NEURON-MICROGLIA INTERACTIONS IN MOTOR NEURON DEGENERATION. THE INFLAMMATORY HYPOTHESIS IN AMYOTROPHIC LATERAL SCLEROSIS REVISITED

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Running title: Neuroinflammation in ALS

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ABSTRACT

Research into the pathogenesis of amyotrophic lateral sclerosis (ALS) has obtained notable gene discoveries, although, to date, only progress with regard to treatment has been very modest. Currently ALS is considered a multifactorial disease that presents diverse clinical presentations, ranging from a monogenic inherited disease to an autoimmune pathology, and develops with misfolded protein aggregation and neuroinflammation. An important factor related to ALS pathogenesis is the microglial activation associated with degenerative motor neurons. This activation leads to changes in the expression of a wide range of genes related to phagocytosis and inflammation, and to profound modifications in the dynamic interactions between neurons and glial cells. Overactivation and deregulation of microglial activity causes deleterious effects and leads to neuronal death. However, the involvement of microglia in non-inflammatory functions challenges our concept of neuroinflammation and opens up new possibilities for the study of the pathophysiological mechanisms of ALS. In this review we summarize the current knowledge on the adaptive interactions between neurons and microglia in ALS. We also discuss the hypothesis that controlling the extent of microglial activation and neuroinflammation may have clinical and therapeutic benefits for the condition.

KEYWORDS: Amyotrophic lateral sclerosis, motor neuron disease, neuroinflammation, microglia, astroglia, neuron-glia interaction.

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1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common adult motor neuron disease. Known in the US as Lou Gehrig's disease, after the legendary American baseball first baseman (1903-1941). ALS progresses rapidly and is invariably fatal. ALS afflicts some 350,000 people a year worldwide, with a prevalence of 2-4 per 100,000 individuals. Before the age of 65, slightly more men than women develop ALS, with the only striking female predominance appearing in bulbar-onset patients. This sex difference disappears after age 70 [1–3]. There is no curative treatment, and the drugs currently available have an imperceptible action on the clinical course [4].

ALS was described for the first time 140 years ago [5]. Despite the identification of several single gene mutations with more than 10 loci, mostly in autosomal dominant familial cases (fALS), ALS is a multisystem neurodegenerative disease whose origin and progression are not clearly understood [6]. For the familial forms, fifteen years after the initial discovery of mutations in the enzyme Cu/Zn superoxide dismutase (SOD1), a diversity of new genetic mutations have been progressively identified, including TAR DNA binding protein-43 (gene TARDBP, protein TDP-43) and the C9orf72 gene, until now the most common mutation underlying fALS. Recent data from family histories suggest a continuum of heritability between fALS and sporadic ALS (sALS), with a progressive decrease in the frequency of mutations in ALS genes [7,8].

Except for the rare juvenile form (especially the very aggressive fused in sarcoma (FUS) gene mutation form [9]) the age range for developing ALS is quite broad, between 45 and 60 years [10]. The progressive atrophy and loss of upper (corticospinal) and lower (spinal and bulbar) specialized neurons lead to muscle atrophy, weakness and spasticity, and rapidly to paralysis [11]. In a diversity of phenotypes, the extrapyramidal, cerebellar, sensory or autonomic systems are also affected [12]. (See

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the ALS Research Forum webpage [13] for detailed information). The approximately 20 per cent of ALS patients who develop frontotemporal dementia (FTD), adds another dimension the disease's genetic and phenotypic variability [14-17]. Together with its large clinical heterogeneity (Figure 1a) even within the familiar forms [18-20] the absence of specific biomarkers delays ALS diagnosis and strongly limits an early neuronal therapeutic approach.

Until now, the higher ALS prevalence in specific groups of patients (football players [21-23], veterans of the Gulf War [24,25] or farmers with elevated pesticide and/or fertilizer exposure [26–29]) has not helped unravel the cascade of events or the driving force underlying the disease. Several pathological mechanisms such as genetic factors, environment, autoimmunity, oxidative stress, excitotoxicity, microglial activation, impairment in filament organization and neuronal transport, and misfolded proteins have been proposed as possible explanations of ALS heterogeneity [30,31]. Among them, misfolded protein aggregation and neuroinflammation are the only common mechanisms present in most ALS patients (Figure 1b). The recent implication of microglia in non-inflammatory functions challenges our concept of neuroinflammation and opens up new avenues in the study of ALS pathophysiology. In this review we discuss the current knowledge of these mechanisms, placing special emphasis on the non-inflammatory dynamic interactions between neurons and microglia in ALS.

2. GENETICS OF ALS

The hunting for genetic mutations causative for ALS has accumulated substantial body of research, with a wide range of results. According to the ALSoD database [32], 126 genes had been associated with ALS by September 2015. Of these, defects in six genes have been found to be main causes of the disease [33], and a growing number of genes

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such as the fractalkine receptor (CX3CR1), UNC13A, KIFAP3, EPHA4 and SLC11A2 have been associated with ALS susceptibility, progression or prevalence. [34–38] (Table 1) Multiple mutations of a sole gene also lead to graded phenotypic affectations. For example, the 166 widely dispersed exonic mutations of the Cu/Zn superoxide dismutase (SOD1) gene coding for a mitochondrial enzyme of only 153 amino acids that neutralize toxic superoxide radicals are associated with some 20% of fALS and 1-3% of sALS, all characterized by a large heterogeneity in their presentation [39-43].

However, toxicity of SOD1 exon mutations is related not to altered enzyme activity but to the formation of SOD1 misfolded protein aggregates [44-47]. Characterized in motor neurons and glial cells, wild-type and mutant SOD1 aggregates [39,48] correlate with a similar ALS phenotype [49,50] and also in mice with wild-type SOD1 causing ALS [51]. Inflammation and neuronal death represent a cascade events that are also present in other neurodegenerative diseases characterized by misfolded protein aggregates like the protease-resistant prion protein, Amyloid-β, Tau, or α-synuclein [52,53].

This toxic formation of protein aggregates applies to many other native and mutated misfolded proteins characterized in ALS patients, like the vesicle associated membrane protein (VAMP)-associated protein (VAPB), TARDBP, FUS, and angiotensin (ANG) genes [33,54], and has been extended by the discovery of frequent combined C9orf72 gene mutations that facilitate native protein aggregation (see below). The recently discovered TBK1 gene mutations modify two main pathways, inflammation and autophagy [55], and may alter microglial adaptation to neuronal damage, in particular the engulfment of dying spinal cord neurons [56].

Different VAPB mutations combined with other ALS-relevant mutations, like those in C9orf72 [57,58], have been identified in fALS patients. The formation of mutant VAPB cytoplasmic aggregates triggers Ca^{2+} dependent aberrant functioning of the endoplasmic

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reticulum (ER) and Golgi apparatus. This also occurs in surrounding cells in which wild-type VAPB aggregation takes place, leading to motor neuron degeneration [59,60]. The recent discovery in patients with and without fALS history that the C9orf72 gene defect can cause ALS [61,62] has added a new dimension to the disease's complexity. It challemged the present fALS and sALS classification (Figure 1b), and offered a convergent explanation of ALS pathogenesis that includes RNA dys-metabolism and proteinopathy [63]. In fact, the common genetic variations found in both genetic and sporadic ALS patients by genome-wide association studies [64,65] include a diversity of factors that support ALS susceptibility and heterogeneity, and argue for subgroups of both fALS and sALS along an ALS continuum.

3. ALS AS A MISFOLDED PROTEIN DISEASE

The genetic data suggest that alterations in RNA processing (modified protein synthesis) and proteinopathy (modified protein degradation) are key to both fALS and sALS pathogenesis. For example, 97% of all sporadic cases present TDP-43 proteinopathy, characterized by cytoplasmic TDP-43 ubiquitinated aggregates [66]. In 45% of all sporadic FTD cases, and in numerous genetic mutations of fALS-FTD, TDP-43 proteinopathy also occurs [16,67]. Multiple forms of ALS may then be viewed as different manifestations of a misfolded protein disease (MPD), in which a misfolded protein like SOD1, VAPB or TDP-43 develops insidiously and initiates an early neurodegenerative process that manifests clinically in middle or late life in both sALS and fALS.

In all MPDs, the pathogenic mechanism represents the initial aggregation and CNS deposition of a misfolded protein in specific neurons and/or astroglia that grow following a crystallization-like process [68–70]. Many of these proteins are

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metalloproteins in which the ionized metal participates directly in the protein conformation and function. Besides the mutation hotspots of the coding genes identified in fALS and sALS and associated with a proteinopathy, the age-related metal dyshomeostasis allows variations of metalloprotein conformation and activity that may also result in intracellular aggregates. For example, the variety of binding affinities and binding sites reported for copper to SOD1 indicate that, through these numerous possible interactions, the copper level may directly drive the diversity of the states and functions of these proteins, in particular in an insoluble state [71]. This possibility may pave the way for new therapeutic strategies able to control the misfolded protein metal dyshomeostasis (see below).

Elaborate systems have evolved to protect cells from misfolded proteins. An example is the ubiquitin-proteasome system, which degrades the newly translated proteins that failed to fold correctly [72,73]. In PMD, misfolded proteins are not removed and they aggregate within the cell. As seen in the frontal cortex and spinal cord from sALS patients and in the $SOD1^{G93A}$ spinal cord in mice, these aberrant intracellular accumulations result in ER stress, proteasomal impairment and increased energy demand, progressive Golgi damage, and mitochondrial dysfunction with massive oxidative stress [53,74–76]. In addition, these aggregates from dying neurons/astrocytes or mediated by exosomes activate a microglial phagocyte phenotype to remove aggregates and cellular debris [77,78] (figure 2) and extends the damage to the surrounding cells mostly via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated excitotoxicity in spinal motor neurons [79]. Similar results are observed with the Guam population diet which is rich in β-methyl-amino-L-alanine (BMAA). It chronically activates AMPA receptors, and in combination with other insults or with genetic predisposition, leads to ALS [80,81].

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Glia participation in the neurodegenerative process depends on increased energy consumption [82,83]. Increased energy consumption is also required for neurons to engulf aggregates, repair damage or enter in apoptosis [84–86]. In these conditions, glucose transport to CNS is not sufficient to cover demand, and lactate is formed within astrocytes and sent to neurons for its complete oxidation [87,88]. As a result, the tissue pH decreases, which alters protein folding and induces aggregate formation, as observed with Amyloid-β [89]. In ALS, this dynamic adaptation of the glial phenotype to the graded metabolic and structural modifications results in an overproduction of neurotoxic molecules and chronic neuroinflammation (Figure 2) which drives, at least in part, the disease progression [77,90-92] .

The pleiotropy of the ALS genes coding for proteins involved in this pathogenic process represents, in fact, a convergent mechanism that helps explain the broad spectrum and peculiar complexity of ALS clinical manifestations [93]. The presence of alterations in interrelated metabolic pathways has also been confirmed by blood analysis of ALS patients [94]. Currently, there is a growing consensus among clinicians and researchers that the degeneration and death of upper and lower motor neurons relies on the convergence of various altered metabolic processes in which neuroinflammation is always present and related with high energy consumption and misfolded protein formation (Figure 1b). For these reasons, energy metabolism and nutritional status are factors that help to explain the penetrance variability of ALS genes [95,96]

4. ENERGY METABOLISM AND NUTRITION IN ALS

Whole body energy metabolism is impaired in ALS, and body mass index and overall nutritional status at disease onset are independent predictors of survival in ALS [94]. A recent metabolomics study identified alterations characteristic of an increased glycolysis

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and lipolysis in order to produce more adenosine triphosphate (ATP) and thus to facilitate energy consuming processes [97]. This overall increase in energy demand reflects major cellular activity in an atempt to control the ALS neurodegenerative process taking place and to activate CNS and muscle repair. To ensure very high levels of ATP production from carbohydrate and lipid oxidation, mitochondria increase their activity and number, resulting in enhanced release of reactive oxygen species (ROS) [98]. In close contact with the nucleus, which also produces ATP from the ADP-ribosyl residues after sudden extensive changes in chromatin [99] (perhaps the ones caused by the heterogeneous ribonucleoproteins (hnRNPs) identified in ALS) proteasome becomes especially important for maintaining cellular homeostasis through degradation of ubiquitinated factors like Drp1, which control mitochondria fusion and fission and the ubiquitinated misfolded ALS proteins [100]. In addition, reduced glucose uptake and metabolism consequent to increased glucocorticoid signaling pathways cannot be discarded [101–103].

In fact, the on-going ALS neurodegenerative process is the result of two dynamic tissue mechanisms, one neurotoxic and an adaptive neuroprotective. The high-energy demand present in ALS must be part of this second mechanism, which remains efficient despite its elevated ROS production and progressive mitochondria and proteasome damage, and the altered ER-mitochondria interactions. In any case, the importance of rich-energy nutrition must be considered a key aspect of the patient care. In this framework, the Guam population, whose etiology of ALS correlates significantly with picking, processing, and eating cycad seed flour rich in neurotoxins such as BMAA, a potent glutamate agonist at both AMPA and kainate receptors [104] exemplifies the relevance of nutritional factors [105,106]. As such recent clinical trials have highlighted the importance of controlling ALS patient nutrition, with interventions from

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complementary approaches (ClinicalTrials.gov identifier: NCT00983983) or with administration of olanzapine, a weight gain drug (ClinicalTrials.gov identifier: NCT00876772).

5. REFINING CONTROL OF NEUROINFLAMMATION IN ALS

Until a few years ago, little was known about microglia adaptation and participation in ALS, and the microglia's direct and permanent involvement in the disease is a recent discovery. ALS pathophysiology includes a chronic microglial reaction in the surroundings of degenerative motor neurons [107]. In patients with ALS, microgliosis appears specifically in the motor cortex, along the corticospinal tract, and in the ventral horn of the spinal cord [108–110]. Although the molecular mechanisms of this neuroinflammation have not been clarified, it has been demonstrated that activation of microglia in the ALS-injured CNS leads to release of cytokines, chemokines, and neurotrophic or neurotoxic factors that stimulate astrocytes and mobilize cells from the peripheral immune system [111–113]. These cells in turn produce new cytokines creating a chronic inflammatory response [111,112].

In the healthy CNS, microglia present a highly ramified morphology, express receptors for neurotransmitters and neuromodulators [116–118], dynamically monitor the neuronal microenvironment and actively respond to neuronal activity as an integrated part of the "quad-partite" synapse [119–121]. This non-inflammatory microglial activity has important roles in CNS development eliminating the excess of neurons and controlling synaptic pruning and maturation of excitatory synapses (see [122] for a review). In the adult brain, the microglia is an important player in the regulation of synaptic plasticity [121,123,124] and in clearing newborn adult hippocampal progenitors [125]. Thus, there is evidence of the involvement of microglia in the control

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of long-term potentiation [126–128] long-term depression [129], synaptogenesis [130,131], and in sensing and eliminating defunct synapses [119].

These non-inflammatory roles of microglia involve dynamic interactions with neurons. Microglia are maintained in surveillance via signaling between fractalkine (CX3CL1) and its G-coupled protein receptor, CX3CR1. In the CNS, fractalkine is expressed by neurons as a membrane-bound glycoprotein, from which a soluble chemokine can be proteolytically released [132,133]. Fractalkine modulates migration, motility, and activation of microglia by interaction with the CX3CR1, which is specifically expressed in microglia [112]. In turn, the interaction fractalkine-CX3CR1 is required for proper maturation of excitatory synaptic transmission [134]; mice lacking CX3CR1 show persistent alterations in hippocampal glutamatergic transmission [135].

When microglia become reactive, their morphology changes, ranging from enlargement of processes and hypertrophy of the cell body to acquisition of an amoeboid shape. Besides these morphological alterations, reactive microglia also release cytokines and upregulate a variety of surface molecules[113,136–138]. Classically, microglia have been considered as resident macrophages of the CNS and the concept of macrophage polarization has been extrapolated to explain reactive microglia activity [139]. According to this paradigm, M1 (or classically activated) microglia present inflammatory activity, whereas M2 (or alternatively activated) microglia have tissue repair functions [107]. Initially a pro-inflammatory M1 phenotype aims to protect and repair damaged tissue but prolonged and excessive inflammation is cytotoxic and induces neuronal death [136–138]. Pro-inflammatory microglia promote a neurotoxic T-cell response and are cytotoxic owing to the secretion of nitric oxide (NO) and classical pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, ROS production and the reduction of protective trophic factor expression

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[142]. In addition, chronically activated microglia may become increasingly dysfunctional and may directly participate in the development of secondary tissue injury [143]. In contrast, M2 neurorestorative microglia activity includes the production of high levels of anti-inflammatory cytokines like IL-4, IL-10, neurotrophic factors, and proteins such as insulin-like growth factor (IGF)-1, arginase-1 and Ym-1 [144–146].

However, the M1 vs. M2 paradigm of macrophage activity has recently been reappraised since it appears to be at odds with the recent data emerging from epigenetics and genome-wide transcriptional studies [112,147]. New findings have also established that microglia are a unique cell population, distinct from macrophages [112,148]. Analysis of acutely isolated microglia from $SOD1^{G93A}$ tg mice revealed upregulation of both neuroprotective and toxic factors related with ALS [112]. $SOD1^{G93A}$ microglia upregulated the expression of IGF-1 and progranulin, two microglial factors known to confer neuroprotection and increase survival in ALS models [149,150], and progranulin mutations are major known genetic causes of ALS-FTD [151]. With respect to the neurotoxic factors, metalloproteinase 12 and optineurin are upregulated in $SOD1^{G93A}$ microglia [112]. Mutations in optineurin cause fALS [152,153] and deregulate interferon signaling [154], while metalloproteinase inhibition increases survival in ALS mice [155]. More interestingly, in the same study $SOD1^{G93A}$ microglia also increased the expression of genes related with Alzheimer's, Huntington and Parkinson's diseases but not of genes related with lipopolysaccharide-induced microglial activation [112].

Thus, the M1-M2 dichotomy does not seem the best-suited concept for explaining the microglial response to motor neuron injury. Rather, microglia become reactive through expression changes in a wide range of genes that mediate the acquisition of new cell functions. This means that, at each moment, the balance of secreted molecules from the

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microenvironment determines a spectrum of microglial phenotypes that also vary with the time and intensity of neuron damage [112,145]. However these phenotypes are classified, microglia respond with a large diversity of signals, some of them detrimental and others restorative, which modulate neuroinflammation and thus interfere with ALS progression (Figure 3).

Astrocytes also participate in the inflammatory mechanisms associated with motor neuron degeneration [156–158] and may play a dual role. Like microglia, astrocytes exert both toxic and protective effects on neurons in a context-dependent manner (Figure 3). In fact, they may represent a diverse population of genetically tractable cells that mediate neural circuit–specific roles in health and disease (see [159] for a review). Astrocyte activation has been described in the spinal cord, motor cortex and subcortical white matter [160,161] of ALS patients. In the $SOD1^{G93A}$ tg mouse, astrogliosis is concomitant with motor neuron loss [161,162].

Astrocytes express MHC molecules and can present antigens to primed memory T cells [163]. They respond to neuronal injury by releasing acute-phase proteins such as α1-antichymotrypsin and α2-macroglobulin, complement proteins, and neuronal growth factors and cytokines [116]. In general, their neurotoxic activity is mediated via activation of pattern recognition receptors such as Toll-like receptors. After receptor activation, astrocytes secrete cytokines like CCL2, IL-6 and other neurotoxic factors that enhance the inflammatory response [108,164,165]. Their neuroprotective effects are mediated by an homeostatic control of neuronal activity [159,166] and the release of neurotrophic factors such as nerve growth factor and IGF-1 [165].

Astroglia also contribute to motor neuron degeneration through non-immune mechanisms that may be associated with abnormal astrocyte activity rather than with astrogliosis. For example, in ALS patients and in ALS animal models, astroglia lead to

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glutamate excitotoxicity due to a decreased expression of glutamate transporter-1 [167,168]. A recent study has related the increased expression of connexin 43 in the motor cortex and spinal cord of ALS patients, and enhanced gap junction coupling in $SOD1^{G93A}$ mice, with the course of the disease [169]. The subsequent increased hemi channel-mediated activity and elevated intracellular calcium levels suggest excessive astrocyte exchange of metabolites, ions and second messengers, which results in an astrocyte-mediated toxic effect on motor neurons.

6. IS ALS A NEUROINFLAMMATORY DISEASE?

Research into the pathogenesis of ALS had conventionally assumed that neuroinflammation was a response to motor neuron injury and extracellular protein deposition. However, studies in SOD1 tg mice evidenced microglial activation even before motor neuron cell death [170,171], which suggested the hypothesis that neuroinflammation might contribute to ALS pathogenesis [107]. Currently, there is increasing evidence linking ALS molecular hallmarks with modifications in microglial activity or the immune system response. For example, TDP-43 and the p65 subunit of the nuclear factor-κB (NF-κB) are increased in the spinal cord of sALS patients when compared with healthy individuals [172]. TDP-43 interacts and co-localizes with p65 in microglia and neurons from ALS patients and mice expressing wild-type and mutant TDP-43 transgenes, but not in cells from healthy individuals or non-transgenic mice [172,173]. Thus, TDP-43 acts as a co-activator of p65, and microglia expressing larger amounts of TDP-43 produced more pro-inflammatory cytokines and neurotoxic mediators after activation [173]. At the functional level, inhibition of NF-κB activity in TDP-43 mice reduces denervation in the neuromuscular junction and ALS disease

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symptoms [174]. TDP-43 deregulation thus contributes to ALS pathogenesis, in part by promoting the inflammatory activity of the microglia.

Similarly, C9orf72 plays a key role in innate immune cell function. Mice lacking C9orf72 develop splenomegaly nad lymphadenopathy and accumulate swollen macrophage-like cells [175]. Loss of C9orf72 in mice also leads to macrophage and microglial dysfunction and age-related neuroinflammation similar to that described in C9orf72 fALS [175]. Again, these new findings raise the possibility of a dual pathogenic mechanism, in which the effects of microglial dysfunction from decreased C9orf72 expression combine with toxic by-products in neurons to promote neurodegeneration (Figure 2).

Immune actions may then precede ALS pathology and may be sufficient to cause it, suggesting that ALS is an autoimmune disease. A growing body of evidence supports this hypothesis. For example, circulating myeloid cells of ALS patients have a pro-inflammatory phenotype [90,176], while regulatory T lymphocytes are decreased, enhancing neuroinflammation and disease progression [177]. Also, the up-regulation of inflammatory genes in arrays on samples from ALS patients suggests the involvement of immune actions in the ALS pathogenesis [178–180]. Some other studies have related motor neuron disease with autoimmune neuromuscular disorders. Around 30% of ALS patients present decrement on repetitive nerve stimulation [181], and the risk of ALS is increased in people with concomitant myasthenia gravis [182]. This combination of ALS and myasthenia gravis has been reported to occur non-stochastically [183] and, although the reasons for the coincidence are unknown, it may involve a link between the immune system and ALS [156]. Taken together these results suggest that immune processes may drive ALS pathology independently of neural processes, exacerbating motor neuron injury and feeding a cyclic process that sustains neurodegeneration.

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As in other aspects of the disease, the nature of neuroinflammation in ALS is heterogeneous. In many cases adaptive immune cells may drive the pathological process, a feature that defines ALS as a neuroinflammatory disease rather than a neurodegenerative disorder [139]. However, this concept is far from being consolidated: it remains unclear how and when the immune system impacts the course of ALS, and it stills needs to be clarified whether an immune mechanism is involved in all patients.

7. MICROGLIA MAY DRIVE ALS PATHOPHYSIOLOGY

A key aspect of CNS function is the dynamic interaction between neurons and microglia. Therefore dysfunction of microglial non-inflammatory activity may contribute to pathological processes [184,185]. For exemple, excessive activation of microglial CD33 was found to upregulate synapse removal in Alzheimer's disease patiens and mouse models [186,187]. Also, altered microglial brain-derived neurotrophic factor signaling has been associated with neuropathic pain [123]. In this line, the axis fractalkine-CX3CR1 participates in the control of microglial dysfunction. Microglial CX3CR1, involved in neurotrophic fuctions, is upregulated in many pathological conditions [188]. The role of CX3CR1 in the control of neuroinflammation has been extensively studied in the CX3CR1-EGFP mouse line. Although the consequences of CX3CR1 deletion in microglia largely depend on the mouse model used, the overall idea is that a lack of CX3CR1 leads to a hyperactivity of microglia in the diseased brain, thereby unleashing potential neurotoxic properties [113,189,190]. However, other evidence suggests that fractalkine sustains neuroinflammation and contributes to neurotoxicity (see [191] for a review). In any case, CX3CR1 signaling impairment has a direct influence on neurodegenerative diseases associated with neuroinflammation, microglia and/or T-cell recruitment [192,193].

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In a cohort of Spanish sALS patients, a genetic association was described between the V249I and T280M variants in the human CX3CR1 gene and the progression rate and survival time [34]. This association has also been reported between these two CX3CR1 gene variants and some other inflammatory diseases, including multiple sclerosis, age-related macular degeneration, AIDS, and coronary disease [194]. V249I and T280M genetic variants affect the activity of the CX3CR1 protein by reducing its affinity to fractalkine [195]. In animal models, CX3CR1-/- knockout mice inbred with the $SOD1^{G93A}$ tg ALS model present a worsened disease outcome, more extensive neuronal loss and increased microglial activation [196]. As CX3CR1 is involved in microglial neurotrophic function (Figure 3), its reduced activity may exacerbate the pro-inflammatory activity of microglia.

What still remains to be established is whether the fractalkine-CX3CR1 axis is affected in the diseased brain. There are few reports of fractalkine concentration in the brain and CX3CR1 expression in microglia during the course of disease. Cardona et al. [188] detected a rather high concentration of free fractalkine in the brain, which suggests a constitutive release under physiological conditions. In multiple sclerosis or epilepsy, the CNS levels of fractalkine and/or CX3CR1 were found either unchanged or increased [197,198], indicating that the inhibitory function of the fractalkine-CX3CR1 axis is not generally weakened under diseased conditions.

Microvesicles (MVs) and exosomes are considered important players in the dynamic interactions between neurons and glia. In the CNS, neurons, microglia, astrocytes and oligodendrocytes release MVs and exosomes with different sizes and contents into the extracellular space. These particles can move freely through the extracellular medium and interact with different cell types by as yet undetermined mechanisms [199]. MVs carry and may shuttle specific proteins and noncoding nucleic acids such as microRNAs

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(miRNAs) [200]. In fact, some miRNA may be found in MVs at higher concentrations than in cells [201]. For these reasons, besides their potential as disease biomarkers [202], MVs and exosomes are considered to have a role in the pathogenesis and dissemination of inflammatory diseases.

The cell-to-cell transmission of TDP-43 and SOD1 misfolding, which is propagated by prionic mechanisms (see [52] for a review), now appears to be mediated by MVs trafficking. A recent study provides evidence for a primarily microvesicular intercellular spread of TDP-43 aggregates [203], and both neuronal and astroglial MVs have been implicated in the propagation of pathogenic misfolded SOD1. In vitro, motor neuron NSC-34 cells overexpressing wild type and mutant SOD1 secreted this protein via exosomes [204]. Astrocytes expressing mutant SOD1 release a high amount of exosomes carrying the protein and promote motor neuron death [205]. Interestingly, MVs from activated astroglia also transfer amino-acid transporter-1 [206], suggesting a possible role for them in the extracellular mechanism that curtails excitotoxicity.

There is growing evidence of the involvement of miRNA deregulation in ALS pathogenesis [207–211]. Changes in miRNA expression have been found in patients and the $SOD1^{G93A}$ mouse [176,212,213] reflecting a dysfunction of the expressed RNA binding proteins involved in ALS. miRNAs are stable in cerebrospinal fluid, serum and other body fluids as exosomal cargos [214] and their concentrations are also modified in ALS [215] suggesting that the dysfunction of RNA binding proteins in ALS is systemic. In serum of fALS patients, specific miRNAs non-related with the mutated gene are downregulated already in presymptomatic disease [216,217], whereas serum miRNA profiles are far more heterogeneous in sALS patients [218]. Other exosomal miRNAs are upregulated in ALS [219]. Some are components of the innate immune system and

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modify the pro-inflammatory NF-κB signaling pathway [220], indicating, again, a role for the immune system in ALS pathogenesis.

Finally, reactive microglia release MVs and exosomes, which may have a dual role in motor neuron degeneration (Figure 3). On the one hand, MVs released by reactive microglia carry pro-inflammatory cytokines [221] and induce an inflammatory reaction in target cells [222]. Microglia-derived MVs also promote precipitation of misfolded proteins in Parkinson's and Alzheimer's disease models [223,224]. On the other hand, microglia stimulated with interferon-γ release exosomes that confer protection on surrounding cells [225]. Thus, different stimuli may trigger the microglial release of MVs with different composition and properties, as already described for macrophages and monocytes [226,227]. If true, microglial activation will lead to increased secretion of MVs, which differ in composition and are associated with ALS pathological processes with distinct roles. This possibility may open up new avenues in the development of treatments for ALS. Currently it is considered that, by engineering MVs, it will be possible to enhance the protective microglial phenotype in order to curtail neuroinflammation and promote tissue repair.

8. NEUROINFLAMMATION AND ALS TREATMENTS

The difficulty of developing effective treatments for ALS is explained, at least in part, by its heterogeneity and the presence of both functional and structural on-going cell damage. For years, the multiple ALS phenotypes and the identification of many mutations in the SOD1 gene that could not explain the disease were not little help in guiding pharmacological research; riluzole, introduced in 1995, is presently the sole authorized treatment, with limited efficacy and a high financial cost [228]. Since the 1990s, clinical trials with many other drugs have mostly yielded negative results, and

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there is an urgent need to define new and effective therapeutic strategies and improve clinical trials [229–231].

Protein aggregation and neuroinflammation are common features of the chronic course of ALS. Therefore the molecular mechanisms underlying these factors are potentially amenable to therapy. In this line, the compound BIIB067, a drug designed to reduce the production of SOD1, is currently in a phase I clinical trial (ClinicalTrials.gov ID: NCT02623699) [231]. With respect to neuroinflammation some patents protecting the development of anti-inflammatory compounds have been registered for use in ALS [232], but to our knowledge, none are being developed as part of current ALS therapeutic strategies [231]. In this context, in the search for valid ALS therapies, the standard view of neuroinflammation, which has focused mainly on the cytotoxic activity of microglia, is not longer valid. Instead this view should be replaced by an integrative approach that involves a wide spectrum of microglial phenotypes and recognizes the dynamic interactions between microglia, neurons, immune cells, and astrocytes.

9. CONCLUDING REMARKS

The causes and pathophysiology of ALS are far from being clarified. Recent years have seen notable gene discoveries in ALS, but only very modest progress in treatment. In spite of the large body of evidence provided, many questions remain unanswered. For example: Why do some individuals carrying a pathogenic SOD1 or C9orf72 mutation never develop ALS or FTD? Are they resistant to the disease? What is the real role of the immune system in the pathogenesis of the disease? Answers to these questions should help us to understand ALS and to find therapeutic solutions for this devastating disease.

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The involvement of microglia in non-inflammatory functions challenges our concept of neuroinflammation and opens up new research posibilities in the neuron-microglia crosstalk. In this new scenario, mechanisms such as the fractalkine-CX3CR1 axis or MVs and exosome trafficking appear as central players in this relationship. As these new functions are progressively characterized, the pathophysiological mechanisms of ALS will hopefully be revelated. In this pursuit, molecular characterization of the different ALS clinical presentations must allow an integrated view of the disease and enable new effective strategies for diagnosing and treating the condition. In the meantime, we can only continue to work hard and hope that we will see soon a giant step forward for humankind in the treatment of this pernicious killer disease.

REVIEW CRITERIA

Articles were identified from publications listed in English on PubMed to October 2016. Combinations of the following key words were used: “amyotrophic lateral sclerosis”, “ALS”, “motor neuron disease”, “neuroinflammation”, “microglia”, “astroglia”, “immune system”, “excitotoxicity”, “environment”, “energy metabolism”, “nutrition”, “risk factor”, “phenotype”, “neuron-glia interaction”,” microvesicle”, “exosome”, “microRNA”.

We also identified articles from the reference lists of the articles found with the above-cited search terms. Other publications were identified through the author’s collections of scientific literature. Clinical trials were selected at the ClinicalTrials.gov database. The final reference list was generated on the basis of originality and relevance to the scope of this Review.

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COMPETING INTERESTS

The authors have no conflicts of interest to declare.

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FIGURE LEGENDS

Figure 1: ALS viewed as a multifactorial disease with diverse clinical presentations and neurodegeneration patterns. a) Overlapping of ALS clinical manifestations and diverse pathologies depends on the main mutated genes, except for C9orf72, common to all diseases. b) Drawing represents ALS genetics as a continuum spectrum, ranging from a monogenic inherited disease (fALS) to idiopathic sALS, and with a decreasing gene penetrance. In this model, misfolded TDP-43 protein aggregation and neuroinflammation are the only pathological mechanisms common to all ALS phenotypes.

Figure 2: Cellular processes involving altered responses of dysfunctional glia in ALS. Misfolded protein aggregates (MPA) induce motor neuron and astrocyte suffering and the neuronal release of glial activation signals. Reactive microglia mediate different pro-inflammatory and neuroprotective processes in coordination with astrocytes and requires increased energy consumption. Extracellular MPA also activates microglial phagocytic activity to remove aggregates, which results in microglial dysfunction that directly participates in neurodegeneration. Gene polymorphisms inducing decreased C9orf72 activity favor misfolded protein aggregation and microglial dysfunction (see text for details).

Figure 3: Signaling systems involved in the ALS neuron-microglia-astrocyte crosstalk. Astrocytes and microglia modulate neuronal activity by sensing neurotransmitter release. Also, microglia have receptors to molecules released by astrocytes and vice-versa. In ALS, reactive microglia and astrocytes mediate different neuroprotective (green arrows) and cytotoxic processes (red arrows) according to a diversity of signals

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from the injured motor neurons. This glial activation mobilizes the immune system, also proposed to trigger motor plaque injury and neurodegeneration. 5-HT, serotonin; Ach, acetylcholine; Ado, adenosine; BDNF, Brain-derived neurotrophic factor; bFGF, fibroblast growth factor-b; EAAT-1, excitatory amino acid transporter-1; GABA, γ-aminobutyric acid; GDNF, glial cell-derived neurotrophic factor; Glu, glutamate; GSH, glutathione; IFNγ, interferon gamma; IGF-1, insulin-like growth factor-1; IL-1β, 4, 5, 6, 10, interleukins 1beta, 4, 5, 6, 10; MVs, microvesicles; NMJ, neuromuscular junction; NO, nitric oxide; NT3, neurotrophin 3; ROS, reactive oxygen species; Tau, taurine; TGFβ, transforming growth factor beta, TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal peptide.

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Table 1: Major genes associated with ALS

Gene	Protein name	Protein description and function.	ALS pathophysiology	ALS association
SOD1	Cu/Zn Superoxide dismutase 1, soluble	Soluble cytoplasmic protein, acting as a homodimer to convert endogenous but harmful superoxide radicals to molecular oxygen and hydrogen peroxide.	Aggregate formation	Cause
FUS	Fused in sarcoma	Multifunctional protein component of the heterogeneous nuclear ribonucleoprotein (hnRNP) complex. The hnRNP complex is involved in pre-mRNA splicing and the export of fully processed mRNA to the cytoplasm.	Altered function and Aggregate formation.	Cause
C9orf72	Chromosome 9 open reading frame 72	This protein plays an important role in the regulation of endosomal trafficking, and has been shown to interact with Rab proteins that are involved in autophagy and endocytic transport.	Blockade of nuclear-cytoplasm transport pore. It facilitates aggregate formation.	Cause
TARDBP	Transactive response DNA-binding protein 43	Transcriptional repressor that binds to chromosomally integrated transactive response DNA and represses HIV-1 transcription. It also regulates alternate splicing of the CFTR gene.	TARDBP over-expression leads to deregulation of ER-mitochondria interactions through VAPB and PTPIP51. Aggregate formation	Cause
UBQLN2	Ubiquilin 2	Ubiquitin-like protein that physically associates with both proteasomes and ubiquitin ligases; and thus, are thought to functionally link the ubiquitination machinery to the proteasome to modify protein degradation.	Protein aggregates activate, chronic ER stress.	Cause / Increased risk
SETX	Senataxin	This protein contains a DNA/RNA helicase domain. This suggests that it may be involved in both DNA and RNA processing.	hnRNP complex deregulation?	Cause / increased risk
SPG11	Spastic paraplegia 11 (autosomal recessive). Spatacsin	This protein is needed for the recycling of lysosomes from autophagic lysosome reformation.	ER stress leading to neurodegeneration	Increased risk
VAPB	VAMP (vesicle-associated membrane protein)-associated protein B and C	Membrane protein found in plasmalemmal and intracellular vesicle membranes. It is found as a homodimer and as a heterodimer with VAPA. It can also interact with VAMP1 and VAMP2 and may be involved in vesicle trafficking.	VAPB cytoplasmic aggregates triggers calcium dependent aberrant ER and Golgi structures and function	Increased risk
ATXN2	Ataxin 2	Protein primarily localized to the Golgi apparatus with unknown function.	Associate with TDP-43. ER stress leading to neurodegeneration	Increased risk
TBK1	TANK binding kinase 1	Protein similar to IκB kinases that mediate NFκB activation in response to certain growth factors.	Control of neuroinflammation?	Increased risk
UNC13A	unc-13 homolog A (C. elegans)	Member of the UNC13 family. These proteins bind to phorbol esters and diacylglycerol and play important roles in neurotransmitter release at synapses.	Excitotoxicity and glutamate-induced neurodegeneration.	Decreased survival
CX3CR1	C-X3-C motif chemokine receptor 1	Receptor for fractalkine, a transmembrane protein and chemokine involved in the adhesion and migration of leukocytes. In the CNS is expressed by microglia and mediates neuron-microglia interactions.	Control of neuroinflammation	Decreased survival
KIFAP3	kinesin associated protein 3	Unknown. It interacts with human chromosome-associated polypeptide (HCAP) and KIF3A/B, a kinesin superfamily protein in the nucleus. It is proposed to play a role in the interaction of chromosomes with ATPase motor proteins.	Vesicle and organelle trafficking ?	Decreased survival
EPHA4	EPH receptor A4	Ephrin receptor subfamily of receptors with protein-tyrosine kinase activity. It is implicated in developmental events, particularly in the nervous system.	Neuroprotective actions?	Decreased survival
SLC11A2	solute carrier family 11 member 2	Also known as DMT1, this protein transports divalent metals and is involved in iron absorption. Mutations in this gene are associated with hypochromic microcytic anemia with iron overload.	Altered metal homeostasis. It may form aggregates with SOD1	Decreased survival

Table shows examples of genes in which polymorphisms cause, increase the risk or modify progression rate of ALS. Not everyone carrying a disease-related mutation in these genes develops ALS, which suggests that other factors are needed to cause disease.

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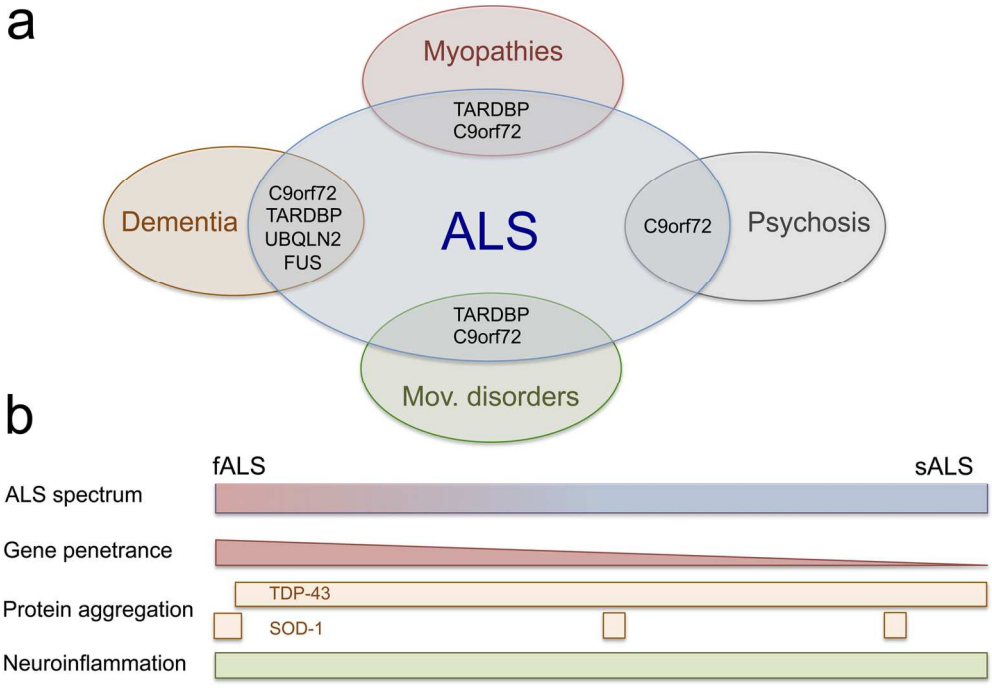

Figure 1
Figure 1: ALS viewed as a mult
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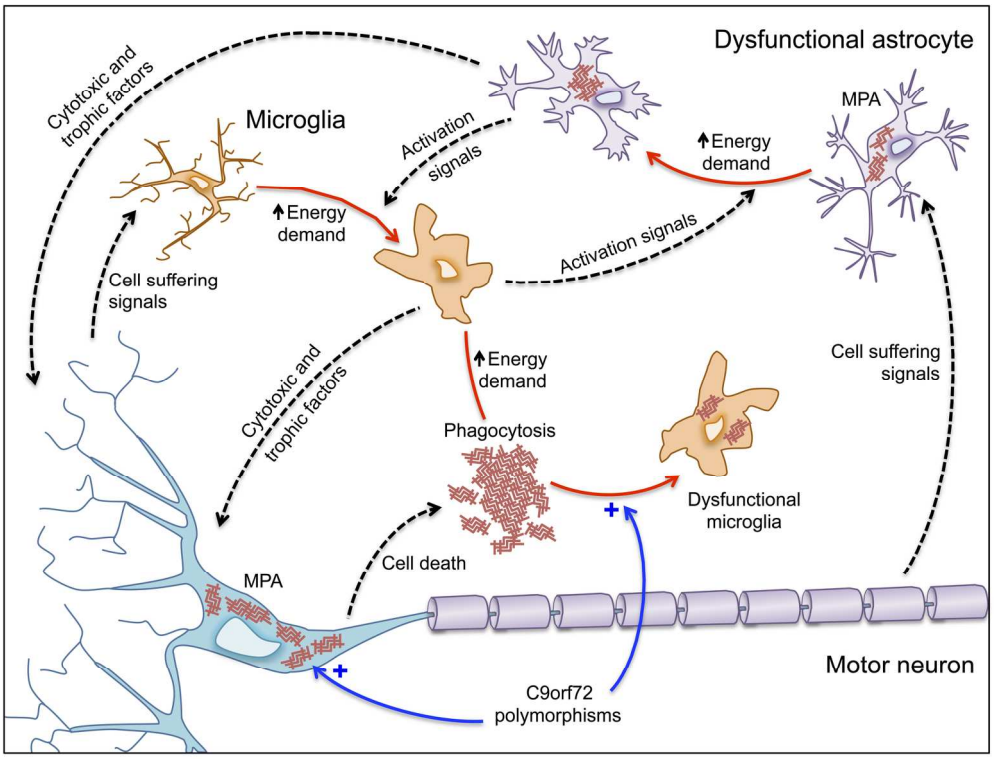

Figure 2: Cellular processes involving altered responses of dysfunctional glia in ALS
Figure 2
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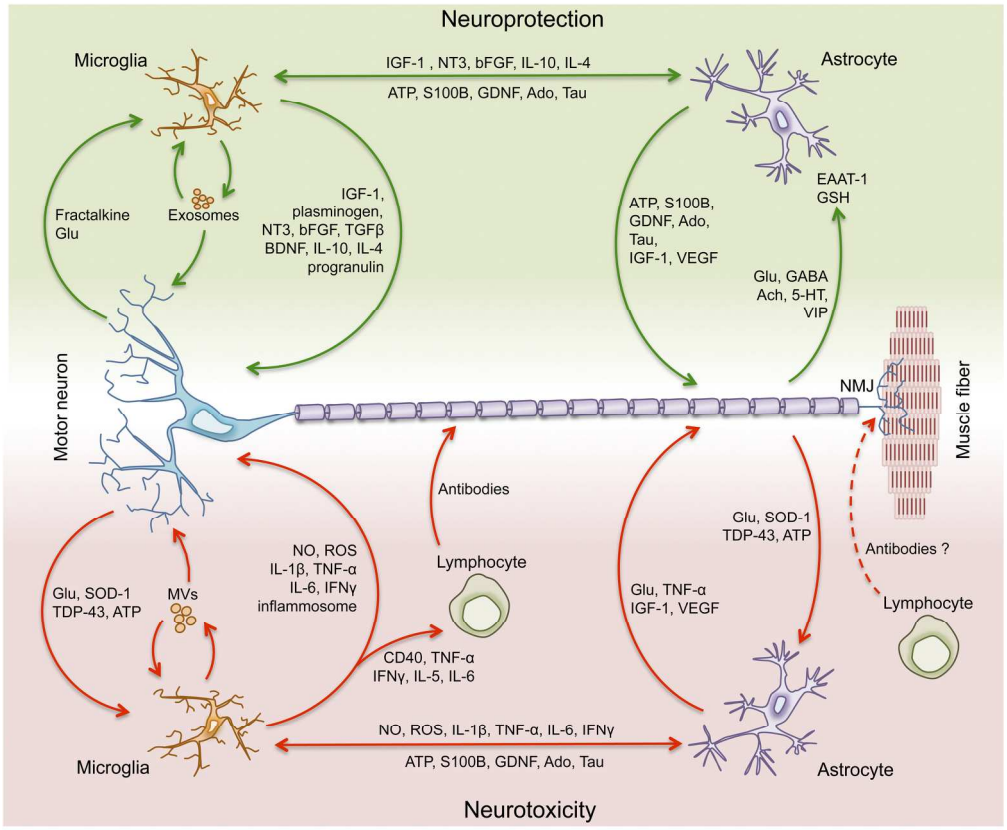

Figure 3: Signaling systems involved in the ALS neuron-microglia-astrocyte crosstalk
Figure 3
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