EXPRESSION OF HEAT-SHOCK PROTEINS IN THE PRESENCE OF OXIDATIVE STRESS IN THE SOD1-G93A AMYOTROPHIC LATERAL SCLEROSIS MOUSE MODEL

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EXPRESSION OF HEAT-SHOCK PROTEINS IN THE PRESENCE OF OXIDATIVE STRESS IN AMYOTROPHIC LATERAL SCLEROSIS MOUSE MODELS

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LIST OF SYMBOLS AND ABBREVIATIONS

HSP	Heat Shock Protein
ALS	Amyotrophic Lateral Sclerosis
NMJ	Neuromuscular Junction
SOD1	Copper/Zinc Superoxide Dismutase
ROS	Reactive Oxygen Species
WT	Wildtype
TA	Transverse Abdominal
EDL	Extensor Digitorum Longus

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that causes death of motoneurons, resulting in paralysis, dysphagia, respiratory distress and ultimately death. The precise causes of this fatal disease are unknown, but the mechanisms contributing to motoneuron death have been researched extensively. Oxidative stress has been established as one mechanism of motoneuron death associated with ALS. Protein misfolding is a cellular dysfunction that occurs more commonly in increased levels of oxidative stress. Large concentrations of misfolded proteins interfere with neuron signaling and trigger apoptotic pathways leading to degeneration. Heat Shock Proteins (HSPs) are chaperones that assist in proper protein folding. The purpose of this research is to determine where and to what extent HSP levels are not being properly upregulated to counter the negative effects of oxidative stress associated with ALS, to determine their corresponding impact on cellular degeneration, and to assess the susceptibility of spinal motoneurons and muscle to oxidative stress. We perform a meta-analysis of HSPs from 11 peer-reviewed experimental journal articles assessing HSP levels in the SOD1-G93A transgenic ALS mouse model. Aggregated analysis of HSP levels in SOD1-G93A ALS transgenic mice revealed that HSPs are downregulated in the limbs at most stages of the disease, and are significantly lower than HSP levels in the spine. In contrast, HSP levels in the spine are significantly upregulated in comparison to wild type or non-ALS mice. Since HSPs combat protein misfolding, the compensatory upregulation of HSPs in the ALS pathology is insufficient to counteract oxidative stress. Our results suggest that the muscle cells are more vulnerable to oxidative-related

degradation than spinal motoneurons. In summary, HSPs as a clinical therapeutic strategy delivered to the muscle and/or spine could be particularly helpful in early stages of ALS, where their effect in delaying oxidative stress induced cellular death is maximal.

CHAPTER 1

INTRODUCTION

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a deadly disease affecting an estimated 450,000 people worldwide [2]. ALS is a neurodegenerative disease, targeting neurons of the spinal cord and brain stem. Those with ALS progressively lose control over their nervous system, initially inhibiting movement of appendages but ultimately resulting in the inability to use respiratory muscles [1]. The average survival of a newly diagnosed ALS patient is two to five years, with 20% of those diagnosed living longer than five years and only 10% living longer than 10 years after symptom onset [33]. About 90% of ALS cases are sporadic, meaning the disease occurs despite a lack of ALS history in the family. The other 10% of cases are familial, meaning the cause of the disease is genetically inherited [30]. While sporadic ALS occurs randomly and has no definitive causes, the etiology of familial ALS is more consistent. About 20% of familial ALS cases have been linked to a mutation in the copper/zinc Superoxide Dismutase (SOD1) gene. This discovery allowed for the genetic engineering of SOD1-mutated mice to be used for research purposes, and namely the SOD1 G93A (glycine 93 to alanine mutation) [4]. The SOD1 G93A transgenic mice replicate the corresponding clinical ALS phenotype, allowing researchers to discover potential causes or mechanisms of cell death. Nonetheless, the exact initiating causes of ALS remain largely unknown, although the resulting mechanisms of motoneuron death have been extensively experimentally examined. There is substantial experimental evidence from the SOD1 G93A transgenic ALS mouse model that implicates oxidative stress as a central cause of motoneuron death[5].

Oxidative Stress

Oxidative stress is a result of an imbalance between the production of reactive oxygen species (ROS) in cells and the inability of the cell to remove ROS or repair the damage caused by excessive ROS levels. ROS are typically produced as byproducts of aerobic metabolism in the mitochondria. In large concentrations, ROS become highly toxic, triggering oxidative stress and resulting in negative effects like inflammation and protein misfolding. Typically, ROS are removed from cells, but mitochondria can "leak" ROS, resulting in an excess build-up of toxic ROS in the cell [4]. Pathological studies have shown that oxidative stress is increased in ALS, but it is unknown if oxidative stress is a result of the effects of ALS, or if it is the initiating cause of the disease. What is known is that, although motoneuron death in ALS is multifaceted and complex, oxidative stress has been established as one of the causes of motoneuron death [5].

One method of cell death that is promoted by ROS toxicity related to oxidative stress is protein misfolding. Protein misfolding is a result of a dysfunctional change in three-dimensional folding, a physiological process that normally enables proteins to fold onto themselves. When proteins are not properly folded, they cannot perform their desired functions. Under pathological conditions, misfolded proteins rapidly accumulate faster than they can be transported or destroyed. The build-up of misfolded proteins interferes with neuronal communications, which triggers apoptotic pathways and the subsequent self-initiated killing of cells [34]. Cells have built-in mechanisms to combat the negative effects of physiological oxidative stress. The Heat Shock response is one of the primary mechanisms used to combat protein misfolding.

Heat-Shock Proteins

HSPs are chaperones that assist in proper protein folding as well as the correction of misfolded proteins. It is known that under conditions of stress, properly functioning cells activate the Heat Shock response to synthesize neuroprotective HSPs. This upregulation of HSPs provides the cell with protection against misfolded and damaged proteins, as the HSPs will chaperone the proteins to their normal, functioning structures [16]. HSP70, in particular, has been identified as a key HSP in correcting misfolded proteins and aiding in proper protein folding [38]. HSPs have been shown to play a major role in neurodegenerative diseases, and it has been suggested that activation of the Heat Shock response is indicative of vulnerability of cells to degeneration.

Location of Initial ALS Degeneration

There have been debates over whether neuronal degeneration in ALS begins at a more central location in the brain and spine (such as the soma of spinal motoneurons); within the long axons of the spinal motoneurons that extend from the spine to the target muscle cell; where the spinal motoneuron axon and muscle cell connect at the neuromuscular junctions (NMJ); or whether ALS begins in the muscle cells, themselves, with the disease retrogradely propagating across the NMJ, up the axon, and towards the spine [9]. Site-specific examination of oxidative stress and the HSP response in the spinal motoneurons and in muscle can provide valuable information comparing the susceptibly of both sites' cells to oxidative stress, protein misfolding, and resultant apoptosis and degeneration. Understanding which cells are most susceptible could provide valuable evidence pointing towards a likely site of initiation as well as discovering potential HSP therapeutic targets.

CHAPTER 2

MATERIALS AND METHODS

Meta-Analysis Procedure

The statistical variation across experimental studies has made drawing specific conclusions on the ALS HSP response difficult. Therefore, to provide more statistically conclusive clarity with a larger sample size of data, we performed a meta-data analysis aggregated from 11 peer-reviewed experimental journal articles examining HSPs in SOD1 G93A ALS mice. Meta-analyses and meta-data analyses are commonly used in clinical practice to reconcile potential differences in a field or treatment strategy. The same general methods can also be applied to experimental data. The general meta-analysis method involved (1) selecting and recapturing published data for SOD1-G93A mouse analysis on HSPs and oxidative stress interactions; (2) normalizing recaptured data to see conditional trends; (3) plotting and analyzing normalized data to create visual trend lines and statistical comparisons for each treatment condition.

Article Selection

Keyword Searches

Articles were found through a series of keyword searches in the SOD1 G93A experimental database owned and maintained by the Laboratory for Pathology Dynamics (Dr. Cassie Mitchell) at the Georgia Institute of Technology. This publicly available database has peer-reviewed publications from ALS transgenic mouse models, namely the SOD1 G93A ALS mouse model [29]. To search for articles, a script was made in the FileMaker Pro application. This script was designed to isolate articles that contained any mention of HSPs and/or oxidative stress in the following locations of each article in the database: abstract; figure captions; treatment description; and data series and response descriptions. Through searches in these article sections using the keywords “HSP” or “heat-shock,” and “oxidative stress,” 66 potential articles were found. The suitability of these initial 66 articles was then determined based on specific study inclusion criteria.

Inclusion and Exclusion Criteria

Articles were excluded if they met ANY of the following criteria: contained data that did not directly or indirectly measure HSP concentration; contained data that involved treatments on the mouse model; exhibited purely *in vitro* data.

For studies to be included in further analysis, they needed to contain data that measured concentration of any HSP, including: HSP70; HSP40; HSP60; HSP90; HSPb8; HSP25; HSP27; HSP105; and HSPh1, at one or more time points in spine or muscle tissue samples from wild-type or B6SJL or C57BL/6 SOD1-G93A congenic mice. Through these criteria, 11 of the 66 articles found via keyword search were determined to have suitable data for analyzing HSP levels in ALS, and the other 55 articles were excluded. **Figure 1** illustrates the breakdown of articles found via keyword searches as well as the general filtering process. **Table 1** shows the total number of data points and articles used in each analysis.

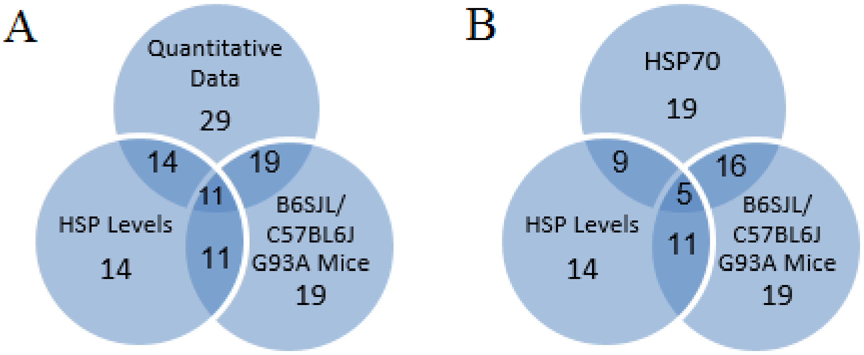

Figure 1. Distribution of Articles Found by Keyword Search. A) Of the 66 total articles, there were 29 articles with quantitative data, a necessity to include in this meta-analysis; 14 articles contained data on HSP levels; 19 articles utilized the desired mice. A total of 11 articles met all three of these inclusion criteria. B) HSP70 contained the most individual articles (19) of any individual HSP. 5 of these articles contained HSP level data and used the desired mouse type in the study. These 5 articles are included in the 11 articles identified in panel A.

Data Recapture

In order to recapture the data from the desired studies, articles were either downloaded using PubMed Central or from e-journal subscriptions available from the libraries of Georgia Institute of Technology and Emory University. Data was recaptured from the following article locations [15], referred to as entities: article title; abstract; figure captions; and data series and response values. Data was transferred from the full-text pdf article to the Laboratory for Pathology Dynamics SOD1 G93A ALS mouse database. To insure accuracy (>99%), every data point was reviewed by an independent quality control team[29].

Data Aggregation and Normalization

The data used from the selected articles all presented HSP quantified levels. However, the procedures that each study used to measure HSP data varied. To account for the variation of HSP level measurement methods between articles, HSP level data were normalized by calculating the ratios of transgenic SOD1-G93A to Wildtype (WT) HSP levels. Data from each study were normalized to their respective WT data, and ratios obtained from each article were weighted equally when calculating averages.

Mouse Models and Tissue Collection

All articles used in this meta-analysis received B6SJL or C57BL/6 SOD1-G93A or WT mice from Jackson Laboratories (Bar Harbor, ME) [6-7,10,12-14,16,21,23-25]. Researchers for each article isolated either the spine or muscles of the mice and recorded HSP levels of tissue samples from these sources. In all studies analyzing HSP levels in muscles, a combination of soleus, transverse abdominal (TA) muscle, and/or extensor digitorum longus (EDL) muscle was used [6,10,13-14,16,23]. For the purpose of this study, data from the three muscle types were combined into a single dataset.

Statistical Analysis

A Mann-Whitney U-test was implemented in Matlab (Mathworks, Inc.) to test for significance of HSP levels between spine and muscle at each time stage as well as between time stages within spine or muscle data sets. Unless otherwise stated, significance was determined at an alpha value of 0.05.

Category	Articles	Data points	References
All HSP	11	57	[7,8,15,18-20,27,36,39-41]
Spine	8	41	[7,8,18,19,27,39-41]
Muscle	7	16	[7,15,19,20,27,36,39]
Hsp70	8	25	[15,18-20,27,39-41]

Table 1. Article and Data Point Breakdown

CHAPTER 3

RESULTS

Heat-Shock Protein 70 Levels in Spine and Muscles

HSP70 was the most frequently analyzed heat shock protein in these studies. HSP70 levels were analyzed in 8 of the 11 articles and comprised 30 of the 57 total data points. SOD1-G93A/WT ratios of concentration data for HSP70 were divided into early and late stages of disease progression. To create the two individual data-sets, data points were sorted into the "early" stage if they were recorded between 30 and 99 days as (30 days being the earliest time point in any article). Data points recorded after 99 days were sorted into the "late" stage of disease progression. Note that, by 100 days, untreated SOD1 G93A ALS mice are considered to have measurable functional symptom onset, which is why 100 days was used as the threshold for binning the two groups. These two sets of data were further divided into "muscle" and "spine" data sets based on the tissue samples analyzed in each report. All data points in each of the four groups were averaged and plotted (**Figure 2**). Differences in HSP70 levels between spine and muscle groups at each time stage were tested for statistical significance (eg. Early stage spine data was compared to early stage muscle data). Results revealed by Mann-Whitney U-Test that, at an alpha value of .10, early-stage spinal HSP70 levels (n=11) were significantly higher (p=.08) than early-stage muscle levels (n=5). While the late-stage spinal HSP70 levels (n=9) are higher than HSP70 muscle levels (n=5), with values of 1.34 for spine and 0.99 for muscle, no significance was observed between these data sets. The significance of HSP70 levels was also assessed between each time stage within each tissue sample group (eg. Early stage muscle data was compared to late stage muscle data). However, no significance was observed between early and late stage HSP70 levels within spinal (early= 1.31, late=1.34) or muscle data (early=0.935, late=0.992). In order to assess the contrasting HSP70 levels in the spine and muscle on a larger scale, the early and late stage data sets were combined. The average HSP70 levels in spinal tissue samples (n=20) were found to be significantly higher (p<.05) than the average HSP70 levels in muscle tissue samples (n=10). The results are in-line with what was stated in articles comparing HSP70 levels in the spine and muscles [40].

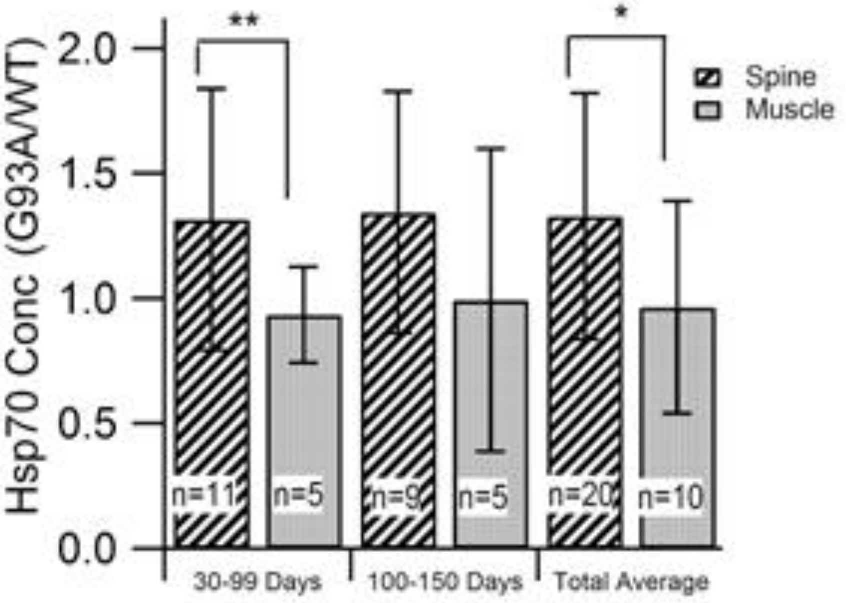

Figure 2. HSP70 Concentrations in Spine and Muscle. HSP70 data was divided into Spine and Muscle categories based on the reported tissue sample location in each article. This data was analyzed both as a total average and at different time bins to represent disease progression. Statistical significance was determined by a Mann-Whitney U-Test. *p<.05. **p<0.10.

Overall Heat Shock Protein Trends in the Spine and Muscles

For assessment of overall trends in HSP levels in the spine and muscles, data from all individual HSPs, including HSP70, were combined into a single, overarching group. This data was then divided into spine and muscle based on the tissue samples analyzed in each report. Analysis of this data via Mann-Whitney U-Test indicated that, at an alpha value of .10, heat-shock protein expression is significantly elevated (p=.0803) in the spine (n=41) compared to the muscles (n=16) of SOD1-G93A mice (**Figure 3**).

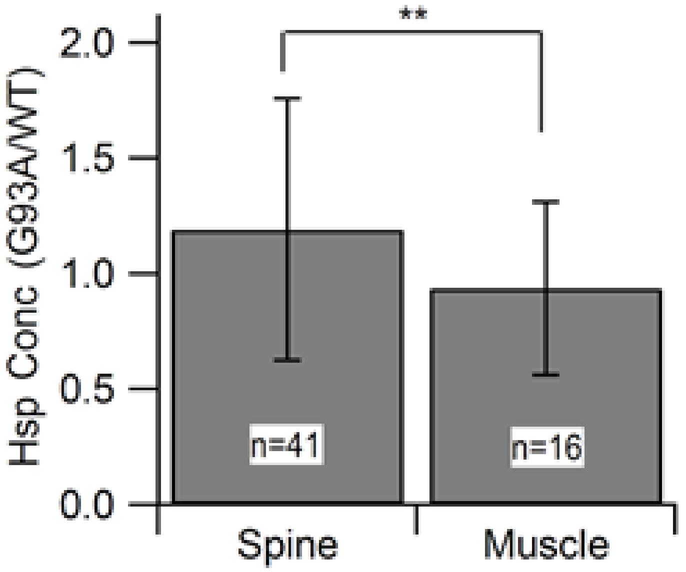

Figure 3. HSP Concentrations in Spine and Muscle. SOD1-G93A/WT Ratios from all HSPs was divided into Spine and Muscle categories based on the reported tissue sample location in each article. Statistical significance was determined by a Mann-Whitney U-Test. **p<0.10.

Heat Shock Protein Trends in the Spine and Muscles at Different Time Bins

HSP levels in each tissue sample location were divided into three separate time stages: 30-69 days (n_{spine}= 9; n_{muscle}= 5); 70-110 days (n_{spine}= 17; n_{muscle} = 6); and 111-150 days (n_{spine}= 15; n_{muscle} = 5). The time intervals were selected in such a way that each stage would cover about the same amount of days and would roughly align with known disease stages from the high copy SOD1 G93A transgenic ALS mouse literature (30-69 days is pre-onset), (70-110 days is onset), and (111-150 days is late or end stage). Results indicated spinal tissue samples have mostly up-regulated HSP levels (30-69 days= 0.99; 70-110 days=1.21; 111-150 days=1.21), higher than in muscle samples (30-69 days= 1.0; 70-110 days= 0.852; 111-150 days= 1.02) at each time interval, but no quantitative statistical significance was observed (**Figure 4**).

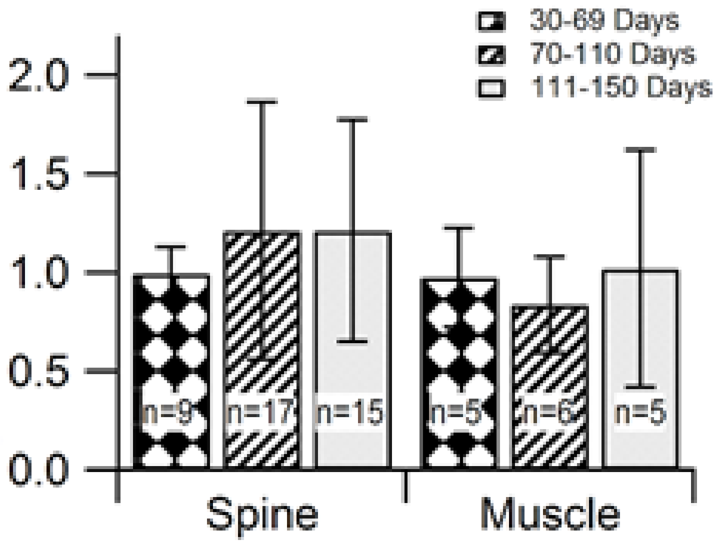

Figure 4. HSP Concentrations in Spine and Muscle at Different Time Stages. HSP data was divided into Spine and Muscle categories based on the reported tissue sample location in each article. This data was analyzed both as a total average and at different time bins to represent disease progression. Statistical significance was determined by a Mann-Whitney U-Test. *p<.05. **p<0.10.

HSP Levels in Transgenic SOD1-G93A Mice Compared to WT Mice

To determine the overall effects of ALS on HSP concentration, data from SOD1-G93A mice was compared to WT data. As all data was aggregated by finding the ratio of SOD1-G93A to WT values, the WT data for these analysis purposes was set to 1. To ensure equal weight of the two data sets, the sample size of the WT data was set equal to the sample size of the SOD1-G93A data (n=54). This data was first analyzed as an average value of all data points (**Figure 5**). SOD1-G93A data (average= 1.10) was found to be upregulated compared to WT (average= 1). However, Mann-Whitney U-Test revealed no quantitative statistical significance in this upregulated value.

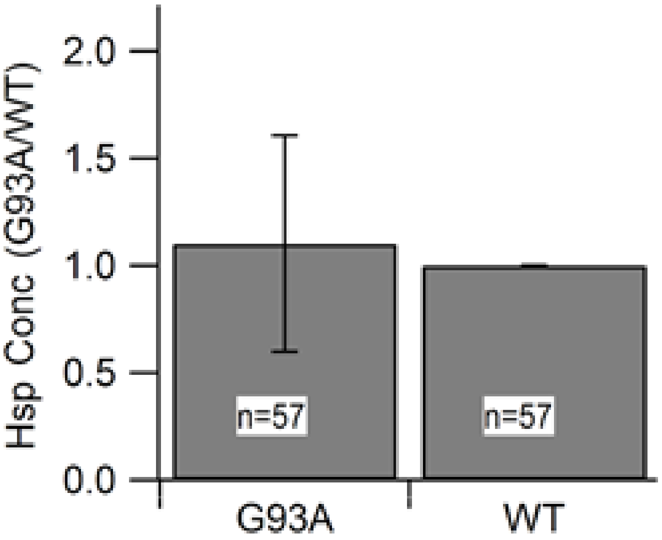

Figure 5. HSP Concentrations in SOD1-G93A Mice. All HSP data was combined into a single SOD1-G93A group. The SOD1-G93A/WT ratios were compared to the WT/WT ratio of 1. Statistical significance was determined by a Mann-Whitney U-Test. *p<.05. **p<0.10.

HSP Levels in G93A Mice Compared to WT at Different Time Bins

SOD1-G93A data was separated into individual time bins of 30-59 (n=12), 60-89 (n=13), 90-119 (n=16), and 120-150 days (n=16) (**Figure 6**). Statistical significance between SOD1-G93A and WT at each time bin was then determined. In all time bins, SOD1-G93A/WT HSP level ratios were found to be higher than the WT value of 1, but no quantitative statistical significance was observed. The change in SOD1-G93A HSP levels at each time bin was also analyzed (eg. 30-59 days SOD1-G93A data was compared to 60-89 days SOD1-G93A data). Average HSP levels increased from 30-59 days (average= 1.01) to 60-89 days (average= 1.08) and from 60-89 days to 90-119 days (average= 1.16), but decreased from 90-119 days to 120-150 days (average=1.13). In all comparisons, no quantitative statistical significance was observed between time bins.

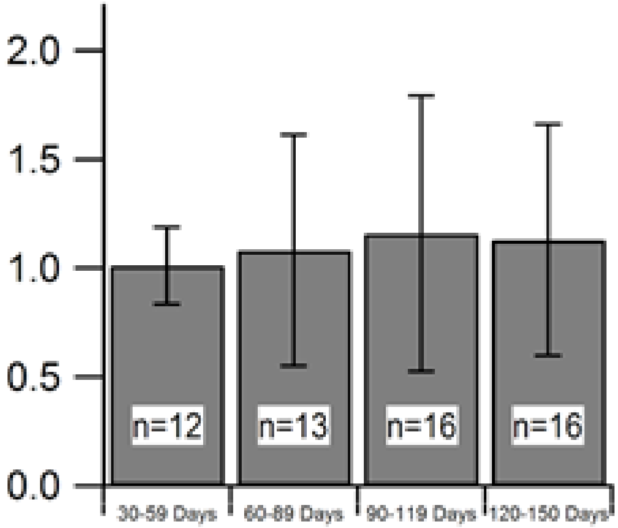

Figure 6. HSP70 Concentrations in Spine and Muscle. All HSP data was combined into a single SOD1-G93A group. The SOD1-G93A/WT ratios were compared to the WT/WT ratio of 1. This data was analyzed at different time bins to represent disease progression. Statistical significance was determined by a Mann-Whitney U-Test. *p<.05. **p<0.10.

CHAPTER 4

DISCUSSION

Heat Shock Response in the Spine and Muscles

The results of the meta-analysis revealed that there are discernable qualitative increases in HSPs in both the spine and muscle of SOD1 G93A ALS mouse, but the increases in HSPs in the spine are more exaggerated, particularly in pre-onset and onset stages. Given that the SOD1 G93A HSPs increases compared to wild type mice range from 1-20%, a much larger sample size is needed to overcome the large experimental error (variance) to illustrate quantitative statistically significant upregulation of HSPs. Nonetheless, the consistent qualitative trends do illustrate that an HSP upregulation is present, although insufficient, to combat the oxidative stress that co-exists in the ALS pathology.

Given the significantly larger concentration of HSPs in the spine compared to the muscles, and the fact that motoneurons have been observed to have a higher activation threshold for the heat shock response [6], it was determined that the heat shock response in the muscles is more susceptible to insufficient production of HSPs to compensate for the negative effects of oxidative stress. In the early stages of the disease, HSP70, which has been determined in the past to be a critical HSP in the defense against protein misfolding caused by oxidative stress [38], was found to be significantly more upregulated in the spine compared to the muscles. This result indicates that there is a discrepancy of HSP levels in the spine and muscle that exists well before functional ALS symptom onset.

Presence of Heat Shock Proteins as Evidence of Retrograde Pathology in ALS

A common characteristic and hypothesized factor of cell death in ALS is protein misfolding due to oxidative stress [40]. Past reports have shown that HSPs, due to the active role they play in assisting with protein folding, are upregulated in SOD1 G93A cells in an attempt to combat the negative effects of oxidative stress [6,40]. The results of this study support the findings of these previous reports as HSP levels were found to qualitatively increase, albeit without statistical significance, as the disease progresses and the effects of oxidative stress worsen. However, HSP levels in the muscles were significantly lower than in the spine, thus leaving muscle cells comparatively more vulnerable to protein misfolding than spinal cord motoneurons. Given that the discrepancy of HSP70 levels, in particular, begins early in disease progression, and the fact that protein misfolding leads to cell death, it appears that muscle cells are more vulnerable to early degeneration than spinal motoneurons. These results are supportive of the retrograde mechanism proposed by the "dying-back" theory, which hypothesizes that ALS begins in the muscle cells or NMJ and that degeneration of spinal cord motoneurons may occur as a consequence of the loss of muscle cells and early degeneration of NMJ [9,40]. The significance of this retrograde mechanism is that it proposes a specific pathological trend for disease progression. Therefore, it is possible that in at least a portion of ALS cases, cell degradation begins in the muscle cells of the limbs, rather than in the spinal motoneurons; in such cases, muscle cells become a key therapeutic target for early ALS intervention. In fact, results suggesting a greater susceptibly of muscles cells to insufficient HSP upregulation could support an initiating mechanism for limb onset ALS, one of the two most common forms of ALS, where muscle weakness begins in the extremities [37].

Even though the results of this meta-analysis qualitatively illustrate that muscles are more vulnerable from the standpoint of insufficient HSP upregulation, it does not mean that HSPs are the root cause of all ALS cases, as ALS is thought to be multi-factorial, with up to 10 different ontological pathophysiology categories contributing to the disease [25]. Nonetheless, these findings do suggest that it is plausible that at least in some cases, retrograde degeneration from the neuromuscular junction could be an initiating mechanism of ALS spread.

Finally, it has also been experimentally determined that the fast-acting motoneuron fibers die first in ALS. Fast-acting fibers (synapsing on Type IIb/x muscle fibers) work cooperatively with fast-resistant (also called intermediate) fibers and slow muscle fibers as part of a motor unit [26]. Among the fibers in this motor unit, fast fibers are known to have a deficiency of mitochondrial superoxide dismutase, leading to an increase in oxidative stress in fast-acting muscles [10]. Previous studies have exhibited that the increase in oxidative stress results in fast fibers having a higher vulnerability to synaptic denervation than intermediate or slow fibers, and loss of Type IIb/x muscle fibers during the early stages of disease progression [3,1,13,32,35]. These findings provide further support for the dying back theory as they present evidence of synapses, including NMJs, degrading before the loss of the entire spinal motoneuron cell body occurs, thus suggesting a potential retrograde mechanism for ALS pathology [12].

Heat Shock Proteins as a Potential Treatment

Previous studies have shown a positive correlation between survival time in mice and HSP concentration [14,19,20]. As the results of this study have shown, the Heat Shock response is weaker in the muscles than in the spine from the early stages of the disease, making muscles more susceptible to protein misfolding and consequently cell death. Coupling this finding with the theory that early degradation of muscle cells can result in the degeneration of spinal motoneurons, there exists promising optimism that early treatment of muscle cells with HSPs could delay or even prevent early muscle cell degeneration. The fact the native HSP upregulation is enhanced earlier in the ALS pathology suggests that, in both muscles and spinal motoneurons, that as the disease progresses, compensatory upregulation begins to fail. Thus, artificial upregulation of HSPs via HSP pharmaceutical treatment could possibly provide the needed compensation to combat ALS-associated oxidative stress. One possible treatment vehicle could be the injection of HSPs near the neuromuscular junction, which would also enable the retrograde axonal transport of injected HSPs to the spinal motoneurons, providing dual protection for both muscle and spinal motoneuron. Preventing early muscle cell degeneration would, in theory, prolong the life of both muscle cells and spinal motoneurons, which in turn could potentially prolong survival of ALS patients or minimally prolong post-onset quality of life.

Future Heat Shock Protein Research

Future research regarding HSPs should be aimed at analyzing the concentrations of independent HSPs. This study performed specific individual analysis on HSP70 as well as aggregated HSP analysis. Studies on the progression and concentrations of independent HSPs would lead to a larger understanding of which specific HSPs, in particular, are downregulated in the spine. For example, this study found that upregulated individuated HSP70 levels in the spine are not as apparent when multiple HSP types are aggregated and collectively assessed. The downregulation of other specific, individual HSPs could be indicative of a wider-spread inefficiency in the Heat-Shock Response in the spine, in addition to what has been seen in the muscles. Therefore, future mapping of temporal individuated HSP levels across different cell types and locations could lead to a better understanding of which HSPs should be artificially upregulated as well as their optimal therapeutic target site.

Homeostatic Instability in ALS

The early pathophysiology of ALS, in its early stages, attempts to compensate for increased oxidants levels by increasing HSPs. However, this response is overall inadequate, and antioxidant levels continue to rise. Regulatory or homeostatic instability can be seen in multiple pathways in ALS [17,28]. However, the compensation seen in HSPs [17] reveals that they are one of the earliest compensation mechanisms in ALS. As such, they could be an effective treatment target for early to mid-stage ALS. However, in reality, HSPs will likely have to be used in combination with other treatment targets to combat the multi-faceted nature of ALS from the sub-cellular through system level. The use of combination therapies in cancer has been quite effective [21,22,23,24]. A similar polytherapy approach tailored to the disease progression stages of ALS and its multiple failed regulatory pathways may also be required to obtain clinically significant therapeutic strategies.

APPENDIX

RAW DATA

Days	Spine	Muscle	Publication	Hsp
30		0.847826	Gifondorwa 1	70
40	1		Yamashita	27
40	1		Yamashita	70
40	1		Yamashita	105
50	0.727273		Wei	90
50	0.9375		Wei	40
50	1.046154	1	Wei	70
50	1.148148	0.833333	Wei	60
56	1.1875	1.4	Crippa 234	B8
60	0.911458	0.785714	Kalmar 339	70
70	1.5		Mimoto	70
70	2.5		Mimoto	70
70		0.87108	Sharp	27
70		0.486885	Sharp	27
72	0.7		Yamashita	27
72	0.75		Yamashita	105
72	0.8		Yamashita	70
75		1.243243	Gifondorwa 1	70
75	1		Kabashi	40
75	1.615		Kabashi	70
80	0.9		Vleminckx	70
95	0.6875		Wei	40
95	0.819672		Wei	90
95	0.871429	0.8	Wei	70
95	0.884615	0.75	Wei	60
98	1.65		Mimoto	70
98	1.666667		Mimoto	70
100	0.859375	0.857143	Kalmar 339	70
110	0.57		D'Arrigo	h1
110	2.81		D'Arrigo	b1
112	1.722222		Crippa 234	B8
117	0.4		Yamashita	105
117	1.3		Yamashita	70
117	1.9		Yamashita	27
120		0.675676	Kalmar 378	70

120		1.014493	Kalmar 378	70
120	1.5		Vleminckx	70
126	1.214286		Mimoto	70
126	2.35		Mimoto	70
130	0.927083	0.414286	Kalmar 339	70
130	0.714286	1	Wei	60
130	0.9		Wei	40
130	0.909091	2	Wei	70
130	1.5		Wei	90
135	0.3		Yamashita	105
135	1.6		Yamashita	70
150	0.95		Vleminckx	70
150	0.95		Vleminckx	70

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