Regulation of an axonal guidance protein, Neuron navigator 3, in Amyotrophic Lateral Sclerosis

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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 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 molecular and protein levels. These results suggest that Nav3 may play a role in disease pathogenesis at the molecular level and not at the protein level, or that Nav3 is differentially regulated at the post-translational level in ALS. Future studies will help to further define the role of Nav3 in ALS pathogenesis.

Introduction

Amyotrophic Lateral Sclerosis is an idiopathic fatal neurodegenerative disease of the human motor system. ALS is a heterogeneous disease with familial (fALS) and sporadic (sALS) forms. About 5-10% of ALS cases are familial, with SOD1, TDP-43, and FUS being the most common identified genes[1]. 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. [1] No studies have been able to establish the genetic basis between such genes and the development of sALS.[1] The classic clinical presentation of ALS includes presence of upper motor neuron and lower motor neuron signs such as progressive weakness, muscle wasting, fasciculations, or spastic dysarthria.

Characteristic pathologic processes in ALS include motor neuron loss and cytoplasmic inclusions in motor neurons.[8] Many molecular mechanisms have been implicated in ALS pathogenesis such as impaired axonal transport, protein aggregation, stress granule formation and RNA processing defects.[8]

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.[2] These proteins have been identified to have roles in cell migration, outgrowth of neuronal processes, and are signal transducers associated with actin filaments, microtubules, and intermediate filaments.

Nav3 has been found to have implications in neuron growth and regeneration in brain injury, neural tumorigenesis in neuroblastoma, and as a tumor suppressor in T cell lymphoma.[3] 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. Shioya et al discovered Nav3 to have a role in neurodegenerative diseases such as AD and ALS. The interaction between miR-29a (microRNA) and Nav3 suggested its role in the neurodegenerative processes of AD.

Rationale:
Previous studies from our lab have shown Nav3 to be increased in sALS cerebrospinal fluid. Given Nav3's known role in axonal transport, we hypothesize that Nav3 is regulated in ALS brain and spinal cord tissue at the molecular and protein level. By using the SOD1G93A mouse model, we can further examine the stage at which Nav3 expression changes corresponding to disease stage and severity.

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. 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.

Immunohistochemistry:
Paraffin-embedded human tissue sections from hippocampus and spinal cord from 21 ALS and 16 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. Qualitative analysis was performed to determine differences in staining intensity between ALS and control.

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.[9] The transgenic mouse model SOD1G93A contains a point mutation at amino acid position 93 (G → A).[11] 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.[10] 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.

Western Blot:
Lumbar spinal cord tissue homogenates from SOD1G93A 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. 20ug of proteins were 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. 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 120 (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).

Neuron navigator 3 (Nav3) is increased in sALS CSF

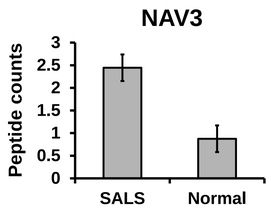

Figure 1. LC-MS/MS identifies alterations in the axonal guidance protein Nav3. Bar graphs are shown for the peptide counts for Nav3. p-value=0.005331.[5]

Nav3 mRNA was also shown to be significantly increased by microarray analysis in laser-capture micro-dissected spinal motor neurons from 12 SALS compared to 10 controls. 1.37 fold increase; p-value=0.0105.[7]

mRNA Nav3 expression in SOD1G93A spinal cords is increased at Day 120 (post-symptomatic) stage

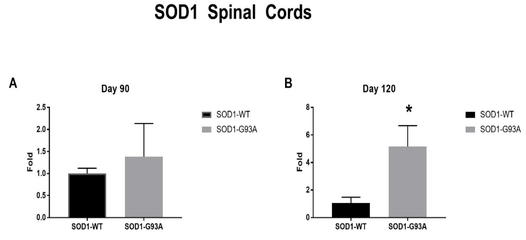

Figure 2. mRNA expression of Nav3 in SOD1G93A spinal cords at Day 90 (symptomatic) and Day 120 (post-symptomatic) stage. A) mRNA expression levels (mean ± SE) of Nav3 in total tissue homogenates of SOD1-WT and SOD1-G93A spinal cord tissue samples measured by RT-PCR at Day 90 (symptomatic). At Day 90, 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 significantly increased at Day 120 ($p < 0.05$).

No difference exists in protein expression of Nav3 in SOD1G93A spinal cords at Day 120 (post-symptomatic) stage

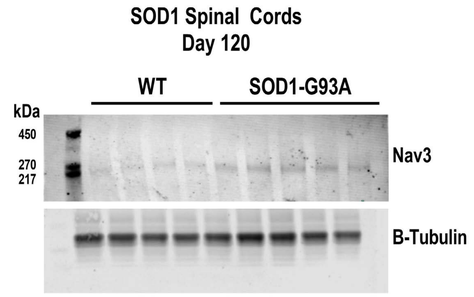

Figure 3. Protein expression of Nav3 in SOD1G93A spinal cords at Day 120 (post-symptomatic) stage. Protein lysates were prepared from 4 wild type and 5 SOD1G93A mice spinal cord samples. Samples were lysed and proteins were processed for western blot analysis for Nav3 using Tris acetate gels. B-tubulin 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 observed.

No difference in Nav3 staining observed between ALS and Control in post-mortem spinal cord tissue

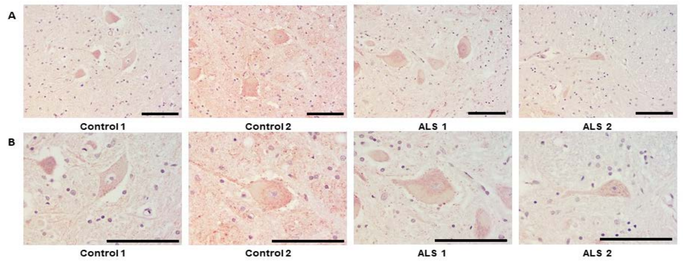

Figure 4. Immunohistochemistry for Nav3 in ALS and control in post-mortem spinal cord tissue. Specific staining for Nav3 is reddish-brown, while all nuclei were counterstained for hematoxylin (purple). Shown are representative images from 2 control and 2 ALS cases. A) Top Row. Images were taken at 20X 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.

No difference in Nav3 staining observed between ALS and Control in post-mortem hippocampal tissue

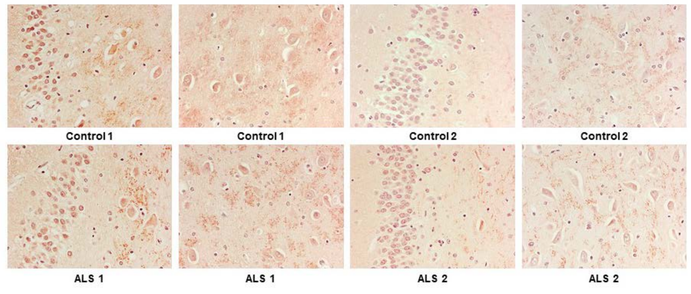

Figure 5. Immunohistochemistry for Nav3 in ALS and control in post-mortem hippocampal tissue. Pictures were taken to show the granular layer of the hippocampus. Specific staining for Nav3 is reddish-brown, while all nuclei were counterstained for hematoxylin (purple). Shown are representative images 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.

Nav3 mRNA levels are increased in response to oxidative stress in primary rat motor neurons

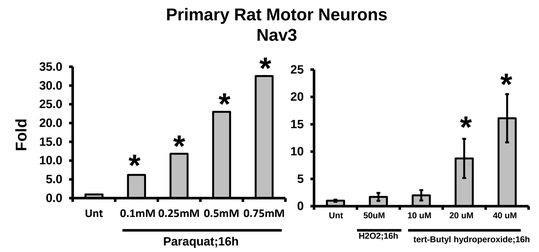

Figure 6. Primary rat motor neurons were cultured from E13.5 embryos and grown on PDL/Laminin-coated plates. Neurons were differentiated for 7 days then treated with the indicated compounds for 16h. Cells were then harvested, RNA extracted and real-time PCR for NAV3 performed. Results were normalized 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 and Conclusions

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 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.

This study aimed to validate increased expression of Nav3 in an SOD1G93A mouse model and human ALS tissue compared to controls. Our results showed a discrepancy between increases in Nav3 regulation at the molecular and protein levels. Results from the RT-PCR experiments using SOD1G93A spinal cord tissue and primary motor neurons individually showed significant increases in Nav3 mRNA. However, results from western blot and IHC experiments using SOD1G93A spinal cord tissue and human spinal cord and hippocampal tissue showed no difference between ALS and control samples.

These results suggest that Nav3 may play a role in disease pathogenesis at the molecular level and not at the protein level. Alternatively, Nav3 could be differentially regulated at the post-translational level in ALS pathogenesis. 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.

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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