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Diagnostic Challenge and Neuromuscular Junction Contribution to ALS Pathogenesis

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Amyotrophic lateral sclerosis (ALS) represents the major adult-onset motor neuron disease. Both human and animal studies reveal the critical implication of muscle and neuromuscular junctions (NMJs) in the initial phase of this disease. Despite the common efforts, ALS diagnosis remains particularly challenging since many other disorders can overlap yielding similar clinical phenotypic features. A combination of further research on the NMJ parameters that are specific for this disease and laboratory tests are crucial for the early determination of specific changes in the muscle, as well as in motor neuron and the prediction of ALS progression. Also, it could provide a powerful tool in the discrimination of particular ALS and ALS-mimic cases and increase the efficacy of therapeutic treatments.

Keywords: amyotrophic lateral sclerosis (ALS), axonopathy, neuromuscular junction (NMJ), dying back hypothesis, ALS-mimic diseases

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS) is a disease characterized by a progressive degeneration of upper motor neurons (MNs) in the motor cortex and lower motor neurons in the brainstem and the spinal cord. The death of these neurons leads to spasticity, weakness, and atrophy of the muscles, progressing to paralysis. The incidence of ALS in Europe is 2–16 per 100,000 each year (1), with respiratory failure being the predominant mode of death in patients within 3 years of diagnosis (2). The onset of the disease occurs prevalently during adulthood (peak age of 58–63 years) (3), though with a small proportion of early-onset disease in certain patients (before 35 years of age). ALS also shares neuropathological and genetic features with another neurodegenerative disorder, frontotemporal dementia (FTD) (4, 5), with many ALS patients showing some cognitive or behavioral changes. This has led to consider ALS and FTD as the ends of the same spectrum of disease (6).

Although the majority of ALS cases occur sporadically (sALS), there is a Mendelian inheritance in about 10% of the cases (familial ALS, fALS), mainly in an autosomal, dominant fashion (7). The two are clinically indistinguishable and a variety of genetic defects in more than 20 genetic loci have been linked with the ALS phenotype (8), with new genes constantly being identified in subsets of ALS patients (9–11). Four major genes which mutations are known to cause ALS are the following: chromosome 9 open reading frame 72 (C9orf72), superoxide dismutase 1 (SOD1), transactive response DNA-binding protein (TARDBP) and fused in sarcoma (FUS) (12–15). C9orf72 has an important role in membrane trafficking and autophagy (16), and SOD1 primary function is thought...
to be as a cytosolic and mitochondrial antioxidant enzyme, converting superoxide to molecular oxygen and hydrogen peroxide (17). TARDBP and FUS encode nucleic acid-binding proteins that reside in the nucleus, and are involved in multiple aspects of RNA processing, such as transcription and splicing [reviewed in (18)].

**NMJ INVOLVEMENT IN ALS**

Despite the progress in our understanding of the molecular pathogenesis linked to these genes, it is still unclear where the motor neuron dysfunction begins and the extrinsic factors that accelerate motor neuron degeneration. This led to the consideration of ALS as either a dying forward process that proposes an anterograde degeneration of motor neurons by glutamate excitotoxicity from the cortex, or a dying back phenomenon in which motor neuron degeneration starts distally at the nerve terminal or at the neuromuscular junction (NMJ) and progresses toward the cell body (3, 19). The NMJ is a tripartite synapse composed by the presynaptic motor neuron, the postsynaptic muscle and the synapse-associated glial cells (terminal Schwann cells, TSC) and allows the transmission of action potentials from motor neurons to muscles [reviewed in (20)]. In this complex structure, besides motor neuron degeneration, glial cells, and muscle fibers play also a major role in ALS onset and progression.

The muscle contribution in ALS development, through NMJ’s disassembly, is still a matter of debate. Nonetheless, increasing evidence points to the critical role of NMJ defects in the early stage of the disease in ALS patients [reviewed in (21)] and a variety of animal models have permitted important advances into exploring this hypothesis.

The human SOD1<sup>G93A</sup> transgenic mouse, the first and most studied ALS model, is the one that has yielded the majority of information about the muscular deficits in ALS (22). Spatiotemporal analysis of NMJs in SOD1<sup>G93A</sup> mouse revealed end-plates denervation before the appearance of clinical symptoms and neuron cell body loss (23), with the fast-fatigable synapses being more vulnerable to denervation (24). Because of its high expression in ALS muscle biopsies, the neurite outgrowth inhibitor Nogo-A was proposed as a factor responsible for motor nerve terminals repulsion and destabilization at the NMJ at very early asymptomatic stages (25, 26). This hypothesis was then confirmed in SOD1<sup>G93A</sup> mouse model, where genetic ablation of Nogo-A in muscle led to marked reduction of muscle denervation and prolonged survival (27). Morphological observation of NMJs in SOD1<sup>G93A</sup> also contributed to reinforce the dying back hypothesis, showing more detailed NMJ alterations prior to functional symptom onset (28). A detailed overview of the findings concerning neuromuscular defects in the SOD1<sup>G93A</sup> mouse model has been reviewed by Dupuis and colleagues (22).

Despite the predominant use of rodent models for studying pathomechanisms and potential therapeutic targets in ALS, the use of smaller animal models, like *Drosophila melanogaster* and zebrafish (*Danio rerio*), is continually increasing. Their advantages lie in their fast development allowing quick generation of lines, their availability and the ease in manipulating gene expression and in drug screening. In drosophilia, studies showed locomotor defects, reduced life span, and anatomical defects at the NMJ, causing impairments in synaptic transmission, in loss and gain of function models of TARDBP (29, 30). Similar results were found for FUS. Gene deficiency and overexpression of FUS in Drosophila models caused decreased synaptic transmission, reduced number of presynaptic active zones, altered postsynaptic glutamate receptor subunit composition at the NMJ, motor neuron degeneration and impaired motor behavior (31, 32). Zebrafish studies have highlighted gain and loss of function mechanisms for TARDBP and FUS, demonstrating shorter axonal projections from motor neurons, premature and excessive branching, impaired synaptic transmission at the NMJ leading to swimming defects (33–35). C9orf72 gene has also been modeled in zebrafish in a loss-of-function model that displayed behavioral and cellular deficits related to locomotion (36). For more details about the different models, all the ALS gene mutations that have been modeled are summarized in a recent review by Van Damme et al. (37).

Altogether, fundamental research supports the crucial role that NMJ could play in ALS pathogenesis and its possible employment as efficient early marker of the disease.

**ALS DIAGNOSTIC CHALLENGES**

The difficulty to diagnose ALS resides mainly in the existence of several mimic syndromes, unrelated to ALS but which present similar clinical features (38, 39).

Motor neuron diseases (MNDs) are classified in four main groups in which ALS represents the most common form (Table 1). Although these diseases affect people in different ways, they share several symptoms due to motor neuron loss of function. All of them present progressive weakening of skeletal muscles, which eventually affects the ability to speak, swallow and breathe. ALS diagnosis is even more difficult if we add to the list other neurological conditions unrelated to MNDs which can mimic its early symptoms. Moreover, increasing evidences point to a possible direct implication of muscle in the early stage of the disease, adding myopathies to the list of ALS-mimic pathologies (Table 1).

Standard diagnostic criteria for ALS have been established in 1991 [El Escorial criteria [EEC] (40)] and were revised in 1997 [AirlieHouse criteria [AHC] or El Escorial Revisited (41)]. Even though the essential requirements for ALS diagnosis were defined by these criteria, many neurologists and neuromuscular clinicians were missing the diagnosis, proving the low clinical accuracy of these diagnostic roles (42).

In 2008, electrodagnostic studies, known as the Awaji criteria (43), were included in the clinical procedure to allow earlier and more accurate assessment of ALS diagnosis. However, the
application of those sets of defined features are still insufficient to rule out other similar and related diseases (44, 45).

Methods for Diagnosis
Although the main ALS evaluation remains the clinical one, laboratory testing, based on advanced techniques of electrodiagnosis, neuroimaging, immunobiochemistry, and neurogenetics, is required for accurate ALS diagnosis.

Tests to rule out other neuromuscular conditions may include:

Electromyogram (EMG)
The needle EMG is the most important study in determining diagnostic certainty of ALS (46). During this test, a needle electrode is inserted through the skin into various muscles, starting with the most severely involved limb (Figure 1). The examination then progresses through four anatomical region: bulbar, cervical, thoracic, and lumbar. At least three anatomical regions have to be positive to this test to define ALS. The fasciculation potential (FP) has been included in Awaji criteria as a hallmark of ALS muscular denervation. In general, a decreased number of motor unit recruitment, with long duration of the motor unit potential, and abnormal spontaneous activity, are measured at the EMG in ALS patients [reviewed in (47)].

Nerve Conduction Study (NCS)
This test measures how fast an electrical impulse moves through the nerve (Figure 1). During the test, one electrode placed on the skin stimulates the nerve of interest with a very mild electrical impulse. Variations in time spent to reach a second electrode can help in identifying a nerve damage. Whereas, EMG measures the electrical activity in the muscles, the nerve conduction study is specific for nerves and helps to localize the disorder among nerve, neuromuscular junction, and muscle. NCS is a powerful tool to discriminate ALS from axonal demyelination or conduction block impairments (48). NCS parameters are generally normal in ALS, albeit the presence of prolonged distal motor latency and slowed conduction velocity could be consistent with the diagnosis of ALS (49, 50). These changes suggest loss of large myelinated fibers, but also motor axons regeneration phenomena (50).

Magnetic Resonance Imaging (MRI)
This technique is able to produce detailed images of the brain and spinal cord, the latter with the advantage of simultaneously investigating the upper and lower motor neurons. During several years, its application was related to the exclusion of other disorders, as tumors or hernias that can display certain of the ALS-mimic symptoms (51). The evolution and improvement of this multimodal tool has recently become essential for the diagnosis of ALS. MRI scans can show cerebral degeneration and gray/white matter atrophy [reviewed in (52)], and also detect abnormalities in ALS muscle, likely due to denervation atrophy process (53).

Blood and Urine Tests
Testing hematological factors is helpful to exclude diseases that are capable of mimicking ALS symptoms. Recently, a population-based study, proposed serum albumin, creatinine levels, and lymphocyte count as markers for ALS, indicating muscle waste and inflammation respectively (54). Other markers potentially related to a better ALS outcome have been proposed: LDL/HDL levels, which are elevated in ALS plasma and represent a general unexplained hypermetabolism (55, 56); serum uric acid levels, which are decreased among ALS patients, further demonstrating the possible role of oxidative stress in the induction and propagation of the disease (57); serum ferritin levels which are elevated in ALS patients and could reflect perturbation in iron metabolism (58); concentrations of certain amino acids, which are decreased in ALS (59); levels of serum proinflammatory cytokines, such as IL-6, which are increased in ALS (60). Finally, high level of circulating AChE and metalloproteinases (MMP) have been reported in ALS plasma (61, 62) and although the exact source of these two classes of enzymes remains uncertain, it could in part reflect a disruption of extracellularly bound AChE at the NMJ and early change in the nerve-muscle integrity.

Spinal Tap (Lumbar Puncture)
Using this particular test, a small amount of cerebrospinal fluid (CSF) is taken from the lower back of the patient for laboratory
FIGURE 1 | Schematic representation of the nerve conduction and muscle contraction studies. Nerve Conduction Velocity (NCV - left) measures the velocity and the quality of conduction of the electrical signal in a nerve. During the test, your nerve is stimulated, with an electrode attached to your skin. One or two more electrodes patches are placed on the skin over your nerve. The electrical impulse of the stimulated nerve pass from the stimulator to the other receiving electrode. The time (in milliseconds) spent by the impulse to move from a point to another, on the order of millimeters, represent the Velocity. In ALS, the impulse conduction is slower respect with control cases and is worsened by the increase of axonal degeneration. The electromyogram (EMG-right) measures the electrical activity of the muscles at rest and during contraction. There are two kinds of EMG: surface EMG and intramuscular EMG. In the first one the muscle activity is recorded by one or more electrodes patched on the skin and it assest the contractile response of superficial muscles. This approach presents severallimination since the result signal is influenced by the depth of the subcutaneous tissue at the site of the recording and by the discharges of adjacent muscles. With the intramuscular EMG, specific deep muscle activity is recorded by using one needle electrode inserted into the muscle. EMG and NCV tests are often done together to give more complete information.

tests. Thanks to its proximity to the central nervous system, the CSF is considered one preferred tissue to search for ALS biomarkers [reviewed in (63)]. Several markers for ALS have been identified in CSF such as Tau, TDP43, Nefl, and MMP levels [reviewed in (64)]. In particular, MMPs with their ability to digest collagen, proteoglycan, and laminin (65), may reflect ongoing destruction of the matrix which wraps synapses (66) and pathological changes at the brain-blood barrier (62).

Muscle Biopsy
With this technique, a small portion of muscle is removed by needle biopsy and sent to a laboratory for histopathological analysis. Rarely performed because of its painful and invasive nature, this tool is useful when ALS diagnosis is in doubt. Generally, ALS muscles present signs of active denervation/reinnervation and an increased number of atrophic fibers (67).

Genetic Testing
People with familial ALS (fALS) background can get an efficient diagnosis through genetic testing (68, 69). This technique may help ALS patients to understand the basis of their condition, and improve the genotype-specific treatments (70). Unluckily, there is a lack of consensus among clinicians above the definition of fALS, since newly genes related to ALS are continuously found (71). Nowadays, genetic testing is not wildly used because of its high cost and the belief that ALS genetics is not well-enough understood to provide a better treatment plan, as reported in 2017 in a study which involved 167 clinicians from 21 different countries around the world (71).

EXAMPLES OF NMJ PATHOLOGIES
ALS-MIMIC

Here we report some examples of ALS-mimic pathologies. The Spinal muscular atrophy (SMA) is an inherited MND that prevalently affects children. Its incidence is 1 per 11,000 live births (72). All forms of SMA are caused by the loss of SMN1, a gene implicated in axonal mRNA transport and snRNP biogenesis (73). Studies involving mice and fly mutants demonstrated that the probable origin of this pathology resides in the early loss of sensory information from proprioceptive neurons (74), which in turn causes degeneration of α motor neurons. In consequence, progressive muscle weakness, and, in severe cases, respiratory failure appear (75). Despite being considered a child's illness, the SMA type 4, that has an adult onset, overlaps with ALS diagnosis (76). Furthermore, like in ALS, several studies reveal the early implication of NMJs in SMA, with synaptic pathology prior to the appearance of clinical symptoms (77–81). However, the first evidence of neuromuscular pathology occurred at different time points of the disease progression, with presynaptic pathology preceding morphological changes at the endplate in ALS, and simultaneous pre and post-synaptic pathologies in SMA, suggesting the possibility to study this particular zone in diagnosis (81). Histochemical skin biopsy comparison was suggested as a powerful diagnostic tool in differentiating ALS and SMA, since
the small collagen fibrils and the increased amount of amorphous material, which are characteristic of ALS, are not in SMA (82).

The Spinobulbar muscular atrophy ([SBMA] Kennedy’s disease) is a X-linked hereditary lower motor neuron disease, where the expanded trinucleotide repeat (CAG > 37) in the androgen receptor gene (AR) causes its nuclear inclusions and impairment of its function (83, 84). The disease affects 1 per 200,000 males in Europe and Asia each year (85). In this pathology, degeneration of anterior horn cells of the spinal cord, where androgen receptors are widely expressed, is observed (86, 87). Although SBMA patients exhibit facial weakness as first sign of the disease, they progressively develop myopathic features, such as muscle atrophy and necrotic myofibers (88). Like ALS, SBMA disease reveals mixed pathological findings, with both myopathy and neurogenic atrophy features, which is the cause of misdiagnosis at the early stage of the disease.

Among the autoimmune syndromes, myasthenia gravis (MG) overlaps ALS syndrome. The annual incidence ranges from 3 to 30 per 1,000,000 people (89). In fact, the binding of autoantibodies to components of the NMJ in MG causes a characterized muscle weakness and fatigability (90). Even if acetylcholine receptor antibodies are considered to be highly specific for the diagnosis of MG, ALS patients can also present these autoantibodies at the blood test (91, 92). In these cases, it is very difficult to define the false positive cases and an experimental treatment with AChE inhibitors is necessary to differentiate MG from ALS (93).

The skeletal muscle disorders are represented with the term Myopathies. Myopathies hold a list of pathologies (Table 1) where muscle weakness can begin in the hands and feet (distal muscles) as well as in the muscles near the center of the body (proximal muscles) sometimes mimicking ALS features, confusing the diagnostic and the treatment decision. Among them, Inclusion Body Myositis (IBM) (94) is the most common ALS-mimic disease. It is the most common adult myopathy in 50 year-old persons and older, and its incidence is 3.5 per 100,000 (95). It is characterized by inflammatory cells surrounding and invading non-necrotic muscle fibers, rimmed vacuoles, congophilic inclusions, and protein aggregates in muscle (96, 97).

In this case, the unique way to exclude ALS is the muscle biopsy combined with quantitative electromyographic analysis, especially in those patients where disease progression is slow and atypical (98).

CONCLUSION

Amyotrophic Lateral Sclerosis (ALS) and MNDs are not yet curable. However, accurate diagnosis is crucial to provide adequate counseling and information about the prognosis and disease course, and to avoid inappropriate therapy. Moreover, a good diagnosis could furnish a more equal stratification of cases and be important in the choice of additional medical support, as for example nutritional intervention strategies or physical therapy.

Currently, there is not a common consensus in the use of laboratory analysis for ALS diagnosis. Basically, clinicians decide for the application of certain techniques based on their experience, expertise and hospital practice. Progress in molecular genetics and identification of specific biomarkers is ongoing, which will translate to a refined diagnostic certitude. Therefore, there is the emerging need to establish a widely accepted protocol for laboratory tests to discriminate the majority of cases that present clinical features resembling ALS.

Increasing human and animal evidence proposed NMJ impairments as possible biomarkers for detection and discrimination of ALS and mimic diseases in an early, preclinical stage. However, further studies are needed to understand how these impairments could be monitored and specifically treated.

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M-LC and A-RB contributed to conception and drafting. EK provided useful comments and reviewed the manuscript.

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