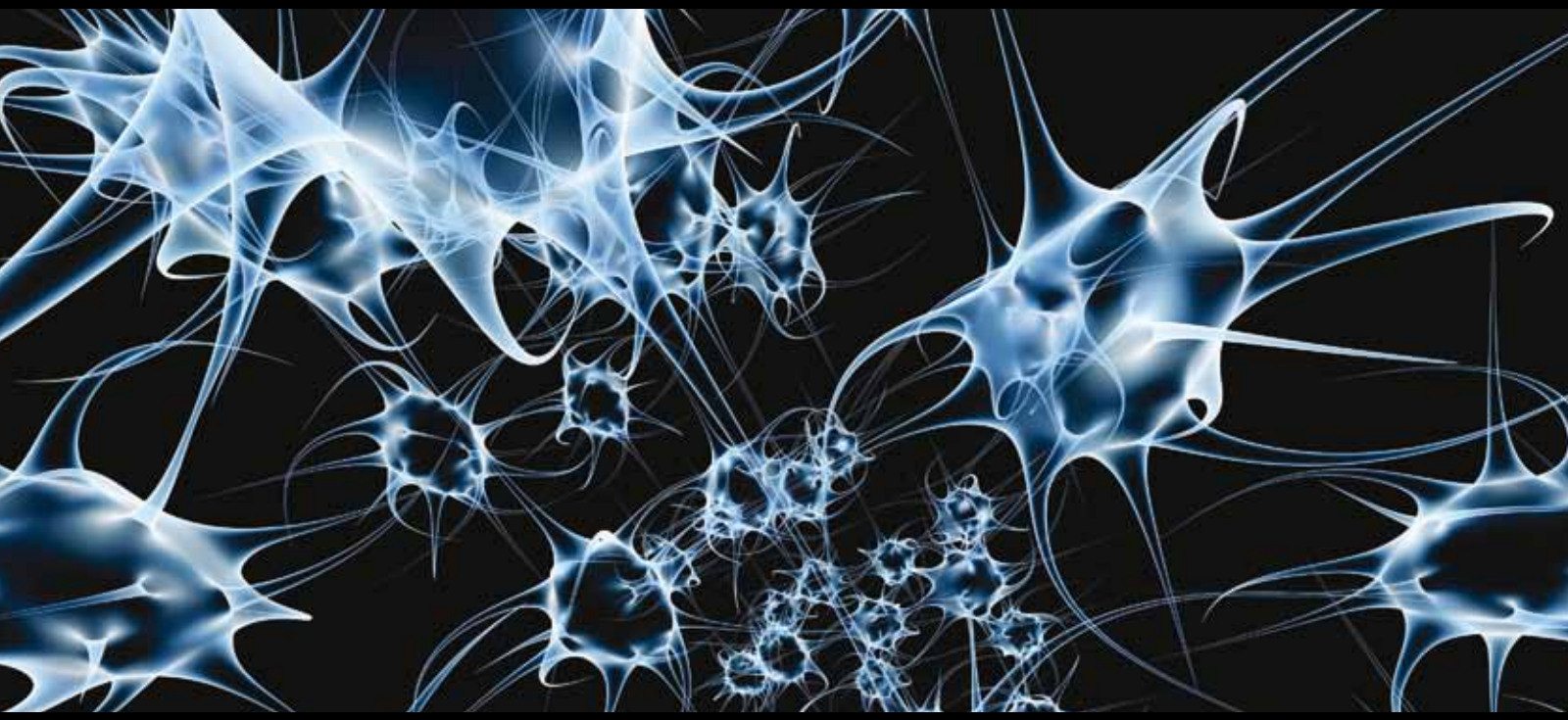
Neurology Research International

Amyotrophic Lateral Sclerosis and Novel Therapeutic Strategies

Guest Editors: Brett Morrison, Kenneth Hensley, Erik P. Pioro, Susanne Petri, and Mahmoud Kiaei



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Editorial Board

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Editorial

Amyotrophic Lateral Sclerosis and Novel Therapeutic Strategies

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Research and key discoveries in the field of amyotrophic lateral sclerosis (ALS) have exponentially increased since the announcement in 1993 of the first ALS-causing mutations in the gene for the well-studied antioxidant enzyme Cu,Zn superoxide dismutase (SOD1). The etiology of sporadic ALS largely remains unknown and the mechanisms of motor neuron degeneration are still being investigated. The only FDA drug approved for the treatment of ALS is riluzole with only modest benefit to patients, but multiple drugs are currently in the development pipeline and in human ALS clinical trials. In this special issue, K. Venkova-Hristova et al. thoroughly reviewed studies of experimental therapeutics in animal models of ALS, including specific examples of those that proceeded into human clinical trials. Since none of the ALS human clinical trials succeeded despite positive results in animal models, the question of "why" has been on everyone's mind, with efforts to develop superior alternatives. This review discusses the potential reasons for the universal failure of preclinical successes to translate into positive clinical outcomes. It has become obvious that the lack of understanding of the precise mechanisms of motor neuron degeneration presents a major obstacle in the development of effective therapy for ALS. This review discusses the details of several major pathogenic pathways in ALS and the efforts of various groups to block one toxic pathway at a time, ranging from oxidative stress to protein aggregation. The authors discuss the pros and cons of ALS models and propose simultaneous targeting of multiple pathways as a more efficient strategy, due to the multifactorial nature of ALS pathology.

Several contributions in this special issue identify cellular physiological pathways that potentially exert neuroprotective effects if rationally manipulated for pharmacological mitigation against ALS. S. Petri et al., therefore, provide a review of a pivotal system called the Nrf2/ARE pathway which normally controls expression of "phase II genes," a gene set relating to antioxidant and electrophilic detoxification, anti-inflammatory, and promitochondrial enzymes. Normal or induced Nrf2/ARE activity reduces neural vulnerability to oxidative stress, inflammatory factors, and mitochondrial dysfunction, but evidence suggests that the regulation of Nrf2/ARE is suboptimal in ALS. Hence, Nrf2 signaling pathway activation is a mechanism against at least three major and interrelated toxic pathways (oxidative stress, neuroinflammation, and mitochondrial dysfunction) in neurodegenerative diseases such as ALS, and it is an attractive and novel target. Accordingly, S. Petri and colleagues examine evidence that two blood-brain barrier penetrable CDDOs (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid), derivatives of the triterpenoid, an oleanic acid compound, function as remarkably potent Nrf2/ARE activators with proof of principal that slowed disease in ALS preclinical models. Further studies are required to investigate the mechanism and the pivotal role of Nrf2/ARE signaling pathway for neuroprotection in the spinal cord, specifically in motor neurons.

A review by E. Y. Achi and S. A. Rudnicki outlines the current state of knowledge about frontotemporal dysfunction and dementia (FTD) in ALS. Increasingly acknowledged in the current ALS literature, the incidence of FTD ranges

from 7 to 22%, depending on the screening tests used. The authors describe the overlapping pathology and genetics seen in ALS and FTD, including the recently described C9orf72 mutations that appear to be a common cause of both familial ALS and FTD. They describe several clinical tests for FTD, while acknowledging that there is no current consensus on the best test to use. This review makes it clear that FTD is an important component of ALS that impacts the quality of life of patients and their caregivers. To date, there have been no clinical trials evaluating treatments of FTD in ALS patients, but based on FTD literature, the authors suggest selective serotonin reuptake inhibitors. It is unclear whether ALS patients with FTD will respond similarly to FTD patients in general, and therefore, there is little doubt that future clinical trials should be undertaken to evaluate the effectiveness of specific FTD treatments in ALS patients. In addition, the prevalence of FTD in ALS patients suggests that future clinical trials in ALS should incorporate measures of cognitive function, in addition to mortality, ALSFRS, and typical motor endpoints.

A major toxic pathway that has been extensively studied in ALS is neuroinflammation. Substantial evidence supports its detrimental effect on neurons in several neurodegenerative diseases. In this special issue, C. A. Lewis et al. discuss the neuroinflammatory responses in ALS and describe the cell and molecular components involved in neuroinflammation. The authors discuss the potential dual role of inflammation on motor neurons as both neuroprotective in the early stages of disease and neurotoxic later in disease and the challenges this presents when devising specific treatments for ALS. The authors specifically discuss a trophic effect of microglia that is induced by T cells, which could be a novel mechanism to alter disease progression by manipulating inflammatory pathways.

The review by J. P. Crow et al. focuses on D-serine, a coagonist of the NMDA receptor which increases glutamate affinity via binding to the NR1 subunit and can, therefore, contribute to excitotoxic cell death, a major pathogenic mechanism in ALS. Based on findings of elevated D-serine level in the spinal cord of ALS transgenic mice and a delay in disease progression in ALS mice lacking the D-serine-producing enzyme serine racemase, it may represent an interesting therapeutic target. J. P. Crow et al. summarize current knowledge on D-serine regulation in vivo, in particular regarding the complex bidirectional function of serine racemase which can both produce and degrade serine. They review the literature on serine racemase knockout in in vivo models with specific focus on their previous and ongoing studies on the extent and function of serine expression in different tissues and cell types of transgenic ALS mice. Ultimately, the paper highlights the crucial importance of using appropriate methodology for reliable quantification of D-serine in tissue. Based on their own results as well as the current literature, they suggest an important role of D-serine in nonautonomous cell death of motor neurons mediated by glial cells.

In the review by D. Krakora and colleagues, the authors examine the evidence for a "dying back" motor neuron injury in ALS. There have been several studies in the SOD1 transgenic mouse models of ALS suggesting that alterations in the muscles and motor axons occur before apparent pathology in motor neuron cell bodies. These pathologic studies are supported by several genetic and pharmacologic manipulations that were able to protect cell bodies while having no impact on the progression of weakness or mortality due to continued axon degeneration and muscle denervation. The authors also discuss studies in these models that found prolongation of lifespan following muscle-specific treatments. Taken together, these studies suggest a role for the peripheral axon, neuromuscular junction, and possibly muscle in the pathogenesis of ALS. The authors discuss several mechanisms that may be involved in this "dying back" motor neuron injury.

Further exploration of these mechanisms is important since effective treatments will need to address toxicity in both the soma as well as the distal axon. In their review, the authors describe several putative treatments, including delivery of growth factors to muscles, either directly or through mesenchymal stem cells, increasing energy supply to potentially hypermetabolic muscle, or exercise programs. Though several of these treatments have been shown to ameliorate the phenotype of SOD1 transgenic mice, they will likely need to be combined with treatments that protect motor neuron somata in order to produce sustained improvement in ALS patients.

Perhaps the tightest bottleneck in translational ALS research now is selection of candidate therapeutics for clinical trials and conduct of the same. T. D. Levine et al. report a biomarker study of 27 ALS subjects taking the current "best practice" drug, riluzole. These subjects were randomized to receive pioglitazone (a PPAR agonist) and tretinoin (a retinoid), or placebo, for six months. Although the experimental treatment did not significantly slow functional decline on the standard ALSFRS-R scale, cerebrospinal tau concentrations decreased in the treatment arm compared to placebo. Interestingly, these researchers also found that phosphorylated neurofilament heavy chain (pNF-H) at baseline predicted faster rate of clinical decline. Taken together these findings suggest that strategies aimed at stabilizing the neuronal cytoskeleton might offer hope for ALS patients in the future and provide tantalizing clues about promising biomarkers to empower future clinical trials.

On a related biomarker topic, K. Kollewe et al. provide a thorough review of current magnetic resonance imaging (MRI) and functional MRI (fMRI) approaches to detect alterations of cortical networks in ALS during activated and task-free resting-state investigations. It is with the latter approach of fMRI-based resting state connectivity that the authors have contributed the original studies in ALS patients and identified disturbances not only in the Sensorimotor Network, as may be expected for a motor neuron degeneration, but also in the Default Mode Network, which interconnects brain regions involved in behavioral and cognitive functions. This is of relevance since ALS is now recognized to cause degeneration not only in motor but also in extramotor regions. They explain the advantages and utility of diffusion-tensor imaging (DTI), which is one of

the most promising current MRI method to detect ALS-related changes in the white matter.

Altogether, papers in this special issue provide a series of comprehensive discussions and updates on the current status of research on pathogenic mechanisms in ALS and potential future therapeutics that may result from this research. Though our treatment options for ALS are currently very limited, advances in understanding the pathogenic mechanisms raise the hope for a more optimistic future for patients diagnosed with ALS.

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Erratum

Erratum to "Progress in Therapy Development for Amyotrophic Lateral Sclerosis"

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Please consider the following changes in Table 1.

Table 1: Mendelian and non-Mendelian loci known to cause FALS or confer risk for SALS. Information in this table reproduces content in Tables 7 and 8 from Lill and Bertram, 2011 ([2], and databases therein). Polymorphisms in the VEGF promoter that were originally associated with increased ALS risk have not been confirmed in subsequent studies but may act as modifiers of disease onset or progression in subsets of ALS cases [58].

Mendelian genes for heritable ALS (FALS)				
Gene	Location	Heritance	Protein	Pathway or effect
ANG	14q11.2	Dominant	Angiogenin	rRNA transcription
ALS2	2q33	Recessive	Alsin	Endosome/membrane trafficking
C9ORF72	9p21.2	Dominant	Uncharacterized	Altered C9ORF72 RNA splicing, formation of nuclear RNA foci
FIG4	6q21	Recessive	FIG4 homolog	Endosomal trafficking
FUS	16p11.2	Both	Fused in sarcoma	Altered RNA processing, formation of inclusion bodies
OPTN	10p13	Both	Optineurin	Golgi maintenance, membrane trafficking and exocytosis, formation of inclusion bodies
SETX	9q34.12	Dominant	Senataxin	DNA and RNA processing
SOD1	21q22.11	Almost always	Superoxide dismutase-1	Protein aggregation, possible gains of redox function, impaired axonal transport
SPG11	15q21.2	Recessive	spatacsin	Impaired axonal transport
TARDPB	1p36.22	Dominant	TAR DNA binding	RNA processing, formation of protein inclusion bodies
UBQLN2	Xp11.231 dominant	X-linked	Ubiquilin-2	Proteosomal protein degradation, inclusion body formation
VAPB	20q13.32	Dominant	Vesicle-associated membrane protein VAMP	Vesicle trafficking
VCP	9p13.3	Dominant	Valosin-containing protein	Proteosomal degradation, endosomal trafficking, vesicle sorting
PFN1	17p13.3	Dominant	Profilin-1	Actin polymerization regulator
Susceptibility loci for sporadic ALS (SALS)				
Gene	Location	Polymorphism	Protein	OR (95% CI)
GWA_9p21.2	9p21.2	rs2814707	Unknown	1.25 (1.19–1.32)
UNC13A	19p13.1	rs12608932 homolog	Unc-13 vesicle protein	1.18 (1.13–1.24)
ATXN2	12q24.12	Poly-Q	Ataxin-2	N.a.

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Review Article

Nrf2/ARE Signaling Pathway: Key Mediator in Oxidative Stress and Potential Therapeutic Target in ALS

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Nrf2 (nuclear erythroid 2-related factor 2) is a basic region leucine-zipper transcription factor which binds to the antioxidant response element (ARE) and thereby regulates the expression of a large battery of genes involved in the cellular antioxidant and anti-inflammatory defence as well as mitochondrial protection. As oxidative stress, inflammation and mitochondrial dysfunctions have been identified as important pathomechanisms in amyotrophic lateral sclerosis (ALS), this signaling cascade has gained interest both with respect to ALS pathogenesis and therapy. Nrf2 and Keap1 expressions are reduced in motor neurons in postmortem ALS tissue. Nrf2-activating compounds have shown therapeutic efficacy in the ALS mouse model and other neurodegenerative disease models. Alterations in Nrf2 and Keap1 expression and dysregulation of the Nrf2/ARE signalling program could contribute to the chronic motor neuron degeneration in ALS and other neurodegenerative diseases. Therefore, Nrf2 emerges as a key neuroprotective molecule in neurodegenerative diseases. Our recent studies strongly support that the Nrf2/ARE signalling pathway is an important mediator of neuroprotection and therefore represents a promising target for development of novel therapies against ALS, Parkinson's disease (PD), Huntington's disease (HD), and Alzheimer's disease (AD).

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease. It causes degeneration of motor neurons in the primary motor cortex, brain stem, and spinal cord which subsequently leads to rapidly progressive paralysis of skeletal muscles and ultimately to death due to respiratory failure, usually within 3 to 5 years after disease onset.

The majority of ALS cases are acquired spontaneously (sporadic ALS; sALS), while only 10%–15% of ALS cases are inherited (familial ALS; fALS) [1]. Recent breakthroughs in genetics have enlarged the number of known mutations causing fALS, among them mutations in genes coding for superoxide dismutase 1 (SOD1), TAR DNA-binding protein (TARDP), fused-in sarcoma/translocation in liposarcoma (FUS/TLS), and, most recently, a repeat expansion of C9orf72, the cause of chromosome 9-linked ALS and frontotemporal lobar dementia (FTLD) [2]. Mutation in the gene for profilin1 (PFN1) was recently reported to be associated with several fALS families. Four mutations (C71G, M114T, E117G, and G118V) were identified in several families [3]. The etiology of sALS is less clear and must be considered multifactorial and polygenic in the majority of cases.

Several interdependent and interacting mechanisms have been shown to induce motor neuron damage in both fALS and sALS: excitotoxicity, aberrant RNA processing, altered axonal transport, protein aggregation, mitochondrial dysfunction, toxicity of nonneuronal (glial) cells and oxidative stress [4]. Even if it is unclear whether oxidative stress is a primary or a secondary cause of neurodegeneration in ALS, data from both human tissue and studies in transgenic animal models suggest that it is a major contributory factor leading to chronic motor neuron death. In mutant superoxide dismutase 1 (SOD1) ALS-mouse models [5] as well as in human familial and sporadic ALS, markers of oxidative damage of proteins, lipids and DNA are elevated in brain and spinal cord [6–9]. A larger number of nonspecific antioxidants

(e.g., creatine, coenzyme Q 10, vitamine E, N-acetyl-cysteine, and others) have been tested in transgenic mouse models for ALS and were efficient regarding survival, disease progression and motor neuron loss in the spinal cord. Clinical trials in ALS patients have not yet been able to prove efficacy of antioxidant treatment in the clinical setting, but ongoing trials such as the dexpramipexole study still use compounds with antioxidant potential which underlines the importance and acceptance of this therapeutic strategy [10].

Inflammation and mitochondrial dysfunction are considered as major pathomechanisms in motor neuron degeneration. These processes both have strong interconnections to oxidative stress cascades. These three pathways are overlapping and interconnected and appear to form a vicious cycle that each could be an initiator as well as a mediator of motor neuron death (Figure 1).

Mitochondrial injury is known to result in an excess of oxygen radicals. Depending on the cell type whose mitochondria are injured, that may trigger different sets of reactions. For instance, glial cells with injured mitochondria can produce proinflammatory molecules that could be toxic to neurons and other cells nearby. These types of inflammatory reactions are stressors for neurons and will be adding to oxidative stress that neurons are subject to. All these in turn will be damaging to mitochondria and other cellular organelles which may lead to further inflammatory reactions in neurons and other cells in CNS (see review by Sun et al. [11]).

A key molecule regulating the cellular antioxidant response is the basic region leucine-zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2). In basal conditions, Nrf2 is bound to the endogenous inhibitor Kelch-like ECH associated protein1 (Keap1). Once activated, it translocates to the nucleus of the cell where it forms heterodimers with other transcription factors such as c-Jun and small Maf proteins (G/F/K), binds to the antioxidant response element (ARE), a regulatory enhancer region within gene promoters. c-Jun is then supposed to act mainly as transcriptional activator while the small Mafs as well as c-Myc inactivate gene transcription after Nrf2 binding [12]. Nrf2-ARE binding regulates the expression of more than 200 genes involved in the cellular antioxidant and anti-inflammatory defense such as phase 2 detoxification enzymes (NAD(P)H quinone oxyreductase, glutathione), enzymes which are necessary for glutathione biosynthesis, extracellular superoxide dismutase, glutamate-6-phosphate-dehydrogenase, heat shock proteins and ferritin, furthermore pro- und anti-inflammatory enzymes such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and heme oxygenase-1 (HO-1) [13–15]. Nrf2 has also been reported to regulate the expression of genes promoting mitochondrial biogenesis such as mitochondrial transcription factors (TFAM) and is therefore directly involved in mitochondrial preservation [16].

Induction of Nrf2 by compounds of different chemical classes was shown to be directly correlated to the inhibition of proinflammatory responses (Cox-2 and iNOS expression), but the anti-inflammatory effects of these molecules are only partially Nrf2-dependent and the exact relation between

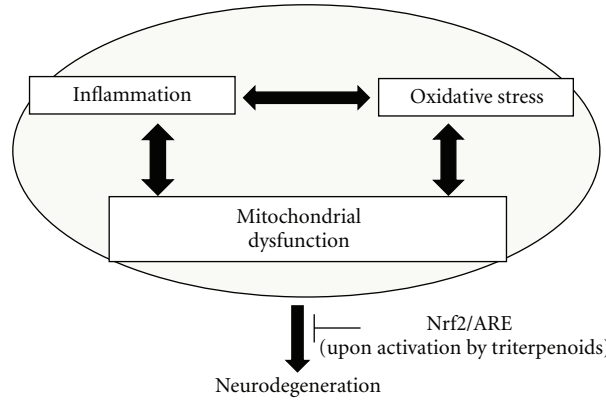

Figure 1: Three major toxic pathways that contribute to neurodegeneration. Nrf2/ARE signaling activation via triterpenoids can reduce oxidative damage, lessen inflammation, and restore mitochondria resulting in more robust motor neurons able to defend themselves against toxic insults.

Nrf2-induction and anti-inflammatory properties remains to be clarified [17, 18]. Probably by regulation of intracellular glutathione content, Nrf2 further has direct cytoprotective effects via the inhibition of Fas-mediated apoptotic pathways [15].

The endogenous inhibitor of Nrf2 is the actin-bound cytoskeletal zinc metalloprotein Keap1. Several models of interaction of Keap1 and Nrf2 have been proposed [18]. Modifications of cysteine residues of Keap1 apparently alter the interaction of Keap1 with Nrf2 and lead to its relocation to the cytoplasm where it is subsequently degraded by the ubiquitin-proteasome system [18–22]. Decreased Keap-Nrf2-binding (via oxidation of sulfhydryl groups or phosphorylation) results in intranuclear shuttling of Nrf2 and subsequent transcription of ARE-driven genes [13, 23–25]. Keap1 and Nrf2 therefore constitute a cellular sensor for damage caused by free oxygen radicals [21]. Some studies reported constant shuttling of Keap1 between the nucleus and the cytoplasm under physiological conditions. Karyopherin-6 (KPNA6) has been identified as a protein which facilitates nuclear import and attenuates Nrf2 signaling [26]. Furthermore, KPNA6 accelerates the clearance of Nrf2 protein from the nucleus, and even promotes the restoration of the Nrf2 protein to basal levels. These findings suggest that KPNA6-mediated Keap1 nuclear import plays an essential role in modulating the Nrf2-dependent antioxidant response and maintaining cellular redox homeostasis.

In addition, it has also been shown that Nrf2 protein stability can be regulated in a Keap1-independent manner by phosphorylation via glycogen synthase kinase-3 (GSK-3β) [27] and that Nrf2 function can further be modified by regulation of its transcription [28].

There are multiple factors that activate Nrf2 in any given cell, for example, environmental stressors such as cigarette smoke, infection, oxidative stress, or inflammation. Several reports have shown that disruption of Nrf2 impairs the induction of the Nrf2/ARE pathway leading to exacerbation of oxidative stress, inflammation, and mitochondrial

dysfunction (reviewed by [40]). Restorative effects of Nrf2 were reported in mice exposed to cigarette smoke [41].

2. Nrf2 in Other Neurodegenerative Diseases

Nrf2 protein expression has already been studied in postmortem brain tissue from patients with neurodegenerative diseases such as Alzheimer's (AD), Lewy body variant of AD (LBVAD), and Parkinson's disease (PD) [25]. Cytoplasmic localization of Nrf2 was found in AD and LBVAD hippocampi and entorhinal cortex. It was therefore concluded that in these diseases nuclear translocation of Nrf2 is impaired and that dysfunction in the Nrf2 pathway leads to decreased cellular defense against oxidative stress [25]. In the PD cases, in contrast, nuclear Nrf2 levels in the substantia nigra were increased which could be interpreted as an appropriate neuronal response to oxidative stimuli [25].

3. Nrf2-Cascade in ALS In Vitro Animal Models

Neuronal and astroglial primary cultures from Nrf2 knockout mice are more vulnerable to oxidative stress than wild-type cells, while overexpression of Nrf2 increases resistance against oxidative and excitotoxic stimuli [14, 42, 43].

Several studies have attempted to clarify the role of the Nrf2-pathway in SOD1-G93A transgenic ALS animal and in vitro models: reduced Nrf2-expression has been described in primary embryonic motor neuron cultures derived from SOD1-G93A transgenic mice. These SOD1-G93A-transgenic motor neurons were more sensitive to apoptosis induced by addition of nerve growth factor (NGF) [44]. In another in vitro model of ALS, motor neuron-like Nsc34 cells which were stably transfected with mutant SOD1, downregulation of genes regulated by Nrf2 was found by microarray analysis. The authors therefore suggested that pharmacological stimulation of the Nrf2-pathway could be a novel therapeutic approach in ALS [45]. Opposed to these in vitro studies in motor neurons, another group detected upregulated Nrf2-expression in astrocytes. This increased Nrf2-expression which was interpreted as reactive attempt to prevent cell death was already observed at disease onset and persisted throughout disease progression [46]. Activation of Nrf2-ARE signaling in ALS mice was studied by cross-breeding ALS-transgenic mice with ARE reporter mice. Thereby, ARE activation could be directly measured via induction of ARE driven hPAP (human placental alkaline phosphatase) activity. In this model, early and intense Nrf2-activation was seen in skeletal muscles. Comparably less activation of Nrf2 was seen in spinal cord motor neurons and astrocytes after symptom onset [47]. The authors therefore concluded that the earliest pathological events in mutant SOD1-associated ALS occur in muscle tissue and that they progress in a retrograde manner during the disease course.

4. Nrf2 in Human Sporadic ALS

While these mutant SOD1 models reproduce pathophysiology of familial ALS, no ideal model of sporadic ALS exists so far. We have recently investigated mRNA and protein expression of the transcription factor Nrf2 and its endogenous inhibitor Keap1 in postmortem brain and spinal cord specimens of sporadic ALS patients and controls. Reduced neuronal mRNA expression levels of Nrf2 were seen in motor neurons of the primary motor cortex and the ventral horn. Immunohistochemistry and Western blot experiments revealed correspondingly decreased Nrf2 protein expression. Astrocytosis was increased in ALS tissues. By colocalized immunohistochemistry, we observed some astrocytic localization of Nrf2 but an overall decreased Nrf2 protein expression as compared to control specimens. For the endogenous inhibitor Keap1, a slight but not significant increase in mRNA expression was observed in the motor cortex but not in the spinal cord. Keap1 protein expression was unchanged in comparison to control tissue [48].

It has previously been shown in rats that transcriptional activity of Nrf2 physiologically decreases with age [49]. One could therefore assume that the reduced Nrf2 mRNA and protein levels in ALS were age-dependent. As we used age-matched control tissues, however, loss in Nrf2 mRNA and protein expression cannot simply be explained by age effects. In contrast to studies in the ALS mouse model, we observed a decrease in Nrf2 expression in sporadic ALS postmortem tissue specimens and therefore suggested that this is associated with reduced cellular defense mechanisms against oxidative stress. A limitation of such postmortem studies is that they represent the terminal stage of ALS. The Nrf2 reduction which we observed does therefore not exclude activation of the Nrf2 pathway during disease onset or early symptomatic stages.

5. Preclinical Studies Using Nrf2 Activators

The Nrf2/ARE signaling system is a powerful defense system that evolved in higher organisms to protect them against an array of insults. The first assay to test the efficacy of Nrf2-dependent NQO1 activation in Hepa1c1c7 murine hepatoma cells was developed in 1988 [50]. Subsequently, 10 chemically distinct classes of Nrf2 activating compounds have been described, among them tert-butylhydroquinone, DL-sulforaphane, lipoic acid, fumaric acid, and curcumin [18]. Problems regarding these Nrf2-inducers include poor penetration of the blood-brain-barrier as well as their multifactorial modes of action not only related to the Nrf2-signaling cascade. Several studies have assessed the effects of different Nrf2-inducing agents in both wild-type and Nrf2-knockout mice and showed that different inducers result in differential gene expression changes and that not all gene expression changes are Nrf2-dependent (reviewed in [51]).

Activation of Nrf2 is neuroprotective in animal models of ALS and other neurodegenerative diseases [29–39], (Table 1). One may predict that manipulation of Nrf2/ARE signaling is a potential novel mechanism for neuroprotection in humans against diseases such as ALS. In a preclinical study in transgenic ALS mice, we assessed a novel class of synthetic triterpenoids, that is, analogues of oleanolic acid which can be considered the most potent Nrf2-inducer to date [22]. We used the G93A SOD1 transgenic mouse model of ALS that is the best model available for ALS to date as it is well

Table 1: Evidence for protective effects of Nrf2-activation in animal models of neurodegenerative disorders.

Disease	Animal model	Method of Nrf2-activation	Reference
ALS	G93A-SOD1 mice	Synthetic triterpenoids (CDDO-ethylamide (CDDO-EA), CCDO-trifluoroethylamide (CDDO-TFEA))	Neymotin et al., 2011 [29]
Alzheimer's disease (AD)	Intracerebroventricular infusion of Abeta peptides in rats	Curcumin	Frautschy et al., 2001 [30]
AD	Transgenic APP/PS1 mice	Tert-butylhydroquinone/adenoviral Nrf2 gene transfer	Kanninen et al., 2008 [31]
AD	Transgenic 19959 mice	CDDO-methylamide (CDDO-MA)	Dumont et al., 2009 [32]
AD	Transgenic APP/PS1 mice	Intrahippocampal injection of Nrf2-expressing lentiviral vector	Kanninen et al., 2009 [33]
Parkinson's disease (PD)	MPTP-toxicity (mice)	Tert-butylhydroquinone	Abdel-Wahab, 2005 [34]
PD	MPTP-toxicity (mice) 3-NP-toxicity (rats)	CDDO-MA	Yang et al., 2009 [35]
PD	MPTP-toxicity (mice)	Astrocytic Nrf2-overexpression	Chen et al., 2009 [36]
PD	Alpha-synuclein expressing *Drosophila*	Nrf2-overexpression, Keap1-downregulation	Barone et al., 2011 [37]
Huntington's disease (HD)	Transgenic N171-82Q mice	CDDO-EA, CDDO-TFEA	Stack et al., 2010 [38]
HD	CAG 140 knock in mice	Curcumin	Hickey et al., 2012 [39]

characterized and widely used to test therapeutic compounds against ALS. These mice develop symptoms of ALS and die from pathologies caused by SOD1 mutation at 85–90 days and 128–132 days after birth, respectively [5]. The synthetic triterpenoids we used in this study are analogs of 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid (CDDO) derived from oleanolic acid. We used two CDDO-analogs, CDDO-EA and CDDO-TFEA, with an astounding potency of 2×10^5 fold higher in activating Nrf2 and inducing phase II genes than the parent compound oleanolic acid. These compounds act at nanomolar levels in vitro and in vivo [29, 35, 52]. We have tested both of these CDDOs in G93A SOD1 mice and observed a significant increase in survival in mice treated with these compounds [29]. We noticed lower Nrf2 expression in the untreated G93A spinal cord sections as compared to the triterpenoid-treated group. This suggests that physiological activation of Nrf2 by oxidative stress does not take place in ALS transgenic mice. This could be due to multiple factors, such as the lack of or damage to other intermediary factors necessary for Nrf2 activation. Our study also used NSC-34 motor neuron-like cells stably expressing G93A SOD1. In this in vitro model, treatment with CDDO-EA or CDDO-TFEA led to increased Nrf2 protein expression as we showed by immunohistochemistry and Western blot. Triterpenoid-induced activation of Nrf2-expression could also be demonstrated in rat primary motor neurons.

Total RNA analysis from CDDO and vehicle-treated NSC-34 G93A cells showed upregulation of classical Nrf2 regulated genes which could be divided into three categories: (1) antioxidants, (2) anti-inflammatory, and (3) genes related to mitochondrial protection. Both CDDOs increased NAD(P)H: quinone oxidoreductase (NQO1), glutathione S-transferase A3 (GSTa3), and heme oxygenase 1 (HO1) which are representatives of antioxidants. Cycloxygenase-2 (Cox-2), inducible nitric oxide synthase (iNOS), Fas ligand (FasL), and tumor necrosis factor-alpha (TNF-alpha) expressions were similarly reduced by both CDDO analogs, showing that the treatment also affected expression of proinflammatory mediators. The third class of genes that we examined were factors involved in mitochondrial repair and biogenesis: here we found treatment-induced upregulation of cytochrome oxidase subunit II (COXII), estrogen-related receptor alpha (ERR-alpha), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha). Consistent to mRNA analysis, Western blot analysis showed increased expression in protein levels for Nrf2, NQO1, HO-1, and glutathione reductase (GR) in NSC-34 G93A SOD1 cells treated with CDDO-TFEA.

In the preclinical in vivo study we showed that CDDOs are bioavailable and penetrate the blood-brain barrier in mice. We also found no obvious toxicity during chronic administration of 80 mg/kg body weight. Treatment of G93A SOD1 mice treated with CDDO-EA and CDDO-TFEA begun at a presymptomatic age resulted in a significant increase in survival of 20.6 and 17.5 days, respectively. Treatment with these two compounds at the onset of ALS which is more relevant with respect to further translation into clinical studies in ALS patients still shows encouraging results: starting treatment at symptomatic age significantly extended the duration from age of onset to age of death by 43% (CDDO-EA) and 38% (CDDO-TFEA) [29].

In both paradigms, treatments attenuated weight loss and preserved motor performance. These Nrf2-activating compounds therefore represent interesting candidates for further clinical evaluation in ALS. Thus far, any investigated approaches for treating neurodegenerative diseases have

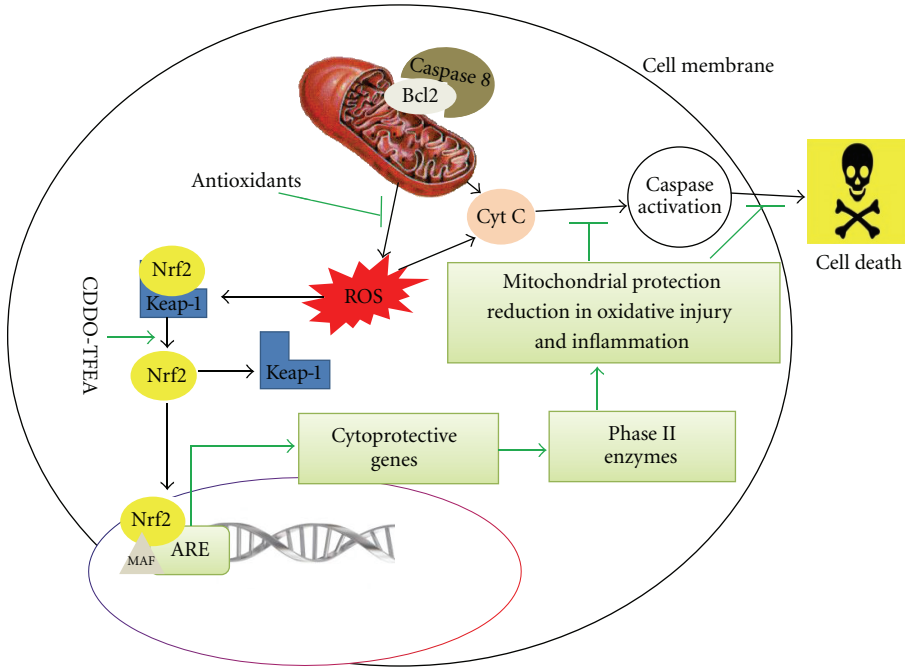

Figure 2: Schematic representation of major pathways involved in motor neuron death. The Nrf2/ARE signaling pathway is a potential target for blocking multiple death pathways. Green arrows represent neuroprotective pathways, and black arrows point to known neuronal death pathways containing potential cellular targets for antioxidant and anti-inflammatory agents.

targeted only one pathway or a specific cell type (e.g., astrocytes) to be blocked, whereas it is known that multiple and cascading pathways act in a noncell autonomous manner that leads to motor neuron death in ALS. To combat pathogenesis with this complexity and magnitude, we must aim at targets that are potentially powerful enough to block multiple pathways. Nrf2 induces over 250 phase II genes, and we have proof-of-concept that multiple genes are active following treatment with CDDO-EA or TFEA, some that produce antioxidant enzymes against oxidative stress, some that produce anti-inflammatory enzymes that target inflammation, and some that produce mitochondrial protective and repair enzymes that target dysfunctional mitochondria (Figure 2). Interestingly, another laboratory consistently demonstrated the neuroprotective effect of Nrf2 in MPTP model of Parkinson's disease [53].

6. Perspectives

Simultaneous blockage of disease-specific broad toxic signaling cascades in motor neurons and glia may ultimately lead to more efficient neuroprotection in ALS. Stimulation of defense mechanisms that modulate neuroprotective genes which affect both neuronal and glial functions is a novel therapeutic approach and holds great promise. A key molecule to affect a variety of defense mechanisms is the transcription factor Nrf2 which activates the Nrf2/ARE signaling program. Nrf2 acts as master regulator of the cellular antioxidant response by stimulation of over 250 phase II genes that should be referred to as "prolife genes" since they save cells from death. Nrf2 activation can at once regulate the expression of multiple cytoprotective enzymes that are capable of simultaneous inhibition of major pathogenic pathways described in ALS such as oxidative stress, neuroinflammation, and mitochondrial dysfunction. Decreased Nrf2 expression was found in motor neurons in ALS postmortem brain and spinal cord. We have established the proof-of-concept that the Nrf2/ARE program is a viable target with excellent therapeutic potential for ALS and have demonstrated that activation of Nrf2 by CDDOs resulted in significant beneficial effects on body weight, motor performance, and survival in the G93A SOD1 mouse model of ALS. While there are still multiple gaps of knowledge on the path from Nrf2 dissociation to nuclear localization and its action as transcription factor, activation of the Nrf2 signaling cascade represents a novel and unique attempt to find a cure for ALS and other neurodegenerative diseases by fortifying the intrinsic defense mechanisms of neurons.

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Review Article

D-Serine Production, Degradation, and Transport in ALS: Critical Role of Methodology

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In mammalian systems, D-serine is perhaps the most biologically active D-amino acid described to date. D-serine is a coagonist at the NMDA-receptor, and receptor activation is dependent on D-serine binding. Because D-serine binding dramatically increases receptor affinity for glutamate, it can produce excitotoxicity without any change in glutamate *per se*. D-serine is twofold higher in the spinal cords of mSOD1 (G93A) ALS mice, and the deletion of serine racemase (SR), the enzyme that produces D-serine, results in an earlier onset of symptoms, but with a much slower rate of disease progression. Localization studies within the brain suggest that mSOD1 and subsequent glial activation could contribute to the alterations in SR and D-serine seen in ALS. By also degrading both D-serine and L-serine, SR appears to be a prime bidirectional regulator of free serine levels *in vivo*. Therefore, accurate and reproducible measurements of D-serine are critical to understanding its regulation by SR. Several methods for measuring D-serine have been employed, and significant issues related to validation and standardization remain unresolved. Further insights into the intracellular transport and tissue-specific compartmentalization of D-serine within the CNS will aid in the understanding of the role of D-serine in the pathogenesis of ALS.

1. Introduction

Of the D-amino acids known to exist in mammals, D-serine appears to be the most abundant, and certainly the most biologically active. Activation of NMDA receptors (NMDA-Rs) requires binding of both glutamate to the NR2 subunit and its coagonist D-serine to the NR1 subunit [1]. (The co-agonist was initially thought to be glycine, hence the older nomenclature of "glycine$_B$" site.) Not only is D-serine binding necessary for receptor activation, but it also increases the affinity of the receptor for glutamate and modulates receptor function by decreasing receptor desensitization while promoting receptor turnover via internalization [2]. Given the widespread distribution of NMDA-Rs in the mammalian CNS, and the abundance of glutamate, the requirement for a co-agonist may represent one of nature's safety mechanisms, designed to prevent overstimulation and excitotoxicity. Thus, it is somewhat ironic that D-serine may actually be a primary cause of excitotoxic neuronal death in ALS. By virtue of its ability to increase glutamate binding affinity, D-serine may produce excitotoxic motor neuron death even in the absence of changes in glutamate levels *per se*.

2. Regulation of D-Serine *In Vivo*

Despite notable progress in the 20 years since D-serine was first identified in rat brain [3], the mechanisms of production, degradation, transport, storage, and release of D-serine remain enigmatic. Serine racemase (SR) is considered to be the primary endogenous source of D-serine (using L-serine as a substrate), while D-amino acid oxidase (DAO) is generally regarded as the primary mechanism of degradation. This otherwise straightforward view is dramatically confounded

by the fact that SR has an alpha, beta-eliminase activity that is 3-4-fold more efficient (Kcat/Km) than its racemase activity [4]. That is, SR produces D-serine from L-serine, but simultaneously degrades both D-serine and L-serine irreversibly to pyruvate and ammonia. To our knowledge, there is no other mammalian enzyme capable of making a product, while at the same time degrading both its product and substrate. Referring to SR as a "strange" enzyme is an understatement [5]. SR knockout mice were originally created to examine the role of D-serine in diseases like schizophrenia [6], but it may be that schizophrenic behavior is best exemplified by SR itself.

Examination of the reaction mechanism reveals how and why SR has simultaneous, constitutive racemase and eliminase activities. Both reaction pathways of SR share the same resonance-stabilized carbanion intermediate [7, 8]. Whether the reaction proceeds to racemize L-serine to D-serine, or to deamination/elimination (producing pyruvate and ammonia) appears to hinge on whether or not the –OH of Ser84 donates a proton to the intermediate. While it remains possible that some cofactor or other allosteric modulator may serve to switch SR from one activity to the other, all such factors identified to date enhance both activities roughly equally [8]. Wolosker [9] demonstrated *in vivo* biosynthesis of D-serine via purification of serine racemase from 60 rat brains, which included a painstaking examination of low molecular weight cofactors and activity modulators [9]. This was initially done by adding back fractionated tissue supernatants, followed by identification of specific factors (e.g., pyridoxyl phosphate, Mg^{2+}, ATP, etc.). Not until three years later did De Miranda et al. (including Wolosker) describe the eliminase activity of SR [10], leading Wolosker to remark in a recent review that "if the elimination reaction had not been discovered *after* the racemization, SR would have been classified as a serine dehydratase enzyme" [8]. The importance of SR-eliminase activity *in vivo* will be discussed later.

3. Impact of SR Knockout on D-Serine Levels *In Vivo*

At least three different SR knockout mouse strains have been created, each using a different strategy, but all targeting the same 37 kD protein [6, 11–13]. In all cases, levels of D-serine in SR knockout mice are lower compared to wild-type mice, but are not zero; in some cases very significant levels remain, particularly in certain brain regions and tissues [6, 11–13]. In the case of the SR knockout mice first reported by Miya et al. [13], no statistically significant change in D-serine levels was seen in cerebellum or in ten other peripheral organs and tissues [14, 15]. Unfortunately, spinal cord was not examined in this study—the most comprehensive to date. Horio et al. [14] did find D-serine levels in cerebellum and peripheral organs to be roughly 10-fold lower than in other brain regions, suggesting that brain regions and organs with lower constitutive levels of D-serine are largely independent of SR. It is noteworthy that cerebellum and spinal cord levels are very similar in humans, and 10-fold lower than in other regions of the CNS [16]. Thus, it is possible that tissues that have constitutively lower levels of D-serine are less dependent on SR-racemase activity for production, at least in the absence of neurological pathology. To our knowledge, our study represents the first measurement of D-serine in spinal cord of SR knockout mice [17]. In that study, knocking out SR had virtually no effect on D-serine levels in spinal cord.

4. SR Knockout and D-Serine Levels in ALS

Mutations to Cu, Zn superoxide dismutase (SOD1) were first reported to be a cause of human ALS in 1993, and the G93A SOD1 mutation of SOD1 was overexpressed in mice two years later, yielding the first mouse model of ALS. All subsequent studies on the role of D-serine in ALS have utilized this well-characterized model. The first measurement of D-serine in spinal cord was that of Sasabe et al. [18], who reported that D-serine was 1.5-fold higher in spinal cords of ALS mice; these results were obtained using a DAO-dependent chemiluminescent assay for D-serine (see Section 12). Using a novel D-serine biosensor (see Section 11), we found that cord levels of D-serine were 2-fold higher in G93A mice than in wild type [17], whereas D-serine in brain of G93A mice were the same as wild type. Partial deletion of SR lowered levels significantly, and complete SR knockout further lowered them, but only to levels normally seen in wild-type mice [17]. Thus, deletion *of SR reversed the pathologic increase in D-serine in G93A mice, but not basal D-serine*. Similarly, knocking out SR in mice that did not express mSOD1 had no effect on cord or brain D-serine levels. In contrast to cord, D-serine was not increased in the brains of G93A mice. Ongoing work is aimed at a better understanding of exactly where the different pools of D-serine originate, and what controls D-serine levels in different organs and tissues. However, the results to date do suggest that spinal cord has an absolute requirement for D-serine, but that levels must be carefully maintained within certain limits, perhaps because cord neurons are exquisitely sensitive to it. Why D-serine is elevated in G93A ALS mice is not clear, but may be a consequence of the generalized glial activation that occurs in these mice (see Section 7 for further discussion).

5. Phenotypic Effects of SR and D-Serine in ALS

Sasabe et al. [18] first showed that primary neurons isolated from G93A mouse spinal cords were more susceptible to NMDA-mediated toxicity, in a D-serine-dependent manner. This result helped establish a potential link between D-serine and excitoxicity in ALS. Perhaps more importantly, Sasabe et al. showed an apparent increase in D-serine in one A4V human ALS patient, and two of three sporadic ALS patient cord samples, suggesting that D-serine may play a role in other, non-mSOD1-mediated ALS [18]. The strong correlation between D-serine and ALS in mice (and humans) warranted further work. Thus, we set out to examine a more direct cause-and-effect relationship by crossing SR knockout mice with G93A mice, as well as treating G93A mice with D-serine, to examine the effects of D-serine on disease dynamics.

Quite unexpectedly, we found that a 50% reduction in SR enzyme (SR+/− : G93A), which lowered D-serine

(mentioned above), resulted in earlier disease onset by 13 days [17]. However, once symptoms of motor neuron disease began, disease progression was actually slowed such that these mice lived approximately 10 days longer than G93A littermates with normal SR expression. When examined in terms of survival interval (time from onset to endstage)—a measure of the rate of disease progression—the SR+/− : G93A mice lived for 60.5 days after onset, relative to 38.8 days for untreated G93A mice. This effect on both onset and progression was even more dramatic in G93A mice with complete SR knockout, these mice showed onset at 61 days of age, and endstage delayed to 146 days of age. In this case (compete SR knockout), the survival interval was 85.8 days, that is, mice progressed very slowly over a much longer period than normal.

Treatment of G93A mice with D-serine was undertaken with the idea that the opposite effects (from SR knockout) would be seen. Despite these expectations, presymptomatic treatment of G93A mice with D-serine (pre-dissolved in chow) had a qualitatively similar effect as with SR knockout; earlier onset and somewhat slower progression. Treatment with D-serine at normal onset (90 days of age) had a "pure" therapeutic effect; progression to endstage was slowed by 19 days. The paradoxical nature of these results began to make sense only when cord levels of D-serine were examined. As mentioned, untreated G93A mice had a 2-fold higher level of spinal cord D-serine. Partial knockout of SR lowered D-serine to levels only slightly higher than those of wild-type mice, and complete SR knockout decreased D-serine levels even more, comparable to those in wild-type mice. Oddly enough, D-serine treatment, either presymptomatically or at onset, had virtually the same effect on D-serine levels as SR knockout, that is, D-serine in cords decreased to those of wild-type mice. Moreover, this effect was only seen in spinal cord, as brains of D-serine-treated mice showed increases of up to 1.5-fold (Thompson, Marecki, and Crow, unpublished). Thus, D-serine added to chow was crossing the blood brain barrier but was somehow being handled differently in cord than in brain.

6. Paradoxical Results in G93A Mice

The question naturally arose: how can D-serine treatment produce the same phenotypic changes and result in the same lowering of spinal cord D-serine as SR knockout? The simplest answer was that D-serine degradation had somehow been induced specifically in spinal cord. However, no significant changes in DAO were seen, which could account for this. Thus, the answer must lie elsewhere. The only other known D-serine handling proteins were the transporters—primarily one called aspartate-serine-cysteine-1 or Asc-1. (It should be noted that transporter nomenclature has yet to be standardized, and "ASC"- and "ASCT"-class transporters exist in the literature under a variety of names.) Alanine-serine-cysteine transporter 1 (Asc-1) is a sodium-independent transporter with a relatively high affinity for D-serine [19, 20]. A sodium-dependent transporter similar to the B-type alanine-serine-cysteine transporter, termed ASCT, has a lower affinity for both L- and D-serine and has been observed in astrocytes [19–21]. Asc-1 was first identified on presynaptic neurons, where it takes up D-serine from the synapse, thereby terminating D-serine neurotransmission [20]. In our study, Asc-1 was found to be upregulated in D-serine-treated G93A mouse spinal cords, but not in their brains [17]. Thus, it seemed likely that D-serine might be localized to different cells/compartments as a result of D-serine treatment. However, while changes in the tissue location of D-serine could alter neurotransmission and potentially excitotoxicity, changes in location *per se* could not account for lower whole cord levels of D-serine, unless that change was also accompanied by degradation. Thus, we inferred that the eliminase activity of SR itself was responsible for degradation of D-serine, and Asc-1 was acting to bring D-serine back into the cells where SR was highest. A recent review by Herman Wolosker, who first purified and characterized SR, offered some additional insight [8].

7. Revised View of Localization of SR and D-Serine: Implications for ALS and Other Disease Conditions

For several years, immunofluorescence studies, based on colocalization of SR with glial markers, have strongly suggested that SR was predominantly found in astrocytes and microglia [22–24]. However, some studies have shown that neurons in some areas of the brain such as the cerebral cortex and hindbrain glutamatergic neurons also contain their own sources of SR [25, 26]. More recent studies, one of which employed an SR knockout mouse as a control [13], as well as an *in situ* hybridization study [27], suggested that SR is predominantly in neurons, not glia [8]. In any case, it appears that neurons cannot make the substrate L-serine and are dependent on astrocytes for a source of L-serine in order to synthesize D-serine *de novo* [28]. Many of the discrepant results could be reconciled if we hypothesized that *glia normally do not express SR, but the presence of ALS-associated SOD1 mutants induces SR expression as a consequence of pathological activation*. This could account for an important finding by Sasabe et al. wherein transfection of isolated microglia with G93A mSOD1 led to apparent expression of SR [18]. This induction would also account for the increase in SR protein [18] and D-serine seen in ALS mouse spinal cord [17, 18], and possible even the lowering of cord D-serine levels following D-serine treatment [17].

The location of SR and D-serine, under both normal and pathological conditions, is important not only in terms of enhanced production and potential excitotoxicity, but also in terms of regulation of D-serine levels via SR-eliminase activity. Wolosker makes a compelling argument that *SR-eliminase will ultimately win out in the concurrent battle to both produce and degrade D-serine* [8]. That is, if D-serine remains in the same compartment as SR—an enzyme that degrades both D-serine and L-serine—then D-serine will ultimately be degraded. If the SR was in neurons, both D-serine and L-serine would ultimately be degraded, requiring resupply of L-serine from glia [28]. Transporters like Asc-1 could act to preserve D-serine levels by physically separating

from SR-eliminase in neurons. However, if SR is expressed by activated glia in pathologic conditions such as ALS, then Asc-like transporters could do just the opposite—they could serve to degrade D-serine by placing it in proximity to SR-eliminase in the glia. Other Asc-like transporters are only now being identified and, based on the conflicting literature regarding precisely where SR is normally expressed, it is too early to draw any absolute conclusions regarding the role of D-serine transporters in overall regulation, but clearly they could be key to both termination of normal neurotransmission and to excitotoxicity. In any event, it is almost certain that SR plays a critical role in the overall regulation of D-serine, by acting as both producer and destroyer.

8. D-Amino Acid Oxidase (DAO)

Because D-amino oxidase (DAO) had been known to exist for many decades, it has come to be viewed as the default mechanism for "detoxifying" D-amino acids. In mammals, DAO is relatively nonspecific, acting upon at least seven D-amino acid substrates [29–31]. However, except for D-serine, the D-amino acids known to exist in mammalian systems do not appear to be particularly toxic [32]. Other lines of evidence argue against DAO being the sole mechanism for regulating D-serine levels: (1) the absence of pathologic phenotypes or compensatory changes in mice lacking DAO [5] and (2) the absence of DAO in forebrain, where D-serine exists at relatively high levels [33, 34]. Indeed, careful characterization of a naturally occurring DAO knockout mouse strain suggests that the primary purpose of DAO is to degrade D-amino acids, whether produced endogenously, obtained from the diet, or from bacterial action in the gut, into keto acids that can then be metabolized as energy sources [35]. That is, DAO may act to obtain useful chemical energy from otherwise "inert" amino acids, as much as to detoxify them.

9. DAO Mutations in ALS

In 2010, Mitchell et al. identified a mutation (R199W) to DAO and reported a high correlation between this mutated DAO and ALS in one family [36]. Because the mutation was found to abolish DAO activity, the immediate inference was that the mutation would result in increased excitotoxic neuronal death via excessive D-serine accumulation. Transfection of NSC34 cells with mutated DAO did reveal significant toxicity, but the D-serine level in the cells was not measured. Thus, no direct correlation between (presumably) higher D-serine concentrations and cytotoxicity could be made. Moreover, an increase in ubiquitinated protein aggregates was seen in the transfected cells. Thus, while this DAO mutation is consistent with the concept of D-serine-mediated toxicity in ALS, it remains to be seen whether spinal cord D-serine levels are indeed altered, as opposed to mutant DAO merely having an increased propensity to misfold and aggregate.

10. Measurement of D-Serine

Further elaboration of the roles of D-serine in health and disease, and indeed all biologically relevant D-amino acids, will depend on rigorous evaluation, and possibly modification and standardization, of the various measurements techniques and methodologies. Some of the discrepant results reported thus far may be due to comparisons of different methodologies, which may contain artifacts; this is particularly true when indirect, secondary assays are employed, such as the commonly used DAO-based chemiluminescent assay for D-serine. Because this assay is frequently used to measure SR activity as well, the implications of any artifacts extend beyond simple tissue levels of D-serine *per se*.

HPLC analysis of D-serine and L-serine, based on chiral derivatization to fluorescent species, is clearly the most sensitive and direct way to identify and quantify these low molecular weight (mass = 87.1) amino acids. Because both serine enantiomers are such small, simple molecules, even chiral HPLC columns cannot readily resolve them. Moreover, they lack any type of chromophore, which would permit UV-visible or fluorescent detection. We have employed an existing derivatization technique involving o-phthalaldehyde and boc-L-cysteine to generate a fluorescent, chiral isoindole [37] and modified the buffer/gradient system (using sodium acetate and acetonitrile) so that D-serine and L-serine could be resolved by more than two minutes (Thompson et al., unpublished). Via the use of authentic D-serine standards added to tissue homogenates, together with DAO pretreatment to remove D-serine (and other D-amino acids), we were able to measure D-serine in mouse tissues and validate an alternate method based on a novel biosensor [17]. Based on our experiences, we feel strongly that HPLC should remain as the gold standard for such analyses and should be used to validate any and all other methodologies, particularly indirect assays that measure secondary enzyme reaction products like hydrogen peroxide or pyruvate.

11. D-Serine Biosensor

When acting on D-serine, DAO produces hydroxypyruvate, ammonia, and H_2O_2. H_2O_2 is a very useful product from the standpoint of biochemical analysis, as it can be measured in many ways, including oxidation by an electrochemical probe to generate an electrical current. By encapsulating DAO obtained from the yeast species *Rhodotorula gracilis*—which is more stable than mammalian DAO and has higher specificity and affinity for D-serine [38]—within the tip of an electrochemical probe, D-serine can be selectively and repeatedly quantified very rapidly. Free D-serine diffuses into the probe, is degraded by yeast DAO to yield H_2O_2 which, in turn, is oxidized on the metal probe, producing a current that is proportional to the concentration of D-serine in solution [39]. Because the H_2O_2 is produced within the biosensor tip, in close proximity (<100 microns) to the electrochemical probe, issues related to H_2O_2 dissipation or degradation are eliminated. Also, the presence of any interfering (diffusible) substances, such as H_2O_2 formed from other reactions in the medium, are easily controlled via the use of a comparable biosensor containing albumin rather than DAO. Unlike most DAO-based measurements of D-serine, the biosensor provides a rapid measurement that is *not dependent on complete consumption of* all *D-serine*

present. Indeed, the amount of D-serine consumed is negligible, such that repeated measurements of the same sample can be made in a very short time. Because the production of H_2O_2 near the electrochemical probe reaches steady-state very rapidly—within a few seconds—the biosensor provides repeated measurements essentially in real time.

12. DAO-Based Chemiluminescence Assay for D-Serine

The most commonly used method for measuring total D-serine in biological samples or *in vitro* reactions is based on DAO-mediated degradation of all D-serine present to hydroxypyruvate and H_2O_2, followed by measurement of total accumulated H_2O_2 via horseradish-peroxidase-(HRP-) mediated oxidation of luminol [8, 18, 40]. Unlike compounds such as dihydrofluorescein or dihydrorhodamine, which are oxidized to stable fluorescent species (fluorescein and rhodamine, resp.), luminol oxidation yields a transient chemiluminescence signal, which must be collected and quantified in real time in a luminometer. While it is possible to precisely quantify chemiluminescence, other issues exist with this assay, which render it problematic. Firstly, unless yeast DAO is used, there is little specificity for D-serine, thus all D-amino acids present will be measured. While the contribution of other D-amino acids in mouse of human tissue samples will likely be small, it can vary from sample to sample.

Secondly, accurate D-serine measurements with the DAO/HRP+luminol chemiluminescent assay *require that the DAO reaction go to completion and consume all the D-serine present*—a process that can take a considerable amount of time given the high Km of most DAO enzymes for D-serine [41, 42]. Unless very careful controls are employed, it is not possible to be sure that the DAO/D-serine reaction has gone to completion and, even if it has, it is equally difficult to know whether or not all the H_2O_2 produced during the long incubation remains intact, that is, that it has accumulated stoichiometrically with D-serine consumption, and not reacted with some other component or otherwise been degraded. Trace contaminants of catalase (or other peroxidases), redox active metals, or reductants can totally invalidate the results, just as other, unrelated reactions which can produce H_2O_2 during or before the extended incubation time, leading to overestimates of D-serine. Quite often the fact that this is a secondary product-type assay is masked when investigators graph results in terms of "D-serine concentration" (y-axis) when, in fact, H_2O_2 is the substance being measured. In most cases, D-serine concentration is simply inferred as being stoichiometrically equivalent to H_2O_2, without employing the controls needed to demonstrate stoichiometry. With regard to controls, this assay must employ dual standard curves—one for authentic H_2O_2 and a second for D-serine (plus added DAO). Only then can the true stoichiometry be assessed. Most accounts in the literature make no mention of such controls.

13. Chemiluminescent Assay as a Measure of SR Activity

The shortcomings of the DAO/HRP+luminol chemiluminescent assay for D-serine are marginally manageable for the purpose of measuring total D-serine content in a deproteinated tissue or cell lysate sample. However, when used to assay SR enzyme activity, additional issues come into play, related to the tedious three-step protocol, contamination of commercial L-serine substrate with D-serine, and competing SR-eliminase activity. By its very nature (three distinct enzymatic steps), the SR/DAO/HRP+luminol chemiluminescent assay does not lend itself well to measuring multiple time points at intervals—something which is essential to establishing a true enzymatic rate, that is, linear increases in D-serine concentration over time. Aliquots from an SR reaction with L-serine must be taken at timed intervals, quenched in some way to stop the SR reaction, then incubated for long intervals with DAO to ensure complete D-serine degradation, followed by the addition of HRP plus luminol to measure accumulated H_2O_2. When used to measure SR (racemase) activity, this assay typically involves single endpoints determinations of accumulated H_2O_2, without consideration for whether production of H_2O_2 was linear, whether extraneous H_2O_2 or degradation might be occurring, or whether it has accumulated stoichiometrically with D-serine, or how much H_2O_2 might have been present at time zero. Regardless of how SR is assayed, multiple time points are essential to determine linearity, or extent of nonlinearity and why. Also, any assay based solely on D-serine will reflect the *net* production of D-serine by SR, that is, the amount produced by racemase activity minus that consumed by SR eliminase activity [4, 43].

14. Validation of Chemiluminescent Assay

The accuracy and reliability of the chemiluminescent assay can only be determined by carrying out simultaneous HPLC analysis on the same reaction aliquots and measure both D-serine production and L-serine consumption. Also, standards curves must be done using authentic H_2O_2 so that the true stoichiometry between D-serine and H_2O_2, under the specific conditions employed, can be determined. Ideally, assays for pyruvate should also be done on the same aliquots. In this way, not only can potential artifacts in the chemiluminescent assay be uncovered, but the relative contributions of SR-racemase and eliminase activities can be quantified. We have carried out such comparisons, and have found the DAO/HRP+luminol chemiluminescent assay to overestimate racemase activity of SR by 10-fold or higher (Marecki, Thompson, and Crow, manuscript in preparation). This appears to be due to high basal levels (at time zero) of H_2O_2 and/or production of H_2O_2 from other sources during incubation. Again, if time points are collected and analyzed at intervals, including time zero, both in the presence and absence of DAO, then many of these artifacts can be controlled. However, it is not clear from many literature accounts that such controls are employed.

Thus, the reliability of many values in the literature cannot be accurately assessed.

15. Chemiluminecsent Assay to Assess SR Kinetics

Unfortunately, many of the kinetic characterizations of SR *in vitro*, both with enzyme purified from tissues and recombinant enzyme, have involved the use of this problematic chemiluminescent assay. Moreover, because of practical limitations for doing multiple time points, it has typically been used as a single endpoint type assay. That is, L-serine is added to SR reaction mixtures, reactions are allowed to run for 30–60 minutes, and then quenched, followed sequentially by DAO and its cofactor (FAD), and then HRP+luminol on a single endpoint sample. The following assumptions are then made: (1) that D-serine production by SR-racemase was linear over time period employed (SR-eliminase activity is typically ignored altogether), (2) that D-serine degradation by DAO was complete, (3) that the amount of H_2O_2 formed is exactly equal to the D-serine present, (4) that no H_2O_2 was present at time zero, and (5) that H_2O_2 accumulation was not affected by degradation or augmented by production from other sources. If any of these assumptions are incorrect, then D-serine production rates, and kinetic constants calculated for SR, are erroneous. The problem is that, *when single endpoints are examined, it is impossible to assess the validity of any of these assumptions*. A basic axiom of enzyme kinetics is that product must increase over time and that one must base calculations on the linear region where pseudo-first-order kinetics govern enzyme behavior, so-called "initial rates". At the very least, the rate of enzyme activity over the time period being measured must be known; one cannot measure something at the end and simply assume that the value at the start was indeed zero or that the change in-between was linear over time. In many cases involving the use of this chemiluminescent assay, investigators report that HPLC was used to validate the results, but chromatograms and controls are typically not shown. Even when chromatograms are shown, it is often unclear whether the entire assay was validated and properly controlled, or whether D-serine was simply measured in a few endpoints samples to demonstrate feasibility.

16. Assay of SR via Pyruvate Production

Mindful of the assay issues described above, some investigators have largely avoided them by measuring SR activity via pyruvate production [18, 44–46]. This is, of course, a measure of SR eliminase activity. When used as a generic measure of "SR activity" in tissues or cells, the use of L-serine as the substrate avoids any interference by DAO, but not SR-racemase activity. If D-serine is used as the substrate, then SR-racemase activity is avoided, but controls for potential interference by DAO must be employed. This is easily done via the use of DAO inhibitors like benzoate [17]. Of course, contaminating DAO would not be an issue when assaying recombinant SR. It should be noted that, although the product of the DAO/D-serine reaction is reported to be hydroxypyruvate, we and others have found that a coupled assay employing LDH and NADH works quite well to measure the "pyruvate" product. Also, derivatizing agents known to react with pyruvate, as well as commercial pyruvate assay kits, all seem to work quite well to measure the DAO/D-serine product. Thus, either hydroxypyruvate has similar reactivities to pyruvate (including acting as substrate for LDH) or perhaps spontaneously dehydrates to give pyruvate.

17. Antibody-Based Assay of "Free" D-Serine

Many investigators have employed an antibody-based technique to visualize D-serine for cell/tissue localization studies (immunohistochemistry) as well as for semiquantitative comparisons [18, 25]. The scarcity of thorough antibody descriptions in more recent publications prompted questions as to how an antibody could be selective for such a small molecule, as well as how a small, soluble molecule would remain inside cells and tissue that had been permeabilized to allow large antibody molecules to enter. These questions were largely answered when it became apparent that the antibody was raised to a glutaraldehyde adduct of D-serine, not to the free amino acid. Thus, in glutaraldehyde-fixed cells and tissues, the antibody appears to be a useful method for visualizing D-serine, both in theory and in practice. It is less clear whether the antibody would work as well when fixatives other than glutaraldehyde are employed, as the resulting D-serine adduct would not be the same. Also, the use of this antibody to quantify D-serine is subject to the same limitations imposed by any type of fluorescent antibody-based technique. That is, careful use of an antibody-based technique can provide semiquantitative comparative data but cannot be relied upon to provide absolute quantitation or to see small changes.

The real value of the antibody-based measurement of D-serine lies in its ability to determine cellular/tissue localization and, when used is this manner, can provide valuable information that would be lost with other techniques that rely on tissue homogenization. Indeed, no measurement of D-serine in whole tissue homogenates can detect changes in local concentration which may be highly significant, for example, changes in synaptic D-serine. This means that a 2-fold change in *total* spinal cord D-serine, as seen with G93A mice [17, 18], could translate into a much larger change locally. The same is true for changes in protein expression such as Asc-1 transporter in D-serine-treated mice [17] or changes in SR protein when mutant SOD1 is present [18]. Perhaps the best hope for future measurement of local concentrations of D-serine, possibly even in live animals, lies in further refinements of the D-serine biosensor.

18. Summary and Conclusions

D-serine appears to be an important player in both the onset and propagation of mSOD1-mediated ALS and potentially in sporadic ALS as well. The fact that exogenous D-serine treatment produced similar changes to SR knockout in G93A mice, both in terms of phenotype and in terms of lowering

cord D-serine levels, strongly suggests that the effects of SR are mediated via its effect on D-serine levels. It remains to be seen whether this is via a true excitotoxic mechanism, but the results to date are consistent with that premise. Although the mechanism is not known, it is clear that D-serine levels are increased by G93A mSOD1, perhaps via generalized glial activation and resulting induction of SR expression. Given what is now known, D-serine may well prove to be a key link between glial activation and motor neuron death in ALS, that is, it may be the elusive mediator of glial-mediated non-cell-autonomous neuronal death. In any case, the production, storage, release, transport, and degradation of D-serine must all be better understood before its complete role in ALS or other neurodegenerative diseases can be fully elucidated. In that regard, the importance of validation and standardization of methodologies for measuring D-serine and the competing activities of SR cannot be overstated. Until such time, questions as to the characterization of SR will linger. In any case, evidence in SR knockout mice indicates that it is not the only source of D-serine in mammals. Ongoing work in our laboratory, both with SR knockout mice (with and without the G93A mSOD1 transgene) and with recombinant SR *in vitro* is aimed at understanding the role of SR-racemase in producing and SR-eliminase in degrading D-serine, as well as other potential sources of D-serine.

Added in Proof. A recent study by Sasabe et al. [47] suggests a significant role for DAO in regulating D-serine levels in G93A mice. Measurements of DAO activity in spinal cords of all six mouse phenotypes in our study (17) showed no statistically significant differences. While this suggested that DAO was not the predominant determinant of D-serine levels in G93A mice, measurements were done only on whole cords at endstage disease. Thus, it is possible that different results could be obtained with select (lumbar) cord regions at different ages. Immunohistochemical data from the 2012 Sasabe et al. study did provide evidence for nonneuronal expression of SR in G93A mice, consistent with the notion that SR is induced by glial activation.

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Review Article

Neuromuscular Junction Protection for the Potential Treatment of Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a neuromuscular disease characterized by the progressive degeneration of upper and lower motor neurons (MNs), leading to muscular atrophy and eventual respiratory failure. ALS research has primarily focused on mechanisms regarding MN cell death; however, degenerative processes in the skeletal muscle, particularly involving neuromuscular junctions (NMJs), are observed in the early stages of and throughout disease progression. According to the "dying-back" hypothesis, NMJ degeneration may not only precede, but actively cause upper and lower MN loss. The importance of NMJ pathology has relatively received little attention in ALS, possibly because compensatory mechanisms mask NMJ loss for prolonged periods. Many mechanisms explaining NMJ degeneration have been proposed such as the disruption of anterograde/retrograde axonal transport, irregular cellular metabolism, and changes in muscle gene and protein expression. Neurotrophic factors, which are known to have neuroprotective and regenerative properties, have been intensely investigated for their therapeutic potential in both the preclinical and clinical setting. Additional research should focus on the potential of preserving NMJs in order to delay or prevent disease progression

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by the loss of both upper and lower motor neurons (MNs) [1–3]. ALS research has primarily focused on mechanisms regarding MN cell death; however, degenerative processes in the skeletal muscle, particularly involving neuromuscular junctions (NMJs), are observed in the early stages of and throughout disease progression [4, 5]. Many studies support a "dying-back" hypothesis in which distal NMJ degeneration precedes and causes proximal cell body death. This paper will describe the NMJ, ALS pathology and the "dying-back" hypothesis (Figure 1). Then, we will discuss growth factor treatments and current progress regarding them.

2. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neuromuscular disease characterized by the progressive degeneration of upper and lower MNs, leading to muscular atrophy and eventual respiratory failure [1–3]. Diagnoses occur most often between the ages of 40 and 60 and the disease is fatal within 5-6 years of clinical diagnosis. ALS is the most frequent adult-onset MN disease with a worldwide incidence rate of 1–3 new cases per 100,000 individuals. About 90% of ALS cases are sporadic and the remaining 10% of ALS cases are familial (FALS). In about 20% of FALS cases, the cause can be attributed to a mutation in the $Cu^{2+/}Zn^{2+}$ superoxide dismutase 1 (SOD1), a ubiquitously-expressed free-radical defense enzyme [6]. The mutations cause misfolding of this normally stable homodimeric protein [7]. Overexpressing the human SOD1 mutant in rodents results in a disease progression similar to that observed in ALS patients, providing a valuable model ($SOD1^{G93A}$ mice or rats) on which a great deal of ALS research has been based [8, 9]. Two other heritable mutations associated with protein mislocalization and aggregation have become important areas of research in ALS: the RNA-processing proteins fused in sarcoma (FUS) and TARDNA binding protein 43 (TDP-43) [10, 11].

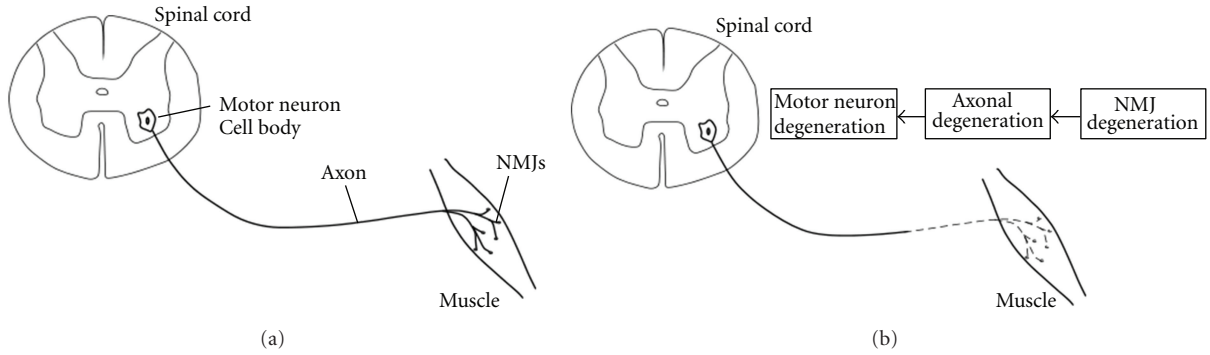

Figure 1: Schematic illustrating the "Dying-Back" hypothesis. (a) In a healthy system, communication and the transport of vital biomolecules occurs normally along the axon connecting MNs and the NMJs they innervate. (b) In ALS, a progressive distal to proximal degeneration occurs, described as "Dying-Back." NMJ degeneration is followed by axonal degeneration and eventually MN degeneration.

Although the SOD1 mutation represents a relatively rare, inherited form of ALS, both inherited and sporadic forms of ALS exhibit the same clinical course and neuropathology. Therefore, SOD1^{G93A} rodent models are important tools with which to better understand and investigate potential therapeutic treatments for ALS.

The mechanism underlying MN death in ALS is still unknown. Multiple mechanisms account for the selective vulnerability of MNs, including abnormal astrocyte and microglial activation, reduced neurotrophic factor secretion, protein aggregations, mitochondrial malfunction, rupture in the axonal passage, destruction in calcium metabolism, changes in skeletal proteins, high levels of excitotoxicity by glutamate and oxidative damage [12–14]. It is widely accepted that ALS is caused by MN degeneration. However, NMJ degeneration precedes and may even directly cause MN loss.

3. The Neuromuscular Junction

The neuromuscular junction (NMJ) is the synapse where the axon terminal of a MN meets the motor endplate, the highly excitable region of muscle fiber plasma membrane responsible for initiating action potentials across the muscle's surface, ultimately causing the muscle to contract. (Figure 2(a)). In vertebrates, the signal passes through the NMJ via the neurotransmitter acetylcholine. Terminal branches expand outward from the motor nerve and emerge from their myelin sheath at the muscle to form terminals. These terminals are filled with synaptic vesicles, mitochondria, and tubules from smooth endoplasmic reticula. Synaptic terminals permit the necessary communication between MNs and their target muscles for muscle contraction. The motor end plate is densely populated by nicotinic acetylcholine receptors. Glial cells, called terminal Schwann cells (TSCs), are also intimately associated with the nerve-muscle connection. TSCs are nonmyelinating Schwann cells that play important roles in the formation, function, maintenance, and repair of the NMJ [15]. In neuromuscular junction diseases such as myasthenia gravis, Lambert-Eaton syndrome, and myasthenic syndrome, normal conduction through the neuromuscular junction is disrupted [16].

4. Presymptomatic Degeneration of the NMJ

Recent studies suggest that distal degeneration in the skeletal muscle plays a key role in the progression of ALS. Several studies using SOD1^{G93A} mice have shown that NMJ degeneration occurs in the early stages of disease progression, long before MN loss [17]. Furthermore, distal axonopathy followed NMJ denervation, but preceded both neuronal degeneration and the onset of clinical symptoms (Figure 1) [18–21]. There is growing evidence suggesting that muscle weakness is not apparent until a large proportion of the motor units are lost [5]. The time differential between NMJ degeneration and muscle weakness is caused by remaining axonal reinnervation of the muscle. This process is able to compensate for denervation at first and no loss in muscle strength is observed. Eventually, reinnervation is not able to keep up with degeneration from the disease and muscle weakness becomes apparent [14, 15].

Presymtomatic NMJ degeneration is supported by a study that used longitudinal magnetic resonance imaging (MRI) of the same SOD1^{G93A} mice. Researchers discovered that the muscle volume in these animals was significantly reduced from as early as week 8 of life, 4 weeks prior to clinical onset [22]. Neuropathological analysis using SOD1^{G93A} mouse samples demonstrated a similar pattern of disease with prominent evidence of axonal degeneration only in muscle [23]. Furthermore, Hegedus et al. (2007) [24] applied electromyography to the SOD1^{G93A} mice and explored the time course of functional loss in motor units. They also explored whether or not a difference existed between the loss of function in fast and slow twitch muscle. A significant decline in the whole muscle contractile force occurred 50

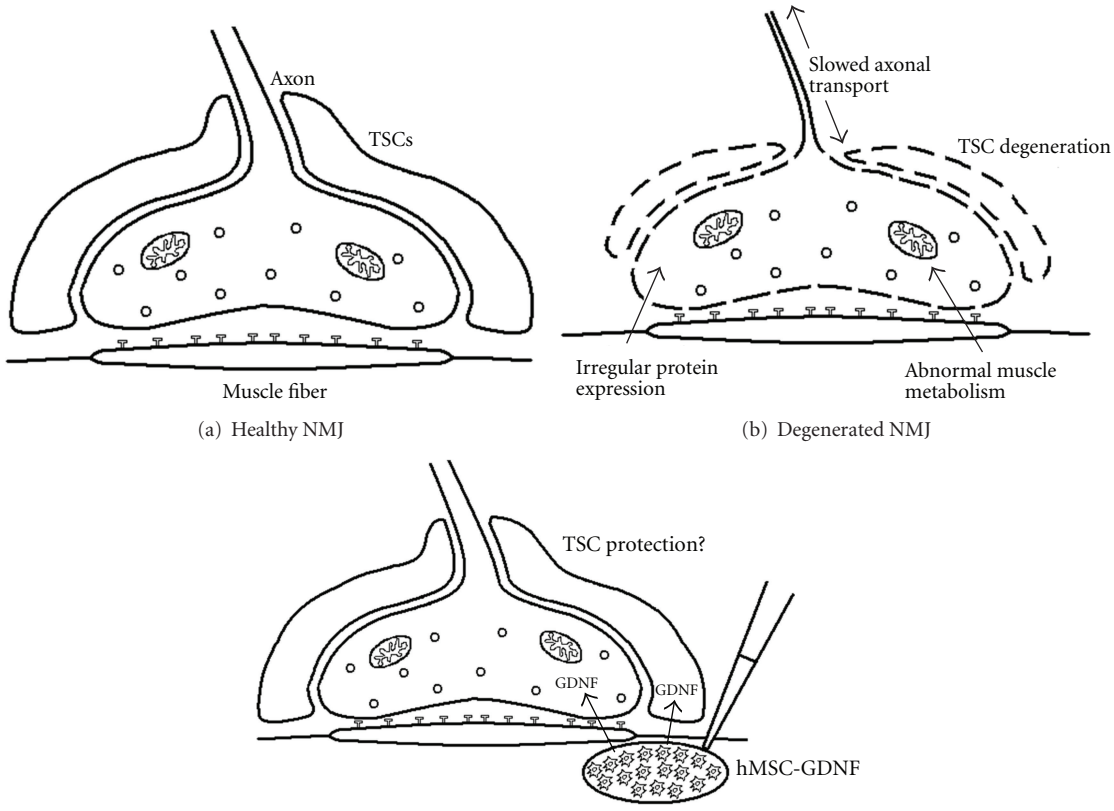

Figure 2: Schematic detailing NMJ degeneration and treatment. (a) A healthy, functioning NMJ, including TSCs and Ach receptors, is a vital point of communication between MNs in the spinal cord and muscle. (b) In ALS, NMJs begin to degenerate due to a number of pathologies, including disrupted axonal transport and irregular mitochondrial metabolism. NMJ degeneration occurs long before MN degeneration in the spinal cord, preceding clinical symptoms. (c) *Ex vivo* delivery of GDNF to NMJ via hMSC-GDNF may help to rescue TSCs from degenerative processes, thereby, delaying or preventing degeneration of the NMJ as a whole.

days before the onset of clinical symptoms. Furthermore, the number of functional motor units decreased in fast twitch, but not slow twitch, muscle. Another study found that skeletal muscle-restricted expression of the mutant SOD1 gene is sufficient to dismantle neuromuscular connections and cause MN distal axonopathy, resulting in MN disease in these mice [25–27]. This suggests that subclinical pathology in skeletal muscle is not merely the consequence of neurogenic atrophy, but initiates additional pathogenic processes.

The "dying-back" hypothesis is further supported by our recent research using $SOD1^{G93A}$ rats. We assessed whether human neural progenitor cells secreting glial cell line-derived neurotrophic factor (hNPC-GDNF) could also maintain neuromuscular connections following transplantation into the spinal cord of ALS rats. The animals were unilaterally transplanted at presymptomatic 70 days with hNPC-GDNF and then sacrificed at the mid-stage of disease (6 weeks after surgery). We confirmed a highly significant increase in MN survival within the hNPC-GDNF group when compared with the non-grafted side. However, hNPC-GDNF did not have a significant effect on the innervation of NMJs in the hind limb muscle [28]. These results suggest that while hNPC releasing GDNF were able to protect MNs, they were no longer connected to the muscle.

5. Possible Mechanisms of NMJ Denervation

In the last few decades, many explanations regarding NMJ degeneration have been proposed. Here, we describe some of the main components of the "dying-back" hypothesis which have been demonstrated (Figure 2).

One circumstance that may contribute to NMJ degeneration is the accumulation of $SOD1^{G93A}$ proteins in neurons, which slows anterograde and retrograde axonal transport, resulting in insufficient maintenance of the distal axon [29, 30]. An increased expression of Sema3A, an axon guidance protein, was found in $SOD1^{G93A}$ mice. It is thought that Sema3A, secreted by the TSCs, may lead to the repulsion of motor axons away from the NMJ, resulting in denervation [5]. Nogo-A, a neurite outgrowth inhibitor that is overexpressed in the slow-twitch fibers of $SOD1^{G93A}$ mice, may contribute to NMJ degeneration [31]. Overexpression of the dynamitin subunit of dynactin also inhibits retrograde transport and causes an α-MN degeneration progression similar to that observed in ALS [32]. Angiogenin (ANG), which is mainly implicated in angiogenesis, also has axonal guidance functions by regulating neurite extension and pathfinding. Mutations in ANG can inhibit neurite outgrowth and negatively affects MN survival [5]. The consequences of

these mutations highlight the importance of anterograde and retrograde transport in maintaining the functionality of MNs. Since the fine-tuning of axonal transport is crucial for the survival of motor neurons, the development of molecular-targeted therapies to maintain axonal transport would be a powerful strategy.

Recent work has also shown that abnormalities in muscle energy metabolism may play a role in initiating NMJ degeneration. Large MNs are susceptible in a caliber-specific order with the largest caliber axons being the most susceptible to degeneration in $SOD1^{G93A}$ mice and human patients [33, 34]. This is supported by the observation that MNs innervating fast-twitch muscle fibers, mainly composed of type IIB and IID/X muscle fibers, showed signs of degradation before MNs innervating slow-twitch type I and IIA fibers in $SOD1^{G93A}$ rodents. Fast-twitch fibers are often innervated by the larger caliber type II MNs and slow-twitch fibers are innervated by the smaller caliber type I MNs [24]. It is generally accepted that the MNs innervating fast motor units have the largest soma sizes, axon calibers, and innervation ratios [35]. It has been proposed that irregular muscle metabolism is the cause of caliber-specific degradation. $SOD1^{G93A}$ rodents experience an increased basal metabolic rate and subsequent weight loss due to decreased levels of cellular adenosine-5′-triphosphate (ATP) [36]. An increased basal metabolic rate in $SOD1^{G93A}$ rats has also been linked to elevated levels of mitochondrial uncoupling protein [37]. Since larger-caliber nerve fibers have the highest metabolic needs, they would be the most susceptible in irregular metabolic conditions [5].

Alterations in trophic factor expression in the skeletal muscle could influence the course of MN degeneration and NMJ denervation. Numerous studies support this idea and demonstrate that the expression of growth factors dramatically changes in the muscle of patients with ALS throughout the stages of the disease. Although increased GDNF mRNA expression was observed in muscle biopsies from ALS patients [38], the other study showed that GDNF mRNA was decreased in the postmortem muscles of ALS patients [39]. These observations imply that GDNF gene expression decreases considerably as the disease progresses. Similarly, decreased expression of insulin-like-growth factor-I (IGF-I) has been observed in the skeletal muscle of ALS patients [40].

Furthermore, TSCs may be intimately involved in the course of ALS pathology. TSCs cap the nerve terminal covering motor terminal branches and synaptic boutons. These cells play key roles in the maintenance of preterminal axon structure and function during development and in adult life [41]. TSCs dysfunction or loss could thus serve as a possible trigger for NMJ degeneration. We recently performed a longitudinal study using $SOD1^{G93A}$ rats to understand the ability of TSCs to protect neuromuscular connections and found that the number of TSCs was significantly reduced following disease progression in ALS rat muscle. Given the importance of TSCs in the maintenance and function of NMJs, further studies are necessary to understand the mediators of TSC plasticity. Then, suitable cellular and molecular targets can be identified for novel treatments for ALS and other neuromuscular diseases.

6. Upper MNs and "Dying-Back"

The connection between upper MN degeneration and the dying-back hypothesis is still uncertain. Some early studies suggest that cortical and lower MN degeneration occur independently and not as a transsynaptic phenomenon [42, 43]. Attarian et al. conducted two studies comparing the responses of motor units in ALS patients to transcranial magnetic stimulation and peripheral nerve stimulation [44, 45]. Although a positive correlation existed between cortical and spinal dysfunction at first, it eventually disappeared, again suggesting that upper and lower MN degeneration occur separately. Furthermore, it has been suggested that the disease starts at a focal point which involves both upper and lower MNs, but that each set of MNs is affected separately as the disease progresses [46]. Some studies even suggest a "dying-forward" hypothesis which places corticomotoneuron degeneration at the earlier stages of disease progression. Corticomotoneuron hyperexcitability, induced by glutamate, may drive the anterior horn cell into a metabolic deficit [47]. However, identifying corticospinal MN degeneration and corresponding subcerebral projection neurons more accurately can now be done with recently identified molecular markers and FluoroGold labeling. Only ~6,000 corticospinal and corticobulbar MNs exist per hemisphere in mice, intermixed with millions of other cortical pyramidal neurons in the same region and layer V of the motor cortex [48]. As the pathology and progression of upper and lower MN degeneration is better understood, we can refine our treatment target and rationale.

7. Possible Treatments Targeting Muscle: How Can We Prevent "Dying-Back"?

Mounting evidence for the "dying-back" hypothesis suggests that the survival of NMJs is imperative in hindering the progression of ALS. Therefore, therapeutic treatments aimed at preserving NMJs may be the most effective.

One therapeutic strategy following this model is the direct delivery of neurotrophic factors to skeletal muscle. Neurotrophic factors are intimately involved in the development and survival of neurons thereby supporting their candidacy as a therapeutic option for ALS. MNs are able to bind, internalize, and retrogradely transport growth factors from muscle in a receptor-dependent manner. Alternatively, injecting viral constructs encoding growth factors directly into the spinal cord avoids the need for retrograde transport of the protein from the muscle. Several growth factors such as GDNF, IGF-I, vascular endothelial growth factor (VEGF), ciliary neurotrophic factor (CNTF), and brain-derived growth factor (BDNF) have been evaluated in experimental models of ALS (for review see [49, 50]). In nearly all cases, these factors have had positive effects on both MN survival and function in $SOD1^{G93A}$ rodents [51–54].

GDNF is important in the branching of neurons at the NMJ and modulating synaptic plasticity [55]. The enhanced

expression of GDNF in the muscle of the SOD1^{G93A} mice delays disease onset, improves locomotor performance, and increases lifespan [51–53, 56–58]. However, delivering GDNF directly to the MNs within the spinal cord had only modest effects on the survival of facial MNs and no effect on lumbar MN survival or function. This was observed even though high levels of GDNF were expressed directly around dying MNs [59]. In support of this study, another report used promoter-driven transgenic mice to overexpress GDNF locally in either the muscle or spinal cord of SOD1^{G93A} animals. GDNF expression in the muscle was able to slow disease progression and onset, but expression in the spinal cord had no effect [60].

In previous years, we demonstrated that intramuscular GDNF delivery using stem cells helps preserve NMJs (Figure 2(c)) [61]. Human mesenchymal stem cells (hMSC) were genetically modified to release GDNF (hMSC-GDNF) and were transplanted into the limb muscles of presymptomatic SOD1^{G93A} rats. These cells survived, released GDNF, and significantly affected innervation of NMJs in the transplanted muscle at 6 weeks post surgery. hMSC-GDNF transplanted rats also survived ~18 days longer than their control littermates when animals were kept until endpoint [61].

IGF-I has been known to play a key role in MN survival, axonal growth, and the maintenance of synaptic connections [62, 63]. This trophic factor is involved in muscle and nerve tissue anabolism and thus induces muscle hypertrophy and promotes neural survival. After intramuscular treatment with adeno-associated virus expressing IGF-I, it was shown that IGF-I can be retrogradely transported from muscle to the spinal cord and led to MN protection in the SOD1^{G93A} mice [52]. This effect was further increased when physical exercise was associated with treatment [64]. Another study reported that muscle-restricted expression of IGF-I isoforms maintained muscle integrity, stabilized neuromuscular junctions, enhanced MN survival, delayed the onset of disease, and slowed disease progression in the SOD1^{G93A} mice [65]. These studies reappraised the potential role of the skeletal muscle and IGF-I signaling as a target for treatment in ALS patients.

VEGF is another trophic factor that contributes to the pathogenesis of ALS and possibly applies to muscle-target treatments. In SOD1^{G93A} mice, increased expression of VEGF by intramuscular viral injections prolongs their survival and enhances motor performance [7, 53]. Also, intracerebroventricular administration of VEGF in a rat model of ALS enhanced MN survival, while an intraperitoneal injection of VEGF led to the preservation of NMJs [66].

Despite the promising effects in preclinical studies, several growth factors, including BDNF, CNTF, and IGF-I, did not yield positive results in clinical trials for ALS patients [67]. However, the failure of these trials may be attributed to factors such as inappropriate delivery routes and doses which were validated in preclinical trials and may have affected the pharmacological concentration of growth factors in target tissues [68]. Therefore, the therapeutic benefits of these growth factors may need to be tested using the direct delivery into skeletal muscle.

NMJ degeneration may also be alleviated by controlling abnormally elevated energy metabolism which occurs in muscle. It has been suggested that hypermetabolism in skeletal muscle drives a chronic energy deficit in SOD1^{G93A} mice which precedes amyotrophy and muscle denervation. SOD1^{G93A} mice show a body weight deficit compared to wild type mice [33]. This body weight deficit was not due to decreased food intake, but rather to an increase in the basal metabolic rate. Energy metabolism, especially lipid metabolism, was strikingly altered in these animals. Furthermore, gene expression changes and increased muscle glucose uptake implicated the muscle as a site of excessive nutrient consumption in SOD1^{G93A} mice. Interestingly, a high-fat diet used to increase energy levels was enough to prolong the life of SOD1^{G93A} rats and reduce muscle denervation, although this strategy might not work well for human ALS patients due to insulin resistance [37, 69].

If an altered metabolic rate in skeletal muscle is critical for NMJ degeneration, exercise would also be expected to benefit ALS patients. Recent studies using ALS mouse models have reported a life span increase in exercised animals [70, 71]. Therapeutic exercise is also feasible, tolerated, and safe for patients with ALS [72, 73]. Clinical trials of ALS patients have suggested that regular physical exercise may be neuroprotective, ameliorate symptoms, and improve functionality [74]. Interestingly, synergistic effects of IGF-I gene delivery and exercise have profound effects on survival function [64]. Therefore, it is possible that combining exercise and stem-cell- or viral-based growth factor delivery may provide a more powerful therapy.

8. Conclusion

ALS is emerging as a "multisystemic" disease in which structural, physiological, and metabolic alterations occur in different tissues and cell types such as MNs, glia, and muscle tissues. The degenerating processes may act synergistically to induce and exacerbate the disease. Recent studies have provided evidence supporting a "dying-back" hypothesis in which distal NMJ degeneration precedes proximal neuronal cell death. It has been proposed that NMJ degeneration is not initially noticeable due to reinnervation processes by remaining axons of the muscle fibers as part of a compensatory mechanism. Eventually, however this process cannot keep up with the disease progression and muscle weakness is observed. Growth factor delivery targeting the skeletal muscle has provided significant results in protecting NMJ innervations, increasing MN survival, and prolonging the survival period of rodent models of ALS. On the other hand, treatments to rescue MNs according to a "dying-forward" model of MN pathology in ALS have shown only limited success in SOD1^{G93A} transgenic rodents as well as humans. Due to the accessibility of muscle tissue, it is much easier to directly deliver growth factors in muscle than in other tissues such as the spinal cord. Perhaps the most powerful approach will be to target both the spinal cord (i.e., cell body) and muscle (i.e., nerve terminals of MNs).

Authors' Contribution

D. Krakora and C. Macrander contributed equally to this work.

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Review Article

ALS and Frontotemporal Dysfunction: A Review

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Though once believed to be a disease that was limited to the motor system, it is now apparent that amyotrophic lateral sclerosis (ALS) may be associated with cognitive changes in some patients. Changes are consistent with frontotemporal dysfunction, and may range from mild abnormalities only recognized with formal neuropsychological testing, to profound frontotemporal dementia (FTD). Executive function, behavior, and language are the most likely areas to be involved. Screening helpful in detecting abnormalities includes verbal or categorical fluency, behavioral inventories filled out by the caregiver, and evaluation for the presence of depression and pseudobulbar affect. Patients with cognitive dysfunction have shortened survival and may be less compliant with recommendations regarding use of feeding tubes and noninvasive ventilation. Evolving knowledge of genetic and pathological links between ALS and FTD has allowed us to better understand the overlapping spectrum of ALS and FTD.

1. Introduction

Amyotrophic lateral sclerosis, or ALS, was first described by Charcot in the nineteenth century. Much of his clinical description continues to hold true to this day. Patients experience progressive, painless weakness that may originate in the arm, leg, or bulbar musculature. Associated with this is atrophy of the muscles; fasciculations may also be seen. In addition to these lower motor neuron manifestations, upper motor neuron findings are found on examination including increased tone, exaggerated deep tendon reflexes, and pathological reflexes such as a Babinski sign or jaw jerk. Sensation and bowel and bladder function are typically spared [1]. In Charcot's description, cognitive changes were not described. Marie, a peer of Charcot's, described emotional lability in patients with ALS in 1892 [2], and reports of ALS patients with cognitive changes including, irritability, delusions, and hallucinations, date to at least the early part of the twentieth century [3–6]. However, for many years it remained entrenched in the teaching of neurology that the body wasted yet the mind was spared in ALS. This may for a while have been a self-fulfilling prophecy; since it was not expected, it was not looked for by physicians and not reported by families. The very nature of the symptoms related to ALS may have also created obstacles to recognizing cognitive symptoms; for example, ALS patients may stop working because of their weakness or slurred speech, so may not be in a setting requiring them to perform complex planning and decision making. The concept of clinics devoted to the care of ALS patients, pioneered by Stan Appel and Forbes Norris, means that ALS patients, once seen at most a few times by their local neurologist and then cared for by an internist until their death, are now being seen regularly throughout the course of their disease by neurologists who were seeing tens of hundreds of ALS patients rather than one or two per year. As reports of patients with motor neuron disease (MND) associated with dementia were published in increasing numbers [7–18] and the clinical features of frontotemporal dementia were better described including consensus criteria first published in 1998 [19, 20], it became clear that the dementia seen in ALS patients is best characterized as FTD [21, 22]. This review will address what constitutes frontotemporal dysfunction in ALS and how frequently it occurs, how to best evaluate cognition in ALS patients, and what is understood about the pathology and genetics of ALS and FTD.

Table 1: Behavioral features in FTLD [23, 24].

Disinhibited type
Increased interest in sexual activity
Lack of judgment
Swearing
Violation of personal space
Impulsive buying
Paranoia
Criminal activity
Grandiose thinking
Ignoring social etiquette
Apathetic type
Blunted emotions
Disinterested and withdrawn
Lack of attention to personal hygiene
Lack of empathy
Stereotypical type
Hoarding
Food fads, overeating
Ritualistic/repetitive behavior

2. Frontotemporal Dementia

FTD has an insidious onset with a slowly progressive course with age of onset typically in the 50's and 60's; hence early on it was referred to as presenile dementia. There is relative preservation of memory, praxis, and visuospatial skills with impairment of behavior, language, and/or personality. Patients characteristically lack insight into their problems. Initial features at presentations may include changes in behavior (behavioral variant or bvFTD), difficulty with expression of language but with relative preservation of comprehension (primary progressive aphasia or nonfluent progressive aphasia), or impaired language characterized by anomia in conjunction with impaired comprehension (semantic dementia) [23]. Patients with bvFTLD may be disinhibited, apathetic, or manifest stereotypical behaviors (Table 1) [23, 24]. Features associated with nonfluent progressive aphasia may include anomia, phonemic paraphasia, grammatical errors, stuttering, oral apraxia, alexia, or agraphia [20, 25, 26]. Semantic dementia, the least common type of FTLD, is characterized by speech that is fluent and grammatically correct but empty of content. Naming of people, both familiar and famous, is frequently impaired, and while confrontational naming is very poor, repetition is generally preserved [27]. Executive dysfunction is common early in FTD; when seen in Alzheimer's disease it typically occurs later [19, 28]. Executive dysfunction is reflected in problems with planning, organizing, abstracting, and prioritizing, along with impaired verbal fluency [19–22].

3. Frontotemporal Dementia and ALS

In one of the earlier reviews of dementia and ALS, Hudson identified 60 families with ALS, including 9 (15%) families who also had dementia. In addition, he reported 42 sporadic cases of ALS with either dementia or parkinsonism, suggesting overlap between various neurodegenerative syndromes. [29] Autopsies in early reports of patients with both ALS and dementia found minimal to marked frontotemporal atrophy but absence of Alzheimer's changes with no granulovacuolar degeneration or tangles [7, 29].

Patients with ALS-FTD typically have onset of symptoms in their 50's, and like ALS without dementia, it is slightly more common in men than women. The ALS symptoms may precede, occur simultaneously, or follow the signs and symptoms of FTD, though the most common finding is to have cognitive change first followed by weakness. Interval between the cognitive symptoms and weakness may be a few months to up to 7 years, with a mean of 2 years [7, 29–35]. Some [30–32, 36] but not all [37, 38] authors found bulbar onset disease more often in patients with ALS-FTD compared to those with ALS alone. At least some patients may have significant upper extremity weakness while lower extremities are relatively preserved, with maintained ambulation even at the time of death [7, 8, 29, 33, 36, 39]. Behavioral changes may include euphoria, indifference, and personality changes, while language impairment includes paucity of speech, echolalia, impaired comprehension, and even mutism [7, 29, 36, 39].

Survival in patients with ALS-FTD is worse compared to those with ALS alone or FTD alone [30, 40]. In those who are cognitively impaired but not frankly demented, the type of frontotemporal dysfunction may influence survival. ALS patients with dysexecutive function may have worse survival but those with abnormalities limited to language or visuospatial skills have similar survival compared to the cognitively normal ALS patient [40, 41]. ALS patients with dementia primarily characterized by poor memory such as seen in Alzheimer's disease, did not have further shortening of survival [37, 40].

EEGs are frequently normal though may show background or focal slowing [8, 9, 42]. SPECT scan may show reduced uptake in the frontal lobes [8, 39, 42]. In a voxel-based morphometry study, MRI's of both ALS and ALS-FTD patients had atrophy in the frontotemporal regions compared to controls, though frontal atrophy was greater in the ALS-FTD patients [43].

Strong and colleagues proposed the following classification system for the frontotemporal syndromes in ALS: ALSci or cognitively impaired, ALSbi or behaviorally impaired, ALS-FTD in which Neary criteria for FTD are met, FTD-MND like in which patients clinically had FTD and pathologically have motor neuron loss but did not manifest signs of motor neuron disease during life, and ALS-dementia in which patients have Alzheimer's or vascular dementia. Patients with ALSci may have impaired verbal fluency or executive dysfunction, and those with ALSbi have the behavioral features associated with frontotemporal dysfunction, but do not meet Neary criteria for FTD [44]. Patients with the behaviorally predominant form of ALS-FTD may also have impaired language, with reduced verbal fluency as the most common language deficit found [21, 45]. Occasional ALS-FTD patients have primary progressive aphasia as the main manifestation of their dementing illness [46, 47]. Many

of these patients become mute as their disease progresses [36, 48]. For those with profound dysarthria secondary to weakness of bulbar musculature, it may be difficult to recognize the degree of their aphasia. However, if they have sufficient hand strength to hold a pen, the degree of language impairment may be apparent through their writing.

Overall, memory is relatively preserved in patients with ALS-FTD, and the memory problems reported are believed by memory problems reported are believed by most neurologists to be related to frontal dysfunction. Patients may have difficulty with retrieving memories and have poor learning strategies [38]. Lack of concentration and poor attention may also play a role in a patient's performance [49]. In keeping with the dementia of ALS not fitting an Alzheimer's-like pattern, the Mini-Mental Status Examination is typically normal in ALS patients [32].

4. Cognitive Testing in Patients with ALS

Following ever increasing papers reporting frank dementia with ALS, investigators started to pursue whether more subtle cognitive changes in patients may be present in some patients. Testing is potentially problematic; slurred speech makes it difficult to do verbal testing, hand weakness may mean written tests are not possible, and timed tests need to be adjusted to accommodate slowness related to the ALS. However, as many patients are now followed in multidisciplinary ALS clinics at regular intervals, some clues of early cognitive changes may come to light in this setting. For example, patients who previously had no problems understanding the instructions on how to do a vital capacity may have difficulty figuring out how to time their breaths for the test as their ALS progresses. Those who communicate by writing and early on had perfect syntax and spelling may start to make spelling and grammatical errors later on in their disease. Such observations piqued the interest of investigators to determine the frequency and extent of cognitive problems in the ALS patient.

In an attempt to minimize the limitations dysarthria and hand weakness create for testing in this population, a neuropsychological battery administered by computer touch screen, the Cambridge Neuropsychological Test Automated Battery (CANTAB), was used to study 69 patients with ALS. In addition to performing the CANTAB, treating neurologists were asked to determine if they believed the patient was demented based upon standard criteria for dementia, and based upon FTD specific criteria for dementia. Using traditional criteria, 7% of patients were classified as demented, and 33% possibly demented. Using FTLD criteria, the number of patients classified as demented jumped to 22%, while 27% where classified as possibly demented. Patients clinically diagnosed with dementia had difficulty with new learning and planning, along with rigid thinking and slowness of processing information in keeping with frontotemporal dysfunction [50].

Using a caregiver questionnaire avoids the limitations posed by the patient's physical disabilities. In addition, because patients with FTD frequently are oblivious to behavioral changes, caregivers can better identify them. The Neuropsychiatric Inventory (NPI) [51], Frontal Behavioral Inventory (FBI) [52], Cambridge Behavioral Inventory (CBI) [53], and Frontal Systems Behavioral Scale (FrSBe) [54] were designed by the cognitive neurologists to help screen for frontotemporal dysfunction. The FrSBe has both a self-rating as well as a caregiver form; the NPI, CBI, and FBI are all caregiver-only forms [51–54]. Having used both the NPI and CBI in our patient population, we found the latter more useful in identifying behavioral changes [55, 56]. Apathy is identified in up to 56% of patients in studies using CBI or FrSBe [57–59] and correlated with reduced verbal fluency [60] but not with arterial blood gasses or the ALS functional rating scale [58]. Besides apathy, other common findings from using one of these caregiver inventories have included stereotypical behaviors in 20% [61], executive dysfunction in 34–48% [41, 59], and disinhibition in 18–27% [41, 61]. Using the CBI, 11% of the patients met criteria for FTD [61]. There have been conflicting results regarding correlating degree of abnormalities found with bulbar onset disease [59, 61]. Depression was found in up to a third of the caregivers; it was related to the patient's behavioral symptoms in one study [62] but not confirmed in a subsequent study [61].

Lomen-Hoerth and colleagues screened 100 patients with ALS for cognitive abnormalities using verbal fluency and the Mini-Mental Status Examination (MMSE). Thirty-one had abnormal verbal or categorical fluency in one minute, defined as a score of fewer than 8 "d" words and less than 13 animals. If they were anarthric, they could write their answers. Half of the patients with bulbar onset disease had an abnormal score while approximately a quarter of patients with limb onset disease had an abnormal score. No patient had an abnormal score on the MMSE. All 100 patients were asked to undergo more extensive testing, 44 agreed and had additional tests of executive function, memory, language, and visuospatial skills. Of these, 18 had results consistent with probable or definite FTD, with 12 having the primarily behavioral type, 4 with primary progressive aphasia, and 2 with semantic dementia. An additional 5 patients had possible FTD. FTD was more likely to be diagnosed in older patients, those with a family history of dementia, Parkinson's disease, or ALS, and had a lower forced vital capacity [32].

Of 160 patients studied with a cognitive battery that evaluated executive function, memory, language, and visuospatial skills, 14% met Neary criteria for FTD, 21% had executive dysfunction, 14% were impaired in one of the other areas tested, and 47% were cognitively normal. Patients with executive dysfunction were older and had more rapid disease progression, but there was no relationship to having bulbar onset ALS [63]. In another study of 279 patients, similar distributions were found; 15% had FTD, 49% were cognitively normal, 32% were mildly impaired, and 13% moderately impaired. Areas most likely to show abnormalities included attention, concentration, and working memory; confrontational naming and memory were also abnormal, though to a lesser degree [64].

In a study of 40 patients, the diagnosis of dementia required memory dysfunction, as well as impairment in at least 2 other domains (executive function, language, visuospatial skills, and attention). Using this definition, 70%

of the patients were cognitively normal, 13% had probable, 10% had possible dementia, and 8% were mildly cognitively impaired. The larger number of cognitively normal subjects reported in this series compared to others is likely a reflection that memory impairment had to be present, while this is usually missing in FTD. Similar to other studies, impairment of executive function including letter and category fluency was the most likely test to be abnormal. There was no association with cognitive function and bulbar onset, respiratory status, or disease duration [37].

There are a relative paucity of studies that have looked at cognitive function in ALS patients over time; those that have been done have showed varying results. In one such study of 52 patients retested every 4 months, while verbal and nonverbal fluency and concept formation were abnormal compared to controls, they did not decline significantly over time [65].

In two small studies with patients undergoing repeat testing at 6 months in one and 9 months in the other, there was no significant decline on the whole [66, 67]. However, at both test times, ALS patients performed in the abnormal range in generating "s" words. At the second test, ALS patients scored at least 1 standard deviation below the scores of controls on the following tests: verbal and written word generation, motor free visual perception test, and recognition memory test of faces. Overall, bulbar onset patients did less well than limb onset patients on cognitive testing, and their performance worsened on repeat evaluation [66]. As individuals, 7/19 patients declined in tests over time, but in only one performance was abnormal for the majority of tests [67].

What about the patients with FTD? How often do they show signs of motor neuron disease or frank ALS? Thirty-six patients with FTD had a detailed neuromuscular examination and nerve conduction studies and electromyogram (EMG). According to the El Escorial criteria, 5 patients had definite ALS and one patient had neurogenic changes limited to a single limb. Six patients had dysphagia of unclear cause. Five patients had fasciculations but EMG revealed no other abnormalities. However, one of these patients went on to develop definite ALS a year later [68]. In a retrospective study of patients who first presented with behavioral variant FTD and were followed until their death, 18 of 61 subsequently developed ALS. Those who developed ALS were more likely to have an onset of symptoms of delusions and word finding difficulties and less likely to have memory problems compared to those that purely had FTD [30]. A study of 319 FTD patients with extended followup found that motor neuron disease only developed in 8 of them [69].

Primary lateral sclerosis (PLS) and progressive muscular atrophy (PMA) are viewed as ALS variants, the former associated with purely upper motor neuron dysfunction and the latter with purely lower motor neuron involvement. Neuropsychological testing in patients with PLS has found mild cognitive impairment tests of frontal function including verbal associative fluency and psychomotor speed [70]. Autopsy of 2 cases of FTD (one with executive dysfunction followed by aphasia, the other with expressive aphasia and apraxia) found TDP-43 and ubiquitin positive inclusions in frontal and temporal neurons as well as neuronal loss in the motor cortex and degeneration in corticospinal tracts. There was no evidence for lower motor neuron pathology. One patient had been diagnosed with PLS premorbidly; the other had Parkinsonism but was not seen for 4 years prior to death [71]. A review of 5 cases of possible FTD-PLS identified only one patient whose autopsy found no lower motor neuron involvement [72]. A minority (17%) of patients with PMA had cognitive impairment defined as 2 standard deviations below the norm in letter-number sequencing and immediate and delayed story recall [73]. Overall, there is a sense that patients with PMA and PLS are more likely to be cognitively normal compared to those with ALS though further testing is warranted.

5. Screening for Cognitive Impairment in the Clinic

Extensive neuropsychological testing in every patient seen in the ALS clinic is not feasible for most clinics. It can be expensive, time consuming, and for patients with marked weakness and dysarthria, not technically realistic. Therefore, developing a screening battery that can be done within these limitations is important. When testing ALS patients, one also needs to take into account respiratory status, pseudobulbar affect, medications, depression, and pain. In a 2009 practice parameter update from the American Academy of Neurology, the authors acknowledged lack of consensus on how to best study cognitive changes in patients with ALS [74]. There is currently an FTD task force as part of the Northeast ALS (NEALS) Consortium working on this, as well as an effort by the NIH to identify common data elements for investigators to use, including studying cognition in ALS patients [75]. Several investigators have developed screening tools to evaluate cognition in ALS patients. The ALS Cognitive Behavioral Screen, developed by Wooley and colleagues, includes a 15-item ALS specific behavioral questionnaire filled out by the caregiver, and an 8-item cognitive assessment of the patient that is estimated to take only 5 minutes; it has been validated [76]. The Penn State Screen Battery of Frontal and Temporal Dysfunction Syndromes takes approximately 20 minutes; and is currently being used in a nationwide study to establish its validity [77, 78]. The UCSF screen battery is the longest of the screens, taking approximately 45 minutes to complete. It includes an ALS specific version of the FBI, written verbal fluency, the ALS Cognitive Behavioral Screen, an emotional lability scale, and the Beck Depression Inventory-II [75]. In my clinic, I screen with category fluency, the CBI, and antisaccade testing [55, 79]. The latter consists of asking the patient to look in the opposite direction to your wiggling finger, and is a measure of frontal lobe inhibition [80]. For patients whose bulbar dysfunction precludes spoken tests of fluency, written verbal fluency can be performed. Patients are given five minutes to write "s" words and four minutes to write "c" words that contain only four letters; an index is calculated taking into account how long it takes the patient to copy the same words they spontaneously wrote [81].

6. Pathology of ALS-FTD

Patients with FTD pathologically may demonstrate a tauopathy; in those without a tauopathy, the pathology was originally described as FTD-U for ubiquitin positive but tau-negative inclusions [82]. In 2006, 2 groups reported that the ubiquitinated inclusions seen in FTD with ALS were TAR-DNA-binding protein 43 or TDP-43 [83, 84]. Burden and distribution of the TDP-43 varied depending upon the clinical phenotype. Patient with pure ALS have TDP-43 pathology primarily in the spinal cord, those with pure FTD have TDP-43 pathology primarily in the cortex, while those with FTD-ALS have TDP-43 pathology in both areas [85, 86]. However, TDP-43 inclusions are not only found in the expected areas for ALS and FTD, but can also be seen in the cerebellum, parietal, and even occipital lobes although to lesser degrees, suggesting TDP-43 is part of a multisystem neurodegenerative process [87]. TDP-43 levels in CSF are higher in patients with ALS or FTD, but there is overlap between the patient groups as well as with controls [88].

In a patient with ALS, FDT, and Parkinsonism, TDP-43 pathology was found not only in the motor system, hippocampus, and amygdala; it was also prominent in the globus pallidus, caudate, and putamen [89]. This spectrum of TDP-43 burden that reflects clinical manifestations give further proof that ALS and FTD may share similar mechanisms of protein misfolding.

SOD-1 mutations are responsible for approximately 20% of the familial cases of ALS [90].

Although ubiquitin positive neuronal inclusions were found at autopsy of patients with SOD1-associated ALS, the inclusions were not immunoreactive with TDP-43 [91].

7. The Genetics of ALS and FTD

The link between ALS and FTD has been further forged as we have learned more about the genetics of both diseases. The majority of ALS is sporadic, but 5–10% is inherited. The first gene identified as playing a role in familial ALS was the superoxide dismutase gene, or SOD1, and is believed to be responsible for about 20% of the familial ALS cases; mutations are also found in approximately 2% of sporadic ALS [90, 92]. There is no association between the SOD1 gene and dementia; patients with the SOD1 mutation compared to those with sporadic ALS performed better on cognitive testing and at a level similar to controls [93]. FTD has a higher familial incidence than ALS; with 42–45% of FTD patients having a positive family history for a similar disorder [94, 95]. Within the same families there were cases of ALS, FTD; and ALS-FTD identified. In 2000, a genome wide-linkage analysis of two large data sets consisting of over seven hundred families, found a genetic locus between D9S301 and D9S167 on chromosome 9q21-q22 linked to ALS with FTD [96]. A previously identified senataxin or SETX gene mutation, also located on chromosome 9 (9q34), has been linked to the rare juvenile-onset dominant FALS, however, the two loci above do not overlap, and the pattern of the disease is very different in each [97]. A second genome-wide linkage study in a large ALS/FTD kindred in 2006 also showed linkage to chromosome 9, but at 9p13.3-21.2 [98]; similar results were found by other researchers as well [99, 100]. In 2011, two groups of researchers were able to uncover the specific mutation within this locus leading to ALS/FTD. It was an expansion of a GGGGCC hexanucleotide repeat in the intron of protein *C90RF72* leading to an alternative splicing of this protein that is the responsible mutation [101, 102]. Less than 23 repeats corresponded with a wild-type allele, while more than 30 repeats corresponded with individuals expressing the disease. The US-based study found a prevalence of this expansion in 12% of familial FTD and 22.5% of FALS [101]. The study involving the European population, found higher prevalence rates: 46% in familial ALS, 21% in sporadic ALS, and 29% in familial FTD [102]. Clinically, the patients differ from the typical patient with FTD because of prominent psychiatric symptoms, including delusions, paranoia, and irrational thoughts [103]. While TDP-43 deposition is found in patients with this mutation, p62 positive cytoplasmic inclusions in the hippocampus and cerebellum are another hallmark pathological feature [104, 105].

Discovery that TDP-43 accumulated in neurons in both ALS and FTD [83, 84] led to sequencing of the TARDBP gene in ALS patients. Mutations in the gene were found in 1–3% of patients with both sporadic and familial ALS and patients with the mutations had TDP-43 pathology at autopsy [92, 104–107].

A recent study in Nature involving 19 individuals within a five-generation family uncovered a new gene, UBQLN2 mutation underlying X-linked dominant inheritance of ALS/FTD. This mutation led to ubiquilin 2 protein pathology in the spinal cord and brain. Functional analysis showed an impaired degradation of ubiquinated proteins, a well-known function of the ubiquilin 2 proteins [108].

Motor neuron disease associated with FTD and Parkinsonism, that is, disinhibition-dementia-parkinsonism-amyotrophy complex (DDPAC), is a rare entity that has been linked to a microtubule-associated protein tau gene mutation (MAPT) on chromosome 17q21. This disease is a separate entity from the ALS/parkinsonism dementia complex specific to the Guam population, and pathologically is characterized by a large amount of cytoplasmic accumulation of filamentous tau inclusions [109, 110]. Tau stabilizes and conducts transport of vesicles along cellular microtubules, and neurodegenerative disease has been theoretically linked to these cytoplasmic tau inclusions.

8. Conclusions

When cognitive change was first discussed at ALS meetings, it was met with some skepticism; over time, it has become well accepted that there may be a spectrum of cognitive changes in patients with ALS [74, 86, 111]. Some patients have normal cognition throughout their disease; others have mild cognitive impairment, while relatively few are frankly demented. Abnormalities identified are typically ones that imply involvement of the frontotemporal lobes, including dysexecutive syndrome, behavioral changes, and language dysfunction. The frequency and severity of cognitive changes

in ALS has varied widely in reports to date, likely in part related to the tests used and how dementia was defined [29–35, 37, 38]. The challenges of trying to evaluate cognition in patients who may have trouble speaking and writing cannot be ignored. Does it matter that patients with ALS may not be cognitively intact? It is true that some patients would rather not know that cognition is affected, and this may influence the number of patients who are willing to enroll in studies investigating this topic. Family members, however, may be reassured when they learn some of the things they have noticed in their loved one can be explained by cognitive decline that is part of the disease process. It may also influence treatment and equipment needs. For example, a patient with cognitive impairment may not be able to learn to use a computer-based augmentative language device. Patients who lack insight into the severity of their disease may not understand the importance of trying to adapt to using noninvasive ventilation, or may not be appropriately cautious when walking with leg weakness. Patients with ALS-FTD were less likely to be compliant with recommendations for percutaneous endoscopic gastrostomy or use of non-invasive ventilation compared to cognitively intact patients [41, 112]. Patients with ALS may have difficulty interpreting the emotions associated with facial expressions, even when they are otherwise cognitively normal; this may impact their relationships with their caregiver and possibly influence medical decision making [113]. Caregivers of those with behavioral symptoms have lower quality of life, higher caregiver burden, and higher rates of depression [59]. Behavioral problems may need to be managed; the selective serotonin uptake inhibitors have been found to be beneficial in treating patients with FTD, and so likely should be tried in the ALS patients who have behavioral management issues as well [114].

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Review Article

Magnetic Resonance Imaging in Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a rapidly progressing neurodegenerative disorder which is incurable to date. As there are many ongoing studies with therapeutic candidates, it is of major interest to develop biomarkers not only to facilitate early diagnosis but also as a monitoring tool to predict disease progression and to enable correct randomization of patients in clinical trials. Magnetic resonance imaging (MRI) has made substantial progress over the last three decades and is a practical, noninvasive method to gain insights into the pathology of the disease. Disease-specific MRI changes therefore represent potential biomarkers for ALS. In this paper we give an overview of structural and functional MRI alterations in ALS with the focus on task-free resting-state investigations to detect cortical network failures.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease which affects not only motor function but also involves extramotor systems. According to the revised El Escorial criteria for the diagnosis of ALS the presence of signs for the affection of both upper motor neurons (UMN) in the primary motor cortex and lower motor neurons (LMN) in brain stem and spinal cord is mandatory, and the disease must be progressive [1]. ALS, has a wide variety of clinical phenotypes, and it is therefore sometimes difficult to differentiate ALS from other ALS-mimicking conditions. For the detection of LMN involvement in different body regions, electromyography (EMG) can be used in addition to the clinical examination. UMN signs, on the other hand, must be visible at the clinical examination while electrophysiological transcranial motor stimulation (TMS) abnormalities are not accepted for the diagnosis of ALS according to the El Escorial criteria. Therefore it would be very advantageous to have an additional technical method which sensitively monitors UMN involvement. Magnetic resonance imaging (MRI), and here in particular diffusion tensor imaging (DTI), represents a promising technique for early detection of alterations in the motor cortex and pyramidal tracts. Different other MRI techniques are also currently being developed to serve as biomarkers for earlier and more accurate diagnosis of ALS. A biomarker for UMN affection would further be useful to monitor the neurodegenerative process and therefore disease progression, that is, within clinical trials. Guidelines for the use of neuroimaging in the management of motor neuron diseases have recently been published by the European Federation of Neurological Societies (EFNS) [2]. In 2010, an international group of experts has met at Oxford University, UK, to define essential parameters for future research needed to promote MRI as a biomarker for ALS.

It was concordantly proposed to initiate longitudinal and multicenter studies and thus to analyze larger sample sizes so that results can be optimized and MRI can become a more specific diagnostic tool. Within MRI, one must distinguish between structural and functional MRI techniques. Structural MRI detects morphological changes in grey and white matter. The diffusion tensor imaging method can be used for tractography (e.g., imaging of pyramidal tracts) or to study the connection between different cortical grey matter areas. Structural MRI at present mainly serves to rule out other diseases mimicking ALS but is also supposed to be useful in finding cortical atrophy in ALS. Functional MRI (fMRI) can detect cortical activations corresponding to a task (e.g.,

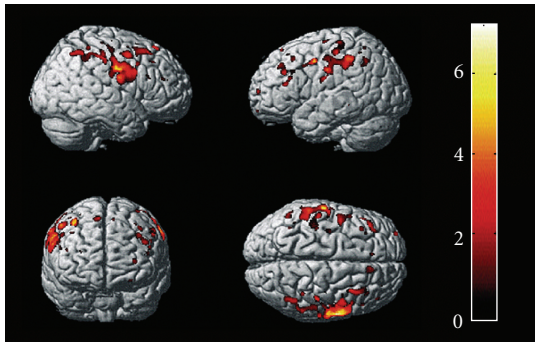


Figure 1: Regional grey matter atrophy in ALS patients compared with controls: group comparison of ALS patients versus healthy controls showed regional grey matter atrophy in the precentral and postcentral gyrus bilaterally, which extended from the primary motor cortex to premotor, parietal and frontal regions bilaterally. The colour bar indicates the statistical strength of the regional atrophy (yellow-white is most significant). Adapted from Grosskreutz and coworkers [4].

motor task) performed by the participant during scanning. The resting-state technique detects fluctuations in different cortical areas during rest (no task performance needed) and visualizes different functional networks such as the sensorimotor network, visual network, and others. FMRI methods are therefore capable of detecting ALS-related differences in brain activation, compensation, and reorganisation.

This paper describes the structural and functional MRI alterations which have been found in ALS to date, with a particular focus on task-free resting-state investigations to detect cortical network failures.

2. Structural Magnetic Resonance Imaging

2.1. Voxel-Based Morphometry. The voxel-based morphometry (VBM) technique can be used for the analysis of volumetric changes in gray or white matter (GM; WM) in the brain [3–5]. It is an automated analysis of changes in brain volume using high-resolution three-dimensional T1-weighted MRI scans. During the statistical process, potential structural changes in individual patients are compared to a template of age-matched controls. By this approach, neuroanatomical differences can be detected with much greater sensitivity [2, 6–8].

2.1.1. Motor Cortex. Several VBM studies have described atrophy of the primary motor cortex (Figure 1) but this has surprisingly not been a consistent finding in all studies published so far [3, 4, 9, 10]. Marked decreases in the grey matter in the bilateral paracentral lobule were also detected, indicating that the premotor cortex is also involved in degenerative processes in ALS [11].

2.1.2. Extra-Motor Involvement. Regional gray matter loss measured by VBM extends to the frontal, temporal, parietal, occipital and limbic regions of the brain and has also been described for the corpus callosum and the cerebellum which is line with clinical and neuroanatomical data [3, 5, 10, 12–14].

3. Diffusion Tensor Imaging (DTI)

It is known from postmortem studies in ALS brain specimens that there are extensive white matter abnormalities in the region of the central sulcus and the corticospinal tract (CST), extending across the corpus callosum and into the frontal lobes [15, 16].

To investigate white matter and the directionality of fiber tracts, diffusion tensor imaging (DTI) detects alterations in the degree (axial diffusivity, AD) and directedness (fractional anisotropy, FA) of proton movement. It is sensitive to the direction of water movement *in vivo.* As the diffusion properties of water molecules, demonstrated by DTI, are restricted by the presence of barriers (e.g., cellular membranes), the water molecules tend to diffuse preferentially in orientations along axons, leading to an anisotropic diffusion. Therefore, DTI is used to detect pathology within neuronal white matter tracts and reflects microstructural tissue changes [13]. FA is reduced with loss of neuronal pathway integrity; mean diffusivity (MD) is increased with a loss of neuronal pathway integrity [7].

3.1. Corticospinal Tract (CST). The CST is the structure most frequently studied by DTI in ALS [3, 4, 17] and decreased fractional anisotropy (FA) values in this area have consistently been reported [18–22]. To date, correlation of disease severity and decreased FA has been controversially debated, as some studies found a relation between these factors [20, 23, 24] whereas others did not [25–27]. It is also discussed controversially if increased mean diffusivity (MD) correlates with disease duration, as this was reported by [24, 26, 28], but not by other authors [23]. One other group demonstrated a correlation of a lower mean FA in the CST with rapid disease progression [29].

3.2. Corpus Callosum (CC). Neuropathological studies have shown involvement of the corpus callosum (CC) in ALS [15, 16] and so did several DTI studies, which observed FA changes within the CC of ALS patients [30–36]. The largest FA changes were observed in the posterocentral portion of the CC which is known to link the two motor cortices [9, 16]. The involvement of the CC at an early disease stage would be in line with recent clinical studies [37, 38] and provide an explanation for the focal onset followed by a rapidly spreading progression of the disease.

Unfortunately, the changes in the CC are not specific and were also found in patients with other diseases of the upper motor neuron such as hereditary spastic paraparesis [39, 40] while not detectable in a lower motor neuron syndrome as Kennedy's disease [41].

3.3. Extramotor Involvement. FA was shown to be decreased in the premotor white matter (WM), in the prefrontal white matter, and in the temporal white matter [11, 28, 30, 34–36].

3.4. Spinal Cord. The small diameter of the spinal cord and its surroundings and breathing-mediated movement artefacts make it difficult to investigate the spinal cord by DTI [42]. In one study, the cervical cord has been investigated and compared to controls; ALS patients showed significantly lower FA of the cervical cord while MD did not differ between the two groups [18]. But during the course of the disease (9 months followup) [43], FA showed a significant decrease and MD showed a significant increase in the spinal cord of ALS patients. A further study supports the hypothesis that the degenerative process in ALS is mostly a "dying-back" mechanism, as the distal part of the spinal cord was the most altered one [7, 44].

3.5. Summary I (Structural MRI). According to the consensus guidelines on MRI protocols for studies in ALS patients, DTI is the most promising structural MRI method to detect ALS-related changes not only in the primary motor cortex and the pyramidal tracts but also in brain regions beyond the motor system. DTI scans with a minimum of 12 gradient directions (isotropic voxels with a maximum of 2.5 mm slice thickness) have previously been recommended [42], although, especially for longitudinal studies, 20–30 directions would be preferable in order to permit robust diffusivity measurements [45]. Studies in larger patient cohorts and repeated measurements in the same patients throughout disease progression are necessary to develop DTI as a potential biomarker for preclinical UMN involvement or as a tool to monitor disease progression and the response to therapy in ALS. Beside FA and MD, measuring the strength of connectivity between different anatomical clusters of grey matter can reveal alterations in cortical networks in ALS patients compared to healthy controls. Using DTI one can calculate the connectivity between cortical areas as shown in the following figure (Figure 2, [46]). This novel approach may contribute to an increase in sensitivity and specificity of DTI in ALS.

4. Functional Magnetic Resonance Imaging

Functional MRI (fMRI) means the visualization of brain regions in action and is typically done using BOLD-weighted MRI.

BOLD—fMRI takes advantages of the oxygenation level of blood, which is different during rest and activity of the brain when the brain is active, despite the increase in oxygen consumption, there is a subsequent increase in local blood flow that paradoxically results in a decrease of concentration of deoxygenated haemoglobin in the local microvasculature of the activated region. Oxygenated hemoglobin is weakly diamagnetic, while deoxygenated hemoglobin is strongly paramagnetic, thus an increase in the relative concentration of oxygenated hemoglobin results in a lengthened T2*, giving an increase in local MRI signal for T2*-weighted MRI.

This change leads to an increase in the fMRI signal approximately 4 seconds after the neural event in the brain. Thereafter, an equilibration of oxy- and deoxyhemoglobin succeeds the "deactivation phase." This contrast alone is too weak to show differences to the surrounding brain regions. Comparisons with the same region at rest have to be done followed by special analysis methods [6, 47–49]. The advantages of the BOLD technique are evident: it is noninvasive, provides high resolution, and has a wide accessibility.

Studies with fMRI using a motor task have shown increased cortical activity in ALS patients in the ipsi- and contralateral sensorimotor cortex, supplementary motor area, basal ganglia, and cerebellum [50–53]. This has been discussed as being either the result of cortical adaptation due to peripheral weakness [52] or of cortical reorganisation [50]. In a recent study we have demonstrated that increased cortical activation can be detected even when the performing hand was clinically not affected and interpreted this as a sign of cortical reorganisation in clinically early stages of disease. In this study we could show that early and late phases of neuroplastic changes in ALS can be distinguished according to different disease stages [54]. In another fMRI study we have described for the first time that the pattern of cortical activation during tongue movements differs in ALS patients with and without bulbar signs [55]. We have further investigated this finding by repeated measurements during disease progression in ALS patients with limb and/or bulbar signs, using two different motor tasks (vertical tongue movement and movement of the right hand). In this study, we detected two different patterns of cortical activation changes which were dependent on the presence or absence of bulbar signs. This observation suggests fundamental differences in the neurodegenerative process and subsequent reorganisation mechanisms according to the affected body regions, which apparently can exist in parallel in the same patients [56].

As it is difficult to control task performance in patients with motor deficits, the analysis of "functional connectivity" of spatially remote brain regions has recently gained increasing interest in neuroimaging research in ALS. The idea is that during rest spontaneous coherent fluctuations of the BOLD signal exist in different brain areas which are functionally connected [57]. Resting-state imaging of discrete cortical networks provides a new technique to explore ALS as system failure of interconnected networks [42]. This method only take minutes to acquire and does not suffer from performance confounds that may be present in patients with cognitive or motor impairments [58–60]. It is therefore more suitable for clinical use and in particular for multicentre studies.

There are different typical resting-state networks which can be recovered from the BOLD signal with high reliability across individuals and studies (Figure 3) [58, 61–63]. One of the consistently recovered networks is the default-mode network (DMN) which is conceptualized as a stand alone cognitive network [64, 65]. It comprises a large frontal area including the ventral anterior cingulate cortex (vACC), the medial prefrontal cortex (MPFC) and the orbitofrontal cortex (OFC)), the posterior cingulate cortex (PCC), the inferior parietal cortex (IPC), and one temporal region, the parahippocampal gyrus (PHG) [62, 66, 67]. Another often reported network is the sensorimotor network [58, 61, 62]

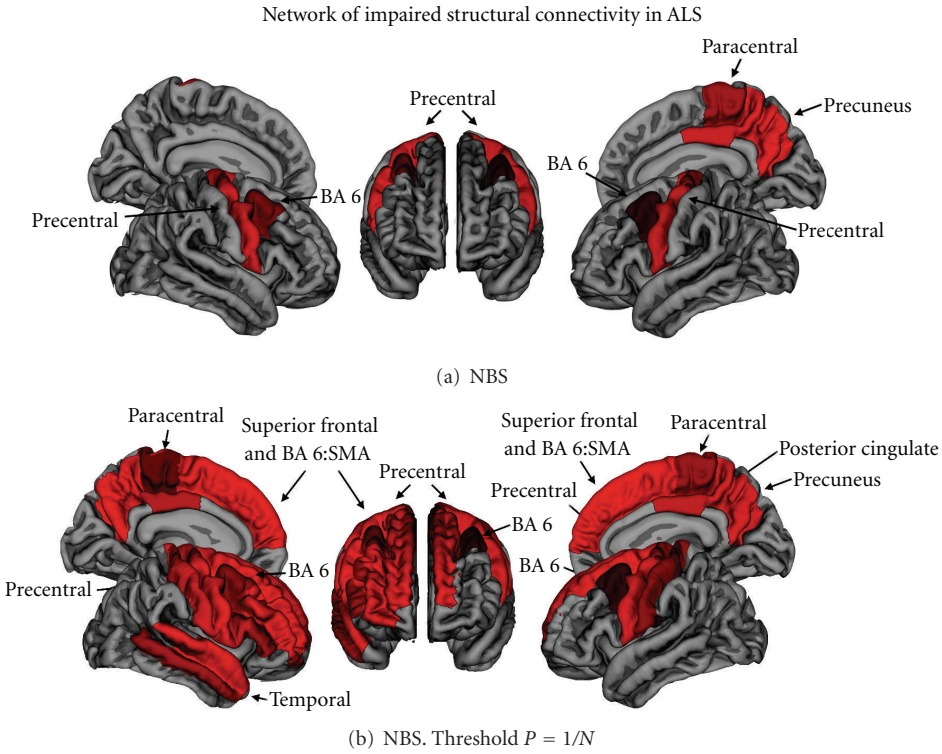


FIGURE 2: Cortical brain regions with impaired structural connectivity in ALS patients. (a) The network-based statistic procedure revealed a subnetwork of brain regions showing significantly reduced structural connectivity in ALS patients, compared to healthy controls. (b) Using an NBS threshold of $P = 1/N$ (N being the number of nodes of the network) a similar but more extended network was revealed. The model-free approach revealed a sub-network consistent with known motor regions, including precentral and paracentral gyri (primary motor), and caudal middle frontal and superior frontal gyri (supplemental motor areas, BA 6). Adapted from Verstraete and coworkers [46].

which includes the primary motor cortex (PMC), the anterior part of the cingulate cortex (ACC), the somatosensory region (SSC), and the auditory cortex (Aud. C) [62, 66–68]. In addition, several other networks such as a visual executive network have been described [58, 61, 62].

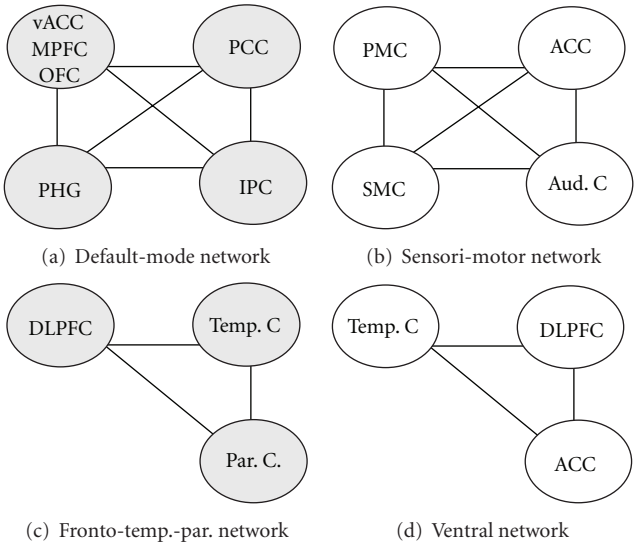
ALS is a neurodegenerative disease which involves mainly the motor system, but already early descriptions [70] and more recent neuropsychological [71–74], electrophysiological [75–77], neuropathological [14], and neuroimaging [78–80] studies pointed out that other than the motor regions of the nervous system are involved in the degenerative process.

We analyzed for the first time the resting-state networks in ALS patients [57]. Given the definition of ALS as a motor neuron disease, we expected most prominent differences between ALS patients and healthy controls in the sensorimotor network. In view of the increasing knowledge about extramotor involvement in ALS as described above, we also suspected differences between ALS patients and healthy controls in the default-mode network.

We investigated 20 patients suffering from ALS and 20 healthy age-matched controls in a 3-Tesla Siemens Magnetom Allegra Scanner (Erlangen, Germany). The first group consisted of 20 patients, who fulfilled the diagnostic criteria for probable or definite ALS during the course of the disease according to the revised El Escorial criteria of the World Federation of Neurology [1]. The control group comprised 20 healthy volunteers. During the data acquisition for functional connectivity the subjects were instructed to neither engage in cognitive nor motor activity. Analysis and visualization of the data were performed using BrainVoyager QX (Brain Innovation BV, Maastricht, The Netherlands) software.

Applying independent component analysis (ICA), different robustly reproducible functional networks could be extracted from the resting state in both groups [81–85]. Only in two networks, the default-mode and the sensorimotor networks, we found significant differences between ALS patients and healthy controls.

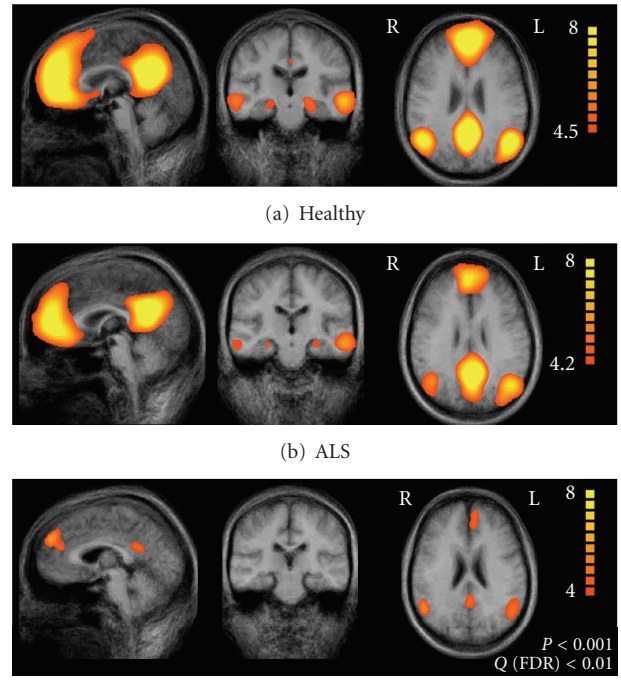
4.1. Default-Mode Network (DMN). This network has received considerable attention over the past few years. In the study presented here, we found distinct differences of the default-mode network comparing healthy subjects with ALS patients; in ALS patients we found a significantly decreased connectivity in the lateral prefrontal cortex (BA9), PCC (BA 23), and IPC (BA39) (Figure 4). The PCC, MPFC, and the bilateral IPC are seen as "core hub" of this network and showed a strong intraregional correlation with each other and a weaker correlation with the remaining regions such as the temporal cortex and the medial temporal lobe [57, 86]. Considering our data, functional connectivity is decreased in the core hub of the default-mode network in ALS patients



(a) Default-mode network (b) Sensori-motor network

(c) Fronto-temp.-par. network (d) Ventral network

Figure 3: Schematic presentation of 4 reliable recovered networks, adapted from Kollewe and coworkers [69]. (a) *Default-mode network*: this network has been reviewed by Raichle and Snyder [65] who have described that activity in this network is high during rest and reduced during cognitive activity. It comprises a large frontal area, including ventral anterior cingulated cortex (vACC), medial prefrontal cortex (MPFC) and orbitofrontal cortex (OFC), the posterior cingulated cortex (PCC), the inferior parietal cortex (IPC), and a temporal region involving the parahippocampal gyrus (PHG). (b) *Sensori-motor network*: this network has been previously identified by a number of authors [62, 66–68]. This network comprises the primary motor cortex (PMC), premotor cortex (PMC), anterior section of cingulated cortex (ACC), the somatosensory region (SSC), and auditory cortex (Aud. C). (c) *Fronto-temporo-parietal network*: this network includes prefrontal (BA9, BA10, BA11), temporal (BA20, BA27), and parietal (BA7, BA39, BA40) regions. (d) *Ventral network*: this network comprises the middle temporal gyrus (Temp. C, BA21), parts of the frontal cortex (DLPFC, BA9, BA47), and parts of the cingulated gyrus (ACC, BA31, BA24). The *posterior network* is not shown; it comprises mainly visual areas in the occipital cortex including BA18 and BA19.



(a) Healthy

(b) ALS

(c) Healthy > ALS

Figure 4: Default-mode network. Upper row (a) illustrates the result of the group ICA analysis for the healthy control participants. The middle row (b) illustrates the results for the ALS patients. The statistical comparison is shown in the lower row (c). Adapted from Mohammadi and coworkers [57].

without affecting subcortical (PHG) or temporal regions. In the prefrontal region we found decreased connectivity in BA 9 which is typically involved in working memory tasks, in tasks of sustained attention, and (bilaterally) in tasks demanding problem solving [87]. In IPC we found reduced connectivity in ALS patients located in BA 39. Left BA 39 is known to be involved in perception, recognition, and recall of written language as well as in problem solving [88].

All in all, the particular pattern of differences between ALS and control subjects for the default mode network (DMN) bodes well with previous neuropsychological studies suggesting an impairment of higher level executive functions [72–74, 89–91].

4.2. Sensori-Motor Network. Regarding the sensori-motor network, our study detected differences between ALS patients and controls only in the premotor area (BA6) (Figure 5). All other regions of the sensori-motor network, in particular the precentral gyrus, did not show significant differences between the two groups. Alterations in premotor cortex activity have been demonstrated in a number of functional imaging studies in ALS [50–53, 92, 93] but it has been discussed that these changes might be due to the fact that the same task might be more difficult for ALS patients (and hence associated with increased activation) rather than due to genuine functional changes. As in the present approach no task is imposed on the subject, our data favour a primary functional involvement of the premotor cortex in ALS.

Taken together, we demonstrated significant changes in the DMN and the sensori-motor network. This suggests a disease-specific alteration of these two networks. The DMN has been linked to cognitive processes whereas the latter has been shown to be involved in motor control. The present results once again support extra-motor involvement in ALS.

In the mean time, several further studies have addressed the issue of functional connectivity in ALS by analysis of resting-state data, with partially conflicting results. In a study in 20 ALS patients and healthy age-matched controls, alterations in sensori-motor network in the ALS patient group were detected, similar to our data, but significant changes were seen only in a subgroup of ALS-patients in the DMN, and in the right frontoparietal network [94].

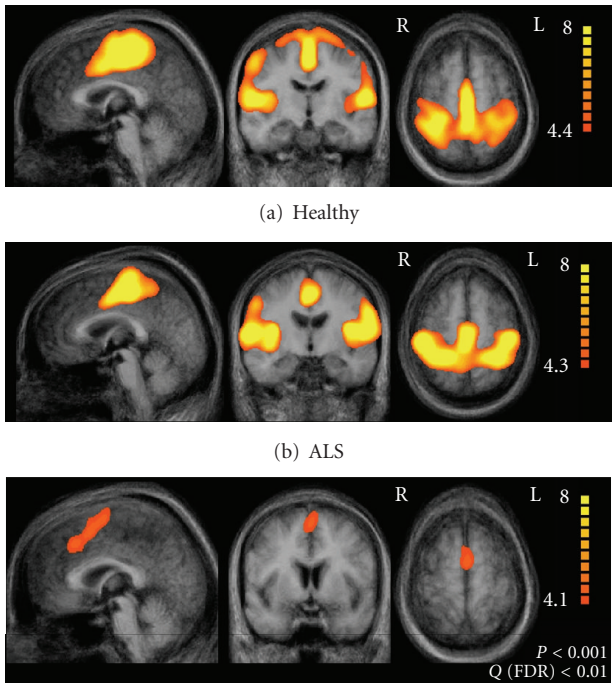


Figure 5: Sensori-motor network. Upper row (a) illustrates the result of the group ICA analysis for the healthy control participants, the middle row (b) illustrates the results for the ALS patients. The statistical comparison is shown in the lower row (c). Adapted from Mohammadi and co-workers [57].

In another study, 25 ALS-patients and age-matched healthy controls were investigated using a combined method to study both structural and functional connectivity [95]. This integrated approach identified apparently dichotomous processes characterizing the cerebral network failure in ALS with increased functional connectivity within regions of decreased structural connectivity. This may point out an interaction between functional and structural connectivity. The atrophy of white matter between distinct cortical areas (reduced structural connectivity) correlates to a higher synchronization of BOLD and an increased interplay between these areas (functional connectivity).

In a third study by the so-called "seed-based analysis" no significant changes in the functional connectivity of the motor system were seen [96]. By this method one searches for a simple correlation between two predefined areas. These partially controversial results of the different studies which investigated resting-state network changes in ALS might be due to methodological differences or to different compositions of the patient groups with different grades of disease severity and therefore different pathological stages. As recently recommended [42], further studies with greater numbers of patients including sufficient numbers of patients in different disease stages could provide better insight into changes of the distinct cerebral networks and their relation to the disease process. In the future, it will be important to pursue multimodal approaches looking for grey matter changes, structural connectivity and functional connectivity, and their correlation with different clinical scores (ALSFRS, neuropsychological parameters, motor performance).

5. Conclusion

Of the currently available structural and functional MRI techniques, a combination of DTI and resting fMRI might provide the most promising early screening protocol to identify subjects "at risk" for developing ALS. However, further validation studies in larger patients' samples are required before these techniques can enter the clinical routine [7].

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Review Article

Progress in Therapy Development for Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that cannot be slowed substantially using any currently-available clinical tools. Through decades of studying sporadic and familial ALS (SALS and FALS), researchers are coming to understand ALS as a complex syndrome with diverse genetic and environmental etiologies. It is know appreciated that motor neuron degeneration in ALS requires active (gain of function) and passive (loss of function) events to occur in non-neuronal cells, especially astrocytes and microglia. These neuroinflammatory processes produce paracrine factors that detrimentally affect motor neurons, precipitating protein aggregation and compromising cytoskeletal integrity. The result is a loss of neuronal homeostasis and progressive die-back of motor axons culminating in death of the afflicted motor neurons. This review will discuss experimental therapeutics that have been tested in murine ALS models, with an emphasis on those that have progressed to human clinical trials. Reasons will be considered for the frequent failure of preclinical successes to translate into positive clinical outcomes. Finally, this review will explore current trends in experimental therapeutics for ALS with emphasis on the emerging interest in axon guidance signaling pathways as novel targets for pharmacological support of neural cytoskeletal structure and function in order to slow ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS; colloquially referred to as Lou Gehrig's disease in American English and Motor Neurone Disease in British English) is one member of a family of anterior (ventral) horn diseases that cause progressive, irreversible degeneration and ultimately death of spinal motor neurons and their cortical efferents [1]. Other anterior horn diseases include Charcot-Marie-Tooth disease, spinal muscular atrophy, progressive motor atrophy (PMA), poliomyelitis, and West Nile virus. ALS is anatomically distinguished from other anterior horn diseases and motor neuropathologies by involvement of both upper and lower motor tracts with a relative sparing of sensory neural degeneration, though sensory involvement is present in a subset of ALS patients.

ALS is also distinguished from other motor neuron diseases by its frustrating lack of definable genetic causes and generally enigmatic etiology. Approximately, one fifth of ALS cases are hereditary, but even within this subset there are currently thirteen confirmed Mendelian mutations encoding proteins in disparate pathways that appear at first glance to be minimally interconnected (Table 1) ([2]; also see the ALSoD database, http://alsod.iop.kcl.ac.uk/, [3]). It may be significant that most of the Mendelian factors associated with FALS code for proteins involved in cellular mass transport (either axonal transport or vesicle trafficking), or else code for proteins whose malfunction results in macromolecular aggregates that could impede these transport processes. This is a concept that will be explored in more depth later in this paper.

Amongst the remaining majority of sporadic ALS (SALS) cases, there are few confirmed genetic or environmental risk factors. To date, several independent genomewide association studies have been conducted in an effort to identify genetic contributors to SALS but these have resulted

Table 1: Mendelian and non-Mendelian loci known to cause FALS or confer risk for SALS. Polymorphisms in the VEGF promoter that were originally associated with increased ALS risk have not been confirmed in subsequent studies but may act as modifiers of disease onset or progression in subsets of ALS cases [4].

Mendelian genes for heritable ALS (FALS)				
Gene	Location	Heritance	Protein	Pathway or effect
ANG	14q11.2	Dominant	Angiogenin	rRNA transcription
ALS2	2q33	Recessive	Alsin	Endosome/membrane trafficking
C9ORF72	9p21.2	Dominant	Uncharacterized	Altered C9ORF72 RNA splicing, formation of nuclear RNA foci
FIG4	6q21	Recessive	FIG4 homolog	Endosomal trafficking
FUS	16p11.2	Both	Fused in sarcoma	Altered RNA processing, formation of inclusion bodies
OPTN	10p13	Both	Optineurin	Golgi maintenance, membrane trafficking and exocytosis, formation of inclusion bodies
SETX	9q34.12	Dominant	Senataxin	DNA and RNA processing
SOD1	21q22.11	Almost always	Superoxide dismutase-1	Protein aggregation, possible gains of redox function, impaired axonal transport
SPG11	15q21.2	Recessive	Spatacsin	Impaired axonal transport
TARDPB	1p36.22	Dominant	TAR DNA binding	RNA processing, formation of protein inclusion bodies
UBQLN2	Xp11.231 dominant	X-linked	Ubiquilin-2	Proteasomal protein degradation, inclusion body formation
VAPB	20q13.32	Dominant	Vesicle-associated membrane protein VAMP	Vesicle trafficking
VCP	9p13.3	Dominant	Valosin-containing protein	Proteasomal degradation, endosomal trafficking, vesicle sorting
Susceptibility loci for sporadic ALS (SALS)				
Gene	Location	Polymorphism	Protein	OR (95% CI)
GWA_9p21.2	9p21.2	rs2814707	Unknown	1.25 (1.19–1.32)
UNC13A	19p13.1	rs12608932 homolog	Unc-13 Vesicle protein	1.18 (1.13–1.24)
ATXN2	12q24.12	Poly-Q	Ataxin-2	n.a.

in short, nonoverlapping lists of candidates [2, 5–7]. Three genetic loci have been found that confer significant but small increases in SALS risk (Table 1). Thus, definitive diagnosis of SALS relies upon a combination of primary clinical findings and systematic exclusion of other potential genetic and environmental explanations.

The comparative lack of understanding of ALS etiology has understandably hindered effective therapy development. Nonetheless, there has, for decades, been consistent global research effort to find new targets and therapies for slowing ALS. This passionate effort is driven in part by the severity of ALS clinical presentation, the invariably fatal prognosis, and a growing appreciation that ALS shares many biochemical and cell biological commonalities with other much more prevalent neurological diseases. Thus, understanding the essential neurobiology of ALS could open new vistas for treating neurodegeneration in general. The identification of FALS genes and production of robust animal models (particularly of superoxide dismutase-1 (SOD1) transgenic mice in the early 1990s) have massively accelerated the pace of ALS research and therapy development efforts [8–11]. To date, there have been many preclinical drug tests in these mouse models, with numerous published studies demonstrating modest benefits. Unfortunately, there have been very few replicated preclinical studies that succeeded in producing more than 10% life extension in ALS mice, whereas several promising therapies that were successful in murine trials failed in human clinical trials [10].

This paper will discuss the history of ALS therapy development efforts from the perspective of therapy targets, review the outcomes of major clinical trials, and suggest possible pathways for future preclinical and clinical investigation. Emphasis will be placed on discussion of past therapy development efforts that culminated in human trials, and a specific discussion will be undertaken of probably reasons why successful preclinical strategies have often failed to translate into positive outcomes during human clinical trials.

2. Targeting Motor Neuron Death Pathways by Blocking Glutamate-Linked Excitotoxicity

The central feature of ALS is death of the motor neurons. Thus, the earliest strategies to combat ALS focused on intrinsic neuron death pathways, and to this day there is much active research in this area. The key questions are why do the motor neurons die and what pathways might be pharmacologically manipulated for therapeutic benefit? Even prior to the identification of SOD1 mutations in FALS, it was appreciated that human ALS nervous system tissue exhibited protein aggregation, oxidative stress, and loss of

cellular homeostasis related to defective glutamate transport [12]. This cytological picture was corroborated when the first $SOD1^{G93A}$ and $SOD1^{G85R}$ transgenic mouse models were introduced in the mid-1990s. The involvement of SOD1 implied, at the time, a likely redox component to disease mapping directly to toxic SOD1 gains-of-function [8–10, 13]. Moreover, the mice were found to suffer a loss in the glial glutamate transporter EAAT2 [13]. By this time in history, it was understood that excess extracellular glutamate could trigger harmful Ca^{2+} influx leading to downstream neurotoxicity, including loss of mitochondrial functionality and concomitant oxidative stress. It was therefore not surprising that some of the first experimental treatments applied to these transgenic models involved administering antioxidants such as vitamin E (alpha tocopherol), alone or in combination with the NMDA (glutamate) receptor antagonists [8, 9].

These early studies appeared quite successful: the glutamate receptor antagonists riluzole and gabapentin both were found to slow disease progression and prolong survival in $SOD1^{G93A}$ mice, whereas vitamin E reportedly delayed onset of symptoms and slowed disease progression without prolonging survival [8]. Moreover, riluzole improved motor function as measured by spontaneous wheel-running activity and other motor assays [9]. These early findings in ALS mice actually followed, in time, the first human clinical reports that riluzole slowed SALS progression [14] and were thus actually rather confirmatory in nature rather than predictive. Four subsequent clinical trials noted a general positive effect of riluzole at 100 mg/day as indicated by quality of life measures such as tracheostomy-free survival, resulting in a consensus opinion that riluzole treatment added 2–4 months of life expectancy to ALS patients against the typical 4-5 year survival prognosis [15]. Currently, riluzole (Rilutek) is the sole drug approved by the United States Food and Drug Administration (FDA) for treatment of ALS.

Unfortunately, the early enthusiasm that mutant SOD1 transgenic mice would prove good predictors of clinical drug efficacy proved unfounded. First, the efficacy of riluzole, gabapentin, and vitamin E in murine ALS models proved very poorly reproducible [11]. Moreover, clinical trials of gabapentin [16], vitamin E [17], or the NMDA receptor antagonist memantine [18] failed to significantly benefit ALS patients though there were some nonsignificant trends noted in particular motor functional parameters such as rate of arm strength decline with gabapentin use [16]. In one study, vitamin E use combined with riluzole treatment may have tended to prolong time spent in earlier stages of ALS but had no effect on longer-term disease progression or survival in human ALS patients [17]. More recently, there has been some epidemiological evidence that long-term antioxidant supplementation with vitamin E may reduce risk for ALS [19] but in general the effects of both antioxidant and anti-excitotoxin therapies have been muted both in preclinical animal studies and actual human clinical trials.

The concept of anti-excitotoxins for ALS therapy is still a subject of active research and has yielded one very encouraging recent clinical result. The benzothiazole compound R-pramipexole (dexpramipexole or KNS-760704) emerged from Parkinson's disease research as a drug that could rescue dopaminergic neurons from glutamate excitotoxicity, but not by blocking Ca^{2+} influx; rather, the neuroprotective effects seemed mediated downstream from the glutamate-gated receptors and possibly map to action in the mitochondria [20]. Subsequent studies of dexpramipexole in ALS patients culminated in a recent report that 50–300 mg/day of the drug caused a dose-correlated positive change in functional decline as measured by a standard instrument, the ALS-Functional Rating Scale-Revised (ALSFRS-R) [21]. No other drug previously produced a significant effect on ALSFRS-R. Moreover, interim analysis indicated a near-significant trend ($P < 0.07$) toward increased lifespan in the ALS group receiving dexpramipexole [21]. Further clinical trials of dexpramipexole are ongoing.

3. Targeting Neuroinflammation by Suppressing Glial Activation and TNFα Production

Although the fatal risk in ALS is death of the motor neurons, it has become abundantly clear that this motor neuron death is not cell-autonomous but requires active contributions from surrounding nonneuronal cells (most likely, one or more glial cell types). The evidence for this is twofold. First, transgenic expression of mutant SOD1 restricted to neurons using a neurofilament light-chain promoter fails to produce motor neuron disease [22]. Second and even more convincingly, chimeric mice that express mutant SOD1 in both neurons and glia only develop disease when the mutant protein is heavily expressed in ambient nonneuronal cells, in which case the disease progression is correlated to the amount of glial mutant SOD1 expression [23]. Thus, it would seem that either astrocytes, microglia, or oligodendrocytes/Schwann cells (or some combination) contribute to motor neuron death in ALS.

Understanding the reasons for this could uncover feasible targets for pharmacological exploitation. As noted above, part of the glial involvement is passive, via loss-of-function(s); for instance, loss of EAAT2 expression in astrocytes likely exacerbates excitotoxic stress. A significant component of glial involvement in ALS is likely active, mediating motor neuron damage through active production of toxins or inappropriate release of paracrine factors. It has long been noted that microglial activation and proliferation occur in human and murine ALS [10, 24–26], suggesting that a neuroinflammatory process may be at play. This concept has gained credibility with the finding that particular cytokine and chemokine expression is perturbed in human ALS and animal models [27–33]. Most of the cytokine expression studies to date have been performed in SOD1 mutant animal models, simply because the central nervous system (CNS) tissue is easier to access at various stages of disease than is possible for human ALS where it is extremely difficult to assay neural parenchyma for inflammatory factors before (or even immediately following) death [10, 32–34]. Nonetheless, some studies have found elevated inflammatory

cytokines in human ALS cerebrospinal fluid [27] or blood serum [27–31].

In particular, several studies have found that tumor necrosis factor-α (TNFα) is elevated in human ALS blood serum [30, 31]. In SOD1^{G93A} mice, TNFα message and protein are both elevated in spinal cord tissue, indicating endogenous production in CNS cells [32–34]. Moreover, TNFα is amongst the earliest and most aggressively upregulated gene products in SOD1^{G93A} mouse spinal cord tissue, becoming elevated before the appearance of obvious behavioral anomalies [32, 34]. Interestingly, cell culture studies of primary glia isolated from neonatal SOD1^{G93A} mice indicate that the cells exist in a metastable condition that is prone to fulminant activation. Such neonatal SOD1^{G93A} astrocytes hyperexpress endogenous TNFα as well as its principle receptor TNFRI in response to application of recombinant TNFα ligand or interferon-gamma (IFNγ) [35]. These same SOD1^{G93A} astroglia produce constitutively more prostaglandin-E2 (PGE_2) and induce the leukotriene-producing arachidonate 5-lipoxygenase (5LOX) enzyme in response to inflammogens, to a much greater degree than do nontransgenic astrocytes or astrocytes overexpressing wild-type human SOD1 [35]. Similarly, primary SOD1^{G93A} microglia have been shown to superexpress TNFα in response to lipopolysaccharide (toll-like receptor ligand) stimulation *in vitro* [36]. A principle result of the hyper-inflammatory cellular phenotype of SOD1^{G93A} glia is their enhanced production of reactive oxygen and nitrogen species and enhanced cytotoxicity [35, 36].

The association of TNFα and other inflammatory cytokines with toxic glial activation in ALS has encouraged a number of preclinical and clinical trials of therapeutics selected to either suppress TNFα production or otherwise blunt the neurotoxic activation of microglia. For instance, thalidomide derivatives which suppresses TNFα message translation in addition to other actions are amongst the more effective drugs tested in SOD1^{G93A} mice [10, 37, 38]. Unfortunately, human clinical trials of thalidomide for ALS have been unsuccessful due to lack of patient tolerance and excessive occurrence of sinus bradycardia [39, 40]. Doses of thalidomide that were tolerated by the ALS patients were insufficient to noticeably shift patient cytokine profiles [39]. It remains to be determined whether second-generation thalidomide derivatives such as lenalidomide, which are more active in SOD1^{G93A} mice [37, 38], prove safer and more effective than thalidomide against the human disease.

A surprisingly large number of compounds antagonize microglial activation by TNFα and other inflammogens *in vitro* [10, 41]. The list of such drugs includes cyclooxygenase inhibitors (e.g., classical nonsteroidal anti-inflammatory drugs or NSAIDS) and lipoxygenase inhibitors which apparently interfere with TNFα signaling inside microglia [41], as well as certain tetracyclic antibiotics exemplified by minocycline which act on mitogen activated protein kinase (MAPK) pathways to suppress toxic microglial activation and also stabilize mitochondrial [41, 42]. Unfortunately, to date, these indirectly acting anti-neuroinflammatory agents have not proven efficacious in the human clinic. There is no epidemiological or clinical evidence that NSAIDS generally protect against or slow SALS. Some clinical trials have been undertaken to test particular COX-II selective therapies for neurological diseases including ALS. For instance, the cycloogenase-2 inhibitor celecoxib, which decreased PGE_2 and slowed progression of murine FALS [43], failed to have an effect in human trials [44]. Even more concerning, a formal clinical trial of minocycline actually caused acceleration of ALSFRS-R deterioration and produced neurological and gastrointestinal side-effects in the treatment group [45].

In more recent years, interest in TNFα pathways in ALS has waned somewhat, due largely to reports that crossing SOD1^{G93A} mice with TNFα-knockout mice did not significantly affect disease parameters [46]. There are however cogent reasons not to overinterpret this finding. TNFα is a pleiotropic cytokine that has both protective and toxic effects depending on the milieu in which it is expressed and whether one is speaking of soluble versus membrane-bound variants [47]; knocking out all TNFα constitutively would affect both aspects of cytokine function, possibly canceling out each, with little net effect on the ALS mouse. Also, one must consider the possible compensatory biology that would come to play during development of a complete TNFα knockout mouse. In any event, TNFα may be a useful model stimulus with which to probe both negative and positive aspects of neuroinflammation in ALS mice; the role of increased TNFα presence in human ALS remains subject to debate and further research.

Interest continues to identify safe anti-neuroinflammatory agents that might have some merit to test in ALS clinical trials. It is encouraging that polyphenols or similar compounds such as the 5LOX inhibitor nordihydroguaiaretic acid (NDGA) protect against murine ALS [41] and can be chronically tolerated, at least by healthy humans. In fact, NDGA was used as an antioxidant and anticaking agent in human food until the late 1950s [48]. NDGA is amongst the most potent tested inhibitor of TNFα-stimulated microglial activation *in vitro*, exceeding minocycline potency by 12-fold [41] extends lifespan in SOD1^{G93A} mice when administered orally beginning at symptomatic stages of disease [41] and also seems to promote glutamate uptake both *in vitro* in MN-1 cells and *in vivo* in spinal cord synaptosomes [49]. Interestingly, NDGA administered subcutaneously initially enhanced glutamate uptake in synaptosomes from SOD1^{G93A} mice but then lost efficacy with repeated administration apparently due to induced pharmacoresistance through upregulation of P-glycoprotein [49]. NDGA did not affect lifespan in this administration paradigm [47], in contrast to prior feeding paradigms [41], suggesting that precise dosage and route of administration may be important considerations.

4. Recent Initiatives to Support Neuron Structure and Function by Pharmacological Manipulation of Cytoskeletal Dynamics and Inter-Neuronal Mass Transport

Most ALS preclinical and clinical strategies thus far have fallen into two broad categories: preventing death of the

motor neuron soma by pharmacologically targeting the neuron; or supporting motor neuron health indirectly by targeting the biology of ambient glia. Of course, minocycline garnered enthusiasm because of its apparent multiple targets of action both inside neurons and at the glial cell. Another tactic is gaining momentum in preclinical studies, which are beginning to reconceptualize ALS fundamentally as a cytoskeletal disease. This perspective is based on the fact that motor neuron cell death only happens near the end of an extensive clinical disease and thus likely is a final consequence of other neuronal processes. Indeed, ALS can be thought of as a progressive distal axonopathy [50, 51] beginning with motor end plate denervation and progressing through a period of axonal retraction through the ventral roots. In $SOD1^{G93A}$ mice, motor end plates can be visualized along with their impinging axons [50, 51]. Beginning at 40–50 d, a full month before clinical symptoms or α-motor neuron death, the axons disappear from the motor end plate. This is followed by demonstrable shrinkage and loss of axons at the level of ventral routes and only finally by death of the attached cell bodies in the ventral horns [50, 51]. The denervation of motor end plates is probably cyclical, proceeding through a denervation-reinnervation process, because the affected muscles develop a characteristic fiber-type grouping that is commonly observed in situations where the distal motor nerve is injured. Compensatory axonal sprouting proximal to the muscle leads to clusters of grouped typeI (slow-fatigable, aerobic) and typeII (fast fatigable, anaerobic) fibers [50, 51]. Thus, if one could identify the initial triggers behind neuromuscular junction pathology in early ALS and/or the driving forces behind subsequent motor axon retraction, it might be possible to interfere to the net benefit of the motor neuron.

Progress is being made toward both goals of identifying triggers and identifying driving processes for ALS axonopathy [52]. As for triggers, emphasis is becoming rather rapidly focused on axonal guidance or repulsion cues. Normally, during the course of embryonic development, neural crest cells are guided to migratory locations and growing axons find their appropriate targets through both negative and positive chemotactic cues. Most notably, semaphorins released by glial cells trigger a regulated process of axon growth cone collapse through receptor-mediated cytoskeletal reorganization [52]. As the actin microfilament network and tubulin-based microtubule tracts disintegrate on the side of the growth cone nearest the semaphorin source and extend on the further side, the axon bends away from the source of the repulsion cue. This guidance phenomenon may be of paramount importance in certain adult neuropathologies where the injured nervous system is attempting to heal itself by pruning and regrowing neural connections. In such circumstances, semaphorins and other paracrine factors become upregulated in order to sculpt new neural connections [52].

In 2007, Verhaagen's group published a curious observation that semaphorin 3A (Sema3A) is upregulated in terminal Schwann cells near the fast-fatigable fibers that are earliest to denervate in murine ALS [53]. To date, this observation has not been widely corroborated or built upon, but there are compelling reasons to do so when one considers the molecular mechanism of Sema3A action in relationship to our current knowledge of ALS axonopathy. The primary action of Sema3A is mediated by binding to receptor heterodimers composed of neuropilin-1 (NRP1) and plexin A [52, 54]. This event recruits intracellular kinases including Fyn kinase, triggering downstream activation of glycogen synthase kinase-3β (GSK3β) and cyclin-dependent kinase-5 (Cdk5) [52, 54]. GSK3β and Cdk5 act on a variety of microtubule-associated proteins including the collapsin response mediator protein (CRMP) system. CRMPs, particularly the CRMP2 isoform, function to stabilize both actin microfilaments and microtubules. Phosphorylation, expression downregulation, or sequestration into axonal aggregates can prohibit proper CRMP2 function and trigger severe axon retraction (reviewed in [54]).

There are at least two additional pieces of evidence besides Verrhaagen's finding to suggest that the Sema3A-CRMP2-cytoskeleton signaling pathway is involved at least in murine ALS and potentially amenable to pharmacological intervention. First, Pettman and colleagues recently reported that a variant of CRMP4 is induced in cultured motor neurons by exposure to nitric oxide ($^{\bullet}$NO) [55]. Forced adenoassociated virus (AAV) mediated expression of CRMP4 in wild-type motoneurons triggered axon degeneration and cell death, whereas silencing of CRMP4 in mSOD1 motoneurons protected them from $^{\bullet}$NO-induced death [55]. Thus, ectopic CRMP4 seems to oppose CRMP2 and promote neurodegeneration. If this is the case, then boosting CRMP2 function would be expected to compensate for CRMP4 in order to promote healthy neuritic structure and function.

Second, our group serendipitously discovered that a molecule called lanthionine ketimine (LK) binds CRMP2 to alter CRMP2 : tubulin interaction [56]. A cell-penetrating, synthetic LK-ester derivative (LKE) promotes differentiation of NSC-34 motor neuron-like cells, stimulating axonal extension, which also is seen in primary dorsal root ganglial neurons [54]. Curiously, LKE also potently suppresses TNFα-stimulated microglial $^{\bullet}$NO production in culture which may or may not arise from the drug's apparent action upon CRMP2 [56]. Administration of LKE to $SOD1^{G93A}$ mice beginning at symptomatic disease significantly slowed disease progression and extended lifespan [57]. We are currently exploring the development of LKE and related molecules as novel neurotrophic factors for ALS axonopathy and other indications, but this early data clearly suggests that (1) CRMP2-targeting signal transduction pathways are plausible candidates to explain the noncell autonomous, distal axonopathy component of ALS and (2) these signaling pathways are amenable to deliberate pharmacological intervention.

Other researchers have begun attempting more direct microtubule stabilization strategies in ALS mice, with some success. Notable is a 2007 study by Fanara et al. who reported that the microtubule stabilizing agent noscapine extended lifespan of $SOD1^{G93A}$ mice by >10%, restored axonal transport deficits, and reduced motor neuron death [58]. This is encouraging when one considers that many attempts to prevent somatic death of motor neurons through

antagonism of death pathways and apoptosis have generally met with poor results [10].

At this point, it is worth reconsidering the current list of generally accepted ALS genetic risk factors and Mendelian heritance factors (Table 1). At first glance, there appears to be little in the way of pathway overlap amongst these various genes. However, many of these gene products play roles in intracellular vesicle trafficking or axonal transport. Most of the remainder of the gene products can cause protein aggregation when mutated—thus, potentially creating impediments to cellular traffic along microtubule and microfilament tracks. Recent implications of axon guidance cue signaling and CRMP2 involvement in ALS dovetail remarkably well with the genetic epidemiology in suggesting that ALS is a syndrome driven in large part by defects in cytoskeleton-guided vesicle or axonal transport. If corroborated by future research, such hypothesis could form the basis for a unified theory of motor neuron disease and provide guidance for future drug discovery initiatives.

5. Targeting Protein Aggregation and Oxidative Stress: Common Cell Biological Links

All of the targets mentioned thus far in this paper—excitotoxicity, neuroinflammation, glial activation, and protein cytoskeletal dysfunction—are not mutually exclusive but likely interact with one another quite dynamically and may be mechanistically united either through sharing common origins in protein aggregation disorders or common mechanisms of enhanced oxidative stress (reviewed in [59]). For instance, major proteins including mutant SOD1 and the tubulin-binding/stabilizing protein TCTP (translationally controlled tumor protein) are both excessively carbonylated in $SOD1^{G93A}$ spinal cord at latter stages of disease [59]. The protein degradation system component ubiquitin carboxy-terminal hydrolase-L1 and α, β-crystallin is also demonstrably hypercarbonylated in this mouse [59]. Thus, mutant SOD1 expression directly impinges on protein stability through the phenomena of (directly or indirectly) enhanced protein carbonylation; the oxidative effects upon protein degradation pathways might exacerbate. The oxidative stress to cytoskeletal proteins likely would decrease cells' ability to cope dynamically with stress, and posttranslationally modified protein aggregates likely could exacerbate neuroinflammatory microglial activation. The multitude of inter-related pathological stressors implicated in ALS mouse models may begin to explain why so many drugs produce small benefits, but so few drugs produce large benefits in this creature: there are many productive targets that are involved in disease progression, but suppressing one at a time is ineffective because the pathological current "flows around" every therapeutic barrier that the researcher erects.

6. Lessons from the Past, and Possibilities for the Future of ALS Therapy Development

The past two decades have witnessed a tremendous growth in knowledge about ALS. We now have a much more complete understanding of the genetic causes underlying FALS, though much less is known about risk factors for SALS. ALS transgenic models including mutant SOD1 transgenic mice but more recently including other transgenic constructs have provided powerful tools for exploring the basic biology of motor neuron disease and testing experimental therapeutics. This last point, however, is a pivot about which optimism for ALS therapy development has begun to vacillate. Numerous therapeutic strategies have produced small to moderate lifespan and functional effects in SOD1 mutant mouse studies (reviewed in [10, 11]), but the gains seemed capped at about 10% lifespan extension for reasons that still are not understood [10]. More troubling, efficacy effects in ALS transgenic mouse studies have been notoriously unreproducible [10, 11], and some of the more promising results have failed when translated to human clinical trials, or even worse, have caused harm to patients. What lessons can we learn from past translational efforts in ALS that might be applied productively to improve chances of future clinical trial success?

As others have discussed with great clarity [11], ALS mouse trials should be standardized along basic principles of randomization and observer blinding similar to the norm for human clinical trials. Furthermore attention should be placed to the dose and routes of administration in order to avoid true drug effects in mice that could never be safely recapitulated in humans. Drugs should be selected with respect to the timing of their likely mechanism of action: Drugs that target initial triggers of motor axonopathy may not be logical to test in humans beginning at mid-stage disease, though may be suitable for testing at early disease stages in FALS (however, limited patient availability is a hindrance for such trials); or early in SALS provided further improvements can be made in the speed of clinical diagnosis.

As for lessons regarding cellular targets for promising drug candidates, the only limited success to be achieved thus far in ALS clinical trials has stemmed from riluzole and tentatively, from dexpramipexole: two drugs that are thought to target either glutamate receptor activation or downstream consequences of glutamate excitotoxicity. Thus, priority might be given to drug candidates that have effects in cell or animal models that speak to excitotoxic pathways. On the other hand, not every antiexcitotoxin tested in human ALS or even in ALS mice can produce a benefit as exemplified by the failure of the NMDA receptor antagonist memantine [18]. From what we know of the genetic diversity of FALS combined with the diverse cellular pathway involvement in murine ALS pathogenesis, it would seem that drugs should be selected that target common downstream stress pathways that are activated during excitotoxicity as well as other forms of neurotoxic injury (e.g., oxidative insults or ligand-triggered axonal collapse pathways). On the other hand, caution is warranted when considering drugs that affect important cell physiological junctions such as mitochondrial biology, as such drugs necessarily carry a greater risk of unforeseen adverse events. This may be the case with minocycline, which apparently targets fundamental aspects of mitochondrial biology to the net benefit of ALS animal models but to the detriment of human ALS patients [45].

Continued effort to exploit particular neuroinflammatory pathways for ALS therapy seems warranted at this time, though researchers need to be cognizant that drug efficacy may vary from study to study in a capricious manner that is influenced in mice by pharmacotolerance in some cases (e.g., NDGA [49]). Finally, recent discoveries that axonal guidance and collapse pathways may be triggered near vulnerable ALS neuromuscular junctions and other serendipitous findings that the CRMP2/CRMP4 system is perturbed in ALS may point to an unexpected and exciting new concept in ALS therapy: rational pharmacological manipulation of cytoskeletal restructuring pathways to support the structure and function of motor axons.

Finally, it must be seriously considered that one or even two drugs will not treat ALS due to the multifactorial nature of the pathology. Though difficult to design and implement, trials of "cocktail" therapies consisting of multiple, rationally selected drug candidates may be worth attempting in future ALS mouse and human studies.

In summary, ALS therapy development efforts have been characterized by cycles of enthusiasm and deep disappointment, but scientific progress has been continual and may be nearing new milestones for clinical success if the initial clinical data regarding dexpramipexole effects on ALSFRS-R can be corroborated [21]. Rather than lament past failures, the ALS research community must carefully cultivate and renew its determination to understand and exploit novel, untested, but rational therapeutic targets. Such efforts may prove rewarding in the ALS clinic but in a broader sense may lead to new treatment modalities for other anterior horn diseases or wider classes of neurodegenerative pathologies.

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Clinical Study

A Pilot Trial of Pioglitazone HCl and Tretinoin in ALS: Cerebrospinal Fluid Biomarkers to Monitor Drug Efficacy and Predict Rate of Disease Progression

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Objectives. To determine if therapy with pioglitazone HCl and tretinoin could slow disease progression in patients with ALS. Levels of tau and pNFH in the cerebrospinal fluid were measured to see if they could serve as prognostic indicators. *Methods*. 27 subjects on stable doses of riluzole were enrolled. Subjects were randomized to receive pioglitazone 30 mg/d and tretinoin 10 mg/BID for six months or two matching placebos. ALSFRS-R scores were followed monthly. At baseline and at the final visit, lumbar punctures (LPs) were performed to measure cerebrospinal fluid (CSF) biomarker levels. *Results*. Subjects treated with tretinoin, pioglitazone, and riluzole had an average rate of decline on the ALSFRS-R scale of −1.02 points per month; subjects treated with placebo and riluzole had a rate of decline of −.86 ($P = .18$). Over six months of therapy, CSF tau levels decreased in subjects randomized to active treatment and increased in subjects on placebo. Further higher levels of pNF-H at baseline correlated with a faster rate of progression. *Conclusion*. ALS patients who were treated with tretinoin and pioglitazone demonstrated no slowing on their disease progression. Interestingly, the rate of disease progression was strongly correlated with levels of pNFH in the CSF at baseline.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating progressive neurodegenerative disease. ALS affects people of all ages and both sexes with a reported annual incidence of 1-2 in 100,000. 90% of the cases of ALS occur sporadically, while 10% of patients with ALS have a family history of the disease. The course of ALS is progressive and the majority of the patients succumb within 2–5 years from disease onset. At present, there is no effective therapy for ALS, nor is there a single known cause. After several decades of trials, only one drug with modest disease modifying efficacy, riluzole, has been developed. Therefore, it is imperative for clinical trials to be designed and analyzed quickly. With the disease being rapidly fatal, eligible patients are difficult to enroll in studies, yet the need for compassionate care is essential.

Recent discoveries have identified several single-nucleotide polymorphisms (SNPs) in the anaplastic lymphoma kinase (ALK) gene which have been associated with sporadic ALS [1, 2]. In addition, reduced levels of ALK mRNA have been reported in ALS patients. In 2003, Dangond et al. used spinal cord tissue and microarray analysis and observed a 9-fold downregulation of ALK mRNA in ALS patients [3]. They further substantiated this finding by verifying the change in ALK expression with real-time quantitative RT-PCR. Midkine (MK), a ligand for ALK, was also found to be decreased in patients with sporadic ALS [4].

The expression of MK is induced by retinoic acid signaling, and in addition to activating ALK, MK has the ability to activate neurite outgrowth and increase angiogenesis. It has also been shown that retinoic acid inducible genes improve survival in the G93A-SOD1 transgenic mouse model of

ALS [5]. Further, two of the best-studied and most beneficial gene therapies in animal models of ALS, insulin growth factor (IGF), and vascular endothelial growth factor (VEGF), are induced by retinoic acid.

The importance of retinoic acid responsive genes in modulating spinal motor neuron degeneration has been demonstrated in rodent models [5]. It was demonstrated that astrocytosis, accumulation of neurofilament, motor neuron loss, and a significant loss of motor neuron retinoic acid receptor expression were observed in the lumbar spinal cord of vitamin A deficient animals. Recent findings of the role of retinoid signaling in the pathogenesis of ALS have gained momentum with the development of gene expression analysis in spinal cord from animal models and humans with disease [6]. Retinoid signaling, while crucial in the development and maintenance of the central nervous system (CNS), may also contribute to regenerative mechanisms that occur after CNS injury or disease to mediate plasticity and repair. Additional evidence supporting the role of retinoid signaling comes from proteomic studies of cerebrospinal fluid (CSF). Reduced levels of transthyretin (TTR) and increased posttranslational modifications to TTR have been detected in the CSF of ALS patients [7]. TTR functions to transport and deliver retinol to cells of the CNS. Altered protein levels for multiple members of the retinoid signaling pathway have been detected in spinal cord tissue of ALS patients [4]. Data also suggests that stimulating retinoic acid receptors (RARs) are neuroprotective and therefore pharmacologic agents that target these nuclear receptors may be of value in slowing the progression of ALS [8].

Retinoic acids have been studied extensively in various models of the injured nervous system. These studies have shown that retinoic acids may have three distinct, important roles in ALS. (1) Retinoids may be neuroprotective and support axonal growth. (2) They may modulate the inflammatory reaction by microglia and macrophages. (3) They may regulate glial cell differentiation [9]. These findings suggest that retinoid signaling might slow the progression of sporadic ALS.

Another potential etiology of ALS, as well as other neurodegenerative diseases, is neuroinflammation. In the last decade, the neuroprotective properties of proliferator-activated receptor gamma (PPAR gamma) agonists have received increasing attention and have been examined in a number of preclinical models of neurodegenerative conditions, including Parkinson's disease, Alzheimer's disease, cerebral ischemia, ALS, and spinal cord injury. These diseases share an excess of neurotoxic, proinflammatory immune responses as compared to anti-inflammatory microglia or T suppressor cell responses [10]. Therefore, therapeutic strategies designed to modulate microglial activation, reinstating the physiological shift toward less neurotoxic phenotypes, may represent a neuroprotective goal. PPAR gamma agonists act as potent anti-inflammatory drugs and have been studied in G93A-SOD1 transgenic mice, a mouse model of ALS. These studies demonstrate that pioglitazone HCl-treated transgenic mice have improved muscle strength and body weight, exhibit a delayed disease onset, and survive significantly longer than nontreated G93A-SOD1 mice. Quantification of motor neurons of the spinal cord at day 90 revealed complete neuroprotection by pioglitazone HCl, whereas non-treated G93A-SOD1 mice had lost 30% of motor neurons. This was paralleled by preservation of the median fiber diameter in the quadriceps muscle, indicating not only morphological, but also functional protection of motor neurons by pioglitazone HCl. Activated microglia were significantly reduced at sites of neurodegeneration in pioglitazone HCl treated G93A-SOD1 mice, as were the protein levels of cyclooxygenase-2 and inducible nitric oxide syntheses [11].

While a PPAR gamma and a retinoic acid agent could be studied individually, a drug cocktail may offer the best chance of attaining a significant reduction in disease progression, utilizing currently available FDA-approved agents. Therefore, a trial of riluzole, in combination with pioglitazone HCl, a PPAR gamma agonist, and tretinoin, a retinoid, was undertaken.

For the past few years, there has been growing evidence that throughout the course of ALS, proteins are released from injured/dying axons and cell bodies that can be detected in the CSF. Developing biomarkers for ALS has emerged as one of the most urgent needs in the search for effective treatments, because these proteins may provide prognostic indicators for rate of disease progression, or they may be used to monitor therapeutic efficacy of drugs that reduce motor neuron injury and/or cell death. Of these proteins, cytoskeletal proteins including phosphorylated neurofilament heavy chain (pNFH) and tau have been shown to be elevated in the CSF of neurodegenerative diseases and proposed as biomarkers for ALS [12–15] and neurofilament aggregates have been observed in spinal cord motor neurons of ALS patients [16]. Levels of pNFH were shown to be significantly increased in the CSF of ALS patients when compared to disease mimics [17, 18]. Therefore, we also measured the levels of these candidate biomarkers before treatment and after six months of drug therapy.

2. Methods and Materials

2.1. Subjects. Twenty-eight subjects, who met the El Escorial criteria for probable or definite ALS, between the ages of 18 and 85 and were on a stable dose of Rilutek for at least 30 days prior to the start of the study, were screened. Women of childbearing potential were using an effective method of birth control and had a negative pregnancy test prior to randomization. Subjects who had a history of liver disease, severe renal failure, diabetes, coronary heart disease, clinically significant EKG abnormality at screening or intolerance to Riluzole, or any other comorbid condition which would make completion of the trial unlikely or a FVC of less than 70% were excluded. All procedures were approved by the Western Institutional Review Board (WIRB) and were conducted with the understanding and consent of all subjects. Informed consents were obtained from all individuals in accordance with institutional review board requirements prior to the start of any study-related procedures at screening.

2.2. Study Procedures. Prior to enrollment, potential subjects were evaluated, screened, and consented. A medical history was completed and a physical and neurological exam was performed. An EKG was performed to rule out significant cardiac abnormalities and a FVC test was completed for eligibility. Subjects who met all inclusion criteria and met no exclusionary criteria underwent a lumbar puncture and 6 cc of CSF were collected for analyzing specific proteins such as tau and pNF-H at screening. Subjects were also required to fast prior to screening and prior to every subsequent visit. This ensured accurate results for glucose and lipid testing. Amylase, lipase, and a comprehensive metabolic panel were also monitored monthly. Eligible subjects came back for randomization 2 months after screening. This two-month run-in phase was designed to see if we could determine an individual patient's baseline rate of progression. Subjects randomized to drug received 30 mg/day of pioglitazone HCl and 10 mg BID of Tretinoin. Subjects randomized to placebo received matching placebos for pioglitazone HCl and tretinoin. Drug and matching placebo were manufactured by The Apothecary Shop in Scottsdale, Arizona. An unblinded member of PNA's study team randomized subjects based on screening number and dispensed the appropriate medication to subjects. The PI, study coordinator and the patient were blinded during treatment. Subjects who experienced intolerable side effects abated the tretinoin but continued taking pioglitazone HCl ($N = 5$). 2 patients on placebo reported side effects and had the placebo matching the tretinoin stopped. Subjects were seen 8 weeks after screening for randomization then again at months 1, 2, 3, 4, and 6. The primary outcome measurement, ALSFRS revised (ALSFRS-R), was administered and assessed by the same rater at each visit to insure reproducibility and reduce variability. The last recorded ALSFRS-R score was subtracted from their baseline ALSFRS-R score and was divided by the number of months that the subjects were on treatment to determine a monthly change in ALSFRS-R score. Secondary outcome measures were evaluated at baseline then again at the final visit (after 6 months of treatment), which included lumbar punctures for assessment of protein biomarkers of axonal injury.

2.3. CSF Collection and Analysis. Lumbar punctures were performed at baseline and the final visit. Samples were collected at a similar time of the day for all subjects. 6 ccs of cerebrospinal fluid (CSF) were collected into a low-bind tube and frozen in a -80°C freezer for storage. All samples were coded to maintain patient confidentiality. Total protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Levels of candidate biomarkers were determined using commercial ELISA kits to total tau (Invitrogen, Carlsbad, CA) and phosphorylated neurofilament heavy chain (pNF-H) (BioVendor Research and Diagnostic Products, Candler, NC) following manufacturer instructions. All samples were analyzed in triplicate within each experiment, and all experiments were performed at least twice on separate days.

Patient disease progression was monitored by decline in the revised ALS functional rating scale (ALSFRS-R) and correlated to CSF protein levels using the Pearson correlation test. These correlations were stratified into placebo, tretinoin plus pioglitazone HCl, and pioglitazone HCl-alone treatment groups. For group comparisons, nonparametric Mann-Whitney t-test was used to determine statistical significance, followed by paired t-test for pairwise comparisons within individual patients between baseline and final values. For all data analysis, we set a significance level of $P < 0.05$. All statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc. La Jolla, CA).

3. Results

3.1. Study Population. Twenty-eight subjects were screened, but only 27 enrolled in this double blinded, placebo controlled study (12 females and 15 males). Subjects were randomized in a 2 : 1 fashion to tretinoin and pioglitazone HCL or placebo. Subjects who were randomized to the drug arm received tretinoin at 10 mg BID and pioglitazone HCL at 30 mg QD. Subjects who were randomized to the placebo arm received matching placebos for both medications. Baseline demographics and functional indices are as noted: average FVC was 82%, average time from symptom onset was 5 months, the average baseline ALSFRS-R score was 37, and the mean age was 58. There was no significant difference between the placebo and the active arm in any of these demographic features. The average time from symptom to onset to study entry was 18 months for placebo and 24 months for the active arm.

3.2. Effect of Therapy on Disease Progression. There were 22 subjects who completed more than three months of therapy and these were all included in an intent-to-treat analysis. Of these 22 subjects, 16 were randomized to drug and 6 to placebo. There were five subjects in the active arm who experienced intolerable side effects (1 extreme dry skin, 3 felt they were progressing faster, and 1 had increased fatigue). All five subjects stopped taking the pill corresponding to the tretinoin and two of the five subjects' symptoms abated shortly after discontinuation. 3 patients felt they were progressing more rapidly on therapy and this continued even after stopping all study medication. These patients had a baseline rate of progression in the run-in phase of 1.83, and once starting therapy, their monthly change in ALSFRS-R was 1.75 ($P = 0.71$). The 16 subjects who were on active drug and completed at least 3 months of therapy lost on average -1.02 compared to their baseline rate of progression of -1.13 ($P = 0.13$). The 6 subjects on placebo averaged a loss of -0.86 compared to their baseline rate of progression of 1.11 ($P = 0.38$).

3.3. CSF Biomarkers. The baseline levels of tau in the CSF differed significantly between the two groups. The average tau level for the active arm was 300.7 pg/mL ($n = 10$) as compared to 126.5 pg/mL for the placebo arm ($n = 4$) (Figure 1). After six months of therapy the active arm ($n = 10$) demonstrated a 26% decline in the tau values to 223.9 pg/mL ($P = 0.22$), whereas the placebo arm ($n = 4$) demonstrated

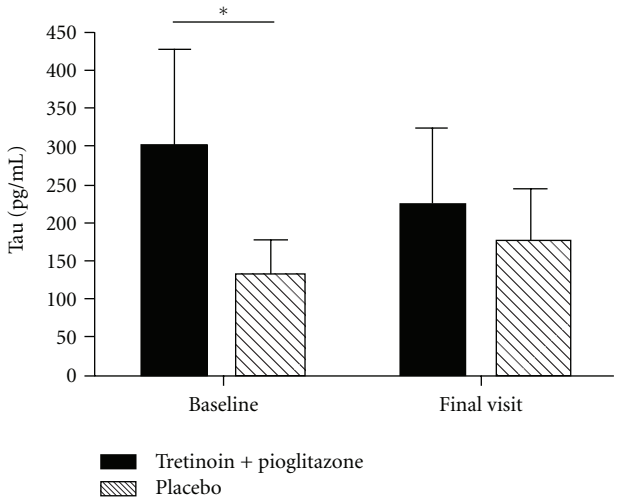

Figure 1: Levels of tau in CSF of patients in active arm versus placebo arm at baseline and after six months of therapy. (*Significant difference in levels of tau at baseline between the two groups, $P = 0.02$ by Mann-Whitney t-test). Error bars denote standard deviation.

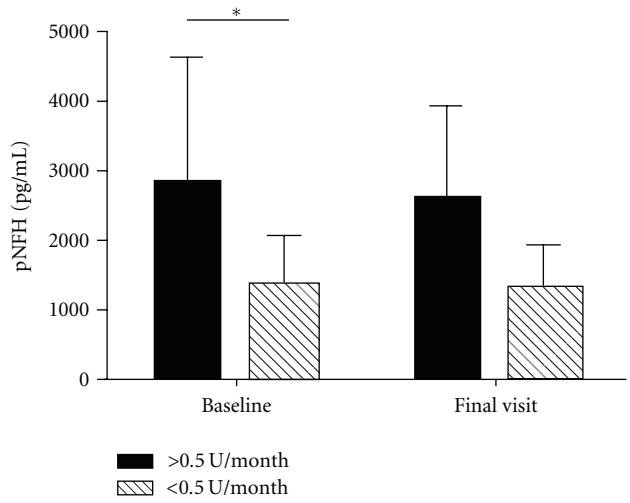

Figure 2: Average pNFH levels in patients who lost <0.5 ALSFRS-R points per month compared to patients who lost >0.5 ALFRS-R points per month. (*Significant difference between the slow and fast progressors, $P = 0.03$ by Mann-Whitney t-test). Error bars denote standard deviation.

a 29% increase in tau values ($P = 0.48$). We did not detect a significant change within individuals over time using a paired t-test (data not shown). There was also no relationship between the levels of tau and patient rate of progression.

The CSF levels of pNFH did not differ between the active and placebo groups and there was no significant change during the course of therapy for individual patients (data not shown). However there was a strong statistical relationship between the baseline value of pNFH and the rate of disease progression (Figure 2). Patients who progressed faster than 0.5 points per months on the ALSFRS-R had an average level of 2860 pg/mL compared to patients who progressed less than 0.5 points per month who averaged 1331 pg/mL ($P = 0.03$).

3.4. Survival. There were 18 subjects who completed the entire 6 months of therapy. One patient dropped out prior to being randomized, and five subjects withdrew after at least two months of therapy. Of the five who withdrew, 3 were on treatment and 2 were on placebo. During the course of the study, 4 subjects died (2 on active drug and 2 on placebo). Three of the four subjects died of respiratory complications and one committed suicide (they had no prior history of depression or suicidal ideation). The average baseline ALSFRS-R score for the subjects who died was 33, which was lower than the average baseline value (38) for those that completed the study ($P = 0.222$).

3.5. Compliance and Safety. Compliance was measured through drug accountability. All subjects were required to return unused medication at each visit. Drug was accounted for by the amount of drug returned subtracted from drug dispensed based on how much drug should have been taken. Tretinoin administered at 10 mg bid was tolerated by 69% and pioglitazone HCl was tolerated by 88% of the subjects randomized to active treatment. There was a 19% dropout rate due to adverse events or intolerability. A DSMB board met shortly after the first four patients were randomized, then every six months thereafter to assure safety, tolerability, and study compliance. Other than one suicide, no other serious events were recorded. At the interim analysis, the majority of subjects tolerated the medication well. Shortly after the last patient completed 1 month on study drug, the FDA reported that the use of pioglitazone HCl increased the rate of bladder cancer. Subjects who were actively taking pioglitazone HCl were contacted and were given the option to withdraw or discontinue use. Everyone continued on treatment, but we felt, in the best interest of others, to stop enrollment at 28 instead of the planned 30.

4. Discussion

This initial phase II trial of tretinoin and pioglitazone HCl used in combination with riluzole showed no significant effect on the rate of disease progression. One of the weaknesses of this study was the small sample size. However, the trial was designed to see if there was a significant and dramatic effect on disease progression. This type of a dramatic treatment effect is the primary goal in ALS trials, as another agent with minimal efficacy is not desirable. If some treatment effect had been found, then this trial would have formed the basis for a larger phase II/III trial. Unfortunately, the results demonstrated lack of efficacy with tretinoin and pioglitazone HCl. Another weakness is the use of two experimental drugs and the use of riluzole in all subjects. These were compromises that were intentionally made to address limitations such as the cost of conducting a larger trial, the difficulty of recruiting subjects, and the difficulty of asking patients with ALS to forgo the only available therapy.

Among the 16 subjects randomized to active treatment who completed more than 3 months of therapy, the average decline in ALSFRS-R was −1.02 points per month. This was higher than the rate of decline in subjects randomized to placebo (−0.86); however, this was not significant ($P = 0.63$). There was also no significant difference between the rate of progression observed during the two-month run-in phase and the rates of progression seen in the placebo and active arms during therapy.

The results from our CSF biomarker studies suggest a correlation between the baseline level of pNFH and disease progression. Subjects who progressed at rates faster than −0.5 points per month on the ALSFRS-R scale had levels of pNF-H that averaged 2860 pg/mL, while subjects who progressed at less than −0.5 points per month averaged 1331 pg/mL ($P = 0.03$) (Figure 2). Our results suggest that elevated levels of pNFH in the CSF may serve as a candidate biomarker for injury or death of motor neurons in patients with ALS. Thus higher CSF levels of pNFH may indicate patients with faster disease progression, a finding that mirrors results of a prior clinical study with memantine [19].

Subjects randomized to combination therapy had a non-statistically significant decrease in tau CSF levels during the course of treatment (an average decrease of 76.8 pg/mL) and 26% decrease ($P = 0.167$) compared to subjects on placebo whose levels increased (average increase of 44.91 pg/mL and 29% increase ($P = 0.412$) (Figure 1). These results may be skewed as there was a significant difference in baseline levels of tau between the active and placebo groups, such that the active arm had 2.4 times the amount of tau at baseline. Patients were not stratified based on these levels, thus this difference was purely coincidental. Therefore, the decline in the active arm and the increase in placebo arm may simply be a regression towards the mean. Further, levels of tau did not correlate to the rate of clinical progression as measured by the ALSFRS-R (data not shown).

5. Conclusion

In summary, our randomized, placebo-controlled trial combining riluzole with tretinoin and pioglitazone HCl failed to alter clinical disease progression over a 6-month time course. CSF-based biomarkers also failed to exhibit significant changes due to drug treatment, though initial levels of pNFH correlated to rate of disease progression. However, it remains possible that other drug treatments that target these same biochemical pathways may be beneficial for ALS.

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Review Article

The Neuroinflammatory Response in ALS: The Roles of Microglia and T Cells

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by upper and lower motoneuron death. Mutations in the gene for superoxide dismutase 1 (SOD1) cause a familial form of ALS and have been used to develop transgenic mice which overexpress human mutant SOD1 (mSOD) and these mice exhibit a motoneuron disease which is pathologically and phenotypically similar to ALS. Neuroinflammation is a pathological hallmark of many neurodegenerative diseases including ALS and is typified by the activation and proliferation of microglia and the infiltration of T cells into the brain and spinal cord. Although the neuroinflammatory response has been considered a consequence of neuronal dysfunction and death, evidence indicates that manipulation of this response can alter disease progression. Previously viewed as deleterious to neuronal survival, recent reports suggest a trophic role for activated microglia in the mSOD mouse during the early stages of disease that is dependent on instructive signals from infiltrating T cells. However, at advanced stages of disease, activated microglia acquire increased neurotoxic potential, warranting further investigation into factors capable of skewing microglial activation towards a neurotrophic phenotype as a means of therapeutic intervention in ALS.

1. Introduction

Neuroinflammation is a pathological hallmark of many neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). It is characterized by the activation and proliferation of microglia (microgliosis) and the accumulation of infiltrating T lymphocytes at sites of neurodegeneration. Although often considered a consequence to neuronal injury and degeneration, the neuroinflammatory response can have protective or deleterious effects on neuronal survival. These disparate effects are elicited by the heterogeneous activation programs of microglia, which in turn are dictated by their surrounding microenvironment and by infiltrating T cells.

2. Amyotrophic Lateral Sclerosis and the mSOD Mouse Model

Typically diagnosed during the fifth decade of life, amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the degeneration of motoneurons in the brainstem and spinal cord and loss of descending motor tracts. Clinical manifestations of ALS include muscle weakness, spasticity, muscle atrophy, and advancing paralysis that culminates in respiratory failure, the usual cause of death in affected patients. ALS is a disease primarily of sporadic etiology with a plethora of aberrant physiological processes implicated in its pathogenesis including excitotoxicity, oxidative damage, the formation protein aggregates, and mitochondrial dysfunction [1]. A pathological hallmark of sporadic ALS is the presence of cytoplasmic ubiquitinated protein inclusions in affected areas of the brain and spinal cord that are predominantly composed of the TDP-43 (transactive response DNA-binding protein 43), an RNA/DNA-binding protein normally found in the nucleus [2].

A small fraction of cases (~10%) termed familial ALS (fALS) are due to a variety of genetic mutations, with 20% of fALS cases due to dominantly inherited mutations in superoxide dismutase 1 (SOD1). SOD1 is a ubiquitously expressed, 32 kDa homodimeric cytosolic protein that

catalyzes the dismutation of superoxide, a by-product of cellular respiration, to hydrogen peroxide. To date, over 125 different mutations that span the entire genomic sequence and protein structure of SOD1 have been identified as causing ALS [3]. In 1994, Gurney et al. [4] developed transgenic mice that overexpress mutant SOD1 (mSOD) and develop a progressive motoneuron degeneration resembling ALS, including cytoplasmic mislocalization of TDP-43 at end-stage of disease [5]. However, after years of investigation, the pathogenic basis of mSOD remains elusive. The majority of SOD1 mutants retain at least partially normal enzyme activity and ablation of the murine SOD1 gene does not culminate in motoneuron pathology [6], indicating that the pathogenic nature of mSOD is through a toxic gain of function rather than a loss of function. Several pathogenic mechanisms of mSOD have been suggested including an increased propensity to form intracellular aggregates, aberrant enzyme activity, ER stress, mitochondrial dysfunction, and glial dysfunction contributing to motoneuron death [7].

An added complexity to mSOD pathogenicity is experimental evidence indicating that motoneuron death in the mSOD model is a noncell autonomous event. Although mSOD expression restricted to neurons is sufficient to cause motoneuron death if expressed at adequate levels [8], mSOD expression in surrounding astrocytes and microglia influences the rate of progression of neurodegeneration. Experiments in which mSOD expression in microglia was reduced [9] or ablated [10] prolonged disease duration and extended survival in mSOD mice but did not affect the time of disease onset. Similarly, the establishment of wild-type astroglial pools via the transplantation of astroglial precursors into the mSOD spinal cord resulted in prolonged survival in mSOD mice [11]. Notably, restricted mSOD expression in astrocytes or microglia is not sufficient to cause dysfunction in wild-type neurons [12]. Together these results suggest that the onset of neurodegeneration in the mSOD mouse is due to mSOD expression in motoneurons but that the rate of disease progression is influenced by mSOD expression in surrounding microglia and astrocytes.

3. Microglia: CNS Resident Macrophages

Within the CNS, populations of macrophages can be distinguished based on their anatomical location. Perivascular macrophages lie between the basal lamina of blood vessels and the glia limitans while meningeal macrophages lie within the leptomeninges that surround the CNS. Microglia are considered the CNS tissue-resident macrophage population and are found within the parenchyma of the CNS. These cells possess a characteristic stellate morphology, with long sinuous processes extending from a round cell body. In their quiescent state, microglia are highly dynamic cells, surveying their surrounding microenvironment through the constant extension and retraction of their processes; it is estimated that the entire extracellular space of the CNS is surveyed every few hours [13].

The phenotype of resting microglia differs from that of other populations of tissue macrophages, being more similar to that of immature myeloid cells; microglia express only low levels of CD45, major histocompatibility complexes (MHCs), and are poor antigen presenting cells (APCs; [14]). The downregulated phenotype of microglia, along with a lack of a conventional lymphatic system and the segregation of the brain parenchyma from peripheral blood by the blood-brain barrier, provides the CNS with a status of immune privilege. This immune specialization enables the suppression and strict regulation of immune responses that could damage surrounding neurons that have only limited regenerative potential. This should not suggest that the CNS is not immune competent, as foreign pathogens, proinflammatory cytokines, or neuronal injury induces microglial activation characterized by morphological alterations including the retraction and thickening of processes and hypertrophy of the cell body [15]. Although these morphological changes are stereotypical with regards to microglial activation, as with other macrophage populations, the phenotype of activated microglia can be highly variable.

Often likened to a double-edged sword in the literature, activated microglia can produce substances that are either beneficial or toxic to surrounding neurons. M1- (classically) activated microglia exhibit a proinflammatory phenotype characterized by the production of interleukin- (IL-) 1β and tumor necrosis factor α (TNF-α) and increased release of reactive oxygen species and nitric oxide through upregulated expression of NADPH oxidase and inducible nitric oxide synthase (iNOS), respectively (Table 1). *In vitro*, cocultured microglia and neurons treated with lipopolysaccharide (LPS), a potent stimulus for M1 activation of macrophages, result in increased microglial production of nitric oxide (NO) and reactive oxygen species (ROS), as well as increased levels of extracellular glutamate which culminates in the excitotoxic death of neurons [16]. Treatment of cultured microglia using IL-4 results in an M2- (alternatively) activated phenotype typified by the enhanced expression of anti-inflammatory cytokines (e.g. IL-10) that dampen inflammation and lead to the release of neurotrophic factors (e.g., IGF-1, GDNF) that support neuronal survival (Table 1). Another feature distinguishing M1 and M2 activation programs is the metabolism of L-arginine; in M1-activated macrophages and microglia, upregulation of iNOS converts L-arginine to NO, while in M2-activated macrophages it converts L-arginine to L-ornithine (Table 1, [17]).

Neurons play an integral role in regulating microglial activation by expressing membrane bound and soluble mediators that enhance microglial production of anti-inflammatory cytokines and neurotrophins. For example, CD200 is a glycoprotein expressed by neurons and its cognate receptor (CD200R) is expressed by all myeloid cells including microglia. In the CNS of mice deficient for CD200R, microglia were observed to possess activated morphologies under steady-state conditions and exhibited an enhanced response following facial nerve axotomy compared to similarly treated wild-type mice [18] suggesting that neuronal expression of CD200 regulates microglial activation. A second example of how neurons regulate microglial function

Table 1: Macrophage activation programs can be distinguished by the associated release of cytokines, arginine metabolism, secreted release of mediators, and antigenicity.

	M1	M2
Cytokines released	TNF-α, IL-1β, IL-6, IL-12, IL-23	IL-10, IL-4, IL-13, TGF-β
Arginine metabolism	iNOS $\longrightarrow$ NO	arginase 1$\longrightarrow$ L-ornithine
Other secreted mediators	NO, ROS	Neurotrophics (GDNF, IGF-1)
Antigenicity	IL-1R, CCR7	IL-1Ra, CD150, CD14, CD163

is through the chemokine fractalkine (CX3CL1) which is expressed on neuronal cell membranes. Following proteolytic cleavage, CX3CL1 is released into the extracellular milieu and affects microglia exclusively as microglia are the only cells within the CNS that express the fractalkine receptor (CX3CR1). CX3CR1$^{-/-}$ mice exhibit dysregulated microglial responses following peripheral injection of LPS, while CX3CR1 ablation in mSOD mice results in increased levels of neuronal loss [19]. Neuronal communication with microglia keeps inflammatory responses in check, preventing neuronal damage by aberrantly activated microglia.

4. T Cells

T cells are the central players in adaptive immunity and can be divided into different subsets based on the expression of cell surface molecules and function (Table 2). Cytotoxic T cells (CTLs) express CD8 and are capable of inducing apoptosis in cells through the expression of Fas ligand and through the exocytosis of perforin and granzymes [20]. The Fas ligand (CD95L) expressed on CD8$^+$ T cells interacts with Fas (CD95) expressed on host cells to induce the downstream activation of caspases, culminating in the apoptosis of host cells. Perforin induces the formation of pores on the target cell membrane, which can result in osmotic cell lysis and provides a means of entry for secreted granzymes [21].

T lymphocytes expressing CD4 include helper T cells (Th) that are further classified according to cytokine production profiles and effector functions and T-regulatory cells (Tregs; Table 2). Compared to CTLs, CD4$^+$ T cells have only limited ability to directly kill cells; they do not express Fas ligand or secrete granulysin and function mainly to activate and regulate the activity of other cells involved in the immune response, including macrophages and microglia [22]. For example, Th1 and Th17 cells can promote M1 macrophage activation through the secretion of the proinflammatory cytokines IL-1 and IL-17, respectively, while Th2 cells secrete cytokines that antagonize proinflammatory mediators and are capable of skewing macrophage activation towards an M2 phenotype through the secretion of IL-4 [23]. Regulatory T cells (Tregs) are characterized by the expression of CD4, CD25, CD62L, CD103, CD152, and the FoxP3 transcription factor which is essential for obtaining the Treg phenotype [21]. For each adaptive immune response launched, a corresponding regulatory response is elicited and mediated by Treg cells that function to regulate the type and level of immune activation [23].

Naive T cells are activated upon recognition and binding of antigen specific to their expressed T-cell receptor; differentiation to a specific effector subtype is determined by the local microenvironment. For CD4$^+$ T cells, antigen is presented on MHC class II molecules on the membranes of APCs, typically dendritic cells, and activated macrophages. CD8$^+$ T cells recognize antigen presented on MHC class I molecules which are expressed on the membranes of all nucleated cells with the exception of neurons and other cell populations within the CNS; however, under neuroinflammatory conditions, neurons upregulate their MHC class I expression, making them potential targets for CTLs [24]. Notably, after the phagocytosis of foreign pathogens or neuronal debris following injury or degeneration, macrophages can cross-present antigens on MHC class I molecules to CD8$^+$ T cells, resulting in their activation and potential reactivity to neuronal cells [20]. Neuronal antigen-specific CD8$^+$ T cells must first be activated within secondary lymphoid organs before migration and extravasation into the CNS. This may be accomplished through antigen drainage of cerebrospinal fluid into the cervical lymphatics or through the migration of APCs residing in the perivascular space to lymph nodes [20]. For both CD4$^+$ and CD8$^+$ T cells, a secondary independent signal elicited through the binding of molecules present on the membranes of host cells is essential for activation and clonal expansion; this secondary signal from APCs may be stimulatory or inhibitory. The types of costimulatory or coinhibitory molecules expressed by APCs confer the nature of their functional activation states, while the density of costimulatory and coinhibitory molecules on T-cell membranes dictates the functional outcome of T-cell activation [25]. In the absence of costimulation, T cells enter a state of anergy and are incapable of activation upon subsequent antigen recognition by their T-cell receptor [21].

Activated T cells are capable of extravasating into the CNS where they perform immune surveillance, and in the steady state, variable numbers of T cells are present within the parenchyma of the CNS [26]; however, very few if any CTLs are observed in the healthy CNS [27]. The healthy CNS parenchyma lacks resident dendritic cell populations but these cells are present within the meninges and perivascular spaces [28], and upon activation, microglia increase their expression of MHC class II molecules, becoming proficient APCs [29].

Once T cells are present within the extracellular space of the CNS, resident parenchymal cells including microglia and neurons are capable of mediating T-cell responses through cell-cell contact, representing a further source of protection from rogue immunological responses. All cells within the parenchyma of the CNS constitutively express Fas-ligand, which upon contact with activated Fas-expressing CD8$^+$ T cells surveying the CNS for their cognate antigen, induces the CD8$^+$ T-cell apoptosis [30]. Microglia are also capable of modulating T-cell responses as they constitutively express B7 homolog 1 (B7-H1) and increase their expression in the

Table 2: The categorization of T cells into subsets is based on cell antigenicity, cytokine profile, and effector function.

	Antigenicity	Cytokine profile	Effector function
Th1	$CD4^+$	IL-2, TNF-α, IFN-γ	M1 macrophage activation
Th2	$CD4^+$	IL-4, IL-10, IL-6, IL-13	Downregulation of M1 macrophage activation
Th17	$CD4^+$	IL-17	M1 macrophage activation
Treg	$CD4^+CD25^+FoxP3^+$	IL-4, IL-10, TGF-β	Damping of proinflammatory response
CTL	$CD8^+$	TNF-α, IFN-γ	Elimination of infected cells

presence of proinflammatory cytokines IL-1 and interferon-γ (IFN-γ; [31]). B7-H1 interacts with the programmed-death receptor 1 (PD-1) expressed on T cells, inhibiting T-cell activation and cytokine secretion [20]. These factors contribute to CNS-associated immune privilege by preventing aberrant inflammatory reactions and the consequent neuronal injury they could impart.

5. Neuroinflammatory Response in the mSOD Mouse Model of ALS

Neurodegenerative diseases including Parkinson's disease, AD, and ALS are characterized by the death of specific populations of neurons accompanied by a neuroinflammatory response that is characterized by microglial activation and T-cell infiltrates being at affected regions. Significant levels of microgliosis have been observed in the spinal cord of ALS patients at autopsy, with T-cell infiltrates found in close proximity to the corticospinal tract [32, 33] as well as in other affected brain regions [34]. Histological examination of CNS tissue from ALS patients is typically limited to advanced stages of disease. However, studies using PET scanning and other imaging techniques permit evaluation of ALS patients at various stages of their disease. Turner et al. [35] administered the radioligand [11C]-(R)-PK11195 to ALS patients, which binds the translocator protein (formerly known as the peripheral benzodiazepine receptor) that is highly expressed on mitochondria of activated, but not resting microglia. This enabled PET detection of cerebral microglial activation *in vivo* over the disease course. Widespread microglial activation was observed in the motor cortex, pons, dorsolateral prefrontal cortex, and thalamus where the extent of microgliosis was positively correlated with the severity of ALS [35].

Notably, patients suffering from sporadic ALS have been reported to have increased levels of circulating inflammatory ($CD16^+$) monocytes in peripheral blood [36], which correlated well with increased levels of plasma LPS [37], a potent inducer of M1 activation in macrophages. These results indicate that the inflammatory response associated with ALS is not limited to the CNS, with systemic immune activation also being observed and potentially influencing disease progression. Furthermore, recent reports by Swarup et al. demonstrated that mRNA levels of TDP-43 and the p65 subunit of nuclear factor κB (NF-κB), a transcription factor involved in the expression of proinflammatory mediators, are upregulated in the spinal cords of ALS patients [38]. When cultured microglia engineered to overexpress TDP-43 were treated with LPS, increased levels of proinflammatory cytokines and neurotoxic factors were produced compared to wild-type microglia [38]. Together, the increased levels of plasma LPS and TDP-43 observed in ALS patients indicate widespread inflammation and suggest that modulation of the inflammatory response may represent an avenue of therapeutic intervention.

As the mSOD mouse model recapitulates many aspects of the neuroinflammatory response observed in ALS patients, this model enables an in-depth analyses of neuroinflammation at different stages of disease. In the mSOD mouse, increased numbers of activated microglia are observed at early presymptomatic stages of disease, and with disease progression to end-stage, microglial numbers in the lumbar spinal cord increase further by nearly 2-fold [39, 40]. Increased numbers of T cells are found in the lumbar spinal cord of mSOD mice beginning at presymptomatic stages and increasing with disease progression to symptomatic and disease end-stage, where T-cell numbers are 10-fold higher than of controls (Lewis unpublished data; [40, 41]). Phenotypical analysis indicated that T cells populating the mSOD spinal cord were limited to the $CD4^+$ subsets until disease end-stage at which point 40% of T cells were $CD8^+$ CTLs ([41]; Lewis unpublished data).

Although neuroinflammation is often considered a consequence rather than a cause of neurodegeneration in ALS patients and in the mSOD mouse model, several studies have demonstrated that modulation of the inflammatory response in mSOD mice alters disease progression [40–44]. Given that reports that anti-inflammatory drugs including minocycline slowed the rate of disease progression and extended survival times in mSOD mice [42–44] and because mSOD-expressing microglia exhibit enhanced neurotoxicity when treated with LPS [45], it was postulated that microgliosis in the mSOD mouse contributed to motoneuron degeneration. However, experiments in which the proinflammatory cytokine TNF-α was ablated in mSOD mice [46] or where the proliferation of microglia was blocked [47] had no effect on the rate of disease progression, suggesting that microgliosis does not exacerbate neurodegeneration in the mSOD mouse mode.

While previous research has focused on the potential neurotoxicity of activated microglia in the mSOD mouse model, recent work has raised the hypothesis that activated microglia might confer neuroprotection. Phenotypical analysis of microglia in mSOD mice using RT-PCR demonstrated that the expression of the neurotrophic factor IGF-1 by microglia increased with disease progression, as did the ex-

pression of the anti-inflammatory IL-1R antagonist which binds to the IL-1 receptor, blocking IL-1 binding and downstream proinflammatory signalling; levels of the proinflammatory cytokine TNF-α did not change with disease progression [40]. Recent work by Beers et al. [48] supports a neuroprotective role for microglia until the end-stage of disease, at which point levels of proinflammatory cytokine IL-1β and TNF-α increase, as do levels of NADPH oxidase [48]. These observations suggest that during initial stages of disease in mSOD mice, microglia exhibit an M2 phenotype supporting neuronal survival. However, as the disease advances, microglial activation becomes skewed towards an M1 phenotype, although the physiological mechanisms eliciting this switch in activation have not been elucidated.

Investigations into the role of T cells in neuroinflammation in the mSOD mouse suggest that these cells influence the phenotypic profile of activated microglia. In two independent studies, ablation of T cells in mSOD mice was achieved by crossing these mice with a $TCR^{-/-}$ strain [40] or with an $RAG2^{-/-}$ strain [41], and disease progression was accelerated in the mSOD mice [49]. In both studies, microglial morphological activation in mSOD mice lacking functional T cells was reduced; however, levels of M1 functional markers such as TNFα and iNOS were increased while markers of alternative activation such as IGF-1, GDNF-1, TGF-B, and IL-4 were reduced [41]. To further identify which T-cell subsets were capable of affecting disease course, the mSOD mice were crossed onto a strain lacking only functional $CD4^+$ T cells [41]. The observed result was similar to that demonstrated by the studies in which all T cells were ablated, indicating that $CD4^+$ T cells in the mSOD spinal cord function to modulate microglial activation and skew it towards an M2 neuroprotective phenotype [41]. Banerjee et al. [50] further refined these observations by comparing the effects of adoptive transfer of activated $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ Teff cells harvested from wild-type mice on disease progression in mSOD mice. The transfer of Treg cells delayed disease onset while transfer of Teff cells prolonged disease progression and the duration of survival [50]. Notably, $CD8^+$ T cells have not been observed in mSOD spinal cord until disease end-stage (Lewis unpublished data; [40]), a time point that corresponds temporally with reduced numbers of $CD4^+CD25^+$ and $CD25^+$ Treg cells in mSOD spinal cord and the skewing of microglial phenotypes towards M1 activation [48].

Findings from these studies suggest that exploiting the neurotrophism of alternatively activated microglia, rather than dampening microglial activation generally, may have some therapeutic benefit in ALS; however, molecular targets enabling this manipulation remain elusive. Recently Beers et al. [51] demonstrated that the passive transfer of $CD4^+$ Tregs into mSOD mice extended the stable phase of disease progression and survival times, suggesting that manipulation of the microglial response through the adoptive transfer of Treg cells or pharmaceutical agents that potentiate M2 activation in microglia may have therapeutic value. In fact, Neuraltus Pharmaceuticals (Palo Alto, CA) is currently conducting phase II clinical trials in patients suffering from ALS, PD, and AD using NP100, a pharmaceutical drug designed to skew macrophage activation towards an M2 phenotype to determine its efficacy in prolonging disease duration.

6. A Role for Bone-Marrow-Derived Microglia

Pharmacological treatments for ALS have largely been ineffective at slowing the disease process, in part because the blood-brain barrier prevents the transmission of the majority of drugs from the blood into the CNS. This has spurred investigations into alternative therapeutic modalities for the treatment of ALS and other neurodegenerative diseases. Microglia are members of the mononuclear phagocyte system which also includes hematopoietic progenitors, blood monocytes, dendritic cells, and other populations of tissue macrophages [52]. Under inflammatory conditions and to a lesser extent during the steady state, circulating monocytes are recruited to tissue compartments where they extravasate and differentiate into macrophages. Although it has been well established that macrophage populations in nonneuronal tissues are maintained to a variable degree through the recruitment of monocytes [53], evidence indicates that only under certain conditions myeloid cells contribute to the maintenance of microglial populations. This highlights the potential for these cells to function as vehicles to transport neurosupportive substances into the diseased CNS.

Investigations into the migration of bone-marrow-derived cells (BMDCs) into the CNS often employ bone marrow (BM) chimeric mice, typically created by exposing rodents to myeloablative levels of radiation followed by the adoptive transfer of labelled BM cells. The results of these studies suggest that while BMDCs contribute to the maintenance of meningeal and perivascular macrophage populations within the CNS, BMDCs make only limited contributions to the parenchymal microglial pool [54–57]. However, in BM chimeric models of neurodegenerative disease including PD, AD, and ALS, increased numbers of BMDCs are observed at sites of neurodegeneration, suggesting BMDCs home to and/or expand at affected sites.

A caveat associated with the irradiation-BM reconstitution protocol employed to create BM-chimeric mice is that it introduces two confounding variables. First, irradiation elicits a widespread inflammatory response including increased levels of cytokines and chemokines within the CNS [58] and has been shown to induce apoptosis of endothelial cells in the rat spinal cord-blood barrier [59]. Secondly, the injection of whole BM into the circulation of mice introduces BM progenitor populations into the blood that would under normal physiological circumstances not enter the circulation [60]. Indeed, studies employing chimeric mice created through parabiosis, a surgical technique in which the vascular systems of two genetically distinct mice are joined, demonstrated that in the absence of irradiation and the injection of BM precursor populations into the circulation, BMDCs do not appreciably accumulate within the healthy CNS, or in models of neuronal injury and neurodegenerative disease [58, 60, 61]. Recent work by Ajami et al. [62] suggests that in irradiated BM-chimeric mice, hematopoietic precursors

contribute to microglial populations while blood monocytes infiltrating the CNS during experimental autoimmune encephalitis (EAE) in chimeric mice represent a transient population of CNS-associated macrophages that turnover upon disease resolution. Therefore, in order to improve the clinical potential of BMDCs as treatment vehicles in neurodegenerative disease, the cell populations within BM capable of infiltrating the CNS and contributing to microglial pools and factors enabling this migration must be identified.

7. Conclusion

Once considered a consequence of neuron death in chronic neurodegenerative disease, neuroinflammation is now recognized to influence disease progression in ALS and the mSOD mouse model. While microglial activation and T-cell infiltration have previously been implicated in exacerbating pathological processes and contributing neuron death in the mSOD mouse, experimental evidence has demonstrated that microglial activation together with the infiltration of instructive T cells has trophic effects on surrounding neurons until late stages of disease. Further investigations into phenomena that induce this phenotypical switch in activated microglia could potentially enable the exploitation of microglial neurotrophism and provide future therapeutic benefits.

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