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GENE EXPRESSION IN MOTOR NEURONS WITH DIFFERENTIAL SUSCEPTIBILITY TO AMYOTROPHIC LATERAL SCLEROSIS (ALS)

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GENE EXPRESSION IN MOTOR NEURONS WITH DIFFERENTIAL SUSCEPTIBILITY TO AMYOTROPHIC LATERAL SCLEROSIS (ALS)

Julia Morton Caponiti

A DISSERTATION

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Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that causes widespread motor neuron degeneration in the brain and spinal cord and eventually leads to paralysis and death. Although most motor neurons are vulnerable to ALS, a few groups survive until late stages of the disease. The oculomotor nucleus (OMN), which innervates the eye muscles, and Onuf's nucleus, which innervates the muscles of the penis and anal sphincter, are both resistant to degeneration in ALS (Mannen et al., 1977; Alexianu et al., 1994). The rodent homologue of Onuf's nucleus is the spinal nucleus of the bulbocavernosus (SNB). Like ALS patients, ALS transgenic rodent models exhibit less cell death in the OMN and SNB than in other motor neuron populations (Nimchinsky et al., 2000; Hamson et al., 2002). My dissertation research is aimed at exploring differences in gene expression between motor neurons that are resistant to degeneration and those that are vulnerable to degeneration with the ultimate goal of identifying genes that can be further explored for their ability to protect against ALS-induced death.

To examine gene expression in motor neurons with differential susceptibility to ALS, I used microarray analysis on the two resistant motor neuron populations (OMN, SNB) compared to two vulnerable motor neuron populations - the trigeminal nucleus (TGN), which innervates face and mouth muscles, and the retrodorsolateral nucleus (RDLN), which innervates the foot muscles. Multiple genes with differential expression between resistant and vulnerable motor neuron populations were identified. After stringent statistical analyses, there were 47 genes with higher expression in the resistant motor neurons and 42 genes with higher expression in the vulnerable motor neurons. Two of these genes were selected for further investigation based on their known functions. Brain expressed, X-linked 1 (*Bex1*) had greater expression in the resistant

motor neuron populations, and ribosomal protein s6 kinase, polypeptide 5 (*Rps6ka5 or Msk1*) had greater expression in the vulnerable motor neurons. I verified the expression patterns of these genes using confocal optical intensity analyses of immunolabeled tissue and western blots to ensure that the differences in gene expression translated into differences in protein expression.

BEX1 and MSK1 were then investigated at a presymptomatic time point in a rodent model of ALS (mutant $SOD1^{G93A}$ transgenic rats) using confocal optical intensity analyses. BEX1 had significantly greater expression in the resistant motor neurons than the vulnerable motor neurons in both the transgenics and the wildtypes, with no significant differences between the two groups of animals. It is possible that BEX1 would be further upregulated after the onset of symptoms, or that it is a protective mechanism that is not modified by the disease process itself. MSK1 had significantly greater expression in the vulnerable motor neurons than the resistant motor neurons, with no difference between groups in the brainstem. However, the wildtype rats had greater MSK1 expression in the spinal cord than the transgenics. It is possible that the disease process does not affect all populations of motor neurons equally. Future studies will need to investigate the expression of BEX1 and MSK1 in later stages of the disease process to fully understand how the disease affects expression levels.

Bex1 and *Msk1* might be suitable targets for gene therapy to modify neuronal survival within the ALS-vulnerable populations of motor neurons. Because the death of motor neurons is a hallmark of ALS, understanding how some motor neuron populations resist degeneration, even in the late stage of the disease, is critical for advancing the treatment of ALS.

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Chapter 1: Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive adult-onset neurodegenerative disease that eventually leads to paralysis and death. Most individuals diagnosed with ALS die within two to three years after the first symptoms are detected (Chiò et al., 2009; Ravitz & La Spada, 2009). ALS causes widespread degeneration of upper and lower motor neurons, leading to muscle weakness and eventually the complete loss of voluntary movement, including the ability to breathe and swallow (Ravitz & La Spada, 2009). Although a century and a half has passed since ALS was first described (Charcot, 1869), neither the underlying cause nor cure has been discovered (Inghilleri & Iacovelli, 2011).

About 30,000 Americans have ALS at any given time, which is a much lower number than those living with other major neurological diseases, such as Parkinson's and Alzheimer's. One reason for the relatively low number of people living with ALS is that the time course of the disease is so rapid that people do not survive for very long after diagnosis. The number of individuals with ALS would be much higher if people with the disease lived more than two to three years, as they do with Parkinson's and Alzheimer's. There are also numerous reports that the prevalence of ALS around the world is increasing, though the cause of this increase is unknown and may be related to various environmental and genetic factors (Maasilta et al., 2001; Georgoulopoulou et al., 2011). Nonetheless, ALS is so debilitating that hundreds of thousands of dollars are spent each year on medical care for each individual with ALS as the disease reaches late stages, including hospitalization, equipment such as ventilators, and home health caregiving. Developing better treatments or effective cures for this disease would ultimately result in fewer individuals suffering as well as decreased overall health care costs.

ALS can be broken into two categories depending on the first onset of symptoms – limb-onset (spinal) form and bulbar-onset (brainstem) form. Two-thirds of patients with ALS have limb-onset form, with the initial symptoms of focal muscle weakness in either the upper limbs (cervical onset) or lower limbs (lumbar onset) (Perry et al., 2010). The other third of patients with ALS have bulbar-onset, which occurs more commonly in women and older individuals and can either be upper motor neuron based (pseudobulbar palsy) or lower motor neuron based (bulbar palsy) (Haverkamp et al., 1995; Forbes et al., 2004). The first symptoms of bulbar palsy are slurred speech, wasting of the tongue, trouble swallowing, and excessive drooling, while the first symptoms of pseudobulbar palsy include excessive yawning and slurred speech (Perry et al., 2010).

Pathological changes in motor neurons, including defects in axonal transport (Kieran et al., 2005), increased ubiquitination (Saxena et al., 2009), and endoplasmic reticulum (ER) stress (Saxena eta al., 2009), are observed before the onset of muscle denervation. Although the primary mechanism(s) contributing to motor neuron degeneration in ALS remain unknown, many perturbations of cellular function and processes have been identified in ALS-affected motor neurons (Nassif et al., 2010). The most prominent hypotheses for the underlying molecular mechanisms of ALS include the buildup and aggregation of intracellular proteins (Bruening et al., 1999; Niwa et al., 2002; Rothstein, 2009), ER stress (Saxena et al., 2009; Sasaki, 2010), oxidative damage (Wiedau-Pazos et al., 1996), glutamate excitotoxicity (Bruijn et al., 1997), growth factor deficiency (Kaspar et al., 2003), and mitochondrial dysfunction (Boillée et al., 2006).

There is not only no existing cure for ALS, there are also no effective therapies to significantly delay progression of the disease or prevent motor neuron death. The main drug used

to treat ALS is riluzole, which inhibits glutamate release from presynaptic terminals and may also inhibit sodium channel conduction (Bensimon et al., 1994; Cheah et al., 2010). Because glutamate excitotoxicity causes excessive activation of glutamate receptors at the synaptic cleft, blocking glutamate release may decrease or delay degeneration (Cheah et al., 2010). However, riluzole only extends life for a few months (Bensimon et al., 1994).

Glutamate excitotoxicity is one of the potential causes of ALS, and one piece of evidence in support of this is the presence of elevated levels of glutamate in the cerebrospinal fluid of ALS patients (Lim et al., 2011). Overactivation of glutamate receptors causes an imbalance in calcium homeostasis, and the increase in calcium influx is toxic to neurons because it leads to the degradation of proteins, membranes and nucleic acids (Dong et al., 2009). An excess of glutamate in the synaptic cleft results in excessive stimulation of glutamate receptors, which leads to an increased intracellular concentration of sodium and calcium ions and excitotoxic cell death (Lim et al., 2011). However, it is still unclear why some groups of motor neurons are more vulnerable to high levels of glutamate than others. Differences in gene expression in ALS-resistant and ALS-vulnerable neurons may cause differential susceptibility to glutamate-induced excitotoxicity. Motor neurons are normally protected from high levels of glutamate by surrounding astrocytes, but astrocytes in the spinal cords of patients with ALS have decreased expression of the focal glutamate excitatory amino acid transporter-2 (EAAT2), which leads to a deficiency in glutamate re-uptake (Rothstein et al., 1995).

Several other pharmacological agents have been examined as treatments for ALS, including celecoxib (Cudkowicz et al., 2006), minocycline (Gordon et al., 2007), and creatine (Rosenfeld et al., 2008). These drugs showed promising results in animal models but were found to be ineffective in clinical trials with ALS patients. Celecoxib is a cyclooxygenase-2 inhibitor

that reduces astrocytic glutamate release, inflammation, and the production of free radicals, but it did not have a beneficial effect on patients with ALS (Cudkowicz et al., 2006). Minocycline inhibits cell death in the central nervous system by reducing activation of enzymes that cause apoptosis and inflammation (Gordon et al., 2007). This drug increases survival in transgenic mouse models of ALS but actually had a harmful effect on patients with ALS. Creatine can enhance mitochondrial function and has neuroprotective properties in a transgenic model of ALS but no impact on disease progression in patients with ALS (Rosenfeld et al., 2008). Although numerous mutations in neurotrophic factors cause motor neuron death and the expression of several of these factors is decreased in ALS, neurotrophic factors have only had modest effects in the treatment of ALS (Gould & Oppenheim, 2011). This lack of success has caused researchers to question whether neurotrophic factors can in fact rescue motor neurons, but ineffective clinical trials could also be due to pharmacokinetic constraints related to drug delivery.

Although individuals with ALS have widespread degeneration of most striated muscles, there is remarkable preservation of muscles of the eye, bladder sphincter, and rectum sphincter (Mannen et al., 1982; Okamoto et al., 1991; 1993; Alexianu et al., 1994; Kihira et al., 1997). Eye movements as well as urinary and fecal continence remain intact even in late stage ALS (Leveille et al., 1982; Schrøder & Reske-Nielsen, 1984). The populations of motor neurons that innervate these muscles, the motor neurons of the oculomotor nucleus (levator palpebrae superioris, superior rectus, medial rectus, inferior rectus, inferior oblique, and sphincter pupillae of the eye), trochlear nucleus (superior oblique muscle of the eye), abducens nucleus (lateral rectus muscle of the eye) and Onuf's nucleus (striated muscles of the rectum and urethral sphincter), are resistant to degeneration in ALS patients (Okamoto et al., 1991; 1993; Alexianu et al., 1994; Kanning et al., 2010). The rodent homologue of Onuf's nucleus is the spinal nucleus of

the bulbocavernosus (SNB), which innervates the muscles of the penis and anus (Breedlove & Arnold, 1981; Breedlove, 1986; Jordan et al., 2002). In the ALS transgenic rodent model (superoxidase dismutase (*SOD1*) mutant G93A), oculomotor, trochlear, abducens, and SNB motor neurons are resistant to death even in the late stages of the disease (Nimchinsky et al., 2000; Haenggeli & Kato, 2002; Hamson et al., 2002; Ferrucci et al., 2010). It is important to note that although the neurons in these nuclei appear to be relatively protected from cell death, they do show some signs of degeneration at very late stages of the disease, such as the presence of ubiquinated inclusions and Bunina bodies (Okamoto et al., 1991; 1993; Kihira et al., 1997). Therefore it is likely that these resistant populations have a significant delay in cell death but not complete immunity to the disease.

The underlying mechanisms that support the preservation of these motor neuron types in the face of mass degeneration and death of all other motor populations are unknown. Identification of the inherent mechanism(s) that confer relative resistance to these two groups of motor neurons could provide valuable insight into the pathology and treatment of ALS. However, very few studies have focused attention on the motor neurons that survive in ALS and instead, the majority of research has aimed at understanding the pathological process that occurs in vulnerable motor neuron populations.

Approximately 90 percent of individuals with ALS have the sporadic form of the disease (sALS), with no known genetic component, while the other 10 percent have familial ALS (fALS). Numerous gene mutations have been identified in ALS (Table 1); however, it is still unclear why these mutations selectively affect certain populations of motor neurons though these mutations are ubiquitous in all neurons. This suggests that inherent differences in the resistant motor neurons allow them to be protected from the effects of the gene mutations. Differential

vulnerability of motor neurons to the disease seems to be independent of the underlying cause, since the pattern of selective degeneration is the same in both familial and sporadic cases of ALS.

The first mutation identified in individuals with ALS was the enzyme superoxide dismutase 1 (SOD1), which accounts for approximately 20 percent of fALS cases (Rosen et al., 1993). In healthy cells, SOD1 removes harmful superoxide free radicals, which can cause protein aggregation and neuronal dysfunction (Burvill, 2009). Mutant SOD1 and the resulting increase in superoxide free radicals causes damage to motor neurons, and reactive oxygen species have been implicated in numerous other diseases as well. It is likely that it is not the loss of normal SOD1 function that causes ALS but rather a toxic gain of function caused by the mutation. *Sod1*-knockout mice show no significant differences in the number and size of motor neurons from wildtypes and, unlike transgenic mice with mutant SOD1, do not develop motor neuron disease (Shefner et al., 1999). SOD1 mutations increase the formation of harmful hydroxyl radicals that damage proteins, organelles, and DNA and result in motor neuron death. Although mutant SOD1 is expressed in all cells, it only causes death in certain motor neuron populations. The question of why vulnerable motor neurons are particularly sensitive to the effects of the mutation remains.

Over 140 individual point mutations in SOD1 have been found in ALS patients, suggesting that protein misfolding may be one of the primary causes (Boillée et al., 2006). Removing misfolded and damaged proteins is important for cell functioning, and the ubiquitin-proteasome system (UPS) is one of the main proteolytic pathways that recycles misfolded or damaged proteins (Burvill, 2009). The unfolded protein response (UPR), also known as ER stress signaling, is a complex set of transcriptional and posttranscriptional events caused by an increase in misfolded proteins in the ER (Kanning et al., 2010). A recent study identified that

mutations in UBQLN2, which encodes the ubiquitin-like protein ubiquilin 2, are associated with ALS (Deng et al., 2011). Mutations in UBQLN2 cause impairments in protein degradation, which leads to abnormal protein aggregation and neurodegeneration. Ubiquilins may be involved in other neurodegenerative disorders as well; for example, ubiquilin 1 is associated with AD (Bertram et al., 2005). There seems to be increasing evidence that protein misfolding and aggregation are an underlying cause of many neurodegenerative disorders, though it is still unclear why this occurs or why it selectively targets specific cells.

The endoplasmic reticulum (ER) is a continuous network of tubules and flattened sacs distributed throughout the cell that serves a variety of functions in the cell, including protein, lipid and steroid synthesis, calcium regulation, and the attachment of receptors on cell membrane proteins. The ER uses high-affinity transporters to take calcium from the cytoplasm into the ER lumen, and receptors distributed throughout the ER are responsible for calcium release from within the ER (Chan et al., 2010). When the concentration of calcium stored within the ER increases to a certain set point, calcium channels open to allow it back into the cytoplasm. These channels are often located close to mitochondria so that they create an influx of calcium into the matrix of mitochondria. When the mitochondria reach their set point of calcium storage, the calcium is returned to the cytoplasm and then either pumped out of the neuron or taken up again by the ER. This ongoing calcium cycle uses up ATP produced by oxidative phosphorylation in mitochondria. The SOD1 protein can be actively transported into the ER lumen and aggregates in the ER-golgi pathway, which can cause ER stress and apoptosis. There is evidence that the UPR is activated in motor neurons of individuals with sALS, and ER stress may be involved in the degeneration process (Sasaki, 2010).

There is a possibility that ALS-vulnerable motor neuron populations are more susceptible to ER stress than ALS-resistant populations. One recent experiment found that vulnerable spinal cord motor neurons in mutant SOD1 (G93A) transgenic mice have higher levels of a luminal ER protein (BiP), which is used as a measure of increased ER stress, starting early in development (Saxena et al. 2009). This is followed by an increase in ubiquitin signal, which may be indicative of an increased ER stress load and an inability for proteins to fold properly (Saxena et al., 2009). These motor neurons also further upregulate BiP after the induction of unilateral sciatic nerve crush in both wild-type and ALS transgenic mice (Saxena et al., 2009). These results imply that even under disease conditions, the resistant motor neuron populations were less susceptible to ER stress than the vulnerable motor neurons. Mutations that cause ER stress and protein misfolding may selectively damage motor neurons that are more susceptible to these types of injuries, and making them less susceptible could slow or halt the disease process.

Another breakthrough in ALS research revealed the involvement of the TAR DNA-binding protein (*TDP-43*), which is a major component of the protein aggregates found in many cases of fALS and sALS (Arai et al., 2006). Numerous other genes involved in fALS, such as alsin (*ALS2*) and fused in sarcoma protein (*FUS*), have been identified using techniques such as linkage analysis and positional cloning (Table 1). There are also tests for mutations that cause ALS in five different genes, including *SOD1*, angiogen (*ANG*), lipid phosphatase (*FIG4*), tar DNA-binding protein (*TDP-43*) and fused in sarcoma (*FUS*) (Bedlack, 2010).

Table 1. Genes involved in ALS (Adapted from Baumer et al., 2010; Nassif et al., 2010; Traub et al., 2011).

Gene	Protein	Function	Rep. References
ALS2	Alsin (ALS2)	-Regulates and activates particular GTPases -Modulates microtuble assembly, membrane organization and trafficking	Hadano et al., 2001
ANG (ALS9)	Angiogenin (ANG)	-Induces neovascularization -Involved in transcription, translation, and response to hypoxia	Greenway et al., 2006 McLaughlin et al., 2011
CHMP2B (ALS16)	Charged multivesicular body protein 2 B (CHMP2B)	-Involved in recycling and degradation of cell surface receptors	Parkinson et al., 2006 Cox et al., 2010
DAO	D-amino acid oxidase gene (DAO)	-A detoxifying agent which removes D-amino acids (including D-serine)	Mitchell et al., 2010
DCTN1 (ALS14)	Dynactin (DCTN1)	-Involved in retrograde axonal transport	Münch et al., 2004 Münch et al., 2005
ELP3	Elongator protein 3 (ELP3)	-Contributes to transcript elongation and regulates the maturation of projection neurons -Involved in transcription and post-transcriptional processing	Simpson et al., 2009
FIG4 (ALS11)	Lipid phosphatase (FIG4)	-Involved in signaling and vesicle trafficking	Chow et al., 2009
FUS/TLS (ALS6)	Fused in sarcoma/ Translocated in liposarcoma (FUS/TLS)	-A multifunctional protein complex involved in pre-mRNA splicing and the export of fully processed mRNA to the cytoplasm -Regulates gene expression -mRNA/miRNA processing	Kwiatkowski et al., 2009 Vance et al., 2009
OPTN (ALS26)	Optineurin (OPTN)	-Involved in pathways mediating apoptosis and inflammation -Involved in membrane trafficking, vesicle trafficking, and transcription activation	Maruyama et al., 2010
PON1, 2, 3	Paraoxonase gene cluster (PON1, PON2, PON3)	-Involved in the response to hypoxia	Ticozzi et al., 2010

TARDBP (ALS10)	Transactive response (TAR) DNA-binding protein 43 (TDP-43)	-Regulates transcription and alternative splicing -One of the primary components of ubiquitin-positive inclusion bodies	Arai et al., 2006 Neumann et al., 2006 Kabashi et al., 2008
SETX (ALS4)	Senataxin (SETX)	-DNA/RNA helicase involved in transcription, splicing, and post-transcriptional processing	Chen et al., 2004 Zhao et al., 2009
SOD1 (ALS1)	Superoxide dismutase-1 (ALS1/SOD1)	-Catalyzes the reaction that breaks down the toxic superoxide anion radical	Rosen et al., 1993
SPG11	Spatacsin (SPG11)	-Involved in axonal transport -A transmembrane protein that is phosphorylated upon DNA damage	Orlacchio et al., 2010
UBQLN2	Ubiquilin-2 (UBQLN2)	-Involved in protein degradation	Deng et al., 2011
VAPB (ALS8)	Vesicle associated membrane protein (VAMP)-associated protein B (VAPB)	-Involved in ER-Golgi transport and secretion -Mutations may cause accumulation of transport intermediates in the form of cytosolic membranous aggregates	Nishimura et al., 2004
VEGF	Vascular endothelial growth factor (VEGF)	-Involved in angiogenesis and the response to hypoxia	Oosthuyse et al., 2001

In the study of neurodegenerative disorders, there is a tendency to study the unhealthy, dying neurons for good reason. Studying dying cells has helped identify the mechanisms underlying degeneration, and much of this information holds promise for devising therapies to halt death of motor neurons. However, this approach has not yet yielded any truly effective therapies, and alternative approaches may be necessary to provide the information needed to make a real breakthrough in this problem. Many studies are focusing on gene mutations that produce neurodegeneration, but this approach does not emphasize the fact that although the mutations are ubiquitous in all cells, the disease process only negatively impacts certain groups of cells. One possible alternative approach is to investigate the characteristics of healthy neurons

that are resistant to each disease. Studying the mechanisms that maintain neuronal function in the face of a devastating disorder may provide novel insight to the degeneration process and potential ways to prevent or reverse neuronal death. In ALS, some motor neurons do not die. These intriguing anomalies raise questions about whether naturally occurring protective mechanisms exist within these cells. Identifying and harnessing such mechanisms may provide clues to developing new strategies to encourage neuronal maintenance in diseased individuals.

The existing studies that have examined the differences between resistant and vulnerable motor neuron populations are limited and use biased approaches. These experiments have used light and electron immunohistochemical methods to examine the expression of antigens that are specifically associated with cell survival and death, such as calcium binding proteins and glutamate receptor subunits. Some studies have found significant differences between resistant and vulnerable motor neurons in calcium binding proteins (Alexianu et al., 1994; Obál et al., 2006) and glutamate receptor subunits (Medina et al., 1996; Laslo et al., 2001), while others have not found perceptible differences (Probst-Cousin et al., 2004). Some studies examined gene expression in spinal cord tissue (Offen et al., 2009) or muscle (Shtilbans et al., 2011) in individuals with ALS or ALS transgenic animals compared to controls, but these approaches highlighted differences in gene expression caused by the disease after onset, not necessarily protective mechanisms that could be encouraged to prevent degeneration.

In Parkinson's disease, a disorder characterized by degeneration of dopaminergic neurons in the substantia nigra, there is relative vulnerability to degeneration even within adjacent populations of dopamine neurons. Several studies have identified differences in protein expression between the resistant and vulnerable populations, and researchers have found significant differences in gene expression between the two groups using laser capture

microdissection and gene microarray (Chung et al., 2005). The methodology used in these studies is similar to the approach and techniques used in the current study. They found that exposing vulnerable cells to the proteins that were generally higher in the resistant neurons, including gastrin releasing peptide (GRP), calcitonin/calcitonin gene-related peptide alpha (CGRP), and pituitary adenylate cyclase activating polypeptide (PACAP), decreases vulnerability to toxicity, suggesting that these genes could be used to make the susceptible cells more resistant to the disease. They also found that overexpressing G-substrate, an endogenous inhibitor of Ser/Thr protein phosphatases, protects dopaminergic cells from toxins (Chung et al., 2007), and that one RAB3 isoform (RAB3B) improves dopamine transmission at presynaptic terminals and protects vulnerable DA neurons (Chung et al., 2009). Techniques similar to this can be applied to other neurodegenerative diseases to provide insight into potential mechanisms underlying the differential susceptibility to degeneration.

Until the mechanisms that underlie relative resistance of some neuronal populations are fully understood, it will be difficult to develop treatments for each neurodegenerative disease that can halt or reverse degeneration in the susceptible populations. Understanding how some motor neuronal populations resist degeneration, in the late stages of the disease, may be crucial for advancing the treatment of ALS. My dissertation research is designed to fill in the gaps in the field of ALS research by examining resistant populations of motor neurons using first an unbiased approach of DNA microarray analysis and then focusing on relevant genes with known functions in other systems to explore further. The ultimate goal of this research is to uncover potential mechanisms that contribute to selective degeneration in individuals with ALS that can then be harnessed to turn vulnerable motor neurons into resistant motor neurons that are protected from the disease. It is also possible that genes that are differentially expressed in motor

neuron populations may provide insight into the relative resistance of some neurons in neurodegenerative diseases other than ALS because of the many overlapping mechanisms in these diseases.

Chapter 2: Identifying Genes of Interest

My research examined gene expression in motor neurons with differential susceptibility to degeneration in ALS in order to provide insight into the disease process and possible protective mechanisms. Two motor neuron populations that are resistant to ALS, the oculomotor nucleus (OMN) and the spinal nucleus of the bulbocavernosus (SNB), were compared to two motor neuron populations that are vulnerable to ALS, the trigeminal nucleus (TGN), which innervates muscles of the face and mouth, such as the masseter muscle, and the retrodorsolateral nucleus (RDLN), which innervates the flexor digitorum brevis muscle in the foot (Leslie et al., 1991). The OMN and TGN are brainstem nuclei and the SNB and RDLN are spinal cord nuclei (Figure 1). The OMN is the resistant brainstem population studied most frequently in the ALS literature, and the TGN is commonly examined as a vulnerable motor neuron population for comparison purposes. The OMN and TGN are located fairly close to each other in the brainstem, and both nuclei are quite conspicuous and easy to identify in coronal sections through the rat brainstem. The SNB and RDLN are well-characterized nuclei located at the same level of the spinal cord that are often compared in the literature (Jordan et al., 1991; 2002). Because the spinal cord nuclei were the most difficult to identify, I conducted preliminary back-labeling studies with the retrograde tracer fluorogold injected into target muscles to ensure the accurate identification of the SNB and RDLN motor neurons.

It was essential to use all four nuclei for this experiment in order to narrow down the number of differentially expressed genes and to ensure that the genes I identified were differentially expressed because of their disease susceptibility rather than because they were located in a particular part of the nervous system (brainstem or spinal cord) or because of inherent differences between the groups of motor neurons. For example, the SNB is a sexually

dimorphic nucleus that is sensitive to circulating levels of androgens, whereas the RDLN is not (Jordan et al., 1991; 2002). The brainstem nuclei were included in this study in part to ensure that differences between resistant and vulnerable motor neurons were not caused by varying levels of hormone sensitivity. Another inherent difference is that the OMN and TGN control mainly reflexive movements, while the SNB and RDLN control voluntary movements. The OMN contains only general visceral efferent fibers (preganglionic autonomic fibers), whereas the TGN contains several types of fibers, including general sensory afferent fibers and special visceral efferent fibers (Büttner-Ennever, 2007; Angeles Fernández-Gil et al., 2010).

Motor neurons in each population of interest were individually dissected from brainstem and spinal cord sections using laser capture microdissection, and the RNA from these neurons was isolated and amplified in order to run a microarray experiment comparing gene expression. Microarrays of these four populations of motor neurons revealed significant differences in gene expression between those that are resistant and those that are vulnerable to degeneration in ALS.

There are several possible interpretations of differential gene expression. A gene with greater expression in the resistant than the vulnerable populations could play an active role in protecting these motor neurons from degeneration. A gene with greater expression in the vulnerable than the resistant populations could contribute to degeneration. However, it is also possible that the vulnerable motor neurons have high levels of a protective gene in a non-disease state, but an ALS-induced decrease in the expression of this particular gene could make these motor neurons more susceptible.

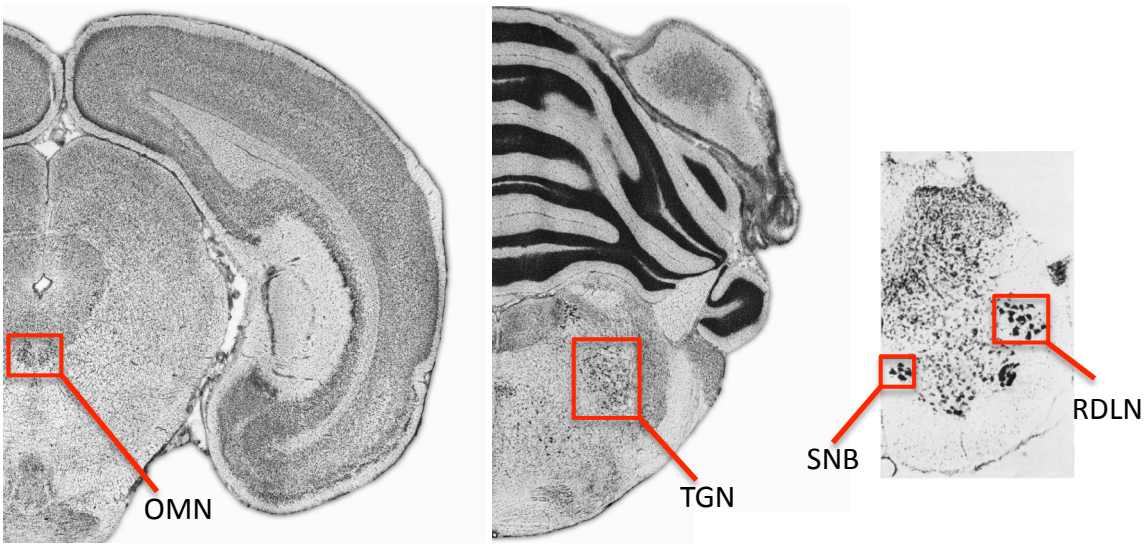


Figure 1. Representative images showing the locations of the motor neuron populations of interest, OMN and TGN in the brainstem and SNB and RDLN in the lumbar spinal cord. The spinal cord section is presented at a much higher magnification than the brainstem sections. The brainstem images were adapted from Paxinos & Watson, 2005 and the spinal cord image was adapted from Breedlove & Arnold, 1981.

Methods

Experimental Animals.

Because I am interested in the baseline gene expression of motor neurons in the undiseased state, I began the analysis by examining wildtype animals. An additional advantage of this approach is that once the disease process sets in, gene expression is likely to change, particularly in the vulnerable motor neurons, thus potentially obscuring any baseline differences that may confer susceptibility. All experiments used group-housed adult male Sprague-Dawley rats (Taconic) around 65 days old with ad libidum access to food and water. Rats were selected for these studies instead of mice because considerable work has been done on this species with regard to the SNB in rats (Breedlove & Arnold, 1981; Breedlove, 1986; Jordan et al., 2002), the nuclei are much larger (approximately 4x) in rats, and a transgenic ALS rat model now exists

(Nagai et al., 2001; Howland et al., 2002). The rats were maintained on a 12 hour light-dark cycle (lights on at 7 a.m.). All procedures were approved by Princeton University IACUC and conformed to guidelines for the care and use of animals written by the US National Institutes of Health.

Fluorogold Tracer Injections.

As a preliminary experiment to help identify the spinal cord motor neurons, the SNB and RDLN motor neurons were backfilled with the retrograde tracer fluorogold (Fluorochrome, LLC). To backfill SNB motor neurons, adult male rats were anesthetized with Nembutal and 5 μl of 10% fluorogold was injected into the bulbocavernosus muscle as previously described (Ottem et al., 2007). To backfill RDLN motor neurons, similar injections were made into the flexor digitorum brevis muscle of the foot. After a three-week post-injection survival time, the rats were anesthetized with pentobarbital and transcardially perfused using 4% paraformaldehyde in 0.1M phosphate buffer. The brainstems and spinal cords were removed, postfixed in the fixative overnight and then cryoprotected in 30% sucrose. They were then embedded in cryo-gel and frozen at -80^{o} C until being cut. 40 μm coronal sections throughout the brainstem and spinal cord were cut with a cryostat into 0.1M PBS. The sections were dehydrated and visualized on a fluorescent microscope to identify the location of the SNB and RDLN motor neuron populations within the spinal cord. After familiarization with the location of these nuclei using retrograde tracer injections, I was able to accurately identify the motor neurons without the use of this dye and did not need to control for potential gene expression changes as a result of the presence of retrograde tracer within the motor neuron.

Laser Capture Microdissection (LCM).

Adult male Sprague-Dawley rats were rapidly decapitated, and brainstems and spinal cords were quickly dissected, embedded in cryo-gel on dry ice, and stored at -80 degrees C until they were processed for LCM. The tissue was sectioned at 6 to 8 μm on the cryostat at -22 degrees C, mounted on slides, dehydrated in graded ethanol solutions (75%, 95%, 100%), and cleared in xylene (Lombardino et al., 2006). Individual motor neurons in the populations of interest were identified by size, morphology and location within the brainstem and spinal cord, and then they were microdissected using LCM (Arcturus PixCell IIe Microdissection System). After dissection, the RNA from the motor neurons was extracted and then frozen at -80 degrees C until the isolation and amplification procedures.

Microarray.

The RNA was extracted and isolated using the Arcturus PicoPure Isolation kit. RNase-free DNase treatment (Qiagen) was used during isolation in order to prevent DNA contamination. After the RNA was isolated, the quality of the RNA from the TGN group was assessed using the Agilent RNA 6000 Pico Kit with an Agilent Bioanalyzer 2100. The TGN group was used as a representative quality check for the overall LCM-acquired samples because this group contained the most collected cells, and therefore a portion of the sample could be taken out without compromising subsequent amplification and labeling.

The Agilent Low Input Quick Amp Kit was used for RNA amplification and labeling. This kit produces fluorescent cRNA from a low starting quantity of mRNA and uses T7 RNA polymerase to simultaneously amplify the RNA and incorporate the fluorochrome cyanine 3 (Cy3) or cyanine 5 (Cy5). Each experimental sample (OMN, TGN, SNB, and RDLN) was labeled with the fluorochrome Cy3 and then purified using the Qiagen RNeasy Kit. Universal rat

reference RNA (Stratagene) was amplified using the same procedure and labeled with Cy5. Universal rat reference RNA was used because it provides hybridization signal at each microarray probe location, decreases variability by normalizing signal output in any two-color hybridization experiment, and allows more reliable comparison of gene expression data within and between groups (Novoradovskaya al., 2004).

After amplification, labeling, and purification, the amount of labeled RNA in each group was quantified on a NanoDrop ND-1000 Spectrophotometer to ensure adequate dye incorporation and quantity of cRNA. The labeled RNA was then hybridized to an Agilent Whole Rat Genome Oligonucleotide 4x44 Microarray with each of the experimental groups hybridized with the rat reference RNA. Two different groups of animals for the brainstem (n=5-7) and two different groups of animals for the spinal cord (n=11-14) were used to create biological replicates. Two technical replicate microarrays were performed within each biological replicate, giving a total of 16 microarrays, in order increase the reliability of my results and to provide additional power for carrying out appropriate statistical analyses. For microarray experiments, technical and biological replicates are necessary in order to ensure consistency and to measure reproducibility (Lee et al., 2000; Chuaqui et al., 2002).

Microarray Data Analysis.

The microarray data was normalized using Agilent Feature Extraction, and the Princeton University Microarray database (PUMAdb) was used for data filtering. Replicate spots were collapsed by Sequence Unique Identifier (SUID), and then filtered based on spots with red (Cy5) and green (Cy3) intensity well above background and red and green net intensity positive and significant. The genes were retrieved as log (base 2) (red signal/green signal). The reproducibility of each technical replicate was examined using Pearson's correlation.

The brainstem and spinal cord microarray data were analyzed using a two-way ANOVA (de Haan et al., 2007) for each gene, with the factors of susceptibility to ALS (resistant vs. vulnerable) and population location (brainstem vs. spinal cord). Further analyses were only conducted on genes that had a significant main effect of susceptibility to ALS, but not those that had a significant interaction between the two factors. These criteria limited all follow-up studies to genes that showed an effect of ALS susceptibility that generalized across both resistant or both vulnerable motor neuron populations. A consistent criticism of microarray experiments is the high number of false positives generated due to the large overall number of genes being tested. In order to further protect against false positives that may be caused by the multiple ANOVA comparisons, the false discovery rate (FDR) correction was conducted and only genes that still had a significant F-statistic using the cut-off of $p < 0.01$ after the correction were considered (Benjamini & Hochberg, 1995). The genes that passed through these statistical criteria were also examined for the fold change between the resistant motor neuron groups (OMN and SNB) and the vulnerable motor neuron groups (TGN and RDLN). Only genes that had a fold change greater than two were considered for the follow up studies, which is a fold change cutoff that is widely used in the microarray literature (Schena et al., 1996). It is advantageous to simultaneously apply p-value and fold change in order to identify genes with more statistical confidence than the use of either technique alone (Noel et al., 2008).

Allen Brain Atlas.

The distribution and location in the brainstem of the genes of interest identified through the microarray experiment were investigated in the Allen Brain Mouse Atlas (Lein et al., 2007; http://www.brain-map.org/). The Allen Brain Atlas is a database of gene expression images that has been created using in situ hybridization. Since the spinal cord motor neuron populations I

examined are relatively small, they are underrepresented in this atlas and not available for many of the genes of interest. However, the brainstem nuclei of interest were identifiable for most genes, and this was an important resource to confirm gene expression in these populations and further narrow down the list of candidate genes. Genes of interest were investigated in three resistant brainstem populations (oculomotor, trochlear, and abducens nuclei) and three vulnerable brainstem populations (trigeminal, facial, and hypoglossal nuclei). Genes that did not have consistently higher mRNA expression in the group of brainstem nuclei that showed higher expression in our microarray analysis were not included in follow up studies.

Results

Fluorogold.

The location of the SNB and RDLN nuclei were visualized using retrograde tracer injections so that I was able to accurately identify the motor neurons without the use of this dye. Fluorogold-labeled motor neurons had similar morphology to those stained with the motor neuron marker choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine, in the same area of the lumbar spinal cord (Figure 2).

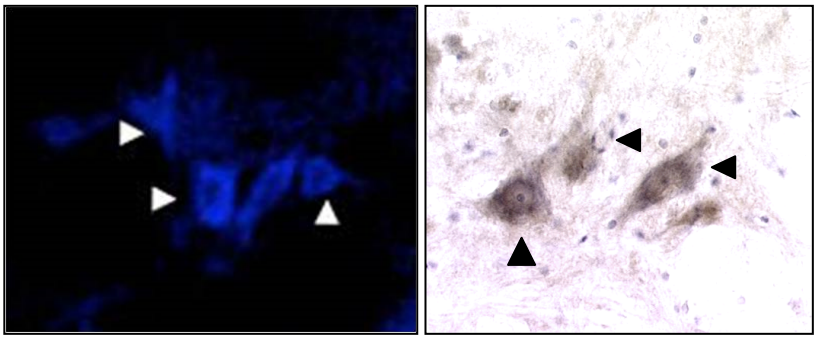


Figure 2. Fluorogold-labeled motor neurons in the SNB of an adult male rat (left, arrowheads) backfilled with tracer injection into the bulbocavernosus muscle. These neurons have a similar morphology to motor neurons stained with ChAT in the same area of the spinal cord (right, arrowheads).

Microarray.

After the RNA was isolated, the quality of the RNA from the TGN group was assessed on the Agilent Bioanalyzer and the resulting RNA Integrity Number (RIN) was 6.9. The labeled RNA in each group (OMN, TGN, SNB, RDLN) was quantified on a NanoDrop ND-1000 Spectrophotometer, and I found that there was adequate dye incorporation and quantity of cRNA for each sample (Table 2).

Table 2. Nanodrop results from each group of motor neurons after isolation, amplification and labeling.

Sample ID	**pmol/µL Cy3**	**ng/µL**	**µg of cRNA yield**	**pmol Cy3/µL RNA**
OMN1	0.76	81.84	2.46	9.29
OMN2	0.85	107.10	3.21	7.94
TGN1	1.10	105.53	3.17	10.42
TGN2	1.33	139.28	4.18	9.55
SNB1	0.71	66.48	1.99	10.68
SNB2	1.71	158.14	4.74	10.81
RDLN1	0.98	83.56	2.51	11.73
RDLN2	1.89	180.3	5.41	10.48

The reproducibility of each technical replicate was examined using Pearson's correlation, and each set of technical replicates showed high reproducibility (all r's > 0.96). After the microarray statistical analysis was complete, there were 47 genes that had significantly higher expression in the resistant motor neuron populations (OMN, SNB) and 42 genes that had significantly higher expression in the vulnerable motor neuron populations (TGN, RDLN) (Table 3).

Table 3. The differentially expressed genes between resistant and vulnerable motor neurons with fold change > 2 and FDR < 0.01.

Gene Name	Symbol	*P*-value	Fold Change
Greater expression in resistant motor neurons (OMN and SNB)			
Cholinergic receptor, muscarinic 3	Chrm3	8.15E-10	7.82
Zinc finger homeobox 4	Zfhx4	8.84E-05	6.49
Calcium channel, voltage-dependent, beta 3 subunit	Cacnb3	5.91E-07	4.72
RAB26, member RAS oncogene family	Rab26	3.49E-10	4.45
Cysteine rich transmembrane BMP regulator 1 (chordin like)	Crim1	4.72E-12	4.41
Guanylate cyclase 1, soluble, alpha 3	Gucy1a3	2.02E-08	4.30
Brain expressed, X-linked 1	Bex1	2.88E-07	3.95
Neurturin	Nrtn	9.97E-07	3.81
Leucine-rich repeats and transmembrane domains 2	Lrtm2	5.27E-05	3.65
Retinoid X receptor gamma	Rxrg	1.11E-04	3.41
Fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific)	Fut4	7.44E-08	3.37
Arginase, liver	Arg1	2.94E-04	3.32
Somatostatin receptor 3	Sstr3	1.45E-04	3.18
Kv channel interacting protein 4	Kcnip4	1.05E-06	3.17
Synaptotagmin XII	Syt12	6.71E-06	3.16
Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	Slc24a3	3.52E-07	3.05
Cytochrome c oxidase, subunit VIIIb	Cox8b	2.77E-05	2.94
Protease, serine, 23	Prss23	3.03E-05	2.84
Synaptotagmin XVII	Syt17	5.55E-09	2.75
Secretin	Sct	4.22E-05	2.65
Visinin-like 1	Vsnl1	9.90E-07	2.63
Myosin XVI	Myo16	8.86E-05	2.60
Phospholipase C-like 2	Plcl2	8.24E-06	2.59
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3	Smarcd3	1.89E-07	2.58
Regulator of G-protein signaling 9	Rgs9	6.17E-05	2.56
Epsin 3	Epn3	3.34E-05	2.45
Myosin, light chain 9, regulatory	Myl9	1.37E-06	2.38
Ring finger protein 152	Rnf152	1.99E-06	2.37
Protein tyrosine phosphatase, receptor type, E	Ptpre	1.83E-04	2.36
Fc fragment of IgG, low affinity IIb, receptor (CD32)	Fcgr2b	8.84E-05	2.31
Zinc finger protein 536	Znf536	9.08E-05	2.30
CD83 molecule	Cd83	1.72E-05	2.29
Mitochondrial protein 18 kDa	Mtp18	1.31E-05	2.27
Ca++-dependent secretion activator 2	Cadps2	3.63E-06	2.26
Doublecortin	Dcx	1.94E-05	2.25

Calcium channel, voltage-dependent, alpha2/delta subunit 1	Cacna2d1	6.80E-06	2.22
Sarcolipin	Sln	1.62E-06	2.21
Tropomyosin 2, beta	Tpm2	5.89E-05	2.19
Interferon regulatory factor 5	Irf5	9.88E-06	2.16
Chemokine (C-X3-C motif) ligand 1	Cx3cl1	2.70E-05	2.15
Inositol polyphosphate-4-phosphatase, type II	Inpp4b	1.18E-05	2.14
Coiled-coil domain containing 65	Ccdc65	1.39E-04	2.14
Insulin-like growth factor 2	Igf2	2.51E-05	2.12
WW domain binding protein 5	Wbp5	4.20E-06	2.12
Proenkephalin	Penk	3.35E-05	2.10
Arginine vasopressin	Avp	1.75E-04	2.09
Huntingtin-associated protein 1	Hap1	7.49E-07	2.03

Gene Name	**Symbol**	***P*-value**	**Fold Change**
Greater expression in vulnerable motor neurons (TGN and RDLN)			
Kinesin family member 7	Kif7	8.85E-10	4.02
Eph receptor A4	Epha4	1.12E-06	3.92
Placental growth factor	Pgf	6.41E-07	3.75
Multiple C2 domains, transmembrane 1	Mctp1	1.09E-08	3.53
Phosphatidylcholine transfer protein	Pctp	1.46E-05	3.28
5-hydroxytryptamine (serotonin) receptor 2A	Htr2a	3.90E-07	3.12
Neurotrophic tyrosine kinase, receptor, type 1	Ntrk1	5.52E-05	3.00
InaD-like (Drosophila)	Inadl	7.15E-06	2.98
RAS-like, family 10, member A	Rasl10a	2.44E-07	2.86
Tetraspanin 18	Tspan18	6.55E-05	2.84
Cd24 molecule	Cd24	6.92E-06	2.82
Neuronal guanine nucleotide exchange factor	Ngef	1.44E-04	2.76
Chondroitin sulfate N-acetylgalactosaminyltransferase 1	Csgalnact1	9.43E-10	2.70
Integrin, alpha 7	Itga7	4.13E-09	2.66
Stromal interaction molecule 2	Stim2	1.43E-04	260
Vav 3 guanine nucleotide exchange factor	Vav3	1.45E-05	2.59
ATP-binding cassette, subfamily G (WHITE), member 2	Abcg2	1.21E-05	2.56
Ataxia telangiectasia and Rad3 related	Atr	9.38E-05	2.54
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 13 (GalNAc-T13)	Galnt13	2.42E-04	2.51
Family with sequence similarity 111, member A	Fam111a	9.63E-05	2.46
Sulfide quinone reductase-like (yeast)	Sqrdl	5.54E-07	2.38
Hemopoietic cell kinase	Hck	3.53E-05	2.37
Low density lipoprotein-related protein 12	Lrp12	3.76E-06	2.36

Myosin light chain kinase	Mylk	2.83E-05	2.35
5' nucleotidase, ecto	Nt5e	7.26E-05	2.33
Doublecortin-like kinase 1	Dclk1	1.65E-08	2.29
Kv channel interacting protein 3, calsenilin	Kcnip 3	5.38E-06	2.26
Death associated protein-like 1	Dapl1	3.34E-04	2.25
Signal transducer and activator of transcription 5A	Stat5a	6.21E-06	2.24
Selenoprotein P, plasma, 1	Sepp1	1.74E-04	2.17
Unc-5 homolog C (C. elegans)	Unc5c	1.75E-04	2.16
Ecto-NOX disulfide-thiol exchanger 2	Enox2	3.92E-04	2.13
EF-hand domain (C-terminal) containing 2	Efhc2	7.15E-05	2.12
Prostaglandin E synthase	Ptges	7.65E-06	2.11
Neural precursor cell expressed, developmentally down-regulated 4-like	Nedd4 l	8.93E-08	2.11
Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	Mthfd 2	7.44E-06	2.10
Neuroligin 1	Nlgn1	3.59E-04	2.05
Ribosomal protein S6 kinase, polypeptide 5	Rps6k a5	1.49E-06	2.05
Stearoyl-Coenzyme A desaturase 1	Scd1	8.26E-08	2.02
Serine (or cysteine) peptidase inhibitor, clade A, member 3N	Serpin a3n	1.06E-04	2.02
Phospholipase C, delta 1	Plcd1	2.74E-04	2.02
Necdin-like 2	Ndnl2	1.64E-05	2.02

I investigated the pattern of expression of each gene of interest (89 total) in the Allen Brain Atlas, and excluded any genes that were not supported by the in situ hybridization data. Of these 89 genes, 35 had gene expression data consistent with the microarray results, 14 had approximately equal expression in resistant and vulnerable populations, 14 had expression patterns inconsistent with microarray data, and 26 were inconclusive based on lack of gene expression data available.

I examined the known functions of each of the candidates from the final list of genes that were still significant after FDR-corrected p-value of $p < 0.01$, greater than two-fold change in expression, and validation by the Allen Brain Atlas. Genes that had higher expression in the resistant motor neurons were investigated for their association with cell survival, neurotrophic

factors, and regeneration whereas the genes that had higher expression in the vulnerable motor neurons were investigated for their involvement in cell death, oxidative stress, and excitotoxicity.

Brain expressed, X-linked 1 (*Bex1*) was chosen as a gene of interest with significantly higher expression in the resistant motor neurons (OMN and SNB), and ribosomal protein s6 kinase, polypeptide 5 (*Rps6ka5* or *Msk1)* was chosen as a gene of interest with significantly higher expression in the vulnerable motor neurons (TGN and RDLN). BEX1 is a small adapter protein that interacts with the p75NTR neurotrophin receptor and calmodulin, and it is involved in axonal regeneration (Vilar et al., 2006; Koo et al., 2007; Nattkämper et al., 2009). MSK1 is a downstream kinase of various MAPK signaling pathways and may be involved in neurotoxin-induced cell death (Deak et al., 1998; Hughes et al., 2003; McCoy et al., 2005; Clark et al., 2007). *Bex1* and *Msk1* are both novel candidates in the field of ALS research, and their involvement in the disease process has not previously been examined.

Discussion

This study identified genes that are differentially expressed in resistant and vulnerable motor neurons by first using a relatively unbiased approach of gene microarray analysis. Individual motor neurons from two resistant motor neuron populations (OMN and SNB) and two vulnerable motor neuron populations (TGN and RDLN) were dissected using laser capture microdissection, and their RNA was amplified for comparison on the microarray. The genes that had differential expression in the resistant and vulnerable motor neurons were also examined in the Allen Mouse Brain Atlas to see whether this in situ hybridization expression database was consistent with the microarray findings.

None of the genes previously found to be involved in mutations associated with ALS (Table 1) were found to be differentially expressed in the resistant and vulnerable motor neuron populations. It is possible that the genes involved in mutations associated with ALS are not differentially expressed in healthy motor neurons that have varying levels of susceptibility to the disease, but instead that the mutations affect specific populations of motor neurons differently. The differences in gene expression found in this study may explain why previously identified mutations impact certain motor neurons more than others. There is something inherently different about the resistant motor neurons that allows them to survive the effects of these ubiquitous mutations, and the purpose of this research is to identify these differences.

I chose two genes to focus on in follow-up studies based on their differential expression and known functions in other systems. Brain expressed, X-linked 1 (*Bex1*) was chosen as the gene of interest with significantly higher expression in the resistant motor neurons, and ribosomal protein s6 kinase, polypeptide 5 (*Rps6ka5* or *Msk1)* was chosen as the gene of interest with significantly higher expression in the vulnerable motor neurons. By choosing these genes, I am now taking a highly biased approach based on the known functions of these genes in other systems and as a result, potentially ignoring many other genes that could be of interest. However, because the next set of studies will require intensive examination of protein expression, the list must be narrowed down. Our microarray data will be posted on a microarray repository website such as BASE (BioArray Software Environment) after the findings are published in order to share the full set of results with the public and help progress the overall field of ALS research. BASE is a MIAME (Minimum Information About a Microarray Experiment guidelines) compliant website used for microarray data management and analysis.

In addition to the differences found in this study between the resistant and vulnerable motor neuron populations, there are also possibilities for differences that would not show up in the microarray results. These false negatives could include molecules that only differ at the translational level, posttranslational and epigenetic differences, and environmental influences on gene expression (Walsh & Henderson, 2004; Hurd & Nelson, 2009). Because these experiments were only done in males, it is possible that certain mechanisms of ALS contribute to the disease specifically in females, and these would not be elucidated in the current study. The Allen Brain Atlas is a useful dataset, but any inaccuracies in this atlas could have caused real gene differences in our experiment to be disregarded rather than examined further. In addition, because this experiment only investigated two particular populations of resistant and vulnerable motor neurons, it is possible that the mechanisms determining susceptibility differ in other resistant populations. Though it is important to consider these potential false negatives, these microarray experiments provided the opportunity to examine overall differences in gene expression and identify two genes that have not previously been studied in relation to ALS, possibly giving insight into the mechanisms underlying differential susceptibility.

Because BEX1 has greater expression in the resistant motor neurons, it is predicted that high levels of BEX1 protect motor neurons from degeneration in individuals with ALS, and the absence of BEX1 could cause degeneration. Because MSK1 has greater expression in the vulnerable motor neurons, it is predicted that high levels of MSK1 contribute to degeneration, and the absence of MSK1 could protect motor neurons. It is also possible that high levels of MSK1 in vulnerable motor neurons suggest a reliance on that molecule for survival. The disease process could result in a drop in a survival factor or an increase in a factor that is harmful.

Additional studies will be necessary to determine the role of BEX1 and MSK1 in these motor neurons.

Brain expressed, X-linked 1 (Bex1).

Bex1 displayed 3.95-fold higher expression in the resistant motor neurons compared to the vulnerable motor neurons. BEX1 is an adapter protein that interacts with the intracellular domain of the p75NTR neurotrophin receptor ($p75^{NTR}$), which is one of the two main receptors for neurotrophic factors and growth factors (Vilar et al., 2006). $p75^{NTR}$ signaling is involved in neurotrophin-mediated survival, growth, and neural differentiation, but can also mediate cell death and inhibit regeneration, depending on the activating ligands (Vilar et al., 2006). BEX1 may play a specific role in cell cycle regulation and cell differentiation, though the mechanisms underlying this role are not yet clear.

There is evidence that BEX1 is involved in axonal regeneration, possibly by interacting with the $p75^{NTR}$ receptor molecule on axons to provide a switch for regeneration decisions mediated by this receptor (Nattkämper et al., 2009). BEX1 is upregulated in motor neurons in transgenic mice with altered peripheral myelin protein 22 (Pmp22), which causes demyelinating neurodegeneration such as in Charcot-Marie-Tooth disease. BEX1 is over-expressed after peripheral axonal damage, and plays a role in overcoming the inhibitory effects of myelin-associated glycoprotein (MAG) and other factors that inhibit axonal regeneration through interactions with $p75^{NTR}$ (Khazaei et al., 2010). There is evidence that there are neural mechanisms that sense injury and therefore upregulate *Bex1* expression in order to antagonize molecules such as MAG and promote regeneration.

BEX1 expression is elevated five days after cardiotoxin-induced muscle injury (Koo et al., 2007). Although *Bex1*-knockout mice have a normal phenotype, they show compromised

exercise performance and a slower rate of skeletal muscle regeneration after injury (Koo et al., 2007). *Bex1*-knockout mice also show lower capability for regeneration after peripheral nerve injury than wildtype animals, which supports the idea that BEX1 is necessary for regrowth (Khazaei et al., 2010). Although there are no functional deficits before the sciatic nerve injury in these two groups of mice, there are significant differences in their ability to recover motor functions post-injury. BEX1 has a calcium-dependent calmodulin binding domain, showing that in the presence of calcium, BEX1 interacts with calmodulin, a ubiquitous calcium-binding molecule, and this interaction may be involved in regeneration (Koo et al., 2007). These studies taken together provide evidence that increased levels of *Bex1* could protect OMN and SNB motor neurons from degeneration in ALS through interactions with the $p75^{NTR}$ receptor and calmodulin.

Ribosomal protein s6 kinase, polypeptide 5 (Rps6ka5 or *Msk1).*

Rps6ka5 displayed 2.05-fold higher expression in the vulnerable motor neurons compared to the resistant motor neurons. RPS6KA5, also known as mitogen- and stress-activated protein kinase 1 (MSK1), phosphorylates a range of substrates including the transcription factor cAMP response element binding protein (CREB). MSK1 is expressed in the nucleus of neurons, it is activated via phosphorylation, and it is an important downstream kinase of mitogen-activated protein kinase (MAPK) signaling pathways (Deak et al., 1998; Hughes et al., 2003; McCoy et al., 2005; Clark et al., 2007). The p38 MAPK and the extracellular-signal-regulated kinase (Erk) pathways are two MAPK signaling pathways that activate MSK1 (McCoy et al., 2005; Clark et al., 2007). Misregulation of these MAPK signaling pathways is involved in numerous diseases, ranging from cancer to inflammatory diseases (McCoy et al., 2005). Overstimulation of the MAPK/ERK pathway can promote neuronal cell death in hippocampal

cell cultures exposed to okadaic acid-induced neuronal death (Rundén et al., 1998). Increased MAPK/ERK activation also causes glutamate-induced oxidative toxicity in neurons (Stanciu et al., 2000). Inhibition of the MAPK/ERK pathway can be neuroprotective and prevents neuronal damage and death caused by these insults (Rundén et al., 1998; Stanciu et al., 2000).

Excessive glutamate is the cause of neuronal death in several types of brain injury, possibly through signal transduction pathways such as MAPK. Studies have shown that glutamate induces phosphorylation of MSK1, and inhibitors of the ERK and p38 MAPK pathways inhibit glutamate-induced MSK1 activation (Brami-Cherrier et al., 2007). Magnesium removal induces hyperexcitability, which increases MSK1 and causes the phosphorylation of CREB, culminating in increased death in cultured hippocampal neurons (Hughes et al., 2003). This form of neuronal cell death is blocked in hippocampal neurons from *Msk1*-knockout mice, showing that MSK1 is an essential component of this cell death process. Calcium influx, which can lead to neurodegeneration, also activates MSK1 phosphorylation (Clark et al., 2007).

Surprisingly, there are also several studies showing that MSK1 may play a role in neurotrophin-regulated survival and synaptic plasticity (Arthur et al., 2004; Brami-Cherrier et al., 2007), showing that further research is necessary to elucidate the role of MSK1. In mice deficient for *Msk1*, two neurotrophic factors (BDNF and NT3) are unable to induce CREB phosphorylation, providing evidence that MSK1 is required for neurotrophin-mediated CREB phosphorylation. *Msk1*-knockout mice show spontaneous striatum atrophy as they age, suggesting that it may have a neuroprotective role in this brain region (Martin et al., 2011). Huntington's disease causes selective degeneration in striatal neurons, and there are decreased levels of MSK1 in the striatum of individuals with Huntington's disease (Roze et al., 2008). It is possible that MSK1 plays differing roles in cell survival depending on the location in the brain or

that there is an inverted U relationship for the effects of MSK1, in which there is an optimal amount of MSK1, but too much or too little has deleterious effects.

Several studies have shown that there is increased activation of MAPK signaling pathways in degenerating neurons in individuals with Alzheimer's disease (Arendt et al., 1995; Zhu et al., 2003). There is also evidence of increased levels of activated MSK1 in vulnerable neurons in individuals with Alzheimer's disease compared to age-matched controls, possibly making these neurons more susceptible to degeneration (Webber et al., 2005). These studies taken together suggest that neurons with increased levels of *Msk1*, such as the RDLN and TGN, may be more vulnerable to ALS-induced cell death.

Chapter 3: Verification of differential protein expression of BEX1 and MSK1 in motor neurons that are resistant and vulnerable to ALS

Brain expressed, X-linked 1 (*Bex1*) was identified through the microarray experiments as being significantly higher in the resistant motor neurons, and ribosomal protein s6 kinase, polypeptide 5 (*Rps6ka5 or Msk1*) was identified as being significantly higher in the vulnerable motor neurons. In this study, the expression pattern of BEX1 and MSK1 was validated using confocal optical intensity analyses of immunolabeling and western blots to ensure that the differences in gene expression translated to differences in protein expression. It is crucial to examine protein expression because previous studies in other systems have shown that changes in mRNA levels do not always correspond to similar changes in protein levels (Pascal et al., 2008). Gene expression differences that do not cause protein expression differences are less likely to be functionally relevant.

The detection technique of immunofluorescence labeling combined with confocal optical intensity analysis was used to visualize the specific pattern of expression within the motor neurons in each population of interest, and western blotting was used to quantify the levels of protein expression in each group of motor neurons. In addition to BEX1 and MSK1, protein expression of MSK1 phosphorylated at Ser360 (pMSK1) was also examined because studies in other systems have indicated that MSK1 is regulated by multiple phosphorylation sites and is most active in its phosphorylated state. Ser360 is a proline-directed phorphorylation site on MSK1 that is a direct phosphorylation target of p38 and ERK1/2 pathways rather than autophosphorylation (McCoy et al., 2005). Phosphorylated BEX1 was not investigated because there is much less known about BEX1 phosphorylation and it does not seem that BEX1 activation is directly related to its phosphorylation state (Vilar et al., 2006).

Methods

Experimental Animals.

All experiments used group-housed adult wildtype male Sprague-Dawley rats (Taconic) around 65 days old with ad libidum access to food and water. They were maintained on a 12 hour light-dark cycle (lights on at 7 a.m.). All procedures were approved by Princeton University IACUC and conformed to guidelines for the care and use of animals written by the US National Institutes of Health.

Immunofluorescence Reactions.

I investigated the distribution of BEX1, MSK1, and pMSK1 proteins in the four motor neuron populations of interest using immunofluorescence labeling with well-characterized antibodies (Saper, 2005; 2009). I combined BEX1 and MSK1 immunolabeling with labeling for the cholinergic synthetic enzyme choline acetyltransferase (ChAT), a definitive marker of motor neurons. Male rats (n=8-10) were perfused, and brainstems and spinal cords were removed and processed for immunofluorescence, as previously described (see Chapter 2). 40 μm coronal sections throughout the brainstem and spinal cord were cut with a cryostat into 0.1M PBS. Free floating sections were rinsed in 0.1M TBS, blocked in 3% normal donkey serum for 1 hr, and incubated with goat anti-ChAT (1:100, Millipore AB144P) and either rabbit anti-BEX1 (1:2K, courtesy of Dr. Frank Margolis), rabbit anti-MSK1 (1:100, Santa Cruz sc-25417), or rabbit anti-phosphorylated (Ser360) MSK1 (1:200, Epitomics #2154-1) in TBS with Tween for 2 days at 4 degrees C. Sections were rinsed, incubated with donkey anti-goat Alexa 568 (1:500) to visualize the ChAT and donkey anti-rabbit Alexa 488 (1:500) to visualize the BEX1, MSK1, and pMSK1, then rinsed and mounted on slides. ChAT double-labeling was used to locate the populations of

interest on the microscope and to confirm that BEX1-, MSK1- and pMSK1-labeled cells were motor neurons. The BEX1 antibody stains the cytoplasm of the cell body and the MSK1 and pMSK1 antibodies stain the nucleus of the cells. The specificity of the BEX1 antibody was previously determined using western analysis, immunoprecipitation and preabsorption of the antibody with recombinant Bex1 (Koo et al., 2005; 2007). The specificity of the MSK1 antibody was previously determined with positive controls of UV induced HeLa cell lysate (sc-2221) and JAR (human choriocarcinoma) whole cell lysate (sc-2276) (Santa Cruz datasheet; Pathak et al., 2006; Lorenz et al., 2009). The specificity of the phosphorylated MSK1 antibody was previously determined on HEK293 cell lysate treated with alkaline phosphatase (Epitomics datasheet).

Confocal Optical Intensity Analyses of Immunofluorescence.

Immunofluorescence analyses were done on 1 μm thick optical sections at 40x using a Zeiss Axiovert confocal microscope with Argon 458/488 and HeNe 543 lasers and 510LSM software. For BEX1, 20 motor neurons were scanned for all brainstem and spinal cord nuclei while holding the scanning parameters constant (Leuner et al., 2009). For MSK1 and pMSK1, 20 motor neurons were scanned for the brainstem nuclei and 5 to 20 for the spinal cord nuclei because these populations are much smaller and a relatively low number of SNB motor neurons expressed MSK1. Neurons were systematically selected for analysis based on even staining throughout the cell with definitive motor neuron morphology. Neurons without clear boundaries were excluded from the analysis.

Outlines of all BEX1-stained neurons and MSK1- and pMSK1-stained neuronal nuclei (because of the nuclear localization) were traced in the confocal scans using the measurement tool in the LSM software to determine the mean optical intensity. Because of the observed localization of pMSK1 in the neuropil as well as the nucleus of each cell, an additional optical

intensity measurement was taken for the pMSK1-labeled tissue. For each motor neuron population, 30 optical intensity measurements of 1 mm^2 rectangles immediately adjacent to a motor neuron cell body were taken for each animal. For all reactions, background optical intensity measures of approximately the same size and shape were taken from an adjacent region that did not contain any positive cell bodies.

Signal was defined as the mean intensity value for the cell bodies (for BEX1), the nucleus of the cell (for MSK1 and pMSK1), or the neuropil areas (for pMSK1). The background was defined as the mean intensity value for the comparably sized regions containing no stained cell bodies. Optical intensity was calculated according to the following formula: (Signal – Background) / SD Background (Leuner et al., 2009). The optical intensity data were analyzed using similar statistics to those applied for my gene microarray analysis – a two-way ANOVA for each protein of interest (BEX1, MSK1, and pMSK1), with the factors of susceptibility to ALS (resistant vs. vulnerable) and population location (brainstem vs. spinal cord).

Western Blots.

Western blots were used to quantify the levels of specific proteins in the motor neuron populations of interest. Adult male Sprague-Dawley rats (n=3-6 for each group) were rapidly decapitated and brainstem segments were dissected, embedded in cryo-gel, and stored at -80 degrees C. Brainstem sections were cut on the cryostat at 250 μm, placed on slides on dry ice, and then 1 mm diameter tissue punches were taken from the OMN and TGN nuclei. Western blots were not conducted on spinal cord nuclei because those populations are too small and located too close to one another to have adequate resolution for the tissue punches. The tissue punches were placed in RIPA lysis buffer and then homogenized using an ultrasonic processor. Protein quantity was obtained using the Pierce BCA protein assay. After quantification, 2X

Laemmli buffer was added to the samples and then they were boiled for 10 min. 120 µg protein of each sample was electrophoresed on the gel and transferred to a PVDF membrane.

The membrane was cut into strips, blocked with 5% non-fat milk (for actin, BEX1, and MSK1), or 3% BSA (for pMSK1) for an hour, and then incubated in either mouse anti-β-Actin (1:1000, Sigma A5411), rabbit anti-BEX1 (1:1K, courtesy of Dr. Frank Margolis), goat anti-MSK1 (1:200, Santa Cruz sc-9392), or rabbit anti-pMSK1 (1:K, Epitomics #2154-1) overnight at 4 degrees C. Blots were rinsed in TBST, incubated in anti-mouse HRP secondary (1:10K, Rockland), anti-goat HRP secondary (1:10K for MSK1), or anti-rabbit HRP secondary (1:7.5K for BEX1 and 1:10K for pMSK1, Rockland) for 1 hr, rinsed, and incubated with Pierce SuperSignal West Pico ECL. The blots were then developed on film and analyzed using ImageJ for densitometry. Actin was used to as a loading control to normalize for differences in the amount of total protein loaded to each gel. Any western blots with high background that interfered with the bands were excluded from analysis. The western blots were analyzed with paired one-tailed t-tests comparing expression in the OMN and TGN. One-tailed t-tests were used because there were a priori predictions that the OMN would be greater than the TGN (for BEX1) or that the TGN would be greater than the OMN (for MSK1 and pMSK1).

Results

BEX1.

The BEX1 antibody is a cell body stain, and every BEX1-labeled cell was co-labeled with the motor neuron marker ChAT. Optical intensity measurements were used to assess the differences in BEX1 expression between resistant motor neurons (OMN and SNB) and vulnerable motor neurons (TGN and RDLN) in wildtype rats. As expected given the microarray

analysis and confirmation using the Allen Brain Atlas (see Chapter 2), BEX1 expression was significantly higher in the resistant motor neurons than the vulnerable motor neurons, $F(1,32) = 40.11$, $p < 0.0001$ (Figure 3).

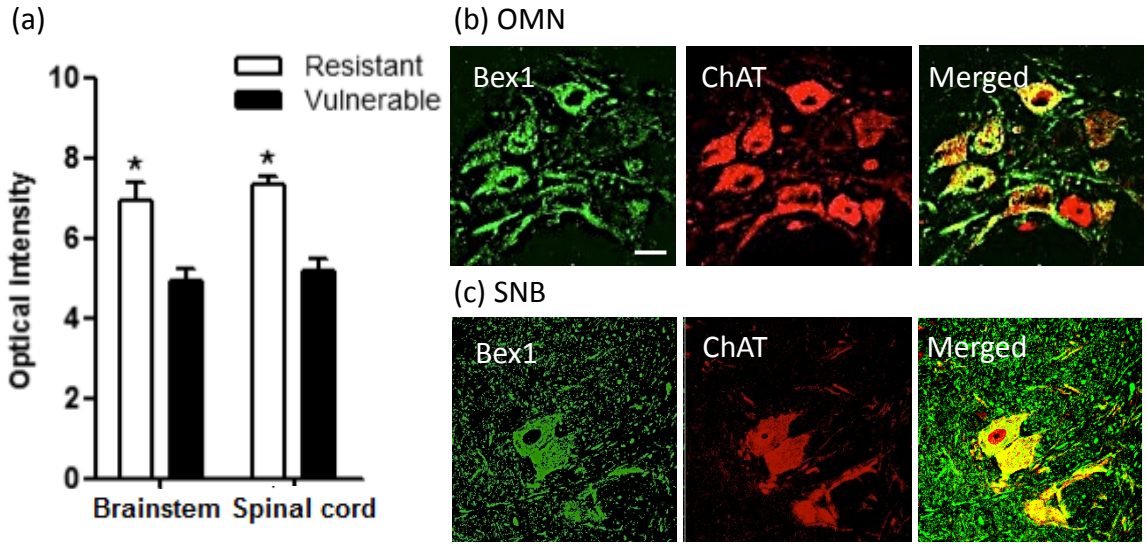


Figure 3. Optical intensity of BEX1 in resistant and vulnerable motor neuron populations in the brainstem and spinal cord. (a) BEX1 had significantly higher expression in the resistant (OMN and SNB) than the vulnerable (TGN and RDLN) motor neuron populations. Representative confocal images of the high expression of BEX1 (green) in ChAT-labeled (red) motor neurons in the (b) OMN and (c) SNB. Error bars represent SEM. Scale bar represents 20 μm and applies to all confocal frames. * $p < 0.05$

Western blots were conducted in order to quantify the amount of BEX1 protein in the brainstem nuclei. Six trials of western blots confirmed the optical intensity findings by showing that BEX1 was significantly higher in the OMN than the TGN, $t(5) = 5.17$, $p < 0.005$ (Figure 4).

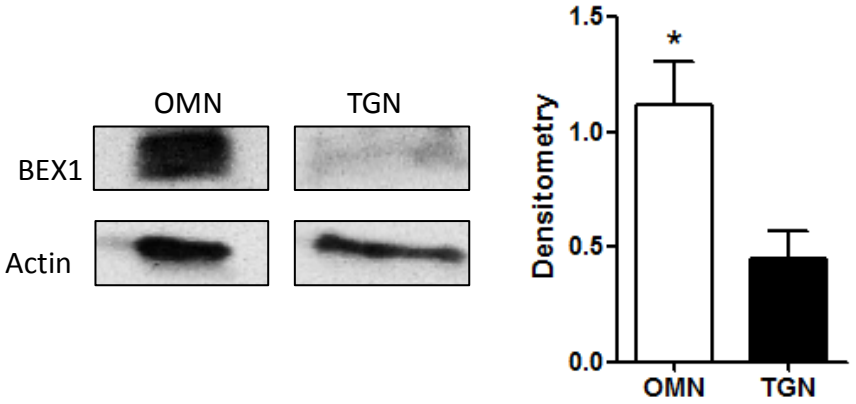


Figure 4. Western blots of BEX1 in brainstem motor neuron populations of interest show that there is significantly higher expression in OMN than TGN. Error bars represent SEM. *$p <$ 0.05

MSK1.

MSK1 staining was localized to the nucleus of each cell, and not all MSK1-positive nuclei were colocalized with the motor neuron marker ChAT. The phenotype of the MSK1-positive/ChAT-negative cells is unknown. As expected given the microarray analysis and confirmation using the Allen Brain Atlas (see Chapter 2), MSK1 expression was significantly higher in the vulnerable motor neurons (TGN and RDLN) than the resistant motor neurons (OMN and SNB), $F(1,28) = 5.865$, $p < 0.05$ (Figure 5).

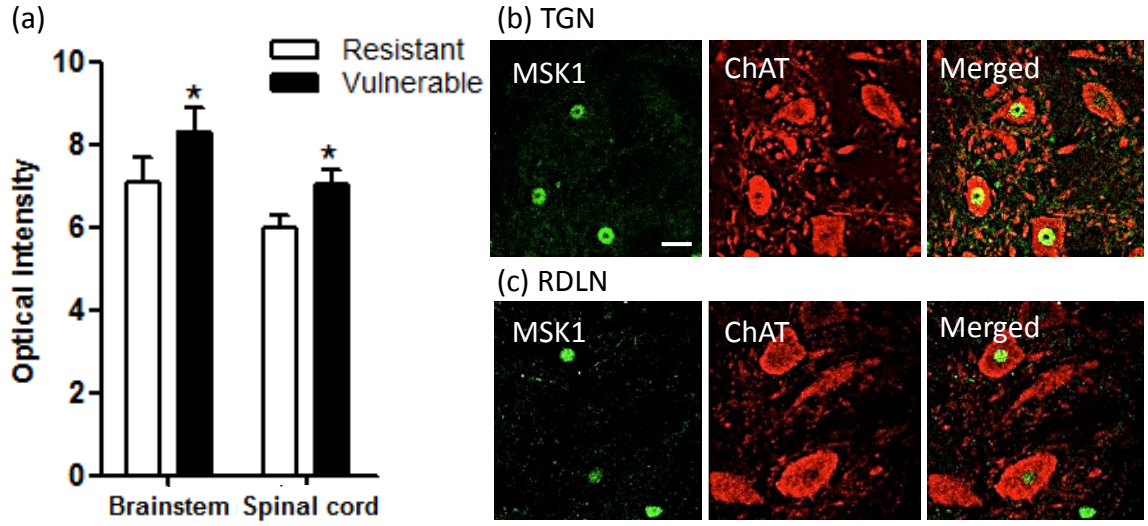


Figure 5. Optical intensity of MSK1 in resistant and vulnerable motor neuron populations in the brainstem and spinal cord. (a) MSK1 had significantly higher expression in the vulnerable (TGN and RDLN) than the resistant (OMN and SNB) motor neuron populations. Representative confocal images of the high expression of MSK1 (green) in ChAT-labeled (red) motor neurons in the (b) TGN and (c) RDLN. Error bars represent SEM. Scale bar represents 20 µm and applies to all confocal frames. * $p < 0.05$

Western blots confirmed the results of the optical intensity analysis for MSK1 in the brainstem nuclei. Four trials of western blots showed that MSK1 was significantly higher in the TGN than the OMN, $t(3) = 2.62$, $p < 0.05$ (Figure 6).

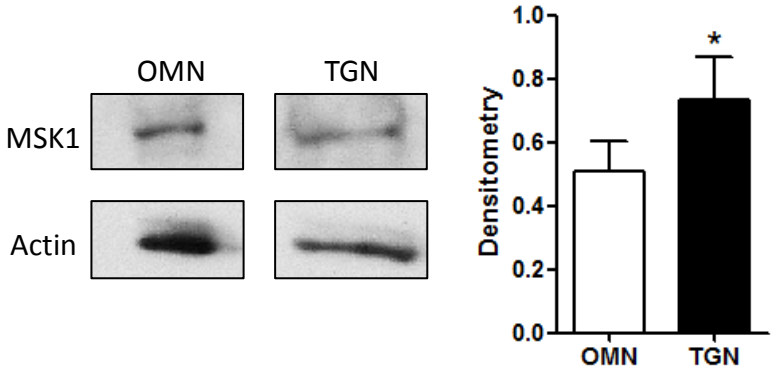


Figure 6. Western blots of MSK1 in brainstem motor neuron populations of interest show that there is higher expression in TGN than OMN. Error bars represent SEM. * $p < 0.05$

Phosphorylated MSK1 (pMSK1).

pMSK1 has a nuclear localization similar to MSK1 and was also found in cells that were not colocalized with the ChAT motor neuron antibody. However, in contrast to MSK1, the staining pattern of pMSK1 was not just localized to nuclei but also showed consistent and intense staining in the neuropil. Because there were two subcellular regions that expressed this protein within the nuclei of interest, two optical intensity analyses were carried out – one on nuclear pMSK1 and the other on neuropil pMSK1. For nuclear pMSK1, there were no significant differences in expression between the resistant and vulnerable motor neurons, $p > 0.05$. For neuropil pMSK1, there was significantly greater expression in the vulnerable motor neurons compared to resistant motor neurons, $F(1, 32) = 82.60$, $p < 0.0001$ (Figure 7). There was also significantly more pMSK1 in the spinal cord neuropil overall than in the brainstem neuropil, $F(1, 32) = 72.14$, $p < 0.0001$.

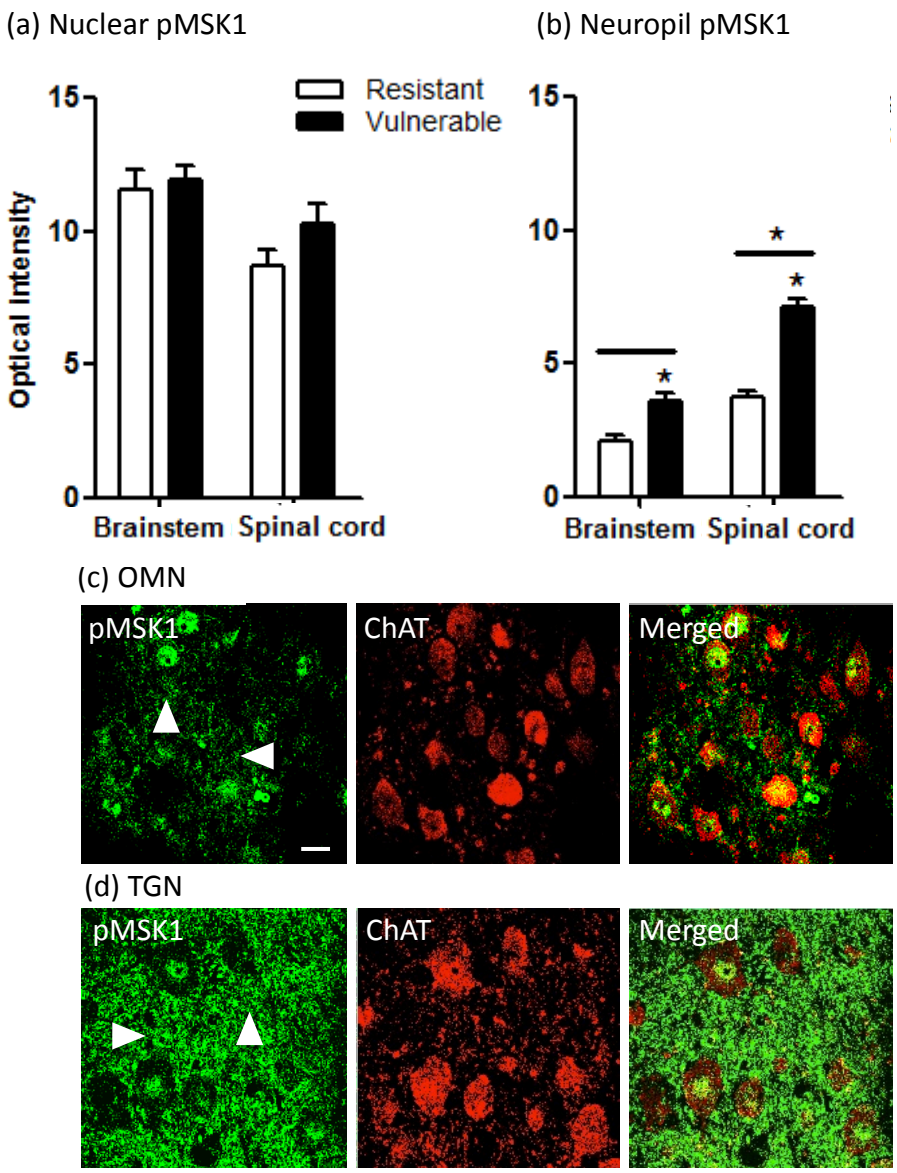


Figure 7. Optical intensity of phosphorylated MSK1 in the nuclei and neuropil of resistant and vulnerable motor neuron populations in the brainstem and spinal cord. (a) Nuclear pMSK1 did not have significantly different expression in resistant and vulnerable motor neurons. (b) Neuropil pMSK1 had significantly higher expression in the vulnerable (TGN and RDLN) than the resistant (OMN and SNB) motor neuron populations. Neuropil pMSK1 expression was higher in the spinal cord than the brainstem. Representative confocal images of the low expression of pMSK1 (green) in neuropil of the (c) OMN compared to high expression in the (d) TGN. Arrowheads indicate pMSK1 neuropil staining. Error bars represent SEM. Scale bar represents 20 μm and applies to all confocal frames. * $p < 0.05$

Western blots were conducted in order to quantify the amount of pMSK1 protein in the brainstem nuclei. Although pMSK1 showed a trend toward an increase in the TGN compared to the OMN across four trials of western blots, the effect did not reach statistical significance, $t(3) = 1.79$, $p = 0.09$ (Figure 8).

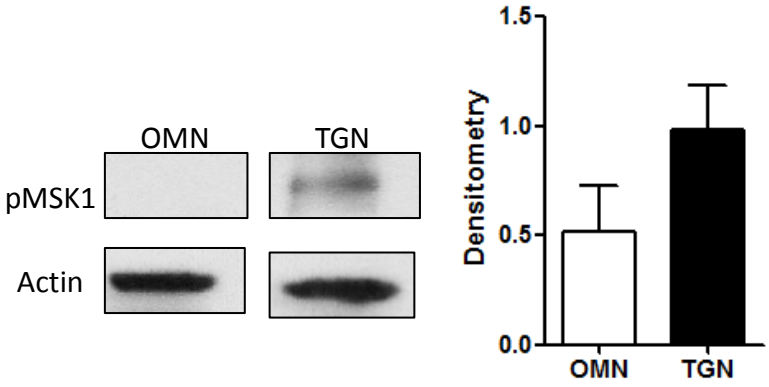


Figure 8. Western blots of pMSK1 in brainstem motor neuron populations of interest show that there is higher expression in TGN than OMN. Error bars represent SEM. $p = 0.09$

Discussion.

These experiments examined the baseline differences in BEX1, MSK1, and phosphorylated MSK1 protein expression in the brainstem and spinal cord nuclei of interest in wildtype male rats. I found that *Bex1* was higher in ALS-resistant motor neurons than ALS-vulnerable motor neurons through the microarray experiments (see Chapter 2), and these findings were validated using confocal optical intensity analyses and western blots. BEX1 was expressed in the cytoplasm of the motor neurons, and all BEX1-positive cells were confirmed as motor neurons by double-labeling with the motor neuron marker ChAT. I found that *Msk1* was higher in ALS-vulnerable motor neurons than ALS-resistant motor neurons through the microarray experiments (see Chapter 2), and these findings were also validated using confocal optical intensity analyses and western blots. MSK1 expression was localized to the nucleus of the cells, and a proportion of MSK1-positive cells were not motor neurons as evidenced by their lack of ChAT colocalization.

There is evidence that age-related changes in the brain that begin around age 50 may make people more susceptible to neurodegenerative diseases (Esiri et al., 2007). The average age of onset of ALS is 55 years old, and it is possible that some of the changes that occur during natural aging may cause the brain to be more sensitive to mutations associated with ALS. As people age, there is an overall decrease in neuronal metabolism, an increase in the number and size of astrocytes and microglia, and an increase in the amount of iron in the brain, which can contribute to oxidative stress (Esiri et al., 2007). There is some evidence that there is a reduction in the numbers of motor neurons and muscle fibers with increased age that selectively affects the largest motor neurons (Vandervoort, 2002), though this may be due to decreases in physical activity rather than a spontaneous decrease in the number of neurons. The pattern of motor neuron loss with normal aging parallels the differential susceptibility to degeneration in ALS. For example, the oculomotor nucleus is completely intact in aged mice whereas the adjacent facial nucleus declines in number (Sturrock, 1991). These studies suggest that the resistant motor neurons may have fundamental properties that protect them from multiple types of stress and injury.

Motor neurons are large cells with extremely long axons, up to one meter in length in humans, and therefore they have increased energy demands compared to other types of cells (Soo et al., 2011). Age-related decreases in metabolism and efficiency affect the cells with the highest energy demands most. Axonal transport is required to carry proteins, lipids, and organelles from the cell body to synaptic terminals or neuromuscular junctions, and also to transport neurotrophins and survival factors from axons back to the soma. There is evidence of impaired axonal transport associated with ALS, including dysfunctional fast and slow axonal transport in the transgenic mice with mutant SOD1 (Zhang et al., 1997) as well as swollen axon

segments and axonal disorganization in human ALS and transgenic mice (Collard et al., 1995; Hirano, 1996). However, it should be noted that the resistant motor neurons are not necessarily resistant because their axons are shorter – the SNB and RDLN motor neurons have long axons, whereas the OMN and TGN motor neurons have shorter axons because they are located closer to the muscles they innervate.

BEX1 is associated with axonal regeneration and regrowth after injury (Nattkämper et al., 2009; Khazaei et al., 2010). If axonal dysfunction is one of the key pathogenic events in ALS, increased expression of BEX1 in specific populations of motor neurons may provide these neurons protection from cell death caused by axonal dysfunction. It is possible that BEX1 helps the axons of the OMN and SNB neurons recover from ALS-induced damage, while motor neuron populations such as the TGN and RDLN do not have high enough levels of BEX1 to receive this benefit.

MSK1 is a downstream kinase of MAPK signaling pathways and it is most active in its phosphorylated state (McCoy et al., 2005). Misregulation of MAPK signaling pathways is involved in several diseases, and overstimulation of these pathways can promote neuronal cell death and cause glutamate-induced oxidative toxicity in neurons (Rundén et al., 1998; Stanciu et al., 2000). Excessive glutamate is the cause of neuronal death in several types of brain injury, and studies show that glutamate stimulates phosphorylation of MSK1 (Brami-Cherrier et al., 2007). Inhibitors of the ERK and p38 MAPK pathways block glutamate-induced MSK1 activation (Brami-Cherrier et al., 2007). Another study showed that inducing hyperexcitability in neurons increases MSK1 and causes neuronal death (Hughes et al., 2003). Calcium influx, which can lead to neurodegeneration, also activates MSK1 phosphorylation (Clark et al., 2007).

Increased expression of MSK1 in specific groups of motor neurons could cause these neurons to be more susceptible to ALS-induced insults, causing degeneration in these neurons.

The expression pattern of phosphorylated MSK1 was different from that of MSK1, indicating that the phosphorylated version of this protein is transported to other parts of the cells. MSK1 is localized exclusively to the nucleus whereas pMSK1 is localized to both the nucleus and the neuropil adjacent to the motor neuron cell bodies. Optical intensity analyses indicated that as with MSK1, pMSK1 was more abundant in the vulnerable motor neuron populations compared to the resistant motor neuron populations, but this effect was localized to the neuropil. The western blots were done on samples taken from a tissue punch of the entire population of brainstem motor neurons, thereby also including neuropil and other cells in that area. The western blots showed that there was a trend toward significance with vulnerable populations having greater expression of MSK1 than resistant populations, and these western blots will be repeated in order to further investigate this apparent difference. One reason for the less robust effect of pMSK1 in the western analysis may be that because the difference in protein levels is localized to the neuropil, much of which radiates from the motor neuron population, the tissue punch dissection may have eliminated some of these neuronal elements.

Although there is not much information in the literature on the expression of phosphorylated MSK1 in neuropil, there is some evidence of localization of this protein outside of neuronal nuclei. A previous study of phosphorylated MSK1 found that pMSK1 is found primarily in the cytoplasm of hippocampal neurons rather than the nucleus (Webber et al., 2005).

The differing levels of expression of BEX1 and MSK1 in certain motor neuron populations discovered in these studies could contribute to the selective pattern of degeneration in individuals with ALS. One of the characteristics of ALS that is least well understood is why

the disease process targets specific motor neurons, even though many of the gene mutations that have been implicated in ALS are ubiquitously expressed. Understanding more about the differences in these populations of motor neurons may provide novel insight into targets that might confer resistance. Now that I have established through confocal optical intensity analyses and western blots that BEX1 and MSK1 are differentially expressed in these motor neurons in wildtype animals, these proteins must be investigated in ALS transgenic animals in order to determine whether the pattern of their expression changes throughout the disease process.

Chapter 4: Differential protein expression in resistant and vulnerable motor neurons in an ALS transgenic rat model

The first mutation identified in individuals with ALS was superoxide dismutase 1 (SOD1), which causes about 20% of familial ALS cases (Rosen et al., 1993). SOD1 normally removes harmful superoxide free radicals from cells, and mutant SOD1 causes an increase in protein aggregation and motor neuron dysfunction (Burvill, 2009). Mutant SOD1 mice were the first rodent model of ALS, and they develop adult-onset progressive motor deterioration similar to human ALS that leads to paralysis and death (Gurney et al., 1994; Wong et al., 1995). These transgenic ALS mice have provided important contributions to the understanding of how mutant SOD1 causes neuronal death and have facilitated therapeutic trials (Gurney et al., 1996). However, certain experimental manipulations are limited in this model due to the innate small size of the mouse, such as administering compounds into the cerebrospinal fluid or obtaining enough tissue to perform biochemical analyses. The development of the transgenic rat model of ALS expressing mutations in SOD1 has been advantageous to the field of ALS research. mSOD1 rats have increased the range of experiments that can be done due to the rats' large size compared to previously used mouse models (Nagai et al., 2001; Howland et al., 2002).

The mutant $SOD1^{G93A}$ transgenic rats are hemizygous and contain a high copy number of the G93A mutant human superoxide dismutase gene (Howland et al., 2002). This line is created by breeding hemizygous transgenic male rats with wild-type Sprague-Dawley females from Taconic. In this mutant SOD1 (mSOD1) transgenic ALS rat line, motor neuron disease begins as abnormal gait and hindlimb weakness and can progress to full paralysis within one to two weeks of symptom onset. Maximum body weight has been identified as a reliable measurement of presymptomatic disease onset. The disease process causes the transgenic rats to decline in weight

due to axonal retraction from neuromuscular junctions, which causes a loss of muscle volume and decreased mobility (Matsumoto et al., 2006). Subjective onset of the disease is generally determined by observable functional deficits such as general muscle weakness or limb paralysis. The end-stage of disease can be defined by failure of the righting reflex, at which point the rats are generally sacrificed due to ethical concerns (Matsumoto et al., 2006). It has been shown that these disease stages correlate well with motor neuron loss, and the onset of muscle weakness generally occurs after 50 percent of spinal motor neurons have been lost (Matsumoto et al., 2006).

Bex1, which appears to be associated with neuronal survival (see Chapter 2), was identified through the microarray experiments as having higher expression in the ALS-resistant motor neuron populations. Protein expression of BEX1 was validated with confocal optical intensity analyses and western blots to ensure that the differences in gene expression translated to differences in protein expression. *Msk1*, which appears to be associated with degeneration (see Chapter 2), was identified through the microarray experiments as having greater expression in the ALS-vulnerable motor neurons, and the protein expression of MSK1 was also confirmed. While these baseline differences in gene and protein expression are relevant to differential susceptibility to degeneration, it is also important to examine these proteins in ALS transgenic mSOD1 rats to see whether BEX1 and MSK1 levels change with the disease process.

Confocal analyses of optical intensity using immunofluorescence were used to compare protein expression of BEX1 and MSK1 in the ALS mSOD1 transgenic rat to an age-matched wildtype control. Future studies will examine MSK1 phosphorylated at numerous sites (Ser360, Ser376, and Thr581) in the transgenic rat to see how pMSK1 expression changes in the disease model.

Gene microarray studies were not carried out for mSOD1 transgenic rats because it is known that the disease process itself alters gene expression such that the relevant differences between the resistant and vulnerable motor neuron groups may be obscured by the massive overexpression of cell death genes in the vulnerable motor neurons. Protein expression of BEX1 and MSK1 in the mutant $SOD1^{G93A}$ transgenic rats (Howland et al., 2002) was compared to a separate set of age-matched Sprague-Dawley wildtype rats.

There are several possible interpretations of differential protein expression in the mSOD1 transgenic rats. Increased expression of BEX1 in the resistant populations in the transgenics compared to the wildtypes could indicate that BEX1 is actually upregulated by the disease process to confer resistance. Decreased expression of BEX1 in the vulnerable populations in the transgenics compared to the wildtypes could indicate that the loss of BEX1 in these motor neurons makes them more vulnerable. Increased expression of MSK1 in the vulnerable populations in the transgenics compared to the wildtypes could indicate that MSK1 is upregulated by the disease process and contributes to degeneration. Decreased expression of MSK1 in the vulnerable populations in the transgenics compared to the wildtypes could mean that MSK1 actually plays a protective role in these motor neurons, and decreases in MSK1 caused by the disease process make them more susceptible to degeneration.

Methods

Experimental Animals.

Male mutant hemizygous $SOD1^{G93A}$ (mSOD1) transgenic Sprague-Dawley rats (n=6, Taconic, Model # 2148-M) and age-matched wildtype Sprague-Dawley rats (n=6, Taconic) were group housed with ad libidum access to food and water. For this experiment, SOD1 rats that

were fully adult (>3 months) but not yet symptomatic were used to avoid the potential confound of differential gene expression related to the cell death process itself. As reported by Taconic (http://www.taconic.com/wmspage.cfm?parm1=3475), the onset of symptoms has shifted in recent years and occurs between 140 to 165 days, with paralysis around 155 to 170 days. Therefore the time point chosen for this study (114 days) was well within the presymptomatic stage of the disease. The rats were maintained on a 12 hour light-dark cycle (lights on at 7 a.m.). All procedures were approved by Princeton IACUC and conformed to guidelines of the US National Institutes of Health.

Body Weight.

mSOD1 transgenic rats and age-matched wildtype rats were weighed throughout the week-long period that they were in our animal facility to determine whether the transgenics differed from the wildtypes and whether they were experiencing loss of body weight that may predict impending disease.

Motor Behavior.

Overall spontaneous locomotor activity was monitored in an open field. Single rats were videotaped in an open field box (52cm x 40cm) for 10 minutes to observe mobility. Videos were scored by an observer blinded to genotype for number of crossings, rearings, groomings, diggings, uncoordinated movements, and percent time spent walking.

Immunofluorescence Reactions.

The rats were perfused, and brainstems and spinal cords were processed for immunofluorescence as described earlier (see Chapter 3). Fluorescent double-labeled reactions of ChAT and either BEX1 or MSK1 were performed as previously described.

Confocal Optical Intensity Analyses of Immunofluorescence.

Immunofluorescence optical intensity analyses were done on 1 μm thick optical sections at 40x using a Zeiss Axiovert confocal microscope with Argon 458/488 and HeNe 543 lasers and 510LSM software. For the brainstem, 20 motor neurons were scanned from the OMN and TGN for each animal while holding the scanning parameters constant. For the spinal cord, 5 to 20 motor neurons were scanned from the SNB and RDLN because these nuclei contain fewer stained cells than the brainstem nuclei. The reactions were analyzed for optical intensity as previously described (see Chapter 3). The optical intensity data were analyzed using a two-way ANOVA for each protein of interest (BEX1 and MSK1), with the factors of susceptibility to ALS (resistant vs. vulnerable) and group (wildtypes vs. mSOD1 transgenics). Analyses were done separately for brainstem and spinal cord data.

Motor Neuron Size.

To determine whether any differences existed in the overall morphology of motor neurons in presymptomatic mSOD1 transgenic rats and wildtypes, comparisons of motor neuron size were done on the 1 μm thick optical scans obtained for the optical intensity analyses on the confocal microscope. ChAT-positive neurons were traced in the confocal scans using the measurement tool in LSM software to determine the cross sectional cell body area (31-99 cell bodies of brainstem motor neurons (OMN and TGN) and 10-65 cell bodies of spinal cord motor neurons (SNB and RDLN) in each animal). Neurons were selected for analysis based on even ChAT staining throughout the cytoplasm. Incomplete neurons or neurons without clear boundaries were excluded from the analysis. The motor neuron sizes were analyzed using a t-test comparing wildtypes and mSOD1 transgenics within each motor neuron population.

Results

Body Weight.

The body weight of the mSOD1 transgenic rats was slightly but significantly lower than their age-matched wildtypes, both at the time of arrival at our facility, $t(10) = 7.47$, $p < 0.0001$, and at time of sacrifice, $t(10) = 7.32$, $p < 0.0001$ (Figure 9). All 6 of the wildtype rats gained weight during this time, whereas only 3 of the mSOD1 transgenic rats gained weight. Two of the mSOD1 transgenic rats lost weight and one rat remained the same. The average change in weight for the mSOD1 transgenics was a gain of 0.5 g and the average change in weight for the age-matched wildtypes was a gain of 30 g. It should be noted that the wildtype rats were one week younger than the mSOD1 transgenics, making this difference in body weight more substantial.

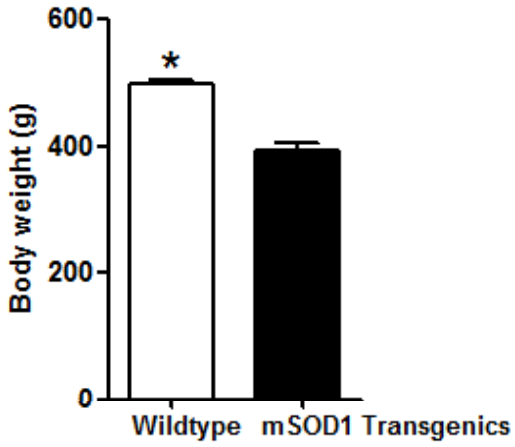


Figure 9. Body weight of wildtypes (age = 107 days) and mSOD1 ALS transgenic rats (age = 114 days). The wildtypes weighed significantly more than the transgenic rats. Error bars represent SEM. *$p < 0.05$

Motor Behavior.

There were no significant differences in the number of crossings, rearings, groomings, diggings, uncoordinated movements, or percent time spent walking between the wildtypes and mSOD1 transgenic rats in the open field box (all p's > 0.05).

Motor Neuron Size.

No qualitative differences were observed in the appearance of any of the motor neuron populations between wildtype and mSOD1 transgenic rats. To determine whether subtler differences existed, the cross-sectional areas of the motor neuron cell body of all ChAT-positive cells in OMN, TGN, SNB and RDLN were measured. No significant differences in cell body area were detected between wildtype and mSOD1 rats in the TGN, SNB or RDLN motor neurons, all p's > 0.05. A small but significant decrease in motor neuron size was observed in the OMN of mSOD1 rats compared to wildtype rats, $t(22) = 3.34$, $p < 0.005$ (Figure 10).

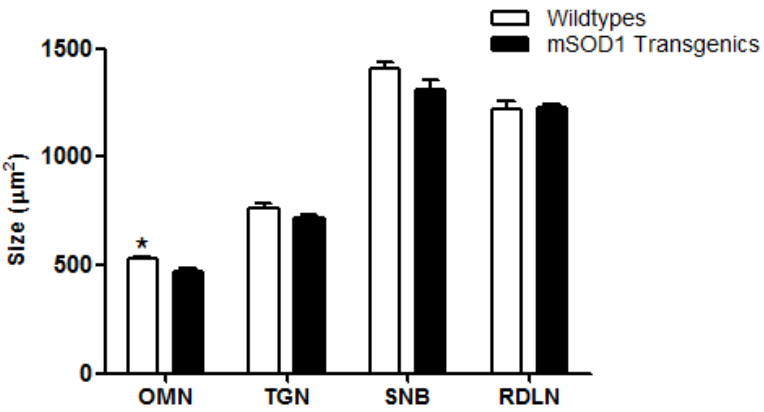


Figure 10. Differences in motor neuron size between wildtypes and ALS mSOD1 transgenics. There were no significant differences in size in the TGN, SNB, and RDLN but there was a small decrease in motor neuron size in the OMN in mSOD1 transgenics compared to wildtypes. Error bars represent SEM. * $p < 0.05$

BEX1.

The cytoplasmic staining pattern of BEX1 in the motor neurons that was observed previously (see Chapter 3) was also seen in the mSOD1 transgenic rats and the age-matched wildtypes, with no differences in location or appearance of staining between the two groups of animals. As previously found for slightly younger wildtype rats, OMN had significantly higher BEX1 expression than TGN in the brainstem nuclei of both wildtypes and mSOD1 rats, $F(1,20) = 55.31$, $p < 0.0001$. Likewise, in the spinal cord nuclei, the SNB had significantly higher BEX1

expression than RDLN, $F(1,18) = 26.11$, $p < 0.0001$. These findings again suggest that BEX1 is expressed at higher levels in protected motor neuron populations compared to vulnerable motor neuron populations. There were no significant differences in the abundance of BEX1 in motor neurons between the wildtype and mSOD1 transgenic rats in either the brainstem or spinal cord, p's > 0.05 (Figure 11).

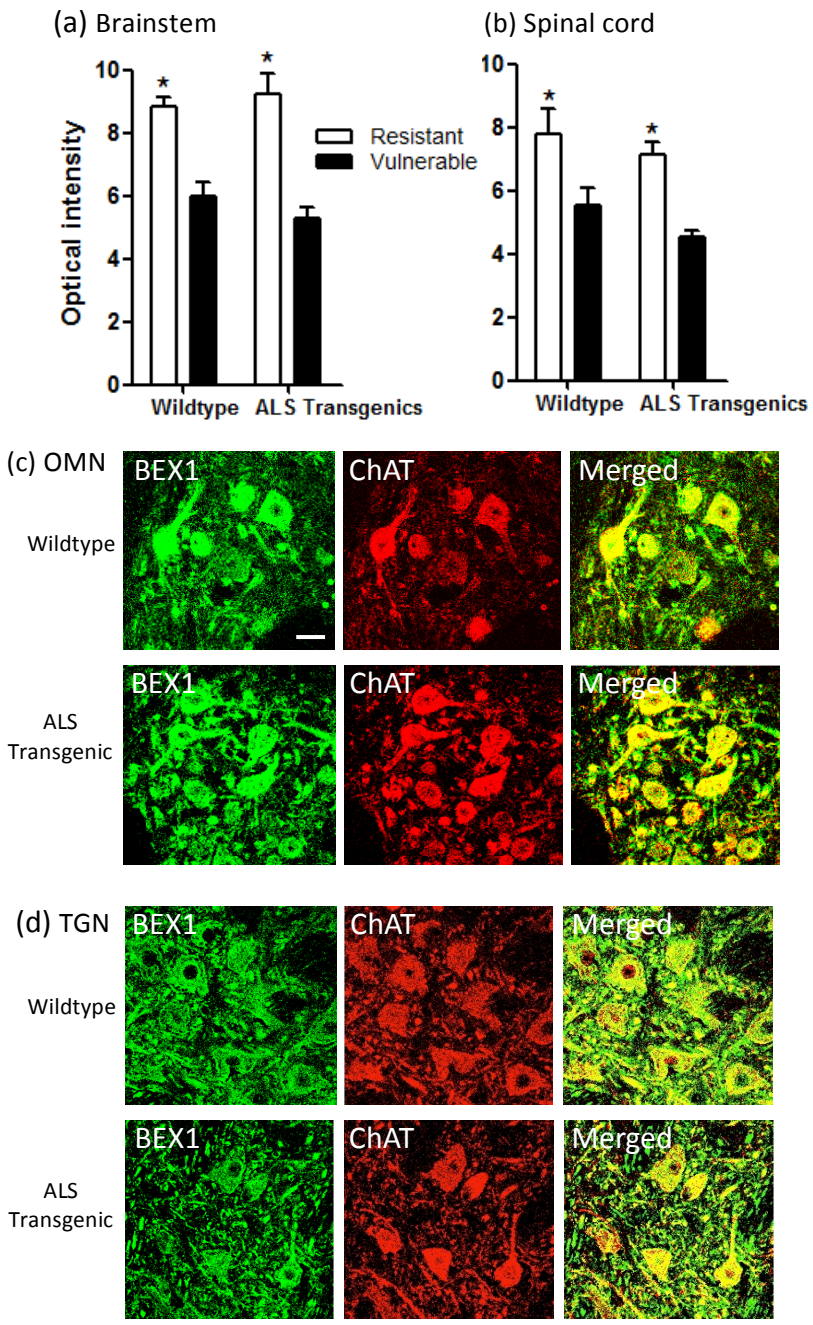


Figure 11. Optical intensity of BEX1 in resistant and vulnerable motor neuron populations in the brainstem and spinal cord. BEX1 had greater expression in the resistant than the vulnerable motor neurons in both the (a) brainstem and (b) spinal cord, with no significant differences between wildtypes and mSOD1 ALS transgenic rats. (c) Representative confocal images of the high expression of BEX1 (green) in ChAT-labeled (red) motor neurons in the OMN motor neurons compared to (d) the lower expression in the TGN motor neurons in both wildtypes and mSOD1 transgenics. Error bars represent SEM. Scale bar represents 20 μm and applies to all confocal frames. *$p < 0.05$

MSK1.

The nuclear staining pattern of MSK1 that was observed previously in slightly younger wildtype rats (see Chapter 3) was seen again in the mSOD1 transgenic rats and age-matched wildtypes as well, with no differences in location of staining between the two groups of animals. In the brainstem nuclei, TGN had significantly higher MSK1 expression than OMN, $F(1,20) = 581.08$, $p < 0.001$ (Figure 10). There was not a significant difference between the wildtype and mSOD1 transgenic rats in the brainstem, $p > 0.05$. In the spinal cord nuclei, the RDLN had significantly higher MSK1 expression than the SNB, $F(1,18) = 41.45$, $p < 0.0001$ (Figure 12). The wildtype rats, however, had a significantly greater expression of MSK1 than the mSOD1 transgenics in both spinal cord nuclei, $F(1,18) = 13.13$, $p < 0.005$. These findings again suggest that MSK1 is expressed at higher levels in vulnerable motor neuron populations compared to resistant motor neuron populations in both the wildtype and mSOD1 transgenic rat.

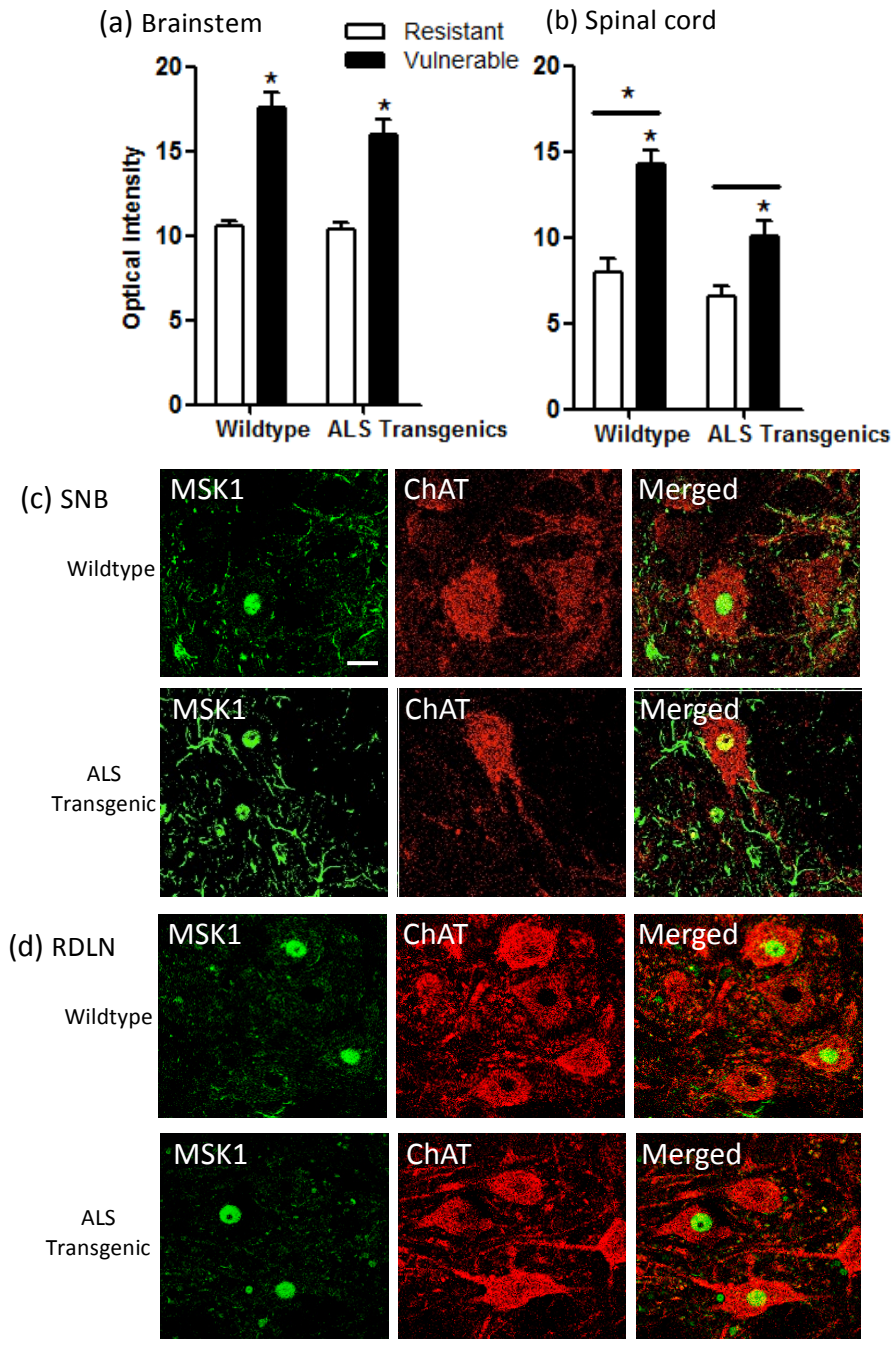


Figure 12. Optical intensity of MSK1 in resistant and vulnerable motor neuron populations in the brainstem and spinal cord. MSK1 had greater expression in the vulnerable than the resistant motor neurons in both the (a) brainstem and (b) spinal cord. In the spinal cord, the wildtype rats had higher MSK1 expression than the mSOD1 ALS transgenic rats. (c) Representative confocal images of the low expression of MSK1 (green) and ChAT-labeled (red) motor neurons in the SNB motor neurons compared to (d) the higher expression in the RDLN motor neurons in both wildtypes and mSOD1 ALS transgenics. Error bars represent SEM. Scale bar represents 20 μm and applies to all confocal frames. *$p < 0.05$

Discussion

This study again verified that BEX1 is preferentially expressed in resistant motor neuron populations compared to vulnerable motor neuron populations and that the converse is true for MSK1. These relative expression patterns did not differ between the wildtype and mSOD1 transgenic rats, suggesting that at presymptomatic stages gene expression in motor neurons of this ALS animal model is relatively normal. Small but statistically significant expression changes in MSK1 in spinal cord nuclei of mSOD1 transgenic rats compared to age-matched wildtypes may be an early indicator that the disease process is about to set in.

For the first several years of the existence of the mutant $SOD1^{G93A}$ transgenic rat model, the onset of symptoms occurred between 100 to 118 days of age. However, a phenotypic drift has occurred in the last two to three years (http://www.taconic.com/wmspage.cfm?parm1=3475), and the onset of gait problems now occurs later (140-165 days). The literature suggests that disease onset and time course is variable in mSOD1 transgenic rats (symptom onset ranging from 127-168 days, end stage disease ranging from 138-188 days) (Matsumoto et al., 2006; Thonhoff et al., 2007). For the purpose of this study, the presymptomatic time point chosen was 114 days at time of sacrifice (before the onset of symptoms), and the rats were monitored closely for any motor deficits leading up to this time point.

Previous studies have shown that maximum body weight can be used as an indication of presymptomatic disease onset in mutant $SOD1^{G93A}$ (mSOD1) transgenic rats, but weight needs to be tracked over a prolonged period of time and this onset can only be determined retrospectively (Matsumoto et al., 2006). In the current experiment, all six of the wildtype animals continued to gain weight during the study but only three of the mSOD1 transgenics gained weight during this period of time. Two of the transgenic rats lost weight and one rat remained the same. It is

difficult to tell whether this indicates a presymptomatic disease onset in these animals without more data points. Nonetheless, the group of mSOD1 transgenic rats used for the current study did not have any visible motor deficits or differences in activity in the open field when compared to the wildtype rats so they were clearly presymptomatic.

Motor neurons in the brainstem and spinal cord shrink in size before they die in individuals with ALS, possibly due to reduced protein synthesis and enzymatic degradation of the cytoskeleton (Kiernan & Hudson, 1992). When obtaining optical intensity measurements of the wildtype and mSOD1 transgenic rats, I did not observe any qualitative differences in the appearance of any of the motor neuron nuclei between groups. The cell body areas of ChAT-positive neurons in OMN, TGN, SNB and RDLN were measured in order to determine whether there were any more subtle changes happening that could be predictive of impending impairment. No significant differences in motor neuron area were detected between wildtype and mSOD1 transgenic rats in the TGN, SNB or RDLN. A small but significant decrease in motor neuron size was observed in the OMN of mSOD1 transgenic rats compared to wildtypes. This difference was surprising because the OMN is protected from cell death in ALS. There are at least two possible explanations for this finding. The first possibility is that early stage ALS was beginning in these animals and the OMN reacted by reducing its cell body size, potentially to conserve energy and enhance survival. It is also possible that the smaller cell body size is a feature of these transgenic rats, perhaps related to their lower body weight, and is independent of the disease process. The latter possibility seems plausible, although it is important to note that not all motor neurons were smaller in the transgenic rats. Future studies will be necessary to examine the time course of motor neuron cell body shrinkage as it relates to cell death in the mSOD1 trasngenic rat.

As expected, in both the brainstem and spinal cord, BEX1 expression was higher in the resistant than the vulnerable motor neurons. There were no significant differences in BEX1 expression between the wildtype and transgenic rats, indicating that presymptomatic mSOD1 transgenics have similar BEX1 expression levels as the wildtype rats. It is possible that no differences were seen between the two groups because it is early in the disease process, and that BEX1 would be further upregulated in the resistant motor neurons after disease onset. It is also possible that differential gene expression that confers a survival advantage to OMN and SNB motor neurons may not change throughout the disease process, but this does not mean that the differential gene expression is functionally irrelevant for ALS. It may instead mean that the disease process itself does not modulate the expression of BEX1, though it is possible that BEX1 is still relevant to the resistance of OMN and SNB to degeneration.

Also as expected based on my earlier findings, in both the brainstem and spinal cord of wildtype and mSOD1 transgenic rats, MSK1 expression was higher in the vulnerable than the resistant motor neurons. In the spinal cord, the wildtype rats had significantly higher expression of MSK1 than the mSOD1 transgenics, although there were no significant differences between groups in the brainstem. This finding that there was higher expression in wildtypes than transgenics was surprising and will be further investigated at later time points after the onset of disease in mSOD1 transgenic rats to determine whether this is a replicable and functionally meaningful difference.

There are at least two potential ways that differential gene expression in TGN and RDLN motor neurons could make them more susceptible to degeneration. One mechanism could be that certain molecules such as MSK1 cause increased vulnerability when they are expressed at high levels. It is also possible that these neurons are more functionally dependent on the expression of

certain molecules, such as MSK1, and therefore any type of insult or pathological condition that reduces the levels of these molecules could encourage neuronal cell death. Thus, it is possible that vulnerable motor neurons rely on MSK1 for maintenance/survival and that during the ALS disease process, MSK1 may drop in the vulnerable populations, making these motor neurons susceptible to death. Protected motor neurons may have other mechanisms in place for their maintenance until late stages of the disease, such as greater expression of BEX1, and may be able to withstand a decrease in MSK1 expression.

The reason for the difference in MSK1 expression between transgenics and wildtypes in the spinal cord but not brainstem nuclei is not clear. It has previously been reported that there is variability in the onset of paralysis among $SOD1^{G93A}$ transgenic rats and they can be classified into three different clinical groups, including forelimb type, hindlimb type, and general type (Matsumoto et al., 2006). These phenotypes may mimic the spinal onset and bulbar onset in ALS patients (see Chapter 1) and provide insight into the mechanisms underlying these different disease courses in humans. It is possible that motor neuron degeneration may not occur uniformly in the brainstem and spinal cord nuclei in the transgenic rats, and therefore there could be differences in protein expression patterns in these groups of motor neurons throughout the disease process. If the disease affected the spinal cord before the brainstem in certain groups of rats, it is possible that there would be differences observed in the spinal cord that are not yet seen in the brainstem.

This is the first report examining BEX1 and MSK1 expression in motor neurons of wildtype or mSOD1 transgenic rats. My findings on their differential expression patterns as well as the known functions of these genes in other systems strongly suggest the possibility that these molecules may be causally linked to the ALS disease process.

Future Studies

The current study focused on ALS transgenic rats before the onset of motor symptoms in order to examine baseline differences in protein expression in a disease model. Future studies will investigate BEX1 and MSK1 in ALS transgenic rats after the onset of symptoms to see how the disease process affects the expression of these proteins.

It is possible that BEX1, which seems to be associated with cell survival, would be further upregulated in the ALS-resistant motor neurons and that MSK1, which seems to be associated with degeneration, would be further upregulated in the ALS-vulnerable motor neuron populations in symptomatic ALS transgenic rats when compared to age-matched wildtype rats. If MSK1 is actually a neuroprotective mechanism that has fluctuating levels in the disease state and causes certain motor neurons to be more susceptible to death, it is possible that MSK1 would instead be downregulated more as the disease progresses. Future studies will also include phosphorylated MSK1 at several activation sites (Ser360, Ser376, and Thr581) to investigate the expression of these activated forms of MSK1 in the disease model.

Protein expression of BEX1 and MSK1 will also be investigated in human brainstem and spinal cord tissue to ensure that the patterns of expression seen in rats in this study translate to differences in human motor neuron populations as well. Confocal optical intensity analyses of BEX1 and MSK1 will be examined in resistant and vulnerable motor neurons in control non-neurological autopsy tissue to see whether there are baseline differences in protein expression. The patterns of expression seen in the control motor neurons will also be compared to autopsy tissue from ALS patients to see whether the disease causes any changes in the levels of these proteins. Although it is not possible to examine protein expression at a presymptomatic time

point in ALS in humans, it will still be informative to measure the levels of BEX1 and MSK1 in surviving motor neurons at end-stage points of the disease.

The information learned from these experiments about the role of BEX1 and MSK1 in the ALS disease process can hopefully be used to develop viral vectors to manipulate gene expression to test the causal role of these genes in the survival of motor neurons in mSOD1 transgenic rats. Because viral vectors can be retrogradely transported (Kato et al., 2007; Zheng et al., 2010), they could be injected into the flexor digitorum brevis muscle of the foot to affect the RDLN and the masseter muscle to affect the TGN in order to rescue these populations from degeneration.

Chapter 5: Discussion

ALS is a devastating neurological disease that afflicts thousands of Americans each year and for which there is no cure. These experiments investigated the molecular mechanisms that allow certain motor neurons to be protected from degeneration and that cause certain motor neurons to be susceptible to degeneration in ALS. The genes identified in these experiments could be future targets for gene therapy in order to significantly delay or prevent the degeneration that occurs in patients with ALS. Many mutations that cause ALS have been identified, but these discoveries have not translated into any successful treatments for the disease. Perhaps focusing on the motor neurons that die is not the best approach, and I might be able to augment the information already known by looking at the disease from the prospective of the protected cells. The ALS-resistant motor neurons seem to have an intrinsic characteristic that allows them to be protected from the detrimental effects of the mutations that cause ALS. The purpose of this research is to elucidate the differences between the ALS-resistant and ALS-vulnerable motor neurons that cause them to respond to the disease process in different ways.

My research first used a relatively unbiased approach of gene microarray analysis to examine the differences in gene expression between resistant and vulnerable motor neurons. I then focused the analysis on two genes whose differential expression was verified using confocal optical intensity analyses and western blots to ensure that the differences in gene expression translated into differences in protein expression. Brain expressed, X-linked 1 (*Bex1*) had greater expression in the resistant motor neuron populations (OMN and SNB), and ribosomal protein s6 kinase, polypeptide 5 (*Rps6ka5 or Msk1*) had greater expression in the vulnerable motor neurons (TGN and RDLN). BEX1 and MSK1 were investigated further in the ALS transgenic rat model (*SOD1*^{G93A}) at a presymptomatic time point in the disease model.

Using confocal optical intensity analyses and western blot methodology, I found that BEX1 has greater expression in the OMN and SNB, the groups of motor neurons that are resistant to degeneration. Previous studies have shown that BEX1 is involved in axonal regeneration and regrowth after injury, and BEX1-knockout mice show reduced capacity for regeneration after nerve injury compared to wildtype mice (Nattkämper et al., 2009; Khazaei et al., 2010). If axonal dysfunction is one of the key pathogenic events in ALS, increased expression of BEX1 in specific populations of motor neurons may provide these neurons protection from cell death caused by axonal dysfunction. Higher baseline levels of BEX1 in these motor neurons may help the axons of the OMN and SNB neurons recover from ALS-induced damage. The role of BEX1 in other neuroprotective capacities has not yet been studied, and further investigations of the role of this protein in motor neurons are needed.

BEX1 was also examined in the mutant $SOD1^{G93A}$ transgenic rat model of ALS using confocal optical intensity analyses to determine whether this protein was differentially expressed in the disease model. Protein expression was examined at a presymptomatic time point in the ALS transgenic rat and a group of age-matched wildtype rats in order to examine baseline differences in protein expression in the disease model. BEX1 had significantly greater expression in the resistant motor neurons than the vulnerable motor neurons in both the transgenics and the wildtypes, with no significant differences in expression between the two groups. Previous studies showed that levels of BEX1 increase after peripheral axonal damage, perhaps to antagonize MAG, a protein that normally inhibits axon growth (Khazai et al., 2010), and therefore it is possible that BEX1 would be further upregulated in the ALS-resistant populations after the onset of disease. It is also possible that BEX1 is a protective mechanism that is needed for survival but not modified by the disease process itself.

Again using confocal optical intensity analyses and western blots, I found that MSK1 has greater expression in the TGN and RDLN, the groups of motor neurons that are vulnerable to degeneration. Studies have shown that glutamate induces phosphorylation of MSK1, and inhibitors of the ERK and p38 MAPK pathways inhibit glutamate-induced MSK1 activation (Brami-Cherrier et al., 2007). MSK1 contributes to excitotoxic-induced cell death in hippocampal neurons, and neuronal cell death is blocked in MSK1-knockout mice (Hughes et al., 2003). However, there are also several studies showing that MSK1 may be involved in neurotrophin-regulated survival and synaptic plasticity (Arthur et al., 2004; Brami-Cherrier et al., 2007). It is possible that MSK1 plays differing roles in cell survival or death depending on the area of the brain or type of neuron, or that there is an inverted U relationship for the effects of MSK1, in which there is an optimal amount of MSK1 that should not be too high or too low.

Phosphorylated MSK1 seemed to be localized to a different part of the motor neuron than MSK1, which suggests that the phosphorylated version of this protein may be transported to other parts of the cells. MSK1 was localized exclusively to the nucleus whereas pMSK1 was localized to both the nucleus and the neuropil adjacent to the motor neuron cell bodies. pMSK1 showed the same differential expression pattern as MSK1 with higher expression in the vulnerable motor neuron populations compared to the resistant motor neuron populations, but this effect was only significant in the neuropil.

When comparing the ALS transgenic rats and age-matched wildtype rats, MSK1 had significantly greater expression in the vulnerable motor neurons than the resistant motor neurons. There were no differences in MSK1 expression between the transgenics and wildtypes in the brainstem, but the wildtype rats had greater MSK1 expression in the spinal cord than the transgenics. Although it was an unexpected finding for the wildtypes to have higher levels of

MSK1 than the transgenics, it is possible that vulnerable motor neurons rely on MSK1 for maintenance and survival. The disease process may cause MSK1 to drop in the vulnerable populations, making them susceptible to death, while other motor neuron populations, such as OMN and SNB neurons, rely on additional mechanisms for survival. The reason for this difference in the spinal cord but not the brainstem is unknown, but perhaps the disease process does not affect all populations of motor neurons equally or MSK1 plays diverse roles in specific groups of neurons. Future studies will examine pMSK1 in mSOD1 transgenic rats to see whether the expression of the phosphorylated version of this protein changes throughout the disease process.

As neurons in the brain increase in age, their efficiency decreases, partially caused by aging mitochondria. Mitochondria are crucial to the survival of cells because they produce ATP through oxidative phosphorylation and they regulate the intracellular calcium levels and the production of reactive oxygen species (ROS). Because of these roles, recent research has begun to investigate whether mitochondria are involved in neurodegenerative disorders. Mitochondria seem to be more delicate than previously thought because they function close to their physiochemical limit and can easily break down under conditions of stress (Nicholls & Budd, 2000). Mitochondrial dysfunction can cause neuronal death in numerous ways, including by changing calcium homeostasis, increasing the production of free radicals, or releasing proteins that promote apoptosis.

Calcium signaling plays a crucial role in many neuronal processes, and mitochondria can accumulate calcium if the cytoplasmic concentration reaches a set point, causing permeabilization of the inner membrane and a flow of calcium into the mitochondria. Aged mitochondria are more likely to be depolarized, which changes calcium homeostasis and the

gradient across mitochondrial membranes. This decreased ability of mitochondria to store calcium causes an increase in intracellular calcium levels. Studies have shown that high levels of calcium can be neurotoxic and may cause increases in MSK1 activation (Clark et al., 2007).

Calcium storage within mitochondria is crucial to the regulation of calcium levels in the neuron, and when this storage decreases, the increased intracellular calcium often activates calcium-activated proteases called caspases (Esiri, 2007). Caspases are a family of proteins that are involved in the apoptotic process, and they damage neurons and make them more susceptible to other stressors such as hypoxia. Mitochondria are more likely to experience oxidative damage as they age due to a decrease in efficiency and energy generation, which increases their production of superoxide ions. These oxygen free radicals are toxic and damage proteins, nucleic acids, and lipids, and they activate the p38 MAPK pathway, causing an increase in MSK1 activity (El Mchichi et al., 2007). There is also evidence that MSK1 activates caspase-8, either through regulating the transcription of activators of caspase-8 or through direct or indirect interactions between the two molecules (El Mchichi et al., 2007).

Glutamate excitotoxicity has been identified as a potential mechanism underlying neuronal degeneration in ALS, and research investigating the distribution of glutamate transporter subtypes in the brainstem of monkeys found that the astroglial-specific GLT-1 glutamate transporter has greater expression in ALS-vulnerable than ALS-resistant motor neurons (Medina et al., 1996). This could mean that a loss of the GLT-1 glutamate transporter in sporadic ALS may cause glutamate to increase more in the ALS-vulnerable motor neuron populations, and this increase could cause excess calcium in motor neurons. Because of their increased expression of parvalbumin and CaBP, motor neurons in the ALS-resistant populations may be better equipped to resist the damage caused by increased calcium (Alexianu et al., 1994;

Reiner et al., 1995). It has also been found that there is greater expression of a metabotropic glutamate receptor (mGluR1a) protein within vulnerable motor neuron populations, which may contribute to a predisposition to neurodegeneration (Laslo et al., 2001). mGluR1a is a G-protein coupled receptor that can trigger calcium release from intracellular stores and activate protein kinase C, both of which contribute to neuronal death. Increases in glutamate have been shown to increase MSK1 activation (Brami-Cherrier et al., 2007), and it is possible that increases in MSK1 in motor neurons that already have high baseline expression of this protein could make them even more susceptible to degeneration caused by caspase activation and excitotoxicity.

MAPK/ERK pathways are increased in response to several types of stressors (Rundén et al., 1998; Stanciu et al., 2000), but there is contradictory evidence on whether the resulting increases in MSK1 have a neuroprotective or neurodegenerative impact. MSK1 is increased in neurons susceptible to degeneration in Alzheimer's disease (Webber et al., 2005) but seems to protect neurons in the striatum from degeneration in Huntington's disease (Roze et al., 2008). Perhaps MSK1 only contributes to cell death after certain types of insults, which can vary depending on the type of neurodegenerative disease and even the specific upstream kinase involved in MSK1 phosphorylation.

Future studies will need to investigate the expression of BEX1 and MSK1 in later stages of the disease process in order to fully understand how the disease affects their expression levels. It is possible that these two proteins work together with some of the other underlying mechanisms of ALS in order to determine the relative susceptibility of each group of motor neurons. Tests of causality should be conducted to investigate whether overexpressing BEX1 or knocking out MSK1 in vulnerable motor neurons protects them from degeneration.

Gene Therapy in ALS

The ultimate goal of this research is for the genes identified in these experiments to be used in gene therapy to alter gene expression in susceptible populations of motor neurons. There are several types of gene therapy, including RNA interference (RNAi), antisense therapy, and viral vector-based gene delivery. RNAi is when noncoding micro RNA (miRNA) inhibit and regulate gene expression by binding mRNA. Several studies examining SOD1 silencing in transgenic mice have found improved motor neuron survival and delayed onset of ALS symptoms, though there is some concern about the efficacy of these treatments in humans, especially those without a known mutation (Nizzardo et al., 2011). Antisense oligonucleotides (AONs) reduce expression of a particular gene by altering splicing or causing degradation of RNA, and there are currently phase 1 clinical trials investigating this approach as a treatment for ALS patients with SOD1 mutations (Nizzardo et al., 2011).

Adeno-associated virus (AAV) vectors that silence mutant SOD1 in mice seem to be a potential treatment because they reduce mSOD1 levels in muscle and motor neurons and can protect against muscle wasting; however, they do not actually seem to modify disease progression (Towne et al., 2011). Although these gene therapy techniques are promising, the main difficulty is that the blood-brain barrier often prevents the therapy from reaching the motor neurons. AAV vectors seem to have the most potential for success because of their ability to cross the blood-brain barrier and target neurons (Nizzardo et al., 2011). It is my hope that my research will bring us one step closer to providing an effective therapy for ALS patients.

The Role of Glia in Neurodegenerative Diseases

Although the hallmark of neurodegenerative disorders is the death of neurons in the brain or spinal cord, it is becoming increasingly clear that glial cells also play a crucial role in the disease process (Clement et al., 2003; Boillée et al., 2006). Autopsy studies have found that misfolded SOD1 is present in glial cells of spinal cord tissue from ALS patients carrying the SOD1 mutation and even sporadic and familial cases lacking the mutation (Forsberg et al., 2011). These results mainly included astrocytes but also some microglia and oligodendrocytes. Studies in ALS transgenic mice have shown mutant SOD1 aggregates in astrocytes, microglia, and oligodendrocytes as well (Bruijn et al., 1997; Jaarsma et al., 2008).

Although glial cells show high levels of the mutant SOD1 aggregates, there is no evidence for extensive glial cell death in ALS (Forsberg et al., 2011). Therefore, it is likely that harmful effects of the misfolded protein in glial cells cause death to nearby motor neurons instead, and studies have shown that astrocytes with mutant SOD1 protein release a toxic substance onto nearby motor neurons (Nagai et al., 2007). Because MSK1 was expressed in cells that were not immunoreactive for the motor neuron marker ChAT, it is possible that increased levels of MSK1 in non-neuronal cells within vulnerable motor neuron populations may actually play a role in the disease process, though this would need to be specifically investigated.

Other Neurodegenerative Diseases

Similar to ALS, Huntington's disease (HD), Alzheimer's disease (AD) and Parkinson's disease (PD) were all first reported more than 100 years ago, and there are still no cures or effective treatments for any of these equally devastating neurodegenerative diseases (Harper, 2002; Forman et al., 2004). All of the aforementioned neurodegenerative diseases selectively

affect specific populations of neurons, and the mechanisms underlying these patterns of degeneration are still unclear. Neurons in the hippocampus and cholinergic neurons of the basal forebrain are most severely affected by AD, PD targets dopaminergic neurons in the substantia nigra pars compacta, and HD causes degeneration of the medium spiny neurons of the striatum. Even more surprising is the fact that within specific diseases, even within the class of neurons that is vulnerable, there appear to be resistant populations similar to certain motor neurons in ALS. In PD, for example, some substantial nigra neurons do not die.

The symptomatology of each disease varies depending on the affected populations of neurons, but there may be similar mechanisms that produce neurodegeneration. For example, converging research over the past decade has revealed that the aggregation and misfolding of fibrous proteins plays a role in numerous diverse neurodegenerative disorders (Forman et al., 2004; Burvill, 2009). Though many of the mutations causing these diseases exist throughout a person's lifetime, most neurodegenerative disorders do not manifest clinically until middle or late life. This suggests that these diseases may require progressive damage to certain regions of the brain and also may be related to aging-related impairments in neural metabolism (Forman et al., 2004). Because of these similarities, studying the neurodegenerative disorders as a group could provide additional insight into overlapping disease processes. Although most research only focuses on one particular disease, it is valuable to compare these diseases to each other because they have many similar features and it is likely that some of the same mechanisms are responsible for the degeneration.

PD and AD, two of the most common neurological disorders associated with aging, affect cells that are particularly vulnerable to the affects of normal aging (Dong et al., 2009; Chan et al., 2010; Kapogiannis & Mattson, 2011). The main symptoms of PD, tremors and difficulty with

walking, movement, and coordination, are caused by the selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc). There are two main groups of dopamine neurons within the ventral midbrain, the A9 group in the substantia nigra (SN), which are preferentially affected in PD, and the A10 group in the medial and ventral tegmentum, which are selectively spared from the disease. Several mutations associated with PD have been discovered, including LRRK2, PARK2, and alpha-synuclein (α-syn) (Sulzer, 2010). Although α-syn mutations leading to PD are relatively rare, it is likely that α-syn is involved in all cases of PD because it is found in the Lewy bodies in neurons. There is some evidence that the protein can damage lysosomal function, block normal protein degradation, and disrupt mitochondria. The relationship between α-syn and neuronal toxicity is still unclear, since levels of α-syn are higher during development rather than older age when PD normally manifests, and the amount of α-syn mRNA is similar in neurons that are susceptible to PD and those that are unaffected (Sulzer, 2010).

PARK2 is a gene that encodes the protein parkin, and mutations of it have been associated with PD. Parkin regulates excitatory glutamatergic synapses and is part of the ubiquitin-proteasome system that is involved in targeting proteins for degradation, the same system that is affected by SOD1 mutations in ALS patients. Mutations in parkin increase the number of glutamatergic synapses, which causes elevated intracellular calcium and leads to excitotoxicity (Dong et al., 2009). Calbindin is a calcium-binding protein that is expressed in some dopaminergic neurons, and the fact that these neurons are selectively preserved in PD supports the idea that increases in calcium are associated with neuronal death. It is still unclear why some dopamine neurons are more susceptible to the disease, especially since none of the genes that have been associated with familial forms of PD are expressed more in this neuronal population.

Similarly, none of the genes that have been associated with familial forms of ALS were expressed more in the vulnerable motor neurons in the microarray study. However, this may mean that the mutations selectively impact the vulnerable motor neurons more than the resistant motor neurons because of inherent differences between the two populations

Dopamine neurons in the SNc are autonomously active and produce action potentials without outside input, unlike most other neurons in the brain (Chan et al., 2010). Whereas most neurons use channels that allow sodium across the membrane to control action potentials, these neurons use L-type calcium channels that are open most of the time. The amount of calcium influx into the neuron is greater and therefore more damaging, especially to the ER and mitochondria. This may cause these neurons to have a greater baseline susceptibility to subsequent increases in calcium.

Neuronal death estimates related to normal aging show that neurons in the SNc die at a significantly higher rate than neurons in many other areas (Chan et al., 2010), which is similar to the finding that the ALS-vulnerable motor neuron populations seem to die more than ALS-resistant populations in normal aging (Sturrock, 1991). These studies provide further evidence that the susceptible motor neuron populations have fundamental properties that make them more vulnerable to the insults caused by the disease process. Neurons in the SNc have higher levels of mitochondrial DNA (mtDNA) mutations and decreases in mitochondrial functions, which are both considered signs of aging. One common theory of aging is that oxidative phosphorylation produces reactive oxygen species and free radicals, which causes an accumulation of damaged mtDNA. This theory implies that PD is related to aging and that everyone would get it if they lived long enough; however, genetic mutations and polymorphisms may cause individual

differences in the rate of cellular aging and the efficiency of mitochondrial oxidative phosphorylation (Chan et al., 2010).

Several groups have investigated the differences in gene and protein expression between neurons that are resistant and vulnerable to PD (Yamada et al., 1990; Chung et al., 2005). CaBP, which has increased expression in the resistant populations in ALS, also has greater expression in resistant neurons in PD (Yamada et al., 1990). Autopsy studies of individuals with PD have shown relative preservation of CaBP-positive, dopaminergic neurons (Yamada et al., 1990). Another protein that may be involved in selective susceptibility to PD is the G-protein-gated, inwardly rectifying potassium channel (Girk2), which has greater expression in the SNc than other areas of the midbrain (Schein et al., 1998; Chung et al., 2005).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that damages neurons and leads to a loss of memory and other cognitive processes. AD is the most common form of dementia, and accounts for 60 to 70 percent of dementia in people over the age of 65 (Hynd et al., 2004). The two molecular hallmarks of AD are senile plaques of insoluble beta-amyloid (Aβ) and neurofibrillary tangles made of hyperphosphorylated tau protein. The entorhinal cortex and hippocampus are affected most severely, but in advanced AD, the whole cortex shrivels up due to massive cell loss. Recent groundbreaking studies have shown that the tau protein pathology spreads from the entorhinal cortex to other connected and vulnerable neurons, almost like an infection (Liu et al., 2012).

Numerous studies have shown that one of the main abnormalities in individuals with AD is a decrease in metabolism (Blass, 2000; Zhu et al., 2007b). Imaging studies of individuals at early stages of AD have shown loss of volume and decreases in metabolism in the hippocampus and regions such as the temporoparietal and medial lateral prefrontal cortices (Kapogiannis &

Mattson, 2011). There are detectable abnormalities in metabolic rate before there are any deficits in neuropsychological testing (Blass, 2000). These pathological manifestations then spread into regions that are most susceptible to increased excitation associated with age-related and disease-related mitochondrial dysfunction and oxidative stress. The decreases in metabolism that seem to spread through the brain may be caused by the spread of tauopathy between vulnerable neurons in the brain. Neurons in the hippocampus are necessary for learning and memory and have higher energy requirements than most other neurons, thus making them more susceptible to the disease (LaManna & Harik, 1985).

Aging is associated with decreases in GABA release and reduced insulin signaling, especially in the hippocampus (Cole & Frautschy, 2007; Kapogiannis & Mattson, 2011). Decreases in GABA release in the hippocampus cause an excitatory imbalance in the brain (Kapogiannis & Mattson, 2011). Insulin is involved with neuronal development and survival, and also has neuromodulatory and neuroendocrine functions. Insulin resistance is central to Type II diabetes and also implicated in AD. Type II diabetes is a significant risk factor for developing AD, possibly due to defects in insulin signaling, accumulation of pathological Aβ, or hyperphosphorylated tau. Recent studies show that AD may involve a resistance to insulin and neurotrophic factors rather than a loss of them (Watson & Craft, 2003). While insulin is not a good treatment for AD, increases in insulin-like signaling may have therapeutic effects (Cole & Frautschy, 2007). Similarly, insulin-like growth factor 1 (IGF-1) has been investigated as a therapeutic agent in the treatment of ALS, with promising results in animal models but ineffective clinical trials (Borasio et al., 1998; Sorenson et al., 2008).

Other changes associated with normal aging, such as neuronal glucose uptake, mitochondrial dysfunction, and increased activation of glucocorticoid pathways, make neurons

especially vulnerable to damage caused by glutamate. The decrease in inhibitory transmission and calcium binding proteins might make neurons vulnerable to calcium overload. There is also a growing body of evidence that suggests that dysfunctions in the glutamate system that lead to excitotoxicity may be one of the underlying causes of degeneration in several neurodegenerative diseases, including ALS, AD, Huntington's disease (HD), and PD (Bruijn et al., 1997; Miguel-Hidalgo et al., 2002; Hynd et al., 2004; Liang et al., 2005; Helton et al., 2008). The excessive activation of NMDA receptors may enhance the localized vulnerability of neurons, and the regional distribution of NMDA receptor subtypes may contribute to the region-specific degeneration seen in individuals with AD (Dong et al., 2009).

There are many studies showing that abnormal mitogenic changes and oxidative stress play a role in AD, and there is increased activation of p38 and ERK MAPK signaling pathways in individuals with AD (Zhu et al., 2004; 2007a; Webber et al., 2005). Phosphorylated MSK1 is increased in the vulnerable hippocampal neurons in AD autopsy tissue compared to age-matched controls, providing evidence that activation of MAPK signaling pathways and the resulting increase in MSK1 expression may play a role in AD pathology (Webber et al., 2005).

Microglial cells have been found in the senile plaques of individuals with AD, suggesting that there could be a microglial immune response that produces free radicals. Microglial activation can be induced by Aβ and results in the release of toxic factors that cause apoptosis. Therefore, neuronal cell death in individuals with AD could be directly initiated by toxic events caused by Aβ or it could be indirectly caused by an interaction between neurons, microglia and toxic factors (Yuan & Yanker, 2000).

Although the neurons affected by each neurodegenerative disorder differ, there are many overlapping mechanisms, such as protein aggregation and oxidative stress, which can provide valuable insight and opportunities for future breakthroughs. There are currently millions of people suffering from neurodegenerative disorders in the United States, but there are still are no cures for any of these diseases. ALS is a devastating disease that leads to paralysis and death within only a few years, and none of the research being done on this disease has led to an effective treatment.

By using the approach of examining neurons that are protected from cell death in comparison with those that are not, my dissertation research pinpointed individual genes that could be involved in differential susceptibility to motor neuron death in ALS. It is possible that altering the expression of *Bex1* or *Msk1* in motor neurons could provide protection from degeneration. The ultimate goal of this research is to provide the basis for studies that aim to manipulate gene expression to protect neurons that are typically vulnerable to degeneration in order to bring us one step closer to finding a cure for this debilitating and devastating disease.

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