Review

Synaptic Failure: Focus in an Integrative View of ALS

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Abstract. From early description by Charcot, the classification of the Amyotrophic Lateral Sclerosis (ALS) is evolving from a subtype of Motor Neuron (MN) Disease to be considered rather a multi-systemic, non-cell autonomous and complex neurodegenerative disease. In the last decade, the huge amount of knowledge acquired has shed new insights on the pathological mechanisms underlying ALS from different perspectives. However, a whole vision on the multiple dysfunctional pathways is needed with the inclusion of information often excluded in other published revisions. We propose an integrative view of ALS pathology, although centered on the synaptic failure as a converging and crucial player to the etiology of the disease. Homeostasis of input and output synaptic activity of MNs has been proved to be severely and early disrupted and to definitively contribute to microcircuitry alterations at the spinal cord. Several cells play roles in synaptic communication across the MNs network system such as interneurons, astrocytes, microglia, Schwann and skeletal muscle cells. Microglia are described as highly dynamic surveying cells of the nervous system but also as determinant contributors to the synaptic plasticity linked to neuronal activity. Several signaling axis such as TNFα/TNFR1 and CX3CR1/CX3CL1 that characterize MN-microglia cross talk contribute to synaptic scaling and maintenance, have been found altered in ALS. The presence of dystrophic and atypical microglia in late stages of ALS, with a decline in their dynamic motility and phagocytic ability, together with less synaptic and neuronal contacts disrupts the MN-microglia dialogue, decreases homeostatic regulation of neuronal activity, perturbs "on/off" signals and accelerates disease progression associated to impaired synaptic function and regeneration. Other hotspot in the ALS affected network system is the unstable neuromuscular junction (NMJ) leading to distal axonal degeneration. Reduced neuromuscular spontaneous synaptic activity in ALS mice models was also suggested to account for the selective vulnerability of MNs and decreased regenerative capability. Synaptic destabilization may as well derive from increased release of molecules by muscle cells (e.g. NogoA) and by terminal Schwann cells (e.g. semaphorin 3A) conceivably causing nerve terminal retraction and denervation, as well as inhibition of re-connection to muscle fibers. Indeed, we have overviewed the alterations on the metabolic pathways and self-regenerative capacity presented in skeletal muscle cells that contribute to muscle wasting in ALS. Finally, a detailed footpath of pathologic changes on MNs and associated dysfunctional and synaptic alterations is provided. The oriented motivation in future ALS studies as outlined in the present article will help in fruitful novel achievements on the mechanisms involved and in developing more target-driven therapies that will bring new hope in halting or delaying disease progression in ALS patients.

Keywords: ALS, muscle, microglia, motor neuron, synapses, neuromuscular junction

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HISTORY, PATHOLOGICAL FEATURES AND ALS-ASSOCIATED GENETIC MUTATIONS

Jean-Martin Charcot in 1869 [1] was the first physician to clearly isolate amyotrophic lateral sclerosis (ALS) from the other sclerosis by linking symptoms to motor neurons (MNs) that originate in the spinal cord (SC) [2, 3]. At the moment, even if we have no marker for diagnosis or prognosis, the disease is recognized by the severe degeneration of lower (spinal and bulbar) and upper (corticospinal) MNs [4] although clinical presentations may differ. It is considered a rare disease with an incidence from 1.2 to 4 per 100,000 patients in Caucasians [5, 6]. Approximately two thirds of ALS patients have a spinal form of the disease (limb onset) and evidence symptoms related to focal muscle weakness and wasting which may start either distally or proximally in the upper and lower limbs. Gradually, spasticity may develop in the weakened atrophic limbs, affecting manual dexterity and gait. ALS patients with bulbar onset usually complain from dysarthria and dysphagia for solids or liquids, with limb symptoms developing either simultaneously with bulbar symptoms or within 1–2 years. Paralysis is progressive and leads to death due to respiratory failure within 2–3 years for bulbar onset cases and within 3–5 years for limb onset [7]. Although initially characterized by this progressive muscle atrophy, the term "lateral sclerosis" refers to a distinctive anatomo-pathological trait of hardening of the anterior and lateral corticospinal tracts, the major efferent motor conduits to the spinal cord, and alterations in the corpus callosum, [8] suggesting that it is a complex multi-component disease.

This simple vision of considering ALS as a type of MN disease has been challenged nowadays due to the evidences resulting from different sources of research. Applications of neuroimaging such us magnetic resonance imaging (MRI), magnetic resonance spectroscopy or diffusion tensor imaging have revealed the involvement of diffuse brain presentations. At cortical level, premotor and motor regions seem dominant although frontal and anterior temporal regions, in addition to subcortical pathways, are also key structures [9]. Because motor planning, motor abstraction, emotional expression, and language are all affected in ALS, some authors demand that ALS should be considered as a result of a system degeneration due to network failure instead of focusing on individual neuronal populations [10].

From the systemic point of view, ALS seems to be restricted not only to the nervous system but also to include whole-body physiology. In particular, energy homeostasis is compromised in patients with ALS, which has notable clinical implications such as weight loss, hypermetabolism, and hyperlipidaemia. These abnormalities correlate with the duration of survival [11]. Finally, more recently, some authors highlight alterations in all the compounds of the neurovascular unit [12], comprising the existence of interactions between the vascular and neural cells [13] in ALS patients and animal models [14–16] as contributors to the disease pathogenesis. Despite MN cell loss is the critical hallmark during the course of the disease [17], other cells are considered major determinants in the onset and ALS progression such as astrocytes [18], microglia [19], oligodendrocytes [20], muscle cells [21], or accepted as contributors, such as lymphocytes [22, 23], pericytes [24] and interneurons [25].

Besides these changes in the physiopathological perspective of ALS, another important source of new evidences comes from genetic research. Mutations in the gene encoding the ubiquitously expressed Cu/Zn-superoxide dismutase (*SOD1*), the first discovered in 1993 [26], were found to cause the familial ALS (fALS) in approximately 10 to 20% of ALS cases [27]. Since then, 164 mutations have been found in such gene [28]. It is at present unclear whether all mutations are pathogenic but studies producing at their understanding have produced a large amount of data thanks to the generation of ALS transgenic models carrying those mutations [29]. The transgenic *SOD1* mouse carrying the substitution of glycine at residue 93 by alanine (tgSOD1^{G93A}) is the most widely studied model of ALS [30]. These mice are normal at birth, but begin to develop MN dysfunctions at 11 weeks of age that progress from hindlimbs to forelimbs presenting as weakness, tremors, reduced extension reflex, total paralysis, and, finally, death [31, 32]. Several transgenic mouse strains expressing the human *SOD1* gene with different mutations, have been generated to date for studying the disease pathogenesis [33] that also present embryonic and neonatal abnormalities in MNs and SC circuitry far earlier than any symptomatic manifestation appear [34, 35]. Later on, other genes were reported to be implicated as well, which have been recently reviewed [36]. To date, 47 missense and one truncating mutations were found in fALS and sporadic (sALS) patients [27]. Among these genes, mutations in TAR-DNA binding protein

(*TARDBP* or *TDP-43*) and fused in sarcoma (FUS) [27] have revealed its importance in the degeneration of MNs and in the so called continuum between ALS and Fronto-Temporal Lobar Degenerationa (FTLD), which is present in 45% of cases of ALS [37]. Recently, it was suggested that TDP-43 dysfunction is mainly localized in SC astrocytes, which will trigger MN degeneration [38]. Indeed, astrocytes expressing mutant SOD1 and TDP-43 were shown to cause the death of MNs trough a common pathogenic pathway that involves nitrosative stress [39]. Intriguingly, measurements of TDP-43 in cerebrospinal fluid (CSF) and blood revealed its limited role as a diagnostic tool [40]. Finally, the most common genetic abnormality in in families with FTLD or ALS has recently been mapped to a non-coding repeat expansion in the gene *C9ORF72* at 9p21 (reviewed in [41]) Strikingly, this hexanucleotide repeat accounts for up to 40% of fALS and 7% of sALS [5]. The contribution of all these mutations to the pathology is mentioned in the section below.

Thus, ALS turns out to be a multi-systemic, non-cell autonomous and complex neurodegenerative disease that requires an integrative vision. In the last decade the huge amount of knowledge acquired has shed insights into the pathological mechanisms underlying ALS from different perspectives such as genetics and proteomics, and longitudinal studies have contributed to improve our understanding on disease progression. However, more than ever, is time to have a whole vision on the several determinants implicated in ALS to better focus future research on identifying new targets and discovering novel efficacious therapies that make a substantial difference for the outcome of ALS patients. Although pretending to draw an integrative vision to ALS pathology and complexity we always kept in mind the synaptic failure as an important contributor to the etiology of the disease.

ETIOPATHOGENESIS: DYING BACK, DYING FORWARD AND SYNAPTIC FAILURE HYPOTHESIS

Determining the nature of the etiopathogenesis in ALS is a difficult task. This is an issue under intense debate nowadays due to the importance of targeting a specific tissue for therapy. The debate has been focused in the direction of the spreading of the disease in the central nervous system (CNS) since both cortical and spinal regions are affected. The so

named "Dying-Back" and "Dying-Forward" hypothesis co-exist to explain the pathogenic pathways for degenerative spreading. Both are based on a network perspective that explains a decline of structural integrity with preserved functional organization of the motor network in ALS. While "Dying-Forward" hypothesis claim that cortical hyperexcitability in cortical motor areas might trigger anterograde degenerative process towards the SC, the "Dying-Back" theory of pathological spread in ALS suggests that early degeneration is more likely to be captured at the spinal anterior horn due to weakness in neuromuscular connectivity rather than in the brain [42]. Cortical regions mostly affected include the precentral gyrus, especially in subregions with maximal clustering of cortical MNs (Betz cells), whose degeneration is considered to be a pathological hallmark of ALS [43]. Regions functionally connected with the motor cortex are deeply affected with a reduced microstructural organization of the efferent conduits that form the corticospinal tracts, interconnecting the motor network. This functional connectivity may allow the rapid spread of the disease. Indeed, it has been recently hypothesized that disease might spread contiguously or non-contiguously following a 'domino-prion-like' propagation between neighboring neurons in a cell autonomous or non-cell autonomous manner. This supposition is inspired in the sticky properties of mutant SOD1, and normal TDP43, which can undergo a seeded aggregation similar to prions. Both native and mutant forms of SOD1 associated with each other easily form aggregates and fibrils under denaturing conditions [44]. Importantly, removal of the misfolded seeds does not stop aggregation of endogenous SOD1, indicating that the newly formed aggregates can act as templates for the subsequent misfolding of additional native SOD1 [45] (reviewed in [46]). However, Dying-Forward hypothesis might not be easy to demonstrate in mouse models due to significant differences in connectivity between mice and human. Betz cells in humans connect monosynaptically to spinal MNs through ventral corticospinal track. However, this association is very scarce in mouse whose corticospinal track course mainly through the dorsal funiculi and end on interneurons at lamina V of the dorsal horn at the SC. Only few axons run also through dorsolateral and ventral funiculi which may contact directly to some MNs [47]. Although several alterations have early been observed in cortical MNs in parallel to spinal MNs, it might be improbable that this will
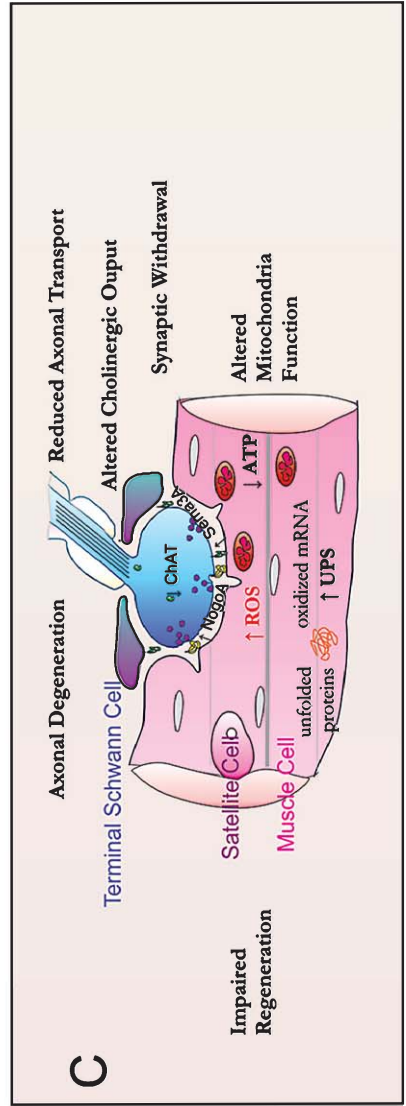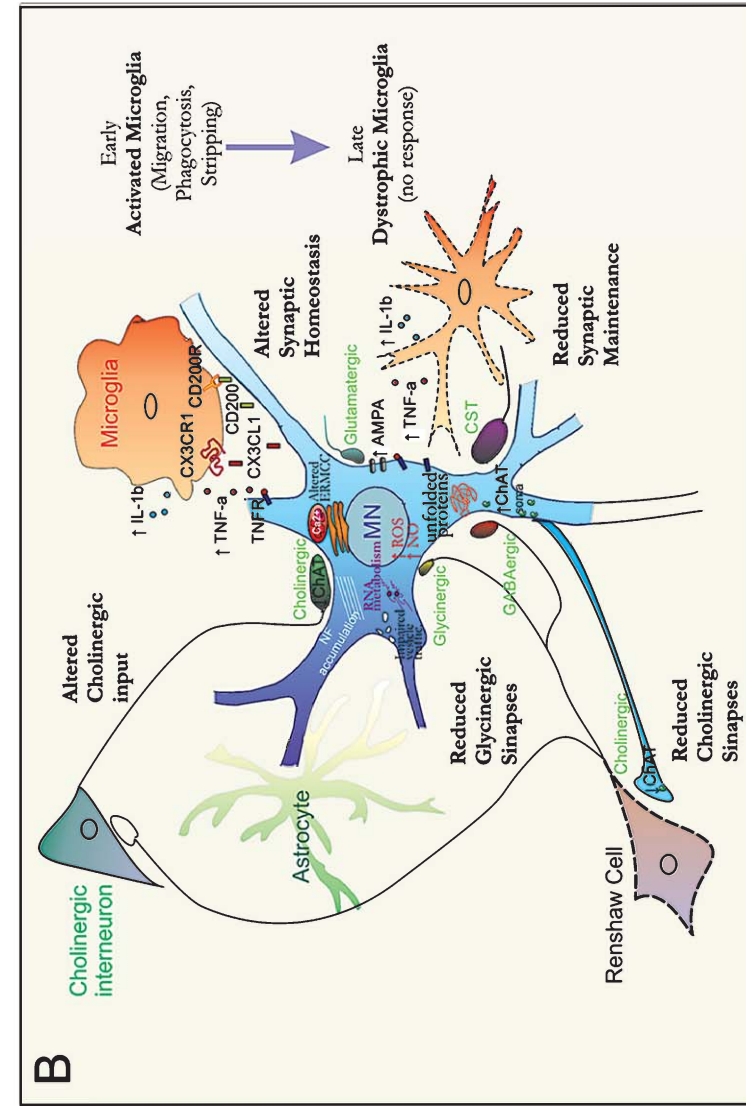
occur as one being the consequence of the other in mouse models of ALS. It would be more plausible that noxa induction, with a crucial role in apoptosis and MN degeneration [48, 49] will occur specifically and importantly mainly in MNs, independently of their location. This favors the hypothesis of a complex genetic-environmental interaction as the causal factor for MN degeneration. Factors may not only affect MNs, but also other cells in a less degree, which together certainly account for a toxic neuronal and system microenvironment. Among these factors, several have been reported to be likely contributors to the disease such as the exposure to tobacco or some pesticides or the intense practice of athleticism or soccer as determined by epidemiological analysis [5].

Synaptic terminal degeneration is a pathological hallmark in human ALS progression. Synapse elimination occurred even in patients who had no MN and corticospinal tract impairment [50, 51]. The working model whereby loss of synapses at lower MNs plays an important role in ALS pathogenesis is commonly referred to as the 'synaptic stripping' hypothesis. Direct evidence in support of this hypothesis comes from studies showing a decrease in the expression of synaptic vesicle proteins like synaptophysin and synapsin and syntaxin and synaptotagmin in ALS and animal models [52, 53], which were evident in remaining MNs and suggest a loss of synapses before neuronal loss. Nagao and colleagues showed that expression of the synaptic marker vesicle-acetylcholine-transferase (VAChT) was reduced in the ventral horn of an ALS patient, reinforcing the loss of cholinergic synapses in lower MNs as an early event in ALS [54]. In agreement with that, it has been recently described that there is a marked reduction in choline acetyl-transferase (ChAT) mRNA and protein content in the cholinergic synaptic terminals apposed onto lumbar spinal MNs at the early asymptomatic phase in the tgSOD1^{G93A} ALS model [55]. Besides, this reduction was also apparent in the cholinergic synaptic terminals of the connections from MNs onto Renshaw cells. Renshaw are glycinergic and gabaergic inhibitory cells that receive input from motor axon collaterals and synapse in turn on the soma of MNs in a negative-feedback fashion (homonymous recurrent inhibition). These inhibitory terminals in MNs have been observed to be reduced, preferentially the

Fig. 1. Integrative view of ALS. A. ALS is a result of the degeneration of the motor functional circuitry mainly. In humans, the corticospinal tract (CST) allows monosynaptic connection of cholinergic Betz cells in motor cortex to spinal motor neurons (MNs). At the spinal cord, MNs forme a microcircuitry with interneurons and other MNs to accomplish the efferent motor signaling. Cholinergic interneurons at lamina X synapse in MNs regulate motor behavior and Renshaw inhibitory interneurons receive inputs form MN axon collaterals to synapse, that turn to MNs and conforms a negative feedback regulation. B. ALS can be considered as a multi-component disease that affects synaptic communication. Several terminal boutons that make synapses on MNs are structural or functional affected, such as glycinergic and cholinergic ones. The number of glycinergic synapses from inhibitory Renshaw cells is reduced and the density of these cells has been found decreased in later stages of ALS. Choline acetyltransferase (ChAT) content is early diminished from boutons apposed on MNs and from their terminals on Renshaw cells. Altogether these variations match with the altered recurrent inhibition found in patients and contribute to imbalance in inhibitory/excitatory homeostasis. MNs present altered molecular environment with incapability to accomplish several neuroprotective programs (such as autophagy, unfolded protein response (UPR), proteosomal activity) that lead to a failure in handling protein homeostasis and aggregation, which are related to a reduced neuronal survival. There exists a persistent dysregulation in the endoplasmic reticulum (ER)-mitochondria calcium cycle (ERMCC) that couple energy from mitochondria to protein processing in the ER and participates in AMPA-mediated synaptic activity. This leads to excitotoxicity and abundant reactive oxygen species (ROS) and nitric oxide (NO) production. In addition, difficulties in vesicle trafficking may occur in soma and along the axon that difficult synaptic communication with the muscle and other cells. Thus, the machinery for local protein translation in dendrites and synapses present alterations that involves the mRNA silencing foci at which TDP-43 and FUS/TLS participate with consequent mRNA metabolism alterations. Microglia contribute to synaptic scaling and maintenance by cross-talk to MNs through TNFα/TNFR1, CX3CL1/CX3CR1 and CD200/CD200R axis. Activation of CX3CR1 in microglia are part of the "off" signals that switch microglia from the activated state to the resting one, which may be interrupted in ALS, at least in some of the ALS stages. Indeed, in later stages, microglia become non-responsive and dystrophic, a phenotype that follows the chronic activation and the release of pro-inflammatory cytokines, such as IL1-beta and TNF-alpha. Activation of TNFR1 in neurons classically lead to apoptosis but also increases AMPA receptors at the membrane. Besides, the composition and editing of AMPA receptors which is altered in ALS contribute to excitotoxicity due to changes in their ion permeability. C. Neuromuscular junction (NMJ) fragility is promoted by disturbances in several cellular components and lead to impaired MN transmission and regeneration. At the axonal terminal, the transport is altered and, in particular, there is a reduced ChAT transport which can lead to a decrease in immediately available transmitter pool, as well as to a reduced spontaneous acetylcholine release that weakens NMJ. Synaptic withdrawn may be induced by the release of inhibitors of regeneration like Nogo A or Semaphorin 3A from muscle or terminal Schwann cells, respectively. Myofiber atrophy is one of the earliest events in ALS. Muscle cell metabolism and regeneration is altered. There exists mitochondria function defects promoting ATP reduction, disturbances of calcium buffering capacity and oxidative stress exarcerbated by muscle training and muscle hypermetabolism that leads to a rise in oxygen species, which contribute to oxidation of mRNAs and altered miRNA biogenesis. These features contribute to a reduced capacity for muscle regeneration accompanied by an impaired proliferative potential of satellite cells that lead this capacity. Finally, the presence of protein aggregates in the muscle may overshoot protein clearance systems, such as proteasomal system, which contribute to muscle wasting and energy depletion. UPS, ubiquitin-proteasome system.

glycinergic ones [56], compromising the functionality of the local SC motor circuitry. Indeed, alterations in inhibitory neurotransmission have been reported in ALS patients as well [57, 58], which extensively contribute to the imbalance between inhibitory and excitatory synaptic drive to MNs. Excitatory signaling gain and/or an inhibitory loss on MNs will increase Ca^{2+} influx and depolarization, favoring excitotoxicity [59, 60]. It is not only a specific characteristic that happens in the SC but also in cortical areas. Functional studies, using transcranial magnetic stimulation of the motor cortex, showed an increase in the excitatory postsynaptic potentials recorded from the contralateral extensor digitorum communis muscle, a feature that Authors attribute to a marked loss of inhibition at cortical MNs in patients with ALS [61]. PET scanner studies [62] and *in situ* hybridization [63] revealed a decrease in both the density of the GABAA receptor and the expression of GABAA receptor subunit mRNA, respectively, in the motor cortex of ALS patients. Thus, all these evidences point to a loss of synaptic homeostasis in MNs, involving both lower and upper MNs and resulting from a decreased activity of the inhibitory circuits controlling MN excitability. Early alteration in cholinergic input from interneurons and output towards the NMJ and Renshaw cells concomitant to loss of inhibitory glycinergic synapses and Renshaw cells itself [64] may favor excitotoxicity definitely compromising regulatory microcircuitry function.

GLIA CONTRIBUTION TO SYNAPTIC DYSFUNCTION IN ALS

Astrocytes and microglia accompany MNs and react in consonance to neuronal function. The emerging concepts of the "tripartite" synapse [65], that includes astrocytes, and even the "quadripartite" synapse [66, 67] that add microglial cells as well, may better describe recent evidence indicating that there are multiple cell types working together at remodeling synaptic plasticity. One of the major functions of microglial cells in the brain is to survey for damage and protect neural material by orchestrating the innate immune response [68]. Microglia present an extremely cellular plasticity switching from a "resting" or "surveillant" cell phenotype with several fine processes to a globular "amoeboid" activated microglia with the ability to migrate to sites of damage and to phagocyte cellular debris [69]. Several evidences point to ramified "resting" microglia

as highly dynamic surveyors of the healthy brain and determinant contributors to synaptic plasticity [70]. In particular, microglia contact with dendritic spines occur in a transient manner and linked to neuronal activity and functional experience as shown by Tremblay & collaborators [71]. Several molecules that mediate microglia-neuron cross-talk have been related to these synaptic events including some cytokines with additional functions beyond their role in inflammation. One of these cytokines is tumor necrosis factor-α (TNF-α), whose secretion has been recently specifically assigned to microglia but not to astrocytes [72]. Two transmembrane receptors, TNF receptor 1 (TNFR1) and TNFR2, were discovered 25 years ago [73]. TNFR1 is expressed ubiquitously on almost all cell types, while TNFR2 is more limited to specific cells, as those of the immune system [74]. However, activation of both TNFR1 and TNFR2 expression in neurons, astrocytes and oligodendrocytes was observed after traumatic CS injury [75]. While TNFR1 is predominantly linked to apoptosis and neurodegeneration, TNFR2 was shown to be involved in tissue regeneration and neuroprotection [76, 77].

However, TNF-α levels also control basal synaptic function and synaptic scaling plasticity [78]. Synaptic scaling is a form of non-associative homeostatic plasticity allowing equilibrium between excitatory and inhibitory inputs that works to stabilize neuronal firing rates by adjusting the strength of all the synapses of a neuron to keep firing rates roughly constant. TNF-α increases expression of surface AMPA receptors in both primary and slice cultures, hence increasing synaptic strength and synaptic scaling [79, 80]. Both mRNA of TNF-α and its pro-apoptotic receptor TNFR1 were shown to be elevated in late presymptomatic stage and to increase progressively along disease progression in the lumbar SC of tgSOD1^{G93A} mice [81]. Interestingly, circulating levels of TNF-α and TNFR1 were found elevated in ALS patients, although no correlation with disease severity was not observed [82]. In ALS, the AMPA receptors presented an altered composition in subunit stoichiometry and editing that render them more permeable to calcium ions [83]. For this reason it has been considered a promising target and several drugs have been used to drive clinical trials although without success, probably because the excitotoxic phenomenon represents a too early phenomenon in disease onset. Besides the role of microglia on the plasticity of existing synapses it may also contribute to more profound structural changes

by phagocyting specific subsets of synapses as it does during developmental synaptic pruning and in injury-related synaptic stripping events [84]. Moreover, as TNF-α is released by activated microglia one can consider neuroinflammation and excitotoxicity as mechanistic links for microglia-astrocyte and microglia-neuron cross-talk [85]. In these processes, other molecular axis of neuronal-microglia dialogue may also be implicated. Neuroprotective signaling from MN to microglia can be mediated through at least two cytokines, the chemokine ligand 1 (CX3CL1/fractalkine) and the membrane glycoprotein CD200 that have the correspondent receptors CX3CR1 and C200R, respectively, restricted to microglia [86, 87]. Increased expression of microglial C200R was found in the presymptomatic tgSOD1^{G93A} mice [88] while that of CX3CR1 was observed at disease endstage [89]. CX3CL1 signaling by healthy neurons controls the overproduction of iNOS, IL-1β, TNFα, and IL-6 [90, 91]. In addition, CX3CR1 was also shown to play a role in cognitive function and synaptic plasticity [92]. Indeed, the axis has shown to have an important role in synaptic maturation and function since mice lacking CX3CR1 had deficits in synaptic pruning and decreased frequency of spontaneous excitatory post-synaptic currents, suggesting immature connectivity in the knockout mice [90]. In turn, microglial cathepsin S (CatS), a lysosomal/endosomal cysteine protease, seems to be responsible for the liberation of neuronal CX3CL1 [30]. Thus, CatS-mediated modification of the perisynaptic environment induce a downscaling of synaptic strength [93]. Information about the relevance of CX3CR1-CX3CL1 axis in ALS is scarce apart from that its disruption accelerates neurodegeneration in the double transgenic SOD1^{G93A}/Cx3cr1–/– mice, which showed to survive less than the single SOD1 mutant [87].

Recent findings have underlined that toll-like receptor 2 (TLR2) and TLR4 are critical for glia activation. In the SC of ALS patients TLR2 was predominantly detected in microglia whereas TLR4 was strongly expressed in astrocytes [94]. Interestingly, while TLR2 evidenced to mediate inhibition of neural progenitor cell proliferation [95] and showed to be associated with synaptic loss, TLR4 was suggested to relate with synaptic stability in the SC after peripheral nerve injury [96]. In addition, microglial activation was also related with TDP-43 pathology and indicated to be an important determinant of cognitive impairment in ALS [97]. Finally, dystrophic microglia may have a determinant role to accelerate disease progression. As suggested by Graeber and Streit, chronic activation of microglial cells could lead to overactivation followed by microglial dystrophy and degeneration [98]. This is especially relevant in the context of aging-related disorders since microglia response to injury during early-life could increase the susceptibility to the development of disease in adulthood and aging [99]. Aged microglia present a decline in their dynamic motility through neural parenchyma, less frequent and extensive synaptic neuronal contacts, together with a decreased homeostatic regulation of neuronal activity and perturbations in the "on"/off signals, as well as increased neuroinflammatory response and oxidative stress [70]. The presence of mutant SOD1 acting on microglia is required to cause the disease [100] and its scission exclusively from microglia significantly slow disease progression [101]. Indeed, when the mutant SOD1 microglia was replaced with microglia expressing the wild-type SOD1 a prolonged survival of the ALS mice was observed [102]. Besides, the presence of mutant SOD1 intra- and extracellularly may enhance the production and release of reactive oxygen species (ROS), increase the inflammatory cytokine cascade [103] and accelerate the formation of the senescent microglial phenotype. Therefore, any neurotoxic effect that acts at a cellular level in ALS may affect the main functions of microglia promoting its early senescence and thus reducing synapse contacts, activity and plasticity that impede the possibility to regenerate disconnected MN.

NEUROMUSCULAR COMPONENTS CONTRIBUTING TO SYNAPSE FAILURE IN ALS

Cholinergic transmission towards the neuromuscular junction (NMJ) has also shown to be altered in ALS models. Decreased choline acetyltransferase (ChAT) expression and resultant reduction of acetylcholine (ACh) release from motor nerve ends can lead to dysfunction of motor synapses at axon terminals [104, 105]. Impaired neuromuscular transmission was detected in early sALS patients [106], and a reduced supply of releasable ACh was thought to be the primary cause for the degeneration of motor terminals in a dog model of hereditary spinal muscular atrophy [104, 107]. Several factors can contribute to such issue. Recently, Tateno and collaborators reported an interesting hypothesis based on the alterations in axonal transport towards the NMJ terminal,

in general, and to the transport of ChAT and AChE in particular. These molecules require binding to a motor protein to be transported along the axon, such as the kinesin-associated protein 3 (KAP3), which is specifically sequestered by the presence of misfolded SOD1 species. As a consequence, the transport of ChAT was shown to be interrupted at the pre-onset stage in the tgSOD1[G93A] mice [108] and to accumulate in the MN soma [55]. Considering that misfolded SOD1 has been also linked to sALS, the inhibition of ChAT transport and hence the reduction in acetylcholine (ACh) availability may be one of the common pathways leading to MN-specific dysfunction in ALS. ACh is necessary for the neurotransmission but also activates endplate receptors during spontaneous release. This may explain selective vulnerability observed between fast (more vulnerable) and slow MN types in ALS because fast MNs are more dependent on endplate ACh receptor activation during spontaneous release that acts then as a retrograde signaling system for regulating their electrical properties, maintaining connectivity and promoting regeneration [109]. Accordingly, Naumenko and collaborators have observed a significant decrease in the immediately available transmitter pool in nerve endings of tgSOD1[G93A] mice males [110], likely one of the reasons for the dramatic drop in the spontaneous mediator release and enhanced fatigability of ALS muscles at high rate firing [111]. In this regard, a decrease in spontaneous ACh release would consistently weaken the strength of selective NMJ and hinder regeneration, as observed in ALS [55, 112].

Interestingly, it was recently evidenced that matrix metalloproteinase-9 (MMP-9) is expressed only by fast MNs and its pharmacological inhibition revealed to significantly delay muscle denervation [113]. MMP-9 activity was previously found enhanced in pyramidal neurons in the motor cortex and MNs in the SC of ALS patients [114], as well in their serum [115]. Moreover, other studies revealed that MMP-9 activity also increase in the SC and skin of the tgSOD1[G93A] mouse model, mainly in the later stages of the disease [116]. Later, it was also shown that the extracellular media of differentiating NSC-34 MN-like cells carrying the G93A mutation in human SOD1 also contain increased MMP-9 activation [117]. Most important, these Authors demonstrated that MMP-9 activation was reduced after treatment with the bile acid glycoursodeoxycolic acid, suggesting that it may be a promising therapeutic strategy when considering MMP-9 as a candidate target. NMJ fragility has long been considered a hallmark in ALS. Studies in rodents and humans have demonstrated that axonal retraction occurs well before the loss of cell bodies and that intrinsic axonal mechanisms actively contribute to phenotype in ALS [42, 118]. Actually, in the tgSOD1[G93A] mouse model it was observed that denervation occurred as a very early phenomena, i.e. weeks before MN lost was noticed [119]. Such studies also demonstrated that fast-fatigable motor units presented higher susceptibility to degenerate than slow ones as mentioned [118, 120]. Thus NMJ degeneration represent early pathological targets [112]. Is important to bear in mind that at the peripheral NMJ, three main different cell types, i.e. motor nerve terminal, terminal Schwann cells (TSC) and muscle fibers, collaborate in the assembly and maintenance of the synaptic apparatus. Non-myelinating, terminal Schwann cells (TSC), phenotypically different from those that wrap the axon in the peripheral nerve, cap the nerve terminal covering motor terminal branches and synaptic buttons and have a main role in regeneration [121]. A number of TSCs was noticed to be significantly reduced following disease progression in the tgSOD1[G93A] rat muscle. In addition, it has been reported that TSCs specifically express the chemorepellent semaphorin 3A (Sema3A) at the fast-fatigable-type neuromuscular synapses [122]. The authors propose that this *de novo* expression of Sema3A in the ALS rat model may impede nerve terminal plasticity at specific neuromuscular synapses, thus contributing to their early and selective loss.

Besides Sema 3A, other inhibitors for axonal regeneration such as Nogo-A have been observed originating from skeletal muscles, particularly from deltoid ALS patient biopsies and in tgSOD1[G93A] mice. This factor was proposed as a diagnostic [123] and prognostic [124] marker in ALS, once correlated with disease severity and motor impairment. Consistently, the double transgenic SOD1[G86R] and Nogo-A knock-out mice exhibited MN maintenance, reduced expression of denervation markers AChrna 1 alpha and Tyr kinase and prolonged lifespan [125]. However, the specificity of this factor in ALS disease remains controversial [126–128] as no increment of Nogo-A serum levels has been found in patients [129] and compensatory Nogo-B increment can underlay therapeutic benefits of Nogo-A knocking-out [130].

Failure or dysfunction of either the axonal metabolism itself or that of the skeletal muscle fibers contribute to this NMJ fragility and MN degeneration [131, 132]. The post-synaptic component of

the NMJ, the muscle, plays an essential role in ALS axonopathy since overexpression of mutated SOD1 enzyme exclusively in skeletal muscle showed besides motor deficits such as spasticity and hyperreflexia, also abnormalities in the NMJ and a distal axonopathy [133, 134]. However, apparent controversial results in the literature make difficult to achieve definite conclusions about muscle pathology contribution to ALS. In this regard, intramuscular injection of a lentiviral vector (not retrogradely transported to central nervous system), encoding a siRNA against SOD1 or specific gene excision through the Cre-Lox-P recombinase system under creatine kinase promoter, showing a 50% mRNA and protein reduction of mutant SOD1 exclusively in skeletal muscle provided no benefits of muscle grip strength maintenance and no delayed onset or progression of the disease [135]. However, due to the fact that even low the level of mutant SOD1 in skeletal muscle is able to trigger the ALS phenotype [133], reminiscent mutant SOD1 expression could be responsible for such observations. Several pathological events have been linked to muscle dysfunction in ALS and referred to influence the NMJ connection, such as metabolism and self-regenerative capacity. Dupuis and co-workers, described the up regulation of mitochondrial uncoupling protein (UCP) correlated with ATP reduction specifically in skeletal muscle of ALS patients and animal models [136]; they also demonstrated that muscle mitochondrial uncoupling driven by this protein leads to NMJ and MN dismantling, while exacerbating ALS phenotype [137]. In agreement, several Authors have reported increased lipid and carbohydrate clearance in tgSOD1^{G93A} and tgSOD1^{G86R} individual muscles at asymptomatic stages [138–140], correlating these findings with the muscle hypermetabolism and energy deficit. Consistently, high-fat diets have demonstrated to restore body mass, ameliorate muscle denervation and motor deficits and prolong the lifespan in animal models. Muscle mitochondrial defects have been largely described in skeletal muscle from ALS patients [138, 141, 142]. However, mitochondria are key intracellular organelles regulating not only energy but also other essential cellular functions, such as calcium homeostasis or apoptotic signaling. The expression of aberrant mutated proteins such as SOD1 in skeletal muscle myofibers of ALS models, as previously described in MNs, has been proposed to interfere with the inner membrane mitochondrial potential and the calcium buffering capacity [143] that results in the sequestrate of the anti-apoptotic protein Bcl-2, leading to apoptosis and development of local oxidative stress [144, 145]. Interestingly, Wei R and colleagues did not detect the presence of mutant protein aggregates in gastrocnemius muscle of tgSOD1^{G93A} mice from pre-symptomatic (50 days) to terminal stages (130 days), although a 2 to 4 fold increase in 20 S proteasomal activity was observed. These Authors concluded that the muscle ability to maintain a high proteasomal activity mediated the effective clearance of the toxic protein aggregates [146] as it had been previously suggested in C2C12 myoblasts [147]. The ubiquitin-proteasome system (UPS) is crucial for maintaining muscle homeostasis, as it is required to remove sarcomeric proteins upon changes in muscle activity [148, 149]; excessive up-regulation through increased proteasomal activity, ubiquitin-conjugation or over-expression of transcripts encoding ubiquitin, ubiquitin-conjugating enzymes, ubiquitin-protein ligases or proteasomal subunits, leads to muscle wasting [150]. Importantly, myofiber atrophy is one of the earliest events on ALS. Studies with specific modulators of this pathway would be of interest to establish the optimal UPS activity beneficial for mutant protein clearance but not detrimental for the maintenance of muscle mass on ALS.

Molecular signaling alterations have been also found in RNA processing both mRNA and miRNA. mRNA processing was found disturbed in ALS mouse models; consistently, oxidized mRNA species were detected in the SC of patients, as well as in tgSOD1^{G93A} mice at the pre-symptomatic 45 days [151]. Tight implication of miRNA biogenesis and performance in muscle cells is likely to operate on ALS since miRNA 23a, 455, 29b and 206 were upregulated in vastus lateralis of ALS patients [152]. Both miRNA 29 and 206 are known to promote muscle regeneration and recovery after cardiotoxin or nerve crush injury [153, 154] and ALS mouse model genetically deficient in miR-206 showed accelerated progression of the disease [155].

In adults, satellite cell pool, largely responsible for the regenerative capacity in this tissue, remain quiescent but retain the ability to activate and reenter cell cycle in response to signals from the cellular niche such as muscle injury or intense physical exercise. A portion of these cells proliferate and fuse with each other or with the parental myofiber to fully regenerate the damaged tissue, whereas the rest remain undifferentiated and exit cell cycle to replenish the myogenic cell pool. This process is regulated by highly coordinated transcriptional and translational networks in which myogenic regula-

tory factors play a central role [156–160]. However, in aging [161, 162], Duchenne muscular dystrophy [163, 164] or spinal muscular atrophy [165], successive rounds of regeneration or aberrant stimuli from the cellular niche perturb self-renewal or myogenesis leading to depletion of the satellite cell pool or loss of myogenic potential and muscle wasting. In line with this, it has been recently reported an abnormal number of satellite cells in both fast and slow-twitch myofibers of tgSOD1^{G93A} mouse model since the earliest pre-symptomatic stages of the disease, accompanied by blunted protein translation of myogenic regulatory factors controlling the activity of these cells and *in vitro* impaired proliferative potential [166].

Despite the uncertain mechanisms of MN degeneration driven by the skeletal muscle, several evidences exist based on therapeutic benefits proportionated by strategies supporting muscle homeostasis, regenerative potential and muscle mass. Controlled exercise improved motor function and survival in tgSOD1^{G93A} animal models [167] and reduced spasticity and motor function loss in ALS patients [168]. Mild physical activity has neuroprotective benefits as promotes neurogenesis [168–170] and increases peripheral levels of neurotrophic factors such as BDNF (brain-derived neurotrophic factor) [171–173] and IGF-1 [174, 175]. However, muscle training is an inductor of oxidative stress, as markedly increases the systemic consumption and uptake of oxygen thus enhancing ROS production, including the generation of the superoxide radical from the mitochondrial electron transport chain [176]. Therefore, despite substantial effort to establish the optimal stage, intensity and duration of the training to maximize the potential benefits, the controversy remains [174, 177–179]. These observations, together with the high incidence of ALS cases within professionals developing intense physical activity, such as soccer players [180–183], encouraged clinicians to advice against such practice to preserve muscle mass.

Artificial delivery of neurotrophic molecules, such as muscle-derived but no centrally derived GDNF (glial-derived neurotrophic factor) over-expression, was neuroprotective in the tgSOD1^{G93A} mice [184]. Indeed, intramuscular injections of myoblasts [185] and mesenchymal stem cells [186] carrying vectors encoding GDNF, expanded the survival and motor performance of tgSOD1^{G93A} rodents and delayed MN loss, as shown by levels of acetylcholine receptor postsynaptic clusters and ChAT or agrin-positive

end plates within the lumbar SC region. Additionally, IGF-1 restricted expression in the skeletal muscle of ALS rodents counteracted the symptoms of the disease, exacerbated myogenesis and reduced catabolism, thus prolonging the lifespan [21, 187, 188]. These Authors concluded that skeletal muscle satellite cell activity, mediated by muscle IGF-1, contributed to the ameliorated phenotype of the tgSOD1^{G93A} mice.

Overall these findings provide evidence that ALS skeletal muscle suffer from direct neurotoxic effects of SOD1 or other molecules and definitely affect synaptic connectivity at the NMJ, as well as its metabolism, contributing to the multi-systemic phenotype of the disease.

MN PATHOLOGY AND CONTRIBUTION TO SYNAPTIC DYSFUNCTION IN ALS

Several years ago, some Authors suggested that synaptic dysfunction may precede synaptic loss [51, 189, 190] and, indeed, this is a rather common feature in other neurodegenerative diseases as well [191]. In both sALS and fALS, molecular changes most likely precede the clinical onset. In sALS, an acquired defect in chromatin, DNA, RNA or proteins (such as epigenetic alteration, a somatic DNA mutation, a RNA editing error, and the misfolding of proteins possibly due to ageing effects or environmental stressors) occurs randomly in each cell, and these defects accumulate and converge to initiate the pathogenic process. The mechanisms involved in MN dysfunction in ALS have been accurately detailed recently by Cozzolino and collaborators [192]. The discovery of new genes implicated in ALS and the existence of common mechanisms found in most of the genes have pointed to new insights into the pathogenic pathways. Bioinformatics' analysis of their annotated functions reveal an enrichment in processes such as endocytosis and impaired vesicle (*Alsin*, *VAPB*, *OPTN*, *VCP*), axonal transport and organelle trafficking (spatacsin), compromised autophagy (*CHMP2B*) and protein degradation, in the UPR response (*UBQLN2*, *SIGMAR1*), transcription regulation and RNA metabolism (*C9orf72*, *TARDBP*, *ANG*, *SETX*), as well as in mitochondria-dependent oxidative stress. Increased levels of "classical" markers of oxidative stress, including reactive oxygen species (ROS) and products of protein and lipid oxidation, have been repeatedly and consistently observed in tissues from patients and in *in vitro* and

in vivo mutant SOD1 models, although its origin is still not clear [193]. Since, mitochondria are the major sites of formation of ROS and are particularly susceptible to oxidative stress, the vicious cycle can pushover mitochondrial damage. Besides, connectivity among different organelles reveal some key joined events related to pathogenesis in ALS. As mentioned by Cozzolino and collaborators in their review [192], the interaction between mitochondria and endoplasmic reticulum (ER), termed the ER-mitochondria calcium cycle (ERMCC), couples energy metabolism in mitochondria and protein processing in the ER to the synaptic activity in neurons mediated by AMPA receptor. Therefore, ERMCC seems to mediate AMPA receptor-mediated glutamate excitotoxicity in MNs where a toxic shift of calcium from the ER compartment to the mitochondrial compartment causes chronic calcium depletion of the ER and chronic mitochondrial calcium overload. The former would trigger the protective unfolded protein response (UPR), and the latter, a consecutive release of cell death signals through the mitochondrial permeability transition pore. Persistent ERMCC dysregulation is a key pathway resulting in the accumulation of misfolded proteins, failing UPR and MN death. The failure of protein homeostatic handling in the cell have been revealed by reported defects in UPR, in protective autophagy and in adequate protein quality control in ALS models [194, 195], as well as in SC tissues of human sALS patients [196]. But importantly, SOD1, OPTN, FUS/TLS & TDP-43 are prone to aggregate when mutated, but even in sALS, SOD1 aggregation represents a common hallmark [197]. It has been indicated that the increased aggregation propensity of SOD1 mutants is related to the decrease survival in ALS patients, further suggesting that aggregation could be a major contributor to SOD1 toxicity in such patients [198]. Importantly, several animal models with reduced motor function presented also impaired autophagy. Conceivable, motor defects generate high amounts of stranded cargo that may overwhelm the autophagic apparatus. Besides, some reports implicate these proteins also in synaptic function. TDP43 and FUS/TLS form part of the so-called mRNA-silencing foci that are cytoplasmatic supramolecular aggregates of repressed RNA and inhibitory proteins in charge of local translation induced specifically by synaptic activity in dendrites and synapses [199], while Optineurin is involved in vesicle trafficking needed for synapse maintenance [200]. The assignment of apoptosis to the ended mechanism of neuronal death in ALS has

been for a subject of extensive debate since controversial evidences have been obtained when human samples were analyzed and matched with effects obtained in animal models [201–204]. What seems clear, however, is that if apoptosis exists it occurs only as a later event of a degenerating process that follows a "Dying Back" pattern of MN dysfunction [192].

Analysis of transcriptomic profile in neurodegenerative diseases has advanced considerably in the past 5 years. Increased scientific rigor and improved analytical tools have led to more-reproducible data. Many platforms for transcriptome analysis assay the expression of the entire genome, enabling that a complete biological context is captured. Gene expression profiling pointed to dysregulation of genes related to neuroinflammation, as well as to disruption of RNA splicing and protein turnover, and several studies in ALS also support the cytoskeleton involvement. Certainly, results from omics experiments will contribute to have a more integrative vision in the pathology of MNs and in its interaction with the cellular neighbors. Computational biology will help systems biology analysis to definitely ascertain the optimal combination of therapeutic agents to end up with the neurodegenerative process. This so-called network medicine [205] has been advanced fruitfully nowadays in cancer research and is certainly the next frontier also for complex neurodegenerative diseases such as ALS.

CONCLUSION

Consideration of ALS as a type of MN neuron disease has evolved to be considered as a multi-systemic, non-cell autonomous and complex neurodegenerative disease. Dying forward and dying back hypothesis to explain the etiology of MN degeneration are difficult to demonstrate in experimental models since rodent corticospinal circuitry differs substantially from that of humans. In that regard, it would be more plausible that noxa implicated in degeneration would affect specifically and importantly MNs mainly, independently on their location, favoring the hypothesis that complex genetic-environmental interaction is indeed the causal factor for MN degeneration. Histological and functional analysis on microcircuitry at the SC, brain and neuromuscular levels where MNs, interneurons, glial and muscle cells may interplay already revealed marked synaptic alterations that may compromise tis-

sular homeostasis and that extensively will contribute to the imbalance between inhibitory and excitatory synaptic drive to MNs. Excitatory signaling gain and/or an inhibitory loss on MNs will increase Ca^{2+} influx and depolarization, favoring excitotoxicity largely observed in several regions of the CNS in patients and models. Besides, particular early affectation on cholinergic transmission within the CNS and towards the NMJ might contribute to synaptic dysfunction first, followed by later cellular dysfunction and even selective MN degeneration. Since glial cells, particularly microglia at the CNS and terminal Schwann cells at the NMJ, contribute to synaptic remodeling and maintenance, it will be worth focus the attention on molecules involved in crosstalking with neurons and axons, as well as in aspects regarding the effects of glial senescence in disease environment and its repercussions on neuronal function failure. Finally, special emphasis on metabolism pitfalls present in ALS patients shifted the attention to muscle regenerative capabilities in patients and models, which have been observed as being severely affected. ALS skeletal muscle suffers from direct neurotoxic effects of SOD1, or other molecules, which definitely affect synaptic connectivity at the NMJ, as well as its metabolism, contributing to the multisystemic phenotype of the disease. With all these landscape together with intrinsic abnormalities, MN function evolve towards dysfunction of its main cellular processes related to transcription, translation and vesicle trafficking, considered to be fundamental in synapse maintenance, connectivity and finally in survival.

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