

ALEXANDER DISEASE AND POTENTIAL TREATMENT
THROUGH THE NRF2-ARE PATHWAY

by

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A THESIS

Presented to the Department of Biology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

May 2014

An Abstract of the Thesis of

Alexandra Joanne Small for the degree of Bachelor of Science
in the Department of Biology to be taken May 2014

Title: Alexander disease and potential treatment through the Nrf2-ARE pathway

Approved: 

Monte Westerfield

Neurological diseases have high prevalence globally and most are untreatable. Neurodegenerative diseases like Alzheimer's disease (AD) and Parkinson's disease (PD) are highly common especially with a growing elderly population. Alexander disease (ALX) is a rare neurodegenerative brain disease that typically affects infants, but has paralleling characteristics with AD, PD and Amyotrophic Lateral Sclerosis (ALS) including oxidative stress and neuronal degeneration. As a consequence ALX is much less researched. Using the large volume of research that has been done on AD, PD and ALS, this paper explores the possibility of using cellular antioxidant pathways, specifically the Nrf2-ARE pathway, to treat ALX. By utilizing the different experimental approaches taken in animal and cellular models of AD, PD, and ALS it is proposed that further research regarding the Nrf2-ARE pathway in ALX models is needed for its use for potential treatment.

Acknowledgements

I would like to thank my advisers, Monte Westerfield, Pat Lombardi and David Frank for helping me through this process. I would especially like to thank Monte Westerfield for listening to my ideas and reading through my drafts, and Angela and Zach for reading so carefully through my drafts. Lastly I would like to thank my wonderful roommates and family for this process would have been a lot more difficult without their support.

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List of Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ALX	Alexander disease
APP	amyloid beta-protein precursor
ARE	antioxidant response element
CDDO-MA	2-cyano-3,12-dioxool-eana-1,9-dien-28-oic methylamide
CDDO-TFEA	2-cyano-3,12-dioxool-eana-1,9-dien-28-oic trifluoroethylamide
CX3CL1	chemokine fractalkine
DALY S	disability-adjusted life years
GCL	glutamine cysteine ligase
GCS	gamma-glutamine cysteine synthase
GFAP	glial fibrillary acidic protein
GSH	glutathione
GSK	glycogen synthase kinase
GSSG	glutathione disulfide
Keap1	Kelch-like ECH associating protein
Maf	musculoaponeutrotic fibrosarcoma oncogene
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NFT	neurofibrillary tangles
Nrf2	nuclear factor E2-related factor 2
PD	Parkinson's disease
ROS	reactive oxygen species
siRNA	short interfering RNA

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Introduction

Most neurological disorders and diseases have no prevention or effective treatments. According to the World Health Organization neurological diseases have the largest effect, through the calculation of disability-adjusted life years (DALYs), on populations worldwide in comparison to other systematic pathologies, such as respiratory and digestive diseases. DALYs can be thought of as loss of healthy life; one DALY is one year of healthy life lost. Neurological diseases account for 6.26 percent of the total DALYs. Its effects include but are not limited to financial and caretaker burdens and loss of productivity. Not only are neurological diseases highly prevalent, but their global burden is expected to quadruple by 2030.

Oxidative stress has been attributed as part of the pathogenesis in many diseases including neurodegenerative diseases. Nuclear factor E2-related factor 2 (Nrf2) is a transcription factor that regulates the antioxidant response element (ARE) pathway and is one of the major cellular defenses against neuronal degeneration and oxidative stress. This paper discusses oxidative stress' effect and potential use of the Nrf2 pathway for treatment of Alexander disease (ALX), a rare genetic brain disease, while drawing on information of diseases with similar oxidative stressors: Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic Lateral Sclerosis (ALS). Even though these diseases exhibit different pathogenesis and symptoms they share common features, including neuronal degeneration, mechanisms of injury and their relationship to astrocytes.

ALX, AD, PD and ALS all have a gradual clinical progression and neuronal degeneration that increase over time. All four of these diseases have aggregations

caused from misfolded proteins (17). Oxidative stress is believed to have a role in these abnormal aggregations and is hypothesized either to contribute to increased aggregation formation or the cause of the aggregation originally. Another significant similarity is the diseases' connection to astrocytes. Astrocytes are glial cells that have recently been proposed to be an important factor in pathogenesis with AD, PD and ALS as well as presumably the main defect in ALX.

The goal of this research is to suggest possible treatments for Alexander disease by looking at other brain diseases for which oxidative stress contributes to the underlying pathogenesis. AD, PD, and ALS have been researched to a greater extent due to the high prevalence of these diseases worldwide. Alexander disease on the other hand is not as extensively researched. Through the examination of scientific literature, potential treatments of Alexander disease is proposed by using the data being obtained about Nrf2 receptors through these well researched diseases.

Background

Oxidative Stress

Oxidative stress is highly prevalent in diseases in which age is a risk factor. According to Harman's free radical theory, reactive oxygen species (ROS) cause damage to the mitochondria and other organelles which cause cells to age (48). Oxidative stress occurs when metabolic by-products are reactive to oxygen free radicals, such as singlet oxygen or oxidative secondary metabolites, which cause a disruption to protein folding and other structural abnormalities (13). These metabolic by-products, called reactive oxidative species (ROS), can result in an imbalance of

redox couples. The human body has evolved mechanisms of combating ROS by the use of redox-dependent molecules and enzymes that buffer the excess free radicals.

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species and the body's ability to neutralize the free radicals. One example of a redox couple that is often seen in brain diseases is reduced and oxidized glutathione (see *Figure 1*). If oxidized glutathione is imbalanced and more abundant than its reduced couple than it cannot oxidize other molecules which could cause the cell to become toxic. This mechanism will be discussed further later in the paper.

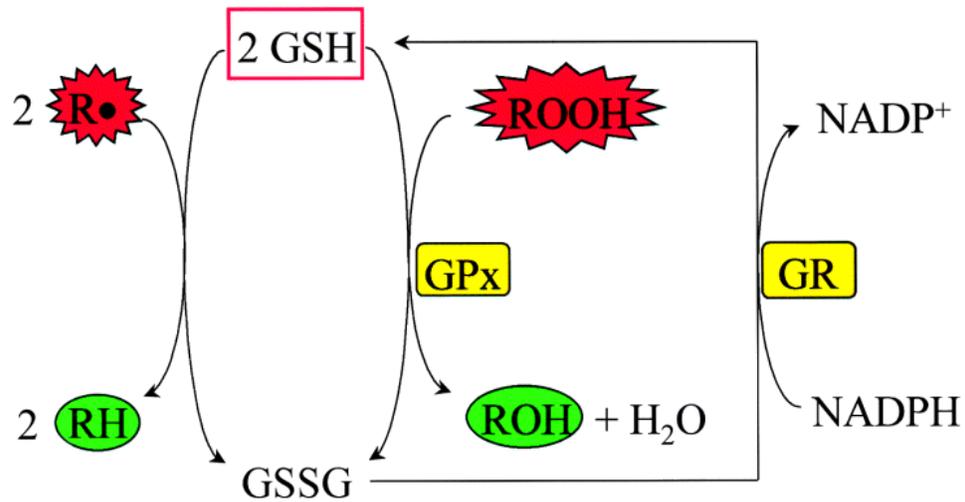


Figure 1: GSH as an antioxidant

GSH reacts with radicals (RH/R) and donates an electron for the reduction of peroxides (ROOH). GSH is regenerated from GSSG through the glutathione reductase (GR) and NADPH (Figure referenced from (50)).

The effect of oxidative stress in the pathogenesis of neurodegenerative diseases is highly pertinent in the discussion of potential treatments. The brain demands a lot of energy in comparison to other organs and has high levels of metabolic activity which results in an increased susceptibility to the formation of ROS. The brain has low levels of antioxidant activity, but is rich in enzymatically active transition metals which can

readily catalyze the formation of radicals. In an aging brain, there is even more risk for the development of ROS due to increasing inflammation and the body's declining ability to degrade ROS (46, 44, 48) .

Astrocytes

Astrocyte dysfunction has been a new focus in neurobiology; its importance is especially seen in the study of neurodegenerative diseases. Astrocytes are specialized glial cells that are responsible for support and structure in neurons. One of the mechanisms of astrocytes neuroprotection is glutathione secretion (29) which is discussed later in the paper. Reactive astrogliosis is an increased amount of astrocytes in response to a disruption of balance in the brain usually triggered when there is neuronal death or injury in the nervous system. Too much astrogliosis can affect brain function and morphology. The role of astrocytes in neurological diseases is increasingly being studied and is becoming recognized as extremely important for brain homeostasis.

The Nrf2 Pathway

Nrf2 is a transcription factor that has been attributed to be a cellular defense against oxidative stress. Exposure to oxidants, electrophiles and other activators stabilize Nrf2 in the cytoplasm allowing Nrf2 to be translocated into the nucleus. Once in the nucleus Nrf2 activates transcription of its target genes through the binding of AREs in its promoter region¹. Through the activation of the Nrf2-ARE pathway hundreds of antioxidant genes are regulated including those involved in glutathione

¹ ARE is a cis-acting regulatory element which is found in the promoter region of many genes. Nrf2 is responsible for activating antioxidant proteins through ARE in response to oxidative stress. At basal levels ARE is still responsible to expression of other genes (59, 82, 83).

synthesis and Phase II detoxification “stress-response” genes (11, 17). In the absence of oxidants and other activators Nrf2 activity is controlled by the cytosolic suppressor protein, Keap1, which promotes the ubiquitination and degradation of Nrf2 before it can be translocated into the nucleus (see *Figure 2*)

Experiments with Nrf2 null mouse models have shown that neurons lacking Nrf2 are more likely to experience oxidative stress and undergo cell death. When overexpressing Nrf2, the antioxidant pathways were ‘saved’ by evidence of increased neuronal resistance to oxidative cell death (11, 47). The possibility of arresting premature oxidative stress using the Nrf2 pathway has been a focus of potential treatment in many diseases. Experimentation on why Nrf2 translocation to the nucleus is affected in some diseases is needed to understand more about the ARE pathway.

Preventative methods and activator drugs for the Nrf2 pathway hold potential treatment options for many diseases including ALX. The overexpression of Nrf2 has limitations thus far. To date, manipulating the Nrf2 pathway has been more successful in prophylaxis than post-pathologic correction. There have been no successful trials of reversing pathological conditions seen in these diseases; data instead show a reduction of progressive neuronal death. Further understanding of oxidative stress and the Nrf2 pathway could lead to more effective methods of protecting neurons and more effective treatments.

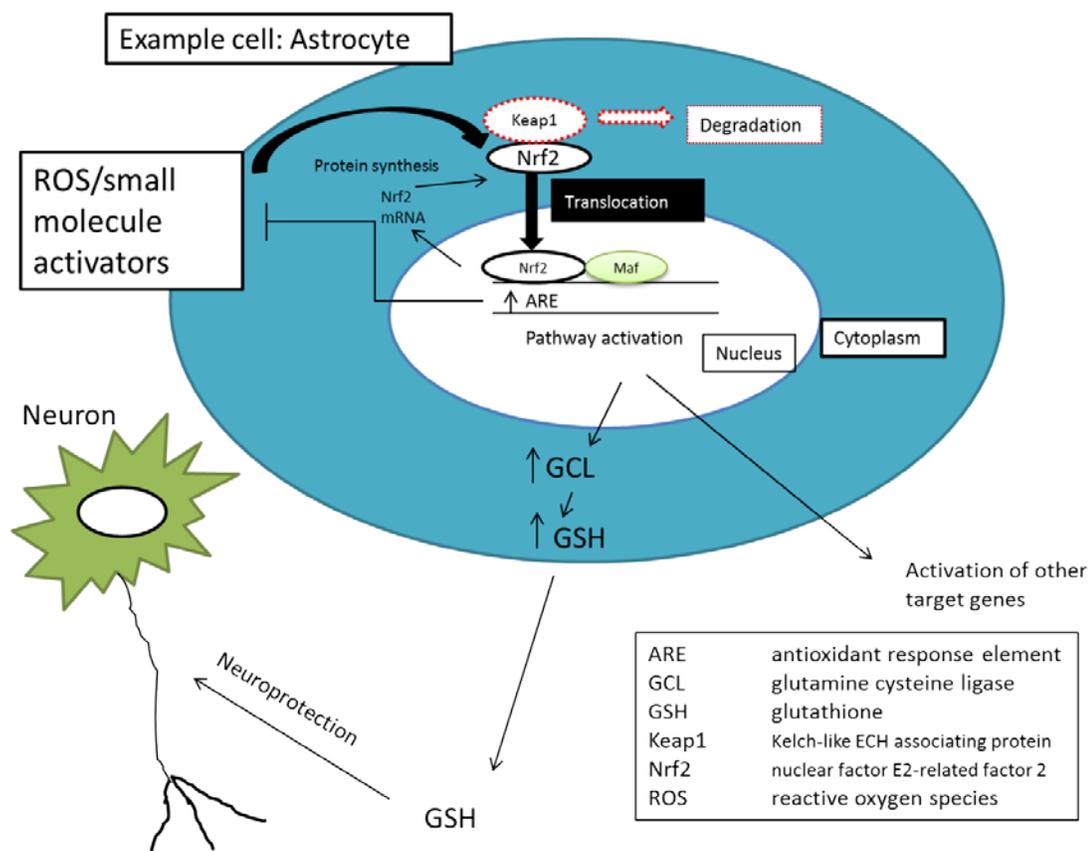


Figure 2: The Nrf2 pathway in astrocytes

If Nrf2 is activated by ROS or small molecule activators Nrf2 will activate the ARE promoter region to allow the expression of antioxidant genes like GSH. Nrf2 binds as a heterodimer with a member of the small Maf (musculoaponeurotic fibrosarcoma oncogene) protein family (46). Based on information from (41, 43, 57, 59, 77)

Glutathione

Glutathione (gamma-glutamyl-cysteinyl-glycine; GSH) is a thiol² that is abundant in cells and primarily is in the cytosol. GSH and glutathione disulfide (GSSG)

² A thiol is a class of organic chemical compounds that are similar to alcohols but instead of an oxygen atom, contain a sulfur atom. The sulfur atom can be oxidized.

in an important redox couple that is important for oxidative defense, nutrient metabolism, and the regulation of cellular events (ie gene expression, cytokine production and immune response, etc.) (see *Figure 1*). Glutathione is synthesized from glutamate, cysteine and glycine which are catalyzed by two enzymes; gamma-glutamylcysteine synthetase and glutathione synthetase (39). GSH is considered an antioxidant due to its ability to be oxidized by electrophilic substances, such as free radicals and ROS, and is also important for activating pathways that are responsible for limiting cell proliferation. Due to its presence in the cytosol GSH can be utilized rapidly if the cell is undergoing oxidative stress, which can deplete GSH levels. Diseased cells, as seen in AD, PD and ALS, will often have depleted levels of GSH in the brain (39).

The basal and inducible levels of the enzymes that synthesize GSH are directly regulated by the Nrf2-ARE pathway (16). Astrocytes and other glial cells have higher levels of GSH than seen in neurons, making astrocytes critical for protection against oxidative stress in neurons (16, 29, 43). Neurons rely on the release of GSH by astrocytes for maintaining sufficient GSH levels. In degenerative diseases like AD, PD, and ALS, neurons need optimal levels of GSH for protection against free radicals released from the activation of microglia and astrocytes (33). Drugs that increase the synthesis of GSH are being targeted to combat oxidative stress in the brain through activation of the Nrf2-ARE pathway for AD, ALS and PD.

Alexander Disease

ALX was first described in 1949 by an Austrian pathologist, Dr. W.S. Alexander, and no more than 550 cases of ALX have been reported (2). ALX is characterized as a leukodystrophy, which is a white matter brain disease. White matter

contains myelinated neuronal axons insulated by myelin which serves to enhance neuronal transmission conduction velocity. Without myelin, these signal transmissions slow down or fail.

The first known case of ALX was found during the autopsy of a developmentally delayed infant. Histological staining revealed protein aggregates found within astrocytes termed Rosenthal fibers. Rosenthal fibers are the foundation of the diagnosis of Alexander disease and can now be visualized by MRI (4).

ALX is more commonly found in infants, but there are some juvenile and adult cases. This disease is fatal and there is no treatment. Doctors can treat the symptoms but there is nothing to be done to stop the progressive destruction of white matter and buildup of Rosenthal fibers. There are two categories of ALX; types I and II. Type I is the early-onset patients that typically show signs of seizures and developmental delays. The average lifespan of a type I patient is fourteen years. Type II is the late-onset patients that typically show signs of neuromuscular degeneration, such as loss of muscular control and coordination, difficulty swallowing and speaking, and excessive vomiting. The average lifespan of a type II patient is 25 years (5).

Rosenthal fibers are composed of different types of proteins including $\alpha\beta$ -crystallin, heat shock protein 27 and glial fibrillary acidic protein (GFAP). GFAP is an astrocytic intermediate filament protein that is important for the structure of astrocytes (17). Ninety-five percent of ALX patients have the disease due to a gain-of-function mutation in the GFAP gene on chromosome 17 that overexpresses GFAP (1, 5). Nearly

all ALX mutations are due to an amino acid substitution and arise *de novo*³, occurring in the parental germ cell as opposed to early in development (5).

In ALX, the toxicity of GFAP is attributed to oxidative stress and protein aggregation induced by a physiological response to mediate the overexpression of GFAP (7). Overexpression of GFAP in astrocytes creates functional abnormalities not only in the astrocyte but also affecting the surrounding neurons resulting in the degeneration of nearby myelinated neurons (4). In an experiment using *Drosophila* as a model organism, Wang *et al.*, 2007 was able to rescue GFAP toxicity with antioxidant vitamin E supporting the hypothesis that oxidative stress has a role in ALX pathogenesis (7).

Alzheimer's disease

Ninety-five percent of dementia cases found in people over the age of sixty-five are classified as late-onset AD. More than five million people in 2013 were living with AD in the US and old age is the number one risk factor. Less than five percent of AD cases are under the age of sixty-five, and are classified as early onset AD. Early onset AD has been attributed to genetics (2, 12). Although there is no cure for Alzheimer's disease, there are treatments that slow down the development of the disease and many experimental treatments are being researched. In 1976 AD was declared a substantial public health challenge by Doctor Robert Katzman. Physical characteristics of AD, beta-amyloid plaques and Tau tangles, were discovered in the 1980s⁴.

³ A *de novo* mutation is a genetic mutation that neither parent possesses, but is due to an error in cell division. This can occur in the parental germ cells or in other cells early in development.

⁴ <http://www.healthline.com/health-slideshow/alzheimers-history#1>. Accessed on 2\5\14.

A common sign of AD is general memory loss. Patients usually visit the doctor once memory loss starts to affect their daily life. Other symptoms that are common include difficulty performing daily tasks, confusion on time or places and problems with speaking or writing. Over time, the symptoms increase to a severe cognitive decline where the patient can no longer control movement and need help with daily activities⁵.

Amyloid beta-protein is the major component of the senile plaques found in AD patients. Amyloid beta-protein is a 39-43 amino acid peptide derived from the cleavage of amyloid beta-protein precursor (APP). Abnormal cleavage of APP is attributed to aggregation of amyloid plaques. Beta-amyloid plaques are often found near degenerated neurons (13). In mouse models, it has been found that elevated beta-amyloid levels cause a loss of synapses and decreased neuronal transmission (14).

Tau protein under normal conditions is a microtubule-associated protein that regulates microtubule assembly and organization. In the brain, Tau is found in axons. It has been correlated with the regulation of axonal transport of organelles. When Tau is not phosphorylated normally, its affinity for microtubules decreases causing an accumulation in neurons which results in neuronal death or degeneration (15). Abnormal Tau accumulation is referred to as neurofibrillary tangles.

Reactive astrogliosis is seen as an important factor in the pathology of AD, associated with amyloid beta plaques. Astrocytes have been implicated in the mediation of amyloid beta peptides. It has been shown in *in vivo* APP/PS1 mouse models that astrocytes under stress will undergo chemotaxis and internalize amyloid

⁵ <http://www.alz.org/>. Accessed on 2/5/14.

beta (16, 17). Nrf2 levels were reduced in AD postmortem brain tissue in hippocampal neurons which was attributed to Nrf2 not translocating to the nucleus as effectively (9, 22). APP/PS1 mouse models showed reduced expression of Nrf2 through lower levels glutamine cysteine ligase (GCL) which is directly synthesized by the Nrf2-ARE pathway (44).

Parkinson's disease

Parkinson's disease (PD) affects about seven to ten million people worldwide, and 60,000 Americans are diagnosed with PD each year with ninety-six percent of the cases over the age of fifty (26). It is the second most common neurodegenerative disorder after AD (19). The cause of PD is still unknown, and old age is the number one risk factor of developing PD- similar to AD. Around twenty percent of the cases have a genetic component, which often leads to development of the disease at an earlier age. There are currently eleven known gene mutations that have been related to PD onset, with the most common one being the alpha-synuclein gene (18). Diagnosis is based on patient symptoms because there is no test to accurately diagnose PD (26). Symptoms include resting tremors, slowed movements, gait impairment and postural instability, with late stages of the disease often causing cognitive decline and behavioral change (19).

Development of the disease is due to dopaminergic cell loss within the substantia nigra. The substantia nigra is an important motor center that is responsible for producing dopamine, a neurotransmitter that functions in motor control and also acts as a hormone. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an inhibitor of complex I of the electron transport chain in mitochondria that shows implications in

dopaminergic pathway damage. Along with dopaminergic cell loss, Lewy bodies, which were first described in 1912 by Freidrich Lewy are another distinguishing hallmark of PD (19). Lewy bodies are alpha-synuclein-immunoreactive aggregations found within neuronal processes (18).

Evidence derived from postmortem analysis of PD brain tissue show increased levels of oxidized protein levels, lipids and nucleic acids implicating oxidative stress involved in the mechanism of injury (34). *In vitro* experiments indicate that accumulation of alpha-synuclein collected within astrocytes often resulted in neuronal death (17). Abnormal levels of Nrf2 have not been seen within PD cells, but Nrf2 null mice in Parkinson models showed increased sensitivity to damage of the dopaminergic pathway, increased protein aggregation and neuronal death (9, 32, 34).

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS), also referred to as Lou Gehrig's disease, is a progressive neurodegenerative disease that affects motor neurons. ALS affects $5/1,000,000$ people worldwide. Patients with the disease typically have asymmetric weakness of the extremities or facial and lingual muscle weakness in the head (difficulty swallowing, talking, etc.). ALS usually does not affect the senses or the ability to think and reason (28). The average age of onset is 56. With a familial history, the average age lowers to 46 years and only ten percent of the cases are linked to genetics. The average lifespan is three years after diagnosis (27). There are no known risk factors for developing ALS except for the small genetically attributed percentage.

There are many gene mutations that are associated with familial and sporadic ALS including SOD1 and C9orf72 (27).

Diagnosing ALS is difficult because there is no test that confirms the disease, and there is still not much known about injury mechanism of motor neurons. Diagnosis is based on symptoms and a series of tests that rule out other diseases. There is no cure for the disease, but patients are treated symptomatically. Currently there is a medication called Riluzole that helps slow the pathogenesis, but only temporarily (28). The disease is classified by the degeneration and loss of motor neurons in the cranial nerves VII (facial), X (vagus), XI (accessory) and the hypoglossal nucleus (IX). Degeneration of motor neurons stops transmission from motor neurons to muscles, causing them to atrophy over time. Eventually, this will lead to respiratory failure due to failure of the diaphragm and chest. Another characteristic of ALS is the loss of axons and decreased myelin as seen in ALX. Histologically Lewy, Bunina⁶ and ubiquitinated bodies are found in the cytoplasm of motor neurons (27). As similar to ALX, PD and AD, ALS is still not fully understood by the medical community and research is being done regarding why and how the disease occurs.

The potential importance of treatments using the Nrf2-ARE pathway is emphasized by the evidence that there is a reduction of Nrf2 mRNA and protein levels and increased levels of Keap1 mRNA in postmortem brain tissue from ALS patients (24, 31, 49). Levels of glutathione are decreased in ALS animal models as well as postmortem brain tissue from ALS patients which could further implicate the Nrf2 pathway in the injury mechanism (29). This notion was consistent with the observation

⁶ Bunina bodies are neuronal inclusions (like Lewy bodies) but with different histological characteristics (27).

that ALS mouse models had neuronal degeneration associated with nearby degenerated astrocytes (17).

Methods

Scientific journal articles that outline the detail and data of experiments regarding the above neurodegenerative disorders and Nrf2 receptors were used to formulate new ways to look at ALX potential treatments. Articles that were used have 1) information on one of the mentioned neurodegenerative diseases, 2) information linking Nrf2 experimental research to the diseases, 3) data from the experiment in the form of tables, graphs and figures and 4) current articles published within the last ten years.

Currently there is very little experimental data on the use of Nrf2 receptors for ALX. When searching for AD, PD and ALS there is at least ten times the amount of information implicating Nrf2 receptors as a future treatment than with ALX. The bulk of these articles may be found on the US National Library of Medicine database through PubMed. Limitations on this method of gathering data include the availability of the articles through PubMed. Some of the article unavailable on PubMed can be found through the University of Oregon's library through the use of interlibrary loans. As a result of relying on the data from other experiments as opposed to collecting my own limited the conclusions that I was able to make on ALX.

Discussion

Nrf2 expression and stabilization is effective for combating oxidative stress and neurodegeneration seen experimentally in AD, PD, and ALS (31). Overexpression can

be achieved through the disruption of the Keap1-Nrf2 interaction or through the genetic overexpression of the Nrf2 pathway. This section will discuss how the Nrf2 pathway has been targeted for therapy in AD, PD, and ALS and how it can be applied to ALX. A summary of the experiments discussed in this paper is outlined in *Table 1*.

Literature Review of ALX and the Nrf2 Pathway

There has been little literature published regarding ALX and the Nrf2 pathway. One of these articles, published in 2012 by Daniels *et al.*, 2007, discusses the reduction of GFAP using overexpression of the Nrf2 transcription factor in R236H GFAP mouse models (8). Overexpression was controlled by a GFAP promoter, which allowed Nrf2 overexpression to be targeted in astrocytes. Five brain regions were examined including the hippocampus, cortex, olfactory bulb, brain stem and cervical spinal cord. Decreased GFAP and Rosenthal fibers due to astrocytic overexpression of Nrf2 were found in all regions tested except the cervical spinal cord when compared to the controls (8).

A finding that was significant is that the overexpression of Nrf2 in astrocytes does not affect GSH levels even though Nrf2 regulates its expression. This is important because GSH depletion is speculated to affect the pathogenesis of other neurodegenerative diseases discussed. There are several potential explanations proposed for this finding: 1) GSH levels do not affect the pathogenesis in ALX, 2) there is enough Nrf2 initially to provide sufficient GSH levels or 3) the beneficial effects of Nrf2 on GFAP seen in this experiment are not mediated through GSH (8). AD, PD and ALS do not have injury mechanisms that affect astrocytes directly as seen in ALX so it is logical to infer that ALX defects do not affect GSH levels.

In another article that was also published in 2012 regarding ALX, the Nrf2 transcription factor was knocked out in GFAP mutant and transgenic mouse models to determine its effect on the pathology of ALX (30). The results demonstrated surprisingly little change. Life span and distribution of Rosenthal fibers were relatively unchanged with the absence of Nrf2. Microglial activation and astrocyte reactivity was significantly reduced in the knockout mice (30). An experiment done by Hubb *et al.*, 2007 investigated how Nrf2 affects the brain (36). The effect of knocking out the Nrf2 pathway was tested on mice. Nrf2- null mice presented widespread astrocytic activation with excess GFAP and myelin degeneration. There was no neuronal cell damage, but formation of spaces in white matter of the brain. It was concluded that Nrf2 has an important role in myelin homeostatic maintenance (36).

Defects in Nrf2 mouse models are synonymous with those seen in ALX models, but with an ALX mutant mouse, a lack of Nrf2 showed no harmful or beneficial effects (30). The lack of response with a loss of Nrf2 suggests that there already is a lack of Nrf2 stabilization in ALX cells, whereas overexpression of Nrf2 showed beneficial effects. These articles are insufficient to support the hypothesis that Nrf2 could provide therapeutic benefits for ALX patients, but both stress that further experimentation may provide insight into potential treatment options.

Disease	Animal Model	Method of Activation	Reference
ALX	R236H GFAP mutant mouse models	Astrocytic Nrf2 overexpression	Daniels, et al., 2012 (8)
AD	SAMP8 (AD) mouse models	Antisense oligonucleotide directed at (GSK)-3-beta	Farr, et al., 2013 (20)
AD	APP/ PSI transgenic mouse models	Tert-butylhydroquinone/ Adenoviral-mediated Nrf2 overexpression	Kanninen, et al., 2008 (44)
AD	APP/ PSI transgenic mouse models	Nrf2 lentiviral vector	Kanninen, et al., 2009 (45)
AD	Nrf2 +/+ and Nrf2 -/- mouse models and APP/ PSI transgenic mouse models	Adeno-associated viral vector of TAU _{p301L} (activation of CX3CL1 to activate Nrf2 pathway)	Lastres-Becker, et al., 2014 (23)
PD	MPTP (PD) mouse models	Astrocytic Nrf2 expression	Chen, et al., 2009 (34)
ALS	GFAP-Nrf2 transgenic mouse models	Astrocytic Nrf2 overexpression	Vargas, et al., 2008 (29)
ALS	C57BL/6 (ALS) mouse models	CDDO-MA	Yang, et al., 2009 (38)
ALS	SOD1 ALS mouse models	Nrf2 lentiviral vector	Nanou, et al., 2013 (49)
----	P1 mouse models (expressing ARE-hPAP reporter)	SiRNA directed against Keap 1	Williamson, et al., 2013 (35)
----	None (cell cultures used)	R-alpha-Lipoic acid, tert-butylhydroquinone, sulforaphane and <i>Polygonum cuspidatum</i> extract	Steele, et al., 2013 (33)

Table 1: A list of experiments utilizing the Nrf2-ARE pathway

Astrocytic Expression of Nrf2

Chen *et al.*, 2009 showed that astrocytic Nrf2 activation, under the control of the GFAP promoter, protects neurons in the MPTP⁷ mouse model of PD (34). MPTP treatment in the mouse model decreased Nrf2-ARE signaling and increased cellular sensitivity to stress. This model also showed that the GFAP-Nrf2 transgene expressed within astrocytes protected against MPTP toxicity and levels of striatal dopamine were increased. Based on these data, astrocytic Nrf2 activation was sufficient to protect against MPTP toxicity (34).

⁷ MPTP is a neurotoxin that causes permanent symptoms of PD in animal models. It is widely used in PD experiments, because it quickly causes PD symptoms (69).

Vargas *et al.*, 2008 found that activating Nrf2 in astrocytes resulted in an up regulation of antioxidant defenses, protection against neighboring neurons in an hSOD1 mouse model of ALS, and a counteraction of neuronal degeneration (29). Nrf2 was overexpressed by using the GFAP promoter in astrocytes, and astrocyte toxicity expressing the hSOD1 mutant was reversed seen through the nearby intact motor neurons. Overexpression of Nrf2 resulted in increased glutathione secretion by astrocytes and increased life span of the mutant mouse model. It was concluded that GSH is a key antioxidant component of the ALS animal model (29).

Up regulation of Glutathione

Steele *et al.*, 2013 compares four known Nrf2 activators to investigate which produced the greatest release of GSH by astrocytes; R-alpha-Lipoic acid, tert-butylhydroquinone, sulforaphane, and *Polygonum cupidatum* extract containing 50% resveratrol (33). It was found that all these activators caused an increase in GSH levels with sulforaphane having the greatest effect by a factor of 2.4 (33). Another part of this experiment was to measure the cleavage of GSH to cysteinylglycine by astrocytes. Sulforaphane again demonstrated the greatest effect increasing cysteinylglycine levels by a factor of 1.7. Cysteinylglycine maintains an optimal level of GSH intracellularly, so it was important to monitor expression of cysteinylglycine as well as GSH (33). Even though GSH expression may not be important in the pathogenesis of ALX, it still provides a means to monitor the expression of Nrf2. This experiment provides evidence that sulforaphane is a potent activator of the Nrf2-ARE pathway.

Delivery of Treatment

Farr, *et al.*, 2014 showed that the use of a phosphorothionated antisense oligonucleotide⁸ directed at GSK-3-beta suppression reduced oxidative stress by increasing Nrf2 transcriptional activity (20). GSK-3-beta, glycogen synthase kinase, is a pleiotropic enzyme⁹ that negatively regulates Nrf2. Its function in the brain is phosphorylation of Tau and when hyperphosphorylated results in NFT generation. SAMP8 AD mouse models were used to test the antisense oligonucleotide through intracerebroventricular¹⁰ delivery. It was found that the antisense not only reduced oxidative damage by upregulation of the Nrf2-ARE pathway, but also improved cognition. The GSK antisense oligonucleotide also demonstrated a capacity to cross the blood brain barrier, indicating that this research may have potential application in peripheral delivery (20).

Williamson *et al.*, 2012 showed evidence that knockdown of Keap1 in astrocytes protects neurons from oxidative stress-induced cell death (35). By using short interfering RNA (siRNA)¹¹ to silence Keap1 in astrocytes, it was noted that a significant increase in levels of genes expressed by the Nrf2-ARE pathway occurred, as well as the upregulation of the Nrf2-ARE pathway in the ARE-hPAP reporter (p1) mouse model. siKeap1 astrocytes also resulted in greater protection of neurons from oxidative stress-induced cell death (35). Results from *in vitro* experiments showed that

⁸ An oligonucleotide is a short nucleic acid polymer that can readily bind complementary nucleotides. Antisense oligonucleotides as described in this paper are single stranded RNA or DNA that are complementary to a targeted sequence to suppress expression of a gene or protein.

⁹ A pleiotropic enzyme means that it is involved in a variety of cellular activities.

¹⁰ Intracerebroventricular delivery is the administration of drugs or chemicals through the ventricular system of the brain.

¹¹ siRNA is a class of small double stranded RNA that can be used to interfere with translated of targeted proteins.

targeting Keap1 using siRNA has therapeutic potential for neurodegenerative disorders, but safe delivery needs to be developed. Intravascular delivery across the blood brain barrier has been attempted and thought possible, but it is still highly experimental (35).

Nanou *et al.*, 2013 used lentiviral delivery¹² to induce overexpression of Nrf2 genes in *in vitro* experiments as well as in ALS mouse models (49). The cellular models indicate delayed disease onset and increased survival time when Nrf2 overexpression was specifically targeted to astrocytes. Cells expressing the virally delivered Nrf2 gene had a significant decrease in oxidative stress levels in comparison to the control. When viral delivery was attempted by injection in muscle tissue of mice *in vivo*, there was little change between the wild type and the ALS model. These results indicate that the administration needs to be more widespread within the central nervous system (49). Other lentiviral deliveries were found successful in AD mouse models by Kanninen *et al.*, 2008, 2009 (44, 45). Lentiviral vector encoding human Nrf2 was protective against amyloid-beta toxicity and was sustained for up to six months. The effects of Nrf2 upregulation were dispersed beyond the direct injection as seen in the ALS model, but muscular injection was not attempted (44, 45).

Lastres-Becker *et al.*, 2014 demonstrated that overexpression of chemokine fractalkine (CX3CL1) with adeno-associated viral vectors¹³ activates the Nrf2-ARE pathway, and attenuates Tau defects induced by microgliosis (23). CX3CL1 is a chemokine that is found in astrocytes and neurons and participates in the prevention of inflammation. In animal models of AD, CX3CL1 is expressed in Tau-injured neurons as

¹² Lentiviruses can deliver viral RNA into the DNA of host cells. One of the unique characteristics of this delivery method is that lentiviruses can infect non-dividing cells.

¹³ Adeno-associated viral vectors are single stranded DNA viruses that insert genetic material into the DNA.

well as other stressed cells. CX3CL1 attenuates microglial activation via Nrf2 activation in order to mediate inflammation. It was hypothesized that this is due to an increased Nrf2 protein stability through suppression of GSK-3-beta by CX3CL1. The results suggest that CX3CL1 as an activator of antioxidant genes by inducing Nrf2 is a viable factor to reduce oxidative stress (23).

Neymotin *et al.*, 2011 examined two triterpenoids¹⁴ that would potentially activate the Nrf2-ARE pathway in cell cultures and mouse models of ALS (31). Treated mouse models were found to have a slowed disease progression using the triterpenoid, CDDO-TFEA (2-cyano-3,12-dioxool-eana-1,9-dien-28-oic-trifluoroethylamide). CDDO-TFEA was able to penetrate the blood-brain-barrier in the mouse models. CDDO-TFEA was delivered through the stomach after being dissolved in oil (31).

Other triterpenoids have been tested for reducing oxidative stress levels, including CDDO-MA (2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid-methylamide) in AD and PD. In a study with AD mouse models there was no direct upregulation of Nrf2, but an upregulation of enzymes that are associated with Nrf2. Decreased signs of microgliosis and oxidative stress were found, as well as reduced levels of amyloid plaques (37). CDDO-MA was also tested with PD mouse models and found to be a potent inducer of Nrf2 effecting GSH oxidation and reduction in a neuronal cell line (38). One benefit of the use of triterpenoids is its ability to safely activate Nrf2 target genes. All experiments done with triterpenoids were delivered orally and found to have high levels in the brain suggesting that it readily crosses the blood-brain barrier.

¹⁴ Triterpenoids come from a class of hydrocarbons and are able to form cyclic structures. It is found in plants and essential oils.

Conclusions

Overexpression of Nrf2 as an activator of the antioxidant pathway is a common theme in the literature regarding its potential use in treatment of neurodegenerative diseases. Abundant Nrf2 may be an effective way to increase expression of antioxidant genes due to titration of Keap1. If Keap1 cannot keep up with degradation of Nrf2, than more Nrf2 will be able to translocate to the nucleus, which allows the synthesis of antioxidant genes (see *Figure 2*). There is no evidence that the Nrf2 pathway is inhibited in ALX, but overexpression of Nrf2 may allow constitutive expression of antioxidant genes and provide protection against oxidative stress.

One thing that is unique to experimentation with Nrf2 in neurodegenerative diseases is that expression is specifically altered in astrocytes. Because GFAP defects in ALX originate within astrocytes, overexpression of Nrf2 in astrocytes will be an important factor to study. Many compounds have been identified that induce Nrf2 transcription in astrocytes, but delivery is a common limitation in treatment application. Many activators that are highly effective have no known mechanism for safe delivery. Compounds like triterpenoids, CX3CL1 and sulforaphane are strong candidates to test in ALX animal models, as well as the use of antisense oligonucleotides and siRNA.

The potential to use the Nrf2-ARE pathway to treat neurodegenerative diseases including ALX is important. Because ALX defects largely affect myelin and astrocytes, the use of increased expression of Nrf2 is viable when there is evidence that a lack of Nrf2 is detrimental to myelin and astrocyte function. Protein aggregations similar to those seen in ALX have been decreased in AD, PD, and ALS by modulating Nrf2 which suggests that similar techniques should be tested on ALX mouse models. Daniels

et al., 2012 provides evidence that overexpression of Nrf2 is successful in ALX, but further experimentation is needed. The potential for astrocytic Nrf2 overexpression is viable and should be further tested using different methodologies of delivery that would be safe for future clinical use.

Gene therapy is a growing field in treatment and prevention in many diseases including genetic diseases and immune deficiencies. One limitation is the difficulty to find a way for the transgene to be expressed in the target body system using a safe method of delivery. Gene therapy through the Nrf2-ARE pathway holds great potential in neurodegenerative diseases, including ALX. Not only may gene therapy be successful in treating ALX, but through ALX's unique connection to astrocytes, it may provide a further understanding of the Nrf2-ARE pathway in the brain and specifically in astrocytes.

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