Amyotrophic Lateral Sclerosis: Problems and Prospects

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Amyotrophic lateral sclerosis (ALS) is a lethal degenerative disorder of motoneurons, which may occur concurrently with frontotemporal dementia. Genetic analyses of the ~10% of ALS cases that are dominantly inherited provide insight into ALS pathobiology. Two broad themes are evident. One, prompted by investigations of the SOD1 gene, is that conformational instability of proteins triggers downstream neurotoxic processes. The second, from studies of the TDP43, FUS, and C9orf72 genes, is that perturbations of RNA processing can be highly adverse in motoneurons. Several investigations support the concept that non-neuronal cells (microglia, astroglia, oligodendroglia) participate in the degenerative process in ALS. Recent data also emphasize the importance of molecular events in the axon and distal motoneuron terminals. Only 1 compound, riluzole, is approved by the US Food and Drug Administration for ALS; several therapies are in clinical trials, including 2 mesenchymal stem cell trials. The challenges and unmet needs in ALS emphasize the importance of new research directions: high-throughput sequencing of large DNA sets of familial and sporadic ALS, which will define scores of candidate ALS genes and pathways and facilitate studies of epistasis and epigenetics; infrastructures for candidate gene validation, including in vitro and in vivo modeling; valid biomarkers that elucidate causative molecular events and accelerate clinical trials; and in the long term, methods to identify environmental toxins. The unprecedented intensity of research in ALS and the advent of extraordinary technologies (rapid, inexpensive DNA sequencing; stem cell production from skin-derived fibroblasts; silencing of miscreant mutant genes) bode well for discovery of innovative ALS therapies.

ANN NEUROL 2013;74:309–316

Amyotrophic lateral sclerosis (ALS) is a lethal disorder characterized by subtle onset of focal weakness, typically in the limbs but sometimes in bulbar muscles, which progresses to paralysis of almost all skeletal muscles. There is significant clinicopathological and genetic overlap between ALS and frontotemporal lobar dementia (FTLD). In ALS, death from respiratory paralysis is typically within 5 years. The cellular pathology is focal at onset and spreads in a pattern suggesting successive involvement of contiguous neuronal populations. Death of motoneurons occurs in conjunction with deposition of aggregated proteins in motoneurons and oligodendrocytes, and neuroinflammation. Whereas most cases of ALS are sporadic (SALS), about 10% are inherited, usually dominantly (familial ALS [FALS]). ALS is designated as an orphan disease, with 1 to 2 per 100,000 new cases and a total of ~5 per 100,000 total cases each year. In the United States and the United Kingdom, ALS accounts for about 1 in 500 to 1 in 1,000 adult deaths. Strikingly, this implies that approximately 500,000 people now alive in the United States will die of ALS. These parameters are largely constant across the globe.

Biological Mechanisms in ALS

Diverse genetic approaches have enabled rapid dissection of the complex genetic and cellular events that underlie initiation and progression of motoneuron death in ALS.3 Clinicopathological characterization has helped drive these advances. Overall, progress in understanding ALS can usefully be divided into 2 eras encompassing investigations before and after the discovery of the role of mutations in the 43kd TAR DNA binding protein (TDP-43) in ALS.

ALS in the SOD1 Era: Protein Toxicity

The identification of mutations in the superoxide dismutase 1 (SOD1) gene in 1993 triggered the first major wave of molecular research in ALS.3 SOD1 is a...
ubiquitously expressed protein that catalyzes the detoxification of superoxide. More than 160 mutations in SOD1 associate exclusively with ALS. Nearly all are dominant missense mutations. Transgenic mice overexpressing mutant SOD1 display an ALS-like phenotype and remain a cornerstone of ALS research. Mutant SOD1 toxicity is mediated through several mechanisms, prominently including protein misfolding and oligomerization. Downstream effects of this gain-of-function toxicity include impaired mitochondrial metabolism, axonal degeneration, axonal transport failure, excitotoxicity, proteasomal disruption, and endoplasmic reticulum stress.

These insights notwithstanding, SOD1-based research has not yet led to clinically applicable therapeutic advances. That the mouse model has facilitated the definition of the pathobiology of ALS is clear. However, its very strength, the consistency of mutant SOD1-induced motoneuron degeneration and lethality, is a potential limitation in therapeutic trials because the overwhelming toxicity of SOD1 overexpression creates a nearly insurmountable therapeutic barrier. A corollary view is that using survival as the endpoint in these mouse studies may have vitiated any opportunity to see interventional benefit. Outcomes more directly related to the primary toxicities of mutant SOD1 (eg, motor endplate denervation, axonal transport failure) might be more sensitive measures of therapeutic efficacy in these mice.

Intriguingly, recent studies have implicated wild-type SOD1 toxicity in the genesis of SALS. Wild-type SOD1 in SALS can assume aberrant conformations that resemble those of mutant ALS; misfolded wild-type SOD1 can reproduce some forms of cytotoxicity induced by mutant SOD1. It has also been postulated that wild-type SOD1, once misfolded, can propagate intercellular pathology in a prion-like manner in which there is induction of toxic misfolding in otherwise normal SOD1 upon exposure to misfolded SOD1. An important implication of these studies is that therapies that mitigate the neurotoxicity of mutant SOD1 may also be effective in SALS in the absence of SOD1 gene mutations.

**ALS after TDP-43: RNA Processing**

Following the SOD1 discovery, genetic linkage analyses and candidate gene screening identified rare ALS-related mutations in the atx1n, senataxin, dynactin, and VAPB genes. However, the next major milestone came in 2006 with the identification of TDP-43 inclusions in ALS brains and spinal cords. In 2008, the discovery of TDP-43 gene mutations in ~4% of FALS confirmed a mechanistic link between TDP-43 and ALS pathogenesis. TDP-43 is a ubiquitously expressed DNA/RNA binding protein with diverse roles including gene transcription, RNA splicing, RNA shuttling and translation, and microRNA biogenesis. Although most abundant in the nucleus, TDP-43 shuttles between nuclear and cytoplasmic compartments and is transported along axons. In disease, TDP-43 is ubiquitinated, hyperphosphorylated, and cleaved to form intranuclear and cytosolic aggregates. There is an overall shift in its localization from the nucleus to the cytoplasm and axons. More than 40 dominant missense mutations have been defined in TDP-43, all except 1 in the C-terminal glycine-rich domain. Mutant TDP-43 may have an increased propensity to cleavage and may be more resistant to degradation than wild-type protein. TDP-43 tightly regulates its own expression through negative feedback exerted by binding its own 3′ untranslated region (3′-UTR). Variants in the 3′-UTR have been found in some ALS patients that predict failure of this feedback loop, suggesting that an excess of TDP-43 could contribute to disease.

Evidence from transgenic worms, flies, zebrafish, and rodents indicates that both loss and gain-of-function mechanisms may underlie TDP-43-mediated neurodegeneration. Mouse models of TDP-43–derived ALS have been challenging. Forcing high expression of wild-type or mutant TDP-43 generates variable phenotypes including tremor and death due to gastrointestinal stasis. However, bacterial artificial chromosome transgenic mice, conditional expression approaches, and rat models more accurately recapitulate the human disease course and pathology. Overall, it is clear that TDP-43 plays vital roles both during development and in the survival of adult motoneurons, and that subtle perturbations in TDP-43 levels are poorly tolerated.

In 2009, mutations in FUS/TLS (fused in sarcoma/translocated in liposarcoma) were identified as a cause of around 4% of FALS. FUS shares similar functional domains to TDP-43 and is also predominantly intranuclear. Under neuronal stress, FUS can exit and subsequently re-enter the nucleus. ALS-related FUS mutations may impair nuclear import, leading to loss of nuclear function and intracytosolic aggregation of FUS. Both TDP-43 and FUS possess prion-like domains. Insoluble TDP-43 has been shown to seed TDP-43 aggregation. This has underscored the hypothesis that, as argued for SOD1, propagation of misfolding between mutant and wild-type TDP-43 or FUS proteins may be critical in the pathogenesis, and spatial spreading, of motor neuron dysfunction in ALS.

**C9orf72: Repeat Expansion Pathology**

The concept that defective RNA processing represents an Achilles heel for motor neurons was further strengthened...
by investigations of C9orf72, a gene of unknown function. In 2006, linkage analysis of families with ALS-FTLD implicated a chromosome 9p locus. The same locus was significantly associated with sporadic ALS in genome-wide association studies. In 2011, the genetic culprit was finally identified as a massive intronic hexanucleotide expansion in C9orf72 expansions are the commonest genetic cause of ALS, accounting for up to 50% of FALS in populations of European descent, up to 25% of familial FTLD, and ~5% of apparently sporadic ALS and FTLD; expansions are also seen in ~0.5% of controls. Haplotype analysis indicates that a common European founder appears to be responsible for all cases. The high population frequency of the expansions has led to frenzied work to develop C9orf72 models of disease, which could provide valuable insight into sporadic disease, given that C9orf72 ALS shares 2 major features with SALS: co-occurrence of ALS and FTLD, and TDP-43 pathology, neither of which are seen in SOD1 ALS.

Four mechanistic models of C9orf72-mediated ALS have been proposed. First, haploinsufficiency is suggested by the finding of reduced levels of C9orf72 transcripts in ALS brains. Zebrafish C9orf72 knockdown also causes motoneuron degeneration. Haploinsufficiency might be anticipated if an aberrant conformation of the hexanucleotide-expanded genomic DNA impaired C9orf72 transcription and/or if expanded RNA transcripts were abnormally spliced or translated.

Second, neuropathological studies indicate that the transcribed expansion forms nuclear RNA foci. Biochemical studies and in silico modeling predict that the C9orf72 expansion can form highly stable G-quadruplex structures and hairpin loops. DNA and RNA G-quadruplexes have physiological roles, such as transcriptional and translational regulation, RNA transport, and telomere stability, which are highly relevant to neuronal biology and ageing and could be disrupted if pathological C9orf72 expansions sequester the proteins to which physiological G-quadruplexes normally bind.

Third, the hexanucleotide repeat may promiscuously bind and sequester transcription factors, by analogy with sequestration of the transcription factor muscleblind by tri- and tetranucleotide expansions in myotonic dystrophy types 1 (DM1) and 2, respectively. Multiple investigators are avidly identifying RNA binding proteins and other factors that may be sequestered in C9orf72 foci.

The fourth potential mechanism for neurotoxicity of C9orf72 expansions is a form of illegitimate protein translation termed repeat-associated, non-ATG (RAN) translation. This mechanism was first identified in DM1 and spinocerebellar ataxia 8. Two groups have recently demonstrated that RAN translation arises from C9orf72 expansions yielding RAN polypeptides that are sequestered in insoluble aggregates. It remains to be established whether these proteins are cytotoxic.

**Axon Biology in ALS and Modifiers of the ALS Phenotype**

ALS research has generally focused on pathological processes affecting the cell body, but it is clear that critical events in ALS pathogenesis implicate the neuronal periphery. The earliest pathological changes in ALS appear to occur in axons, dendrites, and synapses. Pathological studies indicate early peripheral denervation before ventral nerve root or cell body degeneration. In the central nervous system, spinal cords from ALS patients demonstrate distal corticospinal tract inflammatory changes and giant axonal swellings suggesting early distal axonal degeneration, findings supported by antemortem diffusion tensor imaging. TDP-43 aggregates form early within motor axons. Similarly, mutant-SOD1 mice demonstrate presymptomatic muscle denervation and terminal axonal degeneration before anterior horn cell loss.

Genetic findings also strongly implicate the axon in ALS. For example, FALS can be caused in some pedigrees by missense mutations in the gene profilin-1 (PFN1), which mediates actin polymerization. PFN1 mutations impair axonal extension and growth cone elongation, leading to an adult onset, predominantly lower motor neuron deterioration.

Genetic variants that reduce function of EphA4, an ephrin receptor, have been shown to be beneficial in ALS. Lower expression of EphA4 in peripheral lymphocytes correlated with delayed onset of ALS and a more protracted disease course. Among many functions, EphA4 signals cessation of axonal extension of distal neuronal terminals during synaptogenesis. These data strongly point to EphA4 as an important target for therapy development in ALS.

That axonal transport may be implicated in ALS has long been intimated on general grounds (ie, that normal transport is indispensable for a motor neuron, whose axon may be up to 20,000 X larger than the cell body and extend to 1m in length). A role for altered axonal transport in ALS was also suggested by early studies of isolated human motor nerve axons from ALS cases. Recent evidence shows that mutant SOD1 impairs axonal transport in transgenic ALS mice and in isolated axoplasm from squid giant axons.

Given the above, it is intriguing that we now know that axons have a self-destruct mechanism, independent of apoptosis, which can be significantly delayed in vivo given the correct molecular environment. The slow
Wallerian degeneration (WldS) mouse first demonstrated that axons can survive independent of the cell body for weeks after nerve transection.\(^65,66\) Recent discoveries that loss-of-function mutations in the \textit{Sarm1} and \textit{highwire} genes have similarly potent axon-protective effects clearly suggest that axon degeneration is an active process.\(^67,68\) Elucidating the molecular cascade responsible for axon killing holds promise for eventually identifying therapeutic targets for ALS.

In addition to \textit{EphA4}, other genes have been reported to modify the ALS phenotype (Table 1). Genetic studies implicate \textit{UNC13A}\(^38\) (which is also a susceptibility factor) and \textit{KIFAP3}\(^69\) as modifiers of survival in some populations, whereas the single nucleotide polymorphism rs3011225 modifies age of onset,\(^70\) as does the P413L allele of the \textit{chromogranin B} gene.\(^71\) Elegant studies by Henderson and colleagues indicate that the metalloprotease \textit{MMP9} is a determinant of ALS susceptibility; it is expressed more abundantly in motor neurons that are ALS susceptible (e.g., lumbosacral motoneurons) than in those that are ALS resistant (e.g., oculomotor and sphincteric motoneurons; C. Henderson, personal communication).

That non-neuronal cells can modulate the phenotype in animal models of ALS is now clear; mutant \textit{SOD1} in microglia and astrocytes accelerates the disease course after onset, and mutant \textit{SOD1} in oligodendroglial precursor cells hastens death in transgenic mice.\(^72\--\,75\) Similar findings were recently reported for astrocytes in a TDP-43 rat.\(^76\) The mechanisms for these non–cell-autonomous influences are not well defined, although it is intriguing that oligodendrocytes are known to provide significant metabolic support to axons through lactate transport.\(^77\)

**Currently Available Therapies**

The only therapy approved by the US Food and Drug Administration for ALS is riluzole, a small molecule with multiple mechanisms of action, including inhibition of excessive motoneuron excitation. Although modest, the benefit of riluzole (an increase in survival of perhaps 10–20\%) has been seen in multiple studies.\(^78\) Symptomatic/palliative measures are essential for ALS patients, not only at the end-stage of ALS, but during the entire course of the disease to maximize quality of life. Early placement of feeding tubes and the use of noninvasive, positive pressure respiratory assistance devices are important measures that improve quality of life and can prolong survival.

**Therapeutic Pipeline in 2013**

Fortunately, there are several treatment modalities in the ALS pipeline. Small molecules in trial, or soon to be in trial, include Neuraltus’s NP001 (modifies activation of macrophages), Cytokinetix’s CK2017357 (activates troponin to enhance muscle contractility), GlaxoSmithKline’s ozanezumab (blocks inhibition of axonal outgrowth), University of Miami’s arimoclomol (tested only in SOD1-mediated ALS; improves cellular stress responses), mexiletine (reduces excessive neuronal firing, in trial via the Northeast ALS Consortium), and rasagiline (whose neuroprotective properties are being tested in ALS at the University of Kansas).

At least 2 trials of stem cell therapy are underway. A consortium involving the University of Massachusetts Medical School, Massachusetts General Hospital, Mayo

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**TABLE 1: Genes Implicated in Familial and Sporadic ALS**

<table>
<thead>
<tr>
<th>ALS Type</th>
<th>Locus</th>
<th>Familial, n = 24</th>
<th>Sporadic, n = 6</th>
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<td>21q</td>
<td>SOD1</td>
<td>ELP3</td>
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<td>KIFAP3</td>
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Thirty two genes (26 in FALS, 6 in SALS). ALS = amyotrophic lateral sclerosis.
Clinic, and Brainstorm, Inc (Jerusalem, Israel) have devised a study of autologous, marrow-derived mesenchymal stem cells prepared ex vivo using a proprietary protocol. A team of investigators from Emory University and the University of Michigan are testing fetal human stem cells (produced by Neural Stem) administered via direct intraspinal injection to individuals who are receiving a full immunosuppression protocol as used in organ transplantation. Of considerable interest is the observation that 1 individual with early ALS treated with this Emory protocol showed clear improvement over several months, followed by regression. To discern whether this unprecedented response was a consequence of the intraspinal stem cells or the immunosuppression protocol, a team from Emory, Massachusetts General Hospital, and University of Massachusetts Medical School are performing a small study of the immunosuppression regimen alone.

A third exciting treatment modality that has been pioneered by Isis Pharmaceuticals jointly with R. Smith, T. Miller, and D. Cleveland is the use of modified antisense oligonucleotides to silence the SOD1 gene as a therapy for SOD1-mediated ALS. This treatment showed benefit in transgenic ALS rats; an initial pilot, single-dose study in humans found it safe. Further studies are planned to determine the efficacy of SOD1 gene silencing. Alternate approaches using inhibitory RNA, delivered either as a standalone drug, or via viral-mediated delivery to the central nervous system, are under development in several laboratories.

Unmet Needs
In our view, several challenges loom in the quest for meaningful ALS therapies; these suggest new, potentially promising directions for ALS research. A partial list of the central questions includes the following:

1. The daunting heterogeneity of ALS is highlighted by the growing list of genes linked to ALS. Can we find the resources to support research for all genetic causes of ALS, or should we prioritize certain genes for study, and if so how should we prioritize? Will we have to tailor treatments according to individual genotypes, or does a final common pathway link these different forms of ALS? Furthermore, to what degree will studies of genetic defects in FALS illuminate the molecular pathology of SALS?
2. RNA processing is emerging as a common theme in neurodegeneration, but what is the correct approach to this complex field? Our current understanding of TDP-43 and C9orf72 suggests that both loss and gain of function contribute to disease. Is it correct to compare RNA transcripts between transgenic rodent models and human postmortem tissues? There remain major differences in the way different laboratories conduct these studies, with divergent opinions on many stages of the investigations, from clinical phenotyping and accurate genotyping of C9orf72 expansions, to postmortem brain and spinal cord sampling and RNA sequencing approaches.
3. Can we develop biomarkers that meaningfully gauge disease activity and therefore allow trials to be conducted more efficiently?
4. Can we improve on trial design using newer concepts that facilitate earlier detection of failure and accept more risk?
5. Fiscal pressures together with the increasing need for expensive technologies are already forcing us to collaborate in new ways. Can new models for collaborations with pharma beginning in early discovery phases be mutually beneficial, using academics to derisk the lengthy phases of basic investigation while leveraging pharma to facilitate drug screening and the costly process of dealing with regulatory hurdles? As a corollary, can we more fully draw upon the efforts of the National Institutes of Health? The National Institute of Neurological Diseases and Stroke has now created state-of-the-art facilities for drug testing and development that embrace all the expertise required to move from a high-throughput assay to a compound for clinical trial. This centralized facility offers the community of academic neuroscientists economies of scale that are otherwise very difficult to achieve.

New Research Directions
In our view, the unmet needs described above and other considerations point to several important research directions.

Enhanced Candidate Gene Identification
Dramatic improvements in nucleotide sequencing and drops in sequencing costs will permit large-scale exome/genome resequencing (Fig 1). At least 3 projects are currently underway that purport to fully sequence the exomes of >1,000 ALS cases. This will generate hundreds of new candidate ALS genes, each pointing to potential pathways that are targets for therapy development.

New Wave Genetics
A corollary to (1) will be the opportunity to perform analyses of epistatic interactions between multiple genetic variants. Data suggest the possibility that seemingly
The rate of discovery of genes whose mutations cause amyotrophic lateral sclerosis has accelerated, reflecting in part the impact of accessible high-throughput DNA sequencing technology. The total number is depicted on the y-axis versus year on the x-axis. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

**ACKNOWLEDGMENT**

R.B.H. is supported by NIH grants 5RC2NS070342-02, 5R01NS065847-04, 5R01NS67206-04, 1RO1NS073873-03, 5R01NS079836-02, 1R01FD004127-01, ALSA #2003, BiogenIdec, Project ALS, Angel Fund, and ALS Therapy Alliance.

**POTENTIAL CONFLICTS OF INTEREST**

R.B.H.: science advisory board membership, Biogen Idec; consultancy, Kirac Foundation; grants/grants pending, DOD; patents, patented SOD1 as an ALS gene in 1993; royalties, McGraw-Hill; stock/stock options, AviTxl travel expenses, UMass Medical School.

**REFERENCES**


