Amyotrophic Lateral Sclerosis

Toshiyuki Araki, MD, PhD
Editor

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Edited by
TOSHIYUKI ARAKI, MD, PHD
Department of Peripheral Nervous System Research,
National Institute of Neuroscience, National Center of Neurology
and Psychiatry, Japan

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Edited by
Toshiyuki Araki, MD, PhD, Department of Peripheral Nervous System Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan

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FOREWORD

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease, characterized by degeneration of both upper motor neurons including motor cortex of the cerebrum and lower motor neurons in the brainstem and the spinal cord. Because of the rapid progression of muscular weakness and atrophy during the course of the disease and the lack of curative therapy with an estimated mortality of 30,000 patients a year worldwide, ALS is often said to be the most devastating neurodegenerative disorder in adults.

Since the approval of riluzole by the US Food and Drug Administration in 1995, many clinical trials have failed until the recent approval of edaravone. Both riluzole and edaravone are disease modifying drugs with limited benefits, and neither of them are curative. There are several on-going clinical trials with different mechanistic concepts. These include small molecules AMX0035 (combination of sodium phenylbutyrate and tauroursodeoxycholic acid) and mastinib (c-kit inhibitor), antisense nucleotide drug tofersen (antisense for superoxide dismutase 1), humanized monoclonal antibody ravulizumab-cwvz (antibody against C5 complement), and mesenchymal stem cell (MSC)-neurotrophic factor (NTF) cells as cell-based therapy. Furthermore, there are a large number of different potential therapies in basic research stage. Future therapies against ALS may well come out from these endeavors.

Novel groundbreaking therapy against intractable diseases like ALS can only originate from basic research based on the sufficient understanding of clinical features and disease pathophysiology. This book encompasses different aspects from basic research to clinical characteristics of ALS. While the covered areas may be limited as a single book of eight chapters, these chapters, contributed by practicing clinicians and active basic scientists, will inspire ALS researchers in laboratories and clinics, and lead to a further understanding of the disease and development of novel therapies that will eventually help patients suffering from this intractable condition.

Takeshi Iwatsubo, MD, PhD
Director, National Institute of Neuroscience
National Center of Neurology and Psychiatry, Japan.
Professor, Department of Neuropathology
Graduate School of Medicine
The University of Tokyo, Japan
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PREFACE

Amyotrophic lateral sclerosis (ALS) is a fatal, progressive neurodegenerative disorder characterized by motor neuron cell death in the brain and spinal cord. The typical disease symptom is the rapid loss of muscle control, which eventually leads to the complete paralysis of voluntary muscles of the entire body. While there are some treatments to help manage symptoms, there is no curative treatment for ALS. The rarity of the disease and the difficulties in accurate early diagnosis are the major challenges in the proper understanding of the disease and the development of curative therapy. This book brings together a team of experts, both clinicians and basic scientists, to provide a comprehensive understanding of ALS, challenges, and approaches to combat this devastating disease. There are eight chapters in the book.

The first chapter provides a comprehensive review of the clinical manifestation and management of ALS. It discusses the clinical subtypes and the importance of recognition of these subtypes for better prognosis. The pathological features and the management of the disease are also discussed. Early diagnosis of ALS is vital to initiate effective therapies, and diagnostic delay—which can be more than a year from symptom onset—is a major challenge because ALS mimics other neurological disorders. Chapter 2 addresses time to diagnosis, various factors affecting diagnostic delay, and potential interventions to decrease time to diagnosis of ALS.

The role of glial cells in the onset and progression of ALS is increasingly being recognized. Dysfunctional astrocytes in the cerebral cortex and the spinal cord promote neuroinflammation and motor neuron degeneration. Chapter 3 discusses the contribution of dysfunctional cortical and spinal cord astrocytes in the development and progression of ALS. Although the primary feature of ALS is the selective loss of motoneurons in the brain and spinal cord, changes in synaptic transmission and motoneuron excitability are among the first events that take place during development and the subsequent relentless deterioration of motor circuitry. Chapter 4 provides a comprehensive description of our current understanding of defects in intrinsic electrophysiological properties of motoneurons, along with potential therapeutic options to target synaptic transmission and intrinsic features of motoneurons. Since neurons have long neurites, the transport of essential mRNAs and their translation locally in axons are essential to maintain the shape and function of the neurons. Several RNA-binding proteins are involved in the process. Chapter 5 outlines the role of RNA-binding proteins, with emphasis on TDP-43, in axonal transport and local translation of mRNAs in ALS.

In addition to the diagnostic delay as mentioned above and given that the median life expectancy is 3 years, it is important to shorten the diagnostic journey and initiate therapies promptly. Biomarkers may be the key to enhancing early diagnosis, tracking disease progression, and testing target engagement of promising therapeutics. Although clinically validated biomarkers for ALS is lacking, Chapter 6 provides a snapshot of our current understanding of blood-based biomarkers for ALS and discuss the future research directions. To date, there is no

curative pharmacological treatment for ALS. A growing body of evidence show cell therapy as a promising therapeutic alternative for ALS. Chapter 7 discusses the therapeutic potential of various genetically engineered cell types, including induced pluripotent stem cells. The promises and challenges of this approach are also presented. Finally, Chapter 8 directs the reader to a possible new player in ALS—the gut and its microbiota. This chapter outlines the relationship between ALS and the human microbiota, discussing whether an imbalance in intestinal microbiota composition through a pro-inflammatory dysbiosis promotes a systemic immune/inflammatory response and has a role in ALS pathogenesis.

I thank the authors for their contribution, diligence, and professionalism. There is much to learn about ALS. The individual chapters provide excellent views into key topics of ALS. The book is primarily aimed at clinicians and basic scientists; however, it will likely be of interest to a wide audience interested in ALS.

Toshiyuki Araki, MD, PhD
Director
Department of Peripheral Nervous System Research
National Institute of Neuroscience
National Center of Neurology and Psychiatry, Japan
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LIST OF CONTRIBUTORS

ABDULBAKI AGBAS, MSC, PHD

Department of Basic Sciences, College of Osteopathic Medicine, Kansas City University, Kansas City, MO, USA

AMEDEO AMEDEI, BSC

Department of Experimental and clinical Medicine, University of Florence, Firenze, Italy

ANA M. SEBASTIÃO, PHD

Instituto de Farmacologia e Neurociências, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal

ASHOK VERMA, MD, DM, MBA

Department of Neurology, University of Miami Miller School of Medicine, Miami, Florida, USA

CECILE HILAIRE, PHD

INM, Univ Montpellier, Inserm, Montpellier, France

CEDRIC RAOUL, PHD

INM, Univ Montpellier, Inserm, Montpellier, France

DANIELLE RICHARDS, DO

Neuromuscular Center, Neurological Institute, Cleveland Clinic, Cleveland, OH, USA

DORA BRITES, PHD

Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal

ELENA NICCOLAI, BSC, PHD

Department of Experimental and clinical Medicine, University of Florence, Firenze, Italy

ERIK P. PIORO, MD, PHD

Neuromuscular Center, Neurological Institute, Cleveland Clinic, Cleveland, OH, USA

FABIOLA DE MARCHI, MD

ALS Center, Department of Neurology, Maggiore della Carità Hospital, University of Piemonte Orientale, Novara, Italy

FERNANDA GUBERT, PHD

Instituto de Ciências Biomédicas, Brazil

FRANCK AIMOND, PHD

INM, Univ Montpellier, Inserm, Montpellier, France

FREDERIQUE SCAMPS, PHD

INM, Univ Montpellier, Inserm, Montpellier, France

HEATHER M. WILKINS, PHD

Department of Neurology, School of Medicine, University of Kansas Medical Center, Kansas City, USA

JESSICA MANDRIOLI, MD

Department of Biomedical, Metabolic and Neural Science, University of Modena and Reggio Emilia, Modena, Italy

JOHN A. MORREN, MD

Neuromuscular Center, Neurological Institute, Cleveland Clinic, Cleveland, OH, USA

JULIANA FERREIRA VASQUES, PHD

Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil

LEANDRO COELHO TEIXEIRA PINHEIRO, MSC

Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil

LETIZIA MAZZINI, MD

ALS Centre, Department of Neurology, Maggiore della Carità Hospital, University of Piemonte Orientale, Novara, Italy

MAZEN M. DIMACHKIE, MD

Department of Neurology, School of Medicine, University of Kansas Medical Center, Kansas City, USA

RENATA GUEDES DE JESUS GONÇALVES, PHD

Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil

ROSALIA MENDEZ-OTERO, MD, PHD

Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil

SANDRA H. VAZ, PHD

Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal

SARA PINTO, MSC

Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal

SEIICHI NAGANO, MD, PHD

Department of Neurotherapeutics, Osaka University Graduate School of Medicine, Japan

TOSHIYUKI ARAKI, MD, PHD

Department of Peripheral Nervous System Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan

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Clinical Manifestation and Management of Amyotrophic Lateral Sclerosis

Ashok Verma

Department of Neurology, University of Miami Miller School of Medicine, Miami, Florida, USA

Author for correspondence: Ashok Verma, University of Miami Miller School of Medicine, Don Soffer Clinical Research Center, Miami, FL, USA.
Email: averma@med.miami.edu

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Abstract: Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive neurodegenerative disease resulting in death in 2 to 4 years in most cases. There are several clinical subtypes of ALS depending on the degree of upper and lower motor neuron involvement, and recognition of these subtypes is important because certain subtypes have better prognosis. Without a reliable biomarker, ALS is a clinical diagnosis supported by laboratory investigations. The etiology of ALS remains unknown. However, mutations in certain genes cause ALS in about 5–8% of cases and understanding molecular pathogenetic pathways in these cases may pave a way for effective therapies. There is currently no cure or meaningfully effective therapy for ALS. Supportive and palliative measures in multidisciplinary ALS clinics are exceedingly important to maintain and improve the quality of life in patients with ALS. This chapter summarizes the clinical features and management of ALS.

Keywords: amyotrophic lateral sclerosis; motor neuron; muscular atrophy; primary lateral sclerosis; progressive bulbar palsy

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INTRODUCTION

Motor neuron diseases encompass a group of related degenerative disorders of motor neurons in the motor cortex, brainstem, and the spinal cord which manifest clinically by muscular weakness, atrophy, and corticospinal tract signs in varying combinations. Amyotrophic lateral sclerosis (ALS), a prototypic motor neuron disease (MND), is a progressive disease of middle life that leads to death in 2 to 4 years in most cases (1–3).

Jean Martin Charcot (1825–1893), a French neurologist, originally delineated the clinical and pathologic aspects of ALS and recommended the term amyotrophic lateral sclerosis (4). In a series of lectures given in the 1870s, he provided a lucid account of the clinical and pathologic findings of ALS. In the United States, Lou Gehrig, a baseball legend, suffered ALS at age 38 and died 3 years later, and ALS is also named Lou Gehrig disease. Although called Charcot disease in France, MND in the United Kingdom, and Lou Gehrig disease in the United States, ALS has been a preferred term all over the world.

The annual incidence rate of ALS is at 0.6 to 1.8, and prevalence at 4 to 8 per 100,000 population (5–8). The disease occurs in a random pattern throughout the world except for a clustering of patients among inhabitants of Guam, West New Guinea and Kii Peninsula where ALS is often combined with dementia and parkinsonism (9, 10). The ALS is about one-and-half times more common in men than woman (1). Most patients are older than 50 years, and the incidence increases further with later age (5). In 10–15% cases of ALS, an additional diagnosis of frontotemporal dementia (FTD) can be made (7, 11–13). FTD is characterized by the degeneration of frontal and anterior temporal lobes and presents clinically by behavioral changes, impaired executive function, and language dysfunction (12, 13). ALS and FTD are now considered two ends of a spectrum due to the overlap in genetic and molecular mechanisms underlying both these neurodegenerative disorders (12, 13). In 5–8% of cases the ALS is familial (fALS), being inherited in autosomal dominant trait with age-dependent penetrance. A hexanucleotide repeat sequence of C9orf72 gene mutation accounts for approximately 35% of fALS cases (7, 11, 12). Another 15–20% of fALS cases occur from SOD1 gene mutation (14). Over 20 additional genes are linked to fALS, chief among them being *TDP43, FUS, ANG, VCP,* and *OPTN* (7, 15–17). The familial cases as a group differ clinically from sporadic cases in their earlier age of onset, equally affected males and females, and slightly rapid disease progression.

CLINICAL FEATURES: ALS SUBTYPES BASED ON UPPER AND LOWER MOTOR NEURON INVOLVEMENT

ALS in its classic form with amyotrophy (denervation atrophy and weakness of muscles) and lateral sclerosis (corticospinal tract degeneration in the lateral columns of the spinal cord) occurs in approximately 85% of cases. Less frequent are cases in which weakness and atrophy occurs alone, without evidence of corticospinal tract dysfunction, and it is called progressive muscular atrophy (PMA).

When the predominant muscle weakness and atrophy occurs in bulbar territory muscles (muscles of the tongue, pharynx, larynx, jaw, and face), it is called progressive bulbar palsy or progressive bulbar atrophy (PBA). In minority of patients, the clinical state is dominated by pyramidal tract degeneration with spastic limbs and hyperreflexia, with lower motor neuron signs becoming apparent only at a later stage or not at all. This is called primary lateral sclerosis (PLS), an infrequent form of ALS in which the disease process involves only the corticospinal tract pathways, sparing the anterior horn cells in the spinal cord and brainstem. It is important to recognize these subtypes of ALS, because the prognosis in syndromes with the isolated upper or lower motor neuron degeneration is better than in classic ALS with mixed upper and lower motor neuron involvement (2, 3).

Classical amyotrophic lateral sclerosis

The ALS in classic form is insidious in onset and progressive in clinical course and consists of both upper and lower motor neuron involvement (1). Most typically, the disease onset is perceived by the patient as slight weakness in the distal part of one limb. It then progresses and spreads in the adjacent part of the affected limb. For example, it is noted first as an unexplained tripping from slight foot drop with atrophy and stiffness of leg muscles on one side. That is, features of lower motor neuron (weakness and atrophy) or upper motor neuron (stiffness) or both degenerations appear insidiously in one leg. A footdrop with weakness and wasting of the anterior tibial muscles may give an impression of peroneal nerve compression until painless weakness of the calf muscles and thigh muscles, along with normal sensory examination, declares more widespread involvement of lumbosacral neurons. As the disease progresses and spreads, the motor deficit is noted on the opposite side with the subsequent asymmetrical progression in both legs.

In hand-onset ALS, weakness is noted first by mild difficulty in tasks requiring fine finger movements (writing, buttoning, etc.), stiffness of fingers, and slight weakness or wasting of hand muscles on one side. Muscle contraction-induced cramps and fasciculation of the muscles of the shoulder girdle, upper arm, and the forearm may also arise. Thumb and finger abductors, adductors and extensors become weak while the long finger flexors are relatively spared with preserved hand grip. The weakness and atrophy of dorsal interossei and forearm extensor muscles resulting in hallowed intermetacarpal spaces and partial wrist drop may impart a cadaveric or skeletal hand (Figure 1). With further progression and over time, the constellation of atrophic hand and forearm muscles, fasciculations, along with slight spasticity of the arms and generalized hyperreflexia – without sensory or autonomic changes – leaves little doubt as to the ALS diagnosis. Later, the atrophic weakness spreads to the neck, tongue, pharyngeal, and laryngeal muscles and eventually those in the trunk and lower extremities, declaring the devastation of the disease. One of the hallmarks of the disease is despite the amyotrophy, the tendon reflexes are notably active. Babinski and Hoffman signs are variably present.

In about 25 percent of cases, the disease may first start in bulbar (lower brainstem) territory with the attendant difficulty in speaking, swallowing, and handling of saliva (1). Examination in such cases may show atrophic, shriveled and weak tongue (Figure 2) with fasciculation and saliva drooling from the angle of the mouth.

Figure 1. Hand-onset ALS showing asymmetric atrophy and weakness of hand and forearm muscles.

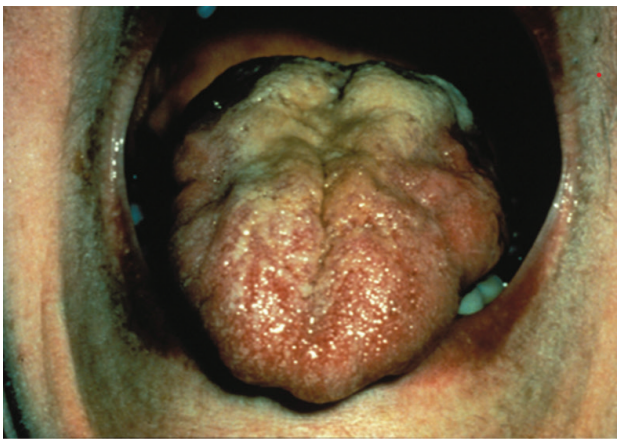

Figure 2. Bulbar onset ALS with tongue atrophy weakness.

Rarely, involvement of thoracic, abdominal, posterior neck muscles, or diaphragm muscle occurs in early course resulting in camptocormia (forward bending of the neck and trunk), head drop, or early respiratory failure in affected individuals (1).

The first and dominant manifestations of ALS may be a spastic weakness of the legs, in which case a diagnosis of PLS is tentatively made (1). Only after months or a year or so, do the hand and arm muscles weaken, waste, and fasciculate, making it obvious that both upper and lower motor neurons are diseased. On occasion, the disease may commence with spasticity of bulbar territory muscles with speech and swallowing difficulty, brisk jaw and facial reflexes, but without muscle atrophy, and it is called pseudobulbar palsy.

Coarse fasciculations are usually evident in the weakened muscles but may not be noticed by the patient until the physician calls attention to them (1–3). The weak and atrophied limb parts may feel cold and achy, but actual numbness or paresthesia, except from poor positioning of the weak limb and focal pressure or compression neuropathies, do not occur in ALS. Sphincter function is well maintained even after both legs have become weak and spastic.

The clinical course of ALS, regardless of its mode of onset and topography of spread and evolution, is progressive. Patient may sometimes observe short periods of stable weakness lasting for weeks or a few months; however, objective changes will be detected in almost all cases. Approximately 50% of patients succumb within 2 to 3 years and 90% within 5 years of disease onset, almost all from respiratory failure (1, 2, 3, 8, 18).

Progressive muscular atrophy

These purely lower motor neuron amyotrophies are more common in men than in women, they progress at a slower rate, and the majority of these patients survive more than 5 years (2, 18). In one large cohort of 155 patients with PMA (18), the authors reported a relatively more benign course in younger patients; 72% of patients with disease onset before age 50 survived over 5 years, compared to 40% of patients with onset after 50 years (18). In about half the patients, the PMA phenotype commences in distal arms with asymmetric weakness and atrophy of hand muscles and then it advances to forearm and arm muscles (Figure 1). Less frequently, the legs and thighs are the sites of the initial atrophic weakness, or the proximal parts of the arms are affected before the distal ones. Fascicular twitching and cramping are common. PMA typically differs from classical ALS in diminished or absent tendon reflexes and undetectable clinical signs of corticospinal tract involvement. However, at autopsy corticospinal tract changes are noted in these cases (19).

The PMA may clinically mimic immune-mediated motor neuropathy that occurs with or without multifocal motor conduction block of electrical conduction and less often inclusion body myositis (described below).

Progressive bulbar palsy

In progressive bulbar palsy, first and dominant symptoms relate to weakness and atrophy of muscles innervated by the motor nuclei of the lower brainstem.

This weakness gives rise to an early defect in articulation and swallowing. As the condition worsens, syllables lose their clarity and run together, until, finally, the patient's speech becomes unintelligible. Usually, the voice is altered by a combination of atrophic and spastic weakness. Defective speech modulation with variable degrees of rasping and nasality is another characteristic. Chewing of food and swallowing become impaired; the food bolus cannot be manipulated efficiently, and this can lead to lodging of food between the cheek and teeth and difficulty in propelling it properly into the esophagus. Liquids and small crumbs of food may find their way into the larynx and trachea with episodes of coughing and choking. Ineffective closure of nasopharynx can result in fluid regurgitation through the nose. Fasciculations and atrophy of the tongue muscle are usually early clinical signs in PBA (Figure 2). Eventually the tongue bulk is lost, and it lies useless on the floor of the mouth.

As the disease progresses in PBA, the pharyngeal reflex is lost, and the palate and vocal cords move imperfectly or not at all during attempted phonation. The jaw jerk may be present or exaggerated at a time when the muscles of mastication are markedly weak. Spastic weakness of the bulbar territory muscles may be the initial manifestation of bulbar palsy without regional muscle atrophy and in such cases the pseudobulbar signs (pathologic laughing and crying) may become a prominent and embarrassing clinical feature. As with other subtypes of ALS, the clinical course of bulbar palsy is relentlessly progressive. Eventually the weakness spreads to the respiratory muscles and deglutition fails entirely. In general, the earlier the onset of the bulbar weakness, the shorter the course of the disease (18).

Primary lateral sclerosis

PLS can be considered another subtype of ALS occurring in 2–4% of cases (1, 20). Most patients, in whom the early signs of corticospinal tract degeneration suggest the presence of ALS, will develop clinical or electromyographic evidence of lower motor neuron involvement within 6–12 months. Some cases, however, have a slowly progressive corticospinal tract disorder that begins with a pure spastic paraparesis; later, the arms and oropharyngeal muscles become involved, and the disease remains one solely of the upper neurons (20).

The typical case begins insidiously in the fifth or sixth decade with asymmetric stiffness in legs with slowing of gait; leg spasticity and imbalance predominates over weakness as the disease progresses. Walking is still possible with the help of a cane for many years after the onset, although falls become frequent. Eventually this phenotype acquires the characteristic features of a severe spastic paraparesis. Over the years, finger movements loose dexterity, the arms become spastic, and, if the illness persists for several years, spastic dysarthria and pseudobulbar palsy is added to clinical features. Infrequently, the PLS may begin with spasticity in one-sided limbs (Mill's hemiparetic pattern) or in bulbar territory muscles (pseudobulbar paresis).

Pathologic studies in a limited number of cases have disclosed a relatively stereotyped pattern of reduced numbers of Betz cells in the frontal and prefrontal motor cortex, degeneration of the corticospinal tracts, also evident on MRI (Figure 3), and preservation of motor neurons in the spinal cord and brainstem (20).

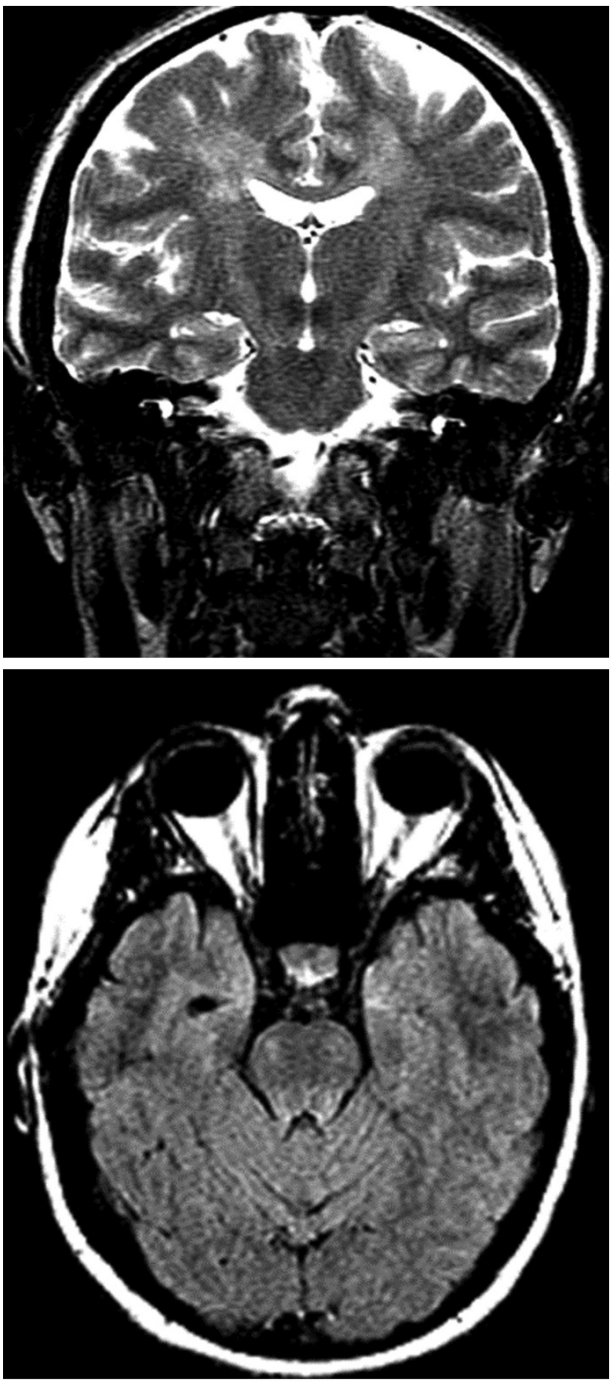

Figure 3. **T2-weighted coronal (3a, top) and FLAIR axial (3b, bottom) MRI showing signal changes that reflect Wallerian degeneration in the corticospinal tracts (Courtesy Dr. Rita G. Bhatia).**

PATHOLOGICAL FEATURES

The pathognomonic finding in ALS is loss of motor neurons in the anterior horns of the spinal cord, motor nuclei of the lower brainstem (lower motor neurons), and motor cortex of cerebrum (upper motor neurons). Large alpha motor neurons tend to be affected before small ones. In addition to neuronal loss, there is evidence of slight gliosis and proliferation of microglia cells. Many of the surviving nerve cells are small and shrunken. In the affected motor neurons, ubiquitin inclusions in threads or dense aggregates can be demonstrated by special stains (1, 3, 12). The anterior roots are thin corresponding to large axon loss, and there is a disproportionate loss of large myelinated fibers in motor nerves (21). The muscles show typical denervation atrophy of different ages.

The lower part of the spinal cord shows the corticospinal tract degeneration most prominently; however, the degeneration can be traced up through the brainstem to the posterior limb of the internal capsule and corona radiata by myelin stains. The loss of Betz cells in the motor cortex corresponds to corticospinal tract degeneration and this is manifested as a slight frontal lobe atrophy on the MRI, but it is not a prominent finding in most ALS cases. In FTD cases, in addition to the usual loss of cortical motor neurons, an extensive neuronal loss, gliosis, and vacuolation involving the frontal premotor area, particularly the superior frontal gyri and the inferolateral cortex of the temporal lobes, is evident (12).

Laboratory investigations and differential diagnosis

ALS is primarily a clinical diagnosis. The lack of a reliable biological marker, highly variable initial clinical presentation, and its clinical overlap with other late-age degenerative disorders make it difficult to diagnose ALS with certainty in early stages. There is an average delay of 6 to 15 months from the onset of symptoms to confirmation of diagnosis (2, 3, 18). The El Escorial criteria for diagnosing ALS was published in 1994 by the World Federation of Neurology (22). The hierarchical diagnostic categories were created chiefly for inclusion standards for patients entering research studies and clinical trials (22). The El Escorial ALS diagnostic criteria were revised to include laboratory features in Arlie House Criteria in 1998 (Table 1). The Awaji-Shima Criteria of 2000 consider electrophysiological features equivalent to clinical lower motor neuron involvement (23). A definitive diagnosis of ALS requires evidence of lower motor neuron and upper motor neuron involvement in at least three of four anatomic regions (cranial, cervical, thoracic, and lumbar regions). Clinically definite ALS shows progression and spread of degeneration or signs within or toward another anatomical regions. More importantly, the laboratory, electrophysiological, and neuroimaging results should not show evidence of other pathological processes that could explain the observed clinical presentation and thus exclude ALS.

Although, there is no definite marker to diagnose ALS, investigations provide useful confirmatory evidence even in the atypical clinical syndrome (1–3). The EMG, as expected, displays widespread fibrillations and positive sharp waves (evidence of active denervation) and fasciculations and enlarged motor units (denoting reinnervation). Motor conduction studies may show drop in combined muscle action potential (muscle atrophy) and only slight slowing, without focal motor

TABLE 1 **Revised El Escorial classification of ALS (22, 23). Four anatomical regions, bulbar, cervical, thoracic, and lumbar are included for disease stratification**

Diagnostic category	Inclusion criteria
Definite ALS	Presence of upper motor neuron and lower motor neuron signs in three anatomical regions
Probable ALS	Presence of upper motor neuron and lower motor neuron signs in at least two regions with upper motor neuron sign rostral to lower motor neuron signs
Probable ALS, laboratory results supported	Presence of upper motor neuron and lower motor neuron signs in one region with evidence by EMG of lower motor neuron involvement in another region
Possible ALS	Presence of upper motor neuron and lower motor neuron signs in one region or upper motor neuron signs in two or three regions, such as monomelic ALS, progressive bulbar palsy, and primary lateral sclerosis

conduction block. If the atrophic paresis is restricted to an arm or hand, raising the question of cervical spondylosis, evidence of denervation in many widely separated somatic segments favors the diagnosis of ALS. Widespread denervation of the thoracic paraspinal muscles and the tongue muscle or facial muscles strongly suggest the disease, as these myotomal involvement is not a feature of cervical or lumbar spondylosis. Sensory nerve action potentials are typically normal in ALS. When in a typical case the amplitudes of sensory nerve action potentials are reduced, there is usually an underlying compression neuropathy or an unrelated neuropathy from diabetes or other cause. Serum creatinine kinase (CK) is moderately elevated in half of patients (1). The CSF protein is usually normal or marginally elevated. A muscle biopsy though helpful in corroborating neurogenic denervation is not needed in ALS.

In patients with prominent corticospinal signs, the MRI may show slight atrophy of the motor cortex and signal changes indicating Wallerian degeneration of the corticospinal tracts (Figure 3). These changes may be diagnostically useful when the presence of severe LMN deficit makes pyramidal tract signs unobvious. Corticospinal tract degeneration appears as an increased FLAIR and T2 signal intensity in the posterior limb of the internal capsule, descending motor tracts of the brainstem, and spinal cord (1–3). These MRI signs however are generally subtle and often missed.

The early clinical picture of ALS is closely simulated by cervical spondylosis or ruptured cervical disc with regional myeloradiculopathy, but with these conditions there is usually pain in the neck and shoulders, limitation of neck movements, sensory impairment, and the lower motor neuron changes are restricted to 1 or 2 spinal segments (1). The EMG showing multi-segmental ongoing active

denervation and reinnervation is particularly helpful in differentiating ALS from these disorders. An isolated and mild hemiparesis or monoparesis because of multiple sclerosis may be difficult to distinguish from early ALS and PLS. Leg-onset PMA may be differentiated from peroneal muscular atrophy (Charcot-Marie-Tooth disease) by asymmetrical clinical course, the complete lack of sensory change, lack of family history, and EMG pattern.

The differentiation of PMA from chronic motor neuropathies, particularly the form that displays multifocal conduction block, poses a major consideration (1–3). An extensive nerve conduction studies and EMG examinations are necessary to distinguish multifocal motor neuropathy from PMA. The presence of an IgM monoclonal paraproteinemia or of specific antibodies directed against the GM1 ganglioside are usually indicative of the immune motor neuropathy, but in half of the cases these laboratory tests are negative (1). A leg form of PMA may be confused with inflammatory myopathy, specifically inclusion body myositis. A rare form of subacute paraneoplastic poliomyelitis in patients with lymphoma or carcinoma that leads to an amyotrophy and progression to death over a period of several months has been reported (24). Another rare condition in young men with localized and asymmetrical amyotrophy of the forearm that became arrested and does not advance over a decade or more is called juvenile MND (25).

The main considerations in relation to progressive bulbar palsy are myasthenia gravis and especially the inherited type of bulbospinal atrophy, the Kennedy's disease (26). The spastic form of bulbar palsy may suggest the pseudobulbar palsy of lacunar disease and can be a prominent part of the progressive supranuclear palsy.

The differential diagnosis of the purely spastic state of primary lateral sclerosis is broad and includes compressive and noncompressive myelopathies, multiple sclerosis, adrenomyeloneuropathy, HTLV-1 associated myelopathy, vitamin B12 or copper deficiency states, familial spastic paraparesis, and lacunar states.

MANAGEMENT

The effect of available treatment for ALS is modest. Two drugs, Riluzole and Edaravone, are approved for ALS; they have modest effect in slowing the disease progression. The antiglutamate agent Riluzole, when given orally, was shown to slow the progression of ALS and improve survival in patients with disease of bulbar onset; it prolonged survival by about 3 months (27). The antioxidant Edaravone has been shown to slow the clinical progress of ALS in select patients in limited trials; but again, the benefit has been marginal (28).

In the absence of curative treatment, supportive and palliative measures are exceedingly important (29–33). Table 2 summarizes the range of symptomatic and palliative treatments in ALS. Regarding symptomatic treatment of spastic leg weakness, anti-spasticity medications, such as baclofen or tizanidine, or subarachnoid infusions of baclofen via an implanted lumbar pump can be helpful. Benzodiazepines may also be used to relieve limb and bulbar spasticity in some cases. These anti-spasticity approaches are most suitable for cases of PLS, which are expected to progress slowly over a long period.

TABLE 2 Symptomatic and palliative management of ALS

Symptoms	Management
Spasticity	• Baclofen • Tizanidine • Intrathecal baclofen pump • Physical therapy
Weakness and physical disability	• Orthotics (leg brace, neck brace) • Mobility aids (cane, walker, wheelchair) • Physical therapy
Dyspnea and poor cough	• Ventilatory support • Cough-assist device • Suction machine • Chest physical therapy • Morphine or benzodiazepine
Dysphagia	• Modified diet • Gastrostomy tube
Dysarthria	• Communication aids
Sialorrhea	• Tricyclic antidepressants • Glycopyrrolate bromide • Botulinum toxin injection • Salivary gland radiation • Suction machine
Emotional lability	• Tricyclic antidepressants • Dextromethorphan hydrochloride/Quinidine sulfate
Depression and anxiety	• Antidepressants • Benzodiazepines
End of life care	• Hospice services

The pseudobulbar syndrome can be ameliorated with dextromethorphan-quinidine compounds.

At all stages of ALS, physical therapy is useful in maintaining mobility. Physical therapy is invaluable, for example, for avoiding contractures of the fingers and shoulders. Occupational therapy is likewise helpful, particularly assessments of the patient's function in the home. A range of personalized orthotic devices, often guided by the physical and occupational therapists, may be of assistance to the patient as the disease progresses.

Important in the management of ALS is periodic monitoring of respiratory function and nutrition (33). Significant practical advances have been made in multidisciplinary ALS clinics with regard to respiratory and nutritional management in ALS. As the respiratory muscle weakness compromises breathing, the use of bilevel positive airway pressure (BiPAP) allows patients to sleep better and

reduce daytime somnolence. With effective noninvasive respiratory support, tracheostomy can be deferred for months or years in most cases. Ultimately, as the disease progresses further and diaphragm fails, BiPAP becomes necessary not only at night but also during the day. When BiPAP use approaches 20 to 24 h per day, patients and their families must address the difficult question of tracheostomy and mechanical ventilation or hospice care.

As oropharyngeal muscles become weak and dysphagia progresses, meals need to be modified to prevent choking, aspiration, and complications. In initial stages, fruits, vegetables and meat should be cut into small pieces and dry foods, such as toast should be avoided. Milk shakes and thicker consistency foods are ideal at this stage. Speech therapists at ALS clinics are helpful in teaching patients and their caregivers methods to adapt to declining bulbar function and minimizing aspiration. Eventually, most ALS patients will need a feeding tube to maintain normal hydration and caloric intake (33).

The American Academy of Neurology has published guidelines for management that have been of aid to patients and physicians; they emphasize the complex and multidisciplinary needs of ALS patients (34, 35)

CONCLUSION

ALS is a progressive neurodegenerative disease resulting eventually in respiratory failure and death in 2 to 4 years or longer in rare cases. Several clinical subtypes of ALS are recognized chiefly depending on the upper and lower motor neuron involvement, and some of these subtypes have better prognosis. The etiology of ALS is unknown, and there is currently no curative treatment of ALS. Supportive and palliative measures are exceedingly important to maintain and improve the quality of life in patients with ALS.

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2

Time to Diagnosis and Factors Affecting Diagnostic Delay in Amyotrophic Lateral Sclerosis

Danielle Richards[1] • John A. Morren[1] • Erik P. Pioro[1,2]

[1]Neuromuscular Center, Neurological Institute, Cleveland Clinic, Cleveland, OH, USA; [2]Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA

Author for correspondence: John A. Morren, Neuromuscular Center, Neurological Institute, Cleveland Clinic, Cleveland, OH, USA. E-mail: morrenj@ccf.org

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Abstract: At present, disease-modifying treatments for Amyotrophic Lateral Sclerosis (ALS) remain limited, with early intervention crucial for maximum potential benefit. A majority of patients will develop dysphagia during the course of their disease, and most will die within three years of the first symptom onset due to respiratory complications. Therefore, early diagnosis is vital to ensure the patient receives appropriate multidisciplinary care and resultant improved longevity as well as quality of life. However, a recent literature review found that ALS patients experience a diagnostic delay of 10–16 months from symptom onset. This chapter examines the factors that contribute to diagnostic delay and potential interventions to decrease time to diagnosis.

Keywords: amyotrophic lateral sclerosis; diagnostic delay; Lou Gehrig's disease; misdiagnosis; motor neuron disease; time to diagnosis

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INTRODUCTION

Currently, for patients diagnosed with amyotrophic lateral sclerosis (ALS), disease modifying treatments remain limited. The typical disease course is marked by a subtle onset and insidious progression, with patients experiencing variable degrees of weakness, spasticity, and muscle atrophy, ultimately resulting in progressive deterioration of limb use, ambulation, speech, swallowing, and breathing. A majority of ALS patients will develop dysphagia during the course of the disease as a result of disease progression involving the bulbar musculature and most will die within three years of the first symptom onset due to respiratory complications (1). This makes early diagnosis and subsequent referral to an appropriate tertiary neuromuscular center/ALS clinic crucial to assure the patient receives appropriate multidisciplinary care and the resultant improved longevity and quality of life. It therefore becomes critical to understand where along the disease timeline diagnostic delays occur and what factors contribute to its prolongation.

DIAGNOSTIC DELAY IN NON-NEUROLOGICAL AMBULATORY MEDICINE

Broadly speaking, diagnostic delay impacts medicine across the spectrum of diseases and subspecialties. In a 2014 study of celiac patients, diagnostic delay of greater than 10 years was reported in 32% of patients surveyed (2). In a 2012 study of missed or delayed diagnoses of breast and colon cancer, which have the potential for positive outcomes if caught early, Poon et al. found that 95% of diagnostic delay involved physician 'cognitive error', which was defined as errors arising from inadequate clinical knowledge or poor clinical judgment. Forty-six percent of these cognitive errors involved an inappropriate workup strategy and 53% were related to misinterpretation of results. In 66% of cases reviewed, researchers found that appropriate application of management guidelines for breast and colon cancer could have prevented further delay (3).

Another meta-analysis of misdiagnosis in various cancer subtypes found a majority of breast cancer diagnostic delay was related to similar cognitive errors of mammogram radiology reviews, where general radiologists lacked specialized training to appropriately assess mammogram studies. Melanoma diagnoses were similarly delayed or altogether missed secondary to a lack of physician experience with or clinical knowledge of the disease (4).

In a 2006 study regarding medical malpractice cases, the most common causes of missed or delayed diagnosis, in descending order, was a failure to order appropriate diagnostic testing, inadequate follow-up plans, failure to collect an accurate history and physical exam, and finally incorrect interpretation of diagnostic testing (5), suggesting limited medical knowledge to be a significant factor in delayed diagnosis. Such delays ultimately result in inappropriate utilizations of resources, patient harm, and potential damage to the physician-patient relationship.

Overall, across a broad spectrum of medical subspecialties, there arise clear similarities in factors that result in diagnostic delay, and the field of ALS is unfortunately no exception. However, there may be more in common when the comparison is between ALS and degenerative conditions with similarly guarded prognosis.

DIAGNOSTIC DELAY IN CHRONIC NEURODEGENERATIVE DISEASE

Similar factors of diagnostic delay are readily found in essentially all subspecialties of neurology, but here we will limit our discussion to dementia, which encompasses a wide variety of chronic neurodegenerative diseases and remains a field where early diagnosis and subsequent medical and social intervention remains paramount for appropriate management. In a 2012 meta-analysis by Aminzadeh et al., only about 50% of cases of mild to moderate dementia were ever correctly diagnosed, with first notable diagnostic delay occurring between symptom onset and initial physician consultation; family members would frequently wait one to two years before seeking any medical assessment. Furthermore, additional delays were caused by subsequent referrals to specialists, as the initial consulted physician was unlikely to be the provider to make the ultimate diagnosis (6).

Research has also noted that a significant barrier to early diagnosis is the limited clinical encounter time often seen with primary care visits, hampering the ability to perform detailed-enough exams, determine appropriate tests, or procedures to detect dementia (6–8). Moreover, even when dementia was suspected, primary care physicians/providers (PCPs) have expressed hesitation about providing the correct diagnosis; they assumed patients and/or their relatives would not want to know, and they even questioned what effect the diagnosis would have on the PCP-patient dynamic (7).

In addition to limited time available for appropriate assessment and workup, Aminzadeh et al. reported other causes for diagnostic delay including limited medical knowledge regarding the disease course, deficits in communication and management skills, and a problematic attitude they termed "therapeutic nihilism" (6). This mindset encapsulates an overall negative view or stigma held by physicians towards dementia and has appeared elsewhere in the literature. In prior studies, physicians have expressed concerns that a diagnosis of dementia would do more harm than good. There was a general perception that there are no available or effective treatments to slow the progression of disease and therefore such a work up would not be worthwhile (7, 8). Similarly, patients' relatives/caregivers reported one of the most common causes of delayed diagnosis related to physician attitudes. In one study, 33% of relatives/caregivers reported that the initial assessing physician did not consider anything to be abnormal with the patient and in another 7% of cases, physicians told relatives/caregivers that pursuing a diagnosis would not be worthwhile (9).

Overall, one can already begin to anticipate similar factors that affect time to dementia diagnosis such as limited physician knowledge or disease stigma being similarly applicable to ALS.

LENGTH OF DIAGNOSTIC DELAY IN ALS

A recently published article that reviewed twenty-one retrospective studies of time from symptom onset to correct diagnosis in the ALS patient population between 1990 and 2020 found that ALS patients experience a delay of about 10–16 months from symptom onset to diagnosis (Figure 1) (10).

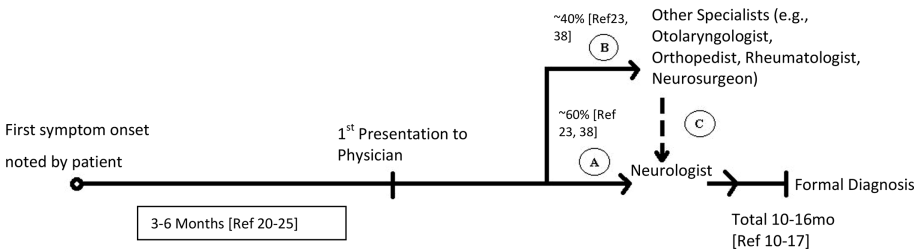


Figure 1. Pathway to ALS diagnosis from first symptom onset to final diagnosis as reported in Richards et al. The initial delay to be evaluatied by the first provider averages 3-6 months. About 60% of patients are then referred to neurologists while the remaining 40% are referred to non-neurologists. Of note, in some studies presenting to a neurologist compared to a non-neurologist does not seem to increase diagnostic delay when the neurologist is the first consultant [Ref 23] or even the third consultant (Ref 21).

This has been confirmed in other research as well (11–14), including more recent studies in 2020 that found a median delay of about 12 months (15–17) and a mean delay of 17 months (15). In the Richards et al. article, the longest delay was 27 months, reported in a study reviewing the United States Centers for Medicare & Medicaid Services database (18) and shortest reported median interval was from a study of a national database used by tertiary ALS clinics in France, reporting a delay of 9.1 months (19).

PATIENT-SPECIFIC FACTORS LEADING TO DIAGNOSTIC DELAY

Much like dementia, the first delay occurs between symptom onset and patients seeking medical attention. Generally, ALS patients will wait anywhere from three (20) to almost six months (21–25) after symptom onset before undergoing a medical evaluation, but other patient-specific factors contribute to the diagnostic timeline (Table 1).

Disease phenotype and diagnostic delay

Clinical presentation plays a significant role in time to diagnosis, particularly with regards to bulbar versus spinal-onset presentation (8, 13, 15, 16, 18, 19, 21, 22, 25–31), with the literature suggesting patients with bulbar-onset ALS experience a delay to diagnosis three to seven months shorter than those with spinal-onset ALS (21, 26–28). The study reporting the longest delay of 2.25 years, as

TABLE 1 **Patient and Physician/Provider factors found to affect time to diagnosis in ALS (10)**

Patient Factors	Physician/Provider Factors
Age	"Cognitive Errors"/Misdiagnosis
Gender	Inappropriate testing/lack of testing
Comorbidities	Initial referral to neurologist vs non-neurologist
Phenotype (region of onset, presence of visible fasciculations)	Inappropriate surgery

mentioned in the review paper by Richards et al. (10), showed a substantial difference between delays in the bulbar-onset group (1.25 years) compared to the spine-onset group (2.5 years) (18). Of note, for patients presenting with both bulbar and spine symptoms, the median time to diagnosis dropped to 0.25 years (10, 18). Patients with spinal-onset presentation also receive more differential diagnoses than those with bulbar presentation (26) and are more likely to be misdiagnosed (27). Compared to spine-onset patients, bulbar-onset patients are also more likely be assessed by a neurologist and less likely by a PCP or orthopedist (28). Multiple studies have also noted shorter diagnostic delay to be associated with shorter survival time (11, 12, 17, 32–36), likely in part driven by the patients with a more rapidly progressive disease who may seek medical attention sooner than those with a more insidious course (12, 32) and bulbar patients who generally experience a more rapid course than their spinal-onset counterparts.

One study by Scialo et al. divided their patient population into two subtypes: those with a diagnostic delay of less than or greater than 36 months. They found that the cohort with a diagnostic delay of greater than 36 months were more likely to present with an atypical clinical phenotype. In another study, patients who presented with clinically-evident fasciculations also experience a shorter delay to diagnosis, though not as short as the bulbar-onset population (23).

Age of onset and diagnostic delay

Multiple studies have also noted age as a factor that prolongs diagnostic delay (22, 24, 26, 37). In one study, patients 65 to 74 years old experienced longer times to diagnosis compared to those 55 to 64 years old, at 12 months and 8 months, respectively (26). Similarly, another study found that the median time to diagnosis was 12.4 months for patients over the age of 60 years compared to 8 months for younger patients. Furthermore, this study showed that diagnostic delay greater than 12 months was about 11 times more likely for patients in the >60 years age group (38). Patients over the age of 60 were also more likely to be initially misdiagnosed compared to younger populations (24). This age-related delay was not consistently found, with Nzwalo et al. reporting significant delay among the younger patient population, which they defined as less than 45 years of age (37). In one Italian multicenter study, older patients were noted to have a shorter diagnostic delay, which authors argued was likely due to more rapid

disease progression as they are also more likely to present with a bulbar phenotype than younger populations (34), as similarly noted by Yates et al. (12). Interestingly, a study by Martinez-Molina et al. found no association with age and length of delay (16).

Gender and diagnostic delay

There is also some evidence of gender differences in time to diagnosis, with male patients experiencing longer delays than females (37, 38) though this could be related to the female predominance of bulbar-onset ALS (38). In one study, men were also more likely to receive a misdiagnosis compared to women by a ratio of 2.5 to 1 (25) , though again this may be because bulbar-onset ALS has a higher female predominance. Interestingly, a study by Iwasaki et al. noted that diagnostic delay in male bulbar-onset patients was 10.5 months compared to 9.8 months in female bulbar-onset patients. In contrast, male spine-onset patients experienced a delay of 13.7 months versus 14.8 months for female spine-onset ALS patients. Martinez-Molina et al. again found no significant association with gender and length of delay (16).

Patient comorbidities and diagnostic delay

Another factor affecting time to diagnosis is the presence of other neurological comorbidities, particularly those diseases with symptoms similar to those of ALS (16, 18, 23), or an overall complex medical history (39). In one study, the presence of comorbidities was associated with nearly twice the length of delay compared to patients without comorbidities, at 19.7 months and 11.1 months respectively (38). Delays have also been reported with patients presenting with frontotemporal dementia (FTD) as the predominant feature of their ALS-FTD, such as two patients in the Househam and Swash study who initially presented with dementia and ultimately experienced a 31.5 month delay from time of first physician assessment to diagnosis of ALS (25).

PHYSICIAN/PROVIDER-SPECIFIC FACTORS LEADING TO DIAGNOSTIC DELAY

As stated before, the first delay in the diagnostic timeline is the period between from disease onset to the patient seeking medical attention, a step that could be argued is generally independent of physicians and providers. However, once the patient is first assessed by a healthcare professional, there arise further physician dependent factors that prolong the time to diagnosis (Table 1).

"Cognitive errors", misdiagnosis, and diagnostic delay

Indeed, it becomes evident in the discussion above regarding patient specific factors that a great deal of this diagnostic delay is likely compounded by, if not a result of, physician 'cognitive error', which was above defined in

non-neurological ambulatory medicine literature as errors stemming from providers' inadequate clinical knowledge or poor clinical judgment, such as inappropriate workup strategy or misinterpretation of results (3). There seems to be an apparent lack of clinical knowledge among physicians regarding spinal-onset ALS, leading to diagnostic delay. This compares to the bulbar-onset patients, or patients presenting with fasciculations, which perhaps raise red flags more broadly known among physicians as ALS symptoms. Two thirds of PCPs self-report that their degree of training regarding ALS is low, with many expressing an overall lack of knowledge of disease clinical signs and symptoms (40). While neurologists are certainly more likely to be exposed to ALS during the course of their clinical experience, they too are at risk of making cognitive errors. In one study, Li et al. asked neurologists in multiple countries to rank the diagnostic importance of MND clinical features and then diagnose known MND case summaries. While in agreement on major MND characteristics, neurologists differed significantly with regard to their final diagnoses of the case summaries. Seemingly, the neurologists may have agreed in "theoretical terms", but applied this diagnostic knowledge in fundamentally divergent ways based on personal clinical experience (41). Misdiagnosis was the another factor that resulted in further delay, with incorrect diagnoses occurring in 13–68.4% of cases (13, 20–22, 24, 25, 37, 42). Such incorrect diagnoses included cerebrovascular disease, cervical myelopathy, neuropathy, radiculopathy, vertebral disc herniation, and myasthenia gravis, among many others (Table 2) (20–22, 25, 38, 42). A pertinent question that then arises is just who is making these misdiagnoses? Surprisingly, anywhere from 7–44.4% of misdiagnoses were made by neurologists (13, 20, 25, 42) with one study finding motor neuron disease (MND) was listed as an initial differential diagnosis in only 30.6% of ALS patients' medical records (38). According to one study, if neurologists are the first providers to assess the patient, only 56% correctly diagnosed ALS; interestingly, this increased to 78% if they were the second provider. However, it should be noted that this rather starkly contrasts with the 1% of patients correctly diagnosed by a primary care provider or other specialist during initial presentation (37). Misdiagnoses leads to more significant delay than those without any diagnosis at all (13, 20, 21, 24, 42), with patients often only receiving the correct the diagnosis once their disease has further progressed (42).

Specialist referrals and diagnostic delay

While the first provider assessment is most frequently with the patient's PCP (13, 15, 21, 23, 37), subsequent referrals to other specialists results in additional delays, with some research showing that neurologists make up 60% of initial specialist referrals (23, 38). Nzwalo et al. reported that 56% of cases will undergo subsequent neurology referral at some point during the course of the disease (37). This was supported by another study that found that while neurologists made up only 28% of the first specialist referral, 62% of patients received a neurology consult at some point within the first three referrals (21). In another study, 49% of ALS patients were referred to other specialists prior to a neurologist, with 54% of this group having been seen by otorhinolaryngologists (30). Other non-neurology

TABLE 2 Rates of specific misdiagnoses prior to formal diagnosis of ALS (10)

Study (Reference)	Overall misdiagnosis rate (%)	Specific misdiagnosis subcategory rate (%)
Palese et al. (38)	49/134 (36.6%)	Myelopathy (14.3%), Radiculopathy (8.2%), Stroke/ Vascular encephalopathy (8.2%), Neuropathy unspecified (8.2%), Nothing pathologic (6.1%), Arthrosis (6.1%), Myasthenia gravis (6.1%), Carpal tunnel syndrome (4.1%), Herniated disc (4.1%), Upper airway infection (4.1%), Musculoskeletal (4.1%), Other (26.5%)
Galvin et al. (21)	20/155 (13%)	Structural (65%): Cerebrovascular disease, Hiatus hernia with reflux, Cervical myeloradiculopathy, and Lumbar radiculopathy.
Paganoni et al. (22)	158/304 (52%)	Neuropathy (28%), Spine Disease (18%), Vascular (11%), Neurodegenerative Disease (11%), NMJ disorder (9%), ENT disorder (7%), Muscle Disease (6%), other (10%).
Belsh and Schiffman (42)	14/33 (42.4%)	Radiculopathy (12.1%), brachial plexus neuropathy (9%), Multiple Sclerosis (3%), Myelopathy (3%), Polyneuropathy (3%), Stroke (3%), Depression (6%), Occult carcinoma (6%), Pulmonary emphysema (6%), Congestive heart failure (3%), Drug induced dysarthria (3%).
Chiò (23)	90/201 (45%)	Discal herniation/medullar compression (12%), Arthrosis/periarthritis (9%), Narrow medullar canal (4%), Cerebrovascular accident (3%), Osteoporosis (2%), Laryngitis/chronic tonsillitis (2%), Thyroid dysfunction (1%), Parkinson's disease (1%), Multiple sclerosis (1%), Other (10%).
Cellura et al. (20)	81/260 (31.1%)	Herniated disc/Cervical myelopathy (32.0%), Vascular pseudobulbar palsy (20.0%), Neuropathy/ Myopathy (8.6%), Myasthenia gravis (7.4%), Carpal tunnel syndrome (6.2%), Depression (6.2%), Alzheimer's dementia (5%), Parkinson disease (5.0%), Arthrosis (2.4%), Thyroid dysfunction (2.4%), Multiple sclerosis (2.4%), Stroke (1.2%), Essential tremor (1.2%).
Household and Swash (25)	39/57 (68.4%)	Vocal cord dystonia, Depression, Laryngeal cancer, Stroke (8.6%), Stress, Thyroid disease, Muscular dystrophy, Frozen shoulder (5.7%), asthma (5.7%), Cervical Spondylosis, Arthritis (14.3%), Cramps, Heart disease, Trapped nerve (8.6%), Recurrent throat infection, Ear infection, Medication side effect, Ligamentous strain, Cervical disc prolapse, Peripheral neuropathy.

specialist referrals included orthopedists, rheumatologists, and neurosurgeons (23, 38), as well as physiotherapy and psychiatry (25).

There are also further differences in length of delay secondary to specialist referral dependent on the type of subspecialist. Palese et al. reported a longer diagnosis delay for patients assessed by a non-neurologists (13 months) compared to those seen by a neurologist (10 months) (38). Nzwalo et al. also reported significantly reduced diagnostic delays for patients who underwent a neurology referral (37). In a study by Househam and Swash, ALS patients who were first referred to a neurologist experienced a shorter delay (10.2 months) compared to those referred to another subspecialty (12.3 months) (25). This was echoed in a later study that found prolonged times to diagnosis among patients referred to non-neurologists (39). Interestingly, spinal-onset patients referred to an orthopedist experienced an additional delay of 10 months compared to those referred to a neurologist (28). Those with bulbar onset experienced a delay to diagnosis of 4.9 months if the referral was to a neurologist, compared to 12.2 months for other specialists (collectively). Diagnostic delay was even more prolonged for patients referred to ENT in particular, at 24.7 months (13).

Differences in delay among subspecialties was not a unanimous finding. Turner et al. reported that a subspecialty referral, specifically to otorhinolaryngologists, did not subsequently result in significant diagnostic delay (30). One study found lower costs associated with neurology referrals but not a significant difference in time to diagnosis (21). In another study, there was no significant difference in diagnostic delay if a neurologist was the first or second physician seen, but this increased when they were seen as the third, fourth, or fifth provider, with time to diagnosis of 17, 19, and 21 months respectively (23). Matharan et al. noted a diagnostic delay roughly ten months longer for those seen by a neurologist versus non-neurologist. Interestingly, they also noted that there was no significant difference in delay depending on whether the patient was referred to a neurologist or sent home without further workup. Authors theorized, at least for the former, this could be due to patients early in the disease course needing serial EMGs or clinical examinations before the disease was advanced enough to be more definitively diagnosed. They also note that bulbar-onset ALS, which showed a shorter diagnostic delay, was more likely to be referred to ENT rather than a neurologist, potentially skewing the specialist referral data (15).

Inappropriate/incomplete testing and diagnostic delay

As stated earlier, those with spinal-onset presented received more differential diagnoses than those with bulbar-onset presentation (26) and are more likely to be misdiagnosed (27). Therefore, it is not surprising that those in the spine-onset subgroup were also more likely to undergo further diagnostic testing, including electrodiagnostic testing (EDX) comprising nerve conduction study (NCS) and electromyography (EMG), as well as neuroimaging such as MRI and CT scans (18). While EDX is certainly an appropriate step in working up MND (as will be further discussed below), neuroimaging which also has a role in ALS investigations may result in incidental findings that potentially introduce confounders and may add unnecessary procedures, thereby prolonging the diagnostic timeline.

Role of electrodiagnosis and neuroimaging in diagnostic delay

While original El Escorial criteria did not allow for EMG findings to serve as a surrogate for clinical features of LMN degeneration, subsequent diagnostic criteria revisions have improved upon this. The 2006 Awaji-Shima criteria now permits fasciculation potentials (without need for positive sharp waves or fibrillation potentials) in the presence of chronic motor axon loss changes as adequate evidence of lower motor neuron degeneration, allowing for earlier diagnosis and classification. Furthermore, these Awaji-Shima criteria exhibit increased sensitivity (43) and equal specificity of an ALS diagnosis when compared to the revised El Escorial criteria (44). In a study by Palese et al., EDX was the most common first investigatory procedure in the pathway to the ALS diagnosis, followed by brain and spinal cord imaging (38). Research suggests that ultimately 75–100% of ALS (15, 21, 30) patients will undergo neurophysiologic/EDX testing at some point during their diagnostic path and 61–100% of patients will undergo brain MRI (21, 30).

Of course, the use of EDX testing does not guarantee a correct diagnosis. Evidence suggests EDX diagnostic sensitivities are lowest in patients categorized as possible ALS and intermediate in patients with probable and probable with laboratory support ALS; the highest sensitivities were found in those with definite ALS (43). In addition, other neuromuscular diseases such as multifocal motor neuropathy or Lambert-Eaton myasthenic syndrome may require a neurologist with a higher degree of expertise in EDX to collect and interpret the data in order to distinguish diseases such as these from ALS. Physicians should always be mindful of any alternative diagnoses to explain patients' presenting symptoms and exam features. Furthermore, results are not absolute and the lack of definite evidence for MND during one investigatory work up does not necessarily predict that future investigations will be similar.

Surgical intervention and diagnostic delay

Misdiagnosis as well as unnecessary or incomplete workups can unfortunately result in exposure to unnecessary procedures. Patients who are misdiagnosed more likely to undergo surgeries as a result with about 12–13% of ALS patients undergoing an inappropriate surgical procedure prior to their correct diagnosis (27, 45). This not only results in increased potential risk but also further delay (27, 37, 38). In one study for example, the 12% of patients who underwent surgery prior to receiving an ALS diagnosis experienced an additional delay of roughly six months compared to the 43% who underwent medical management (27), thereby further compounding upon the misdiagnosis delay mentioned above. Of note, in one study of ALS patients who underwent inappropriate surgery prior to diagnosis, 32% had a pre-operative EMG and of these patients, 72% of reports documented evidence of polyradiculopathy without any mention of the possibility of MND (45), suggesting that timely EDX testing does not always guarantee reduction in delay.

ADVANTAGES OF DECREASING TIME TO DIAGNOSIS IN ALS

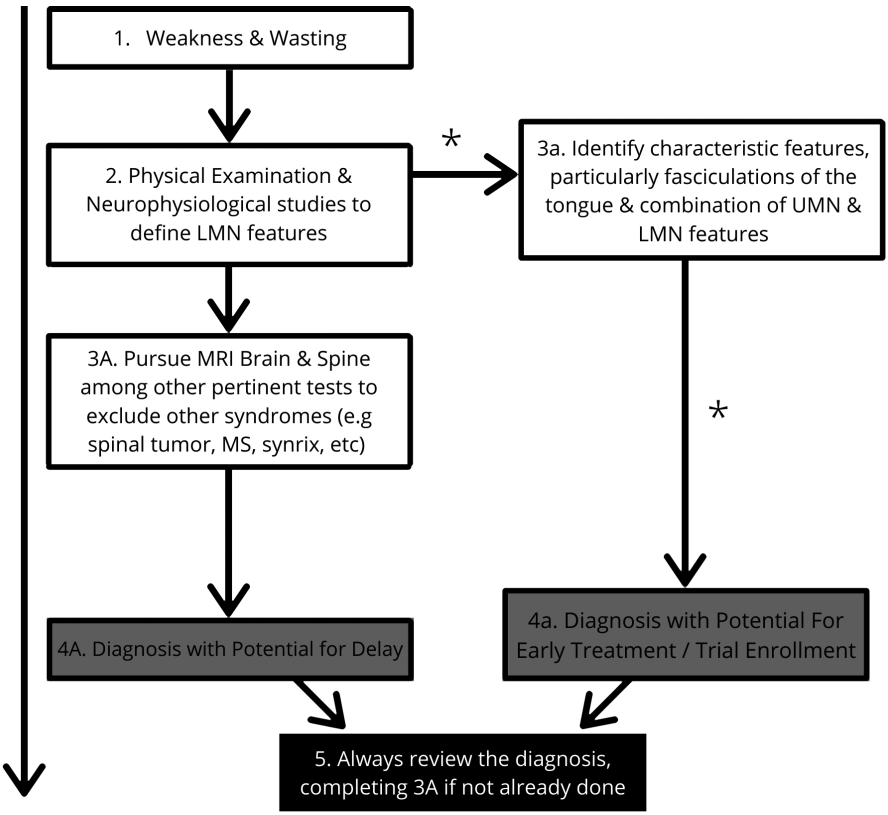
In addition to the numerous unnecessary, costly, and even painful procedures and investigations that arise from delayed or incorrect diagnosis, the results from this work up can be misinterpreted, leading to further prolongation of time to correct diagnosis. Overall, this unnecessary work up and often resultant surgical interventions may also result in delayed referrals to appropriate neuromuscular specialists and multidisciplinary clinics. In these clinics, patients are provided with the appropriate pharmacological and other supportive interventions, such as therapy (physical, occupational, speech and swallowing). Apart from disease-modifying treatments, management involves symptomatic treatment that is specifically tailored to needs of ALS patients. Furthermore, these ALS centers allow for broader access to subspecialty evaluations and management in a single visit, thereby minimizing decentralized and often multiday appointments (46). Importantly, diagnosis and care of patients with ALS in such tertiary centers has been shown to decrease the frequency of hospital admissions, improve quality of life, and increase survival outcomes (47, 48). A more recent study in 2020 found that patients referred to ALS centers also experienced a significantly shorter diagnostic delay of 8.5 months compared to 12 months for those assessed at other facilities (16).

Delays to diagnosis may also affect enrollment into clinical trials and reducing the delay would allow for earlier initiation of disease-modifying treatment candidate agents and extended outcome monitoring periods. These clinical trials typically exclude patients further along in the disease course, as well as those with higher disease burden through the use of strict criteria such as conservative respiratory vital capacity cut-offs or limited timelines from symptom onset. One study assessing rates of exclusion in ALS clinic trials between 2000 and 2017 found that an average of 59.8% of patients are excluded. Respiratory function and disease duration were the second and third most common cited exclusion factors, respectively, with failure to meet a specific El Escorial category as the most common cause for exclusion (49).

Finally, earlier diagnosis and management of ALS can allow patients to more appropriately plan their futures regarding numerous aspects of their lives including financial, social, psychological, and spiritual. Patients will have more time to consider their own goals of medical care and make plans for their inevitable disability. Ultimately, an earlier diagnosis allows for more time to determine a meaningful and dignified end of life. Patients would have additional time to consider and document their own wishes with regard to artificial ventilation, feeding tube placement, and similar terminal care decisions. Furthermore, one cannot minimize the immense psychological toll that arises when a patient initially receives the incorrect diagnosis of a treatable or reversible disorder, only to be subsequently informed that their condition is actually a progressive and ultimately terminal disease. Such humanistic considerations should not be forgotten when formulating potential interventions to curb diagnostic delay.

POTENTIAL DISADVANTAGES OF DECREASING TIME TO DIAGNOSIS IN ALS

Disadvantages to limiting diagnostic delay are largely theoretical but worth discussing, as the diagnosis of ALS remains mostly a clinical one and must be made after the exclusion of alternative and potentially treatable diseases. A 1999 article by Swash proposed a stepwise algorithm (Figure 2) for evaluating weakness and wasting as presenting clinical features, with the aim of minimizing diagnostic delay in ALS, while excluding mimicker conditions (50). He outlined two pathways, noting considerable overlap. The pathway comprising diagnosis by positive



*= major rapid diagnostic pathway- a primarily clinical pathway which does not necessarily emphasize the specification of regions or extent of involvement

MS= multiple sclerosis; UMN= upper motor neuron; LMN= lower motor neruon

Figure 2. A proposed neurological weakness and wasting workup algorithm, modified with permission from Swash (50).

criteria is limited by the absence of a specific biologic diagnostic test. However, the other pathway comprising diagnosis by exclusion of other disorders may promote delay "that can be tempered only by efficiency in the investigative pathway".

One such potentially treatable condition that may present similarly as a progressive weakness is polyradiculopathy. If imaging is suggestive of such an etiology, surgery may be well-indicated. Therefore, shortening diagnostic delay may have the unintentional secondary effect of abating the extent of an appropriate work up for mimics and this may then result in failure to exclude other treatable conditions. Furthermore, it is possible for a patient to have both ALS and additional treatable and more commonly diagnosed neurological comorbidities such as peripheral neuropathy or carpal tunnel syndrome. Properly identifying and managing these conditions through thorough and appropriate investigations could result in improved quality of life, even if the patient is still ultimately diagnosed with ALS.

From a more humanistic perspective, a more extensive workup may ease some degree of psychological impact of receiving the news of a terminal diagnosis, particularly if the certainty of diagnosis is arrived at in a careful, stepwise fashion. This may better satisfy concerns on the part of both the physician and the patient, reassuring each party that no avenue of investigation has been left unaddressed.

IMPLEMENTED MEASURES FOR MINIMIZING DIAGNOSTIC DELAY IN ALS

In the United Kingdom (UK), a goal of the National Health Service (NHS) is to diagnose and initiate treatment of MND within 18 weeks of first referral from primary care providers. In January 2005, the Royal Preston Hospital in the UK introduced a 'fast-track' program for people suspected of having MND, with the ultimate goal to decrease wait times and allow for the final diagnosis to be given in an appropriate tertiary neurological/neuromuscular clinic-based environment. In a review of this program, the NHS goal was met in 91.9% of 'fast-track' patients compared to 57.1% of non-fast-track patients. Furthermore, the mean duration from referral to diagnosis was less than half as long for patients with the fast-track service compared to non-fast-track patients, 50 days compared to 104 days respectively. Interestingly, there was no definite improvement in mean time from initial symptom onset to diagnosis among ALS patients (collectively) after initiating the fast-track program, attributable to an insufficient number of patients through the fast-track pathway to impact the mean time to diagnosis in this specific ALS population (39).

PROSPECTIVE OPPORTUNITIES TO MINIMIZE DIAGNOSTIC DELAY IN ALS

Much of this chapter has discussed the role of primary care practice and its impact on length of time to the diagnosis of ALS. In an era of increasingly subspecialized

medicine, PCPs are increasingly the "gatekeepers" of medicine (51). Unfortunately, a majority of PCPs will see, at most, only one or two ALS cases throughout their entire careers (52). Moreover, most general neurologists will only see a few cases of ALS per year (52) and their knowledge of classic ALS presentations may not be sufficient enough to make the correct diagnosis particularly in cases with more subtle disease onset, or complex presentations.

Intervention strategies similar to those used to improve dementia diagnoses (53), such as practice-based workshops and decision support software, could be applied to improve ALS detection rates at the gatekeeper level, and beyond. Similarly helpful may be diagnostic guidelines and algorithms embedded into electronic medical record software that could alert the user to ALS "red flag" symptoms, and prompt appropriate next steps. To this end, a recently published paper by Matharan et al. proposed an algorithm which may provide guidance regarding when to suspect ALS based on clinical signs and symptoms (Figure 3) (15).

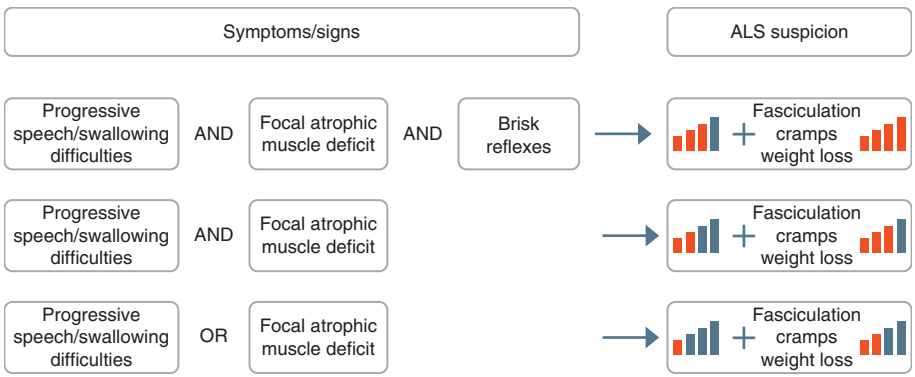


Figure 3. Proposed decision-making algorithm by Matharan et al. with the assistance of Graphandart.com to better screen for ALS based on clinical signs and symptoms (15).

However, ALS-specific provider education directed towards primary care physicians and general neurologists cannot be understated as a key method of intervention to minimize diagnostic errors. As noted in a 1999 paper by Eisen, a potential ALS surrogate marker is only effective if physicians are aware enough about the disease to consider ALS as a differential in the first place (52). Such education must focus on improving recognition of these "red flag" clinical features and correct interpretation of test results. One study found that 70% of patients who presented with a "red flag" symptom such as painless weakness, dysphagia, and gait disturbances did not have ALS as a differential diagnosis in their medical records (21). PCPs may seek a neurology consultation sooner, rather than manage a workup on their own. Furthermore, increased practitioner awareness regarding regional multidisciplinary centers would subsequently promote early referral as well. In addition, there should be education regarding available treatment options, both in terms of disease-modifying therapies and symptom-based management, as well as current clinical trials and those on the horizon, thereby limiting the potential component of "therapeutic nihilism" as noted in dementia literature (6).

Education directed at the general public is also key. To raise the public awareness of ALS, one article proposed changing general terminology about the disease to more accessible phrasing for the average layperson, in much the same way strokes have in a sense been rebranded to "brain attack" and myocardial infarctions to "heart attacks". They also recommended increasing the number of ALS centers so as to be more accessible. However, they acknowledge that given the essential rarity of MNDs, such multidisciplinary centers are typically not cost-effective by conventional standards. Therefore the article proposed creating broader-reaching neurodegenerative centers, where ALS patients may be treated alongside those with dementia, Parkinson's disease, or other progressive neurological conditions (52). Better public/patient understanding about common ALS symptoms may prompt the pursuit of medical evaluation sooner (public education in this regard will have to be necessarily tactful, so as to not promote too low of a threshold for concern). The public should be similarly educated on the availability of disease-modifying treatments and the necessity of intervening early in the disease process.

Ultimately, it is vital to determine where future improvements can be made along the ALS diagnostic timeline, including beyond the contribution of PCPs. Of course, many subsequent referrals are made to otorhinolaryngologists, orthopedists, rheumatologists, and neurosurgeons, among others, and future investigations and interventions pertinent to improving diagnostic delay would be remiss to not include these specialists as well.

CONCLUSION

Diagnostic delay impacts medicine across the spectrum of diseases and subspecialties, but even more so with a progressive neurodegenerative disease such as ALS. Current barriers to minimizing time to diagnosis include referrals to multiple specialists, misdiagnoses, and resultant unnecessary workups and procedures/surgeries. These delays are particularly notable in patients with spine-onset ALS, for whom the differential diagnoses are typically broad.

There is marked potential to reduce these diagnostic delays through improved awareness and clinical education about ALS directed at primary care providers, as well as several other physician/provider types who evaluate these patients before definitive diagnosis is made. There is also a role for tailored education directed at the general public.

The recent literature review (10) found that the typical delay to diagnosis for ALS patients is 10–16 months reviewed studies from 1990 to 2020, which suggests the establishment of clinical diagnostic criteria and growing public awareness of ALS may not have been sufficient to significantly shorten delay (20). While this chapter has addressed the "what" and the "where" with regard to ALS diagnostic delay, there remains the question of why? Is there a reluctance by both PCPs and general neurologists to seek out second opinions from neuromuscular specialists and/or tertiary ALS multidisciplinary clinics? As mentioned previously, the dementia literature aptly notes a certain degree of "therapeutic nihilism" with regard to making the correct diagnosis. Could that also be a significant factor as it pertains to ALS? Perhaps physicians/providers experience some degree of

apprehension in giving patients a terminal diagnosis when other avenues of additional investigation remain open, even if not fully warranted.

There remains an opportunity for broader awareness in the medical field about the role of neuromuscular specialists and tertiary centers in diagnosing and managing ALS. This may lessen the pursuit of unnecessary testing, procedures, and referrals, as may strategically educating the public on common signs and symptoms of the disease. Such education may ultimately result in more expedient referrals to ALS multidisciplinary clinics, followed by overall improvements to quality of life and longevity. Importantly, further dedicated research is needed at the patient and various provider levels regarding reducing the time to ALS diagnosis and hastening referrals to appropriate ALS specialists and multidisciplinary centers.

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Astrocytes in Amyotrophic Lateral Sclerosis

Sandra H. Vaz[1,2] • Sara Pinto[1,2] • Ana M. Sebastião[1,2] • Dora Brites[3,4]

[1]Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal; [2]Instituto de Farmacologia e Neurociências, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal; [3]Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal; [4]Departamento de Ciências Farmacêuticas e do Medicamento, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal

Authors for correspondence: Sandra H. Vaz, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal. E-mail: svaz@medicina.ulisboa.pt; Dora Brites, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal. Email: dbrites@ff.ulisboa.pt

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Abstract: Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder, characterized by the degeneration of upper and lower motor neurons of the motor cortex, brainstem, and ventral horn of the spinal cord. The role of glial cells in the onset and progression of ALS is increasingly being recognized. Dysfunctional astrocytes, with an atypical and neurotoxic phenotype, in the cerebral cortex and the spinal cord promote neuroinflammation and motor neuron degeneration. Indeed, cortical and spinal cord astrocytes from SOD1G93A (mSOD1) mice are neurotoxic, develop early deficits, and lose their neurosupportive properties before disease onset. This chapter discusses the contribution of dysfunctional cortical and spinal cord astrocytes in the development and

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progression of ALS. Differences in astrocyte heterogeneity and reactivity, calcium signaling, neurotransmitters, and in paracrine signaling mechanisms along with implications for novel therapies in ALS are addressed.

Keywords: amyotrophic lateral sclerosis; astrocyte subpopulations; glutamate and homeostatic imbalance; reactive biomarkers; revival of dysfunctional astrocytes

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive and devastating neurodegenerative disorder, characterized by the degeneration of upper and lower motor neurons (MNs) across the corticospinal tract, from the motor cortex to the brainstem, and ventral horn of the spinal cord (SC) (1). The disease progression is aggressive, with a fatal outcome usually within five years of onset. Currently, there is no cure and very few treatments are available for this devastating MN disease. While most cases of ALS are sporadic (sALS) (90–95%), a small subset (5–10%) of patients have a positive familial history (fALS) (2). Mutations in the gene encoding for the Cu^{2+}/Zn^{2+} ion-binding superoxide dismutase (*SOD1*) protein are the most common and represent approximately 20% of fALS cases. SOD1G93A mouse (mSOD1) is currently the most widely used animal model to study ALS. Other mutations associated to ALS are *TARDBP* (also known as *TDP-43*; encodes for TAR DNA-binding protein), *FUS* (encodes RNA-binding protein Fused in Sarcoma/Translocated in Sarcoma), *ANG* (encodes angiogenin, ribonuclease, RNase A family, 5), and *OPTN* (encodes optineurin) (2). The most common mutations associated to ALS and frontotemporal dementia, a variant of ALS, are the gain of toxicity by the nucleotide GGGGCC repeat expansions within the gene *C9ORF72* (3).

The relevance of glial cells on the onset and progression of ALS is now recognized. In genetically modified mice, in which the SOD1 mutation was selectively excised from different central nervous system (CNS) cell types, it was observed that different glial cells significantly promote disease progression (4). Among these, dysfunctional astrocytes, with an aberrant and neurotoxic phenotype in the cerebral cortex and the SC of mSOD1 mice, were recognized as major contributors. ALS astrocytes develop early deficits and lose neuro-supportive properties, secreting toxic factors that directly induce MN cell death (5, 6). In this chapter, we discuss the role of dysfunctional astrocytes in ALS with emphasis on astrocyte reactivity and heterogeneity, neurotransmitter transporters, and dysregulation of autocrine and paracrine mechanisms.

ASTROCYTIC REACTIVITY AND HETEROGENEITY

The exact mechanisms for neuronal degeneration in ALS are still unclear, but astrocytes are recognized as important players in both upper and lower MN loss (4). Neuroinflammation and glial activation are observed at the onset of, and during, disease progression. Astrocytes express differential astrocytic receptors,

transporters, and neurotransmitters, and release neurotrophic factors, inflammatory mediators and cytotoxins. These reactive astrocytes are observed in the cortex and SC of ALS patients, both in sALS and fALS cases (7). Glial cell proliferation and activation are found not only in motor areas, but also in non-motor areas, such as hippocampus, of mSOD1 rats, starting at the presymptomatic stage of the disease (8). SC mSOD1 astrocytes from newborn pups were shown to cause MN toxicity, long before any visible reactive gliosis (5). In ALS, these reactive astrocytes lose their physiological and homeostatic functions and acquire a neurotoxic and aberrant phenotype (5). Transplantation of SOD1 glial-restricted precursor cells into the SC of healthy rodents showed to differentiate into neurotoxic astrocytes and trigger MN degeneration (9), whereas transplantation of normal astrocyte precursors delayed disease progression and extended the survival of mSOD1 rats (10). Astrocytes derived from sALS patients also led to MN degeneration after transplantation into mice (11). Thus, the identification of specific mechanisms and mediators of astrocyte toxicity offers important insights into the pathways of MN degeneration in ALS and the ways to prevent them. Both upper and lower MNs are affected in ALS, and astrocytes reveal regional diverse phenotypes, as depicted in Figure 1.

Cortical and SC astrocytes cause neuronal dysfunction by specific and common pathological mechanisms (12). This is in line with the recent concept of astrocyte heterogeneity, either in the same zone of the CNS or across different regions (13). Reactive astrogliosis and graded reactions depend on microenvironmental cues and interactions between neighboring cells, as well as on autocrine signaling (14). In the mSOD1 mouse model, cortical astrocytes present an early hypertrophic/fibroblast-like morphology and a reactive and inflammatory phenotype. Such phenotype is characterized by decreased expression of glial fibrillary acidic protein (GFAP) and increased cell proliferative capacity, as well as elevated expression of S100 calcium (Ca^{2+})-binding protein B (S100B) and high mobility group box protein 1 (HMGB1). SC astrocytes appear to be more constrained than cortical ones, mainly in the presymptomatic stage, with decreased S100B and HMGB1 expression levels (12, 15). In late stages, a marked proliferative capacity and overexpression of S100B and HMGB1 is observed in astrocytes from the SC of ALS patients, and rodent models (16, 17).

GFAP is the hallmark intermediate filament protein in astrocytes and its upregulation is usually associated with reactive astrogliosis (18). However, GFAP amount in adjacent astrocytes is extremely heterogeneous, as well as its expression in different regions (19, 20). Reactive GFAP-astrocytes were found in the ventral horn of ALS patients (21), with elevated appearance in the cerebrospinal fluid (CSF) relative to other neurologic diseases (22). Astrocytes with increased GFAP content and multiple inflammatory/reactive mediators were also identified in the SC of adult mice (23). While differential distribution of GFAP immunoreactivity was found in the white matter of the SC at early symptomatic transgenic mSOD1 mice, in the gray matter that was found only in the end-stage disease (24). In other studies, GFAP expression did not show differences between mSOD1 and wild-type mice at 40 and 80 postnatal days, but strongly increased at terminal stages in the SC of mSOD1 mice (25). In contrast, our studies evidenced decreased levels of GFAP in the pre-symptomatic stage in the cortical brain (15) and in the SC of mSOD1 mice (17). Decreased GFAP expression levels were also found in astrocytes isolated from the brain cortex of mSOD1 pups, which presented

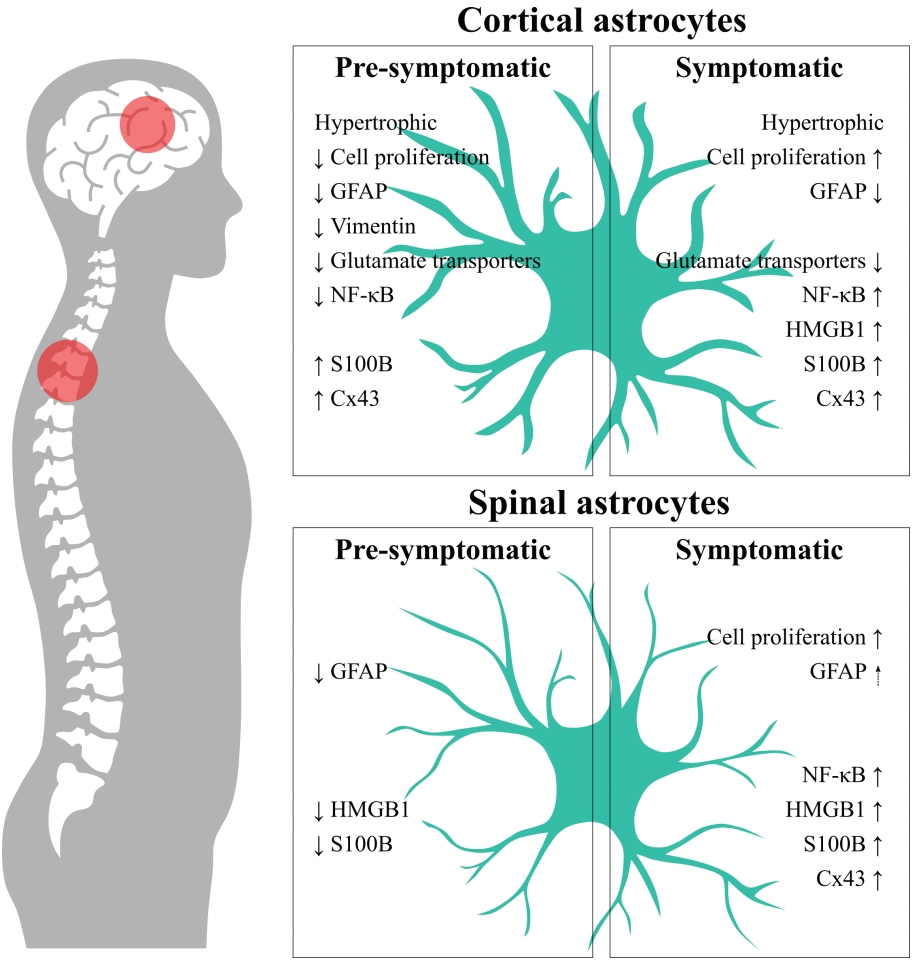

Figure 1. Astrocyte regional diversity and heterogeneity in ALS. Astrocytes in ALS are known to have an aberrant and reactive profile, expressing different astrocytic markers depending on their location in the central nervous system (cortical or spinal astrocytes), and stage of the disease (pre-symptomatic and symptomatic). Cortical astrocytes are less proliferative in the pre-symptomatic stage, where they show a decreased expression of GFAP, vimentin, glutamate transporters and NF-κB, and an increased expression of S100B and Cx43 (12, 15). In the symptomatic stage of the disease, cortical astrocytes are more proliferative, showing an increased expression of NF-κB, HMGB1, S100B and Cx43, together with a reduction of glutamate transporters and GFAP (12, 15). Spinal astrocytes in the pre-symptomatic stage exhibit a decreased expression of GFAP, S100B and HMGB1, while in the symptomatic stage astrocytes present a proliferative profile with increased expression of NF-κB, S100B, HMGB1 and Cx43. The expression of GFAP in the symptomatic spinal astrocyte has been shown to increase or decrease, depending on the model, condition or region used in the study (16, 17, 23, 24). Altogether, these data demonstrate the high diversity and heterogeneity of astrocytes in ALS. ALS, amyotrophic lateral sclerosis; Cx43, connexin-43, GFAP, glial fibrillary acidic protein; HMGB1, high mobility group box protein 1; miRNA-146a, microRNA-146a; NF-κB, nuclear factor kappa; S100B, S100 calcium-binding protein B.

aberrant astrocytic markers [increased S100B, connexin 43 (Cx43), Ki-67, and vimentin, together with decreased GFAP, glutamate transporter-1 (GLT-1) and glutamate/aspartate transporter (GLAST)], as found in the same region at the symptomatic stage (15). Such acquired "immature" or dedifferentiated astrocyte phenotype is neurotoxic and disease-specific in the cortical brain, and probably associated with the bulbar origin of the disorder (12). The early occurrence of such signature in 7-day-old astrocytes from the brain cortex of mSOD1 mice was also observed in other disease models and related with the loss of neuro-supportive functions (26). Decreased expression of GFAP was similarly found in glial cell populations from the SC of symptomatic mSOD1 rats (16). When expression of mSOD1 was virally induced in cortical astrocytes, alterations in cell morphology and density, together with low GFAP immunostaining were obtained (27). However, the loss of GFAP only marginally accelerated disease progression in the SOD1H46R transgenic mice (28). In sum, GFAP is not an absolute marker of reactivity, nor it strictly correlates with the disease severity. Variations in animal models, regional diversity, and specific astrocyte subpopulations may be the reason for the disparate data found in the literature.

Increased expression of HMGB1 in reactive glia may lead to the activation of toll-like receptor/receptor for advanced glycation end-products (TLR/RAGE) signaling pathways, and contribute to the progression of inflammation and MN injury (29). S100B is a Ca^{2+}-binding protein that is highly expressed in astrocytes and, depending on its concentration, can have beneficial or deleterious effects. In ALS, S100B levels are increased in the CSF, positively correlating with a worse prognosis of the disease (30). The inhibition of S100B downregulates the expression of GFAP and cytokines, such as tumor necrosis factor (TNF)-α, C-C motif chemokine ligand 6 (CCL6), and C-X-C motif chemokine ligand 10 (CXCL10), indicating its association to a proinflammatory phenotype in mSOD1 astrocytes. Expression and release of pro-inflammatory cytokines lead to the activation of the nuclear factor kappa B (NF-κB) signaling cascade, a regulator of reactive gliosis and inflammation. In early stages of ALS, NF-κB activation in SC astrocytes can induce a neuroprotective phenotype, by promoting beneficial microglia activation and delaying disease progression. However, prolonged NF-κB activation in later stages exacerbates the immune response with pro-inflammatory microglial activation, gliosis, and disruption of the blood-SC barrier (31). Communication among astrocytes is promoted by connexin-based gap junctions, such as Cx43. Abnormally high Cx43 expression in the cortical and SC astrocytes of mSOD1 mice and ALS patients is associated to astrocyte-mediated neurotoxicity (32).

Astrocytes in ALS release soluble toxic factors that promote MN degeneration (16), but still not clearly identified. Upregulation of the major histocompatibility complex I (MHC-I) in MNs seems to be associated with a slower disease progression (4). However, astrocytes in both mSOD1 mice and ALS patients were shown to downregulate the expression of MHC-I in MNs, by causing endoplasmic reticulum (ER) stress, thus increasing their susceptibility to astrocyte-induced cell death (33). On the other hand, reactive astrocytes secrete increased transforming growth factor (TGF)-β1 that causes MN autophagic dysregulation, abnormal protein aggregation, and cellular toxicity (23). Astrocytes, when exposed to the CSF from ALS patients, release pro-inflammatory cytokines, such as interleukin (IL)-6, TNF-α, and interferon (IFN)-γ, together with increased levels of glutamate, reactive oxygen species, and nitric oxide, causing neurotoxicity. These in turn lead to

a downregulation of neurotrophic factors, such as vascular endothelial growth factor (VEGF) and glial cell line-derived neurotrophic factor (GDNF) (34), as detailed below. In ALS, activated microglia secrete IL-1α, TNF-α, and complement component 1q (C1q), known to induce neurotoxic reactive astrocytes (35). Abrogation of IL-1α, TNF-α, and C1q was shown to reduce astrogliosis and extend mSOD1 mouse survival. In the absence of reactive astrocytes, MN death is significantly delayed (36). The major mechanisms leading to MN degeneration are summarized in Figure 2.

Aberrant TDP-43 aggregation, a pathological hallmark of both ALS and frontotemporal dementia, was found in astrocytes and shown to contribute to neurodegeneration through cell-specific mechanisms (37). Astrocytes expressing mutated C9ORF72 show a deficient expression of antioxidant proteins, such as SOD1, SOD2 and peroxiredoxins (38). Altogether, astrocytes in ALS not only have a reactive and inflammatory phenotype, but also show impaired protective functions, which may be even exacerbated by the activation of other glial cells such as microglia (35). All these features indicate that astrocytes participate in the control and maintenance of homeostatic balance but, when dysregulated, they lead to neuroinflammation and MN death, thus supporting astrocyte's key role in the onset and progression of ALS. It is not known if the appearance of an aberrant astrocyte signature previous to ALS symptom onset, in the brain cortex, results from intrinsic cell deficiencies or whether it is determined by MN paracrine pathological signaling. Dysregulated GFAP, S100B, and the marker of proliferation, Ki-67, in immature cortical astrocytes of mSOD1 pups agree with the first hypothesis. Identification of disease-specific astrocytic subpopulations will have a high impact on the understanding of their pathological role in ALS, and on their targeting toward the recovery of a neuroprotective phenotype.

GLUTAMATE AND GABA TRANSPORTERS

Alterations in excitatory neurotransmission appear to play a role in ALS. Hyperexcitability has been observed in sALS and fALS patients before the onset of symptoms, and also in presymptomatic mSOD1 mouse models. However, other studies showed hypoexcitability, rather than hyperexcitability, prior to degeneration (1, 37, 39). Thus, it is still unclear whether hyperexcitability leads to MN degeneration or if it is a compensatory mechanism resulting from MN loss. Astrocytes sustain homeostatic levels of extrasynaptic glutamate within the synaptic cleft to control synaptic transmission, mainly through specific glutamate transporters, such as GLAST and GLT-1 (40). The GLT-1 transporter is found exclusively in astroglia, both in brain and SC, and is responsible for the uptake of nearly 90% of the glutamate. One of the proposed mechanisms for MN death in ALS is glutamate-mediated excitotoxicity (1), since impaired glutamate clearance was shown in astrocytes expressing mSOD1 and TDP-43, suggesting a common pathological feature in ALS (Figure 3A) (41, 42).

Defects in glutamate uptake by GLT-1 were found in the SC of ALS patients in regions of MN loss (43), in mSOD1 rodents (44), and in TDP-43 mice (45).

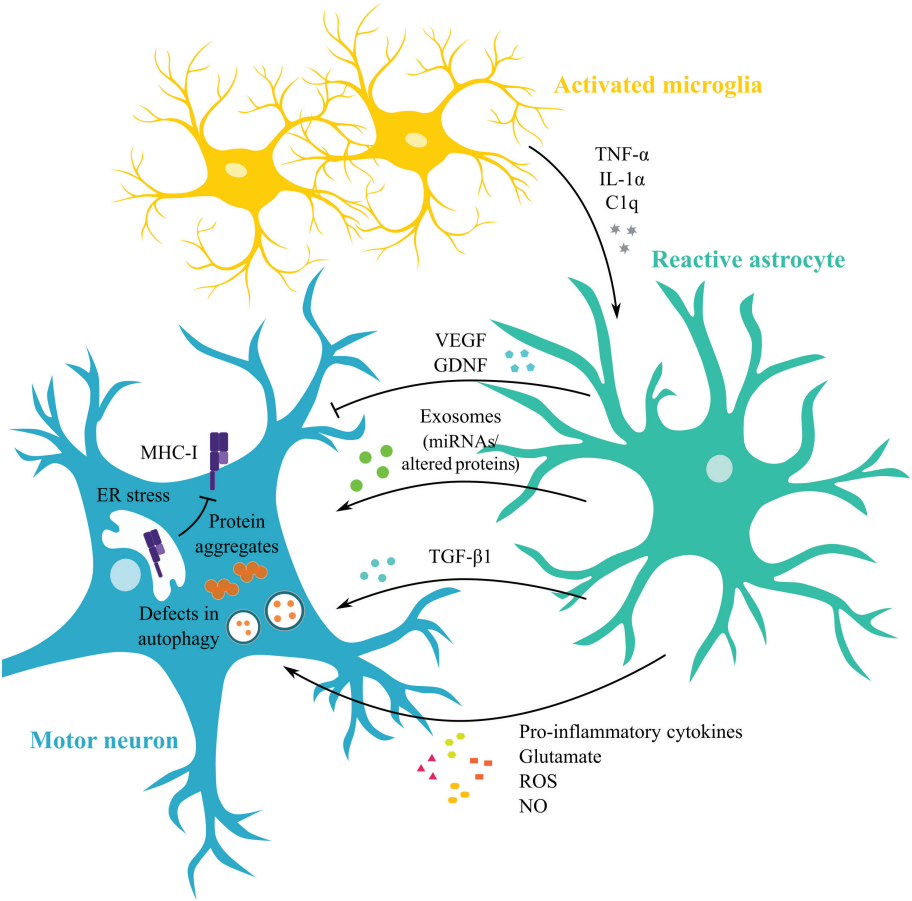

Figure 2. Reactive astrocytes promote MN degeneration in ALS. Astrocytes are known to have an aberrant and reactive profile in ALS, releasing several soluble toxic factors and inflammatory mediators that render MNs more susceptible to degeneration. Astrocytes respond to factors released to the milieu by activated microglia, such as TNF-α, IL-1α and C1q. Reactive astrocytes secrete high levels of pro-inflammatory cytokines, glutamate, ROS and NO, as well as lower levels of neurotrophic factors, such as VEGF and GDNF (34). Moreover, astrocytes can downregulate the expression of MHC-I in MNs, by causing ER stress and making them more susceptible to astrocyte-induced cell death (33). Reactive astrocytes also promote defects in autophagy by secretion of TGF-β1 and activation of the rapamycin signaling pathway in MNs (23). Besides soluble factors, exosomes derived from ALS astrocytes also transfer miRNAs and mutant and misfolded proteins to neighboring cells (35, 36). Altogether, astrocytes can contribute to abnormal protein aggregation and cellular toxicity. ALS, amyotrophic lateral sclerosis; C1q, complement component 1q; ER, endoplasmic reticulum; GDNF, glial cell line-derived neurotrophic factor; IL-1α, interleukin 1 alpha; miRNA, microRNA; MHC-I, major histocompatibility complex I; MNs, motor neurons; NO, nitric oxide; ROS, reactive oxygen species; TGF-β1, transforming growth factor beta 1; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

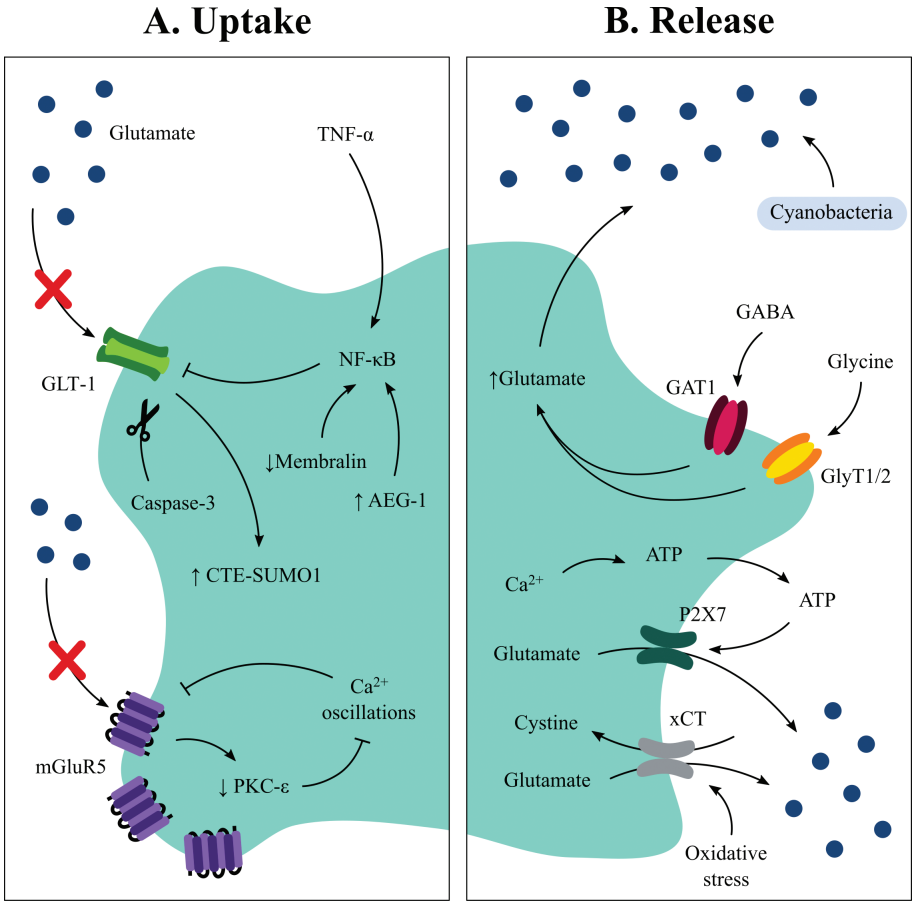

Figure 3. Glutamate-mediated excitotoxicity in ALS. A. In ALS, the astrocytic glutamate transporter GLT-1 is downregulated, leading to an impaired glutamate uptake, and the TNF-α/TNFR1/NF-κB pathway modulates its expression levels (48). Membralin is reduced in the spinal cord of ALS patients and ALS mouse models, and its deletion was shown to suppress GLT-1 expression through the TNF-α/TNFR1/NF-κB pathway (49). AEG-1 is upregulated in ALS and its silencing restores GLT-1 expression and inhibits NF-κB signaling (50). Caspase-3 cleaves GLT-1 and leads to the accumulation of a sumoylated C-terminus fragment, the CTE-SUMO1. The accumulation of this fragment causes astrocytes to alter their phenotypes and secrete toxic factors to MNs (51). Metabotropic glutamate receptors, such as mGluR5, are overexpressed in ALS, but their function was shown to be altered. PKC-ε is reduced in astrocytes, leading to the generation of atypical Ca^{2+} oscillations and impaired glutamate uptake (58, 59). **B.** Glutamate release has been shown to be abnormally increased in ALS. Exposure to GABA or glycine leads to an abnormal GAT1 or GlyT1/2-mediated glutamate release (62). Astrocytes also release high levels of glutamate through the upregulation of cystine/glutamate antiporters (xCT), in response to oxidative stress (65). Elevated symbiotic Cyanobacteria increases glutamate, linking gut microbiota to ALS (66). AEG-1, astrocyte elevated gene 1; ALS, amyotrophic lateral sclerosis; Ca^{2+}, calcium; GABA, gamma aminobutyric acid; GAT1, GABA transporter type 1; GlyT, glycine transporter; GLT-1, glutamate transporter 1; mGluR, metabotropic glutamate receptor 5; MNs, motor neurons; NF-κB, nuclear factor-kappa B; PKC-ε, protein kinase C-epsilon; TNF-α, tumor necrosis factor alpha; TNFR1, tumor necrosis factor receptor 1; xCT, cystine/glutamate antiporters.

Several pathways have been implicated in the modulation of GLT-1 levels: (i) TNF-α and downstream NF-κB signaling have been shown to suppress GLT-1 expression (46); (ii) the specific deletion of the ER-component membralin causes a dramatic accumulation of extracellular glutamate, inducing MN glutamatergic toxicity (47); and (iii) decreased GLT-1 expression and glutamate uptake also occur as result of increased astrocyte elevated gene-1 (AEG-1) in mSOD1 astrocytes (48). Moreover, GLT-1 cleavage may derive from the action of caspase-3, with accumulation of a sumoylated GLT-1 C-terminus fragment early on the disease, causing astrocyte phenotypic aberrancies and release of neurotoxic factors (49). Modulation of GLT-1 to potentially prevent excitotoxicity has been attempted. Although, SC focal restoration of GLT-1 expression in astrocytes was not effective (50), enhanced GLT-1 translation by LDN/OSU-0212320 delayed MN function decline and extended the lifespan of mSOD1 mice (51). MC1568, an inhibitor of the enzymes Class-II histone deacetylases, restored GLT-1 expression and glutamate uptake in the SC of mSOD1 mice, but did not prolong their survival (52). Activation of the metabotropic glutamate receptors (mGluRs), mGluR1 and mGluR5, leads to increased intracellular Ca^{2+} and facilitates glutamate transport (53). In line with this, when the overexpression of dysfunctional mGluR1 and mGluR5 in reactive SC astrocytes from ALS patients and mSOD1 mice was reduced, it prevented excessive glutamate release, improved the function of MNs, astrocytes and microglia, and increased animal survival (54, 55). Deficient glutamate uptake was linked to altered mGluR5-mediated Ca^{2+} signaling profile (56). By restoring Ca^{2+} oscillations in astrocytes from mSOD1 rats, mGluR5-mediated glutamate uptake was recovered (Figure 3A) (57). While glutamate is the major excitatory neurotransmitter in the CNS, gamma-aminobutyric acid (GABA) and glycine are the main inhibitory neurotransmitters. Raiteri *et al*. have shown that the activation of a glycine transporter on SC MNs caused enhanced glutamate release in a mouse model of ALS (58). Moreover, in the SC glutamatergic synaptic boutons of mSOD1 mice, the impact of synaptic vesicle exocytosis on the trafficking of nerve terminal GABA transporter-1 (GAT-1) and of type-1/2 glycine (Gly) transporters (GlyT-1/2) was studied by monitoring membrane expression and function of these transporters. It was observed that the enhanced exocytosis in mSOD1 mice boosts heterotransporter membrane expression, which evokes excessive glutamate release (Figure 3B) (59). GlyT1/2 and GAT1 are widely expressed in astrocytes and gliosomes (60), together with GABA and glutamate transporters. GABA-induced release of glutamate from SC gliosomes is enhanced in mSOD1 mice (61). Astrocytes were also shown to release higher levels of glutamate, through cystine/glutamate antiporters, in response to oxidative stress (62). SOD1 mutation was shown to reduce intracellular lactate levels and its secretion by astrocytes (63). Studies linking ALS with gut microbiota composition identified that elevated symbiotic Cyanobacteria could promote the elevation of glutamate, in contrast to the general *Lactobacillus*, *Bifidobacterium*, and *Odoribacter*—all known to metabolize glutamate (64). These findings open an all-new window of opportunities to characterize microbiota changes as ALS biomarkers and microbial strategies to improve health status quality of ALS patients.

DYSREGULATED AUTOCRINE/PARACRINE SIGNALING MECHANISMS

Astrocytes have a unique form of excitability, which is characterized by intracellular Ca^{2+} oscillations or waves in response to physiological and pathophysiological signals. Intracellular Ca^{2+} elevation is triggered by several mechanisms, such as: (i) activation of Gq-protein-coupled receptors (GPCRs); (ii) GABAB receptor (Gi-coupled GPCRs) activation (65); and (iii) transient receptor potential (TRP) channels (66). Spatially restricted Ca^{2+} transients in astrocyte microdomains are associated with mitochondria (67). Altogether, they promote the release of gliotransmitters, such as glutamate, D-serine, GABA and neurotoxic factors (68).

The pathogenic potential of Ca^{2+} dysregulation in astrocytes may account for disease progression. ALS astrocytes show mitochondrial functional deficiencies and impaired Ca^{2+} homeostasis that promotes MN degeneration (69). In the SC of young mSOD1 mice, the enhanced expression of mGluR5 makes astrocytes vulnerable to glutamate, and causes persistent elevation of intracellular Ca^{2+} concentrations, which are reverted by Bcl-XL, and protein kinase C epsilon (Figure 3A) (57, 70). Administration of the mGluR5 antagonist 2-methyl-6-(phenylethynyl) pyridine (MPEP) slowed astrocyte degeneration, delayed disease onset and extended mSOD1 mouse survival (71). Also, purinergic stimulation of SC and cortical SOD1-expressing astrocytes caused ER Ca^{2+} accumulation and abnormal Ca^{2+} signaling (72). The increased expression of Cx43 in mSOD1 mice also has a significant impact on Ca^{2+} signaling (73). Intracellular Ca^{2+} increase in astrocytes leads to the release of gliotransmitters, including glutamate, D-serine, GABA, brain derived neurotrophic factor (BDNF), as well as neurotoxic factors (68). Since astrocytes in ALS reveal many pathogenic changes, such as disrupted receptor-mediated Ca^{2+} signaling and mitochondrial functional deficiencies, it is anticipated that an impaired gliotransmitter release from ALS astrocytes will play a major role in MN pathology.

The storage and release of bioactive molecules by astrocytes involve mechanisms of exocytosis, diffusion through plasma membrane channels, and translocation by plasma membrane transporters. The soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent vesicular exocytotic release is one of the major pathways for astrocyte secretion. ER Ca^{2+} release induces elevated ATP in mSOD1 astrocytes, which can be inhibited by the overexpression of dominant-negative SNARE to prevent toxicity to MNs and delay disease onset in mSOD1 mice (72). Similarly, pharmacological inhibition of P2X7 receptor abolished astrocyte toxicity towards MNs through degradation of extracellular ATP in mSOD1 mice (74, 75). Since P2X7 receptors form pores under pathophysiological conditions, P2X7 may function as membrane channels that allow the release of glutamate or toxic agents, thus accounting for ALS progression (Figure 3B).

Neurotrophic factors, in particular neurotrophins, are crucial for neuronal differentiation, maturation and survival, as well as for the modulation of synaptic transmission and plasticity (76). They are also potential therapeutic targets for neurodegenerative disorders, such as ALS (77). The neurotrophin family is composed of four members: nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). BDNF is abundantly expressed in the CNS, where it

supports neuronal survival (e.g., MNs) (77). SC astrocytes from mSOD1 mice respond to HMGB1 by decreasing BDNF and GDNF production, in contrast to wild-type astrocytes (78). Also, as previously mentioned, astrocytes exposed to the CSF of ALS patients, besides releasing neurotoxic factors, release lower levels of VEGF and GDNF (34).

ALS pathophysiology is intimately related with neuroinflammatory processes, which include the release of both neuroprotective and/or neurotoxic factors that play a role in MN pathology (79). Several studies have shown that TGF-β signaling is involved in ALS and that TGF-β1 release from astrocytes accelerates disease progression in ALS mice (23). Extracellular vesicles with ~40–160 nm, denominated exosomes, are released from mSOD1 astrocytes and were shown to contain mutant SOD1 and dysregulated cargo in miRNAs, accounting for MN pathology and homeostatic imbalance (80, 81). miRNAs are small non-coding RNAs that control posttranscriptional expression of target genes (82). Dying neurons in ALS release miRNAs, such as miRNA(miR)-218, that can change the phenotype of astrocytes into a reactive one and cause the downregulation of GLT-1 (83). In most cases, exosomal cargo in miRNAs recapitulate their cell of origin (84, 85). Dysregulated expression of miRNAs was found in ALS (86, 87) and proposed as biomarkers (88). Upregulation of miR-155 was identified in fALS and sALS patients, as well as in the SC of mSOD1 mice, in pre-symptomatic and symptomatic stages (17). In contrast, a decreased cargo in miR-494-3p was found in C9ORF72 astrocyte-derived exosomes with harmful consequences in neurite network in ALS (89). Depleted levels of miR-146a were also recognized in exosomes from the cortical astrocytes of mSOD1 mice (15), and its cellular replenishment abrogated the astrocyte aberrant phenotype, characterized by increased S100B and Cx43 levels, together with decreased GFAP, while leading to a secretome with paracrine neuroprotective properties (81). Exosomes from both cortical and spinal mSOD1 astrocytes were deficient in miR-155, miR-21 and miR-146a (12). Exosomes with low levels of miRNAs may lead to paracrine dysregulation and dysfunction of recipient cells, while also activating immune-associated cells (90). Exosomes may serve as potential therapeutic targets in ALS and prognostic markers for therapy in precision medicine through patient stratification.

CONCLUSION

The role of dysfunctional astrocytes in the pathogenesis of ALS indicates that astrocytes may be targeted with strategies for their revival. These strategies may include direct intervention on astrocytes with modulatory medicines, exosomes and miRNA-based therapies, or their replacement (Figure 4). Considering the first approach, activation of the nuclear factor erythroid 2–related factor (Nrf2) was shown to increase glutathione secretion; although some beneficial effects were observed on glial reactivity, it did not affect survival in mSOD1 mouse models (4). Reduction of reactive oxygen species production has been attempted, but again with no effective benefits (91). Another approach was the overexpression of MHC-I in MNs to enhance their resistance to the toxic factors released by the reactive astrocytes; this approach enhanced the survival of mSOD1 mice (33). As the aberrant astrocytes are associated with inflammatory and immune

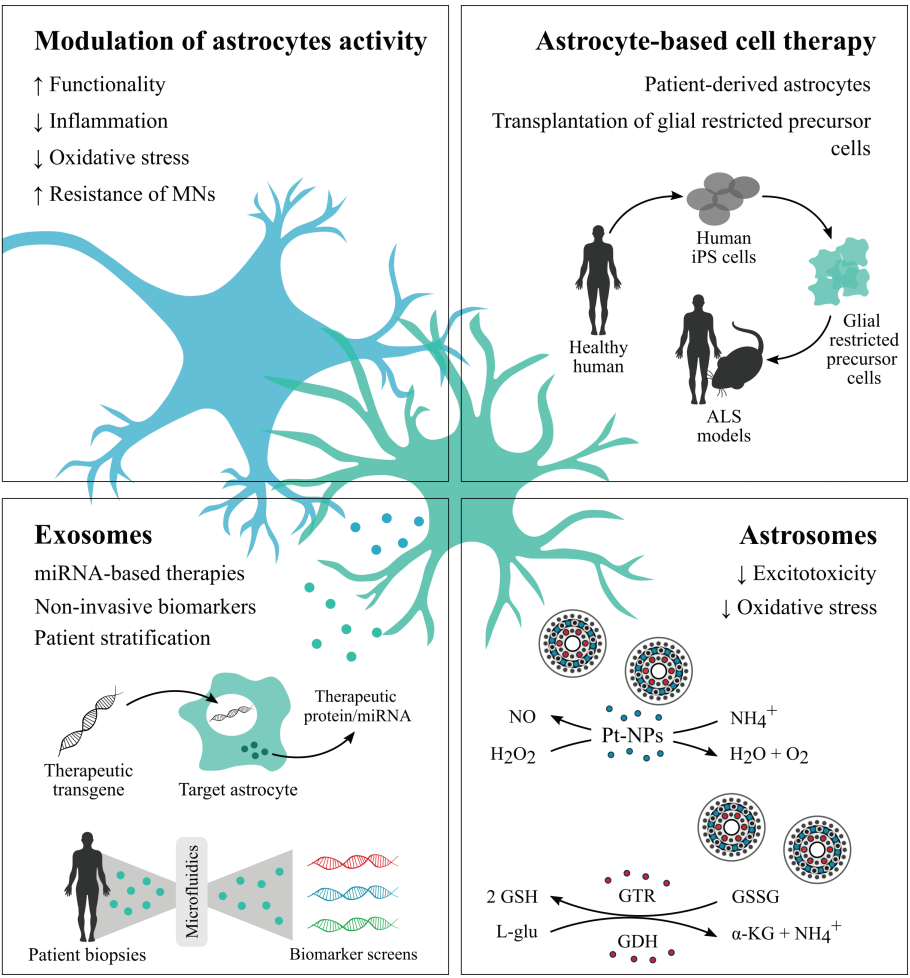

Figure 4. Targeting astrocytes for therapy. For functional recovery, astrocyte intervention strategies for ALS may include the modulation of astrocytic activity by using medicines or exosomes, astrocyte-based cell transplantation, and astrosomes (98). The switch of the phenotypic aberrancies and dysfunctionalities of the cell toward the steady-state astrocyte phenotype may include the inhibition of inflammatory mechanisms and oxidative stress (4, 36), increased resilience to paracrine toxic or inflammatory mediators released by MNs and activated microglia, or the boost of the cell, if senescent. Another possibility is the delivery of miRNA-based therapies, using miRNA mimics and/or inhibitors (36). For astrocyte-based cell therapy, patient-derived astrocytes, and glial progenitor cells/glial restricted precursor cells (4) may be used. The delivery of astrosomes, as artificial astrocytes, is an alternative strategy based on their ability to scavenge hydrogen peroxide, ROS, and ammonia, thus decreasing the excitotoxicity and the oxidative stress associated with ALS (96–98). ALS, amyotrophic lateral sclerosis; GDH, glutamate dehydrogenase; GSH, reduced glutathione; GTR, glutathione reductase; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; L-Glu, L-glutamate; α-KG, α-ketoglutarate; miRNA, microRNA; MN, motor neuron; NH_4^+, ammonia; NO, nitric oxide; Pt-NP, platinum nanoparticle; ROS, reactive oxygen species.

mechanisms (15, 17), modulation of such mechanisms with specific miRNA-based strategies may prevent cell-to-cell paracrine dysregulation and MN degeneration (81, 86).

The use of patient-derived astrocytes by reprogramming techniques brought new possibilities of therapeutic intervention, mainly because drug testing can be done in cells from sALS patients (92, 93). Transplantation of glial restricted precursor cells (94), combined with strategies capable of defending these cells from local toxicity (4), may represent innovative therapeutic approaches for ALS. Transplantation of neural progenitor cells expressing GDNF into the motor cortex of mSOD1 rats showed promise in extending their survival (95). Moreover, Armada-Moreira and colleagues developed an artificial astrocyte ("astrosome") capable of scavenging hydrogen peroxide and ammonia, by using platinum nanoparticles as artificial enzymes, as well as enzymes capable of glutamate degradation (96–98). Therefore, these microreactors have the potential to provide a therapeutic approach for several neurological diseases, such as ALS, in which oxidative stress and excitotoxicity are observed. We now have the possibility to work with human astrocytes differentiated from sALS and fALS patients and soon it will be possible to identify new targets and stratify patient astrocyte phenotypes, by using 3D microfluidic system models, and test promising therapeutics. Ultimately, this will provide a better understanding of the contribution of astrocytes in ALS, and how we might apply novel therapeutic strategies aimed at producing the revival of astrocytes, or even their replacement, and help in halting, or at least delaying ALS progression.

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4

Synaptic Transmission and Motoneuron Excitability Defects in Amyotrophic Lateral Sclerosis

Frédérique Scamps[1] • Franck Aimond[1] • Cécile Hilaire[1] • Cédric Raoul[1,2]

[1] INM, Univ Montpellier, Inserm, Montpellier, France; [2] Laboratory of Neurobiology, Kazan Federal University, Kazan, Russia

Author for correspondence: Cédric Raoul, INM, Univ Montpellier, Inserm, Montpellier, France. Email: cedric.raoul@inserm.fr

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Abstract: Amyotrophic lateral sclerosis is a fatal adult-onset neurodegenerative disease characterized by progressive muscular weakness and atrophy. The primary feature of amyotrophic lateral sclerosis is the selective loss of motoneurons in the brain and spinal cord. However, changes in synaptic transmission and motoneuron excitability are among the first events that take place during development and accompany the relentless deterioration of motor circuitry. This chapter aims to summarize the current understanding of defects in intrinsic electrophysiological properties of motoneurons, local GABAergic and glycinergic inhibitory as well as cholinergic modulatory interneuron networks, and long-range glutamatergic excitatory input neurons that can precede disease onset or occur during the progression of the disease. We summarize evidence that therapeutic options that target synaptic transmission and intrinsic features of motoneurons might represent novel effective strategies for patients with amyotrophic lateral sclerosis.

Keywords: amyotrophic lateral sclerosis; inhibitory transmission; motoneuron excitability; neuromodulation; proprioception

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disorder affecting both upper motoneurons in the cerebral cortex and lower motoneurons in the brainstem and spinal cord. Upper motoneurons are glutamatergic descending neurons that synapse directly or indirectly via interneurons onto lower motoneurons typically through the corticobulbar and corticospinal tracts. Lower motoneurons are multipolar cholinergic neurons whose axons exit the central nervous system to innervate skeletal muscles to produce movement. Consequently, ALS leads to progressive muscle atrophy and weakness and eventual paralysis. Death occurs within 3 years of symptom onset, typically from respiratory failure. Sporadic ALS accounts for approximately 90% of cases; the remaining 10% are hereditary and referred to as familial (1). The discovery of ALS-causing mutations in the *superoxide dismutase-1* (*SOD1*) gene in 1993 led to the generation of transgenic mice that recapitulate the key features of the disease. *SOD1*-mutant mice, with other experimental models that have emerged following identification of a rapidly growing number of ALS-associated genes, have helped to learn about the molecular and cellular processes underlying the disease. Both cell- and non-cell-autonomous mechanisms contribute to the dysfunction and death of motoneurons. The expression of ALS-causing factors in glial cells, which include astrocytes, microglia, and oligodendrocytes, contribute to the selective death of motoneurons, which themselves present with a significant vulnerability due to these same determining factors (2–4). T lymphocytes infiltrating the central nervous system and peripheral macrophages are other cellular factors that participate in the pathogenesis of ALS (5–7). Non-cell-autonomous mechanisms may support dysfunction before the first clinical signs are evident or accompany motor decline during the symptomatic phase. The earliest signs, which are detected during embryonic and postnatal development and that will pave the way for the rest of the disease course in ALS mice, are linked to the electrophysiological properties and circuitry of motoneurons. Moreover, in humans, asymptomatic mutation carriers can exhibit electrophysiological abnormalities such as intracortical facilitation (ICF) transmission deficits, which can be observed 30 years before the onset of symptoms (8). Here, we review alterations of intrinsic electrophysiological features of motoneurons and synaptic transmission, including changes in inhibitory, excitatory, and modulatory signals, observed in patients with ALS and in mice. We discuss how these changes might be considered promising therapeutic targets for new and effective treatments for this devastating disease.

ELECTROPHYSIOLOGICAL PROPERTIES OF MOTONEURONS IN ALS

Motoneurons acquire molecular properties during their differentiation throughout embryonic development as a result of dynamic interplay between spatial and temporal expression of families of transcription factors and diffusible morphogens. In the terminal step of differentiation, the combinatorial activity of terminal effector genes defines the features of individual postmitotic motoneurons.

This battery of terminal identity genes governs the synthesis of neurotransmitters and expression of neurotransmitter receptors, ion channels, and axon guidance and synaptic adhesion molecules (9, 10). At these early stages of development, the first wirings of neuronal circuits proceed with axon outgrowth toward appropriate targets and by the complex interaction of both intrinsic genetic instructions and environmental cues. Spinal motoneurons are organized into motor columns along the rostrocaudal and ventrodorsal axes that project to a single muscle target in the periphery. Long descending premotor projection neurons from the spinal cord and supraspinal centers, as well as proprioceptive afferents, begin to establish the spinal motor circuitry during embryonic development (11, 12). The extensive dendritic arborization of motoneurons that integrates synaptic inputs critical for circuit formation and plasticity is shaped (13), and the diversity of local interneuron subtypes that direct early motor output enables further adaptive motor behavior (14). Among the developmental processes that build a coherent motor circuitry, the early calcium-mediated electrical activity and acquisition of intrinsic electrophysiological properties are critical factors. Expression of ion channels at the plasma membrane determines the intrinsic responses of motoneurons and undergoes dynamic changes from embryonic to postnatal development (15, 16). These multiple components of a developmental program represent a cornerstone establishing movement coordination, control, and skill that will be fundamental throughout the life span. The concept that alterations to these components can occur very early in the life of patients has been explored through animal models of the disease. Although the first clinical signs, and significant loss of motoneurons, appear in adults, early molecular and cellular signs have been documented in ALS mice. In *SOD1*^{G93A} mice, the first motor symptoms appear at around 90 days of age, but activation of cellular stress pathways can be observed in vulnerable motoneurons as early as postnatal day (P)12, and dysfunction of the neuromuscular junction is already noticeable at P50 (17, 18). However, the earliest alterations that evidence a functional defect are those observed during the developmental stages of the motor system and are associated with motoneurons' acquisition of electrophysiological properties and the integration of motoneurons into the motor circuitry.

Electrophysiological changes in motoneurons during embryonic and postnatal development

Some differences in the resting membrane potential (RMP), input resistance, capacitance, and rheobase have been observed in different experimental systems (isolated neurons from mouse or humans, spinal preparations, brainstem and spinal cord slices) at different stages (from embryonic day (E) 17.5 to P10, or after 11 weeks of differentiation *in vitro*) and in the presence of different ALS-causing mutations. However, changes in cell properties do not emerge as a common salient feature of ALS motoneurons (Table 1). The spike features, which include action potential (AP) threshold, delay to AP (i.e., the time interval between current injection and spike onset), AP amplitude, AP duration, rate of AP rise, and repolarization, as well as after-hyperpolarization (AHP) characteristics, are for the most part similar in experimental ALS models and controls. There have been discrepancies in the AP duration and the rate of AP rise between different studies in

TABLE 1 **Alterations of motoneuron electrophysiological features in ALS models**

	Embryonic primary culture	iPSC-derived neuron culture	Embryonic spinal cord preparation	Postnatal brainstem slice	Postnatal spinal cord slice	Postnatal spinal cord preparation
RMP	Unchanged (21–24)	Unchanged (28, 30, 31), Depolarized (27)	Unchanged (25, 34)	Unchanged (26)	Unchanged (20, 23), depolarized (19)	Unchanged (29)
Input resistance	Unchanged (21–23)	Unchanged (27, 28, 30, 31)	Increased (25, 34)	Unchanged (26)	Unchanged (19, 23)	Lower (29)
Capacitance	Unchanged (21)	Unchanged (28, 30), decreased (30), increased (27)	Decreased (25)		Unchanged (20)	Increased (29)
AP threshold	Unchanged (21, 22, 24)	Unchanged (28)	Unchanged (25)	Unchanged (26)	Unchanged (19, 20, 23)	
Delay to AP	Unchanged (21)		increased (25)		Unchanged (19)	
AP amplitude	Unchanged (21–24)	Unchanged (28)	Unchanged (25)	Increased (26)	Unchanged (23), decreased (19)	
AP duration	Unchanged (21–24)	Unchanged (28)	Unchanged (25)	Unchanged (26)	Unchanged (23), decreased (20), increased (19)	Decreased (29)
Rate of AP rise	Unchanged (21, 24)				Increased (20), decreased (19)	
Rate of repolarization	Increased (21)				Increased (20)	
AHP characteristics	Unchanged (22–24)	Unchanged (28)	Unchanged (25)	Unchanged (26)	Unchanged (19, 23), decreased τ (20)	Unchanged (29)

TABLE 1 **Alterations of motoneuron electrophysiological features in ALS models (Continued)**

	Embryonic primary culture	iPSC-derived neuron culture	Embryonic spinal cord preparation	Postnatal brainstem slice	Postnatal spinal cord slice	Postnatal spinal cord preparation
Firing frequency	Increased (21–24)	Increased (27, 28), decreased (27, 30, 31)	Increased (25)	Increased (26)	Unchanged (20), increased (23), decreased (19)	Decreased (29)
Maximum firing rate	Increased (23)		Unchanged (25)			Unchanged (29)
Na^+ current peak	Unchanged (250)	Unchanged (27, 28, 30), decreased (27, 30)				
K^+ current peak		Unchanged (27), decreased (27, 28), increased (30)				
Persistent Na^+ current	Increased (24)			Increased (26)	Increased (20)	
Persistent Ca^{2+} current	Increased (22)				Increased (20)	
HVA Ca^{2+} currents	Increased (22)					
Recovery from fast inactivation (Na^+ current)	Increased (250)					

TABLE 1 **Alterations of motoneuron electrophysiological features in ALS models (Continued)**

	Embryonic primary culture	iPSC-derived neuron culture	Embryonic spinal cord preparation	Postnatal brainstem slice	Postnatal spinal cord slice	Postnatal spinal cord preparation
Spontaneous motoneuron activity	Increased (24)	Increased (28), unchanged (30), reduced (30)				
Spontaneous locomotor outputs						Unchanged (36), Increased burst duration (37)
Evoked rhythmic activity			Slower rhythm period (34)			Unchanged (35), absent in lumbar but not in sacral roots (36)
Noradrenergic sensitivity						Increased (35)

The properties of motoneurons in different experimental ALS models were compared to their respective controls. Embryonic primary culture: neurons were isolated from E12–14 mice and cultured for 2–4 weeks before recording (23, 24), from E15 mice and cultured for 2–3 weeks (21), from E13 mice and cultured for 2–3 weeks (22), from E12–14 mice and cultured for 12–16 days (76), or from E15 and cultured for 8–13 days (250). Embryonic spinal cord preparations were obtained from E17.5 mice (25, 34). Postnatal brainstem slices were obtained from P4-P10 mice (26). Postnatal spinal cord slices were obtained from P0–P12 (20), P7 (23), or P6–P10 mice (19). Postnatal spinal cord preparations were obtained from P1–P3 (35), P3 (37), P3–P6 (36), and P6–P10 mice (29). Regarding iPSC-derived motoneurons, recordings were performed after 14 or 28 days (28), 66–79 days (31), 3–10 weeks (27) of neuronal differentiation. Of note, in (30), electrophysiological properties change with differentiation time: 3–4 weeks versus 7 weeks. RMP depends on ALS patient lines and time in culture, firing frequency varies between time of maturation (27). Of note, an increased amplitude of delay-rectifying potassium (K^+) current peaks can be observed in motoneurons harboring FUS and not SOD1 mutations (30). Evoked rhythmic patterns: the rhythmic activity induced by application of NMA and 5-HT is absent in lumbar (though it induced a tonic activity) but not in sacral segments (36). Noradrenergic sensitivity relates to the noradrenergic-induced amplification of lumbar ventral roots burst amplitude during evoked-fictive locomotion (35). AHP, after-hyperpolarization; AP, action potential; Ca^{2+}, calcium; E, embryonic day; HVA, high-voltage activated; iPSC, induced pluripotent stem cells; K^+, potassium; Na^+, sodium; NMA, N-methyl-D-, L-aspartate; P, postnatal day; RMP, resting membrane potential.

postnatal spinal cord slices. These discrepancies could be because the studies used different genetic models and controls (*SOD1*^{G85R} and *SOD1*^{G93A} mice with non-transgenic controls (19) and transgenic mice expressing the wildtype form of human SOD1 as controls (20)) and performed recordings at different ages (from P0 to P6 (20) and from P6 to P10 (19)). However, both isolated embryonic SOD1^{G93A}-expressing motoneurons (21) and those in slice preparation (20) consistently exhibit an increased rate of repolarization compared to controls.

Analysis of firing frequency-current intensity relationships reveals a common difference between ALS and control motoneurons (Table 1). AP frequency is increased in ALS embryonic motoneurons in culture (21–24), embryonic spinal cord preparation (25), postnatal spinal cord, and brainstem slices (23, 26), as well as in human motoneurons derived from induced pluripotent stem cells (iPSCs) obtained from patients with ALS (27, 28), relative to controls. A closer examination of the studies that show variations in this trend toward an increased AP frequency reveals the developmental dynamics that motoneurons are subject to and that can be altered by the presence of ALS-causing mutations. Indeed, spinal motoneurons from P6–P10 spinal cord slices or preparations show decreased firing frequency compared with wildtype, although by age, the gain is lower in motoneurons from P6–P7 transgenic mice and unchanged in motoneurons from older P8–10 transgenic mice versus those from wildtype mice (29). A broader study in spinal cord slices from P0 to P12 mice showed an overall unchanged frequency-current relationship (20). The maturation of iPSC-derived motoneurons and their progressive acquisition of electrical properties over time in culture also illustrates this differential susceptibility to ALS-causing mutations with respect to motoneuron excitability. A phenotypic switch from early hyperexcitability to late hypoexcitability observed in ALS patient iPSC-derived motoneurons (27, 30) explains the previously reported differences in the firing response of motoneurons (28, 31).

ALS motoneurons show other aberrant properties; elevated persistent sodium (Na^+) and calcium (Ca^{2+}) currents are consistently encountered in different experimental conditions (20, 22, 24, 26). Persistent Na^+ and Ca^{2+} currents that are resistant to inactivation by depolarization play an important role in spike initiation, amplification of synaptic inputs, and increasing firing rate (32, 33). It is noteworthy that Riluzole decreases persistent Na^+ currents in *SOD1*^{G93A} motoneurons and results in reduced excitability (24). Defects in inhibitory synaptic properties are also prominent early defects and are detailed in the next sections.

Analysis of chemically evoked locomotor outputs (rhythmic activity) that emerge from embryonic lumbar spinal cords revealed a slower rhythm period in *SOD1*^{G93A} versus wildtype spinal cords (34). Interestingly, this N-methyl-D-, L-aspartate (NMA)-, and serotonin (5-HT)-evoked locomotor-like slower rhythm period is not observed in *SOD1*^{G93A} postnatal spinal cord preparations. However, *SOD1*^{G93A} postnatal spinal networks display increased sensitivity to noradrenaline (NA)-induced enhancement of burst amplitude (35). Surprisingly, in *SOD1*^{G85R} P3-P6 mice, the rhythmic motor activity evoked by addition of NMA/5-HT was not observed in lumbar roots, whereas rhythmic patterns were observed in sacral roots and were similar to those observed in the sacral roots of wildtype controls (36). In terms of spontaneous rhythmic activity, motor output is similar in *SOD1*^{G85R} and wildtype postnatal spinal cords, while a longer burst duration is observed in *SOD1*^{G93A} spinal cords (36, 37). Interestingly, behavior analysis of postnatal ALS mice revealed early and transient defects in locomotor capacities (26, 36).

Discrepancies exist between different studies, which could be attributable to the use of different genetic models, recording approaches, and conditions, and/or to an effect of the experiment time window. However, altogether this evidence highlights altered motoneuron excitability, inhibitory imbalance, and changes in spinal locomotor networks as salient traits of the earliest origins of the pathology described to date.

Intrinsic features of adult motoneurons in ALS experimental models

To date, only three studies have reported the electrophysiological properties of adult motoneurons in ALS mouse models. In the first, whole-cell patch-clamp recordings were performed in ventral horn slices of 2.5-month-old transgenic mice that express green fluorescent protein (GFP) under the control of the choline acetyltransferase (ChAT) promoter. Based on 11 passive and active intrinsic properties of 42 lumbar motoneurons, the authors performed 11-dimensional cluster analysis from which they defined four clusters of motoneurons with similar properties (38). Table 2 displays the main electrophysiological characteristics.

TABLE 2 **Electrophysiological characteristics of motoneuron clusters in adult mice**

	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Passive				
RMP (mV)	–70.3	–68.8	–74.2	–70.6
Input Resistance (mOhm)	95.4	73.4	48.0	43.8
Membrane time constant (ms)	8.9	6.9	4.9	2.2
SAG ratio (h current)	18.9	11.0	18.2	3.7
Active (500 ms square pulse)				
FIF (Hz) Instantaneous firing, beginning of the pulse	45.9	164.8 (doublet action potentials)	340.6	424.0
SSF (Hz) Steady-state firing end of the pulse	31.9	50.3	74.6	147.7
Muscle innervation				
Muscle	Soleus	Soleus	Tibialis Anterior	Tibialis Anterior
Fiber type	Slow twitch fiber	Slow twitch fiber	Fast twitch fiber	Fast twitch fiber

Motoneuron subtypes were defined using cluster analysis and functional identity was achieved with retrograde labeling of known muscle types (38). Soleus: slow-twitch fiber type and Tibialis anterior: fast-twitch fiber type. RMP, resting membrane potential; FIF, firing frequency; SSF, steady-state firing.

Retrograde labeling of motoneurons from slow-twitch muscle (Soleus) and fast-twitch muscle (Tibialis anterior) demonstrated that clusters 1 and 2 are representative of ALS-resistant slow motoneurons, while clusters 3 and 4 are representative of ALS-vulnerable fast motoneurons. The high input resistance of clusters 1 and 2 is consistent with the high excitability relative to the threshold recruitment of slow motoneurons. In this study, spinal cord slices were prepared from 2–3-month-old (asymptomatic) and ~4-month-old (symptomatic) *SOD1^{G85R}-YFP* transgenic mice (expressing mutant SOD1 fused with yellow fluorescent protein) to assess motoneuron electrophysiology both before and after the development of clinical signs. At 2–3 months, all four clusters were present in the mutant motoneurons, and their electrophysiological properties were similar to wildtype, except that in cluster 4, the RMP was hyperpolarized by 6 mV in mutant versus wildtype motoneurons. At 4 months, however, there was a decrease in the probability of recording mutant motoneurons from clusters 3 and 4, suggesting a loss of these populations. Interestingly, there was also a tendency toward hyperpolarization of the RMP of those motoneurons in clusters 1 and 2. This study suggests that RMP hyperpolarization could be a function of the pathogenic process in ALS mice. This observation supports the possibility that hypoexcitability arises from an increase in threshold current following RMP hyperpolarization.

In a study by Delestree et al., in vivo recordings in the sacrocaudal spinal cords of *SOD1^{G93A}* mice and their non-transgenic littermates from 34 to 82 days (presymptomatic to disease onset) allowed longitudinal analysis of motoneuron excitability during ALS progression in this ALS model (39). Intracellular recordings were performed on motoneurons that were identified by the antidromic APs observed in response to electrical stimulation of their axon in the sciatic nerve. In this study, no attempt was made to analyze according to clusters and so recorded values were distributed over a large range. For example, the recruitment current varied from 1 to 13 nA and the input conductance from 0.1 to 0.8 µS. In line with initial reports in cat motoneurons (40), the recruitment current highly correlated with the input conductance: the larger the input conductance, the higher the recruitment current. *SOD1^{G93A}* motoneurons behaved similarly to wildtype motoneurons, except that the mean input conductance was increased, which should induce increased excitability. As the mean recruitment threshold was not modified and no change in RMP occurred in either genotype, this expected hyperexcitability was probably compensated. As mentioned previously, an increase in persistent Na^+ current has been demonstrated in neonate mutant motoneurons; these results suggest that this increase could persist in the adult state. Remarkably, a greater proportion of mutant than wildtype motoneurons lost their ability to fire. The motoneurons that were unable to produce sustained firing were distributed along the full range of input conductance in *SOD1*-mutant mice, whereas in wildtype mice they were restricted to those with the highest input conductance. This study is in agreement with that of Hadzipasic et al.—it appears that in adult mice, pathogenic *SOD1* mutations lead to motoneuron hypoexcitability before muscle denervation (38).

In contrast to the above study, Jensen et al. showed that adult motoneurons in *SOD1^{G93A}* mice have an increased excitability attributed to a lower rheobase, higher input–output gains, and increased activation of persistent inward currents (41). Therefore, in vivo recordings in adult ALS mouse models lead to conflicting results concerning intrinsic electrical properties, which is presently attributed to

differences in experimental protocols. In any case, in vivo studies in ALS mice suggest that high electrical activity promotes endoplasmic reticulum stress, a marker of disease (42), while an increase in the recruitment threshold (i.e., a decrease in excitability) slows down disease onset and protects against muscle denervation (43). Therefore, the hypothesis that changes in motoneuron inputs could be a major factor in their vulnerability requires further evaluation.

ALS-ASSOCIATED INHIBITORY TRANSMISSION DEFECTS

Neuronal circuits called central pattern generators coordinate locomotion and control skilled movements. These neuronal networks comprise different cell types, such as motoneurons, interneurons, astrocytes, and microglial cells. Most interneurons use GABA or glycine as neurotransmitters and thus present an inhibitory phenotype. These interneurons are also the most abundant neurons in the spinal cord and play a major role in the regulation of neuronal excitability (44, 45).

GABAergic and glycinergic transmission

Among the numerous types of interneurons identified, different classes of inhibitory interneurons have been defined based on the expression of transcription factors. Among the V0 lineage made up of commissural interneurons projecting ipsilaterally or contralaterally (46, 47), inhibitory V0d interneurons participate in left-right alternation (48). The V1 interneuron population, including Renshaw cells and Ia inhibitory interneurons, project rostrally and ipsilaterally on motoneurons and reciprocal inhibitory neurons (49, 50). V2b inhibitory interneurons project ipsilaterally and caudally. Both V1 and V2b interneurons independently participate in alternation of extensor and flexor muscles (51). Neuronal activity is controlled by the balance between excitatory and inhibitory neurotransmission. While motoneuron pathology plays a large role in ALS pathogenesis, accumulating evidence highlights a relevant role for these inhibitory interneurons in the regulation of motoneuron excitability that might contribute to motoneuron pathology.

To date, mainly pharmacological approaches have been used to investigate the role of inhibitory neurotransmission in the control of motoneuron excitability. Both acute and chronic infusion of bicuculline (a $GABA_A$ receptor blocker) generates a dose-dependent and temporary muscular hyperexcitability, motor deficits, and loss of motoneurons, showing that inhibitory GABAergic blockade can generate hyperexcitability of the intraspinal neuronal circuits and motoneuron degeneration (52). In addition, increased motoneuron loss and total paralysis was observed when 4-amynopyridine or a low dose of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid was added (52, 53), suggesting a close functional link between glutamatergic transmission and GABAergic circuits in the regulation of motoneuron excitability. Considering that the use of strychnine (a glycine receptor blocker) has no significant effect and that glycinergic neurotransmission is mainly intersegmental (54), the GABAergic modulatory role appears to be intrasegmental, in line with evidence that ipsilateral flexor–extensor

alternations are governed by GABAergic neurons directly affecting motoneuron activity within each spinal segment.

Postmortem histological studies of ALS tissues have mainly described a decrease in inhibitory GABAergic and glycinergic interneurons. A layer-specific reduction of calbindin $(CB)^+$ neurons has been shown in cortical layers V and VI and in the ventral horn of spinal cords of patients with ALS (55–58). Analysis of the motor cortex of patients with ALS has also revealed a trend toward reduced calretinin $(CR)^+$ cells and a reduction in parvalbumin $(PV)^+$ cells (55, 59). Results from other studies also indicate that there are alterations in GABA homeostasis and transmission in cortical and spinal ALS inhibitory neurons. The motor cortices of patients with ALS exhibit a downregulation and an increase in the mRNA levels of the α1-subunit and the β1-subunit of the GABA receptor, respectively, versus control motor cortices, which could indicate altered receptor function (60). This correlates with the reduced binding of flumazenil (an α1-selective benzodiazepine antagonist) observed in positron emission tomography (PET) scanner studies (61) and the decrease in GABA levels observed by proton magnetic resonance spectroscopy (62) in the cortices of patients with ALS compared with controls.

As in humans, a reduction in CR^+ cells has been observed in the cortex, hippocampus (63), and the spinal cord (64) of $SOD1^{G93A}$ mice. Morrison *et al.* described a decrease in the number of interneurons in the spinal cord of $SOD1^{G86R}$ transgenic mice at early symptomatic stages versus age-matched control mice, with a parallel of onset motoneuron degeneration (65). Interestingly, an early increase in the population of PV^+ interneurons, observed in the motor and somatosensory cortices of ALS mice, could suggest that a transient increase in inhibitory neurotransmission acts as a compensatory mechanism (66).

Other approaches, such as high-resolution magnetic resonance spectroscopy, have revealed a decrease in $GABA_A$ receptors in $SOD1^{G93A}$ transgenic mice versus controls even at presymptomatic stages (67). Finally, gliosomes isolated from the spinal cord of presymptomatic ALS mice exhibit increased expression of the GABA transporter (GAT1) along with a reduction in GABA release versus gliosomes from control mice (68).

Electrophysiological whole-cell patch-clamp recordings in brainstem slices from postnatal $SOD1^{G93A}$ mice revealed an enhanced frequency of inhibitory transmission through an increased amplitude and frequency of miniature inhibitory postsynaptic currents (mIPSCs) mediated by GABA in superior colliculus interneurons (26). Whole-cell patch-clamp recordings of cultured glutamate decarboxylase Gad67-GFP-expressing interneurons from embryonic $SOD1^{G93A}$ mice revealed a significant decrease in the peak outward current and intrinsic hypoexcitability compared with wildtype Gad67-GFP interneurons (69), which could contribute to the attenuated inhibitory function observed in the disease. These results suggest early perturbation of inhibitory neuron populations but do not establish whether synaptic excitability alters to compensate for abnormal firing or whether this is a cause or consequence of perturbed excitatory neuron excitability in the disease (69). Interestingly, subtype-specific investigations have discovered that the largest PV^+ interneuron population in the cortex exhibits similar excitability in wildtype and presymptomatic $SOD1^{G93A}$ mice but that the population is hyperexcitable in symptomatic $SOD1^{G93A}$ mice (70). Interestingly, $SOD1^{G93A}$ PV^+ neurons were found to be more hyperexcitable neonatally than presymptomatically, suggesting that compensatory mechanisms take place at

some stage of the disease. Electromyography performed in end-stage *TDP-43^{A315T}* mice revealed fibrillation potentials and fasciculations (71). mIPSCs and evoked inhibitory postsynaptic currents (eIPSCs) were significantly reduced in layer V pyramidal neurons of 3-week-old *TDP-43^{A315T}* mice versus those of 3-week-old wildtype mice. These neurons also exhibited hyperexcitability that was abolished by picrotoxin (a $GABA_A$ receptor blocker), suggesting that impairments in GABAergic signaling contribute to cortical hyperexcitability (72). It was subsequently proposed that hyperactive somatostatin interneurons can inhibit PV^+ interneurons, inducing a disinhibition of cortical motoneurons (72), although there is yet no convincing evidence showing direct interactions between these interneurons in the motor cortex.

Nieto-Gonzales *et al.* (73) also demonstrated in the wobbler mouse model (74), through electrophysiological measurements of current threshold for input resistance and AP in the presence of picrotoxin, that cortical hyperexcitability could be related to a decrease in tonic GABAergic inhibition, which in turn was related to a reduction in $GABA_A$ receptor-mediated inhibitory currents in layer V pyramidal neurons of the motor cortex.

Electrophysiological studies performed in spinal cords revealed more discrepancies in impaired inhibition transmission: no significant differences in GABAergic mIPSCs and GABAergic currents were observed between *SOD1^{G93A}* and control spinal cord cultured motoneurons (75, 76). However, $GABA_A$ receptors had higher affinity and lower desensitization levels, and α1 subunit expression level were doubled in *SOD1^{G93A}* motoneurons. These differences could be the result of an adaptive process in response to reduced glycinergic inhibition but could also contribute to excitotoxic motoneuron death (75).

A more recent electrophysiological study also revealed impaired chloride homeostasis and a subsequent induction of a more depolarized reversal potential for $GABA_A$ receptors in *SOD1^{G93A}* embryonic motoneurons versus wildtype motoneurons (34). Also observed was a reduction in the frequency of inhibitory synaptic inputs in *SOD1^{G93A}* motoneurons, with less frequent and smaller amplitude mIPSCs. In addition, in *SOD1^{G93A}* motoneurons, inhibitory postsynaptic currents exhibited slower decay time than those in wildtype motoneurons, which correlated with a higher intracellular chloride concentration. Computer simulations projected that this slower relaxation of synaptic inhibitory events could, at the prenatal stage, act as a compensatory mechanism to strengthen GABA/glycine inhibition when E_{GABAAR} is more depolarized in order to maintain well-coordinated, although slightly slower, locomotor activity (34). These results also reinforce the hypothesis that very early inhibitory dysfunction may initiate pathogenesis in ALS motoneurons (77, 78) (Table 3).

Looking more specifically at glycine transmission, *in vitro* binding assays demonstrated reduced binding of strychnine to glycine receptors in the ventral horn of human ALS spinal cord versus controls (79, 80). Lumbar ventral and dorsal horns of patients with ALS were also found to exhibit significantly reduced glycine levels (81). Inhibitory synaptic changes, with reduced binding of strychnine to glycine receptors and a reduction in the inhibitory/excitatory synapse ratio of hypoglossal motoneurons, can be observed from the early symptomatic stages in *SOD1^{G93A}* transgenic mice (82). These changes may also contribute to motoneuron degeneration through both an increase in excitatory synapses and a decrease in inhibitory contacts.

TABLE 3 ALS-associated features of inhibitory currents

	Motoneuron (culture)	Cortical interneuron (culture)	Motoneuron (spinal cord preparation)	Hypoglossal motoneuron (brainstem slice)	Superior colliculus interneuron (midbrain slice)	PV+ interneuron (brain slice)	Layer V pyramidal neurons (brain slice)
Intrinsic excitability		Reduced (69)			Increased (26)	Increased (neonatal)-unchanged (pre-symptomatic)-increased (symptomatic) (70), Reduced (72, 233)	Increased ((72, 73)
GABA-induced current	Unchanged (75, 76)						
GABA-induced current decay τ	Unchanged (75, 76)						
GABAergic mIPSC	Unchanged (76)		Reduced (34)	Unchanged (26)	Unchanged (26)	Unchanged (72)	Reduced (72), Unchanged (73)
GABAergic mIPSC decay τ	Unchanged (75, 76)		Longer (34)				Longer (73)
GABAergic mIPSC frequency	Unchanged (75, 76)		Reduced (34)	Increased (26)	Increased (26)	Unchanged (72)	Reduced (72, 73)
GABAergic sIPSC frequency						Increased (72), Reduced (233)	Reduced (73)
$GABA_A$ receptor desensitization	Reduced (75)						

TABLE 3 **ALS-associated features of inhibitory currents (Continued)**

	Motoneuron (culture)	Cortical interneuron (culture)	Motoneuron (spinal cord preparation)	Hypoglossal motoneuron (brainstem slice)	Superior colliculus interneuron (midbrain slice)	PV+ interneuron (brain slice)	Layer V pyramidal neurons (brain slice)
Glycine-induced current	Reduced (76)						
Glycine-induced current decay τ	Unchanged (76)						
Glycinergic mIPSC	Reduced (76)		Reduced (34), Unchanged (Zebrafish) (85)	Unchanged (26)			
Glycinergic mIPSC decay τ	Unchanged (76)		Longer (34, 85)				
Glycinergic mIPSC frequency	Unchanged (75, 76)		Reduced (34, 85)	Increased (26)			
E_{GABAAR}			Depolarized (34)				
$[Cl^-]_i$			Increased (34)				

Spinal embryonic neurons were isolated from E12–14 mice and cultured for 12–16 days (76), or from E15 and cultured for 8–10 days (75). Embryonic spinal cord preparations were obtained from E17.5 mice (25, 34). Brainstem slices were obtained from P4-P10 mice and midbrain slices obtained from P10-P12 mice (26). Cortical interneurons were isolated from E15.5 mice and cultured for 12 days (69). VP^+ inhibitory interneurons (*Gad67-GFP*) were recorded in brain slices of $SOD1^{G93A}$ (and control) at 6 (neonatal), 26–35 days (pre-symptomatic) and 90–101 days (symptomatic) of age (70). Brain slices were obtained from P3 $TDP\text{-}43^{A315T}$ mice (74), or from P9 $SOD1^{G93A}$ mice (235). Layer 5 pyramidal neurons in brain slices from P15-P25 wobbler mice (76). Exposed spinal cord was prepared from 4 days post-fertilization larvae $Sod1^{G93R}$ zebrafish (87). $[Cl^-]_i$, intracellular chloride concentration; E_{GABAAR}, reversal potential for $GABA_AR$; mIPSC, miniature inhibitory postsynaptic current; PV, parvalbumin.

A progressive and presymptomatic loss of glycinergic synapses on lumbar motoneurons and of CB^+ cells has been observed in $SOD1^{G93A}$ mice (83). A decrease in glycine transporter 2 (GlyT2) and GAD65/67 expression has also been observed in the ventral horn of symptomatic $SOD1^{G93A}$ mice (64). Cell culture models also display a decrease in postsynaptic glycine receptor expression (76, 84). Electrophysiological whole-cell patch-clamp recordings of spinal cord motoneurons revealed an early and specific decrease in the densities of spontaneous glycinergic IPSCs and glycine-induced currents in large-sized $SOD1^{G93A}$ motoneurons compared with wildtype motoneurons (84). A similar decrease in glycinergic currents has been described in a mutant SOD1 zebrafish model; glycinergic neurotransmission is impaired in spinal motoneurons from mutant SOD1 zebrafish. This decrease has been shown to precede the onset of pathophysiological defects in motoneurons, suggesting that motoneuron hyperexcitability may be associated with their loss, or the loss of the recurrent inhibition (85).

Recurrent and cortical inhibition

Motoneurons and Renshaw cells form a recurrent inhibitory circuit in order to adjust the motor output. Renshaw cells were first identified in cats by their high-frequency discharge in response to antidromic motor axon APs (86) and are located in the most ventral regions of laminae VII and IX of the spinal cord (87). They belong to the V1 interneuron subclass and can be identified by their medium to large size, expression of biochemical markers such as GlyT2, CB, and PV, location, and electrophysiological properties such as a high postsynaptic sensitivity to acetylcholine and large glycine- and GABA-evoked currents (88). Their inhibitory action is mediated by both GABA and glycinergic synapses, although synaptic boutons immunoreactive to glycine alone are more numerous than boutons that are immunoreactive to both GABA and glycine (89). Since Renshaw cells release both GABA and glycine, the recurrent inhibition they induce exerts a longer inhibitory synaptic action than the inhibition induced by Ia interneurons, in which neurotransmission is more phasic and solely glycinergic (90, 91). Renshaw cells are the only interneurons that receive direct excitatory synaptic inputs from motoneurons and, in turn, exert inhibitory feedback on them, known as recurrent inhibition (92). However, inhibitory synapses of Renshaw cells are located on dendrites rather than on the cell body (93) and the effectiveness of recurrent inhibition at reducing the motoneuron firing rate is limited (94). This is in keeping with the small amplitude of the postsynaptic inhibitory potential or current generated by Renshaw cells (95). In contrast, the synapses of Ia inhibitory interneurons are close to the motoneuron soma and have a more significant impact in counteracting the excitatory input arriving in the dendrites. Thus, Renshaw cells and Ia interneurons present distinct synaptic connectivity that serves different functions. Individual Renshaw cells receive inputs from particular motor pools and spread their inhibitory output to the same motoneurons, either directly or through inhibition of Ia inhibitory neurons mediating reciprocal inhibition of antagonistic muscles (flexor and extensor alternation activity), to γ-motoneurons controlling muscle spindle length, and to other Renshaw cells (88). Thus, recurrent inhibition is primarily generated by input from motor axon collaterals. However, it may also involve convergent signals from corticospinal origins (96, 97).

In humans, cortical excitability can be investigated using noninvasive procedures such as transcranial magnetic stimulation (TMS) or the nerve excitability test (NET). TMS consists of applying a local time-varying magnetic field that depolarizes neurons beyond their AP firing threshold and stimulates the primary motor cortex. The resulting evoked muscle response is then recorded using an electromyogram. The NET involves directly applying an electrical stimulus to a desired nerve and measuring the evoked response at the appropriate muscle. To differentiate between excitatory and inhibitory circuitries, different TMS stimulation protocols have been developed. To assess motor cortex excitability, this technique is associated with the measurement of motor evoked potentials (MEP), recorded from a contralateral innervated muscle (98), and an increased excitability which is detected following a conditioning stimulus (referred to as ICF). TMS procedures have shown that a transcranial subthreshold stimulus, activating low-threshold inhibitory circuits and thus increasing the stimulus threshold to elicit an evoked response (99), can suppress the response to a later suprathreshold stimulus (100). This inhibitory phenomenon, attributed to GABA-secreting inhibitory cortical interneurons via $GABA_A$ receptors, is referred to as short intracortical inhibition (SICI) (101, 102).

Electroneurography studies revealed marked variability in the hyperexcitability index scores of patients with ALS. The inhibitory effects of TMS on the corticospinal output of patients with ALS demonstrated that the threshold to elicit an MEP was significantly reduced after inhibitory stimuli (103–107). This was accompanied by a reduction in intracortical inhibition (103, 107–114), and lower and less effective SICI in ALS patients with limb-onset disease, suggesting either a dysfunction or a loss of inhibitory interneurons (107, 113, 115–120). However, it must be noted that at the cellular level, electrophysiological alterations, such as alterations of voltage-gated Na^+ and K^+ channels that affect motoneuron AP threshold (24, 121), may also contribute to reduced SICI in ALS.

TMS studies in humans have also demonstrated ICF (111–114, 122), and Vucic *et al*. reported that the measured reduction in SICI represented degeneration of inhibitory cortical circuits combined with hyperexcitation of high-threshold excitatory pathways (123). More recent work has shown that reduced and altered SICI affects motor cortical circuits in ALS; the study also showed that combining two parameters, short-interval ICF and SICI, increases the utility of SICI for identifying patients with ALS (124). Overall, these results demonstrate that in ALS the imbalance between excitatory and inhibitory circuits in the M1 cortex is based on a combination of increased excitability and decreased inhibition (125). One study reported more normal SICI values (126) and also demonstrated reduced late intracortical inhibition, attributed to $GABA_B$ receptors, in patients with ALS compared with control individuals (113, 126). Another showed more frequent and stronger inhibitory responses in cortices of patients with ALS versus those of control patients (127).

More evidence for cortical inhibition dysfunction came from analysis of the duration of the cortical silent period (CSP). Indeed, CSP is thought to reflect both inhibition of anterior horn cells from the spinal cord and cortical influences through $GABA_B$ receptors (128–132). Thus, the observed reduction in CSP duration, predominantly observed in patients with bulbar-onset disease (133), is likely to be associated with disinhibition of anterior horn cells (134, 135) and dysfunction of cortical inhibitory interneurons acting via $GABA_B$ receptors (120).

The late manifestation of overt cortical hyperexcitability (136) could be explained by the incredible capacity of inhibitory circuitry for compensation (137–140) and the high levels of brain reorganization observed in patients with ALS (141, 142). Indeed, these mechanisms of plasticity may slow disease progression. This hypothesis is supported by the observation that patients with preserved intracortical inhibitory circuitry display a slower disease progression (143). However, it remains unclear how interneuronal capacity may selectively fail in patients with ALS over time. As a loss of GABAergic populations is reported during aging in both human and murine studies (144, 145) and is associated with a decline in inhibition in a number of cortical regions (144, 146–149), it is also possible that although inhibitory circuitry can compensate for initial insults, an age-related decline of inhibition leads to failure of further compensation.

Overall, these observations demonstrate that loss of Renshaw cell function could be the result of degeneration of the corticospinal fibers directed to these cells and that the loss of cortical inhibitory influence, in association with ion channel alterations, may participate in increased motor network excitability (125). A better understanding and characterization of subtypes, inputs and outputs, morphology, and electrophysiological properties of the different cortical interneurons would be helpful to better dissect the mechanisms underlying cortical hyperexcitability in ALS.

Renshaw cell circuitry can be studied by combining TMS with the paired H-reflex technique, which produces a response whose amplitude inversely correlates with activity in recurrent inhibitory pathways (150, 151). Raynor *et al*. presented the first evidence for Renshaw cell impairment in patients with ALS (135), reporting an abnormal reduction in recurrent inhibition in patients with ALS compared with control individuals. The collision technique, used in motor axons, can be used to test recurrent inhibition by creating a relatively homogeneous population of motoneurons which are under the effect of both Renshaw inhibitory inputs and post-activation AHP that regulates the AP firing rate of the motoneurons themselves. One of the prerequisites therefore for the correct application of the paired H-reflex method is to produce results whereby the depression of motoneuron activity by Renshaw cells overcomes the depression produced by AHP (152, 153). Unfortunately, in this work (135), the paired H-reflex methodology was not fully implemented (153), and these findings were insufficient to conclude if it was recurrent inhibition, AHP, or both that was decreased. Indeed, even though no changes in AHP have been observed in motoneurons from the *SOD1*^{G93A} and the *SOD1*^{G85R} mouse models (23, 29), the shorter than normal AHP duration observed in earlier stages of ALS (154) could explain the results obtained by Raynor *et al*. (135).

In another study, Shefner and Logigian investigated the mixed nerve silent period (MNSP), the period of motor inhibition observed when the mixed nerve innervating a voluntary activated muscle is electrically stimulated, in patients with ALS and control individuals (155). Patients with ALS exhibited a longer MNSP duration, as well as less complete inhibition in the middle phases of the period, which may also reflect abnormalities in Renshaw cell function. However, the stimulated nerve fibers used in this study originated from the intrinsic muscles of the hand, which are devoid of recurrent inhibition (156).

A more recent study performed by Özyurt *et al*. compared spinal recurrent inhibition and postactivation depression (PAD) on the soleus muscle in

lumbar-affected and nonlumbar-affected ALS patients (157). PAD is another spinal circuit with an effective presynaptic network that tones down the output of the primary afferents on motoneurons. As in the previous studies, this work demonstrated a reduced duration of recurrent inhibition and reduced PAD of the H-reflex in patients with ALS compared with controls, which may lead to excessive excitation of motoneurons. Unfortunately, this work could not provide evidence of whether it is primarily Renshaw cells or motoneurons that are impaired.

Finally, it has been shown that both Renshaw cells and V1-derived Ia inhibitory interneurons, mediating recurrent and reciprocal inhibition of motoneurons, can be excited by V0c cholinergic interneurons to inhibit ipsilateral motoneuron excitability (158). Interestingly, early reduction of ChAT content in the presynaptic boutons of V0c interneurons on motoneuron somas and Renshaw cells has been observed in the *SOD1*^{G93A} mouse model (159). Similarly, it has been reported that cholinergic afferents from motoneurons to Renshaw cells are lost at early stages of ALS, by retraction of the motoneuron collateral (160). Inhibitory boutons from Renshaw cells on motoneurons and the number of Renshaw cells were unaffected at the same stage. In both studies, these changes occurred long before markers of motoneuron degeneration appeared. Therefore, according to these findings, cholinergic dysfunction can also trigger hyperexcitation and neurodegeneration processes in the spinal circuits through decreased excitatory action on inhibitory neurons.

Even though there is accumulating evidence to suggest that the inhibitory circuitry is affected and that interneuron populations are lost in ALS, controversies still exist about the evolution of this altered inhibition. Understanding these processes is of great interest considering that motoneuron hyperexcitability is observed at both the embryonic and presymptomatic stages in ALS models and patients (25, 26, 103) and that interneuron development is an activity-dependent process (161–164). Indeed, attenuating the activity of specific interneuron populations affects their migration and morphology during development (165) and their inhibitory synapse formation on excitatory cells (162, 166). In particular, the complexity of inhibitory innervation field is activity dependent. Thus, in ALS, where hyperexcitability is an early phenomenon (25, 167), aberrant inputs may be created at the motoneuron presynapse long before disease onset (168).

Two hypotheses have been proposed to explain how Renshaw cell alterations may lead to a hyperexcitable state and eventual degeneration of motoneurons. The first hypothesis postulates that the hyperexcitability is caused by loss of recurrent Renshaw cell-mediated inhibition and is based on electrophysiological findings suggesting an impairment of Renshaw cells in patients with ALS (135, 155). It is reinforced by the progressive loss of glycinergic boutons throughout the soma of the motoneurons and loss of CB^{+} cells observed in *SOD1*^{G93A} mice at an early symptomatic stage, before motoneuron degeneration. Since GABAergic terminals are only affected at the final stage, these changes can be assumed to be due to Renshaw cell loss (83). Another study reported early loss of Renshaw cells and revealed that lithium protects against Renshaw cell loss and delays disease progression, leading the authors to suggest that Renshaw cell loss may be the event that makes motoneurons more susceptible to glutamatergic toxicity in ALS (169). In addition, spinal motoneurons from *SOD1*-mutant zebrafish exhibited impaired glycinergic neurotransmission that preceded the onset of

pathophysiological defects in motoneurons, thus also suggesting that motoneuron hyperexcitability may be associated with the loss of these cells, or the loss of the mediated recurrent inhibition (85).

The second hypothesis proposes that the recurrent inhibitory circuit is altered ahead of motoneuron hyperexcitability and neurodegeneration but that this is not a consequence of Renshaw cell loss. Indeed, some studies suggest that the temporal onset of degeneration in motoneurons and interneurons may occur in parallel in patients with ALS (58) and in *SOD1*^{G86R} mice (65, 170). In agreement with the latter hypothesis is the observation that there is an early increase in the population of PV^{+} interneurons in the motor and somatosensory cortex of *SOD1*^{G93A} mice, which suggests that a transient increase in inhibitory neurotransmission could act as a compensatory mechanism to rescue motoneurons from glutamate excitotoxicity (66). Reinforcing this, activation of Renshaw cells has a poor effect on motoneuron soma activity (171) and interneurons are preserved in the symptomatic stage, indicating that progression of motoneuron degeneration is independent of Renshaw cell loss (160). In the same model, immunoreactivity experiments performed on vesicular inhibitory amino acid transporters (VIAATs) have shown a significantly reduced VIAAT expression in the ventral and dorsal horn neuropil, only at late stages, indicating that loss of inhibitory input (mostly Renshaw cells) does not precede but rather follows motoneuron death (172). In line with this, the finding that loss of inhibitory spinal interneurons occurs after loss of motoneurons (64) suggests that motoneuron degeneration may also trigger interneuronal pathology. Finally, as previously mentioned, V1 inhibitory neurons are thought to play a key role in modulating motor output, in part through recurrent and reciprocal inhibition. A more recent study on the fate of these neurons in the ventral spinal cord of *SOD1*^{G93A} mice (173) revealed increased V1 synaptic contacts with motoneuron cell bodies at an early stage of disease, followed by a 50% loss of V1 interneurons at a later stage. Since this loss is delayed compared with motoneurons and V2a excitatory neurons, this also supports the hypothesis that upregulation of inhibition is an early compensatory mechanism, followed by a substantial loss of V1 interneurons later in the disease (173).

These results may explain how Renshaw cell alterations may lead to hyperexcitability and eventually to motoneuron degeneration. However, there is still a debate about whether it is the selective loss of inhibitory interneuron regulation of motoneuron function, loss of inhibitory interneurons, or a combination of both, that contributes to motoneuron degeneration in ALS.

Therapeutic approaches

All these findings suggest that early impairment of GABAergic and glycinergic signaling occurs in ALS patients and animal models. As excitatory and inhibitory regulation are crucially linked from the presymptomatic stage of the disease, alterations in inhibitory circuitry may involve dynamic changes and determine the susceptibility and vulnerability of motoneurons. Therefore, new possible pharmacological neuroprotective strategies aiming to restore normal levels of excitability, potentially by preserving the integrity of inhibitory circuits or restoring inhibition in the spinal cord, may be appropriate for the treatment of ALS.

Therapeutic approaches using pharmaceutical compounds to target the inhibitory system have been successfully used to improve diseases in which

excitability and interneuronal alterations are present (174–176). In ALS, as spasticity, fasciculations, and cramps develop, GABA agonists such as diazepam and baclofen are prescribed to treat these features associated with the disease (177–179). Diazepam has been shown, using paired TMS, to reverse the hyperexcitability observed in patients with ALS compared with control individuals (115). The GABA analog gabapentin reduced fasciculations (180) with promising neuroprotective effects in a chronic model of glutamate toxicity (181) and reached clinical trials. However, later phase trials revealed no beneficial effects (182, 183), which could be explained by the fact that despite sharing structural similarity with GABA, gabapentin may not directly modulate GABA receptors and instead may selectively inhibit voltage-gated Ca^{2+} channels containing the α2δ-1 subunit (184, 185). In *SOD1*^{G93A} mice, administration of lithium prevented Renshaw cell loss and delayed the onset of symptoms (169, 186). Other therapeutic strategies using viral vectors to upregulate the production of GABA could also be employed (187).

To maintain physiological GABA concentrations, the use of drugs that block GABA uptake and catabolism at the synapse may be considered: tiagabine blocks the activity of the GABA transporter GAT1 (188), and vigabatrin blocks GABA transaminase and prevents the degradation of GABA (189). In addition, bumetanide, a drug that can inhibit the Na–K–Cl cotransporter NKCC1 and decreases intracellular chloride concentrations in immature $GABA_A$ receptors (190), and retigabine, which interacts with the KCNQ2/KCNQ3 subunits of K^+ channels and with $GABA_A$ receptors to weakly block sodium and calcium channels and thus decrease excitability (191), may also be considered.

In ALS, specific motoneurons are spared, such as the oculomotor and abducens populations (192), and gene expression studies have identified striking differences in genes responsible for the GABA and glutamate receptor subunits that may contribute to differential vulnerability. Indeed, in disease-resistant oculomotor neurons, α1, β1, β2, e, γ1, and θ $GABA_A$ receptor subunits are upregulated, whereas the α1 subunit is consistently reduced in vulnerable spinal and cortical motoneurons in patients with ALS (60, 62, 193). In addition, the specific vulnerability of ALS-resistant and ALS-vulnerable motoneurons correlates with the subunit composition of $GABA_A$ receptors, Gly/$GABA_A$ receptor density ratios, and the incidence of synaptic versus extrasynaptic $GABA_A$ receptors (194, 195). Considering that the subunit composition of $GABA_A$ receptors determines the location of the receptor as well as its specific pharmacological and electrophysiological properties, differential $GABA_A$ subunit expression will alter GABAergic receptor function (196–198). Thus, considering that an increase in $GABA_A$ receptors could generate a better GABAergic influence and protection, the development of GABA receptor subtype–selective compounds to counteract reduced inhibitory activity and modulate inhibition may be another interesting future therapeutic approach.

Finally, neural stem cell transplantation studies have shown strong evidence that restoration of the inhibitory drive can affect motoneuron survival (199, 200). More specifically, Xu *et al.* demonstrated that neural stem cells transplanted into *SOD1*^{G93A} mice differentiate into neurons presenting a GABAergic phenotype, which form local synapses and positively modify motoneuron survival (201–203), suggesting a future possible therapeutic use for these cells.

ALTERATIONS IN THE MODULATORY TRANSMISSION IN ALS

Neuromodulatory systems complement conventional neurotransmission by influencing neuronal excitability and synaptic efficacy. Abnormalities in this interneuronal signaling, where cholinergic and monoaminergic inputs modulate motor output, have been evidenced in ALS mice and patients.

Cholinergic transmission

Cholinergic C-synapses were identified several decades ago, mainly because of their unusual morphology. They form punctate large clusters (3–7 μm) primarily at the soma and proximal dendrites of α-motoneurons in the trigeminal, facial, and hypoglossal motor nuclei in the brainstem, as well as the α-motoneurons in the ventral horn of the spinal cord (204). C-bouton synapses originate from a small population of cholinergic *Pitx2*$^{+}$ interneurons, the V0c spinal neurons, found in the lamina X near the central canal (205, 206). Identifying that the V0c population forms the C-boutons allowed the *in vivo* function of these synapses to be addressed specifically. This major study revealed that these interneurons are involved in high task demands, such as swimming, that recruit the fast fatigable (FF) and fast fatigue-resistant (FR) motoneurons (206). Consistent with their role in task demand, these interneurons highly express the activity-dependent gene *c-Fos* following locomotion but also following painful sensory stimulation (207).

Interestingly, the motoneurons innervating fast-twitch muscles (those that are the first to degenerate in ALS) have a greater number of C-boutons than those innervating slow-twitch muscles (208). Moreover, C-boutons are not expressed among the motoneurons innervating the oculomotor, trochlear, abducens, and dorsal vagus nuclei, or the spinal gamma motoneurons and the autonomic motoneurons. Given the correlation between motoneurons without these terminals and survival, Ichikawa and Shimizu suggested that C-boutons might be involved in the neuron death that occurs in ALS (209). However, there is an exception to this correlation; the sphincteric motoneurons in Onuf's nucleus, a neuron type that survives in patients with ALS, are contacted by C-type terminals (210).

It is now well established that C-boutons increase the firing rate of motoneurons through a rather well-characterized sequence of cellular events involving activation of the postsynaptic muscarinic M2 receptors and inhibition of the Ca^{2+}-activated K^{+} current, SK channels (204, 211, 212). In addition, we recently demonstrated that muscarinic stimulation is dependent on motoneuron type, with a higher efficacy in the disease-vulnerable FF motoneurons; this further suggests that C-boutons may play a specific role in ALS (43).

Functional analysis of the role of C-boutons in ALS mouse models supports the hypothesis that the increased excitability mediated by C-boutons delays ALS progression, as does inhibition of ER stress (42). In addition, genetically silenced C-boutons in ALS mice exacerbates locomotor deficits (213). On the other hand, decreasing excitability through C-boutons-associated activity reduces motoneuron stress and denervation and thereby maintains muscle strength (43).

Monoaminergic systems

The developmental assembly and function of the locomotor circuits is subject to neuromodulation to provide adaptive behaviors (214). The monoaminergic system that encompasses NA, 5-HT, and dopamine (DA) has been shown to influence the rhythmic firing pattern of motoneurons and contribute to the flexibility of locomotor functions with premotor inputs and sensory afferents. A reduction in descending serotonergic fibers, linked to reduced levels of 5-HT in the spinal cord of *SOD1*^{G93A} mice, has been reported as early as E17.5. 5-HT hyperpolarizes E_{GABAAR} through 5-HT$_2$ receptors in embryonic motoneurons; similar intensities are observed in wildtype and *SOD1*^{G93A} mice (215). During postnatal development, while the levels of DA, NA, and 5-HT in the lumbar spinal cord increase between P1 and P10, only DA is increased in *SOD1*^{G93A} mice compared with wildtype mice, although this difference is mainly due to changes in the dorsal part of the spinal cord (35). DA, NA, and 5-HT increase all exerts marked modulatory activity, by potentiating fictive locomotion in spinal cord preparations. However, NA is the only biogenic amine to differentially enhance motor burst in ALS mice, potentially through modulation of excitatory inputs (35).

In the adult spinal cord, DA levels in patients with ALS were shown to be similar to those in control individuals; NA levels were found to be increased, as was the ratio of 5-HT to its metabolite 5-hydroxyindole-3-acetic (5-HIAA) (216). However, another study in patients with ALS documented a loss of dopaminergic neurons in the substantia nigra (217), which is consistent with the reduced dopaminergic function and nigrostriatal DA deficits observed in patients (218, 219). A study in ALS mice showed that reduced numbers of dopaminergic neurons in the substantia nigra pars compacta and ventral tegmental area were associated with reduced levels of DA (220). PET analysis showed a decrease in the binding of a selective 5-HT$_{1A}$ receptor in motor and extramotor regions of the brain in patients with ALS versus healthy volunteers (221). Another study reported that 5-HIAA/5-HT were elevated only in the lateral white matter of the cervical spinal cord of patients. A reduction in 5-HT$_2$ receptor binding, but not in the 5-HT$_{1A}$ receptor, was also observed in the motor and premotor cortex (222). A more recent study revealed a loss of serotonergic neurons in the brainstem and their projections in the hippocampus and spinal cord of patients with ALS (223). This loss is also found in *SOD1*^{G86R} mice and correlates with reduced levels of 5-HT in the cortex, brainstem, and spinal cord, even at the non-symptomatic stage (223). In ALS mice, reduced serotonergic innervation is associated with upregulation of the 5-HT$_{2B}$ receptor and development of spasticity, which can be abrogated by administration of a 5-HT$_{2B/C}$ receptor antagonist (223, 224). Treatment of *SOD1*^{G93A} neonates with fluoxetine, a selective serotonin reuptake inhibitor that acts at presynaptic terminals to increase 5-HT levels, decreased motor performance and weight of adult mice, without affecting disease onset (225). Analysis of P10 spinal cord slices revealed that 5-HT depolarizes RMP, hyperpolarizes the persistent inward current peak and increases motoneuron excitability, despite wildtype and *SOD1*^{G93A} motoneuron showing similar responses (225). Of note, treatment of adult ALS mice with fluoxetine has no effect on disease course. This underlines the importance of the monoaminergic, and in particular the serotonergic, system during critical stages of development; it will have long-term effects on the motor system. Monoaminergic system changes, which have a critical influence

on the assembly, maturation, and function of motor circuits, represent a pathological characteristic of ALS that remains largely understudied; these changes are therefore also a therapeutic target.

ALS-ASSOCIATED DEFECTS IN EXCITATORY TRANSMISSION

Glutamate is the major excitatory neurotransmitter for lower motoneurons that transmits information from upper motoneurons as well as proprioceptive sensory neurons. Glutamatergic dysfunction has been recognized as an important contributing factor to ALS.

Glutamatergic inputs

In humans, cortical hyperexcitability has been identified as an important pathogenic mechanism in ALS and is mediated through dysfunction of inhibitory and facilitatory intracortical circuits (114). The corticofugal hypothesis proposes that cortical hyperexcitability might cause motoneuron degeneration in ALS via trans-synaptic glutamate-induced excitotoxicity (226, 227). Decreased intracortical inhibition and cortical hyperexcitability can be seen in patients with *SOD1* gene mutations (112, 228). Moreover, cortical hyperexcitability appears to precede spinal motoneuron degeneration (103), supporting the dying-forward hypothesis that disease progression is mediated through glutamate-induced toxicity (229).

ALS mouse models are used to better understand the cellular basis of cortical hyperexcitability. In cultured cortical neurons baring the $SOD1^{G93A}$ mutant, hyperexcitability was attributed to a decrease in the threshold potential and time of the first AP and an increase in the firing frequency. This intrinsic hyperexcitability was attributed to an increase in the persistent inward Na^+ current density (230).

In situ whole-cell patch-clamp recordings of layer V cortical motoneurons in presymptomatic P26–31 $SOD1^{G93A}$ mice revealed increased excitability through increased frequency of spontaneous excitatory postsynaptic currents (231). This was accompanied by an increase in the expression of the vesicular glutamate transporter 2. Moreover, compared with controls, $SOD1^{G93A}$-expressing cortical neurons exhibited a higher output gain (slope of the frequency–current relationship) and lower rheobase.

These results have subsequently been mitigated by the observation that all neurons in $SOD1^{G93A}$ mice exhibit increased activity (whole-cell recordings in brain slices from P90 to P129 versus controls). This result is from a study in which corticospinal and corticocortical neurons were identified following injection of neuronal tracers at specific sites, and inhibitory GABAergic PV^+ neurons were identified by use of cells from *Gad67-GFP* mice (70). Interestingly, the cellular mechanisms leading to hyperactivity varied among the different neuronal populations. Corticospinal neurons exhibited an increase in the output gain, without changes in the rheobase, while corticocortical neurons displayed a decrease in rheobase and an increase in the output gain. It is well established that the activity of layer V pyramidal neurons is strongly inhibited in the perisomatic compartment by PV^+ GABAergic interneurons, which represent 40–50% of layer V

interneurons (232). It was thus unexpected that, in symptomatic *SOD1*^{G93A} mice, inhibitory PV$^+$ neurons became hyperexcitable, with a decrease in rheobase and a leftward shift in their output gain (without change in the maximal frequency of firing). However, this study did not address whether there was a partial loss of these inhibitory interneurons. This longitudinal analysis of cortical excitability highlights that neuronal plasticity occurs during disease progression, beginning with hyperexcitability at the neonatal stage, followed by normal excitability and a return to hyperexcitability at symptomatic stages of ALS. These results supporting an overall hyperexcitability of excitatory and inhibitory cortical neurons were further confirmed and suggested to involve compensatory mechanisms occurring all along disease progression in *SOD1*^{G93A} mice (70). It is interesting to note that spinal motoneurons also display hyperexcitability at embryonic and neonatal stages, which is followed by hypoexcitability in adults without reemergence of hyperexcitability. To further investigate the overall effects of neuronal hyperexcitability on the homeostasis of layer V neurons, intracellular Ca^{2+} levels were assessed using two-photon imaging of GCaMP6s-infected neurons (70). The main conclusion was that basal levels of intracellular Ca^{2+} are increased in SOD1-mutant layer V neurons, supporting a net hyperexcitability and/or an inability to maintain Ca^{2+} homeostasis, a factor that is known to be responsible for neuronal toxicity. It should be noted that spinal motoneurons have a poor capacity to buffer intracellular Ca^{2+} and are thus very sensitive to Ca^{2+}-induced toxicity. Consequently, the hypoexcitability reported at symptomatic stages could be a compensatory mechanism to prevent Ca^{2+} overload.

Similarly, hyperexcitability of layer V pyramidal neurons in 3-week-old *TDP-43*^{A315T} mice (a mouse model of ALS and frontotemporal dementia with profound cortical pathology) was found to be due to reduced mIPSCs, indicative of a reduced GABAergic tone, versus wildtype mice. Consistent with this, PV$^+$ GABAergic interneurons of these mice were hypoexcitable; this was due to hyperactivity of somatostatin interneurons located in the M1 cortex. Interestingly, ablation of somatostatin interneurons restores the PV$^+$ GABAergic inhibition of layer V neurons and protects against excitotoxicity induced by L5 neurons (72). A recent study in late presymptomatic *SOD1*^{G93A} mice confirmed the hypoactivity of PV$^+$ neurons (233). Altogether, these studies support the idea that different cell types contribute to the control of corticospinal layer V neuron activity during ALS progression.

In vivo genetic manipulation is now emerging as a technique that can help us understand the overall effects of changes in cortical activity on ALS onset and progression by allowing modulation of neuronal activity. Chemogenetics—specifically, the chemogenetic tool designer receptors exclusively activated by designer drugs (DREADD) (234)—has been used to increase PV$^+$ neuron activity. Chronic activation of PV$^+$ interneurons at the presymptomatic stage or at symptom onset delays the cortical neurodegeneration observed at the symptomatic P117 stage and delays motor deficits in the *SOD1*^{G93A} ALS model (233).

In addition, genetic ablation of subcerebral projection neurons, including the layer V neurons, has been used to assess the *in vivo* contribution of the cerebral cortex to ALS. This was achieved through ablation of the transcription factor *Fezf2*, which is necessary and sufficient to instruct birth and specification of corticospinal neurons and subcerebral projection neurons. Crossing *SOD1*^{G86R} mice with *Fezf2*$^{-/-}$ mice generates ALS mice entirely lacking both these

neuron populations. The loss of subcerebral projection neurons delayed disease onset and improved motor function in ALS mice (235).

Proprioceptive system

Proprioception is defined as our sense of body position and movement. We are constantly receiving signals from our moving body that allow us to interact with the environment and rapidly adapt to changing circumstances. It is largely the proprioceptors that tell us about the position and movement of our limbs and trunk. The information they provide allows us to bypass obstacles in the dark and handle objects without needing to see them (236, 237). Several types of proprioceptors inform us about different aspects of our body shape.

As an example, in skeletal muscles the spindles associated with the Ia/II afferent fibers encode the muscle length and the velocity of muscle length. These muscle spindles also receive γ-motoneuron efferent innervation that regulates the tension of the spindle. At the junction between muscle and bone, the Golgi tendon organs innervated by Ib afferent neurons encode for muscle strength to control α-motoneuron activity when the strength of contraction may damage the muscle. Consequently, people suffering from major proprioceptive deficits are not able to coordinate movements and become unable to move. They must learn how to use another sensory modality, usually sight, to provide sensitive feedback of movements.

The preceding sections have illustrated that the pathophysiological processes leading to ALS are not circumscribed to motoneuron cell-autonomous features but also affect the motor circuitry in which motoneurons are integrated. The proprioceptive system is part of these sensory motor networks and is thought be one of the systems involved in the pathophysiology of ALS; it also seems to be part of the process of neuronal degeneration (3, 238, 239).

Growing evidence supports the involvement of the somatosensory system in patients with ALS. Most studies have demonstrated the presence of sensory symptoms that can be associated with sensory neuropathy and loss of large-diameter myelinated fibers (240). Interestingly, spinal diffusion tensor imaging coupled with electrophysiological measurements revealed sensory defects in 85% of patients with ALS who had moderate impairment and no sensory symptoms (241). The implications of sensory deficits in the pathophysiology of ALS may have been underestimated; this work provides additional evidence of early degeneration of sensory pathways in patients with ALS.

ALS mouse models confirm the involvement of peripheral sensory abnormalities; in most, sensory deficiencies occur during early stages of the disease (240, 242). Studies in $SOD1^{G93A}$ and $TDP\text{-}43^{A315T}$ mice analyzing proprioceptive nerve ending in muscles reported that peripheral innervation of spindles by Ia and II afferent fibers is diminished in the presymptomatic stages of the disease. The sensory neuron somata are unaffected (243, 244), and central synapses are affected only late in the disease process. Furthermore, $TDP\text{-}43^{A315T}$ mice develop sensory abnormalities even in the absence of α-motoneuron axon lesions (244).

In recent years, several investigators have attempted to address whether degeneration starts with the spinal motoneurons or in other cells of the sensory motor networks. Only two studies have addressed this point through an electrophysiological approach. The first was carried out in a Drosophila $dSod1^{G85R}$ knock-in model

(245) and used structural and electrophysiological measures to reveal early larvae motor deficits. This early reduced locomotion was not due to neuromuscular junction dysfunction, deficiencies in muscle contraction, or to alterations in motoneuron properties. On the contrary, dysfunction of peripheral sensory feedback occurred before any evidence of motoneuron degeneration. These results suggested that the proprioceptors could be affected first in ALS and that their dysfunction could explain the altered motor activity and could ultimately lead to motoneuron degeneration. The second study used the jaw reflex in *SOD1*^{G93A} mice as a model and showed that proprioceptive Ia afferent sensory neurons display electrical abnormalities in the postnatal stage at the beginning of the disease process (246). Proprioceptive neurons were hypoexcitable and more likely to discharge phasically (bursting neurons). Moreover, bursting properties were abnormal and led to an irregular burst pattern. In addition, the existence of a Nav1.6 Na^+ channel deficiency contributed to the arrhythmic burst discharge. Interestingly, examination of other brainstem sensory neurons (tactile, nociceptive, and visual) at 2 weeks of age confirmed that changes in excitability had occurred exclusively in proprioceptive neurons. The authors concluded that such sensory arrhythmia could lead to a disturbance of reflexes causing the muscle fasciculations that are encountered in ALS.

As the disease progresses, sensory motor network dysfunction occurs in an attempt to maintain contractile force for as long as possible, but this ultimately leads to excitotoxicity and death of motoneurons. New therapies targeted toward sensory motor network dysfunction might therefore positively influence disease progression (247, 248).

CONCLUSION

Intrinsic neuronal hyperexcitability in upper and lower motoneurons is the earliest pathogenic defect of ALS to have been identified. Whether this is causative, or aggravating remains to be definitively established, although identification of causative genes in ALS rather supports an aggravating role. The hypoexcitability that emerges during disease progression could be an adaptive process to protect against cell death. A question remains concerning whether the synaptic propagation of aberrant activity could arise from the relationship between upper and lower motoneurons—the so-called forward propagation of excitotoxicity. *In vivo* studies in rodents support a functional correlation between upper and lower motoneurons in disease aggravation (233, 235). However, it should be mentioned that unlike in humans, direct cortical-motoneurons synapses disappear in rodents at postnatal ages (249). The cortical influence on motoneuron excitability in rodents could be more pronounced at early developmental stages, while defects of local circuitry in spinal cord could become more predominant during later stages. In humans, the corticospinal tract could have an even greater influence in ALS progression than it does in rodents. Likewise, the higher sensitivity of lower motoneurons than upper motoneurons to excitotoxicity could explain their earlier death. Therapeutic intervention in circuit dynamic and motoneuron electrophysiological features hold promise of successful therapy for ALS, although it still requires improving knowledge of the complex adaptive changes that occur during development and adulthood.

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5

Axonal Transport and Local Translation of mRNA in Amyotrophic Lateral Sclerosis

Seiichi Nagano[1,2] • Toshiyuki Araki[2]

[1]Department of Neurotherapeutics, Osaka University Graduate School of Medicine, Japan; [2]Department of Peripheral Nervous System Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan

Author for correspondence: Toshiyuki Araki, Department of Peripheral Nervous System Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan. E-mail: taraki@ncnp.go.jp

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Abstract: Since neurons have long neurites, especially axons, the transport of essential mRNAs, and their translation locally in axons, are essential to maintain the shape and function of the neurons. The RNA-binding protein TDP-43 (trans-active response DNA binding protein 43) plays a crucial role in the transport and translation of mRNAs in neurons. In amyotrophic lateral sclerosis (ALS) and fron-totemporal lobar degeneration (FTLD), TDP-43 and other RNA-binding proteins are mis-localized and abnormally deposited in neurons. Mutations of genes regulating these proteins have been identified in clinical cases. Impaired mRNA transport system may be a contributing factor of neurodegeneration in ALS/FTLD. In this chapter, we outline the role of RNA-binding proteins, with emphasis on TDP-43, in axonal transport and local translation of mRNAs in ALS/FTLD.

Keywords: amyotrophic lateral sclerosis; axonal transport; local translation; ribosomal protein; TDP-43

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease in which disorders of motor neurons cause paralysis and atrophy of muscles throughout the body. The disease has a poor prognosis and leads to a life-threatening state in 3–5 years mainly due to dysphagia or respiratory failure. To date, nearly 40 genes, including RNA-binding protein genes such as *TARDBP* (transactive response DNA binding protein), *FUS* (fused in sarcoma), and *hnRNPA1* and *hnRNPA2/B1* (heterogeneous nuclear ribonucleoproteins A1 and A2/B1), have been identified as contributing factors for ALS pathogenesis (1). *TARDBP* codes for TDP-43 (transactive response DNA binding protein 43 kDa). In addition, *C9orf72* has been identified as the most frequent causative gene of familial ALS, in which abnormal expansion of a hexanucleotide repeat sequence (GGGGCC) in the non-coding region of the gene is observed (1). These gene mutations are responsible for tau-negative frontotemporal lobar degeneration (FTLD) and ALS, suggesting that these two diseases share a common pathological mechanism. In addition to familial cases caused by gene mutations, the disappearance of TDP-43 from the nucleus, and the aggregation/deposition of truncated, hyperphosphorylated, and ubiquitinated TDP-43 in the cytoplasm are observed in neurons of most sporadic ALS cases, which is one of the major pathological hallmarks of the disease (2, 3). In addition, abnormal cytoplasmic mis-localization/deposition of FUS, and the co-localization of other RNA-binding proteins with TDP-43 or FUS, have been reported (4, 5). These observations suggest that functional changes in RNA-binding proteins, including TDP-43 and FUS, occur in ALS neurons and that aberrant RNA metabolism caused by these changes may be involved in the pathogenesis of ALS.

FUNCTION OF RNA-BINDING PROTEINS

TDP-43 and FUS are expressed ubiquitously in the body, and they are mainly present in the nucleus of cells. Both TDP-43 and FUS control the transcription of genes and the splicing of transcribed immature pre-mRNAs (6). It is also reported that TDP-43 and FUS are involved in the regulation of miRNA biogenesis (7, 8). In addition, TDP-43 and FUS shuttle between the nucleus and cytoplasm, export mRNAs from the nucleus, transport mRNAs in the cytoplasm, and regulate their translation (6). mRNAs released from the nucleus exist in the form of mRNA-RNA-binding protein complexes, called RNA granules, within the cytoplasm. Each RNA-binding protein has a consensus motif of RNA sequence with high-affinity binding capabilities for specific mRNAs. mRNAs are transported to the required site with their translation suppressed by RNA-binding proteins in RNA granules. Subsequently, mRNAs are released from the granules for translation by ribosomes into proteins (9). There are different types of neuronal RNA granules, including stress granules, transport granules, and P bodies (processing bodies). Stress granules are formed during cellular stress, for example, starvation and oxidative stress. Transport granules transport mRNAs in axons and dendrites, and P bodies are involved in mRNA degradation (9). RNA-binding proteins interact

and complex with each other to form RNA granules, and thus modulate the function of each other. For example, fragile X mental retardation protein (FMRP), the causative gene product of fragile X syndrome, has been reported to form a complex with TDP-43 which alters its aggregation activity and translation of target mRNAs (10).

Neurons have long neurites. Axons can be up to a meter long in motor neurons with an area 1,000 times that of their cell bodies. At the tip of axons, growth cones and pre-synapses exist in developing and mature neurons, respectively, which support binding with other neurons, or effector receptors, to form synapses and communicate with each other. To maintain the axonal morphology and function, neurons actively transport cell components such as proteins and intracellular organelles along axons via motor proteins, for example, kinesin superfamily proteins (11). Most of the proteins required for axonal formation and maintenance were previously thought to be supplied by transporting the translated proteins directly from the cell bodies. However, in recent years, there is growing evidence that a subset of mRNAs is transported along axons as neuronal RNA granules where translation of the proteins occurs locally, at destination. In axons, all the machinery necessary for local translation, such as ribosomes, translation initiation factors, and elongation factors are present (12) to take part in the protein supply locally. The transport and local translation of mRNAs in axons actively take place during axon pathfinding and outgrowth to the projection destination through neurogenesis, formation of networks via synapses, and regeneration of axons and synapses during neuronal injury. They also help to maintain axons in a mature, steady state (13). The advantage of local translation of mRNA in axons is that it can supply proteins more quickly upon demand than transporting proteins along the axons.

PATHOLOGICAL ROLE OF RNA GRANULES

TDP-43 and FUS are constituents of stress granules, which repress the translation of mRNAs (14). Both TDP-43 and FUS are also involved in axonal transport of mRNAs (15, 16). Both RNA-binding proteins have highly hydrophobic amino acid sequence regions called the low-complexity domains (LCD), through which they interact with each other to form multimers. This contributes to the formation of RNA granules with a non-membranous interface called liquid droplets. Mutations of the genes found in familial ALS reside mainly in the LCD of TDP-43, and the nuclear localization signal (NLS) site required for nuclear import of FUS. These mutations affect the intracellular localization as well as aggregation propensity of proteins. Mutant TDP-43 and FUS cause dysregulation of stress granules and trigger the formation and aggregation of inclusion bodies in ALS (14). Furthermore, mutations in TDP-43 increase granule viscosity, confer toxic gain-of-function effects, and cause morphological instability of RNA granules leading to impaired anterograde axonal transport in ALS (15, 17).

As impaired TDP-43 and FUS-mediated pathological conditions of ALS progress, the amount of proteins required for normal physiological transport of mRNAs for local translation in axons decrease. Furthermore, TDP-43 and FUS themselves, accumulated in the axons, inhibit their own function of axonal

mRNA transport (5, 18). Consequently, reduced transport of critical mRNAs for axonal maintenance will cause morphological and functional changes of axons, ultimately resulting in degeneration of motor neurons. Functional deficits at neuromuscular junctions precede the clinical phenotype and motor neuron loss in mutant TDP-43 or wild-type FUS transgenic mice (19,20). Studies in cultured motor neurons and zebrafish indicate that TDP-43 and FUS are involved in axon outgrowth (21–23). These findings imply that axonal degeneration is a primary executor of ALS pathogenesis. TDP-43 transports mRNAs of *NEFL* and *futsch/MAP1B* in axons. Futsch/MAP1B regulates synaptic microtubule organization, and aberrant neuromuscular junctions are observed in TDP-43 mutant *Drosophila* due to a decrease of *MAP1B* mRNA and translated protein at synapses (24, 25). FUS transports mRNA of *Fos-B* in axons, dysregulation of which causes abnormal axon branching (26). It has also been shown that TDP-43 and FUS bind to mRNAs that have structures called G-quadruplex and transport them to neurites (27, 28). ALS-linked mutant TDP-43 lacks the activity of binding and transport of mRNAs bearing G-quadruplex, which correspond to approximately 30% of neuronal mRNAs (27). Thus, decreased levels of functional TDP-43 may cause reduced axonal mRNA transport and resultant axonal degeneration in ALS.

RIBOSOMAL PROTEIN mRNAS AS AXONAL TRANSPORT TARGETS

Ribosomes are involved in the translation of proteins from mRNAs, and there are two types in eukaryotes: cytoplasmic and mitochondrial. Each type is composed of about 80 different ribosomal proteins and four ribosomal RNAs. Ribosomes are present in axons as well as in cell bodies of neurons. mRNAs of translation-related proteins, including ribosomal proteins, are abundant in axons compared to those in cell bodies (29–31), suggesting that the transport of the mRNAs to axons has functional significance. Although some target mRNAs transported by TDP-43 in axons have been reported, there has been no comprehensive study to identify critical TDP-43 targets in relation to ALS pathogenesis. Therefore, we searched for transport target mRNAs of TDP-43 unbiasedly by using compartment culture devices to isolate axon-rich fractions (29). In our analysis, many cytoplasmic ribosomal protein (Rp) mRNAs were reduced in axons, but mitochondrial ribosomal protein mRNAs were not reduced. This means that the sequence specific to cytoplasmic ribosomal protein mRNAs may be important for axonal transport by TDP-43.

TDP-43 and Rp mRNAs are present in a granular pattern in axons, colocalize with each other and move along axons as one, reflecting that Rp mRNAs are transported by RNA granules containing TDP-43. The mRNAs of Rp and translation elongation factors have a unique pyrimidine repeat sequence called 5'terminal oligopyrimidine (5'TOP) in their 5'untranslated regions, which is thought to be the binding site of TDP-43 for axonal transport. Among the RNA-binding proteins known to bind to mRNAs with 5'TOP is La, which was identified as an autoantigen in rheumatic diseases, and controls the translation of 5'TOP mRNAs by binding to them (32). La co-localizes with TDP-43 and Rp mRNAs in axons and

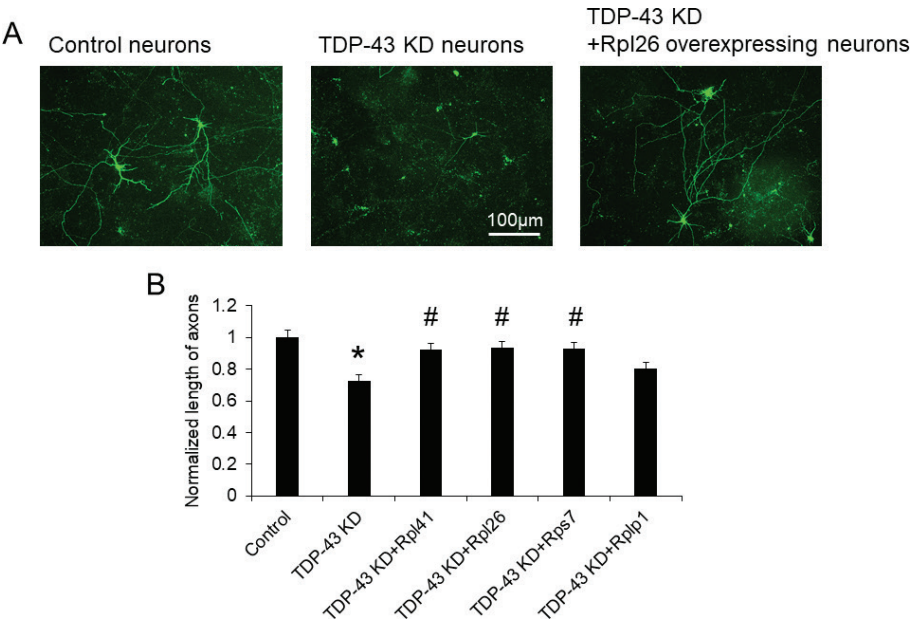

Figure 1. Rescue of axon outgrowth deficit in TDP-43-knockdown neurons by Rp overexpression. **A.** Representative images of neurons in each condition. **B.** Axon length in each group. Rpl41, Rpl26, Rps7 and Rplp1 were examined as representative Rp components. Results indicate mean ± standard error. *P < 0.001 compared with control neurons, and #P < 0.005 compared with TDP-43 KD neurons by one-way ANOVA test. KD, knockdown; Rp, cytoplasmic ribosomal protein.

binds to TDP-43. In ALS patients with pathological changes of TDP-43 localization, RP mRNAs are reduced in the pyramidal tracts of the medulla oblongata where the axons of motor neurons exist (29). Overexpression of several Rps mitigates the deficit of axon outgrowth caused by TDP-43 knockdown (Figure 1), suggesting that Rps may be useful tools for treating ALS and FTLD.

LOCAL TRANSLATION IN AXONS IN PHYSIOLOGICAL CONDITIONS AND ALS PATHOGENESIS

Ribosome assembly occurs primarily in the nucleolus, and it has been thought that ribosomes present in axons are maintained after assembly by transport from cell bodies or supply from glial cells (33). However, in recent years, it has been reported that some Rps are replaced with newly translated ones on aged ribosomes existing in the cytoplasm (34), and Rp mRNAs are locally translated at axon terminals to maintain the function of ribosomes to aid in axonal branching (35). It is also known that mRNAs of the translation initiation factors eIF2B2 and eIF4G2 are transported in axons, where they are translated into proteins, and involved in

the maintenance of overall local translation function (36). More recently, it has been pointed out that ribosomes have heterogeneity depending on the cell types and subcellular compartments and may have a translation function specific to each site (37). These observations indicate that proper functioning of ribosomes and translation factors are essential for maintaining local translation in axons, and the survival of neurons.

Rp and mitochondrial complex-related mRNAs are unstable in fibroblasts and induced pluripotent stem cells of *C9orf72*-mutated ALS patients (38). Also, TDP-43 has been shown to regulate local translation in axons of motor neurons (39). Furthermore, mice expressing mutant FUS have an overall decrease of local translation in axons (16). These findings strongly suggest that Rp mRNA metabolism disorders or ribosome dysfunction may be involved in the pathogenesis of ALS. Therefore, we hypothesize that Rp mRNAs transported in axons by TDP-43 regulate translation function of axonal ribosomes by replacing defective Rps with locally translated ones, the disturbance of which will cause neurodegeneration in ALS and FTLD (Figure 2).

The importance of local translation in motor axons has been demonstrated in another motor neuron disease, spinal muscular atrophy (SMA). SMA is caused by a decrease in the survival motor neuron (SMN) protein due to deletions or mutations of the gene *SMN1*. Although SMN has no evidence of direct binding to mRNA, it is supposed to control transport and local translation of mRNA in axons through binding to RNA-binding proteins (40). SMN protein controls axon growth by modulating localization of *β-actin* mRNA in growth cones (41). Recent reports indicate that SMN protein regulates local translation in axons via axonal transport of the cytoskeletal-related protein growth-associated protein 43 (*GAP43*) mRNA (40). Furthermore, it has been reported that a decrease in SMN protein reduces the translation of *mTor*, which is a key molecule for protein translation by increasing the expression of miR-183 in axons and suppressing local translation (42). These findings indicate that maintenance of local axon translation function is particularly important for motor neuron survival, and that its breakdown is involved in motor neuron degeneration.

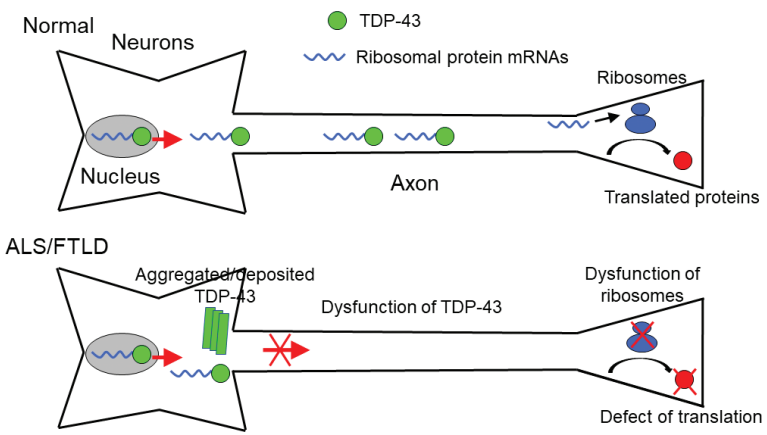

Figure 2. Schematic representation of ALS/FTLD pathogenesis due to defective local translation in axons.

CONCLUSION

In the future, functional analyses of other ALS-causing gene mutations and further analyses using ALS patient samples will clarify the significance of local translational dysfunction in neuronal axons in the pathogenesis of ALS. Furthermore, by identifying the most critical proteins involved in neurodegeneration due to the local translation deficit, new therapeutic targets could be identified.

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6

Blood-based Biomarkers for Amyotrophic Lateral Sclerosis

Heather M. Wilkins[1-3, 5] • Mazen M. Dimachkie[1] • Abdulbaki Agbas[4,5]

[1]Department of Neurology, School of Medicine, University of Kansas Medical Center, Kansas City, USA; [2]Department of Biochemistry and Molecular Biology, School of Medicine, University of Kansas Medical Center, Kansas City, USA; [3]University of Kansas Alzheimer's Disease Center, University of Kansas Medical Center, Kansas City, USA; [4]Department of Basic Sciences, College of Osteopathic Medicine, Kansas City University, Kansas City, USA; [5]Heartland Center for Mitochondrial Medicine, Kansas City, USA

Author for correspondence: Abdulbaki Agbas, Department of Basic Sciences, College of Osteopathic Medicine, Kansas City University, Kansas City, MO, USA; E-mail: aagbas@kansascity.edu

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Abstract: Early detection of amyotrophic lateral sclerosis (ALS) is critical for better therapeutic outcomes. The median time from symptom onset to diagnosis of ALS is 11 months, with a range of 6-21 months. Given that the median life expectancy is three years, it is important to shorten the diagnostic journey, initiate therapies promptly, and facilitate clinical research participation. Biomarkers may be the key to enhancing early diagnosis, tracking disease progression, and testing target engagement of promising therapeutics. Clinically valid biomarkers for ALS are currently lacking, and research has been ongoing to identify appropriate biomarkers. Ideal biomarkers should be minimally invasive, such as blood. In this chapter, we review our current understanding of blood-based biomarker research in ALS and discuss future directions.

Keywords: amyotrophic lateral sclerosis; biomarker; blood; mitochondria; TDP-43

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is mostly a sporadic disease that leads to progressive degeneration of the cortical, bulbar, and spinal motor neurons (1–3). The median age of onset of sporadic ALS is 55, with a male predominance (1.5:1) (2). Diagnosis is based on upper motor neuron signs (spasticity, increased tendon reflexes) and lower motor neuron dysfunction, which may be supported by electrophysiological findings (1). Weakness and atrophy begin either in the bulbar region or in the limb muscles in about a third of cases and spread to the contralateral limb. Respiration is usually affected late in the disease and up to 50% may have evidence of frontotemporal dementia (FTD). Patients with older onset age, bulbar dysfunction, greater clinical disability, and low respiratory function have the poorest prognosis (1, 2). The median life expectancy from symptom onset is approximately three years, with a five-year survival rate of 20–25% and a 20-year survival rate of 5% (2). Most cases are sporadic, but 10–15% are of autosomal dominant inheritance.

Biomarkers can serve as tools for early diagnosis, predictors of prognosis, indicators of target engagement or therapeutic response, and enablers of discovery of future therapeutics for ALS. Biomarker development efforts for ALS have been hampered by a number of issues including small sample size, methodological variation, and lack of standardized techniques. On average, time from symptom onset to clinical diagnosis spans 11 months and this time is critical for life-saving interventions and therapies (4). Biomarkers could hasten diagnosis to allow for earlier introduction of therapies. Prognostic biomarkers are critical due to the heterogeneous nature of ALS and could facilitate prediction of how a subgroup of ALS subjects might progress or respond to a therapy. The low prevalence of ALS is an important issue that negatively affects clinical trials and biomarker development (5–7). In general, recruitment to clinical trials in rare diseases like ALS is a challenge. In ALS, several factors reduce the likelihood of participation in clinical trials including delay or uncertainty in diagnosis, slow progression, respiratory compromise, short life expectancy, and in some cases, dislike of being assigned to the placebo group. Discovery of diagnostic, prognostic, and target-engagement biomarkers are essential for accelerating the research and development of ALS therapeutics. In this chapter, we provide an overview of our current understanding of blood-based biomarkers for ALS.

POTENTIAL BIOMARKERS FOR ALS

The body of knowledge on biomarkers of ALS is limited. Ideally, a biomarker for ALS should be easy to quantify, minimally invasive, specific, reliable with an uncomplicated measurement process, and reproducible across multiple laboratories (8). Figure 1 summarizes the main areas of biomarker research in ALS, all of which target pathological findings in the disease. These aim to measure neurodegeneration, neuroinflammation/systemic inflammation, oxidative stress, excitotoxicity, mitochondrial function, and protein aggregation/proteostasis. Tables 1 and 2 summarize the overall findings of blood-based measures (9–30).

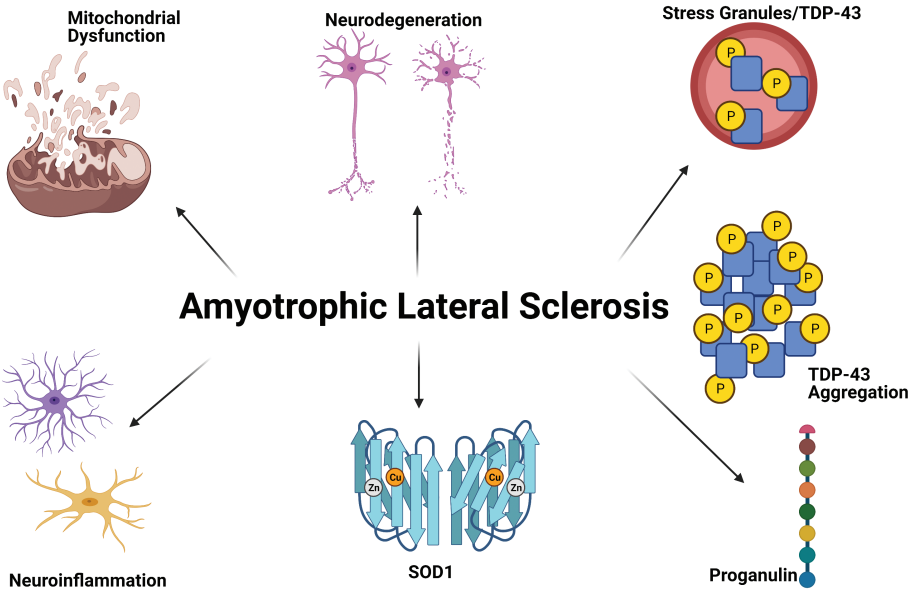

Figure 1. Biomarker Focus in ALS. Areas of biomarker development are focused on pathological findings in ALS. These include neuroinflammation/systemic inflammation, mitochondrial dysfunction, neurodegeneration, and protein aggregation/proteostasis. Created with BioRender.com

TABLE 1 Blood Based Biomarker Studies

Target	Source	Sensitivity/Specificity	n	Source
TDP-43	Plasma	NA	319	(9)
Exosome miRNA	Plasma	NA	40	(10)
Exosome proteomics	Plasma	NA	22	(11)
Proteomics	Plasma	58% and 90%	295	(12)
Glutamate Uptake	Platelet	NA	82	(13)
Mito-Respiration	Platelet	NA	15	(14)
Serotonin	Platelet	NA	114	(15)
NfL	Serum/Plasma	84–100% and 76–97%	248	(16)
NfH	Serum/Plasma	61–80%,72.1–83.7%	157–331	(16–18)
Cytokines	Serum/Plasma	NA	87–183	(19–21)
Ferritin	Serum/Plasma	NA	104–694	(22–24)
Creatine Kinase	Serum/Plasma	63.8% and 54.3%	216–834	(22, 25)
Non-coding RNA	Whole Blood	73.9–93.7%	88	(26)
Chromosomal Confirmation	Whole Blood	83.33–87.5%	58	(27)
Microarray analysis	Whole Blood	87%	1,116	(28)
Immune Cell Profiling	Whole Blood	NA	80	(29)
T-regs	Whole Blood	73.9–76.9%, 69.6–73.1%	217	(30)

TABLE 2 **Blood based biomarkers based on ALS categories (7)**

Familial ALS Biomarkers	Sporadic ALS Biomarkers
TDP-43	TDP-43
FUS	FUS
C9ORF72	Neurofilaments
	Extracellular RNA
	Stress granules
	Progranulin
	TNF-a and range of cytokines
	Metabolites (i.e., creatine kinase, platelet serotonin)
	Mitochondrial biomarkers (i.e., cytochrome oxidase, mitochondrial respiration rate, reduced Complex-II activity)

In this section, our current knowledge on biomarkers for both familial and sporadic ALS are discussed.

C9ORF72 protein

The most common genetic abnormality in frontotemporal lobar degeneration (FTLD) and ALS is the expansion of GGGGCC $(G_4C_2)_n$ repeat in an intron of chromosome 9 open reading frame72, depicted as C9ORF72 (31, 32). GGGGCC repeat expansions are translated through a repeat associated non-ATG (RAN) mechanism that does not require the AUG start codon (33). This non-canonical type of protein translation takes place without frame shifting or RNA editing, resulting in production of dipeptide repeat (DPR) proteins. There are five known DPR proteins, Poly-GA, Poly-GP, Poly-GR, Poly-PA, and Poly-PR (34, 35). These DPR proteins display different profiles across neurodegenerative diseases and could be potential biomarkers. Poly-GA proteins are associated with inclusion bodies when TDP-43 aggregation is lacking (TDP-43-negative inclusions) (35). In the neurons of post-mortem brain, Poly-GA protein aggregates are surrounded by TDP-43 aggregates (36). Poly-GR and Poly-PR DPR proteins cause neurodegeneration in drosophila without TDP-43 aggregation (37). Some studies suggest Poly-GA aggregation can induce TDP-43 phosphorylation and aggregation (35). Thus, the precise role of DPR proteins in TDP-43 aggregation has not yet been resolved. The G_4C_2 repeats can be measured in blood (38) and could serve as a blood-based biomarker for ALS. For familial ALS cases, peripheral blood lymphocyte levels of mutated *SOD1* and mutated C9ORF72 were used to measure target engagement in a clinical trial. Although primary outcomes of clinical trials are focused on cerebrospinal fluid (CSF)-based biomarkers, blood cell profiles appear to be changing as well. For example, SOD1 levels were reduced in peripheral blood lymphocytes in a pyrimethamine clinical trial (39). Poly-GP repeats in C9ORF72-positive ALS cases are detected in peripheral blood mononuclear cells

(PBMCs) (39). The ability to detect these in blood is promising for target engagement in clinical trials with therapies aimed at restoring proteostasis.

Neurofilaments

Neurofilaments function to maintain axon structure and transport (40). Neurofilaments exist in three isoforms; high-molecular-weight subunit (180–200 kDa [NfH]), middle-molecular-weight subunit (130–170 kDa [NfM]), and low molecular-weight-subunit (60–70 kDa [NfL])—all are exclusively expressed in neurons (41). Neurofilaments are considered surrogate biomarkers of neuronal degeneration (42). Aberrant NfL accumulation is observed in both familial and sporadic ALS patients (43–47). CSF levels are considered better than blood levels for the diagnostic confirmation of ALS (48). NfL levels increase during early stages of ALS (18). Further studies show NfL increases as early as 12 months prior to symptom onset in ALS and could be a predictive biomarker (49). Single molecule array technology or SIMOA has enabled the quantification of NfL in serum and plasma at pictogram/mL sensitivity (50, 51). NfL is widely used as a biomarker of ALS. NfL levels in serum are higher in ALS subjects and correlate well with CSF measurements (52). Overall, NfL strongly correlates with survival, but levels are largely steady over time and show no correlation with functional diagnostic scores such as the El Escorial Criteria (7, 16, 53). Using the SIMOA assay, serum NfL may be not only a clinically validated prognostic biomarker for ALS but may also be a biomarker of treatment effect (54). Plasma neurofilament heavy subunit (pNfH) has shown variable results across studies (7, 53). One study showed elevated pNfH levels predict faster progression at 4 months while another study showed it was associated with higher mortality at 12 months (18). Other studies show pNfH levels are neither steady nor reliable longitudinally and are not correlated with disease progression. Overall, the rate of change in blood pNfH is not reliable to predict disease progression and its utility as a diagnostic marker remains to be realized (16–18).

TDP-43

Transactive response (TAR) DNA binding protein 43 (TDP-43) regulates gene transcription, mRNA splicing, stability, and translation (55). Mutations in *TDP-43* cause familial forms of ALS and TDP-43 aggregates are found in most ALS subjects on autopsy (56–58). TDP-43 and its post-translational modifications can be measured across numerous biofluid and could serve as a biomarker for ALS (59–65). Within the ALS field, CSF TDP-43 measurements are preferred over blood-based samples. However, lumbar punctures are invasive, and patients are less likely to agree to this procedure for CSF sampling. Mass spectrometry analysis of post-mortem brain tissue from ALS subjects revealed a number of TDP-43 post-translational modifications including hyperphosphorylation, acetylation, ubiquitination, deamidation, and oxidation (66). Hyperphosphorylation (67, 68) and lysine acetylation increase TDP-43 aggregation (69). Phosphorylation of TDP-43 between amino acids 220-414 is suspected to prevent TDP-43 degradation and increase its expression levels (70). Plasma TDP-43 is higher but is unchanged in serum (9). TDP-43 is mis-localized in cytoplasmic fractions of PBMCs while

overall expression of TDP-43 is not changed. TDP-43 levels in PBMCs correlate with disease burden over time (62, 71, 72). Longitudinal studies showed that TDP-43 plasma levels are highly variable over time, and between individuals (7). These variable findings could be a consequence of blood handling, hemolysis, and coagulation. Classification of TDP-43 expression and post-translation modifications in the blood of ALS subjects could be used as a biomarker for detection/diagnosis and therapeutic outcomes.

Extracellular RNAs, exosomes and stress granules

Extracellular RNAs are found outside the cells in extracellular vesicles (EVs) such as exosomes, micro vesicles and apoptotic bodies, or RNA-binding proteins. Their association with lipids and proteins protect them from degradation and allows for their measurement. Extracellular RNAs are found in many forms, such as tRNA, mRNA, microRNA (miRNA), and circular RNA (circRNA) within EVs. tRNA fragments may be disease-specific and should be considered for biomarker development (73, 74). Next generation sequencing of neural enriched exosomes from plasma of ALS patients identified eight miRNAs that could discriminate ALS from healthy subjects (10). circRNA can be detected in extracellular fluid (75–78). The function of circRNA is largely unknown but regulation of gene expression is a likely function (79). High levels of extracellular circRNA in CSF suggest that the central nervous system (CNS) may secrete them (80–82). The potential of circRNA as a biomarker in ALS was recently reviewed (83).

Exosomes are 50-100 nm extracellular vesicles released from cells. In blood, exosomes are released by erythrocytes, platelets, endothelial cells, and lymphocytes (Table 3). Proteomic analysis of exosomes from ALS and Parkinson 's disease (PD) subjects was able to discriminate between these two diseases (11). Exosomes derived from blood, serum, or plasma show high contamination of blood proteins, which decreases the specificity of proteomic analysis (84).

Stress granules are cytoplasmic RNA complexes that form in response to environmental stress. Several ALS-associated proteins, such as FUS (85), TDP-43(86), Ataxin2 (87), and SOD1 variants (88) have been identified as integral components of stress granules. Currently, measurements of stress granules are limited to cell-based assays.

TABLE 3 Exosome Defining Markers

Exosome Donor Cell	Marker
Platelets	CD31, CD41, CD61, CD42b, GPIIb-IIIa
Endothelial cells	CD31, CD42B, CD51, CD105
Monocytes	CCR2, CD14, CD41a
Neutrophils	CD43, CD16
Lymphocytes	CD4, CD8
Erythrocytes	CD235a

Progranulin

Progranulin (PGRN) is a cysteine-rich secretory protein involved in cell proliferation, inflammation, and tumorigenesis (89). Brain progranulin is implicated in neuronal survival as well as pathogenesis of neurodegenerative diseases (90, 91). Progranulin levels can be measured in both CSF and serum of FTD, ALS, and Alzheimer's disease patients (92). Although no comprehensive study is available to compare progranulin levels in brain with CSF and serum values (92), blood levels are 35 times higher than CSF in ALS subjects with FTD (93). This suggests blood measures of progranulin could serve as a biomarker in ALS.

RNAseq and proteomics

Microarray analysis of blood cells has allowed for machine learning and identification of ALS subjects from the healthy (28) with an accuracy of 87%. Gene expression changes observed in ALS blood cells include increased neutrophil related genes with decreased erythroid lineage-specific genes. The expression of copper chaperone of superoxide dismutase (CCS) and other mitochondrial respiration-linked genes were significantly associated with survival in ALS subjects (28). Further, circulating non-coding RNAs have shown a 73.9-93.7% accuracy in discriminating the healthy from ALS populations (26). Proteomic analysis of ALS blood samples shows changes in proteins involved in the regulation of metabolism and mitochondrial function, particularly carbohydrate, creatine, and lipid metabolism (12). Nitric oxide and reactive oxygen species production are upregulated in macrophages of ALS patients (94). Protein expression of TDP-43, cyclophilin A, and ERp57 in PBMCs were found to associate with disease progression in ALS subjects. A multiprotein expression profile in PBMCs could discriminate ALS from healthy controls with 98% power, and discriminate ALS from other neurologic disease with 91% power. The multiprotein expression profile was further validated in the G93A *SOD1* ALS mouse model using both PBMCs and spinal cord tissue (62). Chromosomal conformation in blood samples can also discriminate between ALS and healthy subjects with a sensitivity of 83.33–87.5% and specificity of 75.0–76.92% (27).

Inflammatory markers

Cytokine expression in blood is altered in ALS subjects but do not change over time. Tumor necrosis factor α (TNF-α) and downstream effector interleukins are increased in ALS subjects (19–21). Data from 25 independent studies examining serum and plasma levels of cytokines show that TNFα, IL-1β, IL-6, IL-8, TNF receptor 1, and vascular endothelial growth factor (VEGF) are elevated in ALS (7). Other inflammatory markers such as complement components, C reactive protein, and chitotriosidase have shown equivocal association with ALS (7). Immune cell profiling has shown that higher levels of lymphocytes, monocytes, and T cell subtypes are associated with longer survival times (29). $CD4^{+}CD25$High T-regs are lower in ALS patients (30, 95), and is a measure of ALS progression. Overall, inflammatory markers have not shown specificity for ALS diagnosis and no association with disease progression has been established yet.

Metabolites

Serum and plasma creatine kinase are elevated in ALS subjects and correlate with the revised ALS functional rating scale (ALSFRS-R) score and other functional outcomes in ALS (22, 25). Plasma and serum ferritin levels are higher in ALS subjects. In some studies, ferritin levels were associated with survival and in others it did not (22–24). Glutamate uptake is impaired in platelets and astrocytes derived from ALS subjects (13). Furthermore, platelet serotonin levels are reduced in ALS subjects and is associated with an increased risk of death (15).

Mitochondrial biomarkers

Mitochondrial dysfunction is observed across numerous tissues in ALS subjects. Spinal cord mitochondrial DNA shows higher levels of mutation, and reduced citrate synthase, complex I+III, II+III and IV activities (96). Induced pluripotent stem cells (iPSCs) derived from ALS patient fibroblasts show reduced mitochondrial function when differentiated into motor neurons. iPSC-derived ALS motor neurons had reduced ATP production and mitochondrial respiration and increased glycolytic flux (97). Muscle samples from ALS patients show a large number of cytochrome oxidase-negative fibers, and some of these patients had reduced enzyme activity (98, 99). Two separate studies of ALS muscles showed reduced mitochondrial respiration and changes in mitochondrial DNA (99, 100). Tissues outside of the spinal cord and muscle also show changes in mitochondrial function. Fibroblasts from ALS patients show reduced basal, uncoupled, and ATP-linked respiration (101). Hepatic mitochondria from ALS subjects show ultrastructural changes with enlarged mitochondria, inclusions, and disorganized structure (102). Lymphocytes from ALS subjects show increased calcium levels and reduced uncoupled respiration (103). These observations show that mitochondrial abnormalities are a systemic finding in ALS. While most mitochondrial respiration indices were reduced in ALS platelets, non-mitochondrial respiration and complex II activities were increased. Complex II activity reduction over three months correlated with decline in function on the ALSFRS-R scale (14). Two separate clinical trials, testing Rasagiline as a therapeutic for ALS, used lymphocyte apoptosis, mitochondrial superoxide, and mitochondrial membrane potential as secondary outcomes (65, 104). Based on abnormal lymphocyte mitochondrial membrane potentials (101), it would seem reasonable to pursue these as potential biomarkers. Blood cell respiration or enzyme V_{max} assays could be used to determine if a drug is engaging its target by altering mitochondrial function.

CONCLUSION

ALS is a rare disease. We estimate the ALS population in the US to be about 17,000 people (13,000–24,000) based on a US population of 329,450,000 (105). This is one of the main reasons affecting biomarker development for ALS. The exact mechanisms underlying motor neurodegeneration and muscle impairment in ALS are unknown. Current hypotheses include neuroinflammation, mitochondrial dysfunction, oxidative stress, excitotoxicity, and protein aggregation (1, 106–112). Lack of understanding of how these mechanisms interact at different stages of the

disease is another issue limiting the progress of biomarker development and subsequent drug development for ALS. The lack of validated biomarkers for ALS has directly affected drug development. There are three FDA approved therapies for ALS: riluzole and edaravone for modulating the course of the disease, and dextromethorphan/quinidine for symptomatic treatment of sialorrhea. The effect of riluzole is modest, extending the lifespan by 2–3 months (113–115). Edaravone appears to slow progression and preserve function in ALS patients (115–117). Like riluzole, edaravone (Radicava) can have some side effects but its intravenous route of administration can be an obstacle at times. Nuedexta targets pseudobulbar symptoms and has no known effect on life span (118, 119). Current clinical trials for ALS are listed on https://clinicaltrials.gov/ [accessed on 17 June 2021]. There are 448 ongoing studies in Unites States, and most of these would benefit from a host of exploratory and confirmatory biomarkers.

Blood-based biomarkers are considered non-invasive and have the potential to be cost-effective. Disagreements exist regarding the utility of blood measures as surrogate for reflecting the status of motor neurons in the spinal cord or muscle. However, as shown in Figure 2, neurodegeneration and reactive gliosis contribute to blood brain barrier (BBB) breakdown. This BBB breakdown can lead to leakage of CNS exosomes/EVs and other molecules into the blood stream. Further studies are required to assess the correlation between blood measures and spinal cord/muscle tissue disease status. Validated biomarker application in people with ALS

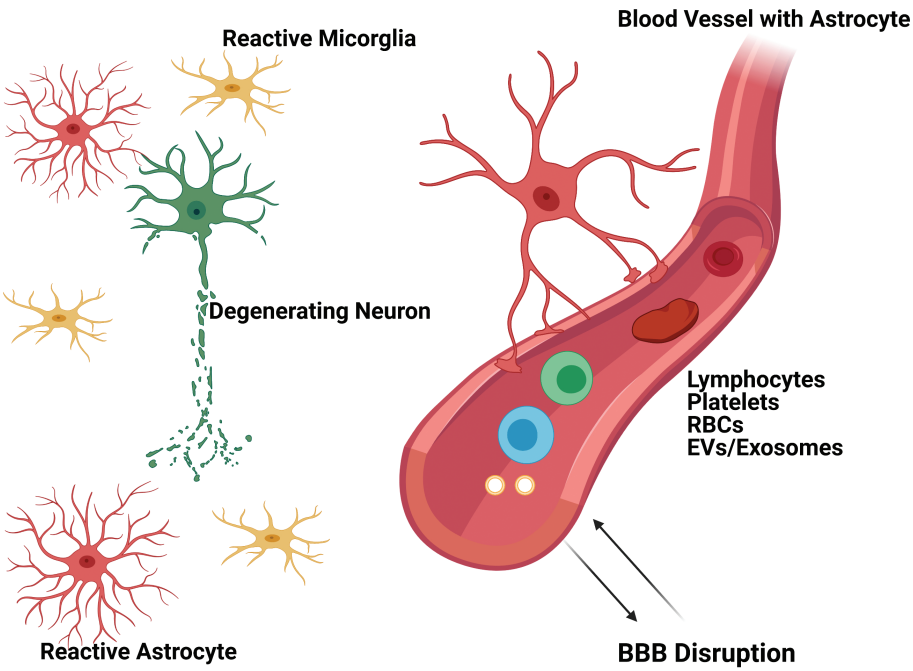

Figure 2. Blood Brain Barrier Breakdown and Circulating Biomarkers. Neurodegeneration and reactive gliosis can lead to blood brain barrier (BBB) disruption (and vice versa). This BBB disruption could allow for CNS derived circulating biomarkers to be measured. Created with BioRender.com

would derive numerous benefits. In addition to shortening the diagnostic journey, disease biomarkers may generate some cost-savings and enhance enrollment in clinical trials. Timely diagnosis will also reduce the time to starting currently available therapies. Biomarkers have the potential to provide valuable information about disease trajectory and critically important early insight into the effectiveness of experimental therapeutics. There is a great unmet need for cost-effective, reliable, accurate, non-invasive and reproducible biomarkers for ALS.

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7

Cell-based Research and Therapy for Amyotrophic Lateral Sclerosis: Promises and Challenges

Juliana Ferreira Vasques[1,2,4,5] • Leandro Coelho Teixeira Pinheiro[1,2,4,5] • Renata Guedes de Jesus Gonçalves[1,2,4,5] • Rosalia Mendez-Otero[1,2,4,5] • Fernanda Gubert[2,3,4,5]

[1]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil; [2]Instituto Nacional de Ciência e Tecnologia em Medicina Regenerativa, Brazil; [3]Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Brazil; [4]RJ Nanotechnology Research Networks Program, Brazil; [5]Translational/Clinical Research Network on Neurodegenerative Diseases, Brazil.

Author for Correspondence: Juliana Ferreira Vasques, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil. Email: julianavasques@biof.ufrj.br

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Abstract: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease which leads to a progressive degeneration of motoneurons. Since the pharmacological options available provide only a slight increase in life expectancy, cell therapy is emerging as a promising therapeutic alternative for ALS. A growing body of evidence from studies using genetically engineered ALS animal models demonstrate the safety and efficacy of therapies based on different cell types such as mononuclear cells, neural progenitors, and mesenchymal stem cells. Despite the encouraging results in preclinical studies, cell therapy-based clinical trials for ALS have achieved only modest results so far, probably due to the genotypic variations seen among ALS patients, which is difficult to reproduce in animal models.

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The advent of induced pluripotent stem cells (iPSCs) has enabled the development of patient-specific cell lines, a valuable tool to investigate in vitro molecular mechanisms of the disease and therapies in different genetic backgrounds. The applications of ALS iPSCs and their future therapeutic potential are also briefly discussed in this chapter.

Keywords: amyotrophic lateral sclerosis; cell therapy; induced pluripotent stem cells; mesenchymal stem cells; stem cells

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fast-progressing neurodegenerative disease that affects motoneurons and results in neuronal death. Although neuronal death is the hallmark of the disease, non-neuronal cells such as astrocytes and microglia play an important role in disease progression (1). Although much progress has been made in the comprehension of ALS pathophysiology, only Riluzole and Edaravone are approved by the FDA (Food and Drug Administration) and have a modest increase in the survival time (2). Cell therapy is emerging as a promising strategy to treat ALS. Several cell types have been suggested, including stem and progenitor cells, and adult somatic cells from different sources, with or without genetic modifications (Figure 1).

Stem cells are defined as cells capable of self-renewal and differentiation into more than one cell type. They are classified as totipotent, pluripotent or multipotent, depending on differentiation capabilities. Totipotent stem cells are the zygotes, that could form the whole individual, while pluripotent stem cells are capable of forming cells from the three germ layers: as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Multipotent stem cells generate only cells from a specific linage or tissue, such as neural stem cells (NSCs) or mesenchymal stem cells (MSCs).

Many factors must be considered to decide the most appropriate cell therapy for a given patient. The clinical problem and the tissue that must be repaired are the primary factors. Cell therapy may aim to regenerate cells or tissue, and in this case pluripotent or multipotent stem cells from the tissue of interest could be used to replace the lost cells. However, cell therapy could also be used to favor the damaged tissue survival or regeneration. MSCs, for example, release paracrine factors that protect host cells that are degenerating, reduce inflammation, stimulate angiogenesis, among others (3). In practice, each stem cell has its advantages and disadvantages for clinical application. For example, ESCs have the advantage of indefinitely proliferation and broad capacity for differentiation but are prone to form tumors or differentiate uncontrollably into undesirable cell types. These cells are of allogeneic origin, requiring immunosuppression when transplanted. iPSCs could overcome this last limitation, once they can be derived directly from the patient. Unfortunately, it is still expensive and time consuming to produce patient-specific iPSCs for therapy. In addition, for diseases such as ALS in which genetic mutations are involved, autologous cell therapy is not the best choice. Multipotent stem cells, that comprises MSCs, could be used autologously, avoiding immunological concerns. However, stem cells from adult tissue are usually present in

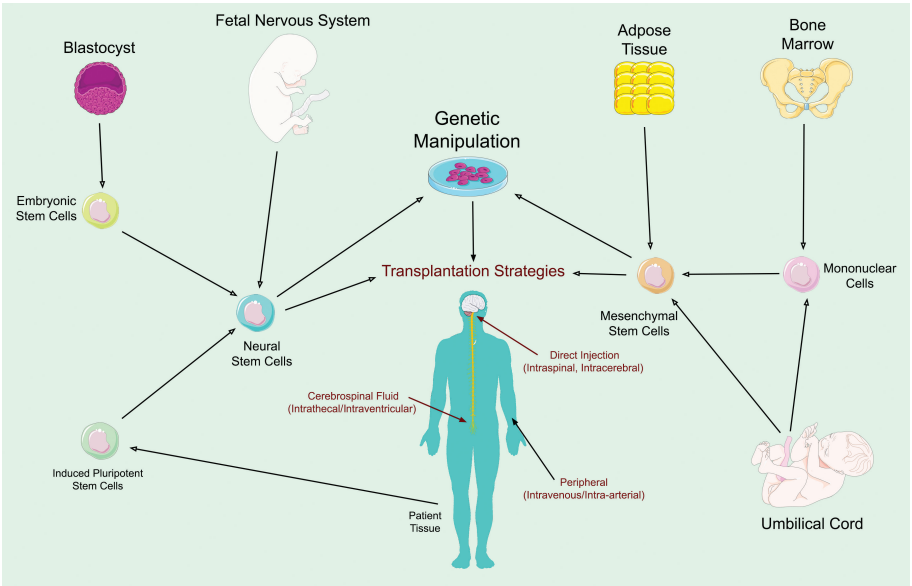

Figure 1. Therapeutic strategies using cells in ALS. Neural stem cells from different sources could be used to replace motoneurons or glial cells, while mesenchymal stem cells or mononuclear blood cells have been tested mainly as immunomodulators. Genetic manipulations, such as growth factors superexpression, can improve cells therapeutic potential. Created using https://smart.servier.com/.

limited quantities and have slow expansion rate, hindering autologous use for the treatment of acute illnesses or traumas, which require immediate treatment. In addition, the number of adult stem cells in most tissues appears to decrease with age (4). In this chapter, the use of cell therapy in ALS is discussed. Different cell types tested in ALS preclinical models and clinical trials are reviewed. Their limitations, and strategies to overcome these limitations are described.

CELL THERAPY IN ALS PRECLINICAL MODELS

Different cell types, doses, and administration routes have been tested in ALS preclinical models, with variable outcomes. The most used cell types in these studies are mononuclear cells, MSCs and NSCs. The main findings of these therapeutic strategies are discussed in the following sections.

Mononuclear cells

The first cell therapy test aimed to replace bone marrow of ALS mice with mononuclear cells from wild-type mice bone marrow or from human umbilical

cord (5–7). Mononuclear cells are a heterogeneous population that comprise both hematopoietic stem cells and MSCs, as well as hematopoietic progenitors, lymphocytes and monocytes. At that time, some groups suggested that ALS could be an autoimmune disease and therefore, such replacement could alter the disease progression (5). Using this approach, Corti and co-workers showed increased animal survival and motoneuron protection, while Solomon and co-workers did not observe alteration in disease evolution despite observing transplanted cells in the spinal cord (6, 7). Other studies suggested that the number of hematopoietic stem cells present in each mononuclear fraction transplanted could be the reason for variable outcomes (5–8). Despite the contradictory results, the use of mononuclear cells continued to be tested, focusing mainly in neuroinflammation modulation (8).

Neuroinflammation has been shown to be an important process in disease pathology. Microglia, astrocytes, and lymphocytes have major roles in ALS (1). Therefore, cell therapy could act as an immunomodulator, ultimately resulting in neuroprotection. Intravenous injection of mononuclear cells derived from human umbilical cord (hUCB-MCs) in a mice model of ALS reduced disease progression and increased lifespan, even when the cells were administrated after the onset of the disease (9, 10). This approach decreased microglia density in spinal cord, reduced pro-inflammatory cytokines in the central nervous system, and increased lymphocytes and decreased neutrophils in peripheral blood, suggesting that hUCB-MC therapy could result in motoneuron neuroprotection by modulation of host inflammatory response (9). Interestingly, hUCB-MCs administrated intracerebroventricularly in murine ALS model also showed positive outcomes. However, transplanted cells were not found in the spinal cord, corroborating the hypothesis that injected cells do not necessarily need to be at the injury site to have a beneficial role (11).

Bone marrow mononuclear cells (BM-MCs) were also tested in mouse models of ALS. Although they are similar to UCB-MCs, BM-MCs can be used autologously, avoiding immunosuppression. Using BM-MCs, different injection routes, such as intraspinal, intramuscular, and intravenous, were tested (12–14). Therapies using these routes individually showed modest positive outcomes, but combined transplantation routes were able to delay disease progression and decrease microgliosis, although there was no change in lifespan (13, 14). These results are in agreement with the multifactorial profile of ALS, suggesting that an intervention in multiple pathways is necessary. In addition, BM-MC therapy in mice model only show positive results when administrated in the presymptomatic phase, an issue that could compromise translation to the clinic (12).

Mesenchymal stem cells

MSCs are part of the pool of cells harvested from bone marrow. Despite representing only 0.001% to 0.01% of total cells (15), MSCs have been extensively studied as key contributors of positive therapeutic effects of BM-MCs. MSCs are versatile cells that strongly respond to different environments, shifting an important paracrine activity that impacts neighboring cells. Therapeutic effects of MSCs are considered to be mostly paracrine, by their ability to secrete a wide variety of growth factors, cytokines, hormones, extracellular vesicles and even mitochondria, that

can act locally or systemically. MSCs also have practical advantages to be used in a clinical setting. They can be harvested from tissues such as bone marrow and adipose tissue for autologous transplantation, or can be isolated from umbilical cord, placenta, dental pulp, and other tissues that are often discarded, and used in allogeneic therapies, since they are low immunogenic (3). They can be expanded *in vitro* and stored in large amounts in biobanks, ready to use when necessary (3). To narrow the types of cells harvested from these tissues, the International Society for Cellular Therapy has defined minimal criteria for MSC characterization (16).

Preclinical studies of MSC therapy for ALS are primarily based on transgenic mice and rats with *SOD1* mutations. Despite being a model that genetically represents only a small portion of ALS patients, it recapitulates critical hallmarks of motoneuron degeneration such as axonal degeneration, apoptosis, and accentuated gliosis (17). These studies vary in their therapeutic approach, testing different MSCs sources, allogenic or xenogeneic origin, therapeutic window, administration routes and dosages.

MSC therapy for ALS relies mostly on it effects directly on motoneurons and/or indirectly on glial and immune cells. MSCs produce and secrete a wide variety of growth factors and cytokines known to be protective to motoneurons, such as GDNF, IGF-1, BDNF, NGF and VEGF (18). For this reason, many preclinical studies injected MSCs directly into the spinal cord, hoping to increase the availability of these factors to motoneurons. After allogenic transplantation at the onset of disease in rats, cells remain in the injection site until end stage of the disease, improving motor capacity, motoneuron survival and increasing lifespan (19), while human MSCs injected long before symptoms onset in mice were no longer detected 70 days after injection, improving motor performance without effect on neuron protection and animals' survival (20). Thus, these data indicate that integration of MSCs to the target tissue can impact therapy outcome.

A less invasive approach to make MSCs secretome available to the spinal cord is delivering cells in the cerebrospinal fluid (CSF) by intrathecal or intracisternal injection. Injected in the CSF, MSCs were shown to survive in the spinal cord (21), spread through the ventricular system reaching the brain (22), and even differentiate into astrocytes (23). Moreover, MSCs reduced astrogliosis, microglia proliferation and inflammation in the spinal cord (21, 23–26). However, systemic administration (less invasive than intrathecal or intracisternal) of MSCs can also reduce inflammation in the spinal cord with limited homing in neural tissue (27), reducing oxidative and glutamatergic stress (28) and increasing neurotrophic factors production by glia (29). The mechanisms by which systemically injected MSCs exert effects in the CNS are still not clear. Terashima and colleagues (30) demonstrated that systemic administration of MSCs expressing HGF, GNDF and IGF-1 were also able to induce the expression of chemoattractants in the spinal cord, increasing the homing of injected bone marrow cells to this tissue.

Given the wide range of approaches to test the effect of MSCs, Zhou and colleagues performed a systematic review and meta-analysis of ALS preclinical studies using these cells. They included 25 studies published until July 2019 and found that MSC therapy in general delayed the age of disease onset, improved motor function, increased lifespan and reduced the estimated hazard ratio for disease. They also analyzed the effect of the different therapeutic approaches. However, given the diversity of the parameters among studies, they found no significant indication of advantage of any specific parameter. However, they point

to an indication to greater benefit of presymptomatic treatments, adipose tissue derived MSCs, and a better general response to treatment in female subjects.

Neural stem cells

While the preclinical studies using MSCs in the presymptomatic stage show a good perspect for future ALS therapies as described above, in the clinical setting, most patients receive their diagnosis long after the appearance of symptoms, indicating that significant motoneuron death has already occurred. Considering this situation, neural stem cells (NSCs) from fetal tissue or induced from ESCs and iPSCs would be an alternative to replace lost motoneurons. Transplanted NSCs were shown to integrate into ALS spinal cord and differentiate into neurons with functional synapses, improving motoneuron survival and motor function (31). However, a newly formed motoneuron in an adult human would have to extend their axon out of the spinal cord to a specific muscle target, and while few studies demonstrated the feasibility of this approach (32), this was not yet demonstrated in ALS models.

Considering that the loss of motoneurons in ALS is not entirely due to cell intrinsic mechanisms, but also due to glial and systemic signaling, this hostile environment would also be detrimental to newly formed neurons. In this context, NSCs can also be used as a source of protective cells, as astrocytes and interneurons that secrete growth factors and act as mediators to reduce local inflammation. NSCs can be induced to produce glial derived trophic factors such as GDNF (33) and differentiate into astrocytes (34). Thomsen and colleagues (35) demonstrated that human NSCs expressing GDNF transplanted into the cortex of ALS rats can improve symptoms and extend survival after differentiating into astrocytes, and they have also demonstrated that these cells can be safely transplanted into the cortex of cynomolgus monkeys (*Macaca fascicularis*), showing a similar pattern of astrocyte differentiation. While the prospect to use NSCs to replace motoneurons is still far away, the use of these cells to generate glia shows a great therapeutic potential in the near future.

CLINICAL TRIALS USING STEM CELLS IN ALS

A variety of cells, doses, and delivery routes/sites have been tested in ALS patients with modest positive results regarding efficacy and safety. Clinical application of stem cells in ALS patients was first reported by Janson and colleagues in a pilot study with 3 subjects submitted to intrathecal transplantation of 2.0×10^7 or 1.0×10^8 autologous peripheral blood stem cells (PBSCs) (36). Two patients experienced speech improvement or muscle strength gain for at least 4 months after the procedure. There were no adverse effects or acceleration of the course of disease over the following 12 months, indicating the safety of the method. A study with 20 patients tested a methodological approach aiming to improve the function of upper motoneurons by injecting $2.5–7.5 \times 10^5$ PBSCs into the frontal motor cortex of enrolled subjects. Compared to control group, the median survival time was significantly higher in the treatment arm, which also showed stable score in the ALS Functional Rating Scale Revised (ALSFRS-R) and the Spitzer

quality of life scale throughout the follow-up period, suggesting a delay in disease progression (37). An additional trial with a cohort of 67 patients confirmed that procedure was well tolerated, safe, and feasible (38).

BM-MCs have also been tested for ALS therapy. A single arm phase I trial conducted in Spain performed autologous BM-MCs transplantation by intraspinal injection in 11 spinal onset ALS patients (39, 40). A median of 462×10^6 cells were infused at thoracic level and subjects were followed up for 1 year. Most of the adverse effects reported were mild and transient, and no acceleration in disease progression was observed, as measured by neurological scales and functional respiratory indexes. Polysomnography showed no significant changes in sleep duration, quality, and ventilation after cell injection, suggesting no cortical diaphragmatic pathway dysfunction. Histopathological examination revealed that in the anterior horn of the grafted segments, motoneurons were significantly more numerous and were surrounded by hematopoietic cells, showing no signs of degeneration, suggesting a neurotrophic action of transplanted cells, as observed by the group in previous preclinical study (41). Sharma and colleagues combined intrathecal and intramuscular autologous BM-MCs transplantation in a cohort of 37 patients and compared them to 20 control subjects (42). The survival duration was significantly higher in the group that underwent cell therapy and the majority of the patients reported improvement in speech, swallowing, respiratory capacity, ambulation, and fine motor activities.

The first FDA-approved stem-cell-based trial for ALS ascertained the feasibility and safety of intraspinal injections of NSI-566RSC, human fetal spinal cord-derived NSCs, in 15 patients. Initially, 12 patients received 5 unilateral or 10 bilateral lumbar injections (1.0×10^5 cells/injection) (43, 44) and then, 5 unilateral cervical injections were performed on 3 new subjects and on 3 who had previously received bilateral lumbar injections (45, 46). Additional 15 patients were recruited to phase II trial to test the safety of escalating doses of NSCs ($2.0–16.0 \times 10^6$) (47). In general, procedure and doses were well tolerated and many of the adverse events were attributed to the immunosuppressant drugs. A similar methodological approach was used in a phase I clinical study conducted in Italy, with 18 spinal onset ALS patients (48, 49). In this case, neural progenitors were isolated from the forebrain of miscarried fetuses. According to ALSFRS-R scores, there was a significant but transient functional improvement within the first 4 months after transplantation. Although these trials demonstrate NSCs safety and some possible efficacy indicators, the use of these cells is often related to ethical and moral concerns and requires an immunosuppressive regimen, which can modify the effect of therapy.

BM-MSCs are among the main cell types used in clinical trials for ALS. Mazzini and collaborators performed two phase I trials with 9 and 10 patients, respectively, to assess the safety of intraspinal transplantation of autologous BM-MSCs (50, 51). Different doses ranging from 7.0 to 152.0×10^6 cells were injected into thoracic spinal cord segments and patients were monitored every 3 months until death. The results of long-term follow-up of the 19 patients confirmed that procedure was safe and feasible, despite the absence of clinical benefits (52). Different groups have shown that intrathecal transplantation of autologous BM-MSCs was also feasible and well tolerated (53, 54). A phase I trial conducted in the Republic of Korea with 7 patients demonstrated the safety of two repeated BM-MSCs intrathecal injections (1.0×10^6 cells/Kg/injection) (55) and the efficacy was tested in

a subsequent phase II study with 64 subjects (56). Changes in the ALSFRS-R scores showed that cell therapy was effective in delaying disease progression, and CSF analysis revealed a significant increase in the levels of anti-inflammatory cytokines as well as a reduction in proinflammatory ones after cell injections. Based on these studies, the Korean government approved in 2015 the use of autologous BM-MSCs for the treatment of ALS, becoming the first country in the world to license the commercialization of a stem cell therapy for the disease. Some studies have also combined intrathecal and intravenous or intramuscular administration of BM-MSCs in an attempt to maximize the possible therapeutic benefits and demonstrated a stabilization of the disease or a reduction in progression speed (57–60). Transplantation of MSCs derived from other sources such as adipose tissue and umbilical cord Wharton's jelly have also been shown to be safe and well tolerated (61, 62).

Despite the encouraging results obtained so far, further randomized controlled trials with large sample sizes are needed to ascertain the efficacy of cell types, doses, and delivery sites/methods so reliable and reproducible therapeutic regimens can be standardized. Stem cell-based clinical trials for ALS are summarized in Table 1.

MODELLING ALS IN VITRO WITH INDUCED PLURIPOTENT STEM CELLS

Animal models have contributed enormously to the understanding of ALS pathophysiological mechanisms (63). However, transgenic animal models represent only a small fraction of familial ALS patients, and about 90% of cases are considered sporadic, without a known genetic component directly associated with the development of the disease. Although motoneuron death is always the final outcome, different molecular pathways can be involved in this degenerative process, depending on the patient's genetic background (64). The lack of variability in preclinical research could explain why therapies with promising results constantly fail or show just modest efficacy results in clinical trials. Therefore, more representative ALS preclinical models, especially for the sporadic form of the disease, are urgently needed.

In 2006, a breakthrough advance in the stem cell field was reported by the Japanese scientists Takahashi and Yamanaka—the genetic reprogramming of adult mice cells into embryonic-like pluripotent stem cells, called iPSCs (65). In the following year, the same feat was achieved with human cells (66). Through this revolutionary technology, it became possible to obtain stem cells capable of differentiating into virtually any cell type from adult somatic cells such as skin, or peripheral blood cells, or even urine (Figure 2).

In 2008, the first iPSCs were derived from an ALS patient, an 82-years old woman carrying a rare mutation in *SOD1* gene. Remarkably, the iPSCs could be differentiated in motoneurons and astrocytes, the two neural cells mainly related to ALS pathology. Differentiated cells carried the same mutation from the donor patient, proving it was possible to reproduce in vitro a genetic profile for which, until then, no study model was available (67). Over the past 15 years, iPSCs have been derived from familial and sporadic ALS patients, with different genotypes,

TABLE 1 Key stem cell-based clinical trials for ALS

Cell Type	Phase	Identifier and Reference	Situation	Delivery Route	Cell Dose	Main Results
PBSCs	N/A	NCT03085706	Completed	Intrathecal	Not provided	No results posted
	N/A	Janson et al., 2001 (36)	Unknown	Intrathecal	2.0×10^7 or 1.0×10^8	Safe; speech improvement and muscle strength gain
	N/A	Cashman et al., 2008 (78)	Unknown	Intravenous	$1.5\text{-}7.6 \times 10^6$/kg	Safe with no clinical benefits
	N/A	Martínez et al., 2009 (37); 2012 (38)	Unknown	Intracortical	$2.0\text{–}7.5 \times 10^5$ or $3.0\text{–}5.0 \times 10^6$	Safe; stabilization of ALSFRS score and increased survival time
BM-MCs	I/II	NCT02286011 Geijo-Barrientos et al., 2020 (79)	Unknown	Intramuscular	Median of 499×10^6	Safe; larger Compound Muscle Action Potential scan curve
	I	NCT02193893	Unknown	Intrathecal	Not provided	No results posted
	I/II	NCT00855400 Blanquer et al., 2012 (39); Ruiz-López et al., 2016 (40)	Completed	Intraspinal	Median of 462×10^6	Safe with evidences of BMMCs neurotrophic activity
	I/II	NCT01254539	Completed	Intraspinal and intrathecal	Not provided	No results posted
	N/A	Sharma et al., 2015 (42)	Unknown	Intrathecal and intramuscular	53.6×10^6 IT + 26.8×10^6 IM	Increased survival duration
	N/A	Sharma et al., 2020 (80)	Unknown	Intrathecal	3 doses: 1.2×10^8, 1.6×10^8 and 3.5×10^8	ALSFRS score stabilization over 16 months and improved ambulation

TABLE 1 Key stem cell-based clinical trials for ALS (Continued)

Cell Type	Phase	Identifier and Reference	Situation	Delivery Route	Cell Dose	Main Results
Fetal NSCs	I	NCT01348451 Glass et al., 2012 (44); Riley et al., 2012 (43); 2014 (45); Feldman et al., 2014 (46)	Unknown	Intraspinal	5 unilateral or 10 total bilateral injections 1.0×10^5 cells/injection	Safe and well tolerated
	II	NCT01730716 Glass et al., 2016 (47)	Unknown	Intraspinal	$2.0 - 16.0 \times 10^6$	Safe and well tolerated
	I	NCT01640067 Mazzini et al., 2015 (48); 2019 (49)	Completed	Intraspinal	3 unilateral or 6 total bilateral injections 75.000 cells/injection	Safe with reduction in ALSFRS decline
NSCs	I/IIa	NCT02943850	Completed	Intraspinal	Not provided	No results posted
MSCs	I	NCT02987413	Completed	Intrathecal	2 doses of 1.0×10^8	No results posted
BM-MSCs	I	NCT02881489	Unknown	Intrathecal	Not provided	No results posted
	I/II	NCT02917681	Unknown	Intrathecal	Not provided	No results posted
	I	NCT01759797 NCT01771640 Nabavi et al., 2019 (54)	Completed	Intravenous or intrathecal	2.0×10^6 cells/kg	Safe and well tolerated
	I/II	NCT03828123 Syková et al., 2017 (53)	Completed	Intrathecal	$15 \pm 4.5 \times 10^6$	Safe and well tolerated with reduction in ALSFRS decline
	I/II	NCT01363401 Oh et al., 2015 (55); 2018 (56)	Completed	Intrathecal	2 doses of 1.0×10^6 cells/Kg	Safe; reduction in ALSFRS decline; immunomodulation

TABLE 1 Key stem cell-based clinical trials for ALS (Continued)

Cell Type	Phase	Identifier and Reference	Situation	Delivery Route	Cell Dose	Main Results
	I/II	NCT00781872 Karussis et al., 2010 (57)	Completed	Intrathecal and intravenous	Mean (SD) of 54.7×10^6 IT + 23.4×10^6 IV	Safe; ALSFRS score stabilization; immunomodulatory effects
	I	Mazzini et al., 2008 (50)	N/A	Intraspinal	Mean of 57×10^6 cells	Safe and well tolerated
	I	Mazzini et al., 2010 (51)	N/A	Intraspinal	Median of 75×10^6 cells	Safe and well tolerated
	III	NCT04745299	Recruiting	Intrathecal	Not provided	No results posted
	N/A	Rushkevich et al., 2015 (58)	N/A	Intravenous and intrathecal	$0.5 – 1.5 \times 10^6$ cells/kg IV + $5.0 – 9.7 \times 10^6$ cells IT	Safe with reduction in ALSFRS decline
MSCs-NTF (NurOwn)	III	NCT03280056	Completed	Intrathecal	Not provided	No results posted
	II	NCT02017912 Berry et al., 2019 (60)	Completed	Intrathecal and intramuscular	IT: 125×10^6 IM: 48×10^6	Safe with reduction in ALSFRS decline
	IIa	NCT01777646 Petrou et al., 2016 (59)	Completed	Intrathecal and intramuscular	3 cohorts: $1 10^6$ cells/kg IT + 24×10^6 cells IM; 1.5×10^6 cells/kg IT + 36×10^6cells IM; 2×10^6 cells/kg IT + 48×10^6 cells IM	Safe with reduction in ALSFRS and FVC decline
	I/II	NCT01051882 Petrou et al., 2016 (59)	Completed	Intrathecal or intramuscular	IM 1×10^6 cells/site- 24 sites; IT 1×10^6 cells /kg	Safe and well tolerated

TABLE 1 Key stem cell-based clinical trials for ALS (Continued)

Cell Type	Phase	Identifier and Reference	Situation	Delivery Route	Cell Dose	Main Results
AT-MSCs	I	NCT03296501 Kuzma-Kozakiewicz et al., 2018 (81)	Active, not recruiting	Intraspinal and intrathecal	16,000 cells 56,000,000 cells	Safe and well tolerated
	I/II	NCT02290886	Active, not recruiting	Intravenous	4 cohorts: placebo; 1.0×10^6; 2.0×10^6; 4.0×10^6 cells/kg	No results posted
	I	NCT01142856	Completed	Intrathecal	1.0×10^6	No results posted
	I	NCT01609283	Completed	Intrathecal	5 cohorts: 1.0×10^7; 5.0×10^7; 1.0×10^8; 2 doses of 5.0×10^7; 2 doses of 1.0×10^8	No results posted
	I	NCT02492516	Completed	Intravenous	2.0×10^6 cells/kg	No results posted
	II	NCT03268603	Recruiting	Intrathecal	4 doses 1.0×10^8	No results posted
	I	Staff et al. 2016 (61)	Unknown	Intrathecal	1.0×10^7 to 1.0×10^8	Safe and well tolerated
WJ-MSCs	I/II	NCT04651855	Recruiting	Intrathecal	Not provided	No results posted
	I	NCT02881476 Barczewska et al., 2019 (62)	Unknown	Intrathecal	0.42×10^6 cells/kg	Safe and well tolerated
UC-MSCs	I/II	NCT01494480	Unknown	Intrathecal	Not provided	No results posted

AT-MSCs, adipose tissue mesenchymal stem cells; BM-MCs, bone-marrow mononuclear cells; BM-MSCs, bone-marrow mesenchymal stem cells; MSCs, mesenchymal stem cells; MSCs-NTF, mesenchymal stem cells stimulated to secrete high levels of trophic factors; N/A, Not Available; NSCs, neural stem cells; PBSCs, Peripheral blood stem cells; UC-MSCs, umbilical cord mesenchymal stem cells; WJ-MSCs, Wharton jelly mesenchymal stem cells.

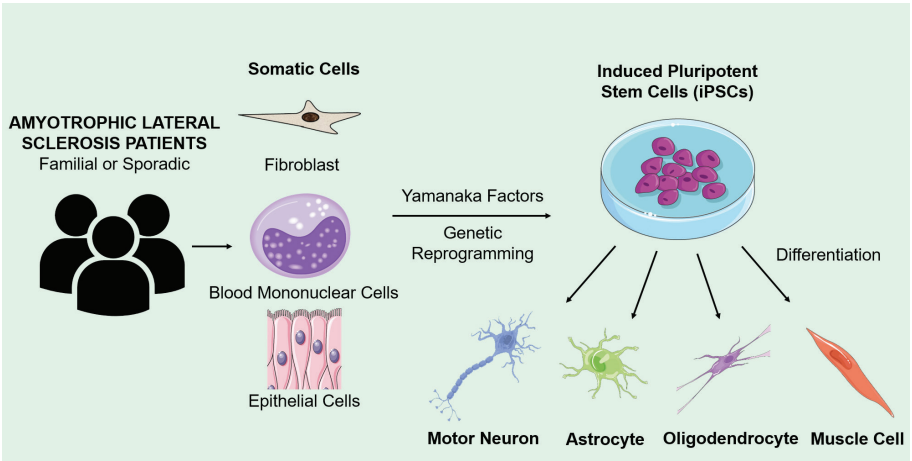

Figure 2. iPSCs can be derived from patient's somatic cells and differentiated in motoneurons, glial cells and muscle fibers, to study ALS pathologic mechanisms in vitro. iPSC-derived cell can also be used in drug screenings and possibly in future cell replacement therapies. Created using https://smart.servier.com/.

and differentiated into cells of interest for the study of the disease, such as motoneurons, astrocytes, oligodendrocytes, and skeletal muscle cells. In these differentiated cells, several important phenotypic alterations were found, which has contributed enormously to the understanding of the pathological mechanisms of the disease. Among the most frequent and relevant findings in motoneurons differentiated from ALS iPSCs are reduction in viability, the presence of intracellular protein aggregates, changes in the electrophysiological properties and mitochondrial function and dynamics (68). Interestingly, several of these features are also present in post-mortem neural tissue from ALS patients. An important ALS histopathological marker is the presence of TDP-43 protein aggregates in spinal cord and motor cortex, and similar aggregates are also consistently found in ALS iPSCs-derived motoneurons (69). These findings corroborate the value of iPSCs as an important tool for ALS modelling.

However, the use of iPSCs and iPSCs-derived differentiated neural cells as an in vitro preclinical ALS model has also important limitations. There is still great variability among the phenotypic changes found in motoneurons differentiated from iPSCs by different research groups. Motoneurons harboring different *SOD1* mutations, for example, have opposite electrophysiological profiles: while motoneurons with the A4V mutation show hyperexcitability, motoneurons with the D90A and R115G mutations are hypoexcitable and have impaired spontaneous activity (70, 71). These contradictory results may be a consequence of using different protocols for iPSCs differentiation, emphasizing the importance of using standardized protocols in the future. Furthermore, ALS-associated mutations are related to different onset age and disease progression. Thus, neural cells differentiated from iPSCs of patients with late onset and/or slow disease progression may need a longer maturation time in vitro to show relevant phenotypic alterations (69). Different strategies are being tested to overcome this limitation.

Pharmacological agents can be used to accelerate cell maturation process. One of these agents is progerin, a truncated protein produced by patients with Hutchinson-Gilford syndrome, whose main characteristic is premature aging. Progerin has already been used in iPSCs derived from patients with aging-associated degenerative diseases, such as Parkinson's Disease, successfully accelerating in vitro appearance of cellular features of the disease (72). Thus, although not yet tested in ALS iPSCs, the use of this drug can be a useful tool in ALS modeling as well. Alternatively, ALS iPSCs-derived neural cells seem to be more sensitive to different types of stressors that can be used to speed up phenotypic alterations onset in vitro (73).

In a translational perspective, the use of cells differentiated from ALS iPSCs could be a useful platform for screening new drugs and therapies, stratifying responsive patients according to their genetic profile. Several drugs with therapeutic potential have already been tested in ALS iPSC-derived neural cells. The FDA-approved antiparkinsonian drug Ropinirole performed well in in vitro studies, but just in cell lines harboring *SOD1* mutations, an important indicative that some pharmacological therapies can be effective only in a specific fraction of ALS patients (69). However, other drugs, such as Bosutinib, originally used for chronic myeloid leukemia, seems to be effective for a broader number of patients; this drug improved motoneuron survival in cell lines derived from patients with mutations in *TARDBP* and *C9orf72* genes, as well as from sporadic patients (74). Bosutinib is now being tested in a clinical trial with ALS patients (75).

Finally, therapies using iPSCs-differentiated neural cells are promising possibilities. iPSCs-derived dopaminergic neurons have recently been transplanted to a Parkinson's patient, with encouraging results (76). However, in ALS, it is necessary for the new motoneuron to expand its axon to the correct target site in the musculoskeletal system, a complicated task, leaving iPSCs-based motoneuron replacement therapies still a hope for the future. Transplantation of iPSCs-derived astrocytes, however, is an easier and interesting approach. This possibility has already been tested in a mouse model, and animals submitted to human iPSCs-derived glial progenitors transplantation into spinal cord had an extension in their lifespan (77).

CONCLUSION

Preclinical studies and clinical trials indicate that cell therapy is a hopeful therapeutic alternative to ALS patients. However, further studies are required to determine ideal cells candidate, doses, and delivery routes. The great heterogeneity in ALS clinical and genetic presentation also makes it difficult to standardize a unique therapeutic protocol for cell transplantation. In this context, iPSCs-derived cells emerge as a promising tool for the optimization of clinical trials, helping to stratify patients and design effective personalized therapies.

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8

Gastrointestinal Status and Microbiota Shaping in Amyotrophic Lateral Sclerosis: A New Frontier for Targeting?

Letizia Mazzini[1] • Fabiola De Marchi[1] • Elena Niccolai[2] • Jessica Mandrioli[3], Amedeo Amedei[2]

[1]ALS Centre, Department of Neurology, Maggiore della Carità Hospital, University of Piemonte Orientale, Novara, Italy; [2]Department of Experimental and clinical Medicine, University of Florence, Firenze, Italy; [3]Department of Biomedical, Metabolic and Neural Science, University of Modena and Reggio Emilia, Modena, Italy

Author for correspondence: Letizia Mazzini, ALS Centre, Department of Neurology, Maggiore della Carità Hospital, University of Piemonte Orientale, Novara, Italy.
Email: letizia.mazzini@uniupo.it

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Abstract: Amyotrophic lateral sclerosis (ALS) is a rare and severe neurodegenerative disease affecting the upper and lower motor neurons, causing diffuse muscle paralysis. Etiology and pathogenesis remain largely unclear, but several environmental, genetic, and molecular factors are thought to be involved in the disease process. Emerging data identify a relationship between gut microbiota dysbiosis and neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and ALS. In these disorders, neuroinflammation is being increasingly recognized as a driver for disease onset and progression. Gut bacteria play a crucial role in

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maintaining and regulating the immune system, and changes in gut microbial composition can influence neural function by affecting neuro-immune interactions, synaptic plasticity, myelination, and skeletal muscle function. This chapter outlines the relationship between ALS and the human microbiota, discussing whether an imbalance in intestinal microbiota composition through a pro-inflammatory dysbiosis promotes a systemic immune/inflammatory response, and has a role in ALS pathogenesis, clinical features, progression, and outcome.

Keywords: amyotrophic lateral sclerosis; gut-brain-axis; microbiota; neuroinflammation; neurodegeneration

INTRODUCTION

The cause of amyotrophic lateral sclerosis (ALS) remains unknown for most patients. An increasing number of susceptibility genes has been recently reported (https://alsod.ac.uk [accessed on 17 June 2021]), but these account for only 10–15% of all cases. Most often, ALS has a sporadic nature, and the onset is the final result of a combination of genetic and environmental factors. The latter include occupational exposure to toxic substances, viral infections, lifestyle habits, habitual diet, and body mass index. However, contrasting results have been reported regarding environmental elements as being risk factors for disease progression (1).

The study of various dietary risk factors is a fascinating topic, but data retrieved from these studies are hard to measure and standardize. A recent Italian study (2), showed that some foods and nutrients, including red and pork meat, proteins, sodium, and glutamic acid, may be risk factors for ALS, while others such as coffee and tea, bread, and raw vegetables can act as protective factors. It has been reported that a higher ALS risk is associated with increased dietary uptake of fat and glutamate (3). Likewise, in the past decades, the imbalance of gut microbiota (GM) has emerged as a new player connected to the diet—linking the type of diet with the potential of developing ALS. Similarly, all reported environmental factors could theoretically impact GM and its functions. This chapter focuses on GM and its potential imbalance as a risk factor for ALS, highlighting the gastrointestinal and metabolic dysfunction, the gut microbiome changes in motoneuron diseases, the possible clinical correlations, and lastly, the potential therapeutic approaches.

THE MICROBIOTA BRAIN-GUT AXIS

The GM is a complex population of microorganisms residing in the intestine, with the highest concentration in the colon (4). Diet is a significant factor influencing microbiota in terms of composition and function. The GM changes throughout various life phases, starting relatively simple, and increasing in complexity based on various environmental and physiological influences (e.g., geographic location, race, hormones, nutrition, diet, lifestyle). The GM includes hundreds of bacterial

species, divided into six phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Verrucomicrobia, and Fusobacteria. Viruses, protozoa, archaea, and fungi are also involved in this environment.

The GM has multiple functions. First, it constitutes the intestinal barrier, promotes itself, stimulates intestinal epithelial cell regeneration, produces mucus, and feeds the mucosa by producing short-chain fatty acids (SCFAs). GM is involved in the maturation of the immune system in childhood, maturation of intestinal lymphoid tissue, development of effective mechanism against pathogens, stimulation of the acquired immune system, intestinal synthesis and metabolism of certain nutrients, inhibition of growth of pathogenic microorganisms, and detoxification of drugs (5). The GM can communicate with the central nervous system (CNS) through the gut-brain axis (GBA), which is a bidirectional communication between the central and the enteric nervous system, linking the brain's higher-order capacities with peripheral intestinal functions (6, 7). Based on these anatomical backgrounds, for the first time, in 2013, the National Institute of Mental Health, USA, launched a project on exploring the mechanisms involved in gut microbiota-brain communication to develop new medications or noninvasive treatments for mental diseases. Since then, studies on the GM's influence on the brain have been increasing, and the gut microbiota-brain axis has become one of the focuses of neurosciences.

ALS AND GASTROINTESTINAL SYMPTOMS

ALS patients may present a wide range of gastroenteric symptoms and other autonomic and non-motor symptoms. Among these, sialorrhea is a well-known and disabling manifestation that may affect up to 50% of patients during the disease course (8). Sialorrhea may be associated with mucous secretions and saliva and an impairment of the ability to swallow secretions, but not due to an increase in saliva production. Tongue spasticity, orofacial and palatino-lingual muscle control failure, facial muscular weakness, and an inability to maintain oral and buccal competence contribute to sialorrhea (9). Sialorrhea can cause skin maceration, worsening of dysarthria, psychological stress, social embarrassment, and worsening of quality of life. Furthermore, throat and bronchial secretion and ineffective cough may impair non-invasive ventilation and may increase the risk of aspiration pneumonia (10), representing a frequent cause of death in ALS (11). Treatment of sialorrhea can include medical interventions like anticholinergics and the tricyclic antidepressant amitriptyline, which is effective in about 70% of patients with mild to moderate sialorrhea. If those treatments are ineffective or scarcely tolerated, more invasive therapy with botulinum toxin A, B, and radiotherapy may be an option (12). Physiologically 1–1.5 liters/day of saliva are produced, constituted by water, electrolytes, antimicrobials, enzymes, and growth factors. Since salivation facilitates mastication, deglutition, and the beginning of digestion and protects the oral mucosa and teeth, a relationship between oral microbiota composition and sialorrhea may be hypothesized, but this is an unexplored field. Similarly, no studies explore why some patients are unresponsive to treatment than others and the possible role of microbiota on this topic.

Concerning intestinal symptoms, constipation has been reported in up to half of ALS patients during the disease course, while stool incontinence is a rare finding (13). Previous studies have also reported delayed colonic transit time and gastric emptying in ALS patients (14, 15). Some factors such as decreased fluid intake due to dysphagia, dietary changes, medications, lack of physical exercise, motor impairment, and psychological stress have to be considered, but an autonomic involvement cannot be ruled out as well (16). Changes in the intermediolateral columns and the Onuf nucleus in ALS have been detected, which could provide an anatomical explanation for these clinical manifestations, as the enteric nervous system and smooth muscle automatism may be unable to modulate the motor functions of the digestive tract (15). Furthermore, roles for the microbiome in luminal fluid, bile acid metabolism (17), generation of SCFAs (18), methane production (19), and on the mucosal layer of the colon (20) for the regulation of the absorption of fluids into the bloodstream have been proposed. Correspondingly, the vagal nerve could be a route for GM and brain communication (16). Of note, GM has been found to interact with ENS-vagus nerve pathways (21) because bacterial-derived neurotransmitters and neuropeptides can directly activate myenteric neurons, which, through vagal nerve ascending fibers, deliver nerve inputs to the brain (22).

Dysphagia is highly prevalent in ALS, being present in about one quarter at onset (mainly in the bulbar phenotype) and in more than 80% of patients during the disease course. Dysphagia is related to tongue atrophy, dysfunction in the closure of the soft palate and the larynx due to the nuclear or supranuclear lesions of the cranial nerves, IX, X, and XII, and diaphragm dysfunction. Dysphagia should be assessed promptly in ALS to prevent complication (aspiration pneumonia, weight loss) and organize proper interventions. Physiological swallowing is a crucial parameter for the proper intake of drugs. Since swallowing problems are often underestimated in ALS patients due to the progressive adaptation to slow deterioration of bulbar function (23), their recognition is an important task in multidisciplinary disease management. Weight loss is strictly related to dysphagia and it is considered a negative prognostic factor for survival, where studies show that patients who had weight loss had a shorter survival time than those who had stable weight (24). High caloric intake and enteral feeding are commonly used to sustain nutrition, but it has not been convincingly shown to improve survival, nutritional outcomes, or quality of life (25). There is no study on the relationship between dysphagia, weight loss, and microbiota composition, although abundances of certain bacterial species (*Akkermansia muciniphila* and *Alistipes obesi*) have been reported in lean individuals, and their abundance increased during dieting. These, as well as others (*Blautia wexlerae* and *Bacteroides dorei*), were the strongest predictors for weight loss when present in high abundance at baseline in healthy people (26). Also, the effect of percutaneous endoscopic gastrostomy insertion on microbiota is unexplored as the only study nearly approaching this topic established that the insertion sites of these catheters in outpatients were frequently colonized (*Candida albicans*, *Staphylococcus aureus*, and *Escherichia coli*), without clinical consequences, although microbiota composition was not studied (27).

In conclusion, gastrointestinal symptoms are part of the disease symptoms, even if they may be underestimated in ALS. They are of clinical relevance since they may reduce food intake and influence survival and quality of life (28).

METABOLIC DYSFUNCTION IN ALS

Energy homeostasis results from a correct balance between caloric intake and energy consumption. In ALS patients, the energy balance can be profoundly altered, resulting in a higher consumption than calorie intake. Indeed, during the disease course, patients tend to lose weight, muscle mass, and fat reserves (29). This condition can be due to direct disease effects, such as dysphagia, loss of appetite, and weakness in the upper limb limiting nutrition autonomy. Furthermore, a second fundamental mechanism is also evident, characterized by increased consumption of energy at rest, due to an increase in basal metabolism and an increased resting energy expenditure (REE). This "hypermetabolic state" can be present in about 50% of sporadic ALS cases (30), while it is higher in familial forms (31). Hypermetabolic patients with ALS have a greater level of lower motor neuron involvement, faster functional decline, and shorter survival; despite this, body weight and BMI changes did not differ between hypermetabolic and normometabolic patients with ALS.

Skeletal muscles have been proposed as a site of origin of this alteration. Some studies have shown that chronic denervation in ALS patients results in increased oxygen consumption. In addition, the skeletal muscles' increase in energy demand can lead to a more significant fat mass depletion (32). These findings are supported by the higher prevalence of lower motoneuron involvement in hypermetabolic patients, which also had a high prevalence of spinal onset disease (33). In the terminal stages of the disease, increased metabolism may be due to higher energy consumption by the respiratory muscles (34).

Several studies investigated the role of BMI in disease progression and survival, suggesting that high-energy reserves at onset can mitigate the increased energy demands occurring during the disease course. Two independent studies suggested a high BMI before the disease was related to better functional outcomes, lower incidence of the disease, and reduced mortality rate (35, 36). Patients with a BMI between 30 and 35 had been found to have a better survival outcome than those with a BMI out of this range (both higher and lower) during the early stages of the disease (37). For patients with BMI lower than 30, higher initial BMI predicted slower functional decline; on the contrary, for patients with BMI greater than 30, higher initial BMI predicted more rapid decline (35).

Lipids

Hyperlipidemia is frequently observed in ALS, but the causes are still unclear; this condition could be partly explained by mitochondrial dysfunction (38) and increased food intake. While weight loss and malnutrition are prognostic factors that negatively impact ALS patients' survival, hyperlipidemia is positively correlated with survival. Dupuis et al. discovered that the frequency of hyperlipidemia, as revealed by increased plasma levels of total cholesterol or LDL, was two-fold higher in patients with ALS than in control subjects, demonstrating that abnormally elevated LDL/HDL ratio significantly increased survival by more than 12 months, as if the increased availability of lipids in circulation is a protective factor (39). In line with the hypothesis of a protective role of elevated LDL/HDL ratio, statins have been associated with worse ALS patients' outcomes (40).

Statins reduce LDL availability for skeletal muscles by inhibiting cholesterol synthesis, leading to reduced muscle nutrients. Statins also reduce insulin resistance, which increases nutrient support for neuromuscular health (39).

Neuroendocrine mechanisms

Metabolism changes in ALS patients can result from an incorrect response to central and peripheral neuroendocrine mechanisms responsible for the entire body's metabolism (41). The hypothalamus plays an essential role in regulating calorie intake and expenditure; indeed, the hypothalamus is affected by circulating hormones and locally produced neuropeptides are able to mediate appetite and eating behavior. In this regard, a recent study (42) observed severe atrophy of the anterior and posterior parts of the hypothalamus, both in patients with sporadic ALS and symptomatic ALS mutations, unrelated to whole-brain volume atrophy or disease stage. Furthermore, the hypothalamic volume was directly correlated with BMI. For the hypothalamus' physiological role, it has been proposed that its atrophy in ALS patients can cause alterations in food intake, an increase in energy expenditure, and, subsequently, a reduction in BMI.

CHANGES IN GUT MICROBIOME COMPOSITION IN NEURODEGENERATION AND ALS

ALS is a very complex disease in which many conditions such as infections or antibiotic exposure, dysphagia, food replacement, motor dysfunction, and lack of movements, could impact the microbiome structure (43). Distinct microbial profiles have been found in many neurological disorders in which the modulation of microbiota (with fecal microbiota transplantation or probiotics administration) has proven to affect brain activity and disease progression (44–46). Evidence linking GM and ALS, collected in animal models and humans, indicate a distinct microbial signature in ALS. The first substantial proof came from the mutant superoxide dismutase $SOD1^{G93A}$ mouse model, which exhibits a leaky gut, an increased number of abnormal intestinal Paneth cells, and altered microbial communities with reduced levels of butyrate-producing bacteria (47). Interestingly, intestinal dysbiosis was identified in $SOD1^{G93A}$ mice well before the onset of motor dysfunction and immune cell activation (48). Zhang et al. demonstrated that mice treated with butyrate restored intestinal microbial homeostasis and decelerated ALS progression (49). Besides, studies on the *C9orf72*-mutant mice provided insights into the microbiota's role in mediating neuroinflammation, since broad-spectrum antibiotics treatment as well as transplanting gut microflora attenuated inflammation and autoimmunity implicated in neural degeneration (50). Recently, Blacher and colleagues confirmed a pre-symptomatic distinct microbiome composition in transgenic SOD1 mice and identified commensals such as, *Parabacteroides distasonis* and *Ruminococcus torques* adversely affected the disease whereas *Akkermansia muciniphila* ameliorated the disease (51). Using a combination of untargeted metabolomic profiling and metagenomics, they found

that *A. muciniphila* increased nicotinamide (NAM) levels in the mice's cerebrospinal fluid, and NAM supplementation was able to improve the mice survival (43). Furthermore, the authors confirmed a distinct microbiome and metabolite configuration in a small group of ALS patients compared to healthy controls (51).

POSSIBLE MECHANISMS UNDERLYING THE EFFECT OF GUT MICROBIOME ON THE PATHOGENESIS OF ALS

Microbiota may influence the CNS and neuronal health either directly via the production of neuroactive metabolites (6) and toxins (7) or indirectly through modulation of immune response (52), dietary compounds, or drugs metabolism (53, 54) (Figure 1). For instance, gut microbes and their metabolites (e.g., SCFAs) can directly stimulate enterochromaffin cells to produce several neuropeptides (e.g., peptide YY, neuropeptide Y, cholecystokinin) or neurotransmitters (e.g., serotonin), which can diffuse into the bloodstream, reach the brain, and influence CNS functions. The intestinal epithelium regulates the translocation of specific bacterial products (e.g., SCFAs, vitamins, or neurotransmitters) into the bloodstream, which, in turn, through the circulatory system, can spread to the CNS (55). In this way, circulating microbiota-derived metabolites, neuropeptides and neurotransmitters can enter the CNS and directly influence its neurobiology.

Blecher et al. recently provided strong evidence for the microbial modulation of metabolites in ALS (51). Noting that the administration of *A. muciniphila* could improve the disease's course in mice, the authors applied an untargeted serum metabolomic profiling to identify a possible mediator. Interestingly, *A. muciniphila* treated mice displayed increased serum levels of NAM, whose direct administration showed beneficial effects, probably through modulation of mitochondrial function and oxidative stress pathways. NAM is a precursor of coenzymes required in energy transduction, signaling pathways, and antioxidant mechanisms that may be impaired in ALS-related neurodegeneration (56). Notably, the authors confirmed their findings in ALS patients, showing lower NAM concentration in their serum and CSF, and reduced expression of NAM synthesis bacterial genes in their stool when compared with healthy subjects (51), supporting the idea that GM can produce compounds capable of permeating the blood-brain barrier and influence neuronal function (57).

Another possible role of GM in ALS pathogenesis is the transformation of dietary and environmental compounds into neurotoxins. Beta-methylamino-l-alanine (BMAA), a well-known neurotoxic amino acid found in the brains of ALS/PDC patients from Guam (58), is thought to be produced in the gut from standard dietary compounds. For example, Cyanobacteria and other bacteria with anaerobic methylation functions can biosynthesize BMAA by methylation of L-serine and L-alanine. Enteric microbes can also convert amino acids such as L-tryptophan into bioactive molecules, such as indole, that once sulfonated can induce neuroinflammation and neuronal damage (59). GM can metabolize choline and L-carnitine into trimethylamine (TMA), and subsequently demethylate them into in dimethylamine (DMA) and formaldehyde (60). According to in vitro and in vivo studies, formaldehyde induces mitochondrial membrane damage, the

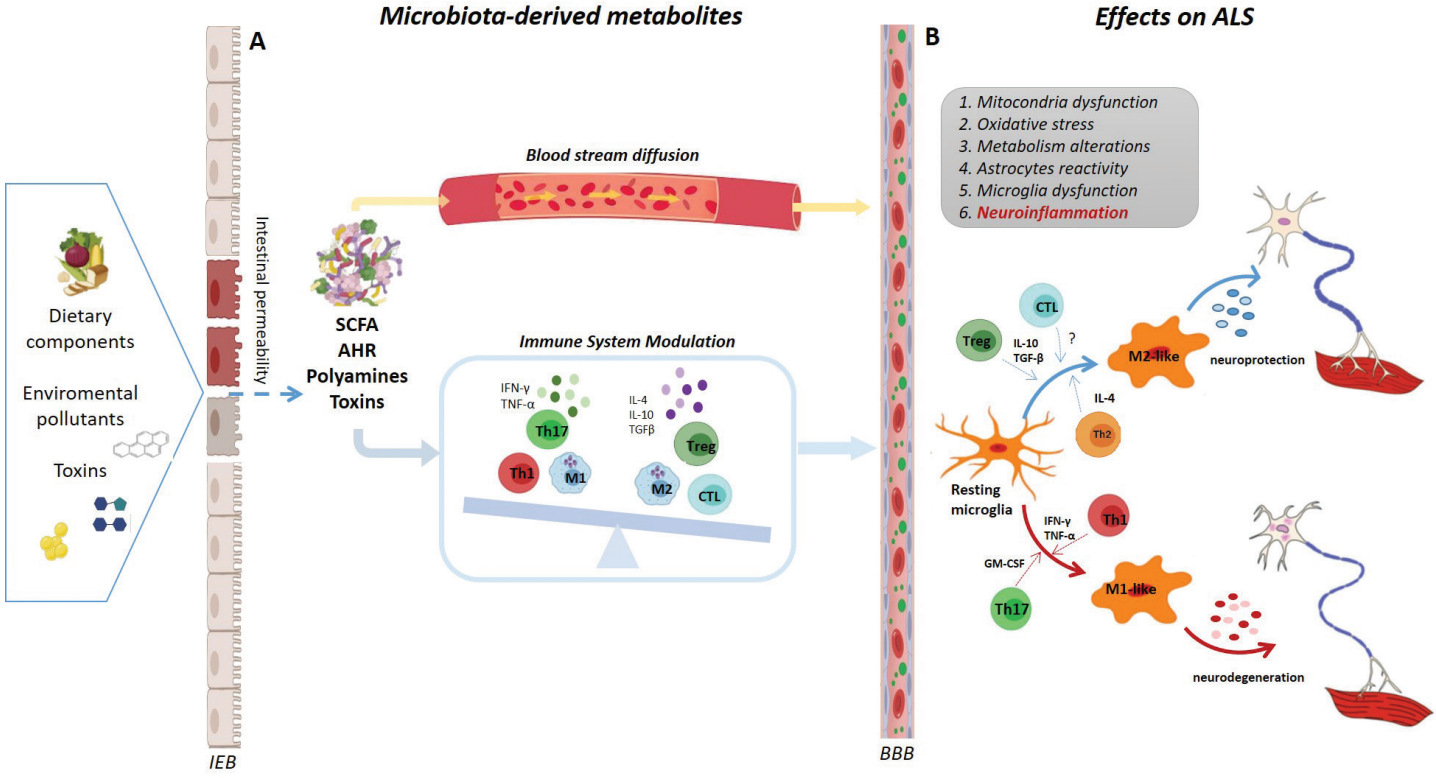

Figure 1. Microbiota-derived metabolites modulation of ALS. A. Toxins and neuroactive metabolites generated by damaged IEB or enteric bacteria can overcome BBB, diffuse to the systemic circulation and impact on ALS pathogenesis; alternatively, microbial metabolic end products may indirectly affect the central nervous system through immune system modulation. **B.** Bacteria-derived metabolites can modify energy homeostasis, promote oxidative stress, and induce mitochondrial dysfunction and neuroinflammation. In particular, peripheral immune T lymphocytes regulate microglia's fate and consequently neuron degeneration or survival. Th1, Th17, and GM-CSF producing $CD4^+$ T lymphocytes favor the microglia M1-like neurotoxic phenotype; Th2, Treg, and certain $CD8^+$ T cell types may contribute to the promotion of the neurosupportive M2-like phenotype. AHR, aryl hydrocarbon receptor; BBB, blood brain barrier; IEB, intestinal epithelial barrier; SCFA, short chain fatty acids.

production of dangerous free radicals, and neuronal Tau protein misfolding and accumulation, thus contributing to ALS pathogenesis (61). Besides, the microbiota can bring on the negative effect of environmental pollutants. Exposure to polycyclic aromatic hydrocarbons (PAHs) is considered a risk factor for ALS (62, 63), and gut microbes can reverse the endogenous detoxification process of PAHs regenerating them as Benzo[a]pyrene (BaP), whose neurotoxic effect has been demonstrated in zebrafish (64). Further, gut dysbiosis may be the cause of metabolic alterations observed in ALS (65). Interestingly, gut dysbiosis and, in particular, the reduction in Firmicutes has been associated with greater REE (66), a possible explanation for the increased energy use displayed by ALS patients.

EFFECTS OF MICROBIOTA-INDUCED INFLAMMATION ON THE PATHOGENESIS OF ALS

An established key point of ALS pathogenesis is neuro-inflammation; it is related to a complex dysregulation of resident and peripheral immune cells (e.g., microglia and astrocytes activation, T cells infiltration, and increased pro-inflammatory mediators) (67). The GM communicates with the intestinal immune system, contributing to maintenance of immune tolerance and shaping immune responses during inflammation (68). Upon pathogen invasion or dysbiotic leaky gut, microbe-associated molecular patterns can stimulate innate cells to produce pro-inflammatory cytokines that, in turn, activate adaptive immune cells, thus contributing to the breakdown of immune homeostasis (69). Besides innate immune cells, intestinal microbes can directly affect the development and differentiation of the adaptive immune system's main components, the $CD4^+$ and $CD8^+$ T cells (70). In addition, GM dysbiosis affects several brain biological processes. Germ-free mice and antibiotic-treated mouse models display a broad range of immunological abnormalities, including altering density, morphology, and maturity of microglia, suggesting that GM can influence both CNS immune cells' development and functions (71).

Interestingly, treatment with SCFAs restored the microglia density and morphology in "depletion of regulatory T cells (DEREG)" mice. SCFAs such as butyric, propionic, and acetic acids are dietary fiber's end-metabolism microbial products, mainly by Bacteroides and Firmicutes (72). They are known to mediate regulatory T cell (Tregs) induction through histone deacetylase inhibition. ALS is characterized by simultaneous activation of distinct lymphocyte subsets, Th1 and Th17, and a decrease of Tregs (73) that have a protective role as demonstrated in both mice and humans; a greater number of Tregs is associated with slow disease progression (74, 75). Tregs have been shown to directly differentiate macrophages from M1 to M2 state (76), and M2 microglia has been associated with the stable disease phase, whereas Th1 and M1 microglia predominate during the rapidly progressing phase suggesting a shift from protection to toxicity (Figure 1). Zhang et al. confirmed it, where butyrate supplementation appeared to reduce the clinical features of ALS and the immunology abnormalities found in the G93A mice's gut (49). Moreover, the longitudinal study by Figueroa-Romero et al. confirmed dysbiosis and spinal cord inflammation in $SOD1^{G93A}$ mice, defining the

chronological timeline, in which GM alterations precede circulating and CNS immune system expansion and activation, and symptom onset and progression (48). The study of Burberry et al. on *C9orf72* null mice suggested that a dysbiosis characterized by immune-stimulating bacteria reduces mice survival by inducing detrimental peripheral inflammation and microglia activation, whereas antibiotic or the microbiota transplantation improved symptoms (50). Intestinal microbiota-driven proinflammatory signals may be essential for glia's physiological functioning, preserving neuronal health. Indeed, the gut microbiome regulates astrocyte activity through an aryl hydrocarbon receptor (AHR)-mediated mechanism involving type I interferon signaling (77).

CLINICAL EVIDENCE THAT GUT MICROBIOME MODULATION IMPACTS ALS

Studies in patients have begun to find a possible link between GM and ALS (Table 1), reporting controversial conclusions (78–87). The first studies conducted were characterized by small and select patient cohorts, with less than ten individuals, although they provided relatively consistent data in favor of dysbiosis in ALS (78–80). In these studies, the cause of pro-inflammatory dysbiosis is associated with the microbial imbalance that could compromise the intestinal epithelial barrier and promote immune/inflammatory responses with consequent alterations and a role in ALS pathogenesis.

Mazzini et al., in 2020, published a prospective longitudinal study on the microbiota composition in ALS (81, 82), demonstrating that the GM of ALS patients are different compared to controls, independent of the degree of disability. Moreover, they observed an increase of Cyanobacteria, noted for a neurotoxic action. Members of the Cyanobacteria phylum were significantly higher in the patients than in the controls, supporting the hypothesis that Cyanobacteria play a fundamental role in the pathogenesis of neurodegenerative diseases such as the ALS (84). Besides, Rowin et al. (79) and Nicholson et al. (85) observed that the glutamate metabolizer bacteria and the dominant butyrate-producing bacteria were, respectively, more abundant, and lower in ALS patients. In contrast, other studies showed that the fecal microbiome of patients with motoneuron disease was not significantly different from healthy controls (86, 87). However, a higher Firmicutes/Bacteroidetes ratio was associated with an increased risk of death and greater species diversity (87). These data support that the microbiota's alterations could modulate the disease's clinical course rather than representing a risk factor for its onset.

CLINICAL CORRELATIONS AND POTENTIAL THERAPEUTIC APPROACHES

In the first longitudinal study assessing GM in ALS (82), disease progression coincided with reduced microbial diversity, probably secondary to dietary changes,

TABLE 1 Studies investigating ALS microbiota

Authors, year, reference	Participants & Methods	Results
Studies in favor of gut microbiota dysbiosis in ALS		
Fang et al., 2016 (78)	- case-control (6 P and 5 C)	- decreased Firmicutes/Bacteroidetes ratio at phylum level in P - significant increased genus Dorea (harmful microorganisms) and significant reduced genus Oscillibacter, Anaerostipes, Lachnospiraceae (beneficial microorganisms) in P
Rowin et al., 2017 (79)	- case-control (5 P and 96 C)	- the genera Lactobacillus, Bifidobacterium, and Odoribacter (glutamate metabolizers) are more abundant in P
Zhai et al., 2019 (80)	- case-control (8 P and 8 C)	- the phylum Firmicutes/Bacteroidetes ratio, genus Methanobrevibacter, showed an enhancive tendency in P - the relative abundance of beneficial micro-organisms (genera Faecalibacterium and Bacteroides) presented a significant decrease tendency in P
Mazzini et al., 2018 (81); Di Gioia et al., 2020 (82)	- prospective longitudinal study - case-control (50 P and 50 C) - probiotic supplementation	- GM of P is characterized by some differences compared to C, regardless of the disability degree - the GM composition changed over the disease course (significant decrease in the number of the observed operational taxonomic units during the follow-up) - probiotic supplementation has no effect on disease progression
Zeng, 2020 (83)	- case-control (20 P and 20 C)	- over-representation of Bacteroidetes phylum and other bacterial genera in P - Firmicutes and Megamonas genus down-regulated in P, with reduced Firmicutes/Bacteroidetes ratio - increased species diversity associated with P compared to C
Nicholson et al., 2020 (85)	- case-control (66 P, 61 C and 12 NC)	- relative abundance of the dominant butyrate-producing bacteria Eubacterium rectale and Roseburia intestinalis was lower in P compared to C - total abundance of 8 dominant species capable of producing butyrate was lower in P, independently from age, sex or presence of constipation
Studies not in favor of gut microbiota dysbiosis in ALS		
Brenner et al., 2018 (86)	- case-control (25 P and 32 C)	- no substantial alteration of the GM composition - significant differences only in the overall number of microbial species and the abundance of uncultured Ruminococcaceae in P
Ngo et al., 2020 (87)	- case-control (49 P and 50 C)	- no correlation between metabolic and clinical features of P and the composition of their fecal microbiome - greater risk for earlier death in P with increased richness and diversity of the microbiome and in those with greater Firmicutes to Bacteroidetes ratio

Overall, ALS seems to be characterized by the reduction of butyrate-producing bacteria, which are important for gut integrity and regulation of inflammation. However, some discrepancies are present. ALS, amyotrophic lateral sclerosis; C, controls; GM, gut microbiota; NC, neurodegenerative controls; P, ALS patients.

highlighting that the interpretation of the results cannot be separated from diet monitoring and other factors that influence the microbiota (e.g., drug use, like antibiotics), and the stage of the disease. Furthermore, antibiotics alter the balance of intestinal microbial species (88); this opens a window on correlations between antibiotic use and unrelated diseases. Retrospective epidemiological studies in Swedish national registries showed that antibiotics, especially if repeated, were associated with an increased risk of developing ALS (89). These results, generated independently of the type of infection and the antibiotics, suggest that this relation was not specific to a particular organ system. After testing several antibiotics classes, only beta-lactamase-sensitive penicillin use was significantly associated with increased odds of developing ALS. The authors concluded that the most probable pathogenic mechanism was antibiotic-induced perturbations of the intestinal microflora (89).

Although evidence on the GM's role in ALS is increasing, the available studies are primarily exploratory; the number of cohorts remains small, which, in consideration of the significant inter-individual variability and clinical heterogeneity that characterizes ALS, could preclude the identification of the relevant characteristics of the microbiome. These results show the importance of large cohorts and multicenter studies, allowing to consider intra-group differences in the ALS population (genetically or phenotypically determined and concerning disease stage) as well as changes between groups between ALS and controls. Microbiota's specific signature may be protective or toxic in different individuals and for diverse genetic backgrounds.

Regarding the implications for ALS patients' treatment, a longitudinal analysis of the microbiota composition after supplementation with placebo or probiotic treatment revealed a significant decrease in the observed operational taxonomic units number during the follow-up, with the predominance of neurotoxic or pro-inflammatory microbial groups such as Cyanobacteria (82). Supplementation with probiotics, though having some effects on the intestinal microflora of ALS patients, did not substantially bring the composition closer to that of healthy subjects (82), implying more drastic interventions are required to reach such a target, as this type of treatment remains a minimal intervention in time and quantitative terms, concerning the abundance of species hosted by the intestine. In this regard, we are coordinating a multicenter controlled clinical trial in Italy that involves transplantation of fecal microbiota in 42 patients with ALS. Fecal microbiota transplantation is planned at baseline and after six months; an extensive immunological profile and microbiota characterization are ongoing (90).

Another relatively unexplored issue regards the fact that microbiota may also influence specific drug availability in ALS. Riluzole is significantly metabolized by GM (54, 90), which may explain interpatient variability in the drug plasma levels (92). It may be argued that similar effects would also be found for experimental drugs tested in ALS patients, which may contribute to hurdles in finding a cure for ALS. Finally, GM may influence non-motor symptoms in ALS such as depression, anxiety, and constipation through peptides and neurotransmitters that could directly impact mood (93), opening the possibility for treatment to improve at least the quality of life of ALS patients.

CONCLUSION

This chapter highlights the possible role of gut microbiome in the pathogenesis of ALS. Many studies on animal models of ALS have revealed changes in the intestinal flora; however, most of the experimental evidence in humans has come from correlation research; many studies mainly describe the alterations of intestinal flora in ALS patients. Emerging evidence shows that GM can influence ALS through hypermetabolism and gastrointestinal abnormalities. Other interesting associations have been reported based on which microbiota could play a role in the interface between environmental and lifestyle factors, and ALS. By studying these associations, we may gain more insight into the complex network of microbiome-host interactions underlying the observed changes in ALS. Longitudinal studies integrating metagenomic, transcriptomic, and metabolomic approaches with clinical parameters may elucidate the relationships between established risk modifiers, gut microbiota, and ALS. Although we still need to establish a "cause and effect" relationship between GM and ALS, the strategy of regulating intestinal microbial flora to treat this devastating disease is intriguing. Further rigorous studies targeting GM may develop novel approaches for the prevention and treatment of ALS.

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Amyotrophic Lateral Sclerosis

Toshiyuki Araki, MD, PhD
Editor

Toshiyuki Araki, MD, PhD, is Director of the Department of Peripheral Nervous System Research at the National Institute of Neuroscience of the National Center of Neurology and Psychiatry (Tokyo, Japan), visiting professor at Tokyo University of Agriculture and Technology (Tokyo, Japan), and lecturer at Waseda University (Tokyo, Japan) and Osaka University (Osaka Japan). He graduated from Osaka University, where he received MD and PhD degrees. He performed postgraduate training at the Washington University School of Medicine in St. Louis (Missouri, USA). The research interest of Dr. Araki has been focused on the neuronal/axonal degeneration mechanisms and application of the knowledge to the therapeutic development. The targets of his research range from peripheral nerve degeneration/regeneration and neuromuscular disorders to neurodegenerative disorders including amyotrophic lateral sclerosis and Alzheimer's disease, and developmental disorders including autism-spectrum disorders. For promoting translational research, he has been collaborating with several pharmaceutical companies. He also serves as the chairman of National Center of Neurology and Psychiatry Ethics Committee for clinical research, and an ethical advisor to Brain/Minds Beyond project. He has authored more than 100 scientific publications. He also serves as an editorial member of scientific journals, and a board member/councilor of several scientific societies.

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