Aberrant Neuregulin 1 Signaling in Amyotrophic Lateral Sclerosis

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Abstract

Neuregulin 1 (NRG1) is a neuron-derived trophic molecule that supports axoglial and neuromuscular development through several alternatively spliced isoforms; its possible role in the pathogenesis and progression of amyotrophic lateral sclerosis (ALS) is not known. We analyzed the relationship of NRG1 isoform expression to glial cell activation and motor neuron loss in spinal cords of ALS patients and during disease progression in the superoxide dismutase 1 (SOD1) ALS mouse model. Microgliosis, astrocytosis, and motor neuron loss were observed in the ventral horns in ALS patients and were increased in SOD1 mice along with disease progression. Type III (membrane-bound) NRG1 expression was reduced in parallel with motor neuron loss, but Type I (secreted) NRG1 expression was increased and was associated with glial activation. Increased NRG1 receptor activation was observed on activated microglia in both ALS patients and in SOD1 mice. This activation was observed at the time of disease onset and before upregulation of NRG1 gene expression in the mice. The downregulation of membrane-bound Type III NRG1 forms may reflect motor neuron loss, but increased signaling by secreted-type NRG1 isoforms could contribute to disease pathogenesis through glial cell activation. NRG1 might, therefore, represent a novel therapeutic target against disease progression in ALS.

Key Words: Amyotrophic lateral sclerosis, Microglia, Motor neuron, Neuregulin, SOD1.

INTRODUCTION

The pathogenesis of amyotrophic lateral sclerosis (ALS) is poorly understood, and there are presently no effective therapies to stop its insidious progression. How and where the disease begins and the molecular mechanisms of disease progression are not known. Although motor neuron loss in the spinal cord has been a central area of research focus for ALS (1, 2), extensive alterations involve neuromuscular synapses, the ventral spinal cord, the lateral corticospinal tract (CST), and the motor cortex (3–9). A major therapeutic strategy has been to try to rescue spinal motor neurons in the ventral horn. Not surprisingly, some of the first clinical trials for patients with ALS focused on neurotrophic growth factors such as brain-derived neurotrophic factor (BDNF) (10, 11), ciliary neurotrophic factor, glial cell–derived neurotrophic factor (GDNF), insulin-like growth factor 1, and vascular endothelial growth factor (11, 12). Unfortunately, the promise of neurotrophic growth factor treatment was not matched by any clear efficacy in human clinical trials (11). Among the reasons that these clinical trials may have failed are an incomplete knowledge of how and where these growth factors work locally (which is needed for optimal drug delivery) and how they interact with other cell types (10).

Research on ALS has traditionally been “neuron-centric,” but the protective and deleterious roles that astrocytosis and microglial activation glial cells have received increased attention (13–15). “Gliotrophic” factors derived from alternatively spliced forms of the neuregulin 1 (NRG1) gene have been shown to be critical for peripheral nerve development, myelination, and, more recently, microglial activation in diverse diseases ranging from peripheral nerve diseases (16, 17) to schizophrenia (18, 19). The NRG1 gene encodes for both secreted (Type I) and membrane-bound (Type III) forms that are highly expressed in spinal motor neurons (20, 21). Several studies suggest a positive feedback loop between this “gliotrophic” factor and neurotrophic factors; for example, BDNF promotes both transcription and the regulated, localized release of soluble NRG1 from neurons during development at the neuromuscular junction and at the peripheral nerve (17, 22). More recently, neuron-derived NRG1 has been implicated in the activation of spinal cord microglia responsible for the generation of chronic pain (23). To date, this important reciprocal signaling pathway has not been explored in ALS or the superoxide dismutase 1 (SOD1) ALS mouse model in which there is also marked glial activation (13–15, 24–26).

In this study, we analyzed NRG1 in the spinal cords of ALS patients and in ALS-SOD1 (G93A) mice at different stages of disease progression. We compared motor neuron loss and glial activation and NRG1 expression patterns and measured the activation of NRG1 receptor signaling as a function of disease progression.

MATERIALS AND METHODS

Human Tissue

Fresh-frozen thoracic and lumbar spinal cords from 6 sporadic and 2 familial ALS subjects (5 males aged 41–84 years and 3 females aged 64–77 years) and 6 control patients (4 males aged 67–84 years and 2 females aged 58 and 76 years) with no pathological evidence of neurological disease were provided by one of the authors (J.R.) (n = 10)
(2) or were obtained from the Human Brain and Spinal Fluid Resource Center (VA West Los Angeles Healthcare Center, Los Angeles, CA) (n = 4). Postmortem time intervals ranged from 2 to 25 hours (mean = 13.5 hours). Genetic testing, including of SOD1 mutations, was not performed on the patient samples.

**SOD1 Mouse Tissue**

Breeding pairs of SOD1 (G93A) transgenic (Tg) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and genotyped as described (27, 28). SOD1 mutant mice were tested weekly for movement behavior. Thoracic and lumbar spinal cord regions were collected from each mouse at Days 35 or 56 (preclinical), Days 90 to 100 (disease onset), and Days 117 to 126 (end-stage disease); 4 to 8 mice (SOD1 Tg and non-Tg littermates) were used per group at each time point (4 time points, 32 mice in total).

**Histopathology**

Fresh-frozen human and fresh mouse spinal cords were fixed in 4% paraformaldehyde for 24 hours, washed overnight in phosphate-buffered saline, 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, placed on SuperFrost slides (VWR, West Chester, PA), and then stained with either Luxol fast blue-periodic acid Schiff (Poly Scientific, Bay Shore, NY) for the presence of myelin or Cresyl violet (Diagnostic Biosoynms, Pleasanton, CA) for motor neuron counts.

**Immunohistochemistry**

Identification and quantification of microglia in human and mouse spinal cord tissue sections were performed using antibodies specific for human CD68 (mouse IgG1, 1:20, catalog no. N1577; DAKO, Carpinteria, CA) and mouse microglia CD11b (rat IgG2b, 1:100; Millipore, Billerica, MA). Astrocytes were labeled with anti-glial fibrillary acidic protein (GFAP) antibodies against human (rabbit polyclonal antibody, 1:100; DAKO) and mouse (mouse IgG 1:100; Chemicon, Temecula, CA). Detection of activation of the NRG1’s erbB2 receptor was performed with either a rabbit anti-phospho-erbB2 antibody (p-Neu, Tyr1248, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA) for both human and mouse sections or a rabbit anti-erbB2 antibody (Neu, C-18, 1:50; Santa Cruz Biotechnology) for mouse sections. Each primary antibody was diluted in blocking solution (10% normal goat serum, 0.05% Triton X-100 in phosphate-buffered saline) overnight at 4°C, followed by incubation with goat anti-mouse or rabbit Alexa Fluor 488 (1:100; Invitrogen, Carlsbad, CA). For CD11b, phospho-erbB2 and CD68 immunostaining, biotin-conjugated goat anti-rat, anti-rabbit, or anti-mouse was used as a secondary antibody; the signal was amplified using a tyramide signal amplification kit (1:250; Invitrogen) following the manufacturer’s instructions.

Monoclonal antibodies specific for the Type III human NRG1 isofrom (also referred to as the cysteine-rich domain [CRD]) were developed in collaboration with the UC Davis/ National Institutes of Health (NIH) NeuroMab Facility, Davis, CA. To test the specificity of the Type III NRG1 antibody, 1.5 × 10⁵ Chinese hamster ovary cells were seeded in each well of a 24-well plate and cotransfected with either human Type I NRG1 or human Type III NRG1 cloned into pFLAG-Myc-CMV-20 (E8783; Sigma, St. Louis, MO) with enhanced green fluorescent protein cloned in the pTriex vector (Novagen, Gibbstown, NJ) using Lipofectamine 2000 (11668-019; Invitrogen). After 24 hours, the cells were fixed in 4% paraformaldehyde for 30 minutes and stained for Type III NRG1 (1:500; NRG1-CRD, N126B/31, 20100712, 73–226; UC Davis/NIH NeuroMab Facility). Nuclei were counterstained with 4,6-diamidino-2-phenylindole.

Neuregulin-CRD (Type III, mouse IgG, 1:200, clone N126B/31) or panNRG1 (SC348, rabbit IgG, 1:100 Neuregulin-1α/β1/2, C-20; Santa Cruz Biotechnology) were incubated overnight at 4°C, followed by a biotinylated goat anti-mouse or anti-rabbit IgG secondary antibody (1:200), streptavidin-horseradish peroxidase, and DAB Vectorstain reagents according to the manufacturer’s recommendations (Vector Laboratories, Burlingame, CA).

**Immunoblotting**

A total of 1.5 × 10⁵ Chinese hamster ovary cells were seeded in each well of a 24-well plate and cotransfected with either human Type I NRG1 or human Type III NRG1 cloned into pFLAG-Myc-CMV-20 (Sigma) or enhanced green fluorescent protein cloned in the pTriex-1.1 vector (70480-3; Novagen) using Lipofectamine 2000 (Invitrogen). After 24 hours, the cells were harvested, and the total protein was extracted using RIPA lysis and extraction buffer (25 mmol/L Tris pH 7.6, 150 mmol/L NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, and Halt protease and phosphatase inhibitor cocktail [78442; Thermo Scientific, Rockford, IL]) for further immunoblot analysis. The total protein of 1 mouse spinal cord and 1 piece of human spinal cord was also extracted using RIPA buffer. The NRG1-CRD antibody was used at a dilution of 1:500. SuperSignal West Pico Chemiluminescent Substrate (34077; Thermo Scientific) was used as directed.

Lumber spinal cords were harvested from either SOD1 (G93A) (n = 5) or non-Tg littermates (n = 5) at disease end stage. Total protein was extracted using RIPA buffer. Protein samples from 5 of the above pairs were used for immunoblotting analysis. Antibodies were used as NRG1-CRD (mouse IgG, 1:500, clone N126B/31), β-actin (1:1000, A5441; Sigma), anti-phospho-ErbB2 (Y1248) (0.5 μg/mL, AF1768; R&D Systems, Minneapolis, MN), and anti-erbB2 Neu C-18 (1:500, SC284; Santa Cruz). SuperSignal West Pico Chemiluminescent Substrate was used as directed. Each blot was probed, stripped, and reprobed with a different antibody. Quantification of band intensity was performed using MetaMorph image analysis software (Universal Imaging Corporation, Buckinghamshire, UK), as previously described (29–31).

**Quantitative Image Analysis**

Digital images were obtained with a Nikon Eclipse 600 epifluorescence microscope (Nikon Instruments Inc., Melville, NY) with a Princeton Instruments Micronox 5-MHz cooled CCD camera or a QImaging color digital camera (Micro Publisher 5.0 RTV, Burnaby, BC, Canada). Mouse motor neurons (i.e. large neurons with clear nuclei and distinctly labeled cytoplasm on Cresyl violet staining) were counted manually by
FIGURE 1. Pathological comparisons in the spinal cords of ALS patients and SOD1 mice. Spinal cords stained with Luxol fast blue-periodic acid Schiff demonstrate myelin loss (top and second rows, pink areas) within the anterior and lateral corticospinal tracts (LCSTs) in ALS, but not in the mouse dorsal corticospinal tract (DCST). Cresyl violet stains show motor neuron loss (large cells) in the ventral horn in both ALS and SOD1 mice along with increased cellularity compared to control (third row). Scale bars = (top row) 500 μm for human and 200 μm for mouse; (second and third rows) 50 μm for human and mouse.

3 individuals, as described (32). Mouse microglia (CD11b positive) (33), mouse reactive astrocytes (GFAP positive), and mouse phospho-erbB2-positive cells were quantified based on the presence of both a glial cell shape and a nucleus labeled with 4',6-diamidino-2-phenylindole. For each cell type counted, 6 to 10 sections at the lumbar spinal cord level were used to analyze each condition in each animal. MetaMorph image analysis software (Molecular Devices, Downingtown, PA) was used to quantify the degree of colocalization by creating thresholded objects in 1 wavelength and measuring the percentage of pixel overlap for each in the second thresholded wavelength. Thresholds were set visually using the criteria of identifying the threshold that best outlined the individual cell types. For each measurement, the average counts of each group for each time point were made from 4 to 8 animals. Cell counting was done manually and blinded by 3 different individuals. For motor neuron counts in the SOD1 animals, there were differences in total numbers for each of the counters, but the relative differences between the groups were consistent. Results from only one of the counters are presented because of the variations.

RNA Isolation and Real-time Quantitative Reverse Transcription–Polymerase Chain Reaction

Mouse spinal cords were dissected, and thoracic spinal cords (T1–T12) were rapidly frozen and stored at −80°C for RNA; the lumbar levels were placed in 4% paraformaldehyde for histology as described above. RNA was extracted using the Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) (34). Quantification of RNA was carried out using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The quality of RNA was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using an RNA 6000 Nano Chip Kit, RNA ladder, and Agilent analysis software (Agilent Technologies). All samples had RNA integrity values above 8.0 and 260/280 ratios near 2.0.

The relative expression of mouse Type I and 3 NRG1, BDNF, and GDNF were measured relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using TaqMan Assays-on-Demand primers (Applied Biosystems, Foster City, CA). Total RNA (1.5 μg) was used in a 20-μL reverse transcription synthesis reaction primed with oligo-dT primers (Superscript First Strand Synthesis System; Invitrogen). Polymerase chain reaction was performed in triplicate using 1× TaqMan Universal PCR Master Mix (Applied Biosystems) with the DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, Waltham, MA) using the following primers and TaqMan probes: Type I NRG1: Mm00626552_m1; Type III NRG1: Mm01212129_m1; BDNF: Mm01334047_m1; GDNF: Mm0059849_m1 and GAPDH: Mm99999915_g1. Cycle threshold (Ct) values were calculated using Opticon monitor software, with the threshold set at 40 SDs above background. The relative expression was calculated by normalizing the expression of individual genes to GAPDH and using the 2−ΔΔCt method (35).
Statistical Analysis

For cell counts and gene expression, a nonparametric analysis of variance with the Tukey post hoc test was performed between non-Tg control and SOD1 at each stage of the disease. In some experiments, comparisons were made between end-stage SOD1 mice and the same sex and age non-Tg littermates. Groups were considered significantly different at p < 0.05 by Student t-test.

RESULTS

Motor Neuron Loss Is Associated With Glial Activation in ALS and SOD1 Mice

SOD1 mice have been the most widely used animal model for ALS (36), but there are both pathologic similarities and differences between human and mouse diseases (9, 37). There is severe lower motor neuron loss in the ventral horn in both, but there are differences in the descending central motor tracts, that is, the lateral CST (LCST) in humans and dorsal CST (DCST) in mice (Fig. 1). Quantification of motor neuron cell number loss in the ALS patients was described previously (2). The motor neuron loss in SOD1 mice in the present study (Fig. 1) is consistent with a published report (36).

In both the ALS cases and end-stage SOD1 mice, there are numerous small cells in the ventral horns that are not present in controls; these small cells consist of a combination of astrocytes and activated microglia (13–15, 24–26). Large numbers of microglia were consistently seen in the ventral horns in the 8 ALS cases (Fig. 2), but (although still increased compared with controls) there were generally fewer reactive

FIGURE 2. Activated microglia accumulate in the ventral horn in amyotrophic lateral sclerosis (ALS) and in superoxide dismutase 1 (SOD1) mice. Human (CD68-positive; green, top row) and mouse (CD11b-positive; green, bottom row) microglia are increased in the ventral horns of an ALS patient and an end-stage SOD1 mouse compared to a control patient and a nontransgenic (non-Tg) control mouse. Reactive astrocytes (glial fibrillary acidic protein [GFAP]-positive, red, top and bottom rows) are increased in the ventral horn of the SOD1 mouse (bottom row) but not markedly in the ALS case (top row). Scale bars = 20 μm.
astrocytes compared with microglia, and the numbers were quite variable from patient to patient (data not shown). In more than 20 end-stage SOD1 mice, there were similar levels of both microglia and astrocytes (data not shown). While our findings showing both astrocyte and microglial activation in the ventral horn agrees with others (36), the relative proportion of these two cell types differs between human ALS and the SOD1 model with more astrocytosis seen in SOD1 mice than the human tissue samples. However, a similar degree of microglial activation was seen in both raising an important question as to whether factors that control microglial activation (such as NRG1) could be involved in the disease pathogenesis of both.

**NRG1 Expression Correlates With Increased Microglial Activation and Motor Neuron Degeneration During Disease Progression in SOD1 Mice**

In SOD1 mice, there was progressive motor neuron loss and activation of both microglia and astrocytes (Figs. 3A, B). Type I (soluble) NRG1 mRNA dramatically increased in the latest stage of the disease (Days 112–126) (Fig. 3C). However, during this period, there was a significant downregulation of Type III (membrane-bound) NRG1 mRNA expression (Fig. 3C). There were changes in both BDNF and GDNF expressions, but these were not as consistent as the NRG1 mRNA changes, where a modest increase in GDNF mRNA at end stage did not reach statistical significance (Fig. 3D).

To determine whether the reduction in Type III NRG1 was due to motor neuron loss, we codeveloped an isofrom-specific monoclonal antibody (with UC Davis/NIH NeuroMab Facility) for Type III NRG1 CRD (Fig. 4A). This antibody does not cross-react with Type I NRG1 in transfected Chinese hamster ovary cells by Western blotting (Fig. 4B) and immunostaining (Fig. 4C) and labels an approximately 50-kDa band in normal human and mouse spinal cord tissues (Fig. 4B). We then compared total NRG1 expression using an established pan-NRG1 antibody (sc348, epitope shown in Fig. 4A) to Type III NRG1 expression in both ALS and end-stage SOD1 mice spinal cords (Fig. 5). Both antibodies labeled ventral horn motor neurons in ALS patients and SOD1 mice, even in advanced diseases in which motor neurons show significant pathological changes. This suggested that Type III NRG1 is not appreciably reduced in individual motor neurons and that the reduction in mRNA might be explained by motor neuron loss. Indeed, the reduction of Type III NRG1 (Fig. 3).

**FIGURE 3.** Motor neuron loss and glial changes are associated with changes in neuregulin 1 (NRG1) isofrom expression in superoxide dismutase 1 (SOD1) mice. (A, B) There were significant reductions in motor neurons in SOD1 mice (A) and increases in activated microglia and reactive astrocytes (B) in the ventral horns of SOD1 mice compared to their nontransgenic (non-Tg) littermates (*n* = 4–8 mice/group/time point; high-power field [HPF] = 0.28 mm² for A; HPF = 0.14 mm² for B; *p* < 0.05; **p** < 0.01; ***p*** < 0.001 for SOD1 mice vs non-Tg mice at the same time point for both A and B). (C) There was an increased in Type I NRG1 mRNA at end stage and a corresponding decrease in Type III NRG1 mRNA in SOD1 mice versus age- and sex-matched non-Tg littermates (*n* = 4/group). (D) The same samples showed little change in brain-derived neurotrophic factor (BDNF) and glial cell–derived neurotrophic factor (GDNF) mRNA (*n* = fold change from 4 SOD1 mice vs 4 non-Tg mice at each time point). *p* < 0.05; **p** < 0.01; ***p*** < 0.001 for the fold changes at Day 36, Day 90, or Day > 126 versus Day 35 for C and D.

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FIGURE 4. Characterization of neurexin 1 (NRG1) isoform-specific antibodies. (A) Schematic of Type I and Type III NRG1 domain structure shows that both Types of NRG1 are expressed as transmembrane precursor proteins that can be recognized by a pan-NRG1 antibody directed against the cytoplasmic tail (SC384). After cleavage, “released” forms of NRG1 (including the Type I form) contain 2 extracellular domains: an immunoglobulin-like (IG) domain that functions as a heparin-binding domain (HBD) and an EGF-like domain (EGF) that is sufficient to bind and activate the erbB receptors. The Type III NRG1 “membrane-associated form” precursor can also be cleaved; however, the hydrophobic cysteine-rich domain (CRD) remains tethered in the membrane. (B) We generated a highly specific anti-CRD domain NRG1 antibody that recognizes the intracellular portion of Type III NRG1. The antibody is specific on Western blots for a 42-kDa protein in Chinese hamster ovary cells transfected with human Type III NRG1 but not for Type I NRG1 or enhanced green fluorescent protein (EGFP). Western blots of human and mouse spinal cords show that the CRD NRG1 antibody also recognizes the Type III NRG1 of approximately 50 kDa. The difference in size could be due to glycosylation or alternative splicing. (C) Immunostaining of the same transfected Chinese hamster ovary cells with either human Type III NRG1 or human Type I NRG show that the anti-CRD NRG1 antibody specifically recognizes only Type III NRG1.
mRNA correlated significantly ($R = 0.995$) with motor neuron loss (Fig. 5C). This is corroborated by Western blotting in Figure 5D showing a significant downregulation of Type III NRG1 in SOD1 mice (30% reduction, 5 mice/group; Fig. 5D and data not shown).

Although the main sources of Type I and III NRG1 isoforms in normal spinal cord are ventral motor neurons (20, 21, 38), the pan-NRG1 antibody also labeled many small cells in the ventral horn (Fig. 5A) that were not labeled by the Type III–specific antibody (Fig. 5B) in both ALS and SOD1 mice (n = 6 samples per group). On the basis of their shapes, they might be glia, but efforts to double stain these cells did not identify them as either reactive astrocytes (GFAP, data not shown) or microglia. One other possibility not yet explored is that they represent NG-2–positive oligodendroglial progenitor cells that have been shown to be increased in ALS (39, 40). Although the identities of these smaller cells are not clear, they could, along with the remaining motor neurons, be a source of soluble NRG1 signaling in both ALS and the SOD1 mouse model that could contribute to the increase in identified glial cells observed.

**NRG1 Receptor Activation on Microglia During SOD1 Disease Progression and in ALS**

Whereas Type III NRG1 remains tethered to the membrane even after proteolytic processing (41), Type I NRG1 is secreted in a regulated fashion, after which it becomes concentrated in regions rich in heparan sulfate proteoglycans because of its unique heparin-binding domain (Fig. 4A) (29). Because there are no highly reliable Type I isoform-specific antibodies, we determined the spatial localization of Type I NRG1 activity by looking for activation of its membrane-bound receptors (42, 43). NRG1 promotes microglial activation in a peripheral nerve injury model of chronic pain through binding to either erbB3 or erbB4 receptors that then heterodimerize with erbB2 to mediate signal transduction through tyrosine phosphorylation (23, 44). Therefore, we determined the level of both erbB2 receptors and activated erbB2 receptors (p-erbB2) in the SOD1 mice and in ALS. Both erbB2 and p-erbB2 expression are increased in the SOD1 mouse ventral horn (Fig. 6A). Although erbB2 is expressed primarily only in motor neurons of non-Tg littermates, the increase in erbB2 expression is due to its presence in many additional cells.

**FIGURE 5.** Reduced Type III neuregulin 1 (NRG1) protein expression correlates with motor neuron loss in the ventral horn in amyotrophic lateral sclerosis (ALS) and in superoxide dismutase 1 (SOD1) mice. (A, B) A pan-NRG1 antibody stains motor neurons and additional small cells in the ventral horn of both ALS and SOD1 mice (A), whereas Type III NRG1 protein expression is only expressed in motor neurons (B). Scale bars = (top rows) 50 μm; (bottom rows) 10 μm. (C) Reduction in Type III NRG1 mRNA expression in SOD1 mice (from Fig. 3C) correlated significantly with motor neuron number. n = 4 SOD1 mice/time point; data taken from Fig. 3A; n = fold change of Type III NRG1 from 4 SOD1 mice versus 4 nontransgenic (non-Tg) mice at each time point. *, p < 0.05 for the fold changes at Day > 112 < 126 versus Day 35. n = 4–8 SOD1 mice/time point. *, p < 0.05; **, p < 0.01 for motor neuron number at Day 90 or Day > 112 < 126 versus Day 35. (D) Type III (CRD) NRG1 protein is reduced in spinal cords from end-stage SOD1 mice versus littermate non-Tg mice by Western blotting.
FIGURE 6. ErbB2 receptor expression and activation (p-erbB2) is increased in the ventral horn of end stage superoxide dismutase 1 (SOD1) mice. (A) Neuregulin 1 (NRG1) receptors (erbB2) and erbB2 receptor activation (p-erbB2) are both increased in the lumbar ventral spinal cord of end-stage SOD1 mice compared to nontransgenic (non-Tg) littermates. Scale bar = 50 μm. (B) Western blots show the specificity of anti-erbB2 and anti-p-erbB2 antibodies as a series of bands at 185 kDa in spinal cords from non-Tg mice.

However, activated p-erbB2 was seen in a subset of these cells in the SOD1 mice but not in non-Tg littermate controls. The specificity of these 2 antibodies is shown by Western blotting in Figure 6B.

Double labeling of p-erbB2 with microglial and astrocytic markers revealed that activated erbB2 receptors were predominately on microglia (70% of CD11b-positive microglia) and, to a lesser extent, on astrocytes (22% of GFAP-positive astrocytes; Figs. 7A, B). Quantification of cells showing erbB2 receptor activation as a function of disease progression in the SOD1 mouse showed that increased NRG1 receptor activation starts from Day 56 and increases over time (Fig. 7C), correlating well with the pattern of microglial activation (Fig. 3B). These changes precede the induction of Type I NRG mRNA, which occurs later in the disease course (Day 90 to end stage).

There was also a marked increase in erbB2 receptor activation in the ventral spinal cord in ALS patients that mostly colocalizes with activated microglia (167 ± 44 CD68+p-erbB2+ cells/mm², n = 3) compared with control spinal cords (18 ± 8 CD68+p-erbB2+ cells/mm², n = 3; p < 0.01) (Fig. 8). erbB2 receptor activation was present at multiple levels (thoracic and lumbar) in 6 of 6 ALS patients but not in any of the 4 control patients. Thus, the results suggest that aberrant NRG1 signaling could underlie microglial activation in both the SOD1 model and the ALS ventral spinal cord.

DISCUSSION

NRG1-Induced Microglial Activation as a Possible Therapeutic Target for ALS

Our data support the hypothesis that NRG1 released from injured neurons and other cells in the spinal cord induce microglial activation, which, in turn, could lead to progressive motor neuron degeneration in ALS. Our parallel findings in both human tissues and the SOD1 mouse model show progressive activation of NRG1’s erbB2 receptor on activated microglia raising the possibility that disease progression in ALS could be targeted therapeutically by disrupting NRG1 signaling. Recently, NRG1 signaling through activation of erbB2 receptors has been shown to be critical for microglial activation in the dorsal spinal cord of rats that develop chronic pain after peripheral nerve injury (23). Using a novel NRG1 antagonist (45) injected intrathecally, the number of activated microglia was reduced together with mechanical and cold pain–related hypersensitivity (23). These findings suggest that NRG1 is an important local signal for microglial activation in the spinal cord after neural injury and that blocking NRG1 signaling could be a novel approach to block the untoward effects that ensue.

Microglial activation has long been argued to be important in the pathogenesis of ALS. In fact, minocycline, an antibiotic drug, showed promise in blocking microglial activation in...
the SOD1 model (46); however, clinical trials with minocycline have not been encouraging (47, 48). One possible reason for this failure is its many off-target effects on cytochrome C, mitogen activated protein kinase, nitric oxide, and caspas that could promote apoptosis and obscure any beneficial effects (49). Another possibility for this failure is that subsets of activated microglia play neuroprotective roles in the different stages of the disease (15). Boillée et al (50) reported that

**FIGURE 7.** Neuregulin 1 (NRG1) receptor activation occurs primarily on microglia and is present from clinical disease onset in superoxide dismutase 1 (SOD1) mice. (A) Confocal images show colocalization of activated p-erbB2 (green, first and second rows) mostly with microglia (CD11b-positive, red, first row), but less so on astrocytes (glial fibrillary acidic protein [GFAP]-positive, red, second row). Scale bar = 10 μm. (B) Quantitation of p-erbB2 positive microglia and astrocytes shows that while microglia show receptor activation, most astrocytes do not (n = 4 mice/group/time point; results are expressed as percentage of total microglia or astrocytes). (C) NRG1 receptor activation is seen in microglia at disease onset and is increased as the disease progresses in SOD1 mice. n = 4-8 mice/group/time point. *, p < 0.05; **, p < 0.01; ***, p < 0.001, for SOD1 mice versus nontransgenic (non-Tg) mice at the same time point for B and C.
FIGURE 8. Neuregulin 1 (NRG1) receptor activation is present on activated microglia in the spinal cord of amyotrophic lateral sclerosis (ALS) patients but not controls. NRG1 receptor activation (p-ErbB2-positive, green) colocalizes with activated microglia (CD68-positive, red) in the ventral horn of an ALS patient. Quantification of the percent overlapping signal revealed 25.00% ± 6.25% overlap versus 2.67% ± 1.15% for controls. The blue channel shows nuclear staining with 4,6-diamidino-2-phenylindole to demonstrate presence of cells. This was similar in the no-primary control shown in the lower panel. Similar results were seen in 3 ALS and 3 control patients (p < 0.01). Scale bars = 10 μm.

diminishing mutant SOD1 levels in microglia slow disease progression in mutant SOD1G93A mice during later stages; however, wild-type donor-derived microglia promote neuroprotection and extend survival in mutant SOD1G93A/PU.1 knockout mice (51). Therefore, a better understanding of the local signals between neurons and glia that both activate and block different subtypes of microglia will be important to modulate their neurodegenerative and neuroprotective roles in ALS.

Different Roles for Alternatively Spliced Isoforms of NRG1

Persistent activation of NRG1 receptors on activated microglia in both ALS patients (Fig. 8) and SOD1 mice was observed at relatively early stages of the disease (Fig. 7). As the disease progresses, a reciprocal increase in Type I NRG1 mRNA together with a decrease in Type III NRG1 was observed in the spinal cord; therefore, early NRG1 receptor activation on microglia cannot be explained by increased NRG1 mRNA levels. One possibility is that increased soluble forms of NRG1 are released through posttranscriptional mechanisms at the early disease stages. Type I NRG1 is synthesized as a transmembrane precursor in neurons (22, 30, 52). The precursor is transported down axons and can be released at sites where it is needed in response to local gradients of neurotrophins, such as BDNF (31). Once Type I NRG1 is released, it becomes concentrated at non-random sites by
binding to specific heparan sulfate proteoglycans in the extracellular matrix (29). Thus, during the degenerative disease process, a combination of local gradients of neurotrophic factors as well as changes in the extracellular matrix composition could be important variables, other than transcription, that contribute to increased NRG1 signaling in disease progression.

Exactly which cells secrete NRG1 is also not entirely clear. Using a pan-NRG1 antibody, NRG1 staining was seen both in injured motor neurons, which also express Type III forms, and in smaller cells in the ventral horn that did not colabel with Type III NRG1 or the glial or microglial markers tested; they might be NG-2 positive oligodendroglial progenitor cells. Sorting out the precise alternative forms of NRG1 is further complicated by a lack of highly specific anti-NRG1 antibodies together with a low abundance and short regions of specific DNA sequences in these forms that make it difficult to detect them by in situ hybridization.

A second possibility is that other ligands may cross talk with the erbB2 receptors producing their activation. Although there are very few ligands known to activate the erbB2 receptors (53), there are a number of homologous NRG genes, some of which are also expressed in the nervous system. For example, NRG2 shares sequence homology to NRG1, activates erbB receptors, and is expressed in brain and spinal cord (54).

The expression and signaling of soluble NRG1 forms increase at end-stage disease, but membrane-bound Type III NRG1 is significantly reduced both in ALS and in the SOD1 model. Using an isoform-specific antibody against its intracellular domain, continued protein expression of Type III NRG1 was seen in surviving human and mouse motor neurons. In fact, the reduction of Type III NRG1 mRNA correlated with the loss of motor neurons, suggesting that this isoform may not be critical in disease pathogenesis. Type III NRG1, also referred to as the CRD form because of its cysteine-rich hydrophobic domain, has been shown to have important roles in peripheral nerve myelination (17, 55) but requires direct cell-cell contact (26) and thus may have limited opportunities to activate receptors on microglial cells unless they are in direct contact with motor neurons.

Differences and Similarities in ALS and the SOD1 Mouse Model

To date, translating therapeutics from the SOD1 model to ALS patients has been disappointing (9, 37). One of the greatest pathological differences between ALS and this model we found is the presence of marked myelin loss in the LCM of ALS patients (Fig. 1) but not in the equivalent DCST (in which approximately 80% of CST axons are located [56, 57]). There are, however, reports of neuronal and descending cortical axon loss by others (58–61).

On the other hand, both ALS patients and SOD1 mice showed motor neuron loss together with increased cellularity in the ventral horn. Some of this cellularity is due to the presence of activated microglia; however, astrocytosis was more prominent in ventral horn of the mice, but relatively less remarkable in the ALS tissues. Schiffer et al (62) reported the presence of astrogliosis in the ventral horns from 70 autopsied ALS cases but did not quantify the data or compare this to the SOD1 mouse (63). Astrogliosis in the SOD1 mice was extremely prominent in our mice and has been frequently observed by others (64–66), but very few of these astrocytes showed NRG1 receptor activation. In summary, while gliosis is present in the ventral horns of both ALS patients and SOD1 mice, extensive microglial activation and sustained NRG1 receptor signaling are common to both and may, therefore, be amenable to targeted therapeutic strategies.

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