MULTIFACTORIAL GENE THERAPY AS A NOVEL APPROACH FOR THE TREATMENT OF MUTANT SUPEROXIDE DISMUTASE-1 LINKED FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS

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A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelors degree
With Honors in

Physiology

THE UNIVERSITY OF ARIZONA

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Abstract

Amyotrophic lateral sclerosis is a rapidly progressing disease characterized by the degeneration and death of upper and lower motor neurons, and it is associated with a lifetime risk of 1 in 1000. The disease not only involves damage to the motor neurons themselves but also to neighboring glial cells. Since the characterization of the disease roughly a century and a half ago, few successes have been seen in the development of a treatment plan that yields viable results and prevents neurodegeneration. The shortcomings of past therapies have been attributed to the narrow focus of treatment plans, targeting a single aspect of the disease pathology. The pathophysiology is complex and multifactorial, integrating aspects such as aberrant reactive oxygen species formation, excitotoxicty, a reduced antioxidant response, and dysfunction of the endoplasmic reticulum and mitochondria. Recent research employing a multifactorial approach to treatment has yielded success in mice engineered with the mutant SOD1 gene, a mutation which mimics the disease progression and pathophysiology seen in ALS patients. Current clinical research for ALS has focused on using gene therapy techniques to introduce therapeutic genes coding functional proteins into affected cells in the hopes of addressing several of the pathological aspects of the neurodegenerative disease.

Introduction

Amyotrophic lateral sclerosis, first described by Jean-Martin Charcot in 1869, is a neurodegenerative disorder that is defined by the selective and progressive degeneration of both upper (UMN) and lower motor neurons (LMN). The upper motor neurons are nerve cells for which the soma is located in the central nervous system (for example the premotor or primary motor cortex of the brain) and which synapse at the spinal cord with the lower motor neurons. These cortical neurons relay efferent information from the brain to these

lower motor neurons. The LMN have their soma located in the ventral horn spinal cord and synapse with muscle fibers at the neuromuscular junction. Although neurons are the building blocks of the nervous system, transmitting information from the CNS to the rest of the body, these cells only make up roughly 10% of the central nervous system. The remaining 90% of cells consists of glial cells, cells which nurture and protect the neurons and maintain homeostasis within the nervous system. Astrocytes are one glial subtype which function in maintaining a favorable environment for neurons, providing them with consistent nourishment and regulating the levels of neurotransmitters and ions in the extracellular space. One of the most significant roles that astrocytes play is the clearing of synaptic glutamate using transmembrane EAAT (excitatory amino acid transporter) glutamate transporters (Lasiene and Yamanaka, 2011).

The upper motor neurons transmit information to the lower motor neurons by releasing neurotransmitters, especially the excitatory NT glutamate, into the synapse. Increased synaptic neurotransmitter concentrations allow the respective receptors on the post-synaptic membrane to be bound, triggering a cellular response in the lower motor neuron. The stimulated LMN's are then able to communicate with muscle cells at the neuromuscular junction (abbreviated NMJ). The neuromuscular junction functions as a site at which signals may be transmitted from the motor neuron to the muscle tissue to control contraction of the fibers. When a particular lower motor neuron is stimulated and initiates an action potential, all of the muscle fibers that are innervated by that motor neuron, collectively called a motor unit, contract when the stimulus reaches them. The end plate region of each muscle fiber, the region of the muscle cell sarcolemma (plasma membrane) that apposes the axon terminal of the motor neuron, contains many invaginations that increase the surface area of the membrane and therefore maximize the cholinergic receptors that can be located on the surface. Upon stimulation, acetylcholine (ACh) is

released from the motor neurons and into the synapse between the neuron and the muscle fiber. Acetylcholine can then bind to its respective receptors on the sarcolemma of the muscle cell, initiating a cellular response that triggers contraction of the fiber. After it has carried out its function at the neuromuscular junction, acetylcholine is degraded by an enzyme called acetylcholinesterase in order to reduce the its synaptic concentration back to baseline (Neuromuscular Junction Lecture Notes, 2011).

ALS, commonly known as Lou Gehrig's disease or Charcot disease, can be generally divided into two types: sporadic and familial. Roughly 90% of ALS cases fall under the category of sporadic, which is considered idiopathic and has seen little success in terms of establishing the mechanism of the complex pathophysiology. The other approximately 10% of ALS cases are familial and have been associated with mutations of several genes at several loci, one of the most well known of which is the mutation of SOD1 (the copper/zinc ion-binding superoxide dismutase). Autosomal dominant mutations of SOD1 alone account for about 20% of all familial cases of ALS. Amyotrophic lateral sclerosis tends to manifest later in life, with the peak of onset for familial forms being 47-52 years of age and the peak for sporadic forms being 58-63 years of age **(**Kiernan et al., 2011). Due to an insidious onset and disease progression, the time of diagnosis averages about 14 months. The disease itself is extremely progressive, with 50% of diagnosed patients dying within three years of the disease onset and only 20% surviving past five years. Although there are some syndromes that display mimicry, ALS can be distinguished by rapid progression and the presence of both upper and motor neurons signs (Mitchell and Borasio, 2007).

There are a few main ways in which ALS may present. About 70% of cases present as limb-onset ALS, in which death of the upper and lower motor neurons results in early signs of muscle wasting in the limbs. This presentation of ALS may manifest as the inability to lift ones arms due to upper limb wasting. Subluxation, or partial dislocation, of the

glenohumeral joint due to wasting of the supraspinatus, infrapsinatus, and deltoid muscles, and also the clinical presentation "split hand" in which there is disproportionate muscle wasting of the four thenar muscles of the thumb are also common signs of limb-onset forms. In roughly 25% of cases, ALS presents as bulbar-onset, in which muscle wasting and symptoms relating to the limbs develop after muscle wasting of the tongue and mouth and the presentation of speech and swallowing difficulties. These cases may present as having significant wasting of the tongue muscles, as indicated by the absence of elevation of the palate during speech, difficulty in opening the mouth, and difficulty swallowing (clinically referred to as dysphagia). Consequently, bulbar-onset is often characterized by slow and distorted speech. Finally, the last 5% of cases represent abnormal cases of ALS presentation. In most patients, regardless of the presentation, dysphagia develops resulting in significant weight loss as well as signs of malnutrition. However, the ultimate cause of death in most cases is respiratory failure due to progressive wasting of the respiratory muscles (Kiernan et al., 2011).

Emerging evidence from recent years seems to support a multifactorial development underlying the mechanisms of the ALS pathophysiology, resulting from complex genetics and the integration of several biochemical pathways. Although the exact mechanism remains elusive, research supports the involvement of many pathways including abnormal protein processing, mitochondrial and ER dysfunction, oxidative damage to cellular components, excitotoxicity, and apoptosis. In the case of familial ALS linked to genetic mutations of the SOD1 enzyme, the disease pathophysiology seems to be centered around a mutation, with cell injury and apoptosis triggered by the excessive generation of free radicals in the cell. Glutamate excitotoxicity has also been implicated as a mechanism that can trigger neurodegeneration due to the overstimulation of ionotropic and metabotropic glutamate receptors, resulting in the increased formation of free radicals

within the neuron. This mechanism has been referred to as the "dying-forward" process as glutamate release by the upper motor neurons facilitates the anterograde degeneration of the lower motor neurons through the excitotoxic cascade mediated by increased synaptic levels of glutamate (due to astrocytic glutamate transporter dysfunction) (Kiernan et al., 2011).

In the case of many neurodegenerative diseases such as amyotrophic lateral sclerosis, the roots of the disease pathophysiology are defective protein products due to genetic mutation (such as in the case of fALS linked to the SOD1 mutation). Therefore, it may seem logical to conclude that if there were a way to introduce functional genes that would produce functional protein products into diseased cells, effective treatment could be achieved. This is the goal and premise of gene therapy, one of the most intensely researched therapies for amyotrophic lateral sclerosis.

Neural Transmission

I. Glutamate Release

Glutamate is the primary amino acid neurotransmitter used by the brain for excitatory activity. The concentration, and therefore activity, of glutamate in the synaptic cleft is kept in balance through tight regulation of glutamate receptors on postsynaptic neuron membranes as well as through astrocytic glutamate reuptake. When the balance of glutamate in the synaptic cleft is disturbed, the unregulated concentrations of glutamate can become neurotoxic, which is the basis of the amytrophic lateral sclerosis pathophysiology. The use of glutamate in glutamatergic synaptic transmission begins with its formation in the presynaptic neuron. One of the main pathways for glutamate formation in the neuron is through transamination of 2-oxoglutarate (also called α-ketoglutarate), an intermediate of the Krebs or TCA cycle. Glutamate is also largely formed in the presynaptic terminal from glutamine using the enzyme phosphate activated glutaminase (PAG) **(Henriksen, 2015)**.

Following its synthesis in the presynaptic terminal, the neurotransmitter is packaged into synaptic vesicles by vesicular glutamate transporters (VGLUTs). vGLUTs are antiporters that harness the vesicular proton gradient to drive the active transport of glutamate into the vesicles. The interior of the vesicles is very acidic, due to a high proton concentration created by the activity of a vesicular H^+ ATPase pump in the vesicular membrane, and therefore the flow of protons out of the vesicle may be coupled with the transport of glutamate into the vesicle in order to make the transport energetically favorable (Henriksen, 2015).

As an action potential travels down the axon and arrives at the presynaptic terminal it causes depolarization of the terminal and, as a result, opening of the voltage gated Ca^{2+} channels in the terminal membrane. Calcium influx through the voltage-gated channels and into the synaptic bouton causes intracellular Ca^{2+} levels to rise, triggering fusion of the synaptic vesicles with the terminal membrane and exocytosis of the neurotransmitter molecules into the synaptic cleft. High calcium concentrations in the cytosol of the presynaptic neuron activate calmodulin protein, which in turn binds to Ca^{2+}/calmodulin-dependent protein kinases. The activated complex phosphorylates several target proteins in the cell in order to bring about a desired cellular response. The SNARE proteins constitute one subset of target proteins that are activated by calmodulin-dependent kinase phosphorylation. This activation promotes vesicular fusion at the presynaptic membrane. The direct action of calcium also plays a vital role in vesicular fusion and neurotransmitter release. Synaptotagmins are vesicular membrane proteins that act as Ca^{2+} sensors. Synaptobrevin SNARE proteins also exist in the vesicular membrane. Together, these two sets of SNARE proteins are labeled “v” SNARE proteins. SNAP-25 and syntaxin proteins are “t” SNARE’s that exist in the presynaptic terminal membrane. The presence of increased Ca^{2+} levels in the synaptic bouton causes nSec1, a regulatory SNARE protein, to release from

syntaxin, lifting the inhibition on syntaxin protein and allowing for the "v" and "t" SNARE proteins to twist together and form a complex. This twisting interaction between v and t SNARE proteins effectively pulls the glutamate-carrying vesicle toward the nerve terminal membrane. Calcium also acts to stimulate synaptotagmins which results in pore formation and fusion of the vesicle with the membrane, ultimately releasing the vesicular contents into the synapse (Henriksen, 2015).

Levels of glutamate are closely regulated not only in the synapse but also in the terminal of the presynaptic neuron. A series of enzymes are used by the cell to modulate the bioavailability of glutamate, depending on the present needs of the cell. The enzyme glutamate dehydrogenase catalyzes the oxidative deamination of glutamate to α-ketoglutarate, using NAD+ as a cofactor. Therefore, glutamate dehydrogenase works to effectively reduce the bioavailability of glutamate inside the presynaptic neuron, so that less of the neurotransmitter is available to the cell for synaptic release. Control of this reaction determines the glutamate that is biologically available for neural use and may be a therapeutic target for neurodegenerative disorders, such as ALS, in which the regulation of synaptic glutamate concentrations is compromised due to inhibition of the clearing mechanisms (Glutamate Dehydrogenase 1, 2017).

II. Glutamate Action

Immediately following release into the synapse, glutamate binds and activates receptors on the postsynaptic membrane. Glutamate-specific metabotropic receptors are present on the postsynaptic neuron and function as G-protein coupled receptors, which activate intracellular signal transduction pathways to elicit a cellular response (Foran and Trotti, 2009). In these receptors, the neurotransmitter (ligand) binding domain is coupled to a heterotrimeric G protein that transmits signals to an effector in the cell and to an ion channel separate from the ligand-binding domain. In this case, the functional link between

the receptor and ion channel is a diffusible second messenger. The glutamate-specific G-protein coupled receptors are labeled mGLUR and are composed of 7 transmembrane alpha helices which associate with a heterotrimeric G protein on the cytoplasmic side of the plasma membrane. In the case of glutamate signaling in the neuron, the G protein is of the type G_q, and consists of three units, named α, β, and Υ. A molecule of GDP is bound to the α subunit in quiescent conditions, and upon stimulation of the GPCR through ligand-binding, a molecule of GTP replaces the GDP on the α subunit. This exchange of GDP for GTP allows the α subunit to dissociate from the heterotrimeric complex and activate phospholipase C (PLC) which in turn cleaves a phospholipid from the plasma membrane to form the molecule PIP2 (phosphatidylinositol 4,5-bisphosphate). PIP2 is then cleaved by PLC to form DAG (diacylglyercol) and IP3 (inositol triphosphate), the latter of which is the second messenger in the signaling pathway. IP3 can then trigger the opening of intracellular calcium channels to elicit a cellular response (Henriksen, 2015).

Glutamate-specific ionotropic receptors are also localized on the postsynaptic membrane and act as ligand-gated ion channels for which glutamate is the ligand that opens the ion-permeable channels. These ionotropic receptors may be organized into three receptor classes: NMDA (N-methyl-D-aspartic acid) receptors, AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, and KA (kainate) receptors. Due to functional similarities, AMPA and kainate receptors are often grouped together. These receptors are permeable to Na^+ and K^+ and in some cases display limited permeability to Ca^{2+}. On the other hand, NMDA receptors have significant permeability to Ca^{2+} ions (Yi and Hazell, 2006). The interaction of glutamate with the AMPA/KA and NMDA receptors modifies the permeability of the postsynaptic membrane to the afore-mentioned cations, resulting in the generation and transmission of excitatory postsynaptic potentials (Magistretti, 2009).

AMPA and kainate channels are usually found to be impermeable to Ca^{2+} ions in the cell, due to the presence of a GluR2 subunit which blocks the Ca^{2+} permeability of heteromeric channels. However, motor neurons represent one specific subset of cells that possesses large numbers of AMPA and kainate channels which lack the GluR2 subunit and are therefore permeable to calcium. This intrinsic feature of motor neurons, confirmed by significantly lower concentrations of GluR2 mRNA in the motor neuron as compared to other cells, therefore makes them more vulnerable to the effects of excitotoxicity and dysregulated calcium homeostasis (Rao and Weiss, 2004).

III. Glutamate Recovery

As with any neurotransmitter that is released into the synapse, the action of glutamate on the postsynaptic neuron must be rapidly terminated after signal transmission in order to prevent overstimulation and toxicity. In the case of glutamate, the excitatory action is terminated by a reuptake system that is present on the processes of astrocytes adjacent to the neurons. Astrocytes interact intimately with motor neurons for trophic support and also to closely regulate the balance of extracellular ion and neurotransmitter levels, as in the case of glutamate (Foran and Trotti, 2009).

Glutamate uptake from the synaptic cleft into the astrocyte is facilitated by the action of high-affinity, Na^{+}-dependent glutamate transporters that span the astrocytic plasma membrane. There are five isoforms of these excitatory amino acid transporters found in human tissues: EAAT1 (GLAST), EAAT2 (GLT-1), EAAT3 (EAAC1), EAAT4, and EAAT5. Two isoforms are located primarily on astrocytes: EAAT1 (also referred to as astrocyte-specific glutamate transporter, GLAST) and EAAT2 (also referred to as glutamate transporter 1, GLT-1). These astrocyte-specific transporters have a polarized distribution in that they are localized specifically to those astrocytic processes that are immediately adjacent to the synaptic cleft (presumably to improve the efficiency of glutamate clearing

from the cleft) (Maragakis and Rothstein, 2001). Of these two receptors localized mainly on astrocytes, EAAT2 is the more abundant, composing roughly 1% of total brain proteins. Consequently, it is considered to be primarily responsible for maintaining the integrity of the synapse and for the removal of glutamate from the extracellular space (accounting for about 95% of uptake) (Foran and Trotti, 2009). It therefore follows that any significant disruption in the activity of this protein may compromise the cell's ability to regulate extracellular glutamate concentrations, resulting in neuronal toxicity.

Glutamate transporters are capable of concentrating glutamate levels in the astrocyte up to 10,000 fold compared with the concentration in the extracellular space. Therefore, the uptake of glutamate from the synapse into the astrocyte is an example of active transport, as the neurotransmitter is being moved against its gradient and into a cellular compartment in which the concentration is higher (Maragakis and Rothstein, 2001). In order to facilitate this movement, the electrochemical gradient of sodium is used as the cellular driving force. Extracellular concentrations of sodium are significantly higher than intracellular concentrations, and therefore movement of sodium down its gradient (into the astrocyte) can be coupled to the movement of glutamate against its gradient (into the astrocyte as well) in order to provide the energy necessary for this process. The stoichiometry required for this cotransport is three sodium ions per each glutamate molecule (Magistretti, 2009).

Normal Processing of Reactive Oxygen Species

I. Introduction to Reactive Oxygen Species

Oxidative stress has been identified as one of the major contributing components to the pathogenesis of amyotrophic lateral sclerosis, and the effects of pathological oxidation are widespread, putting a stress on neurons and astrocytes alike. The human body has

many systems in place to reduce the toxicity of naturally produced reactive oxygen and nitrogen species. One important mechanism that confers protection against excessive oxidative stress is the transcription of genes that encode enzymes for detoxification and antioxidant defense. For example, genes such as superoxide dismutase 1, catalase, thioredoxin, glutathione peroxidase, and many others are vital in reducing the effects of oxidants on the cell. The expression of many of these enzymes involved in antioxidant defense is modulated by the transcription factor Nrf2, and therefore the Nrf2-ARE pathway is considered a master regulator of oxidative stress (Gan and Johnson, 2014). The primary source of physiological oxidative stress is improper reduction of the oxygen species at the end of the electron transport chain in the mitochondria, and to some degree the re-folding of improperly folded ER proteins. The especially high metabolic demand of neurons gives them an inherently high vulnerability to oxidative stress accumulated over time. When there is dysfunction of the mitochondria, this vulnerability of neurons puts them at a high risk for damage or cell death (Barber and Shaw, 2010).

Normal cellular metabolism generates many by-products including reactive-oxygen species (ROS), chemically reactive molecules that play an important physiological role in cell signaling processes. At moderate concentrations, these species are vital for many physiological functions, including regulation of vascular tone in the circulatory system and promotion of the production of growth factors by T-cells, such as Interleukin-2 (Zhang and Gutterman, 2006). Often, these reactive-oxygen species play a physiological role by regulating the activity of signaling proteins through oxidative attack. This oxidation of protein residues, notably the thiol functional groups on cysteine residues, can affect the target in many ways, inducing either a gain of function, loss of function, or imparting a new function on the protein (Trotti et al., 1998). Although there are many sources in the cell, the

mitochondrion seems to be a major contributor to basal levels of ROS through the action of the electron transport chain (Tadic et al., 2014).

There are many types of ROS that are physiologically present in the cell. Superoxide anion, for example, is formed by the reduction of molecular oxygen (O_2) and can be transformed through the action of the enzyme superoxide dismutase into hydrogen peroxide (H_2O_2). Hydrogen peroxide can be further converted into a highly reactive hydroxyl radical (·OH) in the presence of transition metals (such as Fe^{3+} and Cu^{+}). Superoxide anion and hydrogen peroxide are two of the more common reactive oxygen species present in the cell, and although each alone is not highly reactive, they can both be further reacted to produce oxidants that are more potent in their oxidizing abilities and capable of causing extensive cellular damage. As previously elucidated, hydrogen peroxide will decompose to form highly reactive hydroxyl radical. In addition, superoxide anion will rapidly react in the presence of nitric oxide (NO) to produce peroxynitrite ($ONOO^{-}$), the most potent ROS in the cell, which has almost an immediate oxidative action on cysteine sulphydryl groups, its primary target on cellular proteins. Both peroxynitrite and hydroxyl radical are capable of causing significant damage and inducing conformational changes in the structure of proteins, lipids, and DNA in the cell (Barber and Shaw, 2010).

II. Antioxidant Defenses

In order to maintain redox homeostasis in the cell, antioxidant compounds and enzymes are employed. The use of these antioxidants ensures that the rate of ROS clearance is in balance with the rate of ROS production, so that basal levels of ROS are present for cell functions but do not increase to toxic levels, which would potentially disrupt lipid and protein structure and function. When ROS levels are elevated in a cell, the upregulation of the expression of genes encoding antioxidant protein products is induced (Birben et al., 2012). The cell has many mechanisms for sensing disturbances in the redox balance,

notably the redox state of intracellular thiol/disulfide residues. When these amino acid residues are oxidized, there is a structural shift in the residue from displaying free sulphydryl groups to the formation a disulfide bond between two residues. This interconversion enables the residues to have redox-sensing properties, and in the oxidized state this mechanism triggers an antioxidant response in the cell to regulate the rise in ROS (Barber and Shaw, 2010).

Glutathione (GSH)

Due to the inevitability of constant exposure to oxygen radicals in aerobic organisms, cells are equipped with a series of antioxidant defenses to protect themselves against permanent and severe oxidant damage. These defenses can be organized into two primary categories: antioxidant compounds and antioxidant enzymes (proteins). Antioxidant compounds are those which react directly with oxidizing agents to neutralize their oxidizing abilities and deem them harmless to cellular components. There are two systems of antioxidant compounds in place in the cell to counteract the effects of scavenging oxidants: glutathione and thioredoxin. Glutathione is a tripeptide compound composed of a glutamate, cysteine, and glycine amino acid joined together by peptide bonds, with the cysteine amino acid in the middle of the tripeptide. This compound is ubiquitously synthesized throughout the body and has antioxidant properties due to the presence of the sulphydryl group on the cysteine residue, which hangs off of the compound and is accessible from the exterior of the compound (Sies, 1999). In its reduced form, glutathione is designated as GSH, however when electrons are donated from the sulphydryl group on the molecule to reduce another cellular component, the molecule becomes oxidized. Once in its oxidized state, the glutathione molecule can dimerize with another oxidized glutathione through the formation of a disulfide bridge, a covalent bond between the sulfur atoms of two oxidized sulphydryl groups. In this oxidized form, a glutathione dimer is designated as

GSSG. Glutathione can act in the cell in several ways to reduce the deleterious effects of scavenging oxidants. For example, GSH can directly interact with free oxidants, donating a reducing equivalent to these molecules and effectively neutralizing them. Glutathione can also interact with oxidized sulphydryl groups on accessible cysteine residues of cellular proteins, such as those of the excitatory amino acid transporters for glutamate located on astrocytes. In this case, GSH molecules donate a reducing equivalent (H+ and e-) to each thiol group involved in a disulfide bond on a protein, which effectively reduces the sulphydryl groups back to their –SH reduced state. The GSH molecules as a result become oxidized and form disulfide bonds with each other, creating GSSG and depleting the cellular reserve of reduced GSH molecules. In this way, glutathione acts to reverse the oxidation of proteins in the cell and protect them against oxidative damage. Once converted to its oxidized GSSG form, reduced glutathione (GSH) can be regenerated using the enzyme glutathione reductase. A healthy cell maintains a ratio of reduced glutathione forms (GSH) to oxidized (GSSG) of 9:1. Any decrease in this GSH:GSSG ratio is indicative of oxidative stress and an inability of the cell to maintain sufficient antioxidant defenses (Masella et al., 2005).

Thioredoxin (TRX)

The thioredoxin (Trx) system is the second key antioxidant system of defense in the cell. Thioredoxin is a 12-kD oxidoreductase enzyme that plays a role in antioxidant defense by engaging in cysteine thiol-disulfide exchanges in a manner comparable to the glutathione system. Reduced thioredoxin is designated as TRX-(SH)2, and this reduced form confers protection against oxidation to endogenous proteins by donating electrons to the oxidized protein forms and in turn being oxidized to the TRX-S2 form. More specifically, the mechanism of action of thioredoxin is as follows. First, the Cys32 residue of the TRX CXXC motif attacks the oxidized group (disulfide bond) of the substrate. Then, Cys35, the other

cysteine residue of TRX, forms a disulfide bond with Cys32, effectively transferring two electrons to the target substrate, which consequently is converted to its reduced form. In order to maintain a suitable pool of reduced thioredoxin in the cell, the TRX (thioredoxin) reductase enzyme is used to reduce the oxidized thioredoxin form by transferring electrons to thioredoxin from the electron carrier NADPH (which is oxidized to NADP+ in the process) (Lu and Holmgren, 2014).

In addition to the TRX and GSH antioxidant systems, the cell also employs a series of antioxidant enzymes to serve as a defense against oxidative damage of proteins, lipids, and other cellular structures. Many of these antioxidant enzymes are under the control of the Nrf2-ARE pathway, in which the association between the transcription factor Nrf2 and the gene regulatory element ARE modulates the expression of many genes involved in regulation of redox homeostasis in the cell (Barber and Shaw, 2010.

NRF2

Nuclear Factor Erythroid 2-related Factor 2, commonly referred to as Nrf2, is a transcription factor that is a member of the Cap'n'Collar basic-leucine zipper family. Nrf2 coordinates the up-regulation of antioxidant defenses by modulating the transcription of genes that contain a specific cis-acting regulatory element, called Antioxidant response element (ARE). Due to its ability to modulate the expression of hundreds of detoxifying genes, the Nrf2-ARE pathway is considered a primary sensor for oxidative stress and plays a vital neuroprotective role against excessive oxidation. Consequently, the transcription factor is ubiquitously expressed in all human tissues (Gan and Johnson, 2014). Under quiescent conditions, the action of Nrf2 is largely inhibited and negatively regulated through kelch-like ECH associating protein 1 (Keap1), and Nrf2 is responsible for a very low-level of activation of ARE, driving the expression of antioxidant enzymes in unstressed conditions. Under basal cellular conditions, the C-terminal domain of the Keap1 protein is

bound to the Nrf2 transcription factor, while the N-terminal domain is bound to an E3 ubiquitin protein ligase; in this way, not only does Keap1 prevent the translocation of Nrf2 to its site of action, the nucleus, but it also directs the transcription factor to ubiquitination and consequent degradation by the proteasome. In order to activate the Nrf2/ARE pathway and confer neuroprotection against oxidative stress, dissociation of the Nrf2-Keap1 complex must occur. The activity of the Keap1 protein is dependent on the reduced state of three cysteine residues: Cys151, Cys273, and Cys288 (Yang et al., 2015). Oxidative modification of these residues results in a conformational change in the protein and consequent detachment of Keap1 from Nrf2. Oxidation of the cysteines also targets Keap1 for ubiquitination. Upon disruption of the Nrf2-Keap1 complex, Nrf2 begins to accumulate in the cytoplasm and is free to translocate to the nucleus (Gan and Johnson, 2014). Two nuclear export sequences (NES) and three nuclear localization signals (NLS) are vital regulators of the nucleocytoplasmic shuttling of the transcription factor through their interaction with the nuclear pore complex on the nuclear envelope (Yang et al., 2015). After successful shuttling into the nucleus, Nrf2 forms a heterodimer with small musculo-aponeurotic fibrosarcoma (Maf) proteins and is then able to bind to ARE sequences on the target genes (Gan and Johnson, 2014). Once the Nrf2-ARE complex forms, transcription of the protective proteins, such as superoxide dismutase 1, catalase, sulforedoxin, thioredoxin, peroxiredoxin, NAD(P)H Quinone oxidoreductase-1 , and hemeoxygenase-1, is activated (Ma, 2013). The Nrf2-ARE pathway exerts its major protective effect against oxidative stress via increasing glutathione levels, as Nrf2 regulates the expression of both glutamate-cysteine ligase (GCL) and glutathione synthetase (GS), the two enzymes that synthesize glutathione. In fact, Vargas et al. found that Nrf2 overexpression in cultures of motor neurons and astrocytes of transgenic mice with the SOD1 mutation resulted in an approximately 25% increase in the glutathione content (Vargas et al., 2008). This increased

production and secretion of glutathione by the astrocytes improves the antioxidant status of neighboring neurons, conferring protection from oxidative insults and giving them resistance to oxidative stress. In this way, the activation of the Nrf2-ARE pathway actively defends against the progressive loss of neurons in neurodegenerative disorders.

Superoxide Dismutase-1

Possibly the most well known and significant enzyme involved in minimizing oxidative damage is superoxide dismutase. Superoxide dismutases catalyzes the reaction of two molecules of superoxide anion ($O_2^{\cdot -}$), produced from normal cellular metabolism, to form hydrogen peroxide (H_2O_2) and eventually molecular oxygen (O_2) as a by-product. Although the direct product of the dismutase reaction, H_2O_2, is toxic to the cell, it is removed quickly by reduction to water and oxygen through glutathione peroxidases (which utilize glutathione) and catalases. Catalases are detoxifying enzymes that are found almost exclusively in the cell's peroxisomes, where the enzyme reduces the hydrogen peroxide produced as a product of Beta-oxidation of fatty acids in the cell. Due to its usual absence from the peroxisomes, glutathione largely detoxifies cytoplasmic H_2O_2 (Davies, 2000). Members of the SOD family contain one of many transition metals in their active site, typically copper or zinc in mammals. The protein is homodimeric, and each 153-amino acid protein subunit of the dimer binds one zinc atom and one copper atom (Julien, 2001). Superoxide dismutase is a free scavenger of superoxide and is typically found in the cytoplasm, however a fraction of the enzyme has also been located in the mitochondrial intermembrane space, where it targets superoxide anions released from the ETC of the inner membrane and prevents these reactive oxygen species from leaving the mitochondrion and causing oxidative damage elsewhere in the cell (Rizzardini et al., 2005).

Mitochondrial Function

I. The Electron Transport Chain and ROS Formation

As a result of their high metabolic demands, motor neurons are dependent on optimal functioning of the mitochondria. Mitochondria are double-membrane organelles, with an outer membrane facing the cytosol and an inner membrane that has numerous invaginations, termed cristae, and which is relatively impermeable to most ions. Mitochondrial oxidative phosphorylation is the major pathway for ATP synthesis in eukaryotic organisms and is the main mechanism employed to meet the metabolic demands of neurons. In the process of oxidative phosphorylation, electrons are delivered by electron carriers, such as $FADH_2$ and NADH, to the electron transport chain, a series of protein complexes embedded in the mitochondrial inner membrane. The electrons then undergo a series of redox reactions as they are passed from one complex to the next, which consistently have a higher reduction potential to promote this electron "flow". The flow of electrons through intermembrane protein complexes proceeds as follows: Complex I (NADPH Oxidase/Dehydrogenase) where NADH electron carriers are reduced and transfer their electrons to the ETC; Complex II (Succinate Reductase/Dehydrogenase) where FADH2 electron carriers which closely associate with the protein complex are reduced and transfer the electrons that they carry to the transport chain; Complex III (Ubiquinone-Cytochrome C Oxidoreductase) which is preceded by the Q cycle, a series of reactions that converts the two-electron transfer system of the first two complexes to the one-electron transport system of Cytochrome C; and finally Complex IV (Cytochrome C Oxidase) which transfers electrons from Cytochrome C to the final electron acceptor, O_2. These protein complexes make use of iron-containing heme prosthetic groups (in the case of Cytochrome C), Ubiquinone, and Iron-Sulfur Clusters to help facilitate the passing of electrons through the

chain and to act as either electron storage or carriers in the chain (Henriksen, 2015). The free energy that is released in this spontaneous electron transfer from one protein to another of higher reduction potential is then harnessed by proton pumps in complexes I, III, and IV to establish a H+ gradient across the inner mitochondrial membrane. The electrochemical energy from this concentration and voltage gradient can then be used to power the synthesis of ATP via ATP synthase. At the end of the respiratory chain, oxygen molecules act as the final acceptors for the electron pairs that have passed through the chain. The stepwise reduction of the O_2 molecule to its reduced form, H_2O, occurs via several reactive oxygen species intermediates. In the case that the oxygen molecule at the end of the chain is improperly or prematurely reduced, the reactive oxygen species superoxide anion is generated. This faulty reduction of oxygen occurs roughly 1-4% of the time in normally functioning mitochondria and generates basal cellular levels of ROS, which serve physiological functions as previously noted. The absence of these low levels of reactive oxygen species impedes many physiological pathways, however the accumulation of pathologically high levels of ROS, which may be due to an inability to detoxify basal ROS concentrations, can be a source of toxicity to the cell. Although superoxide anion is the primary oxidant species formed by the respiratory chain, H_2O_2 may also be produced through the spontaneous enzymatic action of superoxide dismutase (SOD), and this species may be further transformed to OH^- via the action of metal chaperone proteins (Barber and Shaw, 2010).

II. Calcium Regulation

The Mitochondria play many diverse roles in cell. Not only are they vital for the aerobic production of ATP and for programmed cell death, apoptosis of diseased cells in the body, but they also play a central role in calcium homeostasis and signaling in the cell.

Calcium is an intracellular signaling molecule that regulates numerous vital pathways and is required for some of the most basic biological functions (neurotransmitter release for example). This ion controls so many physiological processes in the body through transient elevations in its cytosolic levels, that it must be tightly regulated until a cellular response is desired. Cells maintain this quiescent cytosolic free calcium level at about 100 nM, achieved and maintained by extruding excess Ca^{2+} through pumps and exchangers in the plasma membrane and by compartmentalizing calcium in the endoplasmic reticulum, which has a concentration that is usually maintained in the 100-800 μM (Staats and Van Den Bosch, 2014). At rest, mitochondrial calcium levels are kept around 100 nM, and at baseline the cytosolic calcium levels fall below the setpoint for mitochondrial uptake, so that little net mitochondrial calcium uptake occurs in quiescent conditions. However when cytoplasmic calcium levels rise from their resting nM levels to μM levels, calcium can be transported into the mitochondria through the activity of a calcium uniporter (MCU). Through the activity of this uniporter, Ca^{2+} can be concentrated in the mitochondrion even up to mM levels. On the other hand, Ca^{2+} is extruded from the mitochondrial matrix via a mitochondrial Na+/Ca2+ exchanger (NCLX), an antiporter membrane protein which couples the active transport of Ca^{2+} ions out of the mitochondrion with the flow of Na^{+} ions from the cytosol into the mitochondrion and down their gradient. High levels of Ca^{2+} can then be extruded from the cell via the function of a Na+/Ca2+ exchanger (NCX) on the plasma membrane and PMCA, a plasma membrane calcium ATPase (Tadic et al., 2014).

Once transported into the mitochondrion, Ca^{2+} increases the rate at which oxidative phosphorylation proceeds, through its activation of many Ca^{2+}- sensitive dehydrogenases of the Krebs cycle and of pyruvate dehydrogenase. By increasing the turnover of the Krebs cycle, Ca^{2+} increases the amount of reduced electron carriers (FADH2 and NADH) in the mitochondrion, which provide the electrons for the electron transport chain. It is therefore

speculated that the transfer of Ca^{2+} from the cytosol into the mitochondria acts as a signal to indicate low ATP supply and high demand. In essence, the cell can increase mitochondrial calcium levels in order to speed up metabolism, however at very high levels can have deleterious effects on the distal protein complexes of the electron transport chain (Tadic et al., 2014).

The Endoplasmic Reticulum

I. Calcium Regulation

The endoplasmic reticulum is an organelle belonging to the endomembrane system, and it consists of a network of membranous compartments inside which many cellular functions are carried out. The ribosome-studded portion of the membranous network plays a role in synthesizing and modifying hydrophobic and amphipathic protein products that are destined to become excreted or integrated into a membrane (i.e. digestive enzymes and membrane receptors). On the other hand, the ER portion lacking ribosomes (smooth endoplasmic reticulum) plays a role in the production and storage of lipids, such as steroids and phospholipids. In addition to these identifying functions, the endoplasmic reticulum plays a role in calcium regulation, by accumulating and storing calcium inside its membranous compartments. Using an ATP-powered pump, ER calcium ATPase, Ca^{2+} can be actively transported from the cytosol into the lumen of the ER, resulting in the significant concentration of the ion inside the ER as compared to the cytosolic levels (Henriksen, 2015).

The endoplasmic reticulum also has a closely regulated mechanism of Ca^{2+} release into the cytosol of the cell through the use of signals from metabotropic glutamate receptors, mGLuR. As mentioned previously in the discussion of the action of glutamate on receptors of the post-synaptic neuron, increased glutamate concentrations in the neural

synapse increase the stimulation of these metabotropic receptors. These receptors are characterized as GPCRs, as they are coupled to a heterotrimeric G protein on the interior of the plasma membrane. Stimulation of the GPCR and consequent dissociation of the α subunit from the G-protein results in the activation of phospholipase C (PLC), a membrane-bound enzyme. Activation of phospholipase C is a means of forming the pathway's second messenger molecule, IP3, in that the enzyme hydrolyzes a molecule of PIP2 to IP3 and DAG. IP3 is an organic molecule and soluble second messenger that upon formation can diffuse through the cytosol of the cell and bind to receptors on the membrane of the endoplasmic reticulum. These Ins3P receptors are ligand-gated Ca^{2+} channels, which upon binding of the IP3 molecule, elicit the opening of the channel subunit, allowing for the flow of Ca^{2+} from the ER store and into the cytoplasm. It is through this mechanism that the binding of glutamate to metabotropic receptors mobilizes calcium from the ER storage (Henriksen, 2015).

As they are both vital cellular stores of Ca^{2+}, the endoplasmic reticulum and mitochondrion form close structural associations so as to carry out a number of cellular functions and facilitate the transfer of calcium between them (Tadic et al., 2014). The transfer of calcium between the two cellular stores is dependent on the relative position and proximity of the two organelles. It has been shown that upon IP3-induced calcium release from the ER, mitochondrial calcium uptake and accumulation can be increased by reducing the distance between the two organelles. In order to facilitate these mitochondria-ER interactions, about 5-20% of the mitochondrial surface is closely associated with the ER at any one point in time, and these ER associated regions are termed MAMs (mitochondria-associated ER membranes). Several protein structures also aid in tethering the ER to the mitochondrion within the cell. For example, PTPIP51 (protein tyrosine phosphatase-interacting protein 5), a protein located in the outer mitochondrial membrane, binds closely with VAPB (VAMP (Vesicle-Associated Membrane Protein) Associated Protein B), an ER

protein, resulting in a strong association which facilitates ER-mitochondria interactions (Stoica et al., 2014).

The VAPB mRNA has six exons that encode a 27.2 kDA homodimer, a dimer of two identical protein subunits. The protein belongs to a family of intracellular vesicle-associated membrane-bound proteins, which are vital players in the regulation of vesicle transport between organelles within the cell (Pasinelli and Brown, 2006). The orientation of these two proteins in their respective organelles allows for the formation of a tether, with VAPB having an anchor in the ER through its membrane spanning domain which projects the protein's N terminus into cytosol of the cell, and PTPIP51 anchored on the outer membrane of the mitochondrion with its C terminus projecting into the cytoplasm. This orientation allows for covalent interactions between the projecting termini of the two proteins through condensation (peptide bond formation) (Stoica et al., 2014).

II. Protein Modification

Aside from its vital role in calcium regulation and homeostasis, the ER is also an entry point for the synthesis of membrane-bound and secretory proteins as well as lipids. Proteins destined for secretion or integration into a membrane begin the process of translation using free ribosomes in the cytoplasm. Upon translation of an N-terminal signal peptide they are translocated to the rough ER membrane where translation continues by membrane-bound ribosomes which feed the protein into the ER lumen. Inside the ER, proteins undergo post-translational modification and are subject to ER mechanisms, which ensure proper folding and assembly of proteins into complexes. Modified proteins then undergo vesicular trafficking from the ER lumen to the Golgi where they are further modified and tagged for their final destination. In addition to its function in tethering the ER and mitochondrion together, VAPB functions in this trafficking of ER-synthesized proteins on to their next destination (Prosser et al., 2008).

After their synthesis into the ER lumen, a high proportion of new secretory and membrane proteins are misfolded and require the intervention of ER mechanisms to correct this folding error. ER chaperone proteins, such as BiP, are utilized by the ER to refold the misfolded proteins or degrade them via the ERAD (endoplasmic reticulum-associated protein degradation) pathway (Barber and Shaw, 2010). In the ERAD pathway, misfolded proteins are targeted for degradation by the proteasome through ubiquitination. The covalent attachment of several ubiquitin molecules (small regulatory proteins) to damaged or improperly folded proteins, in a process called ubiquitination, tags these proteins for destruction by the cell. More specifically, the C-terminus of ubiquitin residues covalently attaches to the N-terminus of lysine residues in the target protein. The ubiquitination process is ATP-dependent, requiring the hydrolysis of one ATP molecule for each ubiquitin that is added to the protein, and also requires the action of three enzymes in order to facilitate this ubiquitin transfer: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-protein ligase). Once a chain of at least five ubiquitin molecules have been added to the target protein, it is capable of being recognized by the proteasome. The proteasome is an ATP-dependent, proteolytic complex composed of multiple subunits. The structure of the proteasome can be described as a 20S core, made up of 28 subunits in a barrel shape, with a cap (19S regulatory particle) on each end of the core. The process of proteolysis itself occurs at sites on the inside of the cylindrical chamber created by the barrel shaped 20S core (Henriksen, 2015).

III. Formation of ROS

Despite the safeguards put in place by the ER to correct misfolded proteins, these proteins still tend to accumulate over time within the ER lumen, which places stress on the organelle. In order to counteract this ER stress, a self-defense mechanism called the unfolded protein response (UPR) is initiated in response. The UPR functions to reduce the

load of misfolded, faulty proteins through the upregulated transcription of ER chaperones to improve the refolding capacity of proteins, and of genes in the ERAD pathway to increase the ER's ability to degrade faulty proteins. In addition, the UPR response triggers the transient suppression of ER-facilitated protein translation, and during a severe response may even initiate apoptotic pathways in the cell (Barber and Shaw, 2010).

As mentioned in the discussion of mitochondrial function, the organelle can be a source of physiological reactive oxygen species. The endoplasmic reticulum, as well, shares in this function and can produce ROS within the cell to maintain baseline levels. One of the folding functions that the ER facilitates is the formation of disulfide bonds between amino acid residues on proteins. During this disulfide formation, electrons from the reduced thiol groups are passed to the chaperone protein Ero1, effectively oxidizing the thiol groups and creating a disulfide bond between them. Ero1 (ER-oxidoreductin 1) is an oxidoreductase enzyme which transfers the thiol electrons onto molecular oxygen to generate ROS in the ER lumen. In addition, unstable disulfide bonds within misfolded proteins that need to be corrected are done so through reduction by glutathione in a manner catalyzed by the Ero1 protein. This reduction of unstable disulfide bonds both depletes cellular reserves of reduced glutathione that are able to function in antioxidant defense and also increases the number of reduced thiol groups which can produce ROS through the action of Ero1 electron transfer to molecular oxygen. In essence, through its protein re-folding functions, Ero1 increases ROS levels in the ER and depletes the reduced glutathione stores. As a chaperone protein, Ero1 experiences upregulated expression upon activation of the UPR response in times of ER stress. This in turn can provide an explanation for the manner in which prolonged stress of the ER and a sustained unfolded-protein response can lead to the production of high levels of ROS in the ER lumen (Barber and Shaw, 2010).

Disease Pathophysiology

I. Mutant SOD1 Toxic Gain of Function

In cases of familial ALS, mutations in the gene coding for Cu/Zn superoxide dismutase are quite common occurring in about 20% of cases. In these fALS patients, more than 100 missense mutations, mutations in which change of a single nucleotide results in a non-synonymous codon substitution, have been discovered (Rizzardini et al., 2005). Mutant SOD1 follows a dominant negative mechanism in that mutant protein not only displays a loss of proper function but also inhibits the function of non-mutant SOD1, which is expressed by the normal allele in subjects with the mutation. This effectively reduces the pool of functional SOD1 protein to nearly zero (Barber and Shaw, 2010). Rizzardini et al. conducted studies on NSC-34 cell lines that were transfected with human G93A-mutant SOD1 in 2005, with the goal of developing a cell cultural model of FALS. In the mutant line of cells containing G93ASOD1, enhanced ROS formation was observed. These pathologically high levels of ROS are thought to come from the toxic gain of function seen in the mutant SOD1 protein, rather than a loss of normal dismutase function. Several mechanisms have been proposed to explain the toxic gain of function by mutant SOD1, with the common themes in these proposals being aberrant oxidation and/or misfolded aggregates of the mutant protein. This higher level of ROS formation in cases of mutant SOD1 may have the potential to cause direct damage to motor neuron cells and proteins and lipids found in the surrounding cells, most importantly astrocytes. In addition, exposure to pathologically high ROS levels could have deleterious effects on the ETC, causing impairment of function and consequently further ROS production. In addition to the toxic gain of function that the mutant SOD1 can exhibit, its mutant structure in itself can be toxic to the cell in that it is prone to forming aggregates of the misfolded protein in the endoplasmic reticulum, an

occurrence which has the potential to cause issues for the ER's unfolded protein response machinery, as will be elaborated on (Rizzardini et al., 2005)

Although the study conducted by Rizzardini et al. did not investigate the source of the pathologically high ROS found in neural stem cells with the G93A mutation of SOD1, they speculate that the ROS originate from the cell's mitochondria. As previously elucidated, the mitochondrion is the primary source of physiological ROS in the cell. Superoxide anion from the ETC is released on both sides of the chain, into the matrix and the intermembrane space of the mitochondrion. From the intermembrane space the ROS can then diffuse out into the cytosol of the cell to play a role in cell signaling and extracellular processes. The small fraction of SOD1 that is physiologically found in the intermembrane space plays a role in limiting the levels of superoxide anion from the ETC that can escape into the cell. However, mutation in the SOD1 gene causes dysfunction of the protein, thereby reducing its ability to prevent ROS from escaping into the cytosol. Without functional SOD1 acting as a guard against the free passage of superoxide anion, these reactive oxygen species are able to leave the mitochondria and cause oxidative damage of cellular structures. This excess of ROS also feeds back to cause oxidative damage of the ETC itself (Rizzardini et al., 2005). Consistent with the hypothesis that the presence of mutant SOD1 results in an increase in ROS and escape of these ROS from the mitochondrion, Rao et al. observed that mutant SOD1 motor neurons display high levels of labeling for the compound nitrotyrosine, a marker for protein modification by reactive oxygen species and reactive nitrogen species. This labeling is often observed in a circular pattern emanating out from the motor neurons, suggesting that ROS escaping from the neurons are capable of causing oxidative damage to cells, such as astrocytes, which surround the neurons (Rao and Weiss, 2004)

Rizzardini et al. also found a decrease in the mitochondrial membrane potential (MMP), causing an increase in the permeabilization of the inner mitochondrial membrane.

This finding is an early indicator of the mitochondrial permeability transition (MPT), a self-amplifying cascade that eventually leads to apoptosis of the cell (Rizzardini et al., 2005). As mentioned in the discussion of mitochondrial function, the inner mitochondrial membrane is relatively impermeable to the passage of most substrates, which aids in creating a potential across this membrane. Oxidation of thiol residues on proteins in the inner mitochondrial membrane, the chances of which increase with increased ROS levels, compromise the nature of the IMM and result in an increase in its permeability. This increase in the permeable nature of the membrane could also play a role in the pathogenesis of mutant SOD1 by increasing the ease with which ROS from the ETC are able to leave the mitochondrion (Henriksen 2015).

Within the cell, the mitochondrion is the primary control center of apoptosis. Calcium overload and high levels of reactive oxygen species in the mitochondrion can stimulate an amplifying cascade called the mitochondrial permeability transition, in which the mitochondrial permeability transition pore (mPTP) exhibits a state of prolonged openness. This mitochondrial permeability transition pore is a pore that spans both the inner and outer mitochondrial membranes and is assembled from proteins that are pre-existing in the membranes (Patergnani et al., 2011). The inner mitochondrial membrane proteins that compose the pore have Ca^{2+} binding sites on the matrix side of the membrane, which regulate the activity of the mPT pore. In quiescent conditions, the pore fluctuates between active and inactive states by flickering open and closed. However, in conditions of Ca^{2+} overload, the binding sites on the matrix side of the IMM are constantly stimulated and the pore exhibits a prolonged “open” state. This extended pore opening, which is also triggered by oxidant stimuli, can have pathological consequences for the mitochondrion and the cell as a whole. As will be mentioned further on in a discussion of the effect of calcium overload on the electron transport chain, ROS-mediated oxidation of cardiolipin results in a

conformational change of the lipid. This change in conformation along with stimulation by the excess of calcium results in the disassociation of cytochrome C from cardiolipin in the inner mitochondrial membrane. The released cytochrome C may then exit the mitochondrion via the mPT pore and bind to the cytosolic protein Apaf-1 to trigger the formation of the apoptosome. Once the apoptosome has been formed, it functions in activating pro-caspase-9 which may then activate effector caspases and trigger the apoptotic cascade, culminating in the programmed death of the cell (Henriksen, 2015).

According to Barber et al. it has been reported that cells expressing the mutant form of SOD1 show down-regulation of genes important for the antioxidant response in the cell, including glutathione, peroxiredoxins, thioredoxins and Nrf2. (Barber and Shaw, 2010). It is still unclear, however, exactly how the expression of Nrf2 comes to be downregulated in the ALS pathophysiology. This down-regulation reduces the cell's ability to remove reactive oxygen species produced through normal metabolism, which results in an accumulation of oxidative stress over time. In addition, the excess of oxidants in the cell due to the excitotoxic cascade compound this oxidative stress and exacerbate the effects observed on the cell due to the Nrf2 loss. It has therefore been suggested that the Nrf2 transcription factor may be a viable candidate for up-regulation via gene therapy, in order to induce transcription of genes involved in modulating the levels of oxidants in the cell (Vargas et al., 2008).

In a recent study conducted by Vargas et al. it was found that increasing expression of Nrf2 led to an increase in the expression of both GCL and GS enzymes. This in turn results in a two-fold increase in the levels of glutathione. Ultimately, with an increase in Nrf2 expression there is increased regulation of ROS levels in the brain, and a restoration of the activity of glutamate receptors, reducing the excitotoxic cycle (Vargas et al., 2008).

II. Excitotoxicity

Glutamate transporters are located in the astrocytic cell membrane and are responsible for regulating extracellular concentrations of the neurotransmitter glutamate. When glutamate is released into the synaptic cleft and has bound to glutamate receptors on the post-synaptic membrane, transporters then play a role in glutamate uptake to reduce the concentration back to baseline. Subjects diagnosed with ALS exhibit significantly decreased activity of the EAAT2 glutamate transporter isoform. Research has found that there is no significant quantitative change in the levels of EAAT2 mRNA in the CNS of ALS patients, despite a decrease in the functional protein up to 95% compared to control patients and a similar decrease in glutamate transport. Therefore, it may be concluded that the dysfunction in EAAT2-mediated glutamate transport is due to post-translational modifications (Foran and Trotti, 2009). The structure, and therefore function, of these transporters can be post-translationally modulated in many ways, specifically through oxidation and reduction of residues on the protein. The transporter structure contains thiol groups with reactive cysteine residues, which can be alternately oxidized and reduced to alter transport activity. In the reduced state, the transporters have free sulphydryl groups and have maximal transport activity. On the other hand, when these groups are oxidized, a disulfide bond forms between the cysteine residues and is accompanied by a decrease in transporter activity, due to a change in the flexibility of the transporter conformation. In this way, it can be said that the glutamate transporter isoforms EAAT1, EAAT2, and EAAT3 exhibit redox-sensing properties and sensitivity to biological oxidants. When concentrations of reactive oxygen species rise, these transporter isoforms are inhibited by the oxidants via direct action on the transporter protein. This inhibition by ROS results in reduced uptake of synaptically released glutamate and, ultimately, accumulation of the neurotransmitter in the synapse. The EAAT2 (also referred to as GLT-1) isoform, in

particular, is concentrated on astrocytic processes adjacent to motor neurons. Therefore, this isoform is at an increased risk of oxidation and inhibition of activity due to ROS released from oxidatively stressed motor neurons (Trotti et al., 1998). As previously discussed, the EAAT2 isoform is responsible for about 95% of glutamate uptake in the synapse, due to its high concentrations on astrocytic processes. Therefore, compromising the function of this isoform has deleterious effects on glutamate uptake and results in the accumulation of extracellular glutamate to toxic levels. In EAAT2-knockout mice, in which the functional EAAT2 gene has been inactivated, researchers found that glutamate uptake into the astrocytes was reduced to 5.8% of the uptake found in wildtype mice. This finding underscores the importance of the functional EAAT2 protein in the CNS and shows the potentially toxic effects that its loss can have in terms of glutamate clearing (Foran and Trotti, 2009).

Glutamate serves as the ligand that "unlocks" the AMPA/kainate or NMDA receptor and therefore opens the ion channel upon binding, allowing for the direct influx of Ca^{2+} into the cell (as well as Na^{+} and K^{+} in the case of AMPA/kainate). When the activity of glutamate transporters on the astrocytic processes is compromised, glutamate levels in the extracellular space can rise to dangerous levels. In fact, glutamate is toxic to the neuron when its extracellular concentration rises above 1-3 μM **(Trotti et al., 1998).** NMDA receptors posses a redox-sensing mechanism similar to that used in the glutamate transporters, which employs the use of sulphydryl groups. When concentrations of reactive oxygen species are high, oxidation of these groups is likely to occur, which alters the receptor conformation and inhibits Ca^{2+} entry through the ion channels. This has been a proposed mechanism to protect against overstimulation of the NMDA receptor under oxidative stress, however excessive extracellular glutamate due to transporter inhibition leads to receptor activation that ultimately overrides this protective mechanism. On the

other hand, AMPA/kainate receptors do not undergo any form of oxidative downregulation, and therefore there is no protective mechanism in place to prevent the excessive influx of calcium through these channels. Following this AMPA/kainate/NMDA mediated Ca^{2+} influx into the postsynaptic neuron, excessive ROS are generated and downstream death signaling pathways are activated. In addition, the neuron may experience calcium-induced calcium release through the mechanism of IP3 action on InsP3R ER receptors. Excessive stimulation of the metabotropic glutamate receptors triggers the prolonged activation of PLC and consequent formation of the IP3 second messenger. This keeps the IP3 signaling pathway constantly "on", resulting in a continuous flow of ER calcium into the cytosol of the cell which depletes the ER stores and causes a spike in intracellular Ca2+ concentrations (**Trotti et al., 1998)**.

One of the hallmarks of amyotrophic lateral sclerosis is an accumulation of the neurotransmitter glutamate in the synaptic cleft. This accumulation may be attributed to excitotoxicity, a phenomenon that occurs when there is an imbalance between the release of glutamate by the pre-synaptic neuron and the reuptake of glutamate, due to the dysfunction of the astrocytic glutamate transporters. Many mechanisms have been proposed to explain why exposure to excess glutamate is neurotoxic and results in neuron cell death, and for the most part, mechanisms focus on excessive stimulation of the excitatory amino acid receptors on the post-synaptic neuron followed by a resultant increased concentration of cytosolic calcium. Inhibition of the high affinity glutamate transporters that occurs as a result of high ROS levels triggers a positive feedback cycle of neurotoxicity. The cycle begins with the aberrant formation of ROS, the origin of which has been previously elucidated. Through oxidation of cysteine residues on astrocytic glutamate transporters and consequent conformational change of the proteins, ROS inhibit the activity of the transporters and prevent the uptake of glutamate from the extracellular space. As glutamate

concentrations rise in the extracellular space, the excitatory amino acid receptors on the postsynaptic neuron begin to demonstrate over-stimulation. This over-activation of receptors leads to increased direct influx of calcium and elevated levels of intracellular calcium in the post-synaptic neuron. The pathologically high concentrations of cytoplasmic calcium activate many Ca^{2+} dependent enzymes and result in the production of ROS. Thus the self-amplifying cycle begins again with the oxidative damage of glutamate transporters (Foran and Trotti, 2009).

III. Mitochondrial Dysfunction

The uncontrolled influx of calcium into the postsynaptic neurons has drastic implications for the cell's calcium buffering systems, notably the mitochondria. When cytosolic calcium concentrations increase uncontrollably, stress is placed on the mitochondria, which can result in pathological ROS formation, an inability to maintain proper electron transport activity, and activation of an apoptotic cascade (Foran and Trotti, 2009). Maintaining low cytoplasmic calcium concentrations is vital to cell signaling, and therefore the mitochondria act as a calcium reserve, taking up excess Ca2+ from the cytosol when the stimulus is not needed. Since motor neurons innately have a poor ability to buffer calcium concentrations in the cytosol, due to low levels of expression of calcium binding proteins such as calbinding D28K and parvalbumin, the job of regulating intracellular calcium falls largely on the mitochondrion (Staats and Van Den Bosch, 2014). However, this "safety-net" of shuttling calcium from the cytosol into the mitochondrion to maintain low intracellular concentrations does not sustain the cell long. When the mitochondria are exposed to calcium levels in the matrix that exceed a certain capacity, they begin to experience calcium overload. Calcium overload then triggers a series of pathways that may result in increased ROS production, membrane permeability, and ultimately the membrane permeability transition and cell death pathways. One of the significant consequences of

calcium overload in the matrix is that it impedes the organelle's ability to function as the major ATP generator in the cell and in doing so drastically increases the rate of ROS formation (Kawamata and Manfredi, 2010).

Calcium plays a vital role in the regulation of activity in the mitochondrion. The presence of Ca^{2+} stimulates both the Krebs (TCA) Cycle and oxidative phosphorylation, therefore making the mitochondrion as a whole work faster and consume more O2 from the electron transport chain. This increase in mitochondrial activity through the action of Ca^{2+} enhances ROS output by the organelle. The ROS generation by mitochondria is correlated with metabolic rate, as a faster metabolism results in greater turnover of the oxidative-phosphorylation cycle and therefore greater electron leakage from the respiratory chain. There are several points along the electron transport chain at which electrons may be prematurely passed to oxygen, the final electron acceptor, to form the reactive oxygen species superoxide. One of the main sites of this premature reduction of the oxygen molecule is at Complex I at the beginning of the respiratory chain. Calcium also acts as a stimulator of the enzyme nitric oxide synthase (NOS), resulting in increased generation of NO· (nitric oxide), which acts to inhibit the activity of complex IV of the electron transport chain. Through cessation of the activity of complex IV, nitric oxide causes a backup in the electron transport chain and does not allow for completion of the Q cycle at complex III. Therefore, the main source of superoxide production in mitochondria is formed at the Q_o site during the Q cycle and manifests as ubisemiquinone radical intermediate (QH·). These reactive oxygen species produced through the Q cycle are made facing the intermembrane space side, and therefore can be transported into the cytosol of the neuron through the VDAC channel. In essence, when the distal respiratory chain experiences inhibition, there is a rise in the production of ROS. In addition, increased activity of the respiratory chain also generates a higher concentration of ROS. As calcium plays a role in both the upregulation of

activity of the respiratory chain and the inhibition of its distal proteins, it may be concluded that excessive influx of Ca^{2+} leads to the excessive production of ROS and accumulation in the cytosol of the cell. These ROS generated in the motor neurons can then cross the neural plasma membrane and cause oxidation of proteins on astrocytes adjacent to the neurons, specifically of glutamate transporters (Brookes et al., 2004).

In addition to directly increasing oxidant concentrations, excessive Ca^{2+} also plays a role in triggering apoptotic pathways. Cytochrome c is a small, positively charged hemeprotein that associates with the negatively charged cardiolipin located on the inner mitochondrial membrane. The presence of Ca^{2+} in the mitochondria enhances the dislocation of cytochrome c from cardiolipin, which simultaneously results in a blockage of the respiratory chain at complex III (increasing ROS production from the Q cycle) and releases cytochrome c to initiate the activation cascade of caspases through interaction with Apaf (apoptotic protease activating factors) (Brookes et al., 2004).

IV. ER Dysfunction

When calcium overload of the cell occurs, several mechanisms are in place in an effort to reduce the intracellular concentration, most notably shuttling calcium into the mitochondrial stores. However, at a certain concentration the mitochondria themselves become overloaded with calcium and their capacity to handle the ion is exceeded. Additionally, the excessive stimulation of metabotropic glutamate receptors due to a lack of synaptic glutamate clearing causes the IP3 pathway to become hyperactive. Consequently, the InsP3 ER receptors receive constant stimulation from the second messenger and facilitate an incessant flow of calcium into the cytosol from the ER lumen, adding to the overload of the cell. In this case of cell overload, the cell would rather increase the flux of calcium into the ER thereby increasing ER stores. However, the signaling pathways activated by an excess of synaptic glutamate oppose this action through IP3 activation. In

essence, the excitotoxicity of the neuron results in the following calcium states: depleted ER calcium stores, calcium overload in the cytosol, and calcium overload in the mitochondrion. As previously discussed, calcium overload in the mitochondrion has deleterious effects on the functioning of the ETC and activates apoptotic cascades. However, the depletion of calcium in the ER has also been shown to have harmful effects to the organelle and calcium regulation in the cell as a whole (Kawamata and Manfredi, 2010). Calcium depletion of the endoplasmic reticulum has been shown to signal the ER stress response and has been found to activate the XBP1 transcription factor, in an attempt to compensate for the ER stress by increasing the efficacy of the unfolded protein response (Celli et al., 2010).

One of the central functions of the endoplasmic reticulum is protein synthesis and modification, and included in this is the role of preventing the accumulation of misfolded proteins, a by-product of normal protein synthesis, through the action of chaperone proteins and the ERAD protein degradation pathway. However, in cases where the accumulation of misfolded proteins can not be prevented, the ER experiences a state of stress which can eventually lead to increased ROS production and the initiation of apoptotic pathways in the cell. The presence of mutant protein aggregates is a feature common to the pathologies of many neurodegenerative diseases, and amyotrophic lateral sclerosis is no exception. Mutant forms of the superoxide dismutase protein are prone to forming high-molecular-weight aggregates called inclusion bodies, which are selectively found in the motor neurons of ALS patients. There are many proposed toxicities of the SOD1 mutant aggregates, including clogging of the proteasome and depletion of chaperone proteins, however the exact mechanism of toxicity remains to be elucidated. Since the degradation of both wild-type SOD1 and mutant SOD1 is proteasome-mediated, the presence of excessive aggregates of mutant SOD1 might overwhelm the proteasome and result in “clogging” of the proteasome machinery. This in turn impairs the process of normal protein degradation in

the endoplasmic reticulum and consequently leads to the accumulation and aggregation of other misfolded proteins**.** In fact, it has been found in mice transfected with the G93A mutant form of SOD1, a form of the protein which was found to accumulate to high levels in their cells, that proteasome activity was downregulated in the lumbar spinal cord motor neurons. In addition, the presence of misfolded aggregates of mutant SOD1 would require heavy chaperone protein activity, especially of BiP, in order to correct the errors in folding. This action would reduce the pool of available chaperone proteins for refolding of normal proteins, effectively "tying-up" the pool of chaperones and reducing their activity in the ER. In concordance with this, an overall downregulation of chaperone protein activity has been displayed in spinal cord extracts of mice with the G93A mutation in SOD1, suggesting that the SOD1 mutant form inhibits normal chaperone function. As a result of the interference of mutant SOD1 aggregates with the ERAD pathway and chaperone function, the exacerbation of ER stress may be a central component of the toxic gain of function that is seen in mutant SOD1 (Boillée et al., 2006).

One of the side effects of increased ER stress is the increased formation of ROS by the organelle. This heightened ROS formation compounds the increase in ROS by the calcium-overloaded mitochondrion, and together these two sources of ROS feedback to exacerbate the positive excitotoxic cycle. As mentioned in the discussion of protein folding in the, Ero1 plays a central role in catalyzing the oxidation of thiol groups on amino acid residues to form disulfide bonds and contribute to tertiary protein structure. Ero1 can therefore be considered a chaperone protein in that it aids in facilitating the proper folding of protein products. Since activation of the UPR increases the expression of genes coding for chaperone proteins, it may be concluded that increased UPR in times of ER stress will increase the expression of Ero1. An increase in the pool of Ero1 in the endoplasmic reticulum suggests increased disulfide bond formation by the protein, via transfer of

electrons from protein thiol groups to molecular oxygen, generating the ROS superoxide anion as a byproduct. Consequently, chronic activation of the UPR may lead to increased production of ROS by the ER through sustained expression of the Ero1 chaperone protein (Barber and Shaw, 2010).

V. Mutant VAPB

In some cases of amyotrophic lateral sclerosis, an additional mutation is present, which compromises the functional and physical link that exists between the two calcium regulating organelles, the mitochondrion and ER. Studies have found a dominantly inherited missense mutation in the gene encoding the protein VAPB, which is located on human chromosome 20 (Nishimura et al., 2004). This missense mutation involves a substitution of a serine for the proline at position 56 on the translated polypeptide, a non-synonymous substitution which in turn has consequences for the function of the protein (Boillée et al., 2006). Considering the elucidated role of VAPB in calcium regulation and organelle crosstalk, it is not surprising that nullification of the function of VAPB disrupts the ER-mitochondria associations. The change in its primary structure, and therefore function, impedes VAPB from interacting in its regular fashion with PTPIP51 on the mitochondrial outer membrane. As a consequence, mitochondria-ER associations are not as close and strong, thereby inhibiting the exchange of Ca^{2+} between the two organelles. In cases where the mitochondrion is nearing calcium overload, shuttling calcium from the matrix to the ER lumen would be a potential mechanism for preventing this overload. However, in the case of ALS with the VAPB mutation, ER-mitochondria interactions are disrupted and this association is not made possible, leaving the mitochondrion to attempt to handle the overload of Ca^{2+} on its own. Due to hyperactive stimulation of InsP3 receptors on the ER membrane, Ca^{2+} stores of the ER are simultaneously depleted, a factor which could potentially be mitigated if the ER-mitochondria associations were intact. However, the

absence of normal VAPB functioning impedes the replenishing of ER calcium stores by the matrix (Nishimura et al., 2004).

In addition to its role in promoting transfer between the mitochondrion and ER, VAPB also has been linked to transport, specifically of proteins, between the ER and Golgi. In the case of mutant VAPB, the transport functions have been inhibited due to a loss of proper structure, more specifically the mutation of the MSP (major sperm protein) domain from the proline to serine switch. This domain is vital for protein-protein interactions, and consequently the ER-Golgi vesicular protein transport is compromised, resulting in an accumulation of proteins in the ER, which inevitably form luminal aggregates (Nishimura et al., 2004).

The mutation in VAPB also has implications for the function of the unfolded-protein response in the endoplasmic reticulum. In the lumen of the ER, there exist three major sensors for ER stress which function in detecting the accumulation of unfolded or misfolded protein products. These sensors include PERK (double-stranded RNA-activated protein kinase-like ER kinase), IRE1 (Inositol requiring enzyme), and ATF6 (activating transcription factor 6) (Tadic et al., 2014). The function of these stressors is central to the ER's ability to detect an excess of unfolded protein in the lumen and to initiate the necessary pathways to manage this accumulation. Luminal domains of the stress-sensing ER proteins bind to GRP78, an ER chaperone, under quiescent conditions. However, in times of ER stress, this chaperone dissociates from the three sensors and binds to misfolded/unfolded proteins instead. This dissociation of the GRP78 chaperone from the sensors triggers their activation, allowing these sensors to activate downstream effectors which upregulate the expression of target genes coding for chaperone proteins and ERAD components. In essence, the activation of these sensors is necessary to trigger the synthesis of proteins involved in refolding and degrading misfolded proteins (Langou et al., 2010).

Both native and mutant forms of the VAPB protein associate with the ER stress sensor ATF6 and impede its function, similar to the manner in which the interaction between the chaperone GRP78 and ATF6 inactivates the sensor. Interaction of VAPB with ATF6 reduces the protein's ability to activate the transcription of a transcription factor called XBP1, x-box binding protein 1. This transcription factor functions during periods of ER stress by promoting transcription of factors that facilitate the unfolded protein response (Langou et al., 2010). XBP1 binds to the UPRE, UPR element, on target genes that encode proteins necessary for carrying out the UPR. This binding activates the transcription of the target genes and, in the end, upregulates the synthesis of ER chaperone proteins and ERAD (ER-associated degradation) protein components, which enhances the ER's ability to properly fold misfolded proteins and prevent their accumulation in the lumen (XBP1 Gene, 2017). The ability of the XBP1 transcription factor to bind to its target genes is therefore central to the maintenance of ER homeostasis under periods of stress. Although native VAPB forms function to prevent the activation of XBP1 by ATF6, the mutant VAPB form is a much more potent inhibitor. Consequently, when the VAPB mutant form is present in cases of ALS, the transcription of genes for components of ERAD and chaperone proteins is highly repressed, exacerbating the accumulation of misfolded protein in the ER lumen and heightening ER stress (Langou et al., 2010).

Gene Therapy

I. Introduction to Gene Therapy

In the case of many neurodegenerative diseases such as amyotrophic lateral sclerosis, the roots of the disease pathophysiology are defective protein products due to genetic mutation. Therefore, it may seem logical to conclude that if there were a way to introduce functional genes that would produce functional protein products into diseased

cells, treatment could be achieved. This is the goal and premise of gene therapy. Gene technology has quickly become one of the most intensely developed areas of clinical research as it offers new possibilities for diseases that previously had no prospective treatments. Gene therapy may be applied to cases in which a mutation in a target gene leads to a defective product that is central to the disease pathophysiology. In addition, this type of therapy can also be a potential treatment for non-genetic diseases by introducing a gene that codes for a therapeutic protein that may compensate for the lack of function causing the disease or counteract a toxic gain of function (National Institutes of Health, 2016).

The aim of the gene therapy technique is simple. A functional copy of a gene is delivered into a target tissue or subset of cells to achieve stable expression of the gene and transcription of its therapeutic product. The simplest method of gene delivery into a cell is synthetic transfer through naked injection. This method of gene transfer, although exhibiting low toxicity to recipient tissues, displays high transiency and low efficiency in terms of achieving stable expression of the transgene. Therefore, vectors have been genetically engineered to act as carriers and facilitate the delivery of the therapeutic gene into the target tissues. Several viruses have been targeted as useful vectors for this purpose, as they can easily deliver genes by infecting target cells. The coding regions of the chosen virus vector are removed and substituted with the genetic information of the gene of interest. Once engineered to carry the gene of interest, the vector can be injected into the tissue or given intravenously directly into the specific tissue. Successful integration of the engineered vector into the host is seen through the production of the corresponding functional protein product by the target cell or tissue (National Institutes of Health, 2016).

Viral vectors can be subcategorized into two types, integrating and non-integrating, based on the virus used. Integrating viruses are capable of integrating their viral genome into the genome of the host cell to achieve prolonged expression of the gene of interest in

the target tissue. These types of virus vectors are based on adeno-associated viruses and retroviruses, the latter of which will be the focus of this discussion. On the other hand, non-integrating viruses merely deliver their viral genome into the nucleus of the target cells, without integrating it into the host genome. Viruses of this subtype are based on adenovirus and herpes simplex virus type I (Kootstra and Verma, 2003). The ability of retroviruses to integrate their viral genome into that of the target cell is a trait conducive to long-term expression of the viral genome in the host, and for this reason, retroviruses are often the virus of choice when engineering vectors for therapeutic use (National Institutes of Health, 2016)

II. Retroviruses

Retroviruses are a family of lipid-enveloped integrating viruses that carry linear, single-stranded RNA genomes. They receive their name from the fact that they reverse the normal process of DNA transcription by reverse-transcribing RNA into DNA during their life cycle in a host cell. This family can be further broken down into three classifications: oncoretroviruses, lentiviruses, and spumaviruses. The retrovirus itself is coated with an envelope of lipids and glycoproteins that are encoded by viral accessory genes. This envelope serves the virus by enabling it to fuse with and enter host cells. The interior of the virus contains two copies of single-stranded RNA which house the genetic information, a series of essential and accessory proteins, and a protease enzyme necessary for cleaving viral proteins into their mature forms. The retroviral RNA genome contains three essential genes called *gag, pol*, and *env* with long terminal repeats (LTRs) found on either end of the genome. These long terminal repeats are repetitive DNA sequences that function in facilitating the insertion of the viral genome into that of the host. The *gag* gene codes for the gag protein, which is a precursor protein cleaved by protease to form capsid proteins (Kootstra and Verma, 2003). The capsid refers to a protein shell that encases the RNA

genome of the virus and contains several different types of polypeptides (Alberts et al., 1994). Together this complex of the RNA genome encased by capsid can be referred to as the nucleocapsid. The capsid is then itself enclosed by a lipid bilayer, forming a membrane or envelope of sorts (Alberts et al., 1994). The *pol* gene codes for viral enzymes, notably reverse transcriptase, protease, and integrase. Finally, the *env* gene codes for envelope glycoproteins which are critical for viral entry into the host cell (Kootstra and Verma, 2003). The viral enzymes coded for by the *pol* gene are critical in the successful replication of the virus within the host cell. Reverse transcriptase enables DNA copies of the viral RNA genome to be made upon entry into the host. Integrase then mediates the viral DNA's integration into the host genome located in the nucleus. Finally, protease plays a role in the maturation of virions following replication of the virus in that it processes the protein precursors of *gag* and *pol* into their mature products (Kootstra and Verma, 2003).

Viruses are obligate intracellular parasites, indicating that they can only reproduce inside a host cell and using the host cell's reproductive machinery. In terms of their structure, viruses are merely genetic elements covered with a protective coat, which allows the genome to be transferred from cell to cell (or organism to organism). The infection of a host cell occurs when an envelope protein on the lipid-bilayer that encloses the virus binds its respective receptor on the host cell plasma membrane. The virus then enters the cell via receptor-mediated endocytosis. Once inside the cell, the lipid envelope of the virus fuses with the membrane of the engulfed endosome, effectively ejecting the viral nucleocapsid into the interior of the host cell (Alberts et al., 1994). Following insertion into the interior of the host cell, reverse transcriptase catalyzes the conversion of the single stranded RNA genome to double-stranded DNA which can then be integrated into the host's double-stranded DNA genome in the nucleus (Kootstra and Verma, 2003). Reverse transcriptase is a special type of DNA polymerase which can utilize RNA as its template. The mature

polymerase enzyme is packaged inside the viral nucleocapsid and is coded for by the viral *pol* gene. Upon fusion of the viral envelope with the cell, the viral proteins and genome are released into the cytosol. Reverse transcriptase then begins its work by making a DNA complement of the single-stranded RNA genome, resulting in the formation of a hybrid helix composed of one DNA and one RNA strand. The enzyme then makes another DNA copy to complete the formation of a double helix composed of two strands of DNA. Following reverse transcription, the viral genome is shuttled into the nucleus, and the viral enzyme integrase recognizes the viral DNA molecule and facilitates its integration into the host-cell genome (Alberts et al., 1994). Integration of the viral genome into the host chromosome is a complex process mediated by integrase that includes many steps including removal of dinucleotides from the 3' ends of the viral DNA molecule, cutting of the host cell DNA strand, insertion of the viral DNA strand, and finally ligation (joining) of the two DNA strands (Wanisch and Yáñez-Muñoz, 2009). Following integration, the host RNA polymerase then begins transcription of the integrated viral DNA to mRNA, which can be translated in the cytoplasm to produce the desired protein product encoded by the virus. In the case of gene therapy, this protein product is product of the therapeutic gene introduced into the viral genome. The integrated viral DNA is permanently incorporated into the host chromosome, and may be repeatedly transcribed by RNA polymerase (Buchschacher and Wong-Staal, 2000).

During a typical case of viral infection, the integrated proviral DNA will employ the use of the host cell transcription machinery, notably RNA polymerase in order to create an mRNA copy of the desired viral genes. This mRNA can then be shuttled to the cytoplasm where the host ribosomal machinery will translate it into viral proteins. These viral proteins will assemble with viral RNA in the cytoplasm to begin the formation of the cores of new viral progeny, referred to as virion. As these progeny bud from the plasma membrane of the

host cell, envelope glycoproteins are incorporated into the membrane to form the new viral envelope. Following budding from the host cell, the precursor viral proteins *gag* and *gag-pol* that were translated in the cytoplasm will undergo modification by protease to become mature viral proteins (Buchschacher and Wong-Staal, 2000).

An important safety feature of retroviral vectors is that they are replication defective, meaning that they can infect the host and integrate into its genome, however they are incapable of multiplying for subsequent infection of neighboring cells. By removing accessory viral genes and keeping only the *cis*-acting sequences (those recognized by viral proteins) intact, the viruses ability to replicate and form progeny (virion) can be inhibited (Buchschacher and Wong-Staal, 2000). Rather, these replication defective viruses merely transcribe and translate arge amounts of viral DNA inside the host cell (which in the case of therapeutic vectors consists of a target therapy gene) without forming new virion. In order to create replication defective viruses, the viral genetic components must be segregated onto separate plasmids, a process which is elaborated on in the discussion of lentiviral vector development (Kootstra and Verma, 2003)

Although retroviruses are the virus-of-choice in many cases of gene therapy due to their ability to achieve prolonged expression of the therapeutic gene in the host (due to genetic integration), they are limited by their tropism. Although retroviruses are very effective at infecting dividing cell types, they are often incapable of infecting non-dividing cell types, which poses a problem when the goal is to infect cells of the nervous system. Non-dividing cells may be differentiated from dividing types in that they are arrested in the G_0 phase also known as the "resting phase" of the cell cycle. Many viruses gain access to the genome of the host, which resides inside the double-membraned nucleus, only when the nucler membrane disintegrates at the beginning of the M phase (mitotic phase) of the cell cycle. Since non-dividing cell types do not undergo this phase, their nuclear envelope

remains intact which forms an impenetrable barrier for many viruses as they are too large or do not have the proper surface proteins to facilitate movement of the virus through the nuclear pores (Fassati, 2006).

The nuclear envelope is a double membrane which separates the interior of the nucleus, which houses the genetic information of the cell, from the cytosol. This envelope acts as a form of protection for the genome against harmful substances which may infiltrate the cell. Nuclear pores, channel-like complexes, are embedded in the nuclear membrane and regulate nucleocytoplasmic shuttling of substances. These pores allow for molecules smaller than 9 nm to pass through them, and if molecules are between 9 nm and 39 nm in size, their passage can be facilitated by the nuclear pores under certain conditions. Importins are nuclear import receptors present on the surface of the nuclear envelope which facilitate the passage of substances through nuclear pores, given that conditions are met. These proteins recognize specific domains on the surface of molecules passing through the pores, called nuclear localizing signals. Due to the lack of these proper nuclear localizing signals on most viruses, including most retroviruses, the virus itself cannot achieve penetration of the nucleus and is ineffective in trafficking its genome into the nucleus of the host (Fassati, 2006).

In order to successfully infect non-dividing cells, the virus must have a pre-integration complex that contains the proper nuclear localization signals (NLS) to be recognized by the Importins of the nuclear pore. The pre-integration complex (PIC) refers to the complex of viral proteins necessary for the virus's integration into the nucleus of the host. Having the proper NLS surface molecules contained within the set of the pre-integration complex allows for recognition by the host nuclear envelope and facilitated shuttling of the viral genome into the nucleus (Yamashita and Emerman, 2006).

III. Lentiviruses

As previously mentioned, retroviral vectors are incapable of infecting nondividing cell types, such as in the central nervous system, heart, liver, eye, pancreas, and hematopoietic stem cell (Cockrell and Kafri, 2007). This limitation of retroviral vectors hinders their usefulness in therapeutic applications of gene transfer to a host. However, the lentiviral subtype is capable of infecting nondividing tissues, and thus lentiviral vector systems are being presently developed in gene technology research as they are capable of circumventing the problem faced by common retroviruses. The pre-integration complex of lentiviruses has been shown to contain nuclear localization signals that are necessary for and facilitate integration into the nucleus (Yamashita and Emerman, 2006). For example, in the HIV-1 lentivirus, several NLS have been identified as vital in the recognition of the viral genome by the host nuclear envelope. The matrix protein (MA), integrase protein (IN), and Vpr of HIV-1 have been shown to be essential NLS for the infection of the virus in non-dividing cells. Is has been proposed that Vpr, which is especially integral in nucleocytoplasmic trafficking of the virus, stimulates docking at the nuclear pores in the host and then proceeds to disrupt the envelope in order to disrupt the permeability of the pores and allow for viral passage. Integrase is also tightly associated with the virus during shuttling between the cytoplasm and nucleus, indicating an important role as an NLS. In addition, following importation of the viral genome into the nucleus, integrase becomes vital in integrating the viral DNA into the chromosome of the host (Fassati, 2006).

Lentiviruses fall under the umbrella of retroviruses due to the presence of the RNA genome, which requires the action of the reverse transcriptase enzyme. These viruses can be distinguished from other retroviruses by the presence of three to six additional viral proteins that are encoded in the viral genome. These accessory proteins are essential for the replication of the virus in the host and also promote persistent infection. Two accessory

proteins, called *tat* and *rev,* are found in all lentiviruses, and any additional proteins are dependent on the specific virus (Kootstra and Verma, 2003). *Tat* is vital in regulating the reverse transcription of viral RNA to double-stranded DNA and in regulating virion budding and release. *Rev* is a protein that regulates the expression and synthesis of other viral proteins (Kootstra and Verma, 2003)

Although several different lentiviruses have been developed for vector use, the engineering of lentiviral vectors has been focused on use of Human Immunodeficiency Virus type 1 (HIV-1) as it had been thoroughly studied and exhibits persistence of infection. Aside from the structural proteins and accessory proteins common to all lentiviruses, HIV-1 also encodes the proteins *vif, vpr, nef*, and *vpu*, which regulate latency and virulence (Kootstra and Verma, 2003).

Lentiviral vectors are developed into at least three plasmids, a packaging plasmid, envelope plasmid, and transfer plasmid, which are simultaneously transfected into the target cells in order to increase biosafety (Wanisch and Yáñez-Muñoz, 2009). The packaging plasmid contains the *cis*-acting elements of the viral genome which may be identified as sequences that are non-coding but necessary for the reverse transcription, efficient packaging, import of the viral genome into the nucleus, and integration of the viral vector. These sequences are segregated from those of the transfer plasmid in order to ensure that the vector is replication defective. The transfer plasmid contains *trans*-elements with encode the accessory and enzymatic proteins which are encoded in the viral *gag* and *pol* genes. Finally, these two cassettes are then segregated from the envelope cassette, which encodes the viral envelope components, in order to increase the number of genetic recombination events that would be necessary for the virus to again become replication competent (Cockrell and Kafri, 2007). These plasmids can be modified in many ways to then further increase the biosafety of the viral vector. For example, to increase the safety of the

packaging plasmid, all viral genes that are not essential for the production and integration of the virus are disposed of (such as HIV-1 accessory genes *vif, vpr, vpu,* and *nef).* Deleting these accessory genes from the construct disables the replicative abilities of the vector. The transfer plasmid is often modified to be self-inactivating (SIN) in which there is elimination of viral promoter sequences from the 3' long terminal repeats. As a consequence, viral expression cannot proceed on its own, and an internal promoter is needed for the viral transgene to be expressed. In this way, it can be ensured that the viral genome will only be expressed following integration into the host genome (and via use of the host promoter) (Wanisch and Yáñez-Muñoz, 2009).

IV. Clinical Application

Despite the recent elucidation of several genetic mutations that are linked to fALS, the pathophysiology of ALS is complex, multifactorial, and still a topic of speculation. Although the exact mechanism of disease onset and progression is largely unknown, recent research findings do provide invaluable information regarding several components of the pathophysiology. It seems logical to argue that a disease with such a complex, multifactorial pathophysiology should be targeted with a multifactorial treatment. This was the therapeutic plan that the research group Benkler et al. proposed for the treatment of ALS, as they figured that the limited success of the past 150-years of ALS treatment had been due in part to the targeting of single aspects of the disease, rather than taking an approach that acknowledges the multifactorial nature of the neurodegenerative disease. Benkler et al. chose three aspects of ALS pathophysiology as the focus of their research: reduced metabolism of glutamate in the pre-synaptic neuron, inhibited glutamate uptake by the astrocyte, and neuronal sensitivity to oxidative stress. The research group then chose three target genes upstream of each pathway, which are capable of influencing that entire pathway through upregulation of their expression. In order to increase the metabolism of

glutamate in the synaptic bouton, the researchers selected glutamate-dehydrogenase 2 (GDH2) as the target gene to be introduced overexpressed in the test subjects. As a result, the researchers hoped to reduce the glutamate bioavailability in the nervous system, so as to counteract the excess of glutamate in the synapse to some extent. In order to target the inhibited uptake of glutamate from the synapse, the group assigned overexpression of the EAAT2 (excitatory amino-acid transporter 2) gene, which would in theory reduce excitotoxicity. Finally, in order to target the neuronal and astrocytic damage mediated by oxidative stress, the researchers chose the Nrf2 gene to be overexpressed in the cell and consequently increase the cellular antioxidant responses. As the target cell types in ALS treatment are neurons and glial cells, specifically astrocytes, the research group chose to use lentiviral vectors to effectively introduce therapeutic genes into these non-dividing cell types (Benkler et al., 2016).

In order to carry out their experiments, Benkler et al. employed the use of mice which were engineered to have the mutant G93A SOD1 gene. The research group then constructed four distinct lentiviral vectors, one carrying the EAAT2 gene, one carrying the GDH2 gene, one with the Nrf2 gene, and finally one carrying the GFP (green fluorescent protein) gene which produces a protein product which fluoresces when exposed to light in the blue to ultraviolet range. The LV-GFP protein served as a control vector for the experiment and allowed the researchers to confirm that their lentiviral vectors and procedure for administration resulted in successful integration of the viral genome into that of the host. To evaluate possible synergistic effects of infecting the mice with all three of the experimental therapy genes, and therefore assuming a multifactorial approach, the researchers inject one subset of mice with a mixture of the three lentiviruses containing the genes of interest. In addition, they created an experimental group which contained only the LV-GFP control and other groups which contained only one of the experimental lentiviruses

(LV-GDH2, LV-EAAT2, LV-NRF2). The viral vectors were administered to the mice via intracisternal and intramuscular injection (into the gastrocnemius of the hind limbs). This method of administration enabled the researchers to target the upper motor neurons via injection into the cisterna magna and the lower motor neurons via injection into the gastrocnemius muscle. Beginning 15 days after injection of the lentiviruses, the mice were evaluated biweekly to assess their neurological state and disease progression. This evaluation included a series of tasks, including ladder testing, measurement of the hindlimb reflex, rotarod testing of motor function, and measurement of weight loss (Benkler et al., 2016).

Prior to use of the lentiviral vectors in *in vivo* experiments, the researchers injected the vectors into a series of primary astrocytic cultures to determine the efficacy of their technique in an *in vitro* ALS model. These astrocytes were derived from the G93A SOD1 mice and therefore expressed the same mutation as the *in vivo* experiments. A mere 72 hours after injection of the astrocytes with the LV-GFP vector, the cells displayed high levels of GFP expression, indicating procedural success in transfer and integration of the viral genome. The group found increased mRNA expression levels for each of the experimental lentiviruses as well, and in the case of LV-NRF2, they also detected significantly increased expression of GCLM and NQO1, genes downstream of NRF2 which play a role in the cell's antioxidant response. The most shocking result from the *in vitro* experiments, however, was the fact that the highest degree of neuroprotection against the ALS pathology was seen in astrocytes transduced with all three target genes, EAAT2, GHD2, and Nrf2, simultaneously. In these particular cultures, neuronal survival reached a rate of approximately 90%, suggesting a strong synergistic effect of the three genes and bolstering the multifactorial approach (Benkler et al., 2016).

Following determination of the synergistic effect *in vitro*, the researchers sought to determine whether this effect would be conserved *in vivo* as well. In fact, they found that only the treatment which simultaneously introduced vectors carrying all three genes preserved the neurological score and weight (clinical onset was determined by a 5% loss of body weight) of the SOD1 mice; this suggests that *in vivo* a multifactorial approach must be taken in the treatment of fALS in order to achieve neuroprotection. This study, published in early 2016, has been the first preclinical study to evaluate the use of a multifactorial approach to gene therapy which addresses the excito-oxidative axis in subjects with ALS (Benkler et al., 2016).

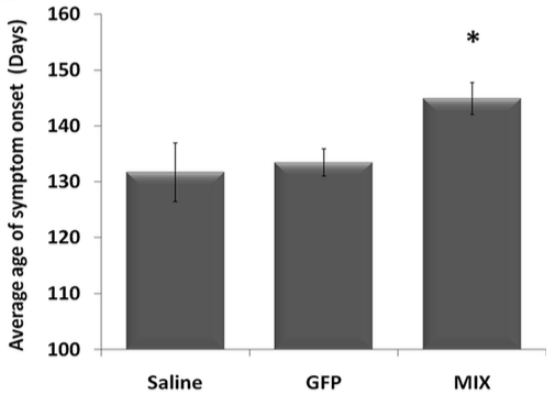

Figure 1
Figure 1 displays the mean age of symptom onset that was found for female mice in the saline (control) group, GFP group, and "MIX" group which received all three lentiviral vectors (Benkler et al., 2016).

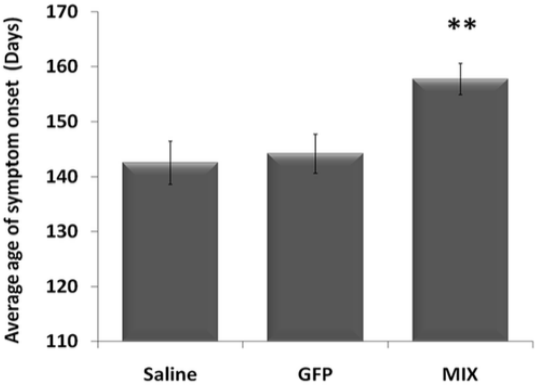

Figure 2

Figure 2 displays the mean age of symptom onset that was found for male mice in the saline (control) group, GFP group, and "MIX" group which received all three lentiviral vectors (Benkler et al., 2016).

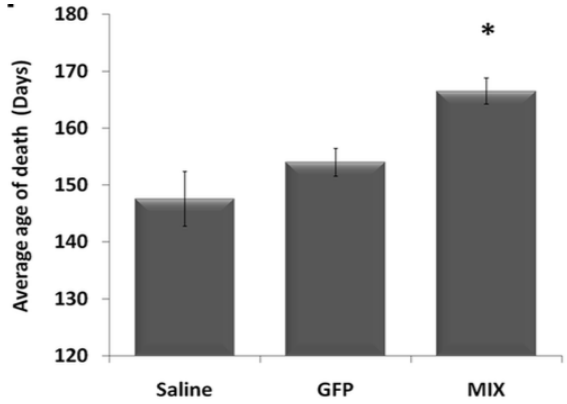

Figure 3

Figure 3 displays the average age of death that was found for female mice in the saline (control) group, GFP group, and "MIX" group which received all three lentiviral vectors (Benkler et al., 2016).

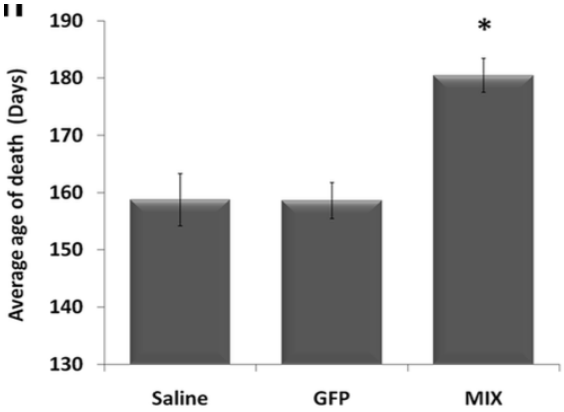

Figure 4

Figure 4 displays the average age of death that was found for male mice in the saline (control) group, GFP group, and "MIX" group which received all three lentiviral vectors (Benkler et al., 2016).

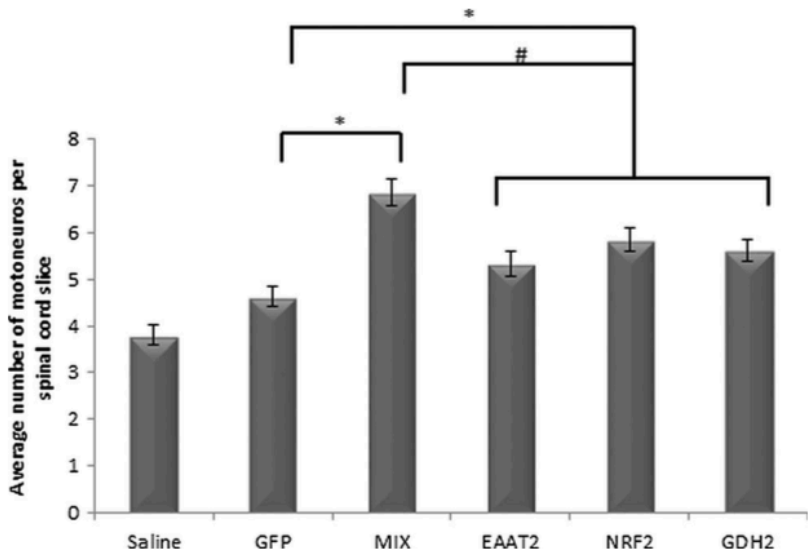

Figure 5

Figure 5 displays the average preservation of motor neurons in mice injected with each of the treatment groups. The mice with the "Mix" treatment containing all three therapeutic viral vectors exhibited a significantly higher preservation of motor neuron viability (Benkler et al., 2016).

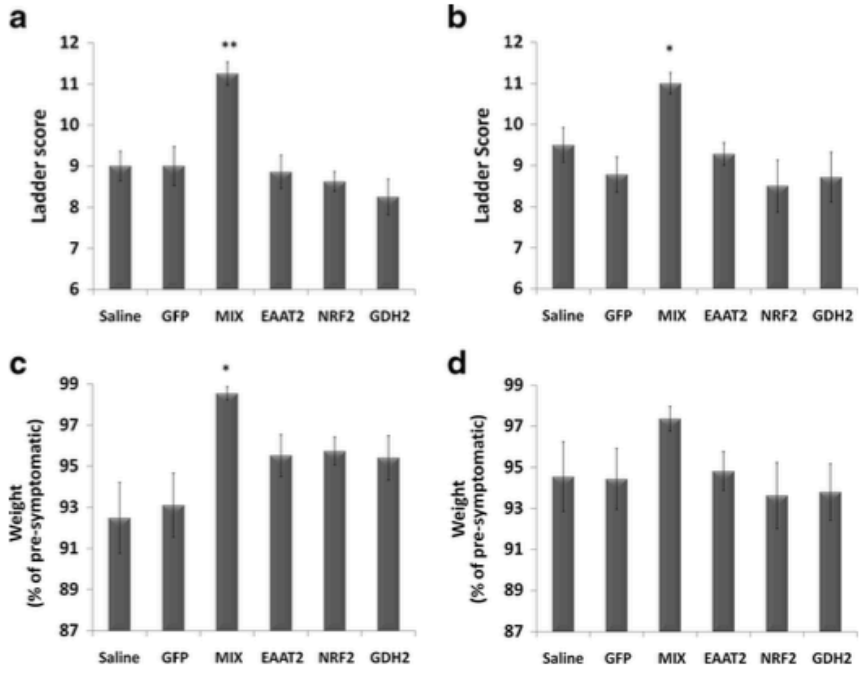

Figure 6

Figure 6 displays the average ladder test score and weight loss for male (**a** and **c**) and female (**b** and **d**) in each of the treatment groups (these categories are evaluation tools for disease progression). The combined treatment group with all three therapeutic genes achieved a combined therapeutic effect that was not observed in the case of single vector administration (Benkler et al.).

Glutamate Dehydrogenase-2

It might seem odd, at first glance, that the researchers decided to target the upstream proteins, GDH2, EAAT2, and Nrf2 in a treatment for ALS expressing the SOD1 mutant form rather than targeting SOD1 itself and introducing a viable gene. However, as mentioned in the earlier discussion of superoxide dismutase 1, the mutant SOD1 form acts by a dominant negative mechanism in which the mutant protein acts antagonistically to the wild-type, inhibiting its proper functioning. Therefore, it would be ineffective to introduce a functional SOD1 gene into the organism as the mutant forms would interfere with the function of this "normal" therapeutic gene. Consequently, the researchers were forced to attack the problem of treating the multi-faceted pathophysiology of ALS by addressing other upstream genes that exert control over pathways involved in the disease progression. The first target gene which Benkler et al. desired to overexpress in the G93A SOD1 mice was glutamate dehydrogenase-2 (GDH2). The reasoning behind targeting this specific gene product was that if the bioavailability of glutamate could be reduced in the nervous system, the neuron would not have as significant a pool of the neurotransmitter to release into the synapse upon stimulation of the cell. This approach attempts to reduce the glutamate that is present in the synapse by downregulating glutamate synthesis at its source, through deamination of the neurotransmitter to α-ketoglutarate by GDH2. Although this pathway does not reduce the toxic levels of glutamate that are present in the synapse in cases of ALS, it does prevent further glutamate release from the pre-synaptic neuron from exacerbating the problem.

Excitatory Amino Acid Transporter-2

The second therapeutic gene that the researchers chose to introduce into the SOD1 mice coded for the glutamate transporter protein EAAT2, excitatory amino acid transporter 2. As previously noted, this transporter isoform accounts for roughly 95% of glutamate

uptake from the extracellular space and into the astrocyte. Heightened levels of reactive oxygen species from the presence of mutant SOD1 have a deleterious effect on the glutamate transporter, placing an inhibition on its function through conformational changes brought about by oxidation of the protein residues. A decreased pool of functioning EAAT2 results in the decreased shuttling of glutamate into the astrocyte, causing a build-up of the neurotransmitter in the synapse as there is no other method of glutamate clearing in the nervous system. This increase in synaptic glutamate then prompts the increased activity of the excitotoxic cascade, in which post-synaptic glutamate receptors are over-stimulated leading to toxicity of the neuron. Engineering a copy of the EAAT2 gene into the viral genome allows for the gene, once integrated into the host DNA, to occupy several loci and therefore experience increased expression in the host cell. Although the production of high levels of functional EAAT2 protein does not combat the production of excessive levels of ROS in the ALS cell, it does increase glutamate clearing in the hopes of reducing the activity of the excitotoxic cascade.

Nrf2

The final target gene which the researchers injected into the SOD1 cell using a lentiviral vector was Nrf2, nuclear factor erythroid 2-related factor 2. To recap on the function of this protein product, Nrf2 is a transcription factor which up-regulates the expression of genes involved in the antioxidant defense via interaction with ARE sequences on the respective genes. Although the expression of Nrf2 up-regulates many protective proteins, such as catalase and thioredoxin, its most significant regulatory effects are on the two enzymes that synthesize glutathione, GCL and GS. By up-regulating expression of these two enzymes in particular, Nrf2 is able to maintain large pools of reduced glutathione in the cell, which are especially useful in conferring protection to the astrocytes (and their EAAT2) from oxidative insult. The goal of the researchers in this pathway was simple: by increasing

the expression of Nrf2 in the host cell, the expression of other antioxidant proteins could be up-regulated, leading to a robust antioxidant response to combat the defects in ROS regulation due to mutant SOD1.

Conclusion

Neurodegenerative diseases such as ALS remain some of the greatest challenges to modern clinical research as many are characterized by a pathophysiology that is largely idiopathic and multifactorial. One of the greatest issues with past attempts to determine a viable treatment for the disease is the focus on a single factor of the disease pathology rather than accounting for the its complexity. The groundbreaking research conducted by Benkler et al. demonstrated the synergistic effect of combining treatments which target different aspects of the pathophysiology, supporting a multifactorial approach as the future of ALS treatment. As previously discussed, gene therapy has quickly become the frontier of ALS research due to its ability to introduce functional genes, which encode functional proteins, into the diseased cell. The recent development of lentiviral vectors in particular has allowed gene therapy to extend to tissues of non-dividing cells, opening up the possibilities for neurodegenerative diseases in which the main targets of gene therapy are non-dividing neuron cells. Despite the displayed success of this approach, gene therapy research is a slowly developing field due to factors related to biosafety that must be considered when introducing a live virus into a host. One significant concern is that of the limited ability to determine the site of viral genome insertion into the host. This limitation does not impede the efficacy of the viral genome in introducing the therapeutic gene into the host, as there is no particular site within the coding region of the host chromosome at which the viral sequence must be inserted in order to be transcribed. However, random and uncontrolled insertion of the viral sequence may pose a danger to the host cell in the case that the insertion disrupts an oncogene. In addition, there is the risk that the introduced

virus may once again become replication competent, meaning that the virus would be able to produce viable virion that could infect neighboring host cells. This concern has been ameliorated to some extent through the segregation of viral components on several cassettes. This segregation, along with the deletion of accessory genes and unnecessary viral components, has improved biosafety by inhibiting the viruses ability to replicate and produce progeny and has increased the number of recombination events necessary for the virus to again become replication competent (Cockrell and Kafri, 2007). The development of lentiviral vectors for the administration of therapeutic genes is constantly advancing, and with these advances several of the biosafety hazards and concerns have been mitigated. The road to gene therapy treatment for ALS is long and will face many challenges in the future, however the successes seen by the Benkler et al. research group give hope for the determination of a multifactorial treatment plan for ALS in the years to come.

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