Regulation of an Axonal Guidance Protein, Neuron Navigator 3,
in Amyotrophic Lateral Sclerosis

A thesis submitted to the University of Arizona College of Medicine – Phoenix
in partial fulfillment of the requirements for the degree of Doctor of Medicine

Arianna Kousari
Class of 2018

Mentors: Robert Bowser, PhD and Nadine Bakkar, PhD

Acknowledgements

I wish to thank my mentors Dr. Nadine Bakkar and Dr. Robert Bowser for their help and mentorship throughout this project. I would also like to thank the members of the Bowser lab for their help and support. The Bowser lab would also like to thank all patients and their families for participation in this study.

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting upper and lower motor neurons. Neuron Navigator 3 (Nav3) is a member of the Navigator family of proteins that function as microtubule-binding proteins. Nav3 is primarily expressed in brain tissue and neuromuscular junctions, and is thought to play a significant role in neuron regeneration and axonal outgrowth. An unbiased proteomic study looking at ALS and control cerebrospinal fluid (CSF) identified Nav3 to be significantly upregulated in ALS compared to controls. This study aimed to validate these findings using immunohistochemistry (IHC), Real-Time PCR, and western blot to determine if Nav3 was increased in brain and spinal cord tissue from ALS patients, primary rat motor neurons, and in the SOD1G93A mouse model. In spinal cords from SOD1G93A mice, RT-PCR showed significant increases in Nav3 mRNA expression at Day 120 (post-symptomatic stage) but not at the earlier Day 90 (symptomatic stage). Spinal cords harvested from SOD1G93A Day 120 mice (post-symptomatic stage) and processed for western blot analysis were found to have no difference in Nav3 protein expression between wild type and SOD1G93A samples. We found no significant differences in IHC staining for Nav3 in post-mortem human hippocampal or spinal cord tissue of ALS compared to controls. Nav3 mRNA levels were found to be significantly increased in response to oxidative stress in primary rat motor neurons. Our results show a discrepancy between Nav3 expression at the RNA and protein levels. These results suggest that Nav3 may be differentially regulated at the post-translational level in ALS. Future studies are needed to further define the role of Nav3 in ALS pathogenesis.

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Introduction

Amyotrophic Lateral Sclerosis (ALS) is an idiopathic fatal neurodegenerative disease of the human motor system. ALS is a heterogeneous disease with both familial (fALS) and sporadic (sALS) forms. About 5-10% of ALS cases are familial, with SOD1, TDP-43, and FUS being the most commonly identified genes.[4] sALS accounts for 90% of ALS cases. Family aggregation studies have shown an overlap between ALS and other neurodegenerative diseases, suggesting the presence of so-called "susceptibility" genes.[41] No studies have been able to establish the genetic basis between such genes and the development of sALS.[41]

The classic clinical presentation of ALS includes presence of upper motor neuron (UMN) and lower motor neuron (LMN) signs in the brainstem and spinal cord. Patients can present with progressive weakness, muscle wasting, fasciculations, spastic dysarthria, or bulbar UMN dysfunction.[4] The incidence is 2.16/100,000 with peak age at onset of 58-63 years old for sporadic disease and 47-52 years old for familial disease.[4] Prognosis is 3-5 years with leading cause of death attributed to progressive weakening of the respiratory muscles leading to respiratory failure, often preceded by pneumonia.

Characteristic pathologic processes in ALS include motor neuron loss and cytoplasmic inclusions in motor neurons. [128] Many molecular mechanisms have been implicated in ALS pathogenesis including impaired axonal transport, protein aggregation, stress granule formation and RNA processing defects.[128]

Neuron Navigator 3 (nav3) is a member of the family of Navigator proteins, which also includes Nav1 and Nav2. The Navigators are mammalian homologs of the *C. elegans* protein UNC-53[5].[5] These proteins have been identified to ~~have~~ play roles in cell migration, outgrowth of neuronal processes, and are signal transducers associated with actin filaments, microtubules, and intermediate filaments. The Navigator family of proteins also play an essential role in the process of axon outgrowth.[5]

Nav3 has been found to be involved in neuron growth and regeneration in brain injury, neural tumorigenesis in neuroblastoma, and as a tumor suppressor in T cell lymphoma.[7]

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Additionally, Nav3 is known to be primarily expressed in brain tissue. Using quantitative RT-PCR and/or in situ hybridization Kishi et al discovered Nav3 to be one of 10 gene products that were not previously known to be localized to the neuromuscular junction.[3]

Shioya et al discovered Nav3 to have a role in neurodegenerative diseases such as AD and ALS.[6] The interaction between miR-29a (microRNA) and Nav3 suggested its role in the neurodegenerative processes of AD. Using immunohistochemistry, they reported that Nav3 expressed a strong signal in the cytoplasm, axons and dendrites of pyramidal neurons in layers III and V of the cerebral cortex of AD and non-AD brains. Protein expression of Nav3 in ALS brain tissue was also confirmed by western blot.

Rationale

Previous studies from our lab have shown Nav3 to be increased in sALS cerebrospinal fluid. Given Nav3's known role in axonal transport in neurodegeneration, we hypothesize that Nav3 is regulated in ALS pathogenesis.

Research Question

This study will aim to determine whether axonal guidance protein Neuron Navigator 3 (Nav3) is regulated during ALS. This study will define whether Nav3 proteins levels are increased in ALS brain and spinal cord tissues compared to controls, as well as in an ALS murine model using Western Blot and Immunohistochemistry methods. The advantage of utilizing the ALS SOD1G93A mouse model is to obtain tissue samples from progressive stages of ALS. The post-mortem human tissues samples only allow us to examine the post-mortem disease state. We predict that mRNA and protein levels of Nav3 will be increased in ALS and spinal cord tissues as well as in the SOD1G93A mouse model tissues, compared to controls, suggesting higher levels of Nav3 expression plays a role in promoting ALS pathogenesis.

Research Materials and Methods

Tissue samples

ALS and non-neurologic disease control post-mortem tissue samples were obtained from the University of Pittsburgh ALS Tissue Bank, the Barrow Neurological Institute ALS Tissue Bank, and the Target ALS Human Postmortem Tissue Core. All tissues samples were collected after informed consent from the subjects or by the subjects' next of kin. The consent process was approved by the University of Pittsburgh Institutional Review Board (IRB)/University of Pittsburgh Committee for Oversight of Research Involving the Dead and the Dignity Health Institutional Review Board. Clinical diagnoses were made by board certified neuropathologists according to consensus criteria for ALS.

Immunohistochemisty (IHC)

Paraffin-embedded human tissue sections from hippocampus and spinal cord from ALS and AD (control) were used for this study. All sections were de-paraffinized, rehydrated, and antigen retrieval was performed using Target Antigen Retrieval Solution, pH 9.0 (DAKO) or a citrate buffer (pH6) for 20m in a steamer. After cooling to room temperature, non-specific binding sites were blocked using Super Block (Scytek) supplemented with Avidin (Vector Labs) for 1h. Anti-Nav3 primary antibody was incubated overnight at 4C in Super Block with Biotin. After 3 washes tissue sections were incubated for 1h in the appropriate biotinylated IgG secondary antibody (1:200; Vector Labs) diluted in Super Block. Slides were washed in PBS or H2O for 15 min and immunostaining visualized using the Vectastain Elite ABC reagent (Vector Labs) and Vector NovaRED peroxidase substrate kit (Vector Labs). Slides were counterstained with hematoxylin (Sigma Aldrich). Sections were visualized using an Olympus BX40 light microscope and images were acquired.

Western Blot

Spinal cord tissue from SOD1 model mice at Day 90 (symptomatic) and Day 120 (post-symptomatic) were used in this study. Protein concentration was determined by Bradford assay kit. Total cell lysates were prepared by homogenizing samples in 25mM HEPES pH7.9, 50mM

NaCl, 1% Triton X-100, supplemented with protease and phosphatase inhibitors. Lysates were subsequently spun at 14,000rpm for 10min, and supernatants were used for western blot analysis. Tris-Acetate gels from Invitrogen were used. 20ug of proteins wasere mixed with 4x SDS dye, boiled at 70 0 C for 10 min, loaded and ran at 100V for 1h. Gels were transferred onto Immobilon FL (Millipore) PVDF membranes, blocked in Odyssey blocking buffer, and probed with primary and secondary antibodies. Membranes were washed in TBS-0.1% Tween 20 after the primary and secondary antibody steps. Primary antibodies for Nav 3 (Abcam) and b-tubulin (Sigma Corp) were used. Signals were imaged using the Odyssey CLx Imager (LiCor), and densitometric analysis was performed using the ImageStudio 4.0 software from LiCor.

Real-Time PCR

Spinal cord tissue from SOD1 model mice at Day 60 (pre-symptomatic), Day 90 (symptomatic) and Day 90 (post-symptomatic) were used for this study. Samples were homogenized in Trizol (Invitrogen), and RNA was extracted using the Ambion PureLink™ RNA Mini Kit. cDNA was synthesized using Superscript VILO (Invitrogen) and real-time RT-PCR was performed using the FastStart Universal SyberGreen master mix (Roche) and primers specific for mouse or rat Nav3. GAPDH levels were simultaneously assayed in all of these samples to normalize Nav3 levels to. Results were plotted as fold changes over wild type (for the SOD1 model), or fold changes over untreated controls (for the primary rat neurons).

Results

In this study, we have shown variable results of the expression of microtubule binding protein Nav3 in SOD1G93A mice spinal cord tissue, primary rat motor neurons, and post-mortem human ALS hippocampal and spinal cord tissue using RT-PCR, western blot and immunohistochemistry.

We first focused on a mouse model of ALS, the SOD1G93A mouse model. Superoxide dismutase (SOD1) is a cytoplasmic and mitochondrial enzyme which functions to catalyze the breakdown of reactive oxygen species, preventing oxidative stress. Mutations in SOD1 account for 20% of fALS cases. Mutant SOD1 is linked to gliosis, ubiquitinated SOD1 inclusions, axonal and motor neuron loss.[139] The transgenic mouse model SOD1G93A contains a point mutation at amino acid position 93 (G → A).[141] Disease phenotype in SOD1G93A mice exhibit progressive locomotor abnormalities with hind-limb weakness and muscle wasting from Day 90 (Symptomatic Stage) and life span is about 150 days.[150] Spinal cord tissue from SOD1G93A mice was harvested at Day 60 (pre-symptomatic), Day 90 (symptomatic) and Day 120 (post-symptomatic) stages. This tissue was used for RT-PCR and Western blot experiments.

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~~INSERT THE PART FROM THE METHODS SECTION ABOUT THE MODEL HERE.~~ We first analyzed the mRNA expression level of Nav3 in spinal cord tissue from SOD1G93A and wild type mice by RT-PCR. We focused on samples from Day 90 (symptomatic) stage, as well as Day 120 (post-symptomatic) stage to examine potential changes during disease pathogenesis. Results from this experiment showed significant increases in Nav3 mRNA expression at Day 120 but not at Day 90 (Figure 1).

In order to check whether there are changes in Nav-3 protein levels in ALS, we used western blot analysis at Day 120 (post-symptomatic) or Day 90 (symptomatic) stages. Proteins were extracted from spinal cord tissue of SOD1G93A mice and wild type samples and these samples were used in western blot analysis. We found no difference in Nav3 protein expression between wild type and SOD1G93A samples after normalizing to the housekeeping gene b-tubulin and performing densitometric analysis (Figure 2).

Since there might be differences between a mouse model of ALS and disease etiology in humans, we used post-mortem human hippocampal and spinal cord tissue to qualitatively analyze difference in Nav3 expression via Immunohistochemistry. No changes in intensity or cellular localization of Nav3 specific staining was observed in post-mortem human spinal cord tissue of 7 control and 12 ALS samples (Figure 3). No changes in intensity or cellular localization of Nav3 specific staining was observed in post-mortem human hippocampal tissue of 9 control and 9 ALS samples either (Figure 4).

To check whether Nav3 might be regulated by oxidative cellular stress, we used primary rat motor neurons, treated with various stressors to mimic the environmental stress neurons experience in ALS. Primary rat motor neurons were treated for 16 hours, either with Paraquat, H2O2, or tert-Butyl Hydroperoxide at variable concentrations, and RNA was extracted to examine Nav-3 levels by RT—PCR. Results showed significantly increased Nav3 mRNA expression in primary rat motor neurons treated with .1mM, .25mM, .5 mM and .7mM concentrations of Paraquat and with 20uM and 40uM concentrations of tert-Butyl Hydroperoxide. No difference in Nav3 mRNA expression was observed in primary rat motor neurons treated with 50uM of H2O2 or 10 uM of tert-Butyl Hydroperoxide (Figure 5). These results indicate that Nav-3 is transcriptionally regulated by stress.

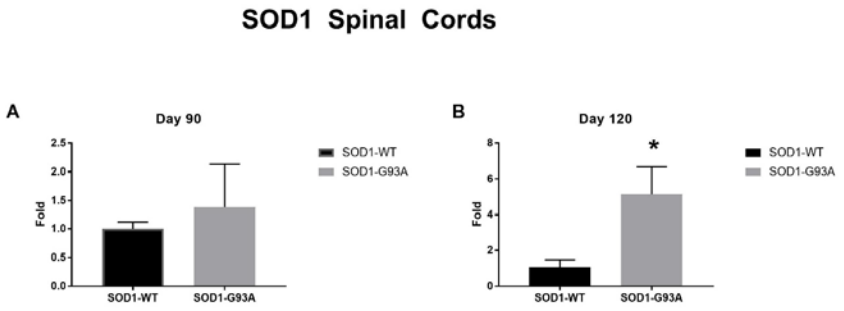

Figure 12. mRNA expression of Nav3 in SOD1 spinal cords at Day 90 (symptomatic) and Day 120 (post-symptomatic) stage. A) mRNA expression levels (mean ± SE) of Nav3 in total tissue homogenates of SOD1-WT and SOD1-G93A spinal cord tissue samples measured by RT-PCR at Day 90 (symptomatic). At Day 90, no significant differences in Nav3 mRNA expression were detected. B) mRNA expression levels (mean ± SE) of Nav3 in total tissue homogenates of SOD1-WT and SOD1-G93A spinal cord tissue samples measured by RT-PCR at Day 120 (post-symptomatic). Nav3 mRNA expression was significant increased at Day 120 ($p < 0.05$).

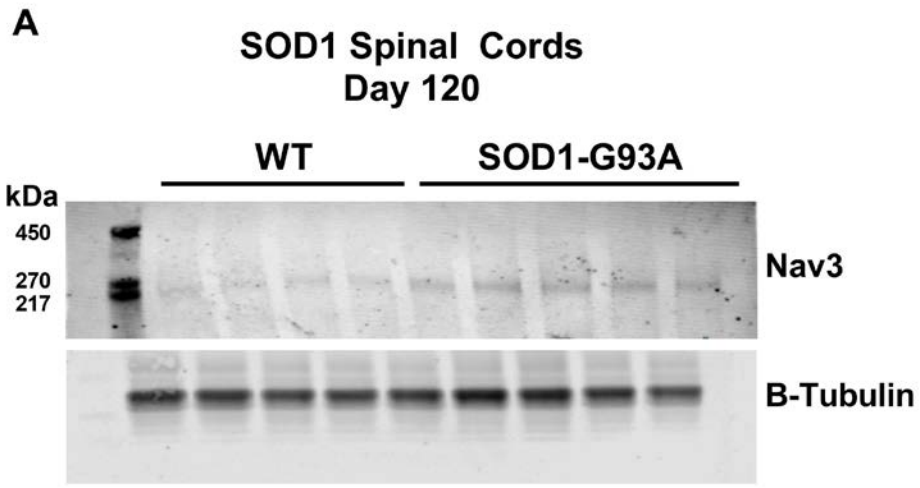

Figure 23. Protein expression of Nav3 in SOD1 spinal cords at Day 120 (post-symptomatic) stage. A) Protein lysates were prepared from 4 wild type and 5 SOD1 mice spinal cord samples. were lysed and proteins were processed for western blot analysis for Nav3 using Tris acetate gels. B-tubulin was ran as a loading control. Densitometric analysis for the Nav3 signal normalized to tubulin was performed and compared between the two groups. No significant differences between wild type and SOD1 G93A were seen.

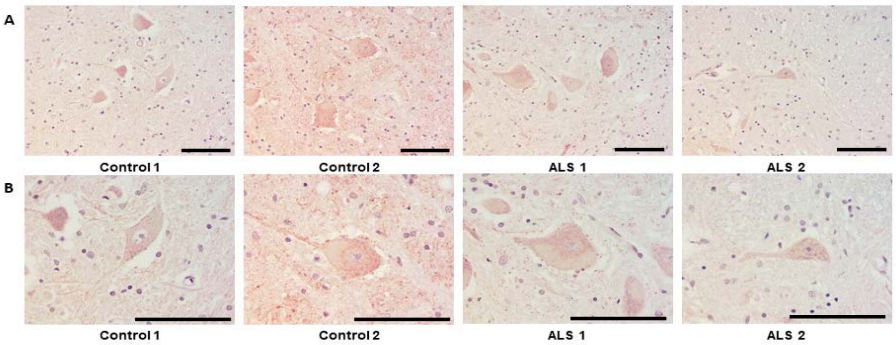

Figure 34. Immunohistochemistry for Nav3 in ALS and control in post-mortem spinal cord tissue. Specific staining for Nav3 is reddish-brown, while all nuclei were counterstained for hematoxylin (purple). Shown are representative images from 2 control and 2 ALS cases. A) Top Row. Images were taken at 20X magnification. B) Bottom Row. Images were taken at 40X magnification. No difference in Nav3 staining was observed between 7 control and 12 ALS spinal cord tissue samples. Scale bars 100uM.

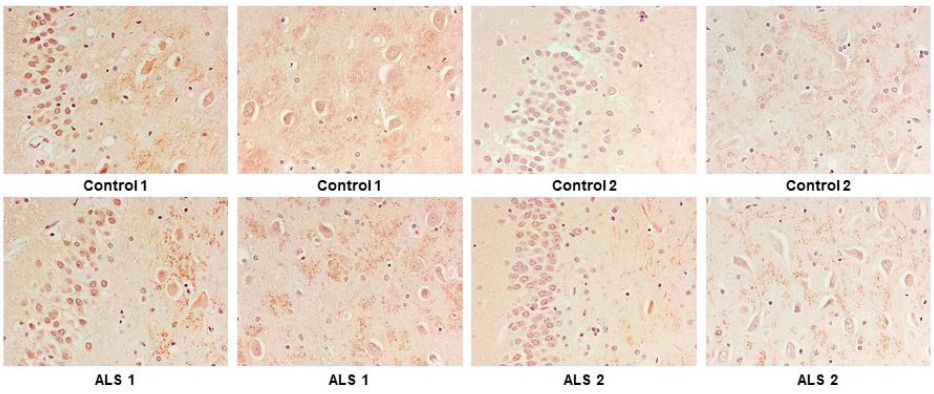

Figure 45. Immunohistochemistry for Nav3 in ALS and control in post-mortem hippocampal tissue. Pictures were taken to show the granular layer of the hippocampus. Specific staining for Nav3 is reddish-brown, while all nuclei were counterstained for hematoxylin (purple). Shown are representative images from 2 control and 2 ALS cases, and images were taken at 10x magnification. No difference in Nav3 staining was observed between 9 control and 9 ALS samples.

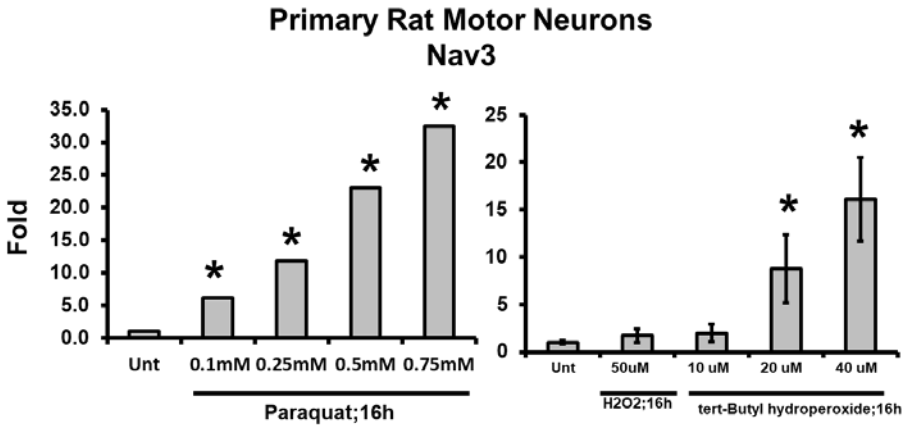

Figure 56. Expression of Nav3 in response to oxidative stress. Primary rat motor neurons were cultured from E13.5 embryos and grown on PDL/Laminin-coated plates. Neurons were differentiated for 7 days then treated with the indicated compounds for 16h. Cells were then harvested, RNA extracted and real-time PCR for NAV3 performed. Results were normalized to cyclophilin levels, and plotted as fold increase over controls. Arrows indicate significance differences over controls, and data from at least 3 independent cultures were averaged.

Discussion

In this study, we have shown variable results of the expression of microtubule binding protein Nav3 in SOD1G93A mice spinal cord tissue, primary rat motor neurons, and post-mortem human ALS hippocampal and spinal cord tissue using RT-PCR, western blot and immunohistochemistry. In SOD1G93A mice spinal cord tissue, RT-PCR showed significant increases in Nav3 mRNA expression at Day 120 (post-symptomatic) stage but not at Day 90 (symptomatic) stage. Spinal cords harvested from SOD1G93A Day 120 mice (post-symptomatic) stage and processed for western blot analysis were found to have no difference in Nav3 protein expression between wild type and SOD1G93A samples. We found no significant differences in Nav3 protein levels and cellular localization in post-mortem human hippocampal or spinal cord tissue of ALS compared to controls -by immunohistochemistry-. Nav3 mRNA levels were found to be significantly increased in response to oxidative stress in primary rat motor neurons.

The role of Nav3 has not been thoroughly elucidated in ALS thus far. However, it has been shown to have implications in other neurodegenerative diseases such as Alzheimer's Disease (AD). It has been shown that Nav3 interacts with miRNAs in AD. Three miRNAs were identified to be up-regulated in ALS brain tissue via microarray analysis but down-regulated in AD brain tissue. This same study also showed that Nav3 expression is most prominent in degenerating pyramidal neurons in the cerebral cortex of AD. Thus, this study concluded that under-expression of miR-29a can affect neurogenerative processes by enhancing neuronal Nav3 expression in AD brains and further elucidated the role of Nav3 in neurodegenerative disease.[6]

The navigator family of proteins are microtubule-binding proteins that are involved in neurodevelopmental and neurodegenerative diseases. Microtubules (MTs) are essential to neuron growth and function such that many neurological diseases stem from defects in the MT cytoskeleton or its regulation.[10] Microtubule binding proteins such as Nav3, tau and Map2 that bind to the + end of MTs, also known as +TIPs, are important regulators of these processes. In AD, abnormal phosphorylation of tau causes its dissociation from MTs and causes tau to aggregate, forming neurofibrillary tangles.[10] Other +TIPs such as tau-tubulin kinase 1 and 2

(TTKB1 and TTKB2) were also shown to phosphorylate TDP-43, causing re-localization of TDP-43 from the nucleus to cytoplasmic inclusions, which are characteristic pathologic features of ALS and FTLD.[11]

Semaphorins, another family of axonal guidance proteins have also been shown to be involved in ALS pathogenesis and more specifically, regeneration failure of injured axons in the CNS.[18] They act as axon repellants and prevent axonal regeneration.[18] Single nucleotide polymorphisms (SNPs) in semaphorins found in ALS patients are thought to influence factors such as disease onset, severity and susceptibility.[16] One study by Korner, et al found Semaphorin 3A (Sema3A) to be increased in human motor cortex tissue and spinal cord tissue of ALS patients compared to controls. This study also showed via in situ hybridization that Sema3A expression was present in motor neurons.[18] Another study conducted in the SOD1G93A mouse model found an increase of Sema3A expression in terminal Schwann cells (TSCs) at the neuromuscular junction, and suggested that increased expression of Sema3A in TSCs leads to de-adhesion or repulsion of motor axons at the neuromuscular junction, thus contributing to axonal denervation and motor neuron degeneration.[17]

Our study aimed to validate increased expression of Nav3 in an SOD1G93A mouse model and human ALS tissue compared to controls. Previous data from the Bowser lab looking at CSF from ALS and unaffected controls had shown increases in the protein levels of Navv 3 by mass spectrometry suggesting that this protein might be involved in ALS pathogenesis or progression ~~(add ref here)~~.[9] Our results in the SOD1G93 mouse model at different stages of disease showed a discrepancy between protein and RNA levels of Nav3~~.~~. Results from the RT-PCR experiments using SOD1G93A spinal cord tissue and the primary motor neurons in vitro model showed significant increases in Nav3 mRNA. However, results from western blot and IHC experiments using SOD1G93A spinal cord tissue and postmortem human spinal cord and hippocampal tissue showed no difference between ALS and control samples. Such a disconnect between protein and RNA levels across different models could be attributed to many factors. Nav3 could be differentially regulated at the transcriptional and post-translational levels in ALS pathogenesis. Another possibility is that the lack of difference at the protein levels is due to an

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antibody epitope recognition issue. The increases in Nav3 mRNA at the Day 120 (post-symptomatic) stage in the SOD1G93A mouse model also suggests that Nav3 may play a role in later stages of disease.

~~The use of antibodies against other distinct epitopes of Nav 3 would help to address whether the lack of changes at the protein levels are antibody-specific. In addition, further studies looking at Nav3 by western blot, as well as Nav3 by RT-PCR in post-mortem spinal cords from ALS and unaffected controls are warranted to investigate whether this will mirror results from the animal model. Using the in vitro model of primary rat motor neurons, we would like to overexpress Nav3 and see whether its localization changes in response to cellular stress.~~

Future Directions

The use of antibodies against other distinct epitopes of Nav-3 would help to address whether the lack of changes at the protein levels are antibody-specific. In addition, further studies looking at Nav3 by western blot, as well as Nav3 by RT-PCR in post-mortem spinal cords from ALS and unaffected controls are warranted to investigate whether this will mirror results from the animal model. Using the in vitro model of primary rat motor neurons, we would like to overexpress Nav3 and see whether its localization changes in response to cellular stress.

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Conclusions

Nav3 remains a protein of interest in ALS and neurodegenerative disease such as Alzheimer's Disease. However, little is known about its role in ALS pathogenesis. Further studies will help to elucidate and define the role of Nav3 in ALS.

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