Selective Loss of trans-Acting Instability Determinants of Neurofilament mRNA in Amyotrophic Lateral Sclerosis Spinal Cord*

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Neurofilament (NF) aggregates in motor neurons are a key neuropathological feature of amyotrophic lateral sclerosis (ALS). We have previously observed an alteration in the stoichiometry of NF subunit steady state mRNA levels in ALS spinal motor neurons using in situ hybridization and proposed that this led to aggregate formation. We have now examined the levels of NF mRNA in whole tissue homogenates of spinal cord using the RNase protection assay and real time reverse transcriptase-PCR and observed significant elevations of NF mRNA level in ALS. Compared with age-matched control, we observed a greater stability of heterogeneously expressed NFL mRNA in the presence of ALS spinal cord homogenates. Heat denaturing or protease K digestion of the control homogenates increased the stability of the NFL mRNA to levels observed in ALS homogenate. Increased NFL mRNA stability was also induced by increasing the percentage of ALS homogenate in an admixture of control and ALS homogenates. These observations suggest the presence of trans-acting NFL mRNA destabilizing elements in control but not in ALS spinal cord homogenates. This was confirmed in gel retardation assays. We also observed that the destabilizing elements interact with the 3′-untranslated region of NFL mRNA. These findings suggest that the trans-acting NFL destabilizing elements are selectively suppressed in ALS homogenates, resulting in an increased stability and level of NFL mRNA.

With death ensuing from respiratory failure within 5 years of symptom onset for the majority of patients, amyotrophic lateral sclerosis (ALS) is among the most devastating of the adult onset neurodegenerative disorders.ALS (also known as Lou Gehrig’s disease), characterized by degeneration of motor neurons in the spinal cord, brainstem, and cortex, manifests as a relentlessly progressive loss of strength and function of virtually all skeletal muscles. Of concern, the prevalence is increasing at a rate greater than can be accounted for by increases in longevity of the population (1, 2). Equally unfortunately, there are no therapies available that will significantly truncate the disease progression.

Intraneuronal aggregates of neurofilament (NF) in degenerating motor neurons are a key neuropathological hallmark of ALS (3–9). Although the exact origin of the NF aggregates remains to be determined, transgenic mouse models in which the overexpression of any of the low molecular weight NF (NFL), middle molecular weight NF (NFM) or high molecular weight NF (NFH) proteins is induced provokes a motor neuronopathy characterized by the presence of abnormal NF accumulations resembling those found in ALS (10–13). Remarkably, the motor neuronopathy in transgenic mice overexpressing human NFH subunits was rescued by the co-expression of a human NFL transgene at levels that restored a correct stoichiometry of NFL to NFH subunits (14).

We have previously observed significant suppressions in NFL mRNA steady state levels in cervical spinal motor neurons in ALS with in situ hybridization (15). Further, we observed a failure of either α-internexin or peripherin mRNA levels to increase to compensate for this suppression of NFL mRNA levels, suggesting an absolute alteration of the stoichiometry of NF expression in ALS. These findings extended the observations of Bergeron et al. (16) of reduced NFL mRNA levels in inclusion-bearing spinal motor neurons in ALS. Using single cell PCR, Menzies et al. (17) have confirmed the observation of spinal motor neuron selective alterations in NF expression. Within brainstem nuclei, Hartmann and Sun observed a suppression of NFM mRNA, although only gross visualization of grain density was undertaken, and the remaining intermediate filaments were not assessed (18). Taken together, these observations have suggested a significant alteration in NF expression in ALS. Given the selective vulnerability of motor neurons to the degenerative process of ALS and the observations that NF protein expression will be critical to maintaining the structure of these large motor neurons, it has been proposed that such alterations in NF homeostasis could be of direct relevance to the pathogenesis of ALS (19). In this study, we have examined the regulation of NF mRNA stability in ALS and specifically observed that trans-acting NFL mRNA destabilizing elements normally expressed in control spinal cord are absent in ALS spinal cord homogenates.

EXPERIMENTAL PROCEDURES

Tissues—Archival spinal cord tissues of sporadic ALS and age-matched control cases were selected. All ALS cases were clinically and neuropathologically definite ALS (20).

Total RNA Isolation, Reverse Transcription—Total RNA was isolated using the TRIzol reagent (Invitrogen), followed by DNase digestion to eliminate genomic DNA contamination. RNA integrity was assessed...
with an AGILENT 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using a RNA Nano LabChip (Agilent Technologies). RNAs were quantified with spectrophotometer A_{260} readings. Synthesis of cDNA was performed using 1 μg of total RNA in a 20-μl system, 25 °C for 10 min, followed by 30 min at 48 °C, and then 5 min at 95 °C.

**Probes for RNase Protection Assays**—[α-32P]UTP-labeled antiense cRNA probes for human NFL (100 nt; 48 nt from exon 3 and 52 nt from exon 4), NFM (150 nt; 42 nt from exon 2 and 108 nt from exon 3), and NFH (200 nt; 101 nt from exon 3 and 99 nt from exon 4) were synthesized by *in vitro* transcription from RT-PCR amplicons, with T7 promoter incorporated within reverse primer. Primers for the transcription templates are listed in Table I. As an internal control, a cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was *in vitro* transcribed from linearized pTRI-GAPDH-Human plasmid (Ambion Inc.).

**RNase Protection Assay**—10 μg of total RNA was combined with 4 × 10^6 cpm of gel-purified NF probe and 2 × 10^6 cpm of GAPDH probe. The samples were incubated at 42 °C overnight and then subjected to RNase digestion for 45 min at 37 °C using RNase A/T1 mix (RPAII kit; Ambion). Protected fragments were separated on 5% acrylamide, 6 μM urea gels.

**Primers for SYBR Green Real Time Quantitative RT-PCR**—Prior sequences were designed using Primer Express Software (PerkinElmer Life Sciences) and are presented in Table I.

**SYBR Green Real Time Quantitative RT-PCR**—Quantitative PCR was performed on an ABI 7900 PCR Instrument (PerkinElmer Life Sciences) in a 20-μl volume, using 2× Universal SYBR Green PCR Master Mix (PerkinElmer Life Sciences). Absolute quantitation assay was adopted. For absolute standard curves, gene fragments that contained fragments to be amplified in real time RT-PCR were ligated into pcDNA3.1(+) vectors and quantified with the *A_{260}*. Spectrophotometer readings, GAPDH and neomycin-resistant gene from heterogeneous expression vector were used as endogenous references for tissue samples and *in vitro* incubation samples, respectively, to ensure the same amount of tissue and the same amount of RNA input in the *in vitro* incubation assays. Each sample was tested in triplicate, and samples obtained from at least three independent experiments were used to calculate the means and S.D.

**Protein Homogenates Preparation**—Protein was extracted from 500 mg of snap-frozen lumbar ventral spinal cord of ALS patients and normal controls. Tissues were homogenized in 2 ml of buffer (25 mM Tris, 0.1 M sucrose, 0.5 mM EDTA plus protease inhibitor mixture (Sigma)) using a Brinkman Polytron PT3000 homogenizer and centrifuged twice (5000 × g, 10 min). The supernatant was collected and stored at −80 °C until use.

**Generation of NFL-1000 mRNA and NFM-CDs mRNA**—Because the full-length NFL mRNA is too long for cloning and heterogeneous expression, the last 1000 bp of NFL cDNA (NFL-1000), which contains the putative stability regulatory elements (21), was RT-PCR-amplified from an ALS patient RNA isolate and cloned into BamHI/NcoI sites of pcDNA3.1(+) vector as the representative for the full-length NFL for *in vitro* stability regulation study. We also engineered NFL cDNA devoid of the 3′-UTR (NFM-CDs) to study the function of 3′-UTR in the stability of NFL mRNA.

**NSC34 cells** were chosen because of their high transfection efficiency using LipofectAMINE (Invitrogen) and were grown in Dulbecco’s modified Eagle’s medium (high glucose, with pyruvate, with t-glutamine) containing 10% (v/v) heat-inactivated fetal bovine serum and 1% penicillin/streptomycin solution (23). Transfection was carried out using LipofectAMINE. Cells were maintained for 24 h, and then RNA was extracted using RNeasy minicolumns (Qiagen). On-column DNase digestion was performed to ensure the absence of DNA contamination from the plasmid DNA.

**Determination of trans-Acting NFL mRNA-stabilizing Elements**—The RNA preparation from transfected NSC34 cells containing heterogeneously expressed NFL mRNA was mixed with protein homogenates of 10 mg of lumbar ventral spinal cord from ALS or normal control and incubated at 37 °C for intervals of 0, 60, 120, and 200 min. At each time interval, an aliquot was removed. RNA was then isolated from each aliquot for real time RT-PCR to determine whether the stability of NFL mRNA was modulated by a protein contained within the homogenates derived from control or ALS patients. The same procedure will be repeated with the homogenates that were either boiled for 3 min or treated with proteinase K (Invitrogen; at concentrations of 0, 25, 50, or 100 μg/ml, at 37 °C for 5 min, and the digest stopped with 10 μM phenylmethylsulfonyl fluoride (Sigma)). To determine whether a direct interaction occurred between the NFL mRNA and components of the spinal cord homogenates, a gel retardation assay was performed. Protein homogenates (2 μg) were preincubated with 0.125 μg of heparin/ml (Sigma), in binding buffer containing 5 mM HEPES (pH 7.6), 25 mM KCl, 2 mM MgCl₂, 3% glycerol, 100 mM NaCl, 0.02 mM dithiothreitol, 2 mM GTP, and 1.5 mM ATP at 30 °C for 10 min. ^32P-Labeled probes (10,000 cpm) were added and incubated at 30 °C for 10 min. Samples were then loaded on a 4% polyacrylamide gel and electrophoresed at 100 V in 1× TBE buffer. The gel was dried and exposed to phosphor image screen for overnight.

**Determination of the Importance of 3′-UTR to the Stability Regulation of NFL mRNA**—Heterogeneously expressed NFL-1000 or NFM-CDs mRNA was mixed with homogenates derived from control or ALS patients, incubated *in vitro* for 2 h, followed by RNA re-extraction and real time RT-PCR. The stability difference of NFM-CDs in control and ALS homogenates was compared with that of NFL-1000 mRNA.

**Statistical Methods**—All experiments were performed in triplicate, and the significance among different groups was determined by using a grouped Student’s *t* test (Microsoft Excel).

**RESULTS**

**Steady State mRNA Level of NFs in ALS Lumbar Ventral Spinal Cord Were Higher Than Those in Normal Controls**—RNA was extracted from the ventral lumbar spinal cord of ALS patients (*n* = 6) and normal controls (*n* = 6) with the removal of genomic DNA contamination with DNase I treatment. RNA integrity was assessed, and only those samples without extensive smearing and having 28 S/18 S rRNA ratios of at least 1:1 were subjected to further analysis. Four ALS and three control samples met these criteria. Using the RNase protection assay (Fig. 1A), all three NF subunits mRNA levels were elevated in ALS relative to normal controls. This observation was confirmed using quantitative real time RT-PCR measurements (Fig. 1B). A 2-fold increase of steady state NFL and NFH mRNA levels and a 1.5-fold increase in NFM mRNA level were observed (*p* < 0.05).

Elevated levels of mRNA may result from either an enhanced rate of gene transcription or from the stabilization of the mRNA. In order to assess mRNA stability, we utilized an *in vitro* incubation assay in which heterogeneously expressed...
NFL mRNA was incubated with protein homogenates of either ALS or control lumbar ventral spinal cord. Because cis-acting NFL mRNA-destabilizing elements have been identified for murine NFL (21, 24), we focused on NFL mRNA stability regulation in the following studies. In order to get efficient heterogeneous expression, the NFL-1000 was used as the applicable representative for the full length of NFL mRNA and heterogeneously expressed from pcDNA3.1(+) vectors in transfected NSC34 cells. Total RNAs were extracted from cells after 24 h of transfection. Protein homogenates were prepared from the identical weight of ventral lumbar spinal cord of ALS patients and normal controls. 1/[H9262]l of RNA containing NFL-1000 was mixed with 50/[H9262]l of tissue homogenates of 10 mg of ALS or control tissue and incubated at 37°C for intervals of time to 200 min. At each interval (0, 60, 120, 200 min), residual RNAs were re-extracted from the incubated mixture, and NFL-1000 mRNA levels were measured by real time RT-PCR. By using the T7 promotor sequence on the pcDNA3.1(+) vector as one of the real time PCR primers, the endogenous NFL mRNA from the spinal cord or NSC-34 cells was not recognized, thus allowing a true measure of the variation in NFL-1000 mRNA level due to alterations in its stability. NFL-1000 mRNA was found to be more stable in ALS protein homogenates than in control homogenates (Fig. 2).

In order to study the determinants underlying the differential stability of NFL-1000 mRNA in ALS versus control homogenates, protein homogenates were boiled for 3 min before incubation with NFL-1000 mRNA at 37°C for 2 h. The stability of the NFL-1000 mRNA was significantly increased in control tissue homogenates, with a modest increase observed in boiled ALS tissue homogenates, such that the stability of the NFL-1000 mRNA was comparable between the two boiled homogenates. There was no significant difference in NFL-1000 mRNA stability when exposed to boiled homogenates of either ALS or control tissue (p > 0.05) (Fig. 3A). Homogenates were also incubated with 0, 25, 50, 100 µg/ml protease K before incubation with RNA. Again, the stability of the NFL-1000 mRNA incubated with control spinal cord protein homogenates significantly increased in a dose-dependent fashion, whereas no change was noted following protease K digest of the ALS homogenates. At the higher concentration of protease K, the stability of NFL-1000 was identical when incubated with either ALS or control homogenates (p > 0.05) (Fig. 3B). The results strongly suggested the presence of protein factor(s) that destabilize NFL-1000 mRNA in control protein homogenates, which were absent from the ALS tissues.

The stability differentiation of NFL-1000 mRNA in ALS versus control homogenates was further investigated using blended homogenates in which the ratio of control- to ALS-derived spinal cord homogenate was varied between 100% control- and 100% ALS-derived homogenate. Heterogeneously expressed NFL-1000 mRNA was incubated with admixed spinal cord protein homogenates, and after 2 h, the amount of remaining NFL mRNA was determined using real time RT-PCR. With increasing amounts of ALS-derived homogenate, an increasing stability of NFL mRNA was observed (Fig. 4). This confirmed the presence of a trans-acting NFL mRNA-destabilizing factor unique to control spinal cord homogenates and not evident in ALS spinal cord.

Because regulatory factors may affect mRNA stability in vivo via various mechanisms, including direct binding to cis-acting stability determinants on the mRNA, we undertook gel retard-
dation assays to determine whether the trans-acting elements observed in control spinal cord homogenates might function in this manner. We showed that the control-derived homogenate contained proteins that bound directly to NFL mRNA and that these elements were absent in ALS-derived homogenates (Fig.

FIG. 2. Enhanced NFL-1000 mRNA stability following in vitro incubation with ALS spinal cord homogenates. Heterogeneously expressed NFL-1000 mRNA was incubated with spinal cord homogenates from either control or ALS tissue at 37 °C (0, 60, 120, and 200 min), and the residual level of mRNA was determined by real time RT-PCR. In the presence of control spinal cord homogenates, NFL-1000 mRNA levels declined more rapidly and to a greater extent than that observed in the presence of ALS-derived homogenates.

FIG. 3. Stability differentiation of NFL-1000 mRNA in ALS versus control homogenates is sensitive to heating or protease K digestion. With boiling of the protein homogenate for 3 min prior to in vitro incubation with NFL-1000 mRNA at 37 °C for 2 h, the stability differentiation of NFL-1000 mRNA in ALS versus control homogenates was lost (A). The digestion of the protein homogenates with increasing concentrations of protease K prior to in vitro incubation also increased NFL mRNA stability such that after 50 μg/ml protease K, no difference was observed (p > 0.05) (B).

FIG. 4. Concentration-dependent effect of ALS over control protein homogenates in stabilizing NFL-1000 mRNA. Heterogeneously expressed NFL-1000 mRNA was incubated at 37 °C with 50 μl of admixed spinal cord homogenate, and after 2 h, the amount of NFL mRNA remaining was determined using real time RT-PCR. The ratio of control to ALS-derived spinal cord homogenate was varied between 100% control and 100% ALS-derived homogenate. With increasing amounts of ALS-derived homogenate, an increasing stability of NFL mRNA was observed.

5). We observed NFL mRNA binding protein factors in control homogenates that were absent in ALS-derived homogenates. Combined with the preceding results, these observations confirm the presence of trans-acting NFL mRNA-destabilizing factors in control spinal cord that bind to the NFL mRNA and facilitate the degradation of the mRNA.

Effect of 3′-UTR Deletion on the Stability of NFL-1000 mRNA in ALS and Control Homogenates—The 3′-UTR is important for the stability regulation of mRNA species. For mouse NFL, several stability determinants have been located within this region (21, 24). By deleting the full length of 3′-UTR from NFL-1000 (NFL-CDS), the differential mRNA stability in ALS and control homogenates was abolished from the different level of enhancement in the mRNA stability (Fig. 6), revealing that the 3′-UTR harbors cis-acting instability element(s) for the differential NFL stability between ALS and control spinal cord homogenates.

DISCUSSION

In this paper, we report elevated steady state mRNA levels for all three NF subunits in ALS lumbar ventral spinal cord using RNase protection assay and quantitative real time RT-PCR. Our observations also demonstrate the presence of trans-acting NFL mRNA-destabilizing elements that interact with the 3′-UTR of NFL mRNA and that are either not expressed in...
expression is developmentally regulated (26). These factors, including p190RhoGEF (27), bind to stability determinants on NFL mRNA (24) in ribonucleoprotein complexes (21). A similar complex may also regulate the stability of NFH mRNA (28) and neuron-specific small BC1 RNA (29). Potentially, the stability regulation of NFL mRNA is thus linked to other cellular events in neurons. The recent observation that NFL mRNA steady state levels are suppressed in NSC34 cells that were stably transfected with mutant human copper-zinc superoxide dismutase (SOD1 G93A) (17) is potentially one such example.

Our current results appear to contradict our previous report, in which, when evaluated by in situ hybridization, NFL mRNA levels were found to be selectively reduced in degenerative motor neurons in ALS (15). The difference may not be as contradictory as it seems, if it is taken into consideration that the in situ hybridization assay focused in motor neurons, whereas RNase protection assay and real time PCR checked the expression level of NFs in spinal cord homogenates, which is the mixture of overall neuronal population. The contradiction may actually imply the existence of specific NF expression regulation within motor neurons relative to the rest neuronal population in ALS. NF expression is naturally heterogeneous among neuronal subtypes throughout the nervous system (30–33); thus, it is not without precedent to see different NFs expression patterns and regulation mechanisms for motor neurons relative to the overall neuronal population.

It has now been established that NF overexpression can have protective effects against motor neuron death in a variety of murine models of motor neurons degeneration (34–36). We have also recently reported that, in vitro, a failure to express NFL places motor neurons at a greater risk for oxidatively mediated cell death (37). We are proposing that, under normal physiological stress, trans-acting NFL instability element expression would be suppressed, resulting in elevated levels of NFL protein. In non-motor neuron populations in the ALS spinal cord, this would appear to be the case. However, within the motor neuron pool, this fails to occur, resulting in a selective vulnerability to the disease process. Experiments are currently in progress to address this issue.

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Fig. 5. Gel retardation patterns showing different abundance of factors in ALS and control protein homogenates that bind to NFL-1000 mRNA. Gel retardation assay was carried out as described under “Experimental Procedures.” Binding bands were seen from the incubation of control tissue homogenates with 32P-labeled NFL-1000 RNA probe (lane 2); prior protease K treatment of control tissue homogenates erased these binding bands (lane 3); faint binding bands as compared with those in lane 2 resulted from the incubation of ALS tissue homogenates with 32P-labeled NFL-1000 RNA probe (lane 4); protease K treatment of ALS tissue homogenates erased these faint bands (lane 5); 32P-labeled NFL-1000 probe alone is shown in lane 1.

Fig. 6. 3′-UTR of NFL mRNA is essential for the NFL mRNA stability. NFL-1000 lacking in 3′-UTR was PCR-amplified, cloned into pcDNA3.1 (+) vector, and heterogeneously expressed in transfected NSC34 cells. in vitro incubation was carried out at 37 °C for 2 h in the presence of either ALS or control spinal cord homogenates. Deletion of the 3′-UTR from the NFL-1000 mRNA (NFL-CDS) resulted in a significant increase in the mRNA stability in the presence of control homogenates and abolished the mRNA stability difference between ALS and control homogenates. **, p < 0.01.

ALS spinal cord or nonfunctional. The observations of the gel retardation assay would favor the former possibility.

Our results suggest that the elevated steady state NFL mRNA levels observed in ALS spinal cord resulted from an enhanced stability of NFL mRNA in ALS spinal cord and that this is due to the absence of trans-acting NFL mRNA destabilizing elements. It has been previously shown that NFL steady state mRNA levels are independent of NF gene transcription and that NF mRNA stabilization is the main factor regulating the steady state levels of NFs mRNA in postnatal rat brain (25). The expression of regulatory factors for NF
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