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Role of Pro-Apoptotic Bcl2-Homology-3 Domain (BH3)-Only Proteins in the Mutant SOD1 Mouse Model of ALS

Anna George Andrianakos
University of Denver

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ROLE OF PRO-APOPTOTIC BCL2-HOMOLOGY-3 DOMAIN (BH3)-ONLY PROTEINS IN THE MUTANT SOD1 MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS (ALS)

A Thesis
Presented To
The Faculty of Natural Sciences and Mathematics
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In Partial Fulfillment
Of the Requirements for the Degree
Masters of Science

by
Anna G. Andrianakos
June 2009
Advisor: Daniel A. Linseman, Ph.D.
Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease involving rapid degeneration of motor neurons in the spinal cord and retraction of their axonal projections to the neuromuscular junctions. Several known mutations linked to some familial cases of ALS have been linked to mutations in Cu/Zn superoxide dismutase (SOD1), resulting in mitochondrial oxidative stress and intrinsic apoptosis. Transgenic mice expressing a G93A mutant of SOD1 provide an in vivo model to investigate motor neuron death during disease progression. The principal regulators of intrinsic apoptosis are the Bcl-2 family proteins. While some members of this family are pro-survival, the Bcl-2 homology-3 domain (BH3)-only proteins are pro-apoptotic. Since cooperation of various BH3-only proteins is often necessary to induce apoptosis, we hypothesized that multiple BH3-only proteins are induced in spinal motor neurons during the progression of ALS. Furthermore, we postulated that these pro-apoptotic proteins act in a coordinated manner to cause motor neuron death. We utilized laser capture microdissection (LCM) to collect highly enriched populations of spinal motor neurons from wild type vs. SOD1 mutant mice. RNA was then isolated from the captured motor neurons and used for quantitative real time PCR analysis of BH3-only transcript expression. We did not detect any significant differences in the expression of BH3-only transcripts between end stage SOD1 mutant mice and age-matched wild type animals. In contrast, immunohistochemical staining for the BH3-only proteins, Bik, Bad, BNip3, Bid, Noxa,
Puma, and Hrk/Dp5, demonstrated selective staining of Hrk/Dp5, Bnip3, and Bid in astrocytes of lumbar spinal cord from end stage mutant SOD1 mice. Hrk/Dp5, Bnip3, and Bid were not observed in astrocytes of wild type mouse spinal cords. These novel findings indicate a potentially important role for astrocytes expressing Hrk/Dp5, Bnip3, and Bid in ALS disease progression.
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CHAPTER ONE: INTRODUCTION

1.1 Spinal Cord and Motor Neurons

The central nervous system (CNS) consists of the brain and the spinal column. The spinal column consists of the spinal cord and vertebral bones. The five regions of the spinal column are the cervical, thoracic, lumbar, sacral, and coccygeal. The nervous tissue is made up of two main cell types: neurons, which transmit signals either from the environment to the brain or from the brain to the environment, and glial cells, which are non-conducting cells that provide support and protection for neurons in the spinal cord. When looking at cross sections of the spinal cord, two main regions are apparent: the more central grey matter, and the peripheral white matter. The grey matter contains cell bodies and dendrites, while the white matter contains bundles of inter-neuronal axons, ascending sensory neurons, and descending motor neurons.

All types of neurons are composed of three essential parts: dendrites, cell body, and axon. Dendrites receive input from other neurons. The signal is then integrated in the cell body and if it reaches a threshold an action potential is generated and propagated down the axon. Motor neurons, also called efferent neurons, function to transmit signals from the spinal cord to muscles by projecting axons out of the CNS to directly or indirectly activate muscle contraction. Thus, death of spinal and cranial motor neurons, as is observed in Amyotrophic Lateral Sclerosis (ALS), results in a loss of muscle strength and eventual paralysis.
1.2 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease causing a muscle weakness and eventual paralysis, ultimately leading to death due to heart and respiratory failure. The first person to describe the progressive motor neuron disease, ALS, was Jean-Martin Charcot in 1869; hence, the disease was originally called Charcot’s Sclerosis (Cleveland 1999). In the United States, ALS is most commonly known as Lou Gehrig’s disease, named after the famous baseball player who died from ALS in 1941 at the young age of 38. ALS has a midlife occurrence, 45-60 years of age, with death typically occurring within two to five years of diagnosis. As of September 2008, there were 20,000 known cases of ALS in the U.S., and it is estimated that 5,000 people are diagnosed with ALS each year (National Institute of Neurological Disorders and Stroke 2008). Diagnosis of ALS in a clinical setting is extremely complicated because the symptoms with which a patient presents, such as muscle fatigue or spasticity, are representative of numerous neuromuscular problems. Further complicating diagnosis of ALS is that ALS does not discriminate, affecting people of all races, ethnic backgrounds, and walks of life.

As first described by Charcot, ALS is defined by the degeneration of the axon portion of the upper motor neurons in the brain; these axons descend to connect onto the lower motor neurons of the spinal cord, and thus the degeneration disrupts normal brain and spinal cord signaling. The devastating pathology underlying ALS includes progressive muscle weakness, atrophy, and spasticity (Boillee et al. 2006). All of these symptoms are characteristic of rapid degeneration of lower motor neurons from the ventral horn of the spinal cord and retraction of their axonal projections that innervate the
neuromuscular junctions. Preceding the diagnosis of ALS, most patients will go to the doctor with complaints of twitching, cramping, stiffness of the muscles, weakness in one or more of their extremities, slurred/nasal speech, or difficulty in chewing or swallowing. However, the chief complaint that the patient presents is largely dependent on the specific muscles in the body that are affected first by this degenerative disease. Unfortunately, regardless of where the symptoms may start, it is only a matter of time before weakness and atrophy spread to other areas of the body. Interestingly, more recent studies on ALS have suggested that patients diagnosed with the disease may also have alterations in cognitive functions such as problems with memory formation/recall and decision making, as well as depression (National Institute of Neurological Disorders and Stroke 2008).

As many as 90% of all reported ALS cases are sporadic, meaning patients had no family history of ALS, while the other 10% of cases are familial. These familial forms of ALS are typically inherited in an autosomal dominant manner; the mutated gene only needs to be passed on from one of the two parents. While some forms of adult onset ALS are inherited primarily through autosomal dominant mutations in superoxide dismutase (SOD1), juvenile onset ALS, also known as ALS2, is inherited in an autosomal recessive manner. Juvenile onset ALS is less prominent as it requires expression of two mutant alleles of the protein alsin, a guanine nucleotide exchange factor for the small GTPases Rac1 and Rab 5 (Otomo et al. 2003; Topp et al. 2004). Despite their differences in origin, sporadic and familial cases of ALS are clinically indistinguishable (Radunovic and Leigh 1996). Both familial and sporadic ALS induce similar pathological hallmarks including substantial loss of motor neurons in the spinal ventral horns and eventual degeneration of brainstem motor nuclei and motor cortex (Ince 1998).
The exact cause of selective motor neuron death is unknown, but experimental evidence implicates many potential factors such as oxidative damage, excitotoxicity, apoptosis, aberrant protein processing/degradation, and mitochondrial dysfunction (Cleveland and Rothstein 2001; Bruijn et al. 2004).

1.3 Copper Zinc Superoxide Dismutase (SOD1)

In 1993, mutations in an enzyme called copper/zinc superoxide dismutase (SOD1) were found to be linked to approximately 20% of familial cases or approximately 2% of all ALS cases (ALS Association Fighting Lou Gehrig’s Disease 2008). In fact, SOD1 was the first ALS-associated gene to be identified and it is on human chromosome 21 (Rosen et al. 1993). SOD1 is a 153 amino acid protein with a molecular mass of 18.5 kD (Sherman et al. 1983). Within SOD1’s active site is a highly reactive copper ion that is required for enzymatic activity; it is held in place by disulfide bonds and one zinc ion that serves to stabilize protein structure. SOD1 exists primarily in the cytoplasm of cells as a homodimer, but 1-2% of SOD1 is found in the inter-membrane space of mitochondria (Mattiazzi et al. 2002). The normal function of SOD1 is to detoxify superoxide to produce water, oxygen, and hydrogen peroxide; the latter is removed by catalase and glutathione peroxidase (Radunovic and Leigh 1996). Therefore, the primary function of SOD1 is to keep cells safe from reactive oxygen species that could cause massive cell damage if not converted to non-reactive forms.

The majority of mutations in SOD1 known to cause ALS are missense mutations, but there is also a small percentage of mutations in SOD1 that are insertion or deletion mutations that can result in prematurely terminated SOD1 polypeptides. The first SOD1 missense mutation was found in 1993, but now there are 114 known mutations in SOD1.
that are found throughout all 5 exons that encode for SOD1 (Figure 1: Cleveland and Rothstein 2001; Rosen et al. 1993; Boillee et al. 2006). Even though the expression of mutant SOD1 in cells is not necessary to cause an ALS phenotype, numerous studies have shown that mutant SOD1 expression is linked to about 20% of familial ALS cases. Phenotypic variation seen in cases of ALS can only partially be attributed to the numerous different mutations in mutant SOD1. As seen from an analysis of 451 blood samples taken from Scandinavian ALS patients with the same SOD1 genotype, variations occurred in age of onset, degree of disease severity, and rate of disease progression within these patients with the same genotype. However, progression of motor signs and symptoms of ALS appear to follow the same disease pattern regardless of the type of mutation in SOD1 that the patient genotypically expresses (Anderson et al. 1997).

Figure 1: ALS Causing Mutations in Superoxide Dismutase (SOD1). Mutations that cause ALS are shown above to be scattered throughout the 3D structure of SOD1. These mutations are located at turns of β-barrels, within β-sheets, within the active site, at copper-coordinating residues within the active site, and at the dimer interface. Mutations in SOD1 are shown as space filling models of amino acid side chains and/or as yellow regions in the peptide chain. (Cleveland and Rothstein, 2001)

There have been many theories proposed as to how mutant SOD1 can lead to ALS. Mutant SOD1 has been found to be a gain of function mutation, which allows for
deleterious reactions causing oxidative stress. Mutant SOD1 was also found to cause abnormal accumulation of non-neuronal cells such as astrocytes in spinal cords of ALS transgenic animals. Mutants of SOD1 have also been shown to be misfolded and form aggregates that can affect common cell functions such as disrupting proteasome machinery, decreasing chaperone activity, and causing dysregulation of organelle function, specifically, the mitochondria.

1.4 Mouse Model of ALS Used in This Study

Transgenic mouse models of ALS have been created for at least nine different forms of mutant human SOD1 (Kunst 2004). Transgenic animals expressing different mutations in human SOD1 vary in age of onset and disease severity in the same way as in human patients with ALS. The transgenic mice that were used for this thesis project express G93A mutant human SOD1 on a FVB background. Generally, transgenic animals are created by taking a mature female mouse and removing fertilized eggs from her uterus. The pronuclei in these eggs are then injected with the gene of interest with an injection needle, in this case G93A mutant hSOD1 was injected. The eggs carrying the mutant SOD1 are then reinserted into a pseudo pregnant FVB female’s uterus for development into offspring (Figure 2, Pierce 2005). The G93A mutation can lead to misfolding of the SOD1 protein and subsequent aggregate formation.
In mouse models of ALS, the mutant SOD1 allele is expressed in the presence of two copies of the mouse wild type SOD1 allele, such that there is either a normal SOD1 expression or increase in SOD1 expression. This indicates that one copy of the SOD1 alleles must be mutant in order to cause symptoms of ALS (Dal Canto and Gurney 1995). G93A transgenic animals have disease onset around 90 days of age and become terminally ill at around 120 days of age. Characteristics of the G93A mutant SOD1 transgenic animal include hind limb paralysis from muscle atrophy and formation of a destabilizing hump. G93A transgenic animals also suffer severe loss of weight and development of crusty eyes and matted fur due to the animal’s inability to feed or groom itself any longer (Figure 3).
Figure 3: G93A Mutant SOD1 Transgenic Animal vs. Age Matched Wild Type FVB Animal. Panel A: Image of an adult wild type animal. It is healthy and has normal muscle function. Panel B: G93A mutant SOD1 Transgenic Animal. This animal has several phenotypical characteristics that are commonly found in end stage cases of ALS, including hind limb paralysis, formation of a destabilizing hump, and crusty eyes and matted fur due to the animal’s inability to groom itself any longer.

Overexpressing wild type SOD1 in animals is not sufficient enough to develop the ALS phenotype (Dal Canto and Gurney 1995). However, in G93A mutant SOD1 mice, wild type SOD1 overexpression can accelerate disease onset and progression of lower motor neuron degeneration (Jaarsma et al. 2000; Tu et al. 1996). The higher the level of G93A mutant SOD1 protein expressed in a given mouse, then the younger the age of onset of disease becomes, as well as more neuronal populations become affected (Jaarsma et al. 2000). Lack of SOD1 was also found to be insufficient to cause ALS because there is an absence of motor neuron dysfunction in mice null for the SOD1 gene, which further suggests that mutant SOD1 has a gain of function effect in ALS disease progression (Reaume et al. 1996).

1.5 Misfolded Mutant SOD1 and Oxidative Stress

Through mutation of wild type SOD1, misfolding occurs and exposes the active site making it more accessible, leading to inefficient incorporation of copper by the chaperone, copper chaperone for superoxide dismutase (CCS) (Kato et al. 2001;
Furukawa et al. 2004). Alternatively, decreased shielding of the copper ion permits various unfavorable reactions that oxidize endogenous antioxidants, such as ascorbate, urate, glutathione, and cysteine, as well as transfer of electrons to oxygen producing superoxides (Estevez et al. 1998, 1999). This unique toxic gain of function mutation in SOD1 is lethal to motor neurons. Other theories postulate that the toxicity of mutant SOD1 results from depletion of zinc, allowing formation of the much stronger toxic oxidant peroxynitrite. Zinc deficient SOD1 also generates superoxides through oxidizing endogenous antioxidants. Due to the fact that nitric oxide equally competes with SOD1 for superoxides, peroxinitrite is formed from nitric oxide and superoxide through diffusion-limited reactions. Loss of zinc in either wild type or mutant SOD1 was sufficient to cause apoptosis in cultured motor neurons, which could be reversed when zinc was reintroduced (Estevez et al. 1999).

Both wild type and mutant SOD1 bind anti-apoptotic protein Bcl-2 in human and mouse spinal cords, providing a direct link between SOD1 and the apoptotic pathway. Bcl-2 was also found to be bound within mutant SOD1 aggregates in spinal cord mitochondria. This entrapment of Bcl-2 suggests that mutant SOD1 may serve to deplete motor neurons of this anti-apoptotic protein (Pasinelli et al. 2004).

1.6 Misfolded Mutant SOD1 and Aggregate Formation

A common theme in neurological disease is the presence of misfolded proteins grouping together to form aggregates. The formation of protein aggregates has been linked to several neurological diseases and disorders. Aggregates of α-synuclein are found in substantia nigra, cerebral cortical and other neurons of Parkinson’s patients. In Alzheimer’s disease there are two types of protein aggregates found in the basal forebrain
and hippocampus. The extracellular Aβ peptide formed through proteolytic processing of amyloid precursor protein (APP), and intracellular neurofibrillary tangles containing Tau protein. Huntington’s disease aggregates containing the mutant Huntingtin protein are found in striatum and interneurons (Ross and Poirier 2004). More recently, ALS has been added to this list. Mutant SOD1 aggregates are found in both familial and sporadic cases of ALS in humans and in transgenic mutant SOD1 animals (Bruijn et al. 1998; Watanabe et al. 2001). Mutant SOD1 aggregate accumulation was found to be specific to motor neurons in the ventral horn of the spinal cord. Aggregates were not found to accumulate near dorsal root ganglion or hippocampus neurons in vitro, even when they expressed the same levels of mutant SOD1 (Durham et al. 1997). This was also found to be true for in vivo cases of human and mouse ALS, where aggregates were found to be restricted to mitochondria of spinal cord tissues (Pasinelli et al. 2004). Mutant SOD1 aggregates have been hypothesized to be the result of defects in chaperone machinery involved in protein folding.

Chaperone machinery, specifically heat shock proteins (HSPs), is responsible for protein folding. Decreased activity of chaperone machinery could be a key in mutant SOD1 misfolding and aggregation found in ALS cases. In transgenic mutant SOD1 ALS animals that were pre-symptomatic, there was a decrease in HSP activity that persisted until the end stages of disease (Tummala et al. 2005). When HSPs were reintroduced into cultures of primary motor neurons expressing mutant SOD1, there was a decrease in aggregate formation, reduced impairment of axonal outgrowth, and diminished apoptosis (Bruening et al. 1999). Increasing expression of HSP70 in vivo through injection into
mutant SOD1 mice resulted in an increase in animal survival, delays in symptom onset, and prolonged motor neuron survival (Gifondorwa et al. 2007). Therefore misfolding of mutant SOD1 plays a significant role in ALS disease initiation and progression. This finding has led to the investigation of different mechanisms by which mutant SOD1 could lead to motor neuron death in ALS. Thus, it is not surprising that mutant SOD1 has also been hypothesized to interfere with proteasome machinery.

The function of the proteasome is to degrade proteins for protein turnover in the cell or to degrade proteins that are defective through proteolysis, a process by which proteins are broken down into single amino acids. In order for the proteasome to recognize a protein that needs to be degraded, the protein must be tagged with ubiquitin. This occurs through the activity of specific ubiquitin ligases, such as E3 ubiquitin ligase, which connects ubiquitin to lysine residues on the target protein forming an isopeptide bond (Ardley and Robinson 2005). Without proper function of the proteasome, misfolded and/or damaged proteins can accumulate, like misfolded SOD1. SOD1 aggregates have been found to also contain ubiquitin, suggesting that mutant SOD1 aggregates are not being turned over by the proteasome correctly. Conversely, it has been found that in transgenic mice expressing mutant G93A hSOD1, there is mutant hSOD1 aggregate formation and downregulation of proteasome activity long before symptoms of ALS develop (Kabashi et al. 2004).

1.7 Mitochondria and Mutant SOD1 aggregates

Another major finding was the discovery that large membrane bound vacuoles formed in the mitochondria of motor neurons in early stage ALS human patients and G93A mutant SOD1 transgenic animals (Higgins et al. 2003; Kong and Xu 1998).
Moreover, vacuole formation is found in transgenic animals prior to any development of signs or symptoms of ALS (Higgins et al. 2003). Even more surprising was the discovery that mutant SOD1 is selectively recruited to the cytoplasmic face of brain mitochondria in ALS transgenic animals (Vijayvergiya et al. 2000). Furthermore, mutant SOD1 is found to be imported into the mitochondria while endogenous wild type SOD1 is excluded (Liu et al. 2004). Uptake of mutant SOD1 into the mitochondria has been hypothesized to cause direct damage leading to cell death, because vacuoles formed in the mitochondria are derived from expansions of outer mitochondrial membrane and extensions of the inner mitochondrial matrix space (Jaarsma et al. 2000; Higgins et al. 2003). Yet, formation of vacuoles derived from mitochondrial degeneration is also found in mice accumulating very high levels of human wild type SOD1 protein, which do not develop disease, and are not formed in animals that develop disease due to inactive forms of mutant dismutase (Jaarsma et al. 2000; Jonsson et al. 2006).

1.8 Mitochondrial Oxidative Stress and Dysfunction in ALS

Oxidative stress is a term used to describe the oxidative and/or nitrosative damage to cellular proteins, lipids, and DNA by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Linseman 2008). Dysfunction of mitochondria leads to decreased ATP production, impaired \(\text{Ca}^{2+}\) buffering, and an increase in ROS generation. Elevated levels of ROS, especially in the presence of calcium, induce mitochondrial permeability transition (MPT), as well as uncoupling oxidative phosphorylation, all of which contribute to cytotoxicity though apoptosis (Beal 2005). Oxidative stress may play a significant role in neurodegeneration by modifying DNA or lipids and proteins involved in important process for cellular function and survival such as signal transduction,
mitochondrial respiration, receptor kinetics, and protein degeneration (Simpson et al. 2003). Normally ROS generated by mitochondria are eliminated by glutathione peroxidase, yet significant amounts can diffuse out of the mitochondria and into the cytoplasm (Keating 2008). Neurons rely heavily on mitochondria for their high energy and activity needs. Therefore, mitochondria are concentrated in synapses and significant alterations to location, number, function, or morphology of synaptic mitochondria could be detrimental to synaptic transmission (Keating 2008).

Mitochondrial oxidative stress and dysfunction have been implicated in many neurological diseases including ALS. Expression of antioxidant proteins Bcl-2, SOD1, and catalase are lower in lymphocytes of sporadic ALS patients compared to wild type age and sex matched controls. However, levels of antioxidant Bcl-2 and SOD1 proteins could be reduced by long time exposure to superoxides in control animals (Cova et al. 2006). The same decrease of SOD1 and Bcl-2 in sporadic ALS lymphocytes was found to occur in lymphocytes of familial ALS, indicating that antioxidant pathways are downregulated in lymphocytes of many ALS patients (Ferrante et al. 1997). Quantification of carbonyl groups as a marker of oxidative stress and examination of electron transport chain (ETC) acitivity (complexes I, II-III, and IV) in postmortem brain tissue showed that the activity of the ETC increased 55% in complex I of familial ALS patients and a similar elevation of carbonyl groups was observed in sporadic ALS patients (Bowling et al. 1993). In ALS patients and ALS mouse models, increased levels of lipid peroxidation and DNA damage were observed in cerebral spinal fluid, as measured by the assay of malondialdehyde and 8-hydroxy 2’ deoxyguanosine, respectively (Oteiza et al. 1997; Bogdanov et al. 2000). The same was found to be true in
spinal cords isolated from mutant SOD1 transgenic mice (Andrus et al. 1998). Oxidative stress was also found to be upregulated in G93A mutant SOD1 animals by measuring malondialdehyde and protein carbonyls in skeletal muscle (Mahoney et al. 2006). Furthermore, spinal cord and brain mitochondria from G93A mutant SOD1 animals were found to have reduced Ca\textsuperscript{2+} loading ability (Susaki and Iwata 1996). Mitochondrial localization was also found to be altered in sporadic ALS patients and mouse models of ALS. Mitochondria were found to be aberrantly localized to axon hillock in proximal axons of anterior horn nuclei (Susaki et al. 2005). Additionally, synaptic transmission changes were found in ALS mouse models prior to onset of neurodegenerative symptoms (Kuo et al. 2004). All of which illustrates a significant role of oxidative stress and mitochondrial dysfunction in initiation and progression of ALS.

1.9 Activation of Intrinsic Apoptosis in ALS

Mitochondria are also known as the power house of the cell because they generate the majority of the cells adenosine triphosphate (ATP) through glycolysis, Kreb’s cycle and electron transport chain. Mitochondria are membrane bound organelles found in all eukaryotic cells. Mitochondrial function has been investigated as a critical component of ALS because amongst its many functions, it also plays a key role in cellular apoptosis. When an apoptotic signal is activated in the cell, the mitochondrion opens a mitochondrial apoptosis-induced channel (MAC) in the outer mitochondrial membrane allowing release of Cytochrome-C into the cytoplasm of the cell. Once released, Cytochrome-C is free to bind with dATP, Apaf-1 and pro-caspase 9 to form a complex called the apoptosome. The apoptosome is able to cleave the initiator caspase pro-caspase 9 creating its active form caspase 9, which then goes on to activate executioner caspases
3 and 7 resulting in apoptosis. Caspase 9 was found to be activated in the spinal cords of transgenic mutant SOD1 mice, and in end stage animals caspase 7 was found to be activated as well (Guegan et al. 2001). Biochemical changes associated with intrinsic apoptosis were found in postmortem spinal cord tissues of ALS patients (Ilzecka et al. 2001). X chromosome-linked inhibitor of apoptosis (XIAP) serves to inhibit the activities of caspases 3, 7, and 9. Expression of human XIAP in spinal motor neurons of mutant SOD1 mice was found to attenuate disease progression but not to delay age of onset (Inoue et al. 2003).

Interestingly, ATP synthesis was found to be significantly reduced in mitochondria isolated from livers, brains, and spinal cords of end stage mice expressing the human G93A mutant compared to age matched wild type animals (Mattiazzi et al. 2002). However, decreases in ATP synthesis do not necessarily lead to apoptosis as seen by the use of oxidative phosphorylation inhibitors (OPIs) in human dopaminergic SH-SY5Y cells. OPIs depleted ATP in dopaminergic SH-SY5Y cells without influencing electron transport chain function; however, OPI’s failed to induce apoptosis (Watabe and Nakai 2007). The role of ATP depletion in apoptosis was also tested in clinical trials by the addition of creatine which functions to alleviate energy deficits, and was found to be ineffective because ATP depletion is probably not the initiating stimulus for apoptosis in ALS (Groeneveld et al. 2003). Collectively, this evidence implicates the mitochondrial apoptotic pathway’s involvement in motor neuron death found in ALS.

1.10 The Bcl-2 Family and BH3-Only Proteins as Regulators of Mitochondrial Oxidative Stress and Intrinsic Apoptosis
Intrinsic regulators of mitochondrial induced apoptosis come from a group of proteins called the BCL-2 family proteins. There are three subgroups defined by the homology shared within four conserved BCL-2 homology domains BH1-BH4 (Kim et al. 2006). The first subgroup is the pro-survival or anti-apoptotic proteins of which there are five members: Bcl-2, Bcl-xL, Bcl-w, A1, and Mcl-1. The other two subgroups are defined as pro-death or pro-apoptotic. One of the pro-apoptotic subgroups includes the two multi-domain proteins called Bax and Bak, both of which exist as inactive monomers. Bax resides in the cytosol and Bak is anchored to the outer mitochondrial membrane. Upon activation, Bax and Bak homo or hetero-oligomerize to form the mitochondrial apoptosis-induced channel (MAC) to allow cytochrome C release into the cytoplasm thus initiating the caspase cascade (Beal 2005; Dejean et al. 2005). The other pro-apoptotic subgroup is comprised of the BH3-only proteins, Bim, Bid, Bad, Bmf, Noxa, Puma, Bik, DP5/Hrk, and BNip3. BH3-only proteins are called such because they only contain the BH3 domain of the BCL-2 conserved homology domains (Figure 4). The BH3-only proteins function to either suppress anti-apoptotic proteins or to directly activate the pro-apoptotic multi-domain Bax and Bak, allowing translocation of Bax to the mitochondrial outer membrane where it undergoes homo-oligomerization or hetero-oligomerization with Bax and Bak leading to the eventual formation of MAC. Consequently, BH3-Only proteins are of interest because they ultimately determine if the mitochondrial apoptotic pathway is going to be induced in a particular cell such as a motor neuron.
Table 1. Bcl-2 family proteins

<table>
<thead>
<tr>
<th>Pro-survival (anti-apoptotic)</th>
<th>Pro-death (pro-apoptotic)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bcl-2</strong> “B-cell lymphoma”</td>
<td><strong>BH3-only</strong></td>
</tr>
<tr>
<td>Bcl-xL</td>
<td><strong>Bim</strong></td>
</tr>
<tr>
<td>Bcl-w</td>
<td>Bid</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Bad</td>
</tr>
<tr>
<td>A1</td>
<td>Bmf</td>
</tr>
<tr>
<td></td>
<td>Noxa</td>
</tr>
<tr>
<td>Bcl-2-like</td>
<td>Puma</td>
</tr>
<tr>
<td>Membrane Anchor</td>
<td>Blk</td>
</tr>
<tr>
<td>BH4</td>
<td>DP5/Hrk</td>
</tr>
<tr>
<td>BH3</td>
<td>BNip3</td>
</tr>
<tr>
<td>BH1</td>
<td></td>
</tr>
<tr>
<td>BH2</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4: Pro-apoptotic and Pro-survival Subgroups of Bcl-2 Family Protein Members.**

This table shows three main subgroups of the Bcl-2 Family proteins. The first subgroup is anti-apoptotic and consists of members Bcl-2, Bcl-xl, Bcl-w, Mcl-1 and A1. The two remaining subgroups serve pro-apoptotic functions. The multi-domain Bcl-2 family member proteins exist as monomers in the cytoplasm, Bax, or in the outer mitochondrial membrane, Bak. Activation of multi-domain proteins induces conformational change and insertion into the outer mitochondrial membrane forming the mitochondrial apoptosis-induced channel (MAC) for cytochrome C release. The other pro-apoptotic subgroup is BH3-only proteins. Theses proteins only contain the BH3 domain and serve one of two functions: either they suppress the activity of pro-survival proteins or they directly activate Bax and Bak.

Interactions between all of the three BCL-2 family protein subgroups are very specific and organized. BH3-only proteins Bim, Puma, and Bid are direct “activators” of Bax and Bak that facilitate MAC formation. Under normal conditions when cells are not being induced to undergo apoptosis, Bcl-2 or Bcl-xl sequesters Bim and Bid, and Mcl-1 sequesters Puma. Upon receiving an apoptotic stimulus, other BH3-only proteins known as “inactivators” or “sensitizers,” such as Bad, can disrupt the interaction between Bcl-2 or Bcl-xl with its BH3-only protein partners Bid or Bim, thereby allowing the latter proteins to freely activate Bax and Bak (Figure 5). Similarly, the BH3-only inactivator,
Noxa, can selectively disrupt the interaction between Mcl-1 and Puma. With its
dissociation from Mcl-1, Puma is free to bind Bax and Bak as well. BH3-only proteins
Bmf and Bik can also inhibit interactions between Bid and Bcl-2 or Bcl-xL. The BH3-
only protein Hrk/Dp5 selectively inhibits Bcl-2 but not Bcl-xl. BH3-only protein’s ability
to antagonize specific anti-apoptotic Bcl-2 family members is not dependent on
interaction between BH3s and pro-survival proteins but rather changes in binding affinity
induced by specific death signals. The cell stressor, or cause for death, is an important
factor in determining which BH3-only proteins become activated because Bcl-2, Bcl-xl,
and Mcl-1 are not functionally equivalent. For example, DNA damage was found to up-
regulate BH3-only proteins Puma and Noxa, while cytokine withdrawal serves to up-
regulate Bim and Bad (Kim et al. 2006).
Figure 5: Bcl-2 Family Proteins as Key Regulators of Mitochondrial Apoptosis. Upper Panel: In the absence of inactivator BH3-only proteins, Bim is being sequestered by pro-survival proteins Bcl-2, Bcl-xl, or Mcl-1, so that Bim is unable to bind to Bax/Bak monomers to induce a conformational change needed to form the mitochondrial apoptosis-induced channel (MAC). Lower Panel: Increasing expression of inactivator BH3-only proteins, Noxa and Bad, either through increased transcription or decreased protein degradation, displaces pro-survival Bcl-2 proteins and allows release of Bim so that it may interact with Bax or Bak monomers causing oligomerization and MAC formation to release cytochrome C into the cytosol, activating the caspase cascade. (Figure created by Emily Schroeder)

More recently, numerous studies have provided significant evidence of a novel antioxidant role that the pro-survival Bcl-2 protein plays in protecting the cell from mitochondrial oxidative stress. Downregulation of endogenous SOD1 in SH-SY5Y neuroblastoma cells through the use of siRNA, caused an elevation in the anti-oxidant glutathione (GSH). However, when endogenous SOD1 was downregulated in conjunction with downregulation of anti-apoptotic protein Bcl-2, the buffering capacity of GSH was not sufficient to prevent apoptosis (Aquilano et al. 2006). Surprisingly, GSH was found to bind to the Bcl-2 homology 3 domain groove of anti-apoptotic protein Bcl-2 as investigated through the use of BH3 mimetics. These BH3 mimetics bind to the BH3 groove of Bcl-2 displacing mitochondrial GSH pools and suppressing transport of GSH into the mitochondria where it is critical to repress oxidative stress in cerebellar granule neurons (Zimmerman et al. 2007). Furthermore, overexpression of anti-apoptotic protein Bcl-2 in GT1-7 neuronal cell lines increased GSH levels and inhibited mitochondrial dysfunction-induced death (Kane et al. 1993). Together these data suggest a novel antioxidant role for the pro-survival protein Bcl-2.
1.11 Role of Bcl-2 and Bax in ALS

Bcl-2 family members are known to be key regulators of mitochondrial induced apoptosis and mitochondrial oxidative stress. Bcl-2 mRNA expression has been shown to be decreased in spinal cord tissues of ALS patients, indicating downregulation of Bcl-2 as a key step in motor neuron degeneration and disease progression (Mu et al. 1996). Overexpression of pro-survival Bcl-2 in mutant SOD1 mice either through intercrossing with Bcl-2 transgenic mice or by intra-spinal injection with a recombinant virus encoding Bcl-2, resulted in a prolonged survival of mutant G93A SOD1 transgenic mice and delays in disease onset (Kostic et al. 1997; Vukosavic et al. 2000). Furthermore, overexpression of pro-survival Bcl-2 proteins was also found to increase the number of surviving motor neurons at end stages of the disease in mutant SOD1 mouse models (Azzouz et al. 2002).

In contrast to the downregulation of Bcl-2 observed in ALS, the pro-apoptotic Bcl-2 family member, Bax, was found to be upregulated in spinal cord tissue of ALS patients and in end stage G93A mutant SOD1 ALS mouse models as well (Ekegren et al. 1999; Gould et al. 2006). In addition, crosses of multi-domain pro-apoptotic Bax-deficient mice with mice expressing mutant SOD1 proved that Bax deficiency can, in fact, extend life expectancy and delay onset of motor neuron degeneration in mutant SOD1 animals (Gould et al. 2006). Other crossing experiments carried out with B6SJL-Tgn (SOD1 G93A) animals and Bcl-2-57 mouse line (which carries more than sixteen copies of the human Bcl-2 gene) was found to attenuate neuronal degeneration and delay activation of caspases in subsequent progeny of the cross (Vukosavic et al. 2000). Furthermore, when in-situ-hybridization was used to measure expression of Bcl-2 and Bax in ALS lumbar spinal cord cross sections, analysis revealed that motor neurons were...
found to have decreased Bcl-2 mRNA expression but increased Bax mRNA expression (Mu et al. 1996). An even more relevant indicator of Bcl-2 family protein’s involvement in mitochondrial induced apoptosis was illustrated by the finding that mutant G93A, G41D, and G85R, SOD1 transgenic animals or humans, sequester pro-survival Bcl-2 into unstable aggregates in the mitochondria of spinal cord neurons (Pasinelli et al. 2004). Therefore, pro-survival Bcl-2 and pro-apoptotic Bax play opposing roles in motor neuron degeneration in the mutant SOD1 mouse model of ALS.

1.12 BH3-only Protein Involvement in ALS

Transgenic mice expressing a G93A mutant of SOD1 provide an in vivo model to investigate motor neuron death during disease progression. As previously stated, the principal regulators of intrinsic apoptosis are the Bcl-2 family proteins. While some members of this family are pro-survival, the BH3-only proteins are pro-apoptotic. BH3-only proteins have been implicated in the neuronal death found in numerous neurodegenerative diseases such as Parkinson’s and Huntington’s disease (Perier et al. 2007; Garcia-Martinez et al. 2007). Therefore, several studies on the function of BH3-only proteins in ALS have been undertaken. A recent study revealed a key role for the BH3-only protein Bim in motor neuron death of the mutant SOD1 mouse. This study reported upregulation of Bim mRNA and protein in whole spinal cord samples of post-symptomatic G93A, G86R, and G85R mutant SOD1 mice and in NSC34 motor neuron cells induced to undergo apoptosis with transient expression of mutant SOD1 (Hetz et al. 2007). This study also found slight increases in Bid and Noxa mRNA levels, yet reported no significant changes in Bad, Bmf, Bik, Puma, Bak, or Bak mRNA. Additionally, a slight increase in protein expression was found for the BH3-only protein Puma, but the
authors did not elaborate on this point. Hetz’s study also crossed mutant SOD1 mice with Bim deficient mice and reported decreased spinal motor neuron apoptosis, delayed disease onset, and prolonged survival overall. One potential shortfall of this study is that the authors did not examine the BH3-only protein Hrk, which is known to work with Bim in a coordinated fashion to induce intrinsic apoptosis (Harris and Johnson 2001).

A subsequent study reported increased ER stress and defects in protein degradation in human sporadic ALS postmortem spinal cord samples and G93A mutant SOD1 mouse whole spinal cord samples, as well as upregulation of BH3-only protein Puma in the G93A mutant SOD1 mouse. Furthermore, genetic deletion of Puma through the crossing of G93A mutant SOD1 mice with Puma knock out mice slightly improved motor neuron survival, delayed disease onset, and reduced motor dysfunction in G93A mutant SOD1 mice (Kieran et al. 2007). Yet, no effect on life span was reported, indicating that other factors are likely involved in later stages of disease progression.

One limitation of the previously cited studies is that by using whole spinal cord, motor neuron-specific RNA is diluted with other cellular sources of RNA such as glial, vascular, and other neurons. Moreover, only around 45% of cells in healthy mouse spinal cords are neuronal, and only a fraction, ~10%, of all neurons are motor neurons (Bjugn 1993). In end stage G93A mutant SOD1 mice there already exists a significant reduction in motor neurons, making accurate analysis of relative levels of motor neuron-specific BH3-only transcripts in whole spinal cord unreliable.

Various other studies have reported significant findings for the roles of BH3-only proteins in neuronal death. Upregulation of Bim though activation of either c-Jun or Forkhead family transcription factors was found to contribute to apoptosis of sympathetic
neurons and cerebellar granule neuron cultures upon either withdrawal of nerve growth factor or removal of depolarizing extracellular potassium, respectively (Harris and Johnson 2001; Whitfield et al. 2001; Linseman et al. 2002; Gilley et al. 2003). The BH3-only protein Hrk was also found to be upregulated in a c-Jun dependent manner during apoptosis inhibited by potassium deprivation in cerebellar granule neuron cultures and knock down of Hrk using siRNA rescued cerebellar granule neurons from subsequent apoptosis (Ma et al. 2007).

The BH3-only protein, Hrk, was also detected in spinal cord motor neurons of sporadic ALS patients where it heterodimerized with the pro-survival protein Bcl-2 (Shinoe et al. 2001). In spinal cords of G93A mutant SOD1 mice, the BH3-only protein Bid was found to be selectively truncated by caspase-8 to its more apoptotic form, t-Bid (Guegan et al. 2001). Gene disruption of Bad or Bim in hypoxic-ischemic neonatal mouse models was found to reduce hippocampal neuronal death induced by hypoxia and ischemia (Ness et al. 2006). The BH3-only protein Noxa was found to be upregulated in axotomized neurons in adult C57BL/6 mice, and in p53-deficient mice expression of Noxa in nerve-injured motor neurons was reduced and progressive neuronal death was suppressed (Kiryu-Seo et al. 2005). Taking all of these studies into consideration, an enhanced expression of BH3-only proteins likely plays a significant role in ALS.

1.13 Hypothesis and Rationale

Taking the findings of previous studies into consideration, in conjunction with the knowledge that multiple BH3-only proteins work in a coordinated manner to induce maximal apoptosis, we hypothesized that multiple activator and inactivator BH3-only proteins are turned on simultaneously to induce intrinsic apoptosis of motor neurons
during the progression of ALS. We also postulated that these pro-apoptotic proteins act in a very intricate and coordinated manner to cause motor neuron death.

As seen previously, several BH3-only proteins are induced in spinal motor neurons during the progression of ALS and these pro-apoptotic proteins contribute significantly to motor neuron degeneration. However, the role of BH3-only proteins in spinal motor neuron death during the progression of ALS has only partially been examined. Therefore, we assessed the expression levels of BH3-only transcripts in spinal cord motor neurons from wild type and G93A mutant SOD1 end stage animals utilizing a technique called laser capture microdissection (LCM). This technique allowed us to collect highly enriched populations of spinal motor neurons from wild-type and SOD1 mutant mice. Obtaining enriched samples of motor neurons by LCM should allow for enhanced sensitivity in detecting BH3-only transcript expression through the use of real time PCR, even if present only sparingly. However, analysis of BH3-only transcript expression in whole spinal cord homogenates, as previously used by Hetz et al. 2007 and Kieran et al. 2007, is not as sensitive due to their diluted expression.

Motor neuron death underlies the pathophysiology of ALS regardless if the initial cause is familial or sporadic. Therefore, we hope to identify key novel death promoting proteins of the mitochondrial apoptotic pathway that could be targeted therapeutically to decrease motor neuron apoptosis and enhance motor function in ALS patients. Identifying roles of BH3-only proteins in motor neuron degeneration will identify novel molecules that could be targeted in ALS cases to slow or halt disease progression.
1.14 Summary of Major Findings

Using quantitative Real Time PCR, we did not detect any significant differences in the expression of BH3-only transcripts between spinal motor neurons laser captured from end stage SOD1 mutant mice and age matched wild type animals. This experiment indicated that BH3-only protein expression was not being altered at the transcriptional level in motor neurons, but it did not exclude changes at the protein level.

To investigate protein levels, we performed immunohistochemical staining for the following BH3-only proteins: Bik, Bad, BNip3, Bid, Noxa, Puma, and Hrk/Dp5. The results demonstrated selective staining of Hrk/Dp5, Bnip3, and Bid in astrocytes of lumbar spinal cords obtained from end stage mutant SOD1 mice. Conversely, Hrk/Dp5, Bnip 3, and Bid were not observed in astrocytes of wild type mouse spinal cords. Collectively, these novel findings indicate a potentially important role for astrocytes expressing Hrk/Dp5, Bnip3, and Bid in ALS disease progression. Future studies examining the role of BH3-only proteins Hrk/Dp5 and Bid in astrocytes expressing mutant SOD1 may establish BH3-only proteins as critical inducers of motor neuron apoptosis and prove that they significantly contribute to disease progression in ALS.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Mating of G93A Mutant SOD1 Transgenic Animal Colony

G93A mutant SOD1 transgenic male animals were crossed with age matched females, wild type FVB. Female wild type animals are utilized because G93A mutant SOD1 transgenic females are sterile. The gestation cycle of the female mouse is 21 days, thus it was imperative that after separation from previous matings a waiting period of at least 21 days was enforced, to insure no litters from previous matings were produced. To ensure successful breeding two main supplements were used to promote mating: love mash, a type of excitatory supplement for the male, and an igloo to promote nesting between G93A mutant SOD1 transgenic males and FVB females.

2.2 Weaning of G93A Mutant SOD1 Transgenic Animal Colony

Once litters are 21 days of age they were weaned away from parent mice. In a sterile environment litter mates were separated into different cages based on gender and number of progeny. It is customary to house between two and six animals per cage. Animals were then pierced with an ear tag containing the first letter of the primary investigator’s name and a personal identification number. A 0.5 cm tail snip was cut after tagging and placed in a sterile 1.5 ml microcentrifuge tube, also labeled with the animal’s identification number, to be used for genotyping.

Animals were assessed on a daily basis to check for signs of pregnancy, injury, or delivery of pups. Once G93A mutant SOD1 transgenic animals approached 90 days of
age, they were checked for any signs of amyotrophic lateral sclerosis (ALS). If the G93A mutant SOD1 transgenic animals could not right themselves within two seconds of being placed on their sides, which usually occurs at end stage or around 120 days of age, they were euthanized and tissue was collected.

2.3 DNA Isolation

The tail sections taken at weaning were incubated in lysis buffer for three hours at 55 °C. Samples centrifuged then supernatant transferred and RNase A added. After a short incubation, genomic DNA was isolated from tail samples using the Invitrogen Charge Switch gDNA Tissue kit (C511203 and C511204). Protocol included steps for isolating DNA, washing DNA, and eluting DNA. In addition to the tissue kit, we purchased a magna rack (Invitogen C515000) to use in conjunction with magnetic beads. Samples were then labeled and stored at -20 °C until used for genotyping.

2.4 Genotyping

Polymerase chain reaction (PCR) mixture was made up as a master mix and 18µl was added to 0.2 ml PCR tubes. To tubes containing master mix, 2µl of previously isolated genomic DNA was added next. The master mix for one PCR reaction contained 2µl 10X Gold Buffer, 1.6µl 25mM MgCl₂, 0.2µl AmpiTaq Gold 250 pack (Applied Biosystems, 4311806), 2µl dNTP’s (2.5mM, Strat a gene 200418-51), 2µl hSOD1 G93A forward primer (5’CATCAGCCCTAATCCATCTGA 3’), 2µl hSOD1 G93A reverse primer (5’CGCGACTAACAATCAAAGTGA 3’), 2µl JaxCon R Maurine Interleukin 2 gene forward primer (5’CATGGCCACAGAATTGAAAGATCT 3’), 2µl JaxCon R Maurine
Interleukin 2 gene reverse primer (5’GTAAGGTGGAAATTCTAGCATCATCC 3’), and 4.2 µl distilled sterilized water. All primers were used at a 1:10 dilution and an internal control, JaxCon R Maurine Interleukin 2 gene, was added to ensure proper set up of PCR. The PCR samples were run in the Bio Rad DNA Engine Peltier Thermocycler (model ALS1296). The thermocycler, set to block method utilizing a hot start, was programmed to run at 94 °C for 10 minutes, 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds, 72 °C incubation for 10 minutes, then to 4 °C indefinitely. The program was set to cycle 35 times through annealing, elongation, and termination steps. Once the PCR program was complete, the samples were run on a 1% agarose gel at 100-120 Volts for 30 minutes.

2.5 Animal Dissection

Animals were euthanized with 250 µl-500 µl isoflourene (Webster Veterinary, 14043-220-05) in a euthanizing chamber. Unresponsive animals were then subjected to the tail drag test and to the toe pinch test. For end stage G93A mutant SOD1 transgenic animals, a toe was pinched on the forelimbs to ensure no reflex occurred. After spraying the dorsal side of the animal with 70% EtOH, animals were decapitated with sterile scissors. The cortex and cerebellum were removed and frozen in Optimal Cutting Temperature (OCT) compound (Tissue-Tek 4583). The skin and hair were removed from the back of the animal to expose the spinal column. The junction between sacrum and lumbar spinal column was found and a razor blade pressed firmly down at the junction, cutting all the way through. The spinal cord was blown out with 1xPBS using a 10 ml syringe and an 18G1/2 precision glide needle (Becton Dickinson, 305196). The lumbar
spinal cord was cut and removed, then frozen in OCT in a cryo-mold over dry ice. The samples were stored at -80 °C until they were cryo-sectioned.

2.6 Cryo-sectioning on Leica CM 1850 UV Cryostat

Tissue frozen in OCT compound (Tissue-Tek, 4583) was cut at -20 °C with microtome blades (DT315X50, Extremus) at a 5 degree angle. The tissue blocks were sectioned at 30 microns until the tissue was visible. Section width then changed to desired microns and the first five sections were discarded. For immunohistochemistry (IHC) sectioning was set at 8 microns, for laser capture microdissection (LCM) sectioning was changed to 12 microns. For laser capture micro-dissection (LCM) the width was changed to 12 microns. Sections cut for LCM were placed on Pen membrane slides (Molecular Devices, LCM0522). Sections cut for IHC were placed on standard Fisher brand superfrost/plus microscope slides (Fisher Scientific, 12-550-15). Exposed tissue in blocks was covered in OCT and re-frozen, then stored along with slides at -80 °C.

2.7 Motor Neuron Rapid Stain For Laser Capture Microdissection

The entire work area and supplies were sprayed with RNase Away (Molecular Bio Products, 7002). Motor neuron rapid staining for LCM was completed using an LCM Staining Kit (Ambion, AM1935). Staining of lumbar spinal cord sections for motor neuron morphology involved subsequent steps of fixing tissue in decreasing ethanol concentrations. Sectioned tissue was circled with a hydrophobic barrier pen (Ambion, 15500G) then stained with Cresyl Violet (Ambion, 8554G). Tissue was then washed with increasing concentrations of ethanol solution then washed again with xylene (MP Biomedicals LLC, Histological Grade 158692) in the fume hood. The protocol used was
adopted from Ambion LCM Staining Kit for Staining for Recovery of intact RNA manual pages 10-12.

2.8 Laser Capture Micro-dissection (LCM)

Laser Capture Micro-dissection was done using Arcturus Veritas Micro-dissection machinery. The entire work area and supplies were sprayed with RNase Away (Molecular Bio Products, 7002). Pen membrane slides and Arcturus Capsure HS LCM caps (Arcturus, LCM0214) were loaded into the LCM machine. A new session was started where road maps were created along with an LCM cap image. 40x magnification was obtained, and laser intensity was lowered to 2mV. Once in focus at 40x magnification, the capture laser intensity was lowered to 2mV and was focused. After focusing the laser, power was adjusted to 70-90mV and UV laser cutting power was set to low and ten for cutting a 2 micron thick path. Each one hour session achieved 300 motor neurons captured from lumbar spinal cord sections of six G93A mutant SOD1 transgenic animals and six age matched wild type animals. After LCM, the cap was checked with 10x magnification to ensure cells were on cap, and then off loaded for further use.

2.9 Cell Removal and RNA Isolation

Caps were removed with sterilized tweezers from the off-loading trays. Polymer around the cells was cut away using a Leica S6E dissection microscope. Lysis solution, DNase, and 50 mg RNase free 250-500 micron glass beads (Glen Mills Inc. Soda Lime lead free glass beads, 7200-000250) were added to polymers with motor neurons adhered in a sterile RNase free micro-centrifuge tube. The micro-centrifuge tube was then placed in a mini bead beater (Biospec Products) and polymer pelleted for two minutes at speed 11.
After checking for dissociation of motor neurons, the polymer was removed and samples were incubated at room temperature for five minutes prior to the addition of stop solution.

Lysates can be stored at -80 °C for up to two months. The protocol was modified from Ambion Taqman Pre Amp Cells to CT kit, page 10 of manual, in that we added twice the amount of lysis buffer, DNase, and stop solution, as well as the addition of pelting polymer with glass beads in mini bead beater.

2.10 Reverse Transcription of Isolated RNA

The master mix for reverse transcription reactions was made as stated in protocol obtained from Ambion Taqman Pre Amp Cells to CT kit, page 11 of manual. All samples were run in duplicate along with negative control samples containing no RNA. Samples ran with a hot start in a Bio Rad DNA Engine Peltier Thermocycler (model ALS1296) set to block method. The thermocycler program used for reverse transcription was incubation at 37 °C for 60 minutes, 95 °C for 5 minutes, then 4 °C indefinitely. Nano Drop spectrophotometry (Nano Drop Technologies) was utilized as a positive control of cDNA preparation in samples after reverse transcribed. Samples were stored at -20 °C until used for pre-amplification.

2.11 Pre-amplification of cDNA

Two pooled Taqman assays were prepared; the first one contained Taqman probe-primer sets from Applied Biosystems for BH-3 only proteins: Bid/Bod (Mn01975020_s1), Dp5/Hrk (Mn01962376_s1), Puma (Mn00519268_n1), and Noxa (Mn00451763_m1). Control house keeping genes in the first assay included Taqman probe-primer sets from Applied Biosystems to GapDh (Mn01253033_ml), β-actin
(Mn00607939_s1), and motor neuron marker choline acetyltransferase (ChAT, Mn0122180_n1). The second assay contained Taqman probe-primer sets from Applied Biosystems for death gene Caspase 1 (Mm012439700_g1) and house keeping gene β-actin (Mn00607939_s1). Assays were set up such that each primer had a final concentration of 0.2X in 1 ml 1xTE.

Reactions for pre-amplification of cDNA were prepared in 0.2 ml RNase free PCR tubes as suggested in protocol from Ambion Taqman Pre Amp Cells to CT kit, pages 12-14 of manual. Samples were run with a hot start in a Bio Rad DNA Engine Peltier Thermocycler (model ALS1296) set at block method. The thermocycler program that was run was incubation at 95 °C for 10 minutes, incubation at 95 °C for 15 seconds, incubation 60 °C for 4 minutes, and then continued at 4 °C indefinitely. Only 10 repetitions of annealing, elongation, and termination were done total. Samples were stored at -20 °C after diluting 1:5 with 1xTE.

2.12 Real Time PCR

Real time PCR mix included 12.5µl 2X TaqMan Universal Master Mix (Applied Biosystems, 4304437), 1.2µl TaqMan probe-primer set to be tested, 6.3µl Nuclease-free water, 5µl of pre-amp cDNA sample, and 6-carboxyfluorescein (6-FAM) blue fluorescent dye. The fluorescent dye used in the TaqMan gene expression assays for the experiment was specified as 6-carboxyfluorescein (6-FAM, blue fluorescence) for real time PCR. MicroAmp Optical tubes/caps (Applied Biosystems, N801-0933 and N4323032) were utilized in an AB1 Prism 7700 sequence detector (Perkin Elmer Corp./Applied Biosystems). The program used was as follows: 50 °C for 2 minutes, TaqGold enzyme
activation 95 °C for 10 minutes, 95 °C for 15 seconds, 60 °C for 1 minute, and 4 °C indefinitely. Only 45 repetitions of annealing, elongation, and termination were done total.

Fluorescence data were normalized and cycle threshold values were determined by setting a threshold above the average fluorescence from the first fifteen cycles of PCR (Gibson et al. 1996; Heid et al. 1996; Livak et al. 1995). Data were evaluated and cycle threshold values for probed genes of interest were recorded.

2.13 Immunohistochemistry (IHC) of BH3-Only Proteins

Previously isolated and cut lumbar spinal cord, cerebral cortex, and cerebellum tissue samples were fixed, blocked, and permeabilized. Primary antibody staining for BH3-only proteins in lumbar spinal cord sections was set up for five G93A mutant SOD1 transgenic animals and four age matched wild type animals. Immunohistochemistry of BH3-only protein staining was completed with antibodies for Bad, Bid, Bim, Noxa, Puma, Bik, Hrk, or Bnip3 (Table 1). Primary antibody dilutions were made up in 2% BSA in 0.2% Triton X-100/1xPBS, at a 1:100 dilution.

<table>
<thead>
<tr>
<th>BH3-Only Protein</th>
<th>Antibody Information</th>
<th>Co-Staining (if applicable)</th>
<th>Antibody Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bad</td>
<td>Anti Rabbit, Polyclonal, ProSci Incorporated, 3343</td>
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<td>Bid</td>
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<td>GFAP</td>
<td>Anti Mouse, Polyclonal, Abcam, ab464-100</td>
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<td>ChAT</td>
<td>Chicken anti Mouse, Monoclonal, Chemicon, AB15468</td>
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<tr>
<td>Bim</td>
<td>Anti Rabbit, Polyclonal, ProSci Incorporated, 2065</td>
<td>GFAP</td>
<td>Anti Mouse, Polyclonal, Abcam, ab464-100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ChAT</td>
<td>Chicken anti Mouse,</td>
</tr>
</tbody>
</table>
Table 1: Primary Antibodies used for Immunohistochemistry of BH3-Only Proteins. This table lists, in the first column, the name of BH3-only protein antibody. In the second column is the primary antibody information. In the third column is the name of primary antibodies that were used for co-staining experiments. In the final column is antibody information of proteins stained in conjunction with BH3-only proteins.

Co-staining was completed for BH3-only proteins Bid, Hrk, and Bim protein expression with Glial Fibrillary Acidic Protein (GFAP) was done by incubating sections in primary antibody dilution using rabbit anti-BH3-only protein at 1:100 and anti-mouse GFAP at 1:100 made up in 2% BSA in 0.2% Triton X-100/1xPBS. Co-staining of BH3-only proteins Hrk and with choline acetyltransferase (ChAT) was done by incubating sections in primary antibody dilution using rabbit anti-BH3-only protein at 1:100 and chicken anti mouse ChAT antibody at 1:250 made up in 2% BSA in 0.2% Triton X-100/1xPBS.

Secondary antibody dilution solution for BH3-only protein staining was prepared in 2% BSA in 0.2% Triton X-100/1xPBS by diluting DAPI 1:500 (Sigma, D9542) and
Cy3-conjugated affinity pure donkey anti-rabbit IgG (H+L) 1:500 (Jackson ImmunoResearch, 711-165-152). Secondary antibody dilution solution for BH3-only protein and GFAP co-staining was prepared by diluting DAPI 1:500, Cy3-conjugated affinity pure donkey anti-rabbit IgG (H+L) 1:500 and Fluorescein (Fitc)-conjugated affinity pure donkey anti mouse IgG 1:500 (Jackson ImmunoResearch, 715-095-150). Secondary antibody dilution solution for BH3-only protein and ChAT co-staining was prepared by diluting DAPI 1:500, Cy3-conjugated affinity pure donkey anti-chicken IgY(IgG)(H+L) 1:500 (Jackson ImmunoResearch, 703-165-155), and Fluorescein (Fitc)-conjugated affinity pure donkey anti-rabbit IgG 1:500 (Jackson ImmunoResearch, 711-095-152).

Slides with primary antibody dilution solutions were incubated overnight at 4 °C in a humidity chamber. Primary antibody was aspirated and slides were washed five times in 1xPBS for five minutes, then incubated with secondary antibody dilution solution for 90 minutes at room temperature. Secondary antibody was aspirated and slides washed five times in 1xPBS for five minutes. 1xPBS was aspirated and three to five drops of anti quench were added to the slides. The slides were cover slipped while removing all bubbles and sealed with clear nail polish. Slides were imaged at 40xAir or 63xOil, by using the Digital De-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAPI, CY3, and FITC. Images were captured on slide book version 4.1.0.11 and formatted in photo-shop version 6.0. Five images were captured per slide, per condition, and per animal and stored at -20 °C.
Negative control for non-specific staining was completed with primary antibody anti-mouse GFAP solution and secondary antibody solution using DAPI 1:500, Cy3-conjugated affinity pure donkey anti-rabbit IgG (H+L) 1:500, and Fluorescein (Fitc)-conjugated affinity pure donkey anti-mouse IgG 1:500. As a secondary control for non-specific staining by pre-binding of primary antibodies for BH3-only proteins Bid and Hrk, with Bid peptide (ProSci, 3353P) and Hrk peptide (ProSci, 3771P) at a ratio of 1:2 was completed. Antibody with peptide solution incubated on a tube rotator set at 30 °C and 500 rpm for one hour. Secondary antibody solution for this control utilized DAPI 1:500 and Cy3-conjugated affinity pure donkey anti-rabbit IgG (H+L) 1:500. All antibody dilution solutions were made up in 2% BSA/0.2% Triton X 100 in 1xPBS solution.

2.14 Rapid Staining of Astrocytes for Laser Capture Microdissection

Pen membrane slides previously cut at 12 microns and standard Fisher brand superfrost/plus microscope slides with sections cut at 8 microns were placed in blocking (10% Bovine Serum Albiumin (BSA) in 0.3% Triton X-100/1xPBS) for three minutes. Primary antibody solution consisted of polyclonal rabbit GFAP antibody [1:50 (Dako)]. After incubation for five minutes at 40 °C in humidity chamber slides were washed for 15 seconds in 1xPBS. Secondary antibody solution consisted of Fluorescein (Fitc)-conjugated affinity pure donkey anti-rabbit IgG 1:25 (Jackson ImmunoResearch, 711-095-152). After incubation in secondary for two minutes at room temperature, slides were washed three times for 15 seconds, and incubated in at 40 °C for 10 minutes (Burbach et al. 2004). Slides could then be used for laser capture microdissection.
2.15 Preparing Primary Astrocyte Cultures

Primary astrocyte cultures protocol was adapted from Gonzalez Deniselle et al. and Hantaz-Ambrosie et al. 1995. Previously dissected spinal cords (see animal dissection protocol) were minced and incubated with 0.1% trypsin for 20 minutes at 37 °C. A mixture of complete medium containing minimal essential medium (MEM) (Gibco, 70%), medium 199 (Gibco, 30%), and 20% fetal calf serum was added to spinal cord samples to halt trypsin activity. Samples were pelleted (300 g, 8 min) and resuspended in complete medium mixture mentioned above. Cells were then plated in culture dishes coated with poly-l-lysine and incubated at 37 °C in a 5% CO₂ atmosphere. The medium was renewed every four days until astrocytes reach 90% confluence (approximately 45). Astrocytes were then lysed and stored for future use.

2.16 Statistical Analysis and Quantification of Data

Recorded cycle threshold (CT) values obtained from real-time PCR on cDNA prepped from motor neurons of G93A mutant SOD1 transgenic animals and age matched wild type animals for BH3 only proteins transcript expression; Bim, Dp5, Noxa, and Puma were analyzed using the Anova single factor data analysis tool in Microsoft Excel. Graphs for standardization of BH3-only protein transcript expression for β-actin and ChAT were created by inverting CT values, (1/CT) of BH3-only proteins transcript expression in order for the amount of transcript to be directly proportional to the numerical value of inverted CT values. The same was done for CT values obtained for β-actin and ChAT. Inverted CT values of BH3-only proteins transcript expression were standardized to inverted CT values of β-actin and ChAT for G93A mutant SOD1 transgenic and wild type FVB animals. Standardized CT values for G93A mutant SOD1
transgenic animals were taken over the mean for standardized CT values in wild type animals with β-actin or ChAT, set to 1, and graphed against wild type values.

Images captured from IHC of BH3-only proteins, Bad, Bid, Bnip3, Bim, Noxa, Puma, Bik, and Hrk were quantified by counting whole numbers of complete astorocytes and motor neurons stained positive for BH3-only protein in lumbar spinal cord sections. Each BH3-only protein was stained in five end stage G93A mutant SOD1 transgenic animals and four age matched wild type FVB animals and five images were taken from each experimental condition. Quantified numbers were subjected to analysis by 2 tailed, type 2, T-test statistical analysis tool in Microsoft Excel. Graphs of averages of motor neurons or astocytes in five images taken per five end stage G93A mutant SOD1 transgenic animals and four age matched wild type FVB animals were generated.
CHAPTER THREE: RESULTS

3.1 Real Time PCR Analysis of BH3-only Transcript Expression in Motor Neurons Captured from Wild Type and End Stage G93A Mutant SOD1 Mice:

Lumbar spinal cord sections were rapidly stained on pen membrane slides, using cresyl violet to show cell morphology. Approximately three hundred motor neurons were captured from each of six wild type and six end stage G93A mutant SOD1 animals. Motor neurons were harvested using laser capture microdissection (LCM) (Figure 6), which allowed us to gather highly enriched samples of motor neurons. RNA was isolated from motor neurons, pre-amplified, and reverse transcribed. cDNA template was used for real time PCR to detect transcript expression of the BH3-only proteins Bim/Bod, Dp5/Hrk, Puma, and Noxa. Real time PCR was also performed for the “housekeeping” genes, GAPDH and β-actin, and the motor neuron marker, choline acetyltransferase (ChAT).
Figure 6: Laser Capture Microdissection (LCM) of Cresyl Violet Stained Motor Neurons in Mouse Spinal Cord Tissue. Panel A: Tissue rapidly stained, within 20 minutes, with cresyl violet to show the large cell morphology of the motor neurons in the mouse spinal cord. Panel B: Motor neurons selected for cut and capture by Arcturus Veritas Microdissection machinery. Panel C: Section of mouse spinal cord tissue post cut and capture of motor neurons. Panel D: Image of Arcturus Capsure HS LCM Cap showing motor neurons captured for RNA isolation.

Cycle threshold (CT) values, the number of PCR cycles required to detect a significant increase in fluorescence over background, were obtained for BH3-only transcripts and were then normalized to either GAPDH, β-actin, or ChAT. Bar graphs were created showing transcript expression of BH3-only proteins in wild type versus end stage G93A mutant SOD1 animals following normalization to either GAPDH (data not shown), β-actin (Figure 7), or ChAT (Figure 8). It was surprising to find that no significant differences in transcript expression of BH3-only proteins Bim/Bod ($f_s=1.64$; $df=2, 12; P>0.05$), Dp5/Hrk ($f_s=1.70$; $df=2, 12; P>0.05$), Puma ($f_s=2.11$; $df=2, 12; P>0.05$), and Noxa ($f_s=1.34$; $df=2, 12; P>0.05$), were observed between wild type and end stage G93A mutant SOD1 animals. Furthermore, a negative control for real time PCR was created by omitting the reverse transcriptase enzyme, prior to reverse transcribing RNA, pre-amplification of cDNA, and real time PCR. As expected, cycle threshold values obtained for this control were very high, close to 50 cycles, suggesting that the values obtained from real time PCR of our generated samples were indeed representative of the levels of transcript expression in the captured motor neurons.
Figure 7: Normalization of Cycle Threshold Values from Real-Time PCR of BH3-Only Transcript Expression to β-actin. 300 motor neurons were collected from six end stage and six age matched wild type animals. RNA was isolated from motor neurons, reverse transcribed, and pre-amplified for Real-Time PCR with Taqman probes to BH3-only proteins Bim/Bod, Dp5/Hrk, Puma, and Noxa, as well as β-actin. Cycle threshold values obtained for BH3-only proteins were normalized to β-actin and analyzed using Anova Single Factor analysis. It was found that no significant difference in transcript expression of BH3-only protein, Bim, Dp5, Puma, or Noxa, exists between motor neurons captured from end stage and age matched wild type animals.
3.2 Immunohistochemistry of BH3-Only Proteins in Wild Type and End Stage G93A Mutant SOD1 Lumbar Spinal Cords.

Upon finding no significant differences in BH3-only transcript expression between end stage ALS mice and wild type age-matched controls, we next analyzed BH3-only expression and localization at the protein level using immunohistochemistry.
Immunohistochemistry (IHC) using primary antibodies to BH3-only proteins was conducted on lumbar spinal cord from four wild type and five end stage G93A mutant SOD1 animals. Five Images were taken using a 40x objective of each staining condition using a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform. IHC of the BH3-only proteins, Bik (Figure 9), Bad, Bim, Noxa, and Puma (data not shown), did not show any discernible differences in protein expression or localization between wild type and G93A mutant SOD1 end stage animals. The expression of these BH3-only proteins in lumbar spinal cord sections of both wild type and end stage animals was largely restricted to the large alpha-motor neurons (Figure 9).

Figure 9: Immunohistochemistry of BH3-Only Protein BIK expression in Motor Neurons of End Stage and Wild Type mouse Spinal Cords. Lumbar spinal cord was sagittally cut 8 microns thick onto standard glass slides. Slides were stained for BH3-only protein Bik, and Dapi (nucleus), red and blue fluorescence respectively. Slides were imaged at 40x air using digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI
Fluorescence up-right microscope platform for DAPI and CY3. Panel A: wild type, arrows pointing to Bik positive motor neurons. Panel B: end stage, Arrows pointing to Bik positive motor neurons.

In marked contrast, IHC of BH3-only proteins Bid (Figure 10), Hrk (Figure 11), and BNip3 (data not shown), in lumbar spinal cord sections, revealed a surprising difference in the morphology of cells positive for BH3-only protein expression in general. Cells that stained positively for Bid, Hrk, or BNip3 in G93A mutant SOD1 end stage animals had a star-like morphology with spine-like projections. This observed morphology was characteristic of astrocytes. However, BH3-only protein expression in lumbar spinal cord of wild type animals was largely restricted to cells with motor neuron morphology (Figures 10 and 11).

Figure 10: Immunohistochemistry of BH3-Only Protein BID expression in Motor Neurons of End Stage and Wild Type Mouse Spinal Cords. Lumbar spinal cord was sagittally cut 8 microns thick onto standard glass slides. Slides were stained for BH3-only protein Bid, and Dapi (nucleus), red and blue fluorescence respectively. Slides were

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3.3 BH3-Only Proteins, Bid and Hrk, are Selectively Expressed in Astrocytes of Lumbar Spinal Cord from End Stage G93A Mutant SOD1 Mice.

Because of the observed morphology of cells that stained positively for BH3-only proteins Bid, Hrk, and BNip3, we next co-stained lumbar spinal cord sections of end stage G93A mutant SOD1 animals for glial fibrillary acidic protein (GFAP). GFAP is a member of the intermediate filament family and is a marker protein for astrocytes.
Therefore, co-staining for GFAP will show if BH3-only protein positive cells in end stage animals are in fact astrocytes. As seen in co-staining of the BH3-only protein Bid with GFAP (Figure 12) and co-staining of the BH3-only protein Hrk with GFAP (Figure 13), Bid and Hrk are indeed primarily expressed in astrocytes of end stage G93A mutant SOD1 animals. Co-staining experiments have not yet been carried out with BNip3.

**Figure 12: Immunohistochemistry of BH3-Only Protein Bid Co-Stained with Astrocyte Marker Glial Fibrillary Acidic Protein (GFAP) in End Stage G93A Transgenic Mouse Spinal Cords.** Lumbar spinal cord was sagittally cut 8 microns thick onto standard glass slides. Slides were stained for Bid and GFAP. Bid expression is shown as red fluorescence, GFAP expression as green fluorescence, and Dapi as blue fluorescence. Slides were imaged at 63x (oil) using a digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAP1, CY3, and FITC. Panel A: arrows pointing to Bid positive cells. Panel B: Arrows pointing to GFAP positive cells. Panel C: Merged image of Bid positive cells and GFAP positive cells. These images show that the BH3-only protein Bid is indeed being expressed in astrocytes of G93A end stage transgenic mice.
Figure 13: Immunohistochemistry of BH3-Only Protein Hrk Co-Stained with Astrocyte Marker Glial Fibrillary Acidic Protein (GFAP) in End Stage G93A Transgenic Mouse Spinal Cords. Lumbar spinal cord was sagittally cut 8 microns thick onto standard glass slides. Slides were stained with Hrk primary and GFAP. Hrk expression is shown as red fluorescence, GFAP expression as green fluorescence, and Dapi as blue fluorescence. Slides were imaged at 63x (oil) using a digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAP1, CY3, and FITC. Panel A: Hrk positive cells. Panel B: GFAP positive cells. Panel C: Merged image of Hrk positive cells and GFAP positive cells. These images show that the BH3-only protein Hrk is indeed being expressed in astrocytes of G93A end stage transgenic mice.

Furthermore, IHC of BH3-only proteins Bid (data not shown), Bnip3 (data not shown), and Hrk (Figure 14) in lumbar spinal cord sections of wild type animals showed protein expression to be selectively located in ChAT positive motor neurons and not in GFAP positive astrocytes (Figure 14, wild type panel). However, co-expression of BH3-only proteins Bid (data not shown), Bnip3 (data not shown), and Hrk (Figure 14), with GFAP was found selectively in astrocytes of end stage animals (Figure 14, end stage panel, and Figure 15). These unique findings in end stage animals were not shared among other BH3-only proteins. Expression of BH3-only proteins Bim (Figure 16), Bad, Bik,
Noxa, and Puma (data not shown), was found to be exclusively within motor neurons in lumbar spinal cord sections of both wild type and end stage G93A mutant SOD1 animals.

Figure 14: Immunohistochemistry of BH3-Only Protein Hrk Co-Stained with Either Choline Acetyltransferase (ChAT) in Wild Type or Glial Fibrillary Acidic Protein (GFAP) in End Stage G93A Transgenic Animal Spinal Cords. Wild Type Panels: Slides co-stained with Hrk and ChAT. Hrk protein expression shown as green fluorescence and ChAT expression is red fluorescence. Image A: ChAT positive cells. Image B: Neurons positive for Hrk expression. Image C: Merged image of Hrk positive cells and ChAT positive cells. Findings show that BH3-only protein Hrk is being expressed primarily in motor neurons of wild type animals. End Stage Panels: Slides co-stained for Hrk and GFAP. Hrk expression as red fluorescence, and GFAP expression as green fluorescence. Image A: Cells positive for Hrk expression. Image B: GFAP positive cells. Image C: Merged image of Hrk positive cells and GFAP positive cells. Findings show that BH3-only protein Hrk is being expressed primarily in astrocytes of end stage animals. Dapi, blue fluorescence, stains the nuclei. Slides were imaged at 40x using a digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAP1, CY3, and FITC.
Figure 15: Immunohistochemistry of BH3-Only Protein Hrk Co-Stained with Glial Fibrillary Acidic Protein (GFAP) in Wild Type and End Stage G93A Transgenic Animal Spinal Cords. Wild Type Panel: Slides co-stained with Hrk and GFAP primary antibodies. GFAP positive cells have green fluorescence and Hrk positive cells have red fluorescence. Image A: Arrows pointing to motor neurons positive for Hrk expression. Image B: Arrows pointing to GFAP positive cells. Image C: Merged image of Hrk positive cells and GFAP positive cells. Finding shows that BH3-only protein Hrk is selectively being expressed in motor neurons of wild type animals. End Stage Panel: Slides co-stained with Hrk and GFAP primary antibodies. GFAP positive cells have green fluorescence and Hrk positive cells have red fluorescence. Image A: Arrows pointing to cells positive for Hrk expression. Image B: Arrows pointing to GFAP positive cells. Image C: Merged image of Hrk positive cells and GFAP positive cells. Finding shows that BH3-only protein Hrk is selectively being expressed in astrocytes of end stage animals. Dapi, blue fluorescence, stains the nucleus. Slides were imaged at 40x using a digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAP1, CY3, and FITC.
Figure 16: Immunohistochemistry of BH3-Only Protein Bim Co-Stained with motor neuron marker Choline Acetyltransferase (ChAT) in Wild Type Animal and Astrocyte Marker Glial Fibrillary Acidic Protein (GFAP) in End Stage G93A Transgenic Animal Spinal Cords. Wild Type Panels: Slides co-stained with Bim and ChAT primary antibodies. Bim protein expression shown as green fluorescence and ChAT expression is red fluorescence. Image A: ChAT positive cells. Image B: Neurons positive for Bim expression. Image C: Merged image of Bim positive cells and ChAT positive cells. Findings show that BH3-only protein Bim is indeed being expressed in motor neurons of wild type animals. End Stage Panels: Slides co-stained for Bim and GFAP primary antibodies. GFAP positive cells have green fluorescence and Bim positive cells have red fluorescence. Image A: Motor neurons positive for Bim expression. Image B: GFAP positive cells. Image C: Merged image of Bim positive cells and GFAP positive cells. Findings show that BH3-only protein Bim is being expressed in motor neurons, but not in astrocytes of end stage animals. Dapi, blue fluorescence, stains the nuclei. Slides were imaged at 40x using a digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAP1, CY3, and FITC.

3.4 Quantification of Immunohistochemistry for Motor Neurons and Astrocytes Positive for BH3-Only Protein Expression in Wild Type and End Stage G93A Mutant SOD1 Lumbar Spinal Cord Sections.

Images taken of IHC for BH3-only proteins Bad, Bid, Bnip3, Bim, Noxa, Puma, Bik, and Hrk were analyzed by counting the numbers of motor neurons and astrocytes stained positively for BH3-only protein expression. Four lumbar spinal cords from wild
type animals were imaged for each BH3-only protein, while five lumbar spinal cords from end stage G93A mutant SOD1 animals were imaged for each BH3-only protein. Five images were taken of each animal for each experimental condition. Twenty images were captured per BH3-only protein for wild type conditions and 25 images per BH3-only protein for end stage conditions. Therefore, a total of 360 images were analyzed for quantification of whole number of motor neurons and astrocytes positive for BH3-only protein expression.

Criteria for identification of motor neurons and astrocytes were set such that motor neurons were defined as any whole round cell around 20 microns in diameter and astrocytes were defined as cells with at least one spine like projection. Motor neurons and astrocytes were counted by using a counting template, which took up one fourth of the entire captured image area. Each image was counted one quadrant at a time; then the total number of motor neurons and astrocytes in all four quadrants was recorded. The total number of motor neurons and astrocytes counted in wild type or end stage animals were averaged and graphed. We were surprised to find that a comparison of the whole numbers of motor neurons stained positively for BH3-only protein expression showed no significant differences between wild type and end stage G93A mutant SOD1 animals (Figure 17, two tailed, two paired T-test P>0.05). Additionally, no significant difference was found to exist in the whole number of astrocytes stained positive in lumbar spinal cord sections of wild type and end stage animals for BH3-only proteins Bad, Bim, Noxa, Puma, and Bik (two tailed, two paired T-test P>0.05). However, there was a significant difference in the whole number of astrocytes stained positive in lumbar spinal cord sections of wild type and end stage animals for BH3-only proteins Bid, Bnip3, and Hrk
(Figure 18, two tailed, two paired T-test P<0.05). In the future, we would like to repeat this experiment by co-staining with astrocyte marker GFAP and re-analyze the numbers of whole astrocytes. In addition, we also plan to perform the same experiments in pre-symptomatic G93A transgenic animals to see if the novel expression of BH3-only proteins, Hrk, Bid, and Bnip3, is present in astrocytes during this time frame.

*Figure 17: Total Number of BH3-Only Protein Positive Motor Neurons Counted in Immunohistochemistry Images of BH3-Only Proteins Bad, Bid, BNip3, Bim, Noxa, Puma, Bik, and Hrk.* Four wild type spinal cord sections and five G93A mutant SOD1 transgenic spinal cord sections were stained for each of the BH3-only proteins Bad, Bid, BNip3, Bim, Noxa, Puma, Bik, and Hrk. Five images were taken per condition per wild type or end stage lumbar spinal cord section, resulting in a total of 20 images per BH3-only protein in wild type animals and 25 images per BH3-only protein in end stage animals. No significant difference in the number of BH3-only protein positive motor neurons was found between wild type and end stage G93A mutant SOD1 transgenic spinal cord sections.
Figure 18: Total Number of BH3-Only Protein Positive Astrocytes Counted in Immunohistochemistry Images of BH3-Only Proteins Bad, Bid, BNip3, Bim, Noxa, Puma, Bik, and Hrk. Four wild type spinal cord sections and five G93A mutant SOD1 transgenic spinal cord sections were stained for each of the BH3-only proteins Bad, Bid, BNip3, Bim, Noxa, Puma, Bik, and Hrk. Five images were taken per condition per wild type or end stage lumbar spinal cord section, resulting in a total of 20 images per BH3-only protein in wild type animals and 25 images per BH3-only protein in end stage animals. Asterisks indicate significant differences in the number of BH3-only protein positive astrocytes found between wild type and end stage G93A mutant SOD1 transgenic animals. Specifically, astrocyte expression of BH3-only proteins Bnip3, Bid, and Hrk was found to be significantly higher in G93A mutant SOD1 end stage animals than in wild type animals.

3.5 Controls to Test the Validity of Results Observed for BH3-only protein Immunohistochemistry.

To ensure that the observed results obtained for expression of BH3-only proteins was not due to non-specific staining of our primary or secondary antibodies, two types of
controls were setup. The first control experiment we performed was to demonstrate that the staining observed in lumbar spinal cord sections of end stage G93A mutant SOD1 animals was not due to non-specific binding of the secondary antibody. This was accomplished by incubating lumbar spinal cord sections from the same end stage animal with either primary antibody to BH3-only protein Bid (Figure 19, panel A) or Hrk (Figure 20, panel A), in combination with GFAP, or with primary antibody to GFAP only. Slides were then incubated with a Fitc-conjugated secondary antibody to GFAP to ensure proper set up of IHC and a Cy3-conjugated secondary antibody to the BH3-only protein. As expected, staining of slides that contained no primary antibody to the BH3-only proteins, Bid or Hrk, but did contain the Cy3 secondary antibody was markedly reduced (Figures 19 and 20, panels B). Therefore, the Cy3 secondary antibody was not non-specifically staining astrocytes in end stage animals. Furthermore, the same secondary antibody was used for all immunohistochemistry experiments carried out on wild type and end stage animals.

The second control experiment we performed was to verify that the staining we observed in lumbar spinal cord sections of end stage G93A mutant SOD1 animals with antibodies to either Bid or Hrk was specific. To test this, we stained the lumbar spinal cord from the same end stage animal with primary Bid or Hrk antibody pre-bound with specific blocking peptides or with primary antibody to Bid or Hrk alone. As predicted, the pre-bound Bid primary antibody (Figure 21, panel B) and the pre-bound Hrk primary antibody (Figure 22, panel B) showed little to no positive staining. While staining observed with the non-bound primary antibody to either Bid or Hrk was similar of results we obtained previously (Figures 21 and 22, panels A).
Figure 19: Immunohistochemistry positive and negative control, GFAP Co-stained With or Without Bid Primary Antibody in End Stage G93A Transgenic Animal Spinal Cords. Panel A. Positive Control: Lumbar spinal cord section from a G93A end stage transgenic animal co-stained with Bid and GFAP primary antibodies. Top left image, Bid positive cells using Cy3-conjugated secondary for red fluorescence. Top middle image, GFAP positive cells using Fitc-conjugated secondary for green fluorescence. Top right image merged. Dapi stains the nuclei in all three, blue fluorescence. Panel B. Negative Control: Lumbar spinal cord section from G93A end stage transgenic animal stained with GFAP primary antibody only. Bottom left image, Cy3-conjugated secondary for red fluorescence only. Bottom middle image, GFAP positive cells using Fitc-conjugated secondary for green fluorescence. Bottom right image merged. Dapi stains the nuclei in all three images, blue fluorescence. Images show that secondary is not staining non-specifically in the absence of Bid primary antibody. Image taken at 40x using a digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAP1, CY3, and FITC.
Figure 20: Immunohistochemistry Positive and Negative Control, GFAP Co-stained With or Without Hrk Primary Antibody in End Stage G93A Transgenic Animal Spinal Cords. Panel A. Positive Control: Lumbar spinal cord section from a G93A end stage transgenic animal co-stained with Hrk and GFAP primary antibodies. Top left image, Hrk positive cells using Cy3-conjugated secondary for red fluorescence. Top middle image, GFAP positive cells using Fitc-conjugated secondary for green fluorescence. Top right image merged. Dapi stains the nuclei in all three, blue fluorescence. Panel B. Negative Control: Lumbar spinal cord section from G93A end stage transgenic animal stained with GFAP primary antibody only. Bottom left image, Cy3-conjugated secondary for red fluorescence only. Bottom middle image, GFAP positive cells using Fitc-conjugated secondary for green fluorescence. Bottom right image merged. Dapi stains the nuclei in all three images, blue fluorescence. Images show that secondary is not staining non-specifically in the absence of Hrk primary antibody. Image taken at 40x using a digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAP1, CY3, and FITC.
Figure 21: Immunohistochemistry Specificity of Primary Bid Antibody. Panel A: IHC using Bid primary antibody and Cy3 secondary, red fluorescence. In this image both astrocytes and motor neurons stain positive for BH3-only protein Bid. Panel B: IHC Bid primary antibody pre-bound to Bid peptide, and Cy3 secondary, red fluorescence. Results show that Bid primary antibody is specifically staining motor neurons and astrocytes in end stage G93A animals. Dapi, added to secondary solution, blue fluorescence. Image taken at 40x using a digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAPI and CY3.
Figure 22: Immunohistochemistry Specificity of Primary Hrk Antibody. Panel A: IHC using Hrk primary antibody and Cy3 secondary, red fluorescence. In this image, both astrocytes and motor neurons stain positive for BH3-only protein Hrk. Panel B: IHC using Hrk primary antibody pre-bound to Hrk peptide, and Cy3 secondary, red fluorescence. Results show that Hrk primary antibody is specifically staining motor neurons and astrocytes in end stage G93A animals. Dapi, added to secondary solution, blue fluorescence. Image taken at 40x using a digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAP1 and CY3.

3.6 Immunohistochemistry for Bid and Hrk BH3-Only Protein Expression in Cerebral Cortex, Cerebellum, and Lumbar Spinal Cord of G93A Mutant SOD1 End Stage Animals.

Next, staining for Bid and Hrk BH3-only protein expression in neuronal tissues other than lumbar spinal cord was tested. Cerebral cortex, cerebellum, as well as lumbar spinal cord were isolated from two different G93A mutant SOD1 end stage animals. Sagittal sections of Cortex, Cerebellum, and transverse sections of lumbar spinal cord were co-stained for the BH3-only proteins Bid (data not shown) or Hrk (Figure 23) and
GFAP. BH3-only protein Hrk was also found to be expressed in cortical neurons of the cortex, as well as purkinje neurons in the cerebellum. Yet, co-expression of Hrk and GFAP was only found to be present in astrocytes of lumbar spinal cord sections taken from G93A mutant SOD1 end stage animals (see Figure 24 for quantification).

Figure 23: Co-staining of BH3-Only Protein HrK and Glial Fibrillary Acidic Protein (GFAP) in Cerebral Cortex, Cerebellum, and Lumbar Spinal Cords of End Stage G93A Mutant SOD1 Transgenic Animals. Transverse sections of cortex and cerebellum, and sagittal sections of lumbar spinal cord sections, 12 microns thick, co-stained with Hrk and GFAP primary antibodies. FITC secondary was used for GFAP expression, green fluorescence, and CY3 secondary used for Hrk expression, red fluorescence. Panel A: Cortex from end stage G93A animal stain positive for Hrk in cortical neurons and positive for GFAP in glial cells. Panel B: Cerebellum from end stage G93A animal stain positive for Hrk in purkinje neurons and positive for GFAP in glial cells. Panel C: Lumbar spinal cord from end stage G93A animal stain positive for Hrk in motor neurons and positive for Hrk and GFAP in astrocytes. Dapi, added to secondary solution, stains the nuclei of cells, blue fluorescence. Slides imaged at 40x air using digital de-convolution microscope imaging system based on a Zeiss Axioplan 2 EPI Fluorescence up-right microscope platform, for DAP1, CY3, and FITC.
Figure 24: Quantification of Co-staining of BH3-Only Protein Hrk and Glial Fibrillary Acidic Protein (GFAP) in Cerebral Cortex, Cerebellum, and Lumbar Spinal Cords of End Stage G93A Mutant SOD1 Transgenic Animals. Two sections of each tissue type, cerebral cortex, cerebellum, and lumbar spinal cord, were analyzed from two different G93A mutant SOD1 end stage animals. It was found that only 7 +/- 3% of GFAP positive cells co-stained for Hrk in cerebellum. In cerebral cortex, 14 +/- 1% of GFAP positive cells co-stained for Hrk. Finally, in lumbar spinal cord sections, 88 +/-0.4 % of GFAP positive astrocytes co-stained for Hrk. Therefore, expression of BH3-only protein Hrk appears specific to astrocytes in lumbar spinal cords of G93A mutant SOD1 end stage animals.

3.7 Identifying Genes that Increase in Transcript Expression in Motor Neurons of End Stage G93A Mutant SOD1 Animals as Disease Progresses.

Finally, we tried to identify a gene whose transcript expression would increase as end stage G93A mutant SOD1 ALS disease progressed. Due to the fact that motor neurons are believed to undergo mitochondrial induced apoptosis, and that BH3-only proteins are known to be key regulators of this pathway, we decided to test for gene
expression of Caspase1. cDNA from previous experiments was pre-amplified and ran for real time PCR using Taqman probes to Caspase1 and housekeeping gene β-actin. Findings showed that there was no significant increase in transcript expression of Caspase1 in end stage G93A mutant SOD1 animals compared to wild type animals (Figure 25).

*Figure 25: Normalization of Cycle Threshold Values from Real-Time PCR of Caspase 1 Transcript Expression to β-Actin. cDNA prepared from the 300 motor neurons collected from six end stage and six age matched wild type animals, was pre-amplified for Real-Time PCR with Taqman probes to Caspase 1 protein as well as housekeeping gene β-actin. Cycle threshold values obtained for Caspase 1 expression were normalized to β-actin and graphed by animal set. No significant difference was shown in caspase 1 transcript expression between wild type and end stage animals.*
CHAPTER FOUR: DISCUSSION

In the present study we were surprised to find no significant differences in transcript expression levels for BH3-only proteins Bim/Bod, Dp5/Hrk, Puma, and Noxa in spinal motor neurons captured from end stage mutant SOD1 mice versus age-matched wild type mice. While traditionally the real time PCR data is interpreted in terms of log scale transcript expression, for our purposes we only analyzed threshold values of transcripts. It should be noted that one level of cycle threshold is equivalent to five times the actual transcript expression present. Therefore, this method of analyzing data would be more accurate. However, we believe that further investigation of our cycle threshold values in this manner would still yield no significant difference in BH3-only protein transcript expression due to the similar cycle threshold values recorded in both wild type and end stage animals. Conversely, we have made the novel observation that BH3-only proteins Hrk, BNip3, and Bid are expressed specifically in astrocytes of lumbar spinal cord sections taken from G93A mutant SOD1 end stage animals. This finding was confirmed by co-staining lumbar spinal cord sections of wild type and G93A mutant SOD1 end stage animals with BH3-only proteins Hrk or Bid in conjunction with astrocyte marker glial fibrillary acidic protein (GFAP). Bid or Hrk BH3-only protein expression and GFAP expression were found to co-localize only in astrocytes of end stage animals. On the other hand, Bid and Hrk BH3-only proteins are solely being
expressed in motor neurons of wild type animals, as was demonstrated by co-staining for motor neuron specific protein choline acetyltransferase (ChAT).

Furthermore, quantification of immunohistochemistry for BH3-only proteins Bik, Bad, Bim, Noxa, Bid, BNip3, Hrk, and Puma showed significant increases in the number of astrocytes stained positively for BH3-only proteins Bid, BNip3, and Hrk, as well as significant increases in absolute numbers of astrocytes in end stage mice. Quantification of co-staining for BH3-only protein Hrk and GFAP in lumbar spinal cord, cerebral cortex, and cerebellum revealed selective expression of Hrk protein in GFAP positive astrocytes of lumbar spinal cord only. Our data showing that the BH3-only proteins Hrk, BNip3, and Bid, are expressed specifically in lumbar spinal cord astrocytes of end stage animals are novel; however, the roles that astrocytes may play in ALS disease progression and motor neuron apoptosis have been examined previously.

Astrocytes are the most abundant non-neuronal cells in the spinal cord. Thus, they are thought of as being partners to motor neurons because they provide trophic support in the form of brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and vascular endothelial growth factor (VEGF), as well as mediate rapid uptake of synaptic glutamate with glutamate transporter EAAT/GLT-1 to inhibit excitotoxicity of motor neurons from occurring (Van Den Bosch et al. 2008). Astrocytes respond to damage by becoming activated in a process termed reactive astrogliosis. During this activation, they increase the assembly of their intermediate filaments and increase the number and size of processes extending from the cell body (Boillee et al. 2006). Autopsies taken from twenty human cases of sporadic ALS were stained with astrocyte marker, glial fibrillary acidic protein (GFAP). Analysis
of cervical, thoracic, and lumbar spinal cord sections through immunohistochemistry and histological methods revealed that reactive astrogliosis was occurring in the ventral horns, dorsal horns, and at transition between gray matter and in anterior and lateral funiculi (Schiffer, et al. 1996). Astrogliosis is a prominent characteristic in ALS animal models as well (Hall et al. 1998). It has been postulated that in ALS cases this crosstalk between motor neurons and astrocytes is impaired resulting in insufficient release of neurotrophic factors from astrocytes, astrocytes acting as a source of extracellular mutant SOD1, or through release of hazardous factors (Van Den Bosch et al. 2008). All of these factors could ultimately contribute to the loss of motor neurons in a non-cell autonomous process found in end stage cases of ALS.

*In vivo* studies using transgenic mice expressing mutant SOD1 showed that expression of mutant SOD1 in neurons or astrocytes alone was not sufficient to cause ALS (Pramatarova et al. 2001; Gong et al. 2000). Yet, more recently it has been reported that neuron-specific expression of mutant SOD1 is sufficient to induce ALS in transgenic mice as well as in wild type SOD1 expressing animals crossed to neuron-specific mutant SOD1 transgenic animals (Jaarsma et al., 2008). However, expression of mutant SOD1 in non-neuronal cells is believed to accelerate motor neuron death in ALS (Van Den Bosch et al. 2008). Chimeric mice expressing wild type or mutant SOD1 provided evidence that toxicity to motor neurons requires damage from mutant SOD1 acting from within non-neuronal cells. It was also found that motor neurons need to be adjacent to non-neuronal cells expressing mutant SOD1 to cause disease symptoms and contribute to disease progression (Yamanaka et al. 2008). In agreement with this, when non-neuronal
cells express wild type SOD1, delays in degeneration and extension of survival of nearby motor neurons expressing mutant SOD1 were observed (Clement et al. 2003).

The above in vivo findings are further substantiated by several in vitro observations. For example, when rodent astrocytes expressing mutant SOD1 are cultured with spinal primary and embryonic mouse stem cell-derived motor neurons expressing wild type SOD1, astrocytes secreted soluble toxic factors that were selectively toxic to motor neurons. Furthermore, astrocytes expressing mutant SOD1 were not found to be toxic to dorsal root ganglion neurons or embryonic stem cell-derived inter-neurons. Also, expression of mutant SOD1 in other types of non-neuronal cells, such as microglia, were not found to induce cell death in motor neurons (Nagai et al. 2007). It has also been found that expression of mutant G93A SOD1 in astrocytes co-cultured with motor neurons induced changes in mitochondrial function in both mutant G93A SOD1 motor neurons and wild type SOD1 motor neurons. In particular, mitochondrial function was altered with respect to reduction of the mitochondrial redox state, a decrease in mitochondrial membrane potential in both wild type and mutant SOD1 expressing motor neurons, and an increase in intra-mitochondrial calcium levels only in mutant SOD1 expressing motor neurons (Bilsland et al. 2008). Collectively, these observations suggest that the neurotoxic role of SOD1 may in fact, act through its expression in astrocytes.

Based on our novel discovery of expression of BH3-only proteins Bid, Hrk, and BNip3 in astrocytes of G93A mutant SOD1 end stage animals, the first plan of action was to test for increases in transcript expression of these BH3-only proteins in a highly enriched sample of astrocytes harvested by laser capture microdissection (LCM). Although many attempts were made to optimize the protocol for rapid GFAP staining and
capture of astrocytes on pen membrane slides, they were not successful. This was largely due to the sensitivity of the LCM system. A colored camera in the LCM instrument is not capable of producing the image seen in Figure 26, because it picks up a broad range of excitation and emission fluorescence making it less sensitive than the deep-cooled camera of the Zeiss Axioplan 2 EPI Fluorescence up-right microscope used to take this image. Furthermore, staining on standard glass slides was found to be far more specific than staining on pen membrane slides (Figure 26), which potentially makes it even more difficult to confidently isolate astrocytes with the LCM system using the pen-membrane slides. This is because pen-membrane slides are coated with an RNAse free plastic polymer that tissue adheres to which can be easily cut by an UV laser for successful acquisition of cells through LCM.

To circumvent the above problem we are attempting to utilize protocols from Gonzalez Deniselle et al. 1999 and Hantaz-Ambrosie et al. 1995 to create primary astrocyte cultures from end stage animals. By creating primary astrocyte cell cultures from whole spinal cords of G93A mutant SOD1 end stage animals, we will be able to conduct many experiments to achieve a better understanding of how specific expression of BH3-only proteins Hrk, Bid, and BNip3 in astrocytes may contribute to motor neuron degeneration and disease progression.
It would also be beneficial to test whether or not BH3-only proteins Hrk, Bid, or BNip3 are binding to mutant SOD1 in astrocytes of G93A mutant SOD1 end stage animals because mutant SOD1 has been shown to bind Bcl-2 in aggregates (Pasinelli et al. 2004). This could be tested through co-immunoprecipitation of primary astrocyte culture lysates from G93A mutant SOD1 animals with antibody to a BH3-only protein of interest and analyzed though western blotting or sequencing. It would also be interesting to test whether or not co-culture of wild type motor neurons and astrocytes from G93A mutant SOD1 end stage animals would result in motor neuron apoptosis (Nagai et al. 2009; Bilsland et al. 2008). We could also take media from primary astrocyte cultures from G93A mutant SOD1 end stage animals and transfer the media to flask containing wild type motor neurons only, to see if perhaps astrocytes are secreting toxic factors that
induce motor neuron degradation. We could also test media from primary astrocyte cultures from G93A mutant SOD1 end stage animals and measure levels of reactive oxygen species, quantify carbonyl groups, lipid peroxidation, and cytokines (Nagai et al. 2009; Bilsland et al. 2008). Furthermore, we could knock out expression of BH3-only proteins Hrk, Bid, or Bnip3 in primary astrocyte cultures from G93A mutant SOD1 end stage animals by using siRNA and test whether or not it has a protective role when these astrocytes are co-cultured with wild type motor neurons.

In conclusion, we describe the novel and specific expression of BH3-only proteins Hrk, Bid, and BNip3 in astrocytes of end stage G93A mutant SOD1 mouse spinal cord. Whatever the function of these BH3-only proteins may prove to be in astrocytes of G93A mutant SOD1 end stage animals, we know that they are not functioning to activate the intrinsic mitochondrial apoptotic pathway in astrocytes. This observation suggests an alternate unknown function of these BH3-only proteins acting in astrocytes of G93A mutant SOD1 end stage animals. Therefore, we propose that expression of Hrk, Bid, and BNip3 in astrocytes plays a significant role in motor neuron apoptosis and disease progression in ALS. Future studies examining the role of BH3-only proteins Hrk/Dp5, BNip3 and Bid in astrocytes expressing mutant SOD1 may establish BH3-only proteins as critical inducers of motor neuron apoptosis and prove that they significantly contribute to disease progression in ALS, allowing identification of therapeutic approaches to halt ALS disease onset or slow progression.
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