Activation of microglial neuregulin1 signaling in the corticospinal tracts of ALS patients with upper motor neuron signs

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Abstract
We recently found neuregulin1 (NRG1) receptors are activated on microglia in the ventral horn of both ALS patients and SOD1 mice, suggesting a common pathological mechanism. However, it is not clear whether this signaling system also plays a role in patients with upper motor neuron (UMN) features, where patients show significant pathological changes in the corticospinal tracts (CSTs). Since the connection between upper and lower motor neuron (LMN) systems in ALS patients is not readily seen in the SOD1 mouse, we examined the lateral and ventral CSTs for NRG1 receptor activation and NRG1 expression in ALS patients with UMN symptoms compared to control patients with no evidence of neurodegenerative disease. We found that ALS patients with UMN symptoms showed increased microglial activation that colocalized with NRG1 receptor activation in the lateral and ventral CSTs. These same regions also showed increased NRG1 protein expression locally but no change in NRG1 mRNA. In conclusion, these data suggest that increased NRG1 protein accumulation could contribute to UMN disease through microglial activation in the CSTs.

Key words: Neuregulin, Microglial Activation, Corticospinal Tracts

Introduction
ALS is a disease affecting the nervous system causing a progressive degeneration of motor neurons that control voluntary muscle movement. ALS causes muscle weakness and wasting throughout the body and most patients die of respiratory failure within 3–5 years of diagnosis (1). No cure yet exists for this debilitating disease. In many patients, the disease progresses through the nervous system and involves both upper and lower motor systems (1–3); however, exactly how upper and lower motor systems are linked during disease progression is unknown.

Under pathological conditions, microglia, resident immune cells in the central nervous system (CNS), become activated and produce reactive oxygen and nitrogen species and pro-inflammatory cytokines, molecules that can contribute to axonal demyelination and neuron death (4). Activated microglia have been found in both the spinal cord (5,6) and the brain in ALS patients (7,8) and inflammatory mechanisms have been hypothesized to induce motor neuron death in ALS and other neurons in multiple sclerosis (5,9–12).

Our laboratory has been working for a number of years to define the communication between motor neurons and non-neuronal cells that they contact (13–16). The NRG1 gene produces both membrane-bound and secreted growth and differentiation factors that regulate glial development and survival, synaptogenesis, and microglial activation (6,16–20). We recently showed that aberrant NRG1 signaling on activated microglia is a common pathological change in the ventral horn of both ALS patients and SOD1 mice (6), raising the possibility that NRG1 may promote disease progression in the ventral horn in ALS. In this study, we focused on patients with UMN signs to measure NRG1 signaling in the degenerating lateral and ventral CSTs compared to control patients with no pathology in the spinal cord.
Materials and methods

Human tissue collection

Fresh frozen thoracic and lumbar spinal cords from four sporadic and two familial ALS patients with marked clinical UMN signs and six control patients with no pathological evidence for neurological disease were provided by Ravits (21) and the Human Brain and Spinal Fluid Resource Center (VA West Los Angeles Healthcare Center, Los Angeles, CA) (Table I). Post mortem time-intervals ranged from 2 to 25 h.

Histopathology

Fresh frozen human spinal cords were fixed in 4% paraformaldehyde for 24 h, washed overnight in PBS, and immersed in 30% sucrose until saturated, all at 4°C. The spinal cords were processed and embedded in OCT (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA). Frozen sections were cut transversely at 20 μm thickness and placed on Superfrost slides (Thermo Fisher Scientific), then stained with luxol fast-blue/periodic acid Schiff (LFB/PAS) (Poly Scientific, Bay Shore, NY) for the presence of myelin. For some experiments, formalin-fixed tissue slides were used.

Immunostaining

Identification and quantification of microglia in human spinal cord tissue sections was performed using antibodies for human CD68 (mouse IgG1, 1:20, DAKO Cat #N1577, Carpinteria, CA). Activation of NRG1’s erbB2 receptor was performed with a rabbit anti-phospho-erbB2 antibody (p-Neu, Tyr1248, 1:50 Santa Cruz Biotechnology, CA). Each primary antibody was diluted in blocking solution (10% normal goat serum, 0.05% Triton X-100 in PBS) overnight at 4°C, followed by incubation with goat anti-mouse or rabbit Alexa fluor 488 (1:100, Invitrogen, Carlsbad, CA). For phospho-erbB2 and CD68 immunostaining, biotin-conjugated goat anti-rabbit (HRP) (clone N126B/31, UC Davis/NIH NeuroMab facility) was as described previously (6).

RNA isolation and real-time quantitative RT-PCR (qPCR)

Human thoracic and lumbar spinal cords were rapidly frozen and stored at −80°C. RNA was extracted using the Qiagen RNaseasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) (24). Quantification of RNA was carried out using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The quality of RNA was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using an RNA 6000 Nano chip kit, RNA ladder and Agilent analysis software (Agilent Technologies). All samples had RIN (RNA integrity number) values above 6.0 and 260/280 ratios near 2.0.

The relative expression of human type I and III NRG1 were measured relative to GAPDH using Taqman Assays-On-Demand primers (Applied Biosystems, Foster City, CA, USA); 1.5 μg of total RNA

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<th>Gender</th>
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SALS: sporadic ALS; FALS: familial ALS; C: cervical; T: thoracic; L: lumbar.
was used in a 20-μl reverse transcription synthesis reaction primed with oligo-dT primers (Superscript First Strand Synthesis System, Invitrogen, Carlsbad, CA, USA). PCR was performed in triplicate using 1X Taqman Universal PCR master mix (Applied Biosystems) with the DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA) utilizing the following primers and Taqman probes: type I NRG1: Hs01108479_m1; type III NRG1: Hs01103792_m1; GAPDH: Hs02786624_g1. Cycle threshold (Ct) values were calculated using Opticon monitor software, with the threshold set at 50 standard deviations above background. The relative expression was calculated by normalizing the expression of individual genes to GAPDH and using the $2^{-\Delta\Delta C_{t}}$ method (24,25).

**Results**

*Myelin loss is associated with NRG1 receptor activation on microglia in the corticospinal tracts (CSTs) in ALS patients with UMN signs*

One of the greatest pathological lesions seen in human ALS is the presence of a marked loss of myelin in the CSTs of ALS patients. However, this is not readily seen in the CSTs of the ALS-SOD1 mouse (6). Figure 1 shows the marked loss of luxol fast-blue staining in the lateral and ventral CSTs compared to normal staining in the dorsal columns of ALS patients with UMN signs. This was seen in six out of six ALS patients we studied.

Microglia have important, though controversial, roles in neurodegenerative diseases and recently have been shown to be increased in the CST of ALS patients with UMN disease (26). NRG1 can promote microglial activation in a chronic spinal cord pain model (19) through binding to either erbB3 or erbB4 receptors that then heterodimerize with erbB2 to mediate signal transduction through tyrosine phosphorylation (19,27). We previously observed a marked increase in NRG1 receptor activation (phosphorylated erbB2) on microglia in the ventral horns of ALS patients suggesting a novel mechanism of lower motor neuron disease progression (6). Here, we asked whether increased erbB2 receptor activation is also present in the CSTs of patients with UMN signs and demyelination. While there is some staining in the control patients, the

Figure 1. Myelin loss in the lateral and ventral CSTs in ALS patients with clinical UMN signs, but not in the dorsal column. Spinal cords from ALS and control patients stained with luxol fast-blue PAS demonstrated myelin loss (pink areas) in ALS spinal cord within the lateral corticospinal tract (LCST) and ventral corticospinal tract (VCST). Scale bar = 20 μm on all images. The images are representative for six ALS patients and six control patients.
pattern and extent is quite different and there is no staining without the primary antibody on either control or ALS tissues (data not shown). Figure 2 shows a marked increase in erbB2 receptor activation that colocalizes with activated microglia in the CST. This was seen in six ALS patients with UMN signs when compared to these ALS patients’ dorsal columns and six control patients’ CSTs as well as ALS patients without UMN signs (data not shown). Interestingly, erbB2 receptor activation is present at multiple levels of degenerated lateral CST (cervical, thoracic and lumbar) in ALS patients with UNM signs.

**Increased NRG1 protein accumulation but not mRNA expression in the CST**

We measured the expression of NRG1 protein in the spinal cords of these same patients to identify the source of NRG1 production using NRG1 antibodies directed against different domains of the protein. Using an antibody that recognizes the entire extracellular domain of both soluble (type I) and membrane-bound (type III) NRG1 forms (ADO3) (18,22,23), there was a significant increase in staining of both the lateral and ventral CSTs in ALS patients with UMN signs, but not in controls (Figure 3A). The pattern of staining with this antibody was mostly extracellular and outside of the myelinated axons (shown with the arrows), suggesting that much of this immunoreactivity is in the extracellular matrix as we have shown extensively for soluble forms of NRG1 that strongly associate with heparan sulfate proteoglycans (17,28). In addition, we used an antibody specific for membrane-bound, type III NRG1 isoforms that also show marked staining of the CSTs. In control tissues, this antibody showed more marked axonal staining that was also present in the ALS patients (arrows), but most of the signal was again outside the myelinated axons and included the staining of small cells, as we have reported previously for the ventral horn (6) (Figure 3B).

Exactly which cells are producing the NRG1 proteins seen in the CSTs is not clear. During development, the major source of both soluble (type I) and membrane-bound (type III) NRG1 isoforms is from the lower motor neurons in the ventral horn (28 – 30). We therefore compared NRG1 isoform (types I and III) mRNA expression levels from the spinal cords from ALS and control patients. No significant difference in mRNA levels for types I and III NRG1 was seen, suggesting that the increase in NRG1 protein levels is due either to increased transcription at higher cortical levels or to an accumulation of NRG1 proteins both in the extracellular matrix and non-neuronal cells between the axons (Figure 4).

**Discussion**

**NRG1-induced microglial activation as a possible therapeutic target for ALS patients with UMN signs**

Our observation of microglial activation in the CSTs is confirmed by other recent findings showing that microglial activation in the lateral CST correlates with disease progression in ALS patients with UMN signs (7,26). Microglia could contribute to myelin loss in the CSTs and LMN loss in the ventral horn in ALS spinal cord through inflammatory mechanisms (1,6,7,10,11,26, 31 – 33). Consistently, anti-inflammatory drug treatments have had promising effects on survival in ALS animal models (34,35), but human ALS clinical trials have not been encouraging (36,37). Therefore, a better understanding of

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**Figure 2.** NRG1 receptor activation is increased in activated microglia in the CSTs of ALS patients with clinical UMN signs, but not in the dorsal column. NRG1 receptor activation measured with specific phosphor-antibodies against its receptor (p-erbB2, green) colocalized with activated microglia (CD68, red) in the LCST and the VCST of ALS but not in the dorsal column and control patients. Nuclei are stained blue with DAPI (blue). Quantitation of the percent p-erbB2 overlap with CD68 staining in each high-powered field revealed a 44 ± 7% overlap in the LCST compared to the dorsal column of 17 ± 2% in ALS patients. No significant difference between the LCST (8 ± 3%) and dorsal column (12 ± 3%) was seen in control patients. Scale bar = 500 μm on the top two rows, and 10 μm on the other images. Similar results were seen in six ALS (p<0.05) and six control patients in two different experiments.
the local signals between CST axons and different subtypes of microglia will be important to modulate their putative neurodegenerative and neuroprotective roles in ALS. It is also possible that these activated microglia serve a phagocytic function based on their rounded morphology that could be independent of NRG1 signaling.

Our present data are consistent with a hypothesis that NRG1 released from central axons or other cell types in the CST induces microglial activation,

Figure 3. NRG1 protein expression is increased in the CSTs of ALS patients with UMN signs. NRG1 antibodies against two different NRG1 epitopes showed increased staining in the LCST and VCST. Whereas AD03 targets NRG1’s extracellular domain (A), the CRD antibody is a type III specific antibody that stains membrane-bound type III forms (B). Each antibody has a different staining pattern suggesting both the presence of matrix-bound (AD03) and membrane-bound (CRD) forms. The arrows show punctuate axonal staining within myelinated axons, suggesting a central source of NRG1 both in controls and ALS patients. Scale bar = 500 μm on the top images, and 20 μm on the other images. These images are representative for six ALS patients with clinical UMN signs and six control patients.

Figure 4. No changes in type I and III NRG1 mRNA levels were seen in ALS spinal cords. Quantitative PCR was performed on full transverse spinal cord sections in triplicate for ALS (n = 5) and control (n = 6) patients using NRG1 isoform-specific primers against types I and III NRG1.
which, in turn, leads to progressive axonal degeneration. This is similar to our previous findings in the ventral horn of ALS patients and SOD1 mice (6), where we saw a robust activation of NRG1’s erbB2 receptors on activated microglia in the CSTs. Taken together, these findings suggest that NRG1 may be a common therapeutic target that could potentially slow disease progression in both the upper and lower motor systems in ALS by disrupting NRG1 signaling. We developed a NRG1 antagonist shown to block microglial activation in the spinal cord of rats that develop chronic pain following peripheral nerve injury which could be tested in ALS models as well (18,19).

Source of NRG1 in ALS patients with clinical UMN signs

The NRG1 gene produces both secreted (type I) and membrane-bound (type III) forms that are highly expressed in spinal motor neurons (28,29). While the mRNA expression of both of these forms did not change in the spinal cords of ALS and control patients, using antibodies against two unique NRG1 epitopes we saw a marked increased in NRG1 protein expression in the CSTs. One of these antibodies (AD03) recognizes all extracellular domains and secreted forms, but its mostly extracellular staining pattern suggests a build-up of soluble forms of NRG1 in the extracellular matrix. The second antibody is highly specific for type III NRG1 forms that are thought to be mostly membrane-bound. This antibody stains central axons in both controls and ALS patients, but has additional staining between axons, perhaps also within small cells present between axons.

The presence of increased soluble and membrane bound NRG1 forms in the absence of any change in NRG1 mRNA levels, raises the interesting possibilities that either NRG1 protein is transported down central motor axons where it accumulates in the extracellular matrix, or is expressed in other cell types within the CST, and that this increase in protein expression is responsible for sustained activation of erbB2 receptors in microglia. Because most secreted NRG1 forms bind specific heparan-sulfate proteoglycans in the extracellular matrix (38), the degenerative disease process in the CSTs could be propagated through changes in the extracellular matrix composition leading to increased NRG1 accumulations and signaling in the CSTs.

Acknowledgements

We thank W. Kupsky for expert neuropathological advice, S. Dettloff for reviewing the manuscript, J. Liu and M. Baughn for technical support.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

The work was supported by grants from the Hiller ALS Center at Wayne State University and the NIH NINDS NS059947.

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Microglial neuregulin1 in the corticospinal tract


