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Boston University

BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**EVALUATING THE ROLE OF THE HIPPO PATHWAY IN THE ONSET AND
DISEASE PROGRESSION OF THE SOD1 MOUSE MODEL OF
AMYOTROPHIC LATERAL SCLEROSIS**

by

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B.S., Tufts University, 2013

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ABSTRACT

The Hippo pathway is a cell signaling pathway involved in organ size regulation and tumorigenesis in mammals. This pathway regulates the activity of Yes-associated protein (YAP), a transcriptional coactivator which binds to the transcription factor TEAD to promote expression of genes controlling growth and proliferation of tissues, as well as inhibition of apoptosis. The Hippo pathway has recently been implicated as a pathogenic mechanism in neurodegenerative disorders. Specifically, mammalian sterile 20 (Ste20)-like kinase 1 (MST1), a protein kinase in the Hippo pathway, has been found to promote neuronal death under conditions of oxidative stress. Moreover, homozygous deletion of MST1 in a mouse model of Amyotrophic Lateral Sclerosis (ALS) significantly delayed onset of neurodegenerative symptoms. We examined the expression levels of key Hippo pathway components in cortex, lumbar spinal cord, and gastrocnemius muscle samples of male and female G39A SOD1 mice using western blots. Our results revealed a significant increase in phosphorylated MST1 (pMST1) in lumbar spinal cord of presymptomatic transgenic animals, and found this increase to be sex and gene copy number dependent. These results suggest that the Hippo pathway is dysregulated in the SOD1 mouse model and that MST1 may play a critical role in pathogenesis and disease progression in ALS.

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LIST OF ABBREVIATIONS

ALS.....	Amyotrophic Lateral Sclerosis
FTD.....	Fronto-Temporal Dementia
FUS/TLS.....	Fused in Sarcoma/Translated in Liposarcoma
LATS.....	Large Tumor Suppressor
MAP1LC3.....	Microtubule Associated Protein 1 Light Chain 3
MST1.....	Mammalian Sterile 20-Like Kinase 1
NINDS.....	National Institute of Neurological Disorders and Stroke
SOD1.....	Superoxide Dismutase 1
TDP-43.....	Transactive Response DNA Binding Protein
TRIAD.....	Transcriptional Repression-Induced Atypical Death
YAP.....	Yes-Associated Protein

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS) is a fatal, progressive neurodegenerative disorder that primarily affects motor neurons in the brain and spinal cord. The onset of ALS is gradual, with symptoms typically starting as mild stiffness or weakness in muscles or difficulty speaking, slowly spreading to affect the entire body, and eventually leading to incapacitation and death, most commonly due to respiratory failure (Rowland 2001). Disease progression appears to depend on the brain region in which it originates: bulbar onset ALS (roughly $\frac{1}{3}$ of cases) begins in the medullary motor neurons and has a worse prognosis, with dysarthria and dysphagia appearing as the earliest symptoms, as compared to limb-onset ALS, which makes up the remainder of ALS cases and begins with weakness in the extremities (Salameh et. al. 2015). ALS currently affects roughly 4 out of every 100,000 people in the United States (NINDS 2015), but its incidence is estimated around 2.08 cases per 100,000 person-years worldwide. The average age of onset is between 54 and 67 years (Moura et. al. 2015), with an average survival period around 24 to 36 months after diagnosis (Moura et. al. 2015). A poorer prognosis is associated with bulbar onset of the disease, age over 75 years, and BMI below 25 kg/m² (Moura et. al. 2015).

Familial and Sporadic ALS

Roughly 5-10% of ALS cases are classified as “familial” (fALS), and have been linked to several known autosomal dominant mutations. The remaining 90-95% of ALS cases are classified as “sporadic ALS,” the basis of which is poorly understood, and is not

linked to any known genetic factor. The etiology of these cases has been suggested to be the result of an interaction between multiple genetic factors and environment (Moura et. al. 2015). One of the most commonly used models for familial ALS involves a mutation in the gene coding for superoxide dismutase 1 (*SOD1*). The *SOD1* mutation accounts for approximately 20% of familial ALS cases (Hardiman 2011).

Neumann et. al. (2006) found transactive response DNA binding protein (TARDBP or TDP-43) to play a role in ALS. A mutant form of this protein was present in protein aggregate plaques of patients suffering from both ALS and fronto-temporal Dementia (FTD), another common neurodegenerative condition. A mutation in the gene coding for TDP-43 was found to be present in a small percentage of both sporadic and familial ALS cases (Sreedharan et. al. 2008). In addition to TDP-43, the RNA binding protein known as fused in sarcoma/translated in liposarcoma (FUS/TLS) was also found to be mutated in about 5% of familial ALS cases, and the mutant form of this protein was also present in protein aggregate plaques of patients suffering from both ALS and Frontotemporal Dementia (FTD), suggesting that there may be a link between the two diseases (Kwaitkowski et. al. 2009, DeJesus-Hernandes et. al. 2011). Both the TDP-43 and FUS/TLS mutations appear to cause impairment in the processing and metabolism of RNA within the cell, leading to ALS pathology (Lagier-Tourenne et al. 2010). Recently, a GGGGCP hexanucleotide expansion in a noncoding region of open reading frame 72 on chromosome 9 (*C9ORF72*) was identified as the cause of a large proportion of ALS cases (Renton et. al. 2011). This mutation has been implicated as the cause of up to 35-40% of familial ALS cases, as well as 5-7% of sporadic ALS cases (Myszczynska and

Ferraiuolo 2016), as well as 18.3% of all cases of TDP-43-related FTD (DeJesus-Hernandes et. al. 2011). The proposed pathological mechanism of this mutation involves expression of a large amount of toxic RNA, which disrupts transcription and RNA metabolism (Renton et. al. 2011), similar to the proposed effects of the TDP-43 and FUS/TLS mutations.

The onset of ALS typically begins with mild stiffness or weakness in muscles or difficulty speaking, and slowly spreads to affect all upper and lower motor neurons. The disease is fatal within a few years, with respiratory failure being the most common cause of death (NINDS 2015). While there is no explicit component of cognitive impairment included in the diagnosis of ALS, Frontotemporal dementia is commonly observed in ALS patients (13.8% of cases), especially those with the TDP-43 mutation, and 34.1% of ALS patients fulfill criteria for more mild cognitive impairments (Phukan et. al. 2012).

ALS Therapies

Therapeutic options for ALS are extremely limited and patients rely mostly on palliative care (Kiernan et. al. 2011). Riluzole is the only FDA approved disease-modifying therapy for ALS, first made available in 1995, and it has been shown to prolong survival by 2-3 months. Riluzole modulates glutamatergic neurotransmission to prevent toxic buildup of glutamate in synapses, preventing excitotoxicity, a hypothesized cause of neuronal death in ALS (Plaitakis et. al. 1990). The drug was found to significantly slow the rate of muscular degeneration (Bensimon et. al. 1994), but the specific biochemical mechanisms by which it creates this effect could not be determined. While modifying synaptic glutamate concentrations appears to have a positive effect on

the course of the disease, its limited therapeutic capacity suggests that it is not the only cause of ALS-related pathology.

The role of SOD1 in ALS

SOD1 is a highly conserved zinc- and copper-containing protein that both allows the cell to resist oxidative stress and regulates the activity of cell proliferation pathways (Crapo et. al. 1992). SOD1 helps to manage intracellular buildup of damaging reactive oxygen species (ROS) including superoxide (O_2^-) by converting them into hydrogen peroxide (H_2O_2), which the cell can metabolize. A gain of function missense mutation in the SOD1 gene, resulting in the replacement of glycine 93 with alanine (G93A), has been identified as a likely cause of some fALS cases (Chiu et. al. 1995). Mutant cells expressing this abnormal SOD1 protein experience a higher degree of oxidative stress, which has been suggested as a cause of ALS symptoms (Lee et. al. 2013). Unequal recombination during meiosis can give rise to difference in the number of copies of the mutant SOD1 gene in an individual as compared to his/her parents (Alexander et. al. 2004.) Importantly, mice with a higher number of copies of the mutant SOD1 gene were found to reach the end-stage of the disease earlier than mice with a lower copy number (Chiu et. al. 1995).

Hippo pathway

The Hippo signaling pathway is involved in cell division and organ size control (Fernandez and Kenney 2010). It was first described in drosophila but is a highly

conserved pathway across species (Harvey and Tapon 2007). The primary function of the Hippo pathway is to regulate several mechanisms involved in cell growth and proliferation. Therefore, the majority of studies focused on this pathway have evaluated its role in tumor suppression (Hao et. al. 2008, Harvey and Tapon 2007, Zhao et. al. 2008). The protein “hippo” is a serine-threonine kinase found in drosophila, named for the characteristic head and eye deformities observed in knockout drosophila lacking the *hippo* gene (Pan et. al. 2007).

Mammalian Sterile 20 (Ste20)-Like Kinase 1 (MST1) is the mammalian paralog of the Hippo protein, and an important regulatory component of the Hippo pathway in mammals. MST is part of a kinase complex that forms the core of the Hippo pathway. It phosphorylates and activates several downstream targets, including the warts analogue Large Tumor Suppressor (LATS), which is involved in the inactivation of the transcriptional coactivators YAP and TAZ (Hao et. al. 2008, Zhao et. al. 2010). LATS was recognized as a tumor suppressor in a study by Justice et. al. (1995), which showed that knockout of the *warts* gene, which acts as an analogue to LATS in drosophila, led to an increase in apical hypertrophy and overgrowth in epithelial cells. Further study determined that the tumor suppressing function of LATS was linked to its interaction with YAP, phosphorylating and inactivating YAP to inhibit its effect on cell growth (Hao et. al. 2008.) Guo et. al. (2015) report that LATS appears to be downregulated in hepatocellular carcinoma, leading to increased YAP activity and a subsequent loss of cell polarity and an increase in cancer cell invasion into healthy tissue. It has also been determined that phosphorylated MST1 (pMST1) phosphorylates the protein WW45,

which is critical for cytoplasmic sequestration of phospho-YAP, as well as MOB1, which enhances the binding affinity of LATS1 for YAP to facilitate phosphorylation (Zhao et. al. 2008). Loss of MST1 function has been linked to loss of cell polarity, which can contribute to the formation of cancer cells (Shroeder and Halder, 2012). However, a study by Lee et. al. (2013) found that MST1 was active at an abnormally high level in motor neurons expressing a mutant form of the *SOD1* gene.

YAP (yes-associated protein) is a transcriptional coactivator which acts by associating with TEAD-family transcription factors to increase expression of genes which promote growth and proliferation of tissues, as well as inhibit apoptosis. The activity of YAP is one of the primary mechanisms controlled by the Hippo pathway (Huang et. al. 2005). YAP is considered an oncogene due to its ability to suppress apoptosis and induce epithelial-mesenchymal transformation, and overexpression of YAP has been observed in certain cancers including mesothelioma, breast cancer, leukemia and hepatocellular carcinoma (Zhao et. al. 2008). When active, YAP localizes to the nucleus and binds to TEAD to induce transcription. However, after phosphorylation of certain serine residues on the YAP protein by the kinases Large Tumor Suppressor (LATS) 1 and 2, the phosphorylated protein is sequestered in the cytoplasm where it can no longer induce transcriptional activation (Zhao et. al. 2010). YAP has a paralog called TAZ which appears to work in concert with YAP but via different mechanisms (Lei et. al 2008), as TAZ knockouts display lung and kidney deformities in drosophila, as opposed to the head and eye deformities observed in Yorkie (YAP) knockouts (Makita et. al. 2008).

While hippo-knockout drosophila displayed overgrowth of tissue in the head and eye regions, knocking out the protein “yorkie” (the drosophila analogue to YAP) caused a significant lack of development in these regions, demonstrating the relationship between these two genes and their related functions. This relationship is further described by Huang et. al. (2005), who found that the activity of yorkie was inhibited in cells expressing the kinase “warts,” a known target for phosphorylation by hippo (Wu et. al. 2003). This inhibition was found to be diminished by treatment of cells with phosphatases, indicating that a phosphorylation event was causing the decrease in yorkie activity (Huang et. al. 2005).

While upstream regulation of the Hippo pathway is not fully understood, it appears to be controlled in part by a G-protein coupled receptor-dependent mechanism. Serum containing the phospholipid lysophosphatidic acid or the sphingolipid sphingosine-1-phosphate, was found to promote dephosphorylation of YAP, while also decreasing phosphorylation of LATS 1/2. Conversely, activation of beta-adrenergic receptors, dopamine receptors, and glucagon receptors had the reverse effect, leading to an increase in the rate of phosphorylation of YAP by LATS 1/2 (Yu et. al. 2012). This suggests that signaling via G-proteins can result in both dephosphorylation of YAP to promote cell division, and phosphorylation of YAP to induce cell death, possibly by promoting MST1 phosphorylation.

Hippo pathway and ALS

MST1 phosphorylation was found to be elevated in motor neurons expressing the G93A SOD1 mutation (Lee et. al. 2013), suggesting that a high degree of YAP/TAZ

inactivation may result in impaired cell growth and proliferation, contributing to the development of the ALS phenotype. This increase in MST1 appeared to be limited to motor neurons, as SOD1 mutant glial cells did not show the same increase in MST1 activation observed in motor neurons (Lee et. al. 2013.) Furthermore, homozygous deletion of the *mst1* gene in mice expressing mutant SOD1 caused a significant delay in the onset and mortality of ALS, suggesting that the function of the MST1 protein contributes significantly to the development of ALS pathology.

The role of MST1 in the management of oxidative stress is further illustrated by its interaction with the redox protein thioredoxin. This interaction limits the degree to which MST1 can be activated in the presence of reactive oxygen species, stopping MST1 from inducing apoptosis under conditions of oxidative stress from which the cell can recover using antioxidant mechanisms like those provided by thioredoxin. However, the excess of ROS in SOD1 mutant neurons appears to impair the association of MST1 and thioredoxin (Lee et. al. 2013), possibly resulting in unchecked activation of MST1, and consequently a high degree of YAP inactivation via phosphorylation, leading to cell cycle exit and cell death. The activity of MST1 is also relevant in pathology of non-neuronal tissues. For example, MST1 expression is upregulated in skeletal muscle following denervation, and homozygous deletion of MST1 significantly slowed the process of muscle wasting after removal of neuronal input (Wei et. al. 2013). This suggests that overexpression of MST1 in ALS may play a role in muscle degeneration as well as neuronal death.

Autophagy is the process of breaking down old or damaged proteins and organelles within a cell to promote turnover and replenish the cell's supply of macromolecules. This is typically done by first marking materials for destruction: in the case of large protein degradation in mammals, the ubiquitin-binding protein sequestosome 1 (SQSTM1), also known as p62, is believed to be a common signal and is as such frequently measured as an indicator of autophagic activity (Jaeger and Wyss 2009). Materials marked for degradation are then enclosed in a membrane-bound vesicle called an autophagosome, which is usually marked with microtubule associated protein 1, light chain 3 (MAP1LC3). The tagged autophagosome is then directed to fuse with a lysosome, destroying its contents and generating macromolecules which can be used for new cellular functions (Jaeger and Wyss 2009). Autophagy in the nervous system is believed to be an important factor in ensuring the longevity of neurons. Since most neurons live for many years, healthy autophagy mechanisms are required to ensure that damaged or senescent organelles can be continuously cycled out in favor of newly created ones, and that the damaging protein aggregates which often accumulate within aging neurons can be cleared away (Jaeger and Wyss 2009). Importantly, overexpression of YAP leads to a decrease in intracellular p62, indicating an increase in the rate of autophagy within the cell (Song et. al. 2015). Given the apparent failure of autophagy mechanisms in many neurodegenerative disorders (Cherra and Chu 2008), and the apparent contribution of the hippo pathway to maintaining effective autophagy in cells (Hoshino et. al. 2006), it is possible that examining this pathway may provide insight into the mechanisms behind cell death in ALS.

Hippo pathway in other neurodegenerative disease

Hoshino et. al. (2006) describe a novel pathological feature observed in many neurodegenerative disorders, which they term “transcriptional repression-induced atypical death” (TRIAD). As its name suggests, TRIAD is a slowly progressing cell death pattern, distinct from apoptosis or necrosis, which appears to be caused by impaired autophagy resulting from a disruption of the cell’s transcriptional machinery (Hoshino et. al. 2006). This study found that neuron-specific isoforms of YAP, referred to as YAP Δ C’s, were expressed in cells undergoing α -amanitin induced TRIAD, coinciding with a decrease in expression of normal YAP. Infection of Huntington Disease (HD) neurons with YAP Δ C DNA via a viral vector slowed the rate of HD related death, while YAP Δ C-specific siRNA accelerated cell death in these neurons (Lee et. al. 2013). This suggests a possible neuroprotective function of YAP, which could potentially be suppressed by the apparent changes in MST expression that occur in ALS. Despite this correlation, the role that MST, YAP, and the Hippo pathway play in the pathology of neurodegeneration remains unclear, and further study into the mechanisms by which these proteins interact under ALS conditions may shed light on new therapeutic targets and strategies.

Objectives

The purpose of this study is to examine the whether dysregulation of the hippo pathway, in particular MST expression and phosphorylation, plays a role in the development and progression of ALS. This pathway is newly discovered, and the majority of related literature focuses on its role in tumorigenesis in several cancers.

However, there is enough evidence to suggest that it may play a pathogenic role in neurodegeneration. SOD1 is expressed in all cells of the body, and the hippo pathway is expressed in many different tissue types at various developmental stages (Crapo et. al. 1992), so a simple analysis of motor neurons was deemed inadequate for thorough study of the pathway's role in ALS.

This study aims to determine whether the hippo pathway presents a viable therapeutic target for ALS by examining its expression and activity in various tissues of transgenic mice expressing mutant SOD1 (G93A) and wild-type littermates. An elevation of hippo pathway activity in the ALS case may indicate a decrease in cell growth and autophagy mechanisms, which could possibly contribute to the development of neurodegenerative symptoms. Expression of Hippo pathway components was assessed in relation to differences in sex, mutant SOD1 copy number, and presence or absence of ALS symptoms. Evaluating the expression of the hippo pathway in the SOD1 mouse will expand our understanding of the pathogenesis of ALS and facilitate the development of novel therapeutic strategies.

METHODS

Animals

In order to obtain animals for this study, male B6SJL-Tg(SOD1*G93A)1Gur/J mice (Gurney et al 1994) (Jackson Laboratory) were bred with female black 6 mice (Jackson Laboratory) to obtain wild-type and transgenic (G93A SOD1) mice. All animals were given access to food and water ad libitum. The onset of ALS symptoms in transgenic animals was assessed using ALS TDI neurological scoring criteria (Hatzipetros et. al. 2015). Animals were assessed using a tail suspension test every 5 days. The mice were suspended by the tail above their home cage for roughly 2 seconds, and the degree of limb splaying was assessed and given a score from 0-4. A score of 0 indicates no motor impairment, a score of 1 was given to mice who had displayed trembling in the leg or partial collapse towards the midline, a score of 2 was given to mice displaying obvious paresis in one or both hindlimbs, a score of 3 indicated that full or partial paralysis of the hindlimbs was present, and 4 indicated rigid paralysis, and was considered to be the humane endpoint of the illness.

RNA Extraction and Copy Number Analysis

In order to determine the mutant SOD1 copy number in the mice that were being used for this study, RNA was extracted from each animal's cortex and was processed using an RNeasy mini-kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The RNA was then incubated with iScript Reverse Transcriptase Supermix (Bio-Rad, Waltham, MA) and used to synthesize cDNA. 50 nanograms of this cDNA was combined with primers for the SOD1 gene (GGGAAGCTGTTGTCCCAAG and

CAAGGGGAGGTAAAAGAGAGC) and SYBR-green PCR Master Mix (Bio-Rad, Waltham, MA), and RT-qPCR was performed using a CFX96 Real-Time PCR System and C100-Touch Thermal Cycler (Bio-Rad, Waltham, MA) to determine the number of copies of the mutant gene present in each subject. The crossing-threshold (CT) value represents the point at which fluorescence in the PCR buffer caused by DNA amplification reached the intensity at which it was detectable. The number of PCR cycles required to reach this threshold is inversely proportional to the number of copies of the gene being amplified in the starting sample. The CT value can therefore be used to accurately determine the SOD1 copy number for each animal. CT values for transgenic animals were in the range of 20.02-26.47, and CT values for wild type animals were in the range of 26.33-38.005.

Western Blotting

Samples of cortex, gastrocnemius muscle, and lumbar spinal cord were collected from both transgenic and wild type mice. The tissue samples were homogenized in a lysis buffer containing sodium dodecyl sulfate, and 20-50ug of protein was suspended in sample buffer, boiled at 95°C for 5 minutes, and fractionated on a 4-20% glycine gel (Invitrogen, Carlsbad, CA) at 120V for 90-120 minutes. The separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using an iBlot Transfer System (Invitrogen, Carlsbad, CA). After the transfer was complete, the gels were placed in a Coomassie stain overnight in order to confirm equal protein loading. The membranes were blocked for 1 hour in 15 mL of a 3% milk-5% bovine serum albumin (BSA) solution, washed 4X (15 minutes, room temperature) with tris-buffered saline containing

Tween 20 (TBST). After blocking and washing, membranes were incubated overnight in a solution of 5% BSA and a rabbit or mouse-derived primary antibody against one of the following proteins: MST1 (Aviva Systems Biology, San Diego, CA), Phospho-MST1 (Cell Signaling, Beverly, MA), YAP (Cell Signaling, Beverly, MA), or p62 (Cell Signaling, Beverly, MA). Once the primary antibody was bound to the target protein, the membranes were washed again in 4 changes of TBST (15 min, room temperature), and incubated for 1 hour with one of the following HRP (horseradish peroxidase)-conjugated secondary antibodies: goat anti-rabbit IgG (Cell Signalling, Beverly, MA) or goat anti-mouse IgG (Bio-Rad, Waltham, MA), which bound to the primary antibody. Finally, the membrane was treated with Western Lightning ECL solution (Perkin-Elmer, Waltham, MA) that caused the HRP-labeled secondary antibody to fluoresce, allowing protein bands to be visualized on film.

Protein bands on film were analyzed using AlphaEase FC software version 4.1.0 (Alpha Innotech Corporation, San Jose, CA), utilizing the spot densitometry function to obtain an integrated density value (IDV) for each band, reflecting the relative concentration of the protein in question in each sample. IDVs were normalized to the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the expression of which was expected to be unchanged across samples from different individuals. The concentration of each protein investigated was expressed as a ratio of that protein's IDV to that of GAPDH (EMD Millipore, Darmstadt, Germany) in the same sample.

Statistical Analysis

Data are presented as the mean +/- the standard deviation for each experimental group. Male and female mice were analyzed separately due to the sex differences observed in the disease profile of the SOD1 model (Scott et al 2008, Heiman-Patterson et al 2005). Two-way analysis of variance (ANOVA) was used to make comparisons of protein expression between wild type, presymptomatic and symptomatic groups, and between sexes. P values below 0.05 were considered statistically significant. Data were analyzed using the statistical analysis program Prism (GraphPad Software, La Jolla, CA).

RESULTS

Samples of cortex, lumbar spinal cord, and gastrocnemius muscle were collected from wild type and transgenic G39A SOD1 mice and tested using western blot to assess the levels of the key components of the Hippo/YAP pathway. In order to examine the possible alteration of Hippo pathway signaling across the course of the disease, transgenic samples were divided into presymptomatic group and a symptomatic group. Presymptomatic samples were taken from transgenic animals expressing mutant SOD1 that had not yet begun to manifest ALS symptoms, while symptomatic samples came from animals showing outward signs of impairment or paralysis before death, according to ALS TDI neurological scoring criteria (Hatzipetros et. al. 2015). Transgenic samples were further divided by G39A SOD1 gene copy number into a high copy number group (CT value<23) and a medium copy number group (CT value between 23 and 25).

First, levels of phosphorylated MST1 were analyzed as an indicator of Hippo pathway activity. In lumbar spinal cord, western blot analysis revealed that the expression of phosphorylated MST1 was significantly increased, as compared to wild type littermates, among presymptomatic transgenic females with medium copy number ($p<0.01$) (Figure 1A). PMST1 was not found to be significantly elevated compared to wild type in symptomatic transgenic medium copy females, or transgenic high copy females (Figure 1B). There were no significant changes in the phosphorylation of MST1 in lumbar spinal cord among transgenic males, and no significant difference was found between males and females (Figure 1A, 1B). Additionally, when all lumbar spinal cord samples, regardless of copy number, were analyzed together, there was no significant

elevation of PMST in presymptomatic or symptomatic transgenic females compared to wild type (Figure 1C).

Phospho-MST1 levels were also assessed in muscle tissue of male and female transgenic presymptomatic and symptomatic SOD1 mice with high and medium copy numbers, as well as wild type mice. None of the transgenic groups showed a significant increase in MST1 phosphorylation compared to wild type, and there was no significant difference between males and females (Figure 2).

Next, overall levels of MST1 were analyzed in lumbar spinal cord of medium copy number animals, to determine if the observed increase in MST1 phosphorylation was accompanied by an increase in MST1 gene expression. Among medium copy females, no significant increase in MST1 expression was found in presymptomatic or symptomatic transgenic animals compared to wild type littermates (Figure 3B). There was also no significant increase in MST1 expression among medium copy male presymptomatic or symptomatic transgenic mice compared to wild type littermates. There were no significant differences in MST1 expression in lumbar spinal cord between sexes (Figure 3B).

Total MST1 expression levels were also assessed in the muscle and cortex of wild type and medium copy number presymptomatic and symptomatic transgenic male and female mice. No significant changes in MST expression were observed between any of these groups, and there were no significant differences in MST expression between sexes (Figure 3A, 3C).

Expression of YAP was measured as an indicator of the downstream effects of increased MST1 phosphorylation. YAP expression levels were assessed in cortex, muscle, and lumbar spinal cord of wild type and presymptomatic and symptomatic transgenic male and female mice. Western blots revealed no significant change in YAP expression among presymptomatic or symptomatic transgenic males or females compared to wild type littermates, and no significant differences between sexes were observed (Figure 4).

Expression of the autophagy marker p62 was assessed to check for impairment of autophagy resulting from increased phosphorylation and activation of MST1. In muscle, no significant changes in p62 expression were present in male or female presymptomatic or symptomatic transgenic animals compared to wild type littermates (Figure 5B). P62 expression was also measured in lumbar spinal cord of G39A SOD1 mutant mice. There was no significant difference in p62 expression among presymptomatic or symptomatic transgenic animals compared to wild type littermates, and there was no significant difference in p62 expression between sexes (Figure 5A).

FIGURES

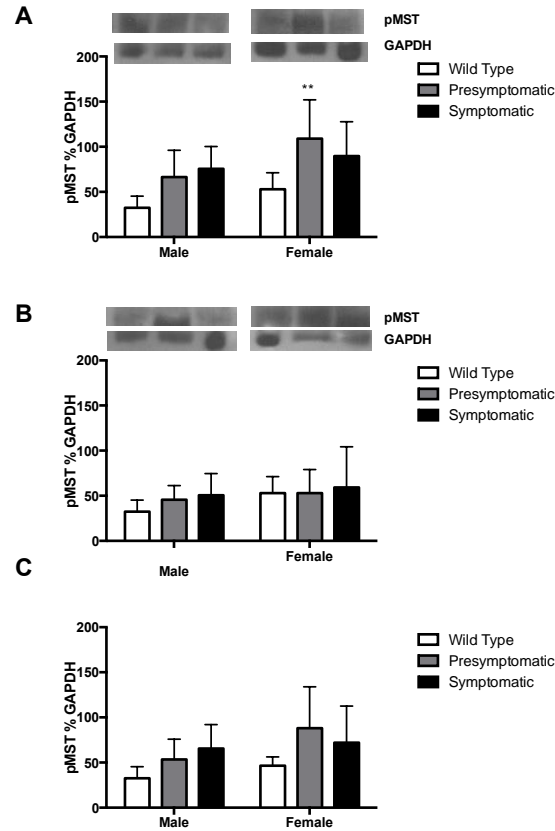


Figure 1: MST1 Phosphorylation in Lumbar Spinal Cord of Male and Female G39A SOD1 Mutant Mice

(A) In lumbar spinal cord, MST1 phosphorylation is increased in medium copy number presymptomatic transgenic females (n=5, $p < 0.01$) but not in symptomatic transgenic females (n=5) among presymptomatic transgenic females with medium copy number, compared to wild type littermates (n=6). There was no significant difference between pMST1 levels in presymptomatic and symptomatic transgenic females. No significant changes in pMST1 levels were observed in transgenic males (n=10, 3 medium copy presymptomatic, 7 medium copy symptomatic) compared to wild type (n=6), or between presymptomatic and symptomatic transgenic groups. There were no significant differences in pMST1 expression between males and females. (B) Among transgenic high copy females (n=7, 3 presymptomatic, 4 symptomatic), there was no significant difference in MST1 phosphorylation compared to wild type (n=6) or between transgenic groups. There were also no significant changes in pMST1 levels among transgenic males (n=10, 5 high copy presymptomatic, 5 high copy symptomatic), compared to wild type (n=5) or between transgenic groups. No significant difference in PMST1 levels was found between males and females. (C) When all lumbar spinal cord samples, regardless of copy number, were analyzed together, there was no longer a significant elevation of pMST1 in presymptomatic transgenic females (n=10). There was still no change in pMST1 in symptomatic transgenic females (n=10) compared to wild type (n=6), or in presymptomatic transgenic males (n=8) or symptomatic transgenic males (n=12) compared to wild type (n=6).

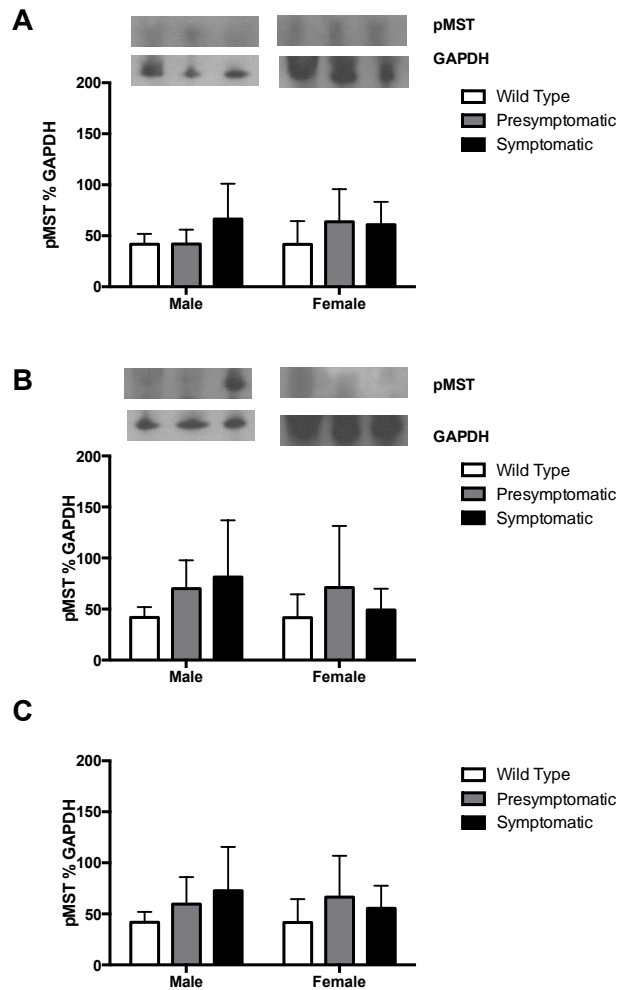


Figure 2: MST1 Phosphorylation in Gastrocnemius Muscle of Male and Female G39A SOD1 Mutant Mice

(A) In muscle, there was no significant increase in MST1 phosphorylation among presymptomatic transgenic males (n=3) or symptomatic transgenic males (n=7) with medium copy number compared to wild type littermates (n=5) or between transgenic groups. There was also no significant difference in MST1 phosphorylation in medium copy presymptomatic transgenic females (n=5) or medium copy symptomatic transgenic females (n=5) compared to wild type littermates (n=6) or between transgenic groups. There was no difference in pMST1 levels between sexes. (B) Among animals with a high G39A SOD1 gene copy number, there was no significant difference in MST1 phosphorylation among transgenic males (presymptomatic n=5, symptomatic n=5) compared to wild type littermates (n=5) or between transgenic groups. There was also no significant increase in pMST1 levels in transgenic females (presymptomatic n=3, symptomatic n=4) compared to wild type littermates (n=6) or between transgenic groups. There was no significant difference in pMST1 levels between sexes. (C) Combining samples of different copy numbers for analysis showed that there was still no significant difference in pMST1 expression in muscle of transgenic males (presymptomatic n=8, symptomatic n=12) compared to wild type (n=5) or between presymptomatic and symptomatic transgenic groups. There was also still no significant change in pMST1 levels among transgenic females (presymptomatic n=8, symptomatic n=9) compared to wild type (n=6) or between transgenic groups. There was no significant difference in pMST1 expression between sexes.

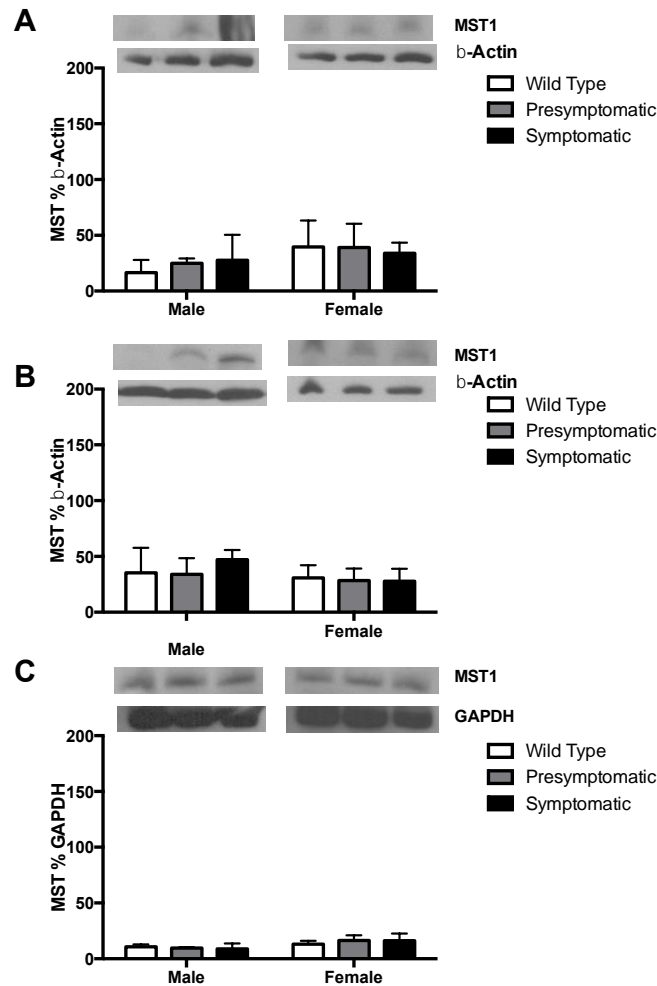


Figure 3: MST1 Expression in Presymptomatic and Symptomatic Male and Female Transgenic G39A SOD1 Mutant Mice with Medium Gene Copy Number

(A) In muscle, MST1 expression was not significantly altered in transgenic male mice with medium G39A SOD1 gene copy number (presymptomatic n=3, symptomatic n=3) compared to wild type littermates (n=3) or between presymptomatic and symptomatic transgenic animals. In female mice, there was also no significant change in MST1 expression in muscle of transgenic animals (n=3 presymptomatic, n=3 symptomatic) compared to wild type littermates (n=3) or between transgenic groups. There was also no significant difference in MST1 levels between sexes. (B) In lumbar spinal cord, there was no significant increase in MST1 expression in medium copy number transgenic male mice (presymptomatic n=3, symptomatic n=3) compared to wild type littermates (n=3) or between transgenic groups. In females, there was also no change in MST1 expression among medium copy number transgenic mice (n=5 presymptomatic, n=5 symptomatic) compared to wild type littermates (n=5) or between transgenic groups. There was also no significant difference in MST1 expression between sexes. (C) In cortex, there was no significant change in MST1 expression among transgenic male mice (n=4 presymptomatic, n=4 symptomatic) compared to wild type (n=4), or between transgenic groups. Among transgenic female mice (n=4 presymptomatic, n=4 symptomatic), there was no change in MST1 expression compared to wild type (n=4) or between transgenic groups. There was no significant difference in MST1 expression between sexes.

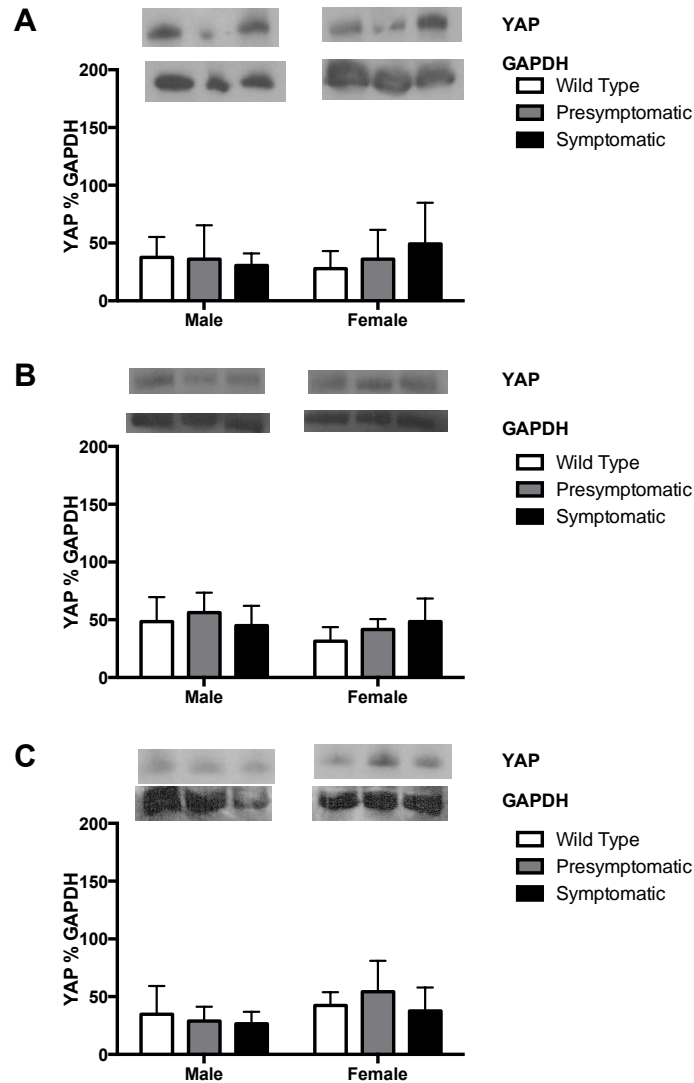


Figure 4: YAP Expression in Presymptomatic and Symptomatic Male and Female Transgenic G39A SOD1 Mutant Mice

(A) In muscle, YAP expression was not significantly altered in male transgenic animals (n=4 presymptomatic, n=4 symptomatic) compared to wild type (n=4) or between transgenic groups. There was also no significant change in YAP expression among female transgenic animals (n=4 presymptomatic, n=4 symptomatic) compared to wild type or between transgenic groups. There was no significant difference in YAP expression between sexes. (B) In lumbar spinal cord, there was no significant difference in YAP expression among male transgenic animals (n=4 presymptomatic, n=4 symptomatic) compared to wild type littermates (n=4) or between transgenic groups. There was no significant change in YAP expression among female transgenic animals (n=4 presymptomatic, n=4 symptomatic) compared to wild type littermates (n=4) or between transgenic groups. There was no difference in YAP expression in lumbar spinal cord between sexes. (C) In cortex, no significant differences were found in YAP expression among male transgenic animals (n=4 presymptomatic, n=4 symptomatic) compared to wild type littermates (n=4) or between transgenic groups. YAP expression was also not significantly changed among female transgenic animals (n=4 presymptomatic, n=4 symptomatic) compared to wild type littermates (n=4) or between transgenic groups. YAP expression in cortex was not found to be significantly different between sexes.

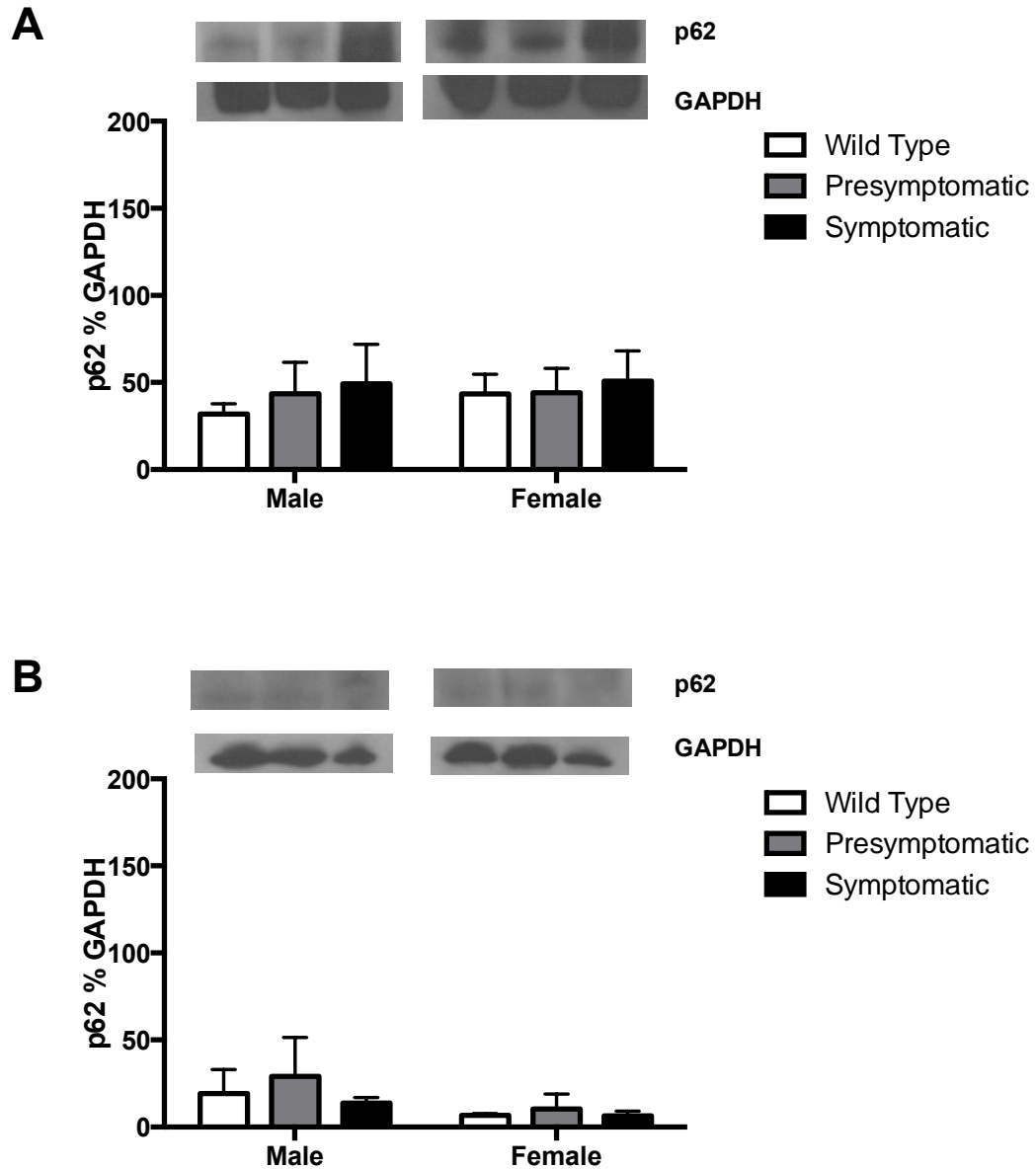


Figure 5: Expression of p62 in Presymptomatic and Symptomatic Male and Female Transgenic G39A SOD1 Mutant Mice

(A) In lumbar spinal cord of G39A SOD1 mutant mice there was no significant increase in p62 expression among transgenic male mice (presymptomatic n=9, symptomatic n=10) compared to wild type littermates (n=5) or between presymptomatic and symptomatic transgenic groups. There was also no significant increase among transgenic female mice (presymptomatic n=8, symptomatic n=8) compared to wild type (n=4) or between transgenic groups. There was no significant difference in p62 levels between sexes. (B) In muscle, p62 was not significantly elevated in male transgenic animals (n=2 presymptomatic, n=2 symptomatic) compared to wild type (n=4) or between transgenic groups. There was also no significant change in p62 levels in female transgenic animals (n=3 presymptomatic, n=3 symptomatic) compared to wild type (n=4) or between transgenic groups. There was no significant difference in p62 expression between sexes.

DISCUSSION

The Hippo signaling pathway has been shown to play a role in neurodegeneration. MST1 appears to mediate neuronal death due to oxidative stress, while homozygous deletion of MST1 delayed onset of ALS symptoms and improved survival (Lee et. al. 2013), and previous studies suggest that YAP activity has a neuroprotective function in the brain (Hoshino et. al. 2008). In this study, we aimed to evaluate the expression of key Hippo pathway components in ALS and investigate their relationship to the development and progression of disease phenotype. Our results demonstrate that there is an increase in phosphorylated MST1 (pMST1) levels in spinal cord of presymptomatic transgenic female mice compared to wild-type littermates, and found this change to be both sex-specific and copy number-dependent.

We hypothesized that if MST1 plays a role in ALS pathology, then MST1 phosphorylation would be increased in transgenic animals compared to wild type littermates. Western blot analysis of pMST1 protein levels revealed a significant increase in pMST1 in the lumbar spinal cord of presymptomatic transgenic female animals, but not in symptomatic transgenic females or any males (Figure 1A). The lack of elevated MST1 phosphorylation in symptomatic transgenic animals suggests that the increase in MST1 activity prior to the onset of ALS symptoms is not sustained during the symptomatic disease phase. This could indicate that increased pMST1 is involved only in the onset of ALS pathogenesis. It is possible that once the neurodegenerative process has

been initiated by pMST1, other pathways begin to mediate cell death while pMST1 levels return to normal, but the mechanisms that would make this possible remain unclear.

MST1 activity has been found to be elevated in skeletal muscle cells following denervation of the muscle, and deletion of the gene coding for MST1 delays muscle wasting (Wei et. al. 2013). Because muscle atrophy in ALS is caused by the death of motor neurons, we hypothesized that pMST1 levels would be elevated in transgenic animals. Our findings, however, show no increase in activation of MST1 in muscle of SOD1 mutant mice (Figure 2), but there was a slight, non-significant trend towards increased pMST1 in symptomatic males and presymptomatic females, regardless of copy number (Figure 2C). These findings suggest that MST1 does not play a role in ALS-related muscle pathology.

The observed increase in pMST1 appears to be specific to the spinal cord of mutant SOD1 mice. These findings suggest that MST phosphorylation is increasing only in nervous tissue cells of the spinal cord, but not in which cell types the change is taking place. It is possible that the observed changes in pMST1 levels in lumbar spinal cord are taking place in motor neurons, the cell type most extensively affected by ALS (Salameh et. al. 2015). Another possibility is that the changes in MST1 phosphorylation observed in the lumbar spinal cord are taking place in glial cells, which provide support and nourishment to neurons and are essential to their survival (Puentes et. al. 2016). It may be possible for a future study to pinpoint the cell types in which MST1 phosphorylation is increased using immunohistochemistry to double-stain slides of spinal cord tissue with

antibodies against pMST1 and either a motor neuron marker or a glial cell marker, in order to determine which cells are expressing the elevated levels of pMST1.

Mutant SOD1 copy number appears to have an effect on the course of ALS. Individuals with a larger number of mutant SOD1 gene copies show a faster progression of symptoms and reach the end-stage of the illness earlier (Chiu et. al. 1995). Based on this, we hypothesized that if MST1 activity contributes to the development of ALS pathology, animals with higher SOD1 copy number will show a greater increase in active MST1 levels. SOD1 copy numbers of transgenic animals were determined by RT-qPCR as described above, and sorted into a “high copy” (CT value below 22) and a “medium copy” (CT value between 22 and 25) group based on the number of copies of the mutant gene present in each animal and analyzed separately. Wild type animals were expected to have a CT value of 30 or above, representing no copies of the mutant SOD1 gene. One wild type sample was found to have a CT value below 30, and was excluded from data analysis. Transgenic animals with CT values above 25 cycles were also excluded from the data, as this reflects a relatively low copy number and by extension an animal which had less severe ALS symptoms, or possibly improper labeling of a wild type sample. Contrary to our hypothesis, the only significant change in MST1 activity occurred in the medium copy transgenic group, while there were no significant increases in phospho-MST1 levels in the lumbar spinal cord or muscle of the high copy transgenic group (Figure 1, Figure 2). This still implies that SOD1 copy number plays an important role in the course of ALS, but not in the way that we predicted. The observed increase in pMST1 levels may in fact be related to the less rapid progression of ALS pathology observed in

low copy number animals (Chiu et. al. 1995), while animals with high SOD1 copy number may undergo neurodegeneration by a different mechanism.

The fact that an increase in phosphorylated MST1 is only observed in females suggests that the activation of MST1 in ALS is sex-specific. This could indicate a hormonal component in the mechanism of hippo/YAP pathway activation and MST1 phosphorylation, or may be due to sexual dimorphism in the structure of spinal cord tissues and the ways in which the related cells cope with increased oxidative stress. Sex differences in the onset and progression of ALS pathology are not unprecedented. Specifically, female SOD1 mutant mice begin to display ALS symptoms later in life than males (Chiu et. al. 1995).

We next examined the expression of the MST1 protein in lumbar spinal cord, gastrocnemius muscle, and cortex of the same set of wild type and SOD1 mutant animals in order to determine whether the observed increase in phosphorylation and activation of MST1 in medium copy females was accompanied by an increase in MST1 protein levels. We found that MST1 expression was not significantly altered in any of these tissues in SOD1 mutant mice (Figure 3). These data imply that the increase in MST1 phosphorylation seen in Figure 1 is caused by an increase in the action of upstream regulators of the Hippo pathway, rather than an increase in total MST1 protein levels. Future studies focused on the effects of upstream regulators of the Hippo signaling pathway, including serum levels of lysophosphatidic acid and sphingosine-1-phosphate, as well as activity of beta-adrenergic, dopamine, and glucagon receptors (Yu et. al. 2012), may help to shed more light on these findings.

YAP has been shown to play a neuroprotective role in some illnesses (Hoshino et. al. 2006, Lee et. al. 2013), and the Hippo pathway regulates its activity. An increase in the expression or phosphorylation of MST1 in a tissue indicates elevated activity of the Hippo pathway, a direct result of which would be an increase in phosphorylation of YAP and its subsequent sequestration in the cytoplasm (Zhao et. al. 2010). This would lead to a decrease in the activity of YAP and TEAD and their associated proliferative and anti-apoptotic functions (Huang et. al. 2005) Therefore, we also hypothesized that YAP activity would be decreased in ALS. Based on this, we expected to measure a decrease in autophagy and an increase in the levels of phosphorylated YAP in the tissue. A suitable antibody for phosphorylated YAP was not available, so we instead measured total YAP expression in lumbar spinal cord, and found that YAP levels were not changed despite the increase in MST1 activation (Figure 4). The apparent lack of a decrease in YAP activity may suggest impairment in the function of other components of the hippo pathway such as LATS or MOB, but analysis of YAP phosphorylation in addition to YAP expression would provide a more conclusive answer. Furthermore, the tissue samples tested for YAP expression in this study were not divided based on copy number due to a small sample size in available tissue. A future investigation of YAP expression in G39A SOD1 mutant animals with varying gene copy number may help us better understand the findings of this study.

Autophagy plays a crucial role in the longevity of neurons, and is impaired in neurodegenerative disorders (Cherra and Chu 2008). This impairment has been linked to an alteration in the activity of YAP and the Hippo pathway (Hoshino et. al. 2006). The

ubiquitin binding protein p62 was examined as an indicator of autophagy in the SOD1 mouse. P62 marks old or damaged proteins for inclusion in autophagosomes, which sequester and transport materials to be degraded within the cell (Jaeger and Wyss 2008). Increased activity of YAP has been found to enhance autophagy, leading to a decrease in p62 levels (Song et. al. 2015). We predicted that if increased pMST levels in ALS impair autophagy, p62 levels would be elevated, reflecting a buildup of unmetabolized autophagosomes within the cell. We further hypothesized that this increase in p62 would coincide with a decrease in YAP activity, given the latter's proposed role in regulation of autophagy. However, no significant changes in p62 were observed in SOD1 mutant lumbar spinal cord or muscle tissue. There was a slight, nonsignificant trend towards an increase in p62 expression in presymptomatic SOD1 muscle tissue while in symptomatic transgenic animals, p62 was roughly at the same level as wild type, but no such trend was apparent in spinal cord. Average p62 expression levels were also higher on average in males than in females (Figure 5), but this difference was not found to be statistically significant. These findings differ from previous studies suggesting that autophagy is altered in ALS (Jaeger and Wyss 2008, Li et, al. 2008). However, a lack of an increase in p62 levels does not necessarily indicate that autophagy is not impaired. MAP1LC3 is a protein found in the membranes of autophagosomes, and is another commonly studied marker for autophagy (Jaeger and Wyss 2008). MAP1LC3 levels been found to be significantly elevated in the spinal cord of SOD1 mice in both the presymptomatic and symptomatic phase (Li et. al. 2008). However, a study by Tanida et. al. (2005) found that

intracellular levels of MAP1LC3 were not an accurate indicator of autophagic activity, meaning that alternative indicators of autophagy will be required for future studies.

In summary, our findings indicate that pMST1 is increased presymptotically in the lumbar spinal cord of transgenic female mice with a medium G39A SOD1 copy number. We also found that the expression of other key Hippo pathway components was not altered in the spinal cord of these animals, and that expression Hippo pathway proteins was not changed in muscle or cortex of these animals. These findings suggest that pMST1 levels are connected to ALS-related spinal cord pathology under certain conditions, but do not provide sufficient evidence that the Hippo pathway represents a viable therapeutic target for ALS treatment. Overall, our study shows that the Hippo pathway plays a specific role in the onset and progression of ALS, and further study is required to elucidate this role.

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Education:

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B.S., Biology-Psychology, 2013

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Research Experience:

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Undergraduate Research Assistant, 2012-2013

- Studied neural mechanisms of stress and drug dependence in CFW mice with Dr. Klaus Miczek
- Second author on abstract submitted to Society for Neuroscience, 11/2013: X.Han, **E. Granucci**, J.F. Debold, K.A. Miczek. Social stress, behavioral sensitization and intravenous cocaine self-administration in CFW mice. 816.24/R2. 2013 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience, 2013.

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Graduate Student, 2015-Present

- Investigated pathogenic mechanisms in the SOD1 mouse model of ALS with Dr. Ghazaleh Sadri-Vakili
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- First author on a poster presented at Massachusetts General Hospital Scientific Advisory Committee poster session, 4/2016: **E. Granucci**, K Glajch, K. Mueller, K. Vakili, G. Sadri-Vakili. Evaluating the role of Hippo pathway in the onset and progression of Amyotrophic Lateral Sclerosis in the SOD1 mouse model.

Technical Expertise:

- Animal Handling (mouse): social defeat, IP drug administration, food training, IV surgery, Microdialysis probe implantation, Rotarod, paw-grip endurance (PaGE) test.
- Molecular biology and biochemistry: HPLC, Bradford assay, cell fractionation, SDS-PAGE, western blotting, H&E staining, immunohistochemistry, RNA and DNA extraction, RT-qPCR, Co-Immunoprecipitation.
- Statistical analysis using ANOVA and T-tests