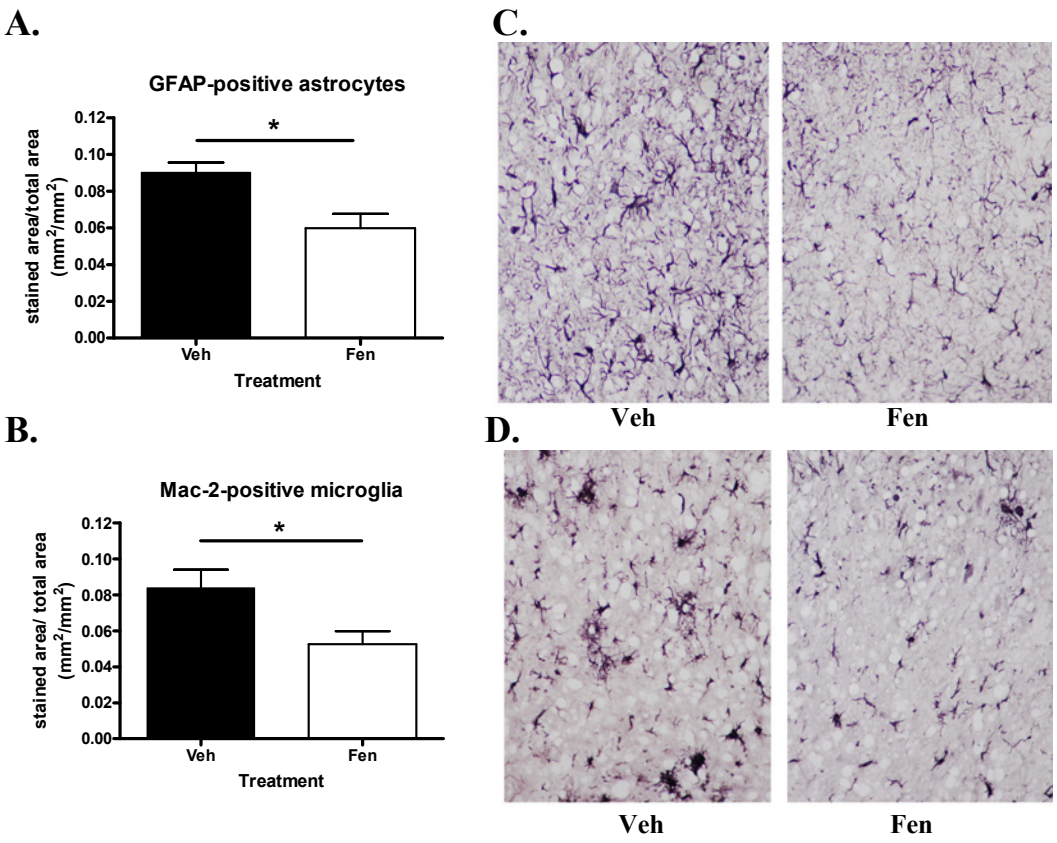


Fig 2.8 Quantification of reactive gliosis at 130 days of age. A. GFAP-positive staining astrocytes occupied significantly less area of the 4th lumbar ventral horn in mice treated with fenretinide (Fen) compared to vehicle treated (Veh) controls. **C.** Qualitative observations reveal that spinal cords from vehicle treated animals possess greater numbers of astrocytes and that these cells are more hypertrophic compared to samples from Fenretinide treated mice. **B.** Mac-2-positive microglia were also significantly less numerous in fenretinide treated mice. **D.** Qualitative observations also revealed more numerous and more hypertrophic microglia in spinal cords from vehicle treated mice. * signifies $p \leq 0.05$.

Chapter Three

3.1 General discussion and conclusions

3.1.1 Summary of results

Currently Riluzole is the only drug approved for treating ALS [171]. While many avenues of investigation are being explored for treating this disease not much attention has been devoted to the role of various phospholipids in ALS. In previous studies, we have demonstrated that treatment with fenretinide can alter membrane lipid profiles and reduce inflammation [143, 144], (Lachance *et al.*, 2008 *submitted*). Recently, we also demonstrated that fenretinide treatment improves recovery in a mouse model of spinal cord contusion injury by targeting these same pathways (manuscript in preparation). In preparation for the present study we performed a preliminary investigation into the lipid status of two transgenic mouse models of ALS. From this work we discovered that both strains display quite striking phospholipid imbalances. In fact, the aberrant ratio between AA and DHA we observed in these two transgenic ALS models was very similar to the imbalance we had observed in SCI models that was corrected by treatment with fenretinide (manuscript in preparation). Based on these findings it seemed likely that the lipid imbalance seen in mouse models of ALS might in fact contribute to the etiology of the disease and we wondered if mice with ALS might also benefit from a similar treatment strategy. A series of experiments were subsequently conducted to determine the treatment outcome and the mechanism of action of fenretinide using a transgenic mouse model of ALS. Specifically, we examined disease onset and progression by measuring the physical capabilities of $SOD1^{G93A}$ transgenic mice on a Rota-rod treadmill. We also assessed the survival

advantage of treated animals, analyzed essential fatty acid profiles and examined markers of oxidative stress. Histological evaluation was performed to explore the link between the known systemic phenotype and what is occurring within the primary affected organ, the spinal cord.

Fenretinide, a semi-synthetic retinoid drug, is largely known for its applications in oncology. It has proved effective in a wide range of cancers and is an especially promising drug because it is administered orally and can be tolerated by both children and adults at very high doses, for many consecutive years, and with only mild or reversible side effects [137, 202-204]. While it has demonstrated excellent success in clinical trials, its mechanism of action is still somewhat poorly understood. In our recent study on SCI we demonstrated that fenretinide therapy possesses anti-inflammatory and anti-oxidant actions evident by decreasing microglial activation in the spinal cord and ameliorating the levels of TNF-α, IL-1β, MDA and NT (manuscript in preparation). In a recent set of *in vitro* experiments we also explored mechanistic pathways that could explain the down regulation of pro-inflammatory cytokines by fenretinide. Our results demonstrated that treatment with fenretinide reduced the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), an important member of the mitogen-activated protein kinase (MAPK) family and a necessary pathway for macrophage cytokine gene expression [205](Lachance *et al.*, 2008 *submitted*). Others showed that fenretinide is a potent inhibitor of prostaglandin synthesis which may also account for its anti-inflammatory actions [206]. Based on this work we felt confident that fenretinide could have beneficial effects for ALS.

This thesis reports the results of experiments designed to assess the ability of fenretinide to improve ALS phenotypes using a transgenic mouse model of ALS disease. We have shown that 5 weekly doses of 5 mg/kg fenretinide, beginning at 30 days of age, was sufficient to significantly improve several disease phenotypes. The data described in this thesis demonstrated that our treatment regimen delayed the onset of functional motor impairment and improved voluntary motor control throughout the life of treated animals in addition to extending their overall survival. We also showed that fenretinide exhibits these effects by increasing the ratio of ω-3: ω-6 PUFA, an environment associated with neuroprotection and the prevention of neurodegenerative processes [207]. Our analysis of oxidative damage revealed significantly lower levels of lipid peroxidation products in mice treated with fenretinide while histological examination showed that the same treatment group also possessed fewer activated microglia and astrocytes. Our evaluation of motor neuron survival revealed that mice treated with fenretinide possessed a greater number of motor neurons in the ventral horn of the spinal cord however the difference detected did not reach a statistically significant threshold so no conclusions can be drawn until further studies are conducted.

3.1.2 Future directions

The work presented in this thesis involved three experimental groups studied in three separate but overlapping experiments. The duration of one experiment involving $SOD1^{G93A}$ mice lasts at least 4-5 months and requires an

additional several months to generate a viable colony. For the studies described in this thesis, there was an insufficient amount of time to perform prolonged studies using several subsequent experiments that would most certainly have improved the statistical significance of some parameters analyzed, which although suggestive did not reach statistically significant differences. The analysis of our results has revealed that some experimental conditions could be improved and further investigation may provide even more promising results. Of most interest would be to obtain histological and organ lipid samples at 120 days of age, which corresponds to the greatest improvement in behavior and all measures of plasma lipids and oxidative stress. There have also been new methods developed to precisely measure the oxidation of DHA in the CNS. Arneson and colleagues have developed a protocol to quantify F_4-neuroprostanes, specific peroxidation products of DHA which are inherently stable, and perhaps this method of analysis, also performed at 120 days, would provide better insight into the oxidation-induced CNS damage [188].

Another important avenue of investigation would be dose-escalation studies. While our results provide exciting evidence for fenretinide's ability to slow ALS progression the improvements were modest and fenretinide is known to be tolerated in humans and rodents in doses 50-fold higher than those used in our studies [183, 202, 208]. Given the aggressiveness of ALS, a higher dose may more effectively combat oxidative stress and inflammation providing more pronounced survival and behavior advantages. It would also be of interest to apply this therapy to a less aggressive disease model such as that of $SOD1^{G37R}$

transgenic mice where treatment may have more power to overcome the disease progression.

Today many pharmacological breakthroughs are being discovered by combining molecules or existing therapies to create drug cocktails. Such treatment strategies can be extremely effective by enabling the therapies to target multiple pathways and effect diverse phenotypes. With the heterogeneity of disease presentation and the diversity of proposed etiologies, ALS is a suitable candidate for such therapies [209]. In fact a number of research groups have begun such investigations with cocktails tailored to several pathways from microglial inhibition to fatty acid supplementation [210, 211]. It is our belief that fenretinide could be extremely effective in a cocktail with the right complementary drugs and we hope to explore such therapies in the future.

Overall the work in this thesis provides new hope for future novel treatment of ALS. While there is a great deal of work necessary to further evaluate this drug and for it to advance to human trials the results from the experiments presented here offer new insight into the contributing factors of this devastating disease. With continued efforts and research more promising drugs and treatment protocols are likely to become available for people suffering from ALS and we hope our findings have contributed to the quest for efficient treatments for ALS and other neurological diseases.

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Appendix One

Other published manuscripts

Guilbault C, De Sanctis JB, Wojewodka G, Saeed Z, Lachance C, **Skinner TA**, Vilela RM, Kubow S, Lands LC, Hajduch M, Matouk E, Radzioch D. Fenretinide corrects newly found ceramide deficiency in cystic fibrosis. *Am J Respir Cell Mol Biol.* (2008) 38(1):47-56.

Submitted manuscripts

Lachance C, **Skinner TA**, Guilbault C, De Sanctis JB, Radzioch D. 4-HPR inhibits inflammatory mediators in FXR1P-ablated macrophages by suppressing phospho-ERK and upregulating arachidonic acid. Submitted to: *Journal of Lipid Research.*

Manuscripts in preparation

López-Vales R, **Skinner TA**, Ghasemlou N, Rathore KI, De Sanctis JB, Radzioch D, David S. Fenretinde promotes functional recovery and tissue protection after spinal cord contusion injury in mice.

Appendix Two

Certificates of compliance

Animal use protocol

Animal training certificates

Radioactivity certificate

Guidelines for completing the form are available at www.mcgill.ca/research/compliance/animal/forms

McGill University
Animal Use Protocol – Research

For Office Use Only:
Protocol #: 5378
Approval End Date: June 30, 2008
Facility Committee: MGH

Title: Determination of the Neurological Component in Respiratory Failure in Cystic Fibrosis Lung Disease
(must match the title of the funding source application)

☒ New Application ☐ Renewal of Protocol # ____ ☐ Pilot **Category** *(see section 11)*: D

1. Investigator Data:

Principal Investigator: Danuta Radzioch. PhD. **Phone #:** 934-1934 ext.44517
Unit/Department: Medicine, Montreal General Hospital Research Institute **Fax#:** 934-8260
Address: Room L11-218 1650 Cedar Ave. Montreal, QC. H3G 1A4 **Email:** danuta.radzioch@muhc.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Jennifer Henri **Work #:** 934-1934 ext.44516 **Emergency #:** (514) 991-9739
Name: Danuta Radzioch **Work #:** 934-1934 ext.44517 **Emergency #:** (514) 331-1284

3. Funding Source:

External ☒
Source (s): Canadian Cystic Fibrosis Foundation
Peer Reviewed for the project proposed in this Animal Use Protocol: ☒YES ☐NO**
Status : ☒ Awarded ☐ Pending
Funding period: 2007-2010

Internal ☐
Source (s): ____
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period: ____

For Office Use Only:

ACTION	✓	DATE
CCs		
DB		July 30'07
APPROVED		

** **All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Form available at www.mcgill.ca/research/compliance/animal/forms**

Proposed Start Date of Animal Use (d/m/y): 07/05/07 **or ongoing** ☐
Expected Date of Completion of Animal Use (d/m/y): 06/04/08 **or ongoing** ☐

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: [signature] **Date:** 24/04/07

Approved by:

Chair, Facility Animal Care Committee:	[signature]	**Date:** May 4/07
RESEARCH ETHICS OFF.	[signature]	**Date:** July 30 '07
Chair, Ethics Subcommittee (as per UACC policy):	[signature]	**Date:** July 31 07
Approved Animal Use	**Beginning:** July 1, 2007	**Ending:** June 30, 2008

☐ This protocol has been approved with the modifications noted in Section 13.

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University Animal Care Committee

Ethics Unit
Office of the Vice Principal (Research)
McGill University
James Administration Bldg
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Comité universitaire de protection des animaux

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June 20, 2006

The McGill University Animal Care Committee certifies that
Tom Skinner has successfully completed the

Advanced Level
of the
Theory Training Course on Animal Use for Research and Teaching
on
June 8, 2006.

The training includes the following topics:

- **Basic Level:** Regulations & Procedures, Ethics, Basic Animal Care, Occupational Health & Safety
- **Advanced Level:** Anesthesia, Analgesia, Euthanasia, Categories, Influencing Factors and Environmental Enrichment

Please note that this certificate does NOT include practical training, which is obtained by successfully completing an Animal Methodology Workshop where another certificate is issued.

Certification is valid for 5 years, starting on the date indicated above.

Deanna Collin
Animal Care Training Coordinator, animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training

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Comité universitaire de protection des animaux

Éthique animale
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www.mcgill.ca/research/compliance/animal/

Oct. 27, 2006

The McGill University Animal Care Committee certifies that
Tom Skinner has successfully completed a
Mouse Workshop on ***July 11, 2006***.

The training included the following procedures:

- ✔ Handling and restraint
- ✔ Gavage (oral dosing)
- ✔ Injections: subcutaneous, intramuscular, intraperitoneal
- ✔ Blood collection: saphenous and cardiac puncture
- ✔ Determination of anaesthetic depth
- ✔ Euthanasia by cervical dislocation

Certification is valid for 5 years, starting on the date of the workshop.

Deanna Collin
Animal Care Training Coordinator, animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training

Le centre universitaire de santé McGill (CUSM)
McGill University Health Center (MUHC)

Service de radioprotection
Radiation Protection Service

Ceci certifie que :
This is to certify that :

Tom Skinner

A réussi avec succès la formation du CUSM en radioprotection à l'intention des travailleurs du secteur des radioisotopes.
Has successfully completed the MUHC radiation Safety Training for Radioisotope Workers

Date of training: **7/28/2006**

RSO's signature: C. Fanichi

Radiation Protection Service