Chapter 9

Selective Vulnerability of Neuronal Subtypes in ALS: A Fertile Ground for the Identification of Therapeutic Targets

Cylia Rochat, Nathalie Bernard-Marissal and Bernard L. Schneider

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Abstract

It is well defined that subpopulations of motoneurons have different vulnerability to the pathology causing amyotrophic lateral sclerosis (ALS). In the spinal cord, the fast fatigable motoneurons have been shown to be the first to degenerate, followed by fatigue-resistant and slow motoneurons. In contrast motoneurons located in the Onuf's and oculomotor nuclei appear to be resistant to disease. With a focus on research mainly done on mice overexpressing the mutated human superoxide dismutase (SOD1) protein, we review recent studies exploring the mechanisms that underlie the selective vulnerability of the various motoneuron subtypes. By comparing differences in gene expression between these populations, it has been possible to identify factors, which critically determine the survival of motoneurons and the neuromuscular function in the pathologic context of ALS. Furthermore, we discuss the contribution of non-cell autonomous processes, involving glial cells and the skeletal muscle, in the neurodegenerative process. Exploring the cause of neurodegeneration from the angle of the selective neuronal vulnerability has recently led to the identification of novel targets, which open opportunities for therapeutic intervention against ALS.

Keywords: motoneurons, SOD1, selective vulnerability, therapeutic target, ER stress

1. Introduction

Amyotrophic lateral sclerosis (ALS) primarily affects the motor system, which controls both voluntary and involuntary movements, including vital functions such as breathing, through

the activity of various types of motoneurons (MNs). Paralysis and death of the patients, which typically occurs within a few years after disease onset, is caused by the progressive dysfunction and degeneration of MNs in the cortex, brainstem and spinal cord. Remarkably, it has been established that the different types of MNs are not equally affected by ALS. This leads to contrasted effects on the motor system. For instance, with disease progression, patients lose their ability to speak, swallow and move, but they keep normal visual, sexual and bladder functions. Indeed, MNs located in the oculomotor and Onuf's nuclei are remarkably resistant to the disease [1]. In contrast, spinal MNs controlling voluntary movements, hypoglossal MNs important for swallowing and breathing, as well as the upper MNs, are typically among the first neurons to degenerate.

Upper MNs, also known as Betz cells or corticospinal MNs (CSMNs), are glutamatergic neurons located in the primary motor cortex and which activate lower MNs in the brainstem and spinal cord. Upon activation, the lower MNs induce muscle contraction via the release of the acetylcholine neurotransmitter in the neuromuscular junction (NMJ), a specialized synapse contacting the skeletal muscle fibers. The ensemble formed by lower MNs and the innervated muscle fiber is called the motor unit.

Spinal MNs are subdivided into α, β and γ MNs, depending on the type of muscle fibers they innervate (reviewed by [2]). In ALS, it is mainly α-MNs that degenerate. However, it is recognized that within the class of α-MNs, there is also a predictable variation in neuronal vulnerability to disease [3, 4]. It is therefore important to distinguish the following subtypes of α-MNs, defined by the contractile properties of the motor unit they are part of: the fast twitch fatigable (FF) MNs, the fast twitch fatigue-resistant (FR) MNs and the slow twitch fatigue-resistant (S) MNs [5]. This classification is also based on other characteristics, such as the size of the neuronal soma (FF MNs have larger cell bodies than S MNs), axonal conduction velocity (FF MNs are faster than S MNs), dendrite branching (FF MNs display a more complex dendritic tree than S MNs) [6], as well as the electrical properties of each of these MN subtypes [7].

The selective vulnerability observed between the different types of MNs provides a remarkable opportunity to explore the factors that specifically contribute to neurodegeneration. Until now, this approach has been mainly based on animal models overexpressing mutated forms of the superoxide dismutase 1 (SOD1) protein. Indeed, in more than 20% of the familial ALS cases (fALS), the SOD1 protein carries point mutations associated with autosomal dominant inheritance. Rodent models overexpressing mutated forms of SOD1 (mSOD1), often under the control of the human SOD1 promoter, faithfully replicate major clinical aspects of ALS [8–10]. Furthermore, MN subpopulations display a selective vulnerability pattern very similar to the one observed in humans [8]. These animal models are therefore instrumental to investigate the molecular and cellular mechanisms underlying the disease process. In this review, we will discuss how the research on ALS has identified novel therapeutic targets by comparing different MN subtypes in SOD1 models of fALS.

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2. Comparing different populations of MNs

A careful analysis of disease progression in the high-copy SOD1^{G93A} mouse models of ALS has allowed defining different stages of the disease. The first behavioral alterations occur as early as postnatal day 10 (P10), with a delay in the righting reflex and an increase in the number of mistakes observed in forelimb placement [11]. Around P50, subtle changes in mouse gait and muscle strength can be observed [12, 13] and by P80, clear impairments in the motor performance can be detected [14]. At 3 months of age, the animals reach disease onset, which is characterized by fine tremors, muscle atrophy and loss of body mass [15]. A severe paralysis of the hindlimbs is observed on average at P120 [16]. Soon after P135, the ALS mice become unable to right themselves when placed on their side, which is considered as the humane disease endpoint [15]. This highly reproducible course of the disease has been used to identify the corresponding neurodegenerative events in the mouse spinal cord. Thorough analysis has revealed a precise sequence in the loss of the different types of MNs, allowing for longitudinal studies to determine molecular and cellular correlates in the disease process.

2.1. Progression of the SOD1 pathology in mouse models of fALS

2.1.1. Spinal MNs

Spinal MNs are responsible for the control of voluntary movements. For instance, MNs located in the lumbar part of the spinal cord control the movement of the hindlimbs (**Figure 1**). These MNs innervate muscles, such as the *gastrocnemius*, which are composed of different types of muscle fibers. The type and the contractile properties of each muscle fiber are defined by the type of the innervating MN [5] (**Figure 2**). During the course of the disease observed in the high-copy SOD1^{G93A} mice, the innervation of the *gastrocnemius* muscle undergoes dramatic changes that can be recorded by electromyography.

A significant decline of the compound muscle action potential (CMAP) is observed around P50 [17, 18], followed by a second decline seen around P100 [18, 19]. In line with the CMAP data, histological analysis has revealed an abrupt loss of muscle innervation at the age of approximately 50 days, corresponding to lower occupation of the NMJ in the fast twitch muscle [3, 14]. Therefore, SOD1 pathology leads first to a loss of the innervation of the type IIb muscle fibers by the FF MNs (**Figure 2B**). The second wave of denervation, which occurs at the late presymptomatic stage of the disease, is defined by the pruning of the FR axons innervating the type IIa muscle fibers [4] (**Figure 2C**). In contrast, the type I muscle fibers remain innervated by the S MNs almost until end stage [18]. Moreover, FR and S MNs have been shown to have a higher capacity for axonal sprouting compared to the FF MNs [3]. They may form new synapses on the denervated end plates [20] (**Figure 2B**, **C**). Although the loss of NMJs is typically observed early during the course of the pathology induced by mSOD1, the degeneration of MN cell bodies in the ventral horn starts at P100 and rapidly progresses, in line with the "dying back" process described by [14].

Overall, these studies highlight a predictable course of degeneration in the SOD1 mouse model, with evident differences in the vulnerability of the different subtypes of spinal MN. Whereas FF MNs are more sensitive to the pathology than FR MNs, the S MNs appear remarkably resistant to the disease. Saxena *et al*. identified these subtypes of MNs using a retrograde tracer locally injected either in the lateral compartment of the *gastrocnemius* muscle, mainly innervated by FF MNs [3], or in the *soleus* muscle innervated by FR and S MNs [13]. The labeled MNs were collected by laser microdissection and their transcriptome analyzed using Affymetrix microarrays. This advanced approach has revealed molecular mechanisms that were undetectable when analyzing the whole ventral spinal cord. In Section 3, we will discuss our current understanding of the mechanisms underlying the observed discrepancies in MN vulnerability.

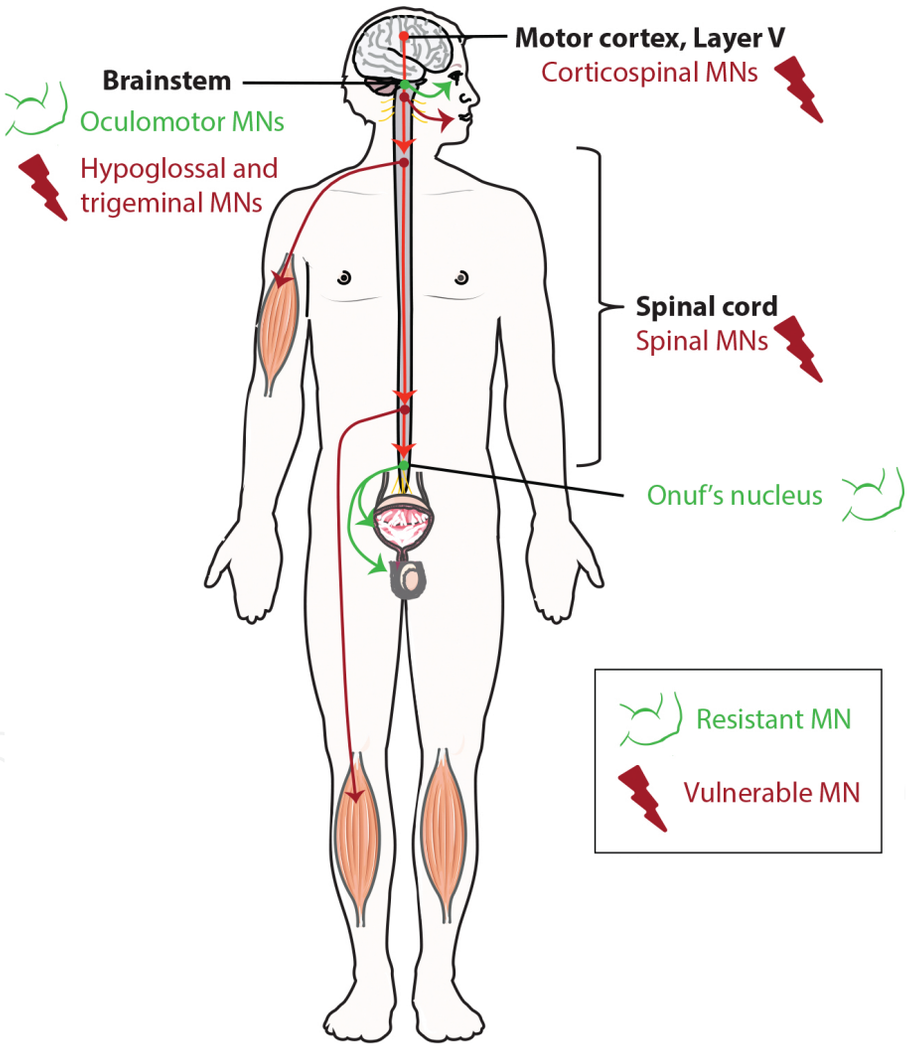

Figure 1. Transversal comparison of the vulnerability of different populations of motoneurons. Corticospinal, hypoglossal and spinal motoneurons (MNs), as well as neurons located in the trigeminal nucleus, progressively degenerate during the course of ALS. In contrast, the oculomotor neurons of the third cranial nerve, located in the brainstem, and which control eye movements, are resistant to disease. Motoneurons located in the Onuf's nucleus in the sacral region of the spinal cord, and which are responsible for the sexual and bladder functions, are also resistant to ALS.

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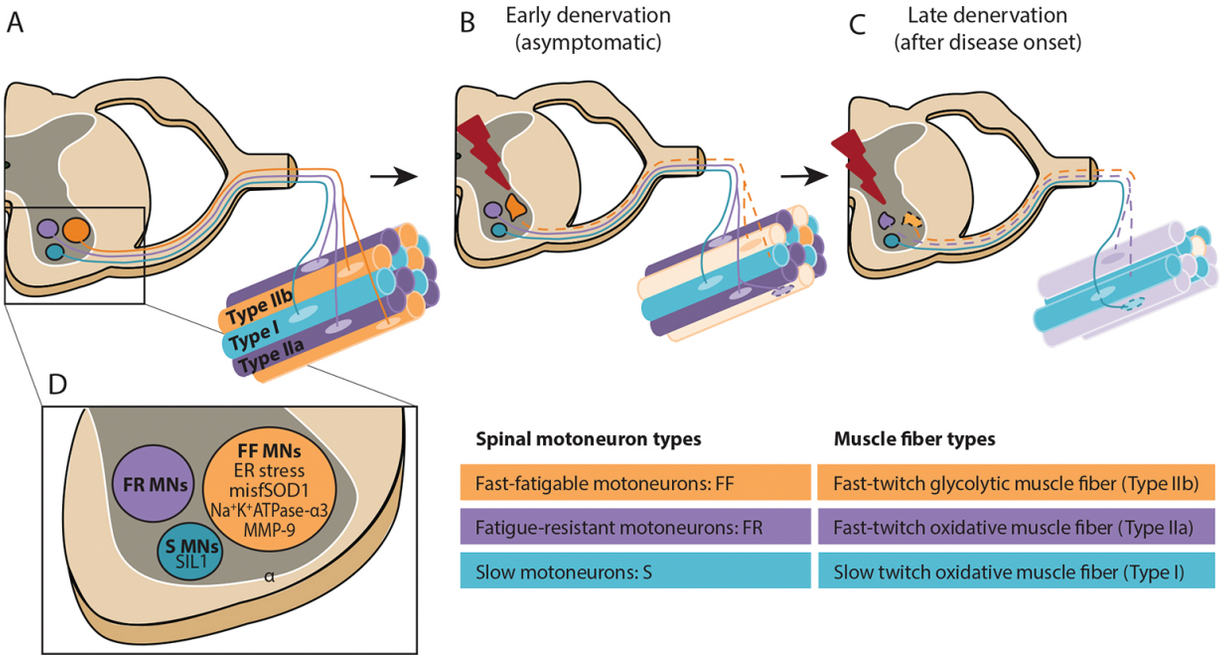

Figure 2. Longitudinal comparison of lumbar motoneurons during progression of the SOD1 pathology. (A) Organization of the motor units in the lumbar spinal cord. (B) The fast fatigable motoneurons (FF) are the first ones to degenerate in the SOD1 ALS mouse model. This degenerative event corresponds to the early denervation of the type IIb muscle fibers, before the onset of symptoms. (C) A second wave of denervation is observed when the fatigue-resistant motoneurons (FR) degenerate, mainly affecting the innervation of the type IIa muscle fibers and further progressing after symptom onset. Note that the axons of the resistant FR and S motoneurons can sprout and reinnervate the vacant neuromuscular junctions. The slow (S) motoneurons are resistant almost until disease end stage. (D) FF motoneurons are characterized by early ER stress and high amount of misfolded SOD1, and they abundantly express MMP-9 and the Na^+/K^+ ATPase-α3 pump. Disease-resistant S motoneurons are characterized by the expression of the ER chaperone SIL1.

2.1.2. Corticospinal MNs

The CSMNs localized in the layer V of the motor cortex are responsible for the initiation of the voluntary movement. These glutamatergic neurons collect, integrate, translate and transmit signals to lower MNs located in the spinal cord (**Figure 1**). CSMNs degenerate and die in ALS patients [21, 22]. There is also experimental evidence for the degeneration of CSMNs in the SOD1 animal models of fALS [23]. However, their role in the $SOD1^{G93A}$ mouse model has been long overlooked; as without specific markers, it was difficult to discriminate these cells from other types of pyramidal neurons located in the layer V of the cortex. Retrograde tracers, as well as some adeno-associated viral (AAV) vectors able to retrogradely transduce neurons from their axonal projections, have proved their utility to study CSMNs [24]. Using retrograde tracers, a degeneration of the corticospinal tract and a loss of CSMNs were observed at end stage in $SOD1^{G93A}$ fALS mice [25]. By combining retrograde tracers together with morphological and molecular approaches, Ozdinler *et al*. could demonstrate that CSMNs degenerate as soon as P30 in $SOD1^{G93A}$ fALS mice [26]. Indeed, they observed a decrease in the size of CSMN somas, which were also found positive for markers of apoptosis. Already at P5, 2% of the neurons positive for CTIP2 (a transcription factor specific for CSMNs in layer V) display

pyknosis. However, the most dramatic degeneration of the corticospinal tract is observed between P60 and P120, accompanied by astrogliosis and microgliosis in the neocortex at a later stage of the disease. Later, CSMNs could be easily identified in a transgenic mouse line expressing eGFP under the control of the ubiquitin carboxy-terminal hydrolase L1 (UCHL1) promoter [27]. By crossing these mice with $SOD1^{G93A}$ mice, it was possible to analyze eGFP-expressing upper MNs in the context of ALS [28]. In the near future, this mouse model could be used for transcriptomic and proteomic analyses, which may provide further insight into the molecular mechanisms occurring in CSMNs during the course of ALS. A recent study by Gautam *et al*. [29] used the UCHL1-eGFP mice crossed with an Alsin KO mouse line to study in CSMNs the impact of ALS2, a gene involved in a small percentage of fALS cases. Intriguingly, Uchl1 null mice completely lacking UCHL1 activity show motor deficits, with a progressive and dramatic loss of CSMNs [30]. Although Uchl1 has not been linked to ALS yet, it will be interesting to further address the role of this gene in ALS. Moreover, analyzing the spinal MNs in this mouse model could shed some light on the impact of the CSMN degeneration on lower MNs.

However, it remains unclear what is the exact contribution of CSMN degeneration to the deficits observed in animal models of the SOD1 fALS pathology. Importantly, the silencing of mSOD1 in the cortex of $SOD1^{G93A}$ rats can delay disease onset, and extends animal survival [31]. Even more, the treatment has neuroprotective effects on spinal MNs, preventing denervation of the NMJs. These data highlight the role of CSMNs in ALS, which may have been underestimated. Further longitudinal studies will be needed to explore the molecular mechanisms leading to CSMN degeneration in ALS and uncover potential therapeutic targets.

2.2. Transversal studies to compare motor nuclei

2.2.1. Onuf/oculomotor versus spinal/hypoglossal MNs

Pools of MNs located in the Onuf's and oculomotor nuclei are considered resistant to the disease. MNs located in the oculomotor nucleus are responsible for most of the eye movements. This function remains mostly intact in ALS patients, although slight defects can be detected toward end stage, in patients maintained with respiratory assist devices (reviewed by [32]). Similarly, the disease does not affect the survival of MNs in the Onuf's nucleus, which controls sexual and bladder functions [1]. In this nucleus, only few degenerating hallmarks have been observed, such as pyknotic nuclei or Bunina bodies [1]. The apparent resistance of the oculomotor and Onuf's nuclei contrasts with the progressive degeneration of spinal, hypoglossal and trigeminal MNs responsible for the locomotion, swallowing and the control of the jaw musculature, respectively [33, 34] (**Figure 1**).

Several research groups have taken advantage of the differential vulnerability between these motor nuclei to investigate the underlying molecular mechanisms [35–37]. These studies used techniques including the local injection of retrograde tracers, laser microdissection of cell subtypes and high-throughput analysis of gene expression.

Using microarrays, Kaplan *et al*. [38] determined in wild-type (WT) mice the gene expression profiles of the oculomotor and Onuf's nuclei, as compared to the vulnerable spinal MNs. A set

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of genes, including the matrix metalloproteinase 9 (MMP-9) and Hydroxysteroid (17-Beta) Dehydrogenase 2 (Hsd17b2), was found to be expressed at higher levels in the vulnerable spinal MNs, as compared to the resistant ones. Conversely, semaphorine3e (Sema3e) showed higher expression in the resistant MNs.

Another study compared oculomotor MNs, hypoglossal MNs and cervical spinal MNs in WT rats, and found that the pattern of expression of a set of genes was specific for each of these MN subpopulations [39]. Based on those results, protein levels were determined for six key genes, comparing their expression in WT and $SOD1^{G93A}$ mice [40]. This study identified a protein expression signature specific to the disease-resistant oculomotor MNs, as compared to hypoglossal and spinal MNs. The $GABA_A$ receptor α1, the guanylate cyclase soluble subunit α3 and the parvalbumin protein were highly expressed in the resistant MNs. Conversely, vulnerable MNs displayed higher protein levels of dynein, peripherin and $GABA_A$ receptor α2. These data suggest that differences in excitability, calcium handling and retrograde transport machinery may underlie the observed vulnerability pattern. Remarkably, these differences in protein expression were found to be conserved in the mouse and human species [40].

In an electrophysiological study using the high-copy $SOD1^{G93A}$ mouse model, Venugopal *et al*. [41] compared the excitability of the vulnerable trigeminal MNs (TMNs) and the resistant oculomotor MNs *ex vivo*, at P8–12. Using a system based on the membrane properties of MNs, and a statistical clustering approach to predict the type of motor unit—i.e. FF, FR or S—, they could determine early perturbations of the firing threshold in the $SOD1^{G93A}$ MNs. TMNs with low excitability had a decreased threshold, whereas the subpopulation of highly excitable TMNs had an increased firing threshold. Remarkably, no such electrophysiological effects of the $SOD1^{G93A}$ pathology were observed in the oculomotor MNs.

Overall, despite the fact that these different pools of MNs have similar physiological roles, they display clear differences in their vulnerability to the ALS pathology. Transversal studies have already identified some of the mechanisms that may confer MN susceptibility to the disease.

3. Possible mechanisms implicated in selective motoneuron vulnerability

Here, we will summarize both intrinsic and extrinsic mechanisms, which may underlie the differences between subtypes of MNs in their ability to cope with the ALS pathology.

3.1. Intrinsic mechanisms

3.1.1. SOD1 misfolding and ER chaperones

One of the leading hypotheses for the cause of ALS is the gain of toxic properties resulting from the accumulation of misfolded proteins including SOD1. In normal conditions, the cytosolic SOD1 protein undergoes several maturation steps to acquire proper structure and

function. These steps include the binding of copper and zinc and the formation of an intramolecular disulfide bond, to form an enzymatically active and stable homodimer [42]. When the SOD1 protein carries pathogenic mutations, it has an increased propensity to misfold (misfSOD1), leading to aggregation and accumulation in organelles such as the Endoplasmic Reticulum (ER) and the mitochondria [43]. Aggregation of SOD1 has been observed in patients with fALS [44, 45] and has also been reported in sporadic cases of ALS (sALS) [45, 46]. Using antibodies that specifically recognize misfSOD1 [47], Saxena *et al*. [7] found an accumulation of misfSOD1 in the FF MNs, as soon as P7 in the high-copy $SOD1^{G93A}$ mouse model (**Figure 2D**). In order to determine the changes in gene expression concomitant with the accumulation of misfSOD1, they performed a longitudinal study to compare the transcriptional profile of FF and FR/S MNs in the lumbar spinal cord [13]. Remarkably, the FF MNs are subjected to ER stress already at 3 weeks of age, which is 20 days before the initial denervation of the corresponding muscle in this animal model. It is only later, at about P50, that the more resistant FR MNs display similar gene expression changes indicative of ER stress. No such response is observed in the S MN almost until end stage, which is consistent with their apparent resistance to disease.

In-depth analysis showed that two ER chaperones have an important role in MN vulnerability: Calreticulin (CRT) and SIL1 (**Figure 2D**). CRT is a protein that binds Ca^{2+} ions. Interestingly CRT has been shown to be two-fold decreased in the FF MNs compared to S MNs, as soon as P38 [48]. *In vitro*, the authors showed that the lower CRT expression contributes to ER stress and calcium homeostasis disturbance, eventually leading to MN death. The role of CRT was confirmed *in vivo*, by crossing $SOD1^{G93A}$ mice with heterozygous $CRT^{+/-}$ mice [49]. The lower level of CRT expression accelerates the progression of the SOD1 pathology, prompting the initial loss of NMJ and leading to early muscle weakness. However, low CRT expression did affect neither the long-term survival of MNs nor animal survival, indicating that CRT is mainly implicated in the most vulnerable FF MNs during the early phase of the disease.

In an effort to find factors regulating the ER stress response in specific pools of MNs, Filézac de l'Etang *et al*. [50] analyzed in WT mice by real-time PCR the expression of a series of genes involved in protein folding, protein quality control and stress sensing, and compared the level of gene expression in FF and FR/S MNs. SIL1 was found to be six-times more expressed in S MNs than in FF MNs. SIL1 is a cochaperone protein, which increases the availability of the ER chaperone BiP by catalyzing the release of ADP from the ADP-BiP complex, and thereby facilitates the dissociation of BiP from its substrates. In the spinal cord of $Sil1^{-/+}$ mice, the ER unfolded protein response (UPR) is restricted to the FF MNs, whereas in Sil1 null mice, ER stress signaling is induced in all MNs. In the $SOD1^{G93A}$ ALS mice, SIL1 expression is reduced in FF MNs, compared to S MNs. However, when SIL1 is overexpressed in MNs using an AAV6 vector, UPR and ER stress signaling are reduced. Moreover, lifespan is prolonged by 84 days in the low-copy $SOD1^{G93A}$ mouse model, and 37 days in the high-copy $SOD1^{G93A}$ mouse model. Conversely, reduced SIL1 activity accelerates the ALS pathology in $Sil1^{-/+}$ $SOD1^{G93A}$ mice, and reduces the animal lifespan. Finally, both *in vitro* and *in vivo* experiments show that SIL1, coupled with BiP activity, controls ER homeostasis.

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3.1.2. MMP-9

By comparing cranial and spinal MNs with the disease-resistant oculomotor and Onuf's nuclei in WT mice, the MMP-9 protein was found to be expressed only in the vulnerable MNs [38]. MMPs are zinc-dependent endopeptidases able to degrade extracellular matrix and basement membrane components. Although the role of MMP-9 in the context of ALS is still poorly understood, it might be involved in the disruption of the neuronal extracellular matrix interaction (reviewed by [51]). MMPs have also been shown to promote the inflammatory process [52]. Kaplan *et al*. [38] found that MMP-9 is highly expressed in the FF MNs, in contrast to the S MNs, which have undetectable MMP-9 levels (**Figure 2D**). Crossing $SOD1^{G93A}$ mice with MMP-9 null mice [53] has significant effects on the disease process [38]. The denervation of the fast twitch *tibialis anterior* muscle is delayed by more than 80 days, and the mouse survival increased by 25%. Conversely, AAV6-mediated overexpression of MMP-9 in the FF MNs accelerates the denervation of the *tibialis anterior* in $SOD1^{G93A}$ mice. However, MMP-9 overexpression in oculomotor neurons does not induce extraocular muscle denervation, suggesting that other factors may contribute to the resistance to disease in this population of MNs.

Although no mutations in MMP-9 gene have been linked to ALS yet, He *et al*. [54] found an association between the C(-1562)T polymorphism in the MMP-9 gene, and risk to develop Parkinson's disease and sALS.

3.1.3. Neuronal excitability

FF and S MNs also differ in their excitability profile. Because of their small soma size, the S MNs have high input resistance, and therefore need less synaptic activation to initiate an action potential. As compared to MNs with a larger soma size, the firing threshold is reached earlier in S MNs, followed by the FR and finally the FF MNs [55]. S MNs are therefore considered to be highly excitable, whereas FF MNs are poorly excitable. S MNs innervate slow twitch muscle fibers, which are typically part of the postural muscles, whereas the poorly excitable neurons control fast twitch fibers, highly present in phasic muscles, and which are used when strength or rapid response is needed. A recent study by Saxena *et al*. [7] explored how neuronal excitability might contribute to the vulnerability profile in each of these MN populations. They were able to genetically modulate MN excitability *in vivo* using a floxed pharmacologically selective actuator module (PSAM), coupled either to the 5HT3-receptor to induce neuronal depolarization, or to the glycine-receptor to induce neuronal hyperpolarization. By activating these channels with a specific ligand, they could show that enhancing neuronal excitability decreases accumulation of misfSOD1 and BiP in MNs, which reduces ALS pathology [7]. Conversely, the reduction of MN excitability enhanced accumulation of misfSOD1 and BiP, accelerating disease progression.

Recently, a study further explored the link between misfSOD1 accumulation and MN excitability. Ruegsegger *et al*. [56] found that misfSOD1 binds the Na^+/K^+ATPase-α3 pump, impairing its ATPase activity. More specifically, misfSOD1 targets a 10 amino acid stretch, which is present in Na^+/K^+ATPase-α3, and not in the closely related Na^+/K^+ATPase-α1 isozyme. Remarkably, Na^+/K^+ATPase-α3 is the main pump expressed in the vulnerable FF MNs. In contrast, FR MNs have similar expression of both Na^+/K^+ATPase-α1 and α3, whereas S MNs

express almost exclusively Na^+/K^+ATPase-α1. Therefore, there could be a link between MN vulnerability and the expression of Na^+/K^+ATPase-α3, which may lead to ion imbalance when exposed to the deleterious effects of misfSOD1.

Le Masson *et al*. [57] used a computational model to demonstrate the dramatic effects that ion imbalance could have in MNs. The low excitable FF MNs have a high-energy demand to trigger action potentials, increasing their need for ATP. Ion pump deficiency caused by misfSOD1 will then consequently increase intracellular cation levels, leading to a constant MN depolarization. The induced burden on the mitochondrial function may affect ATP production, leading to a deficit in ATP used to restore ion homeostasis. Overall, the instability is increased, spreading ion imbalance within the MNs. Overall, these studies highlight the role of neuronal activity in each subpopulation of MNs exposed to the ALS pathology. These findings can be exploited to pinpoint key targets present in some neuronal subtypes to develop novel therapies.

3.2. Extrinsic mechanisms

3.2.1. CNS compartment

Over the 20 past years, it has been long debated whether ALS should be considered as a cell autonomous disease, mainly taking place in the MNs. It is now well established that astrocytes, oligodendrocytes and microglia also play a role in the pathology (reviewed by [58, 59]). Animal models of the SOD1 pathology have been intensively used to address this question. First, when the mSOD1 was selectively expressed in neurons using a panneuronal promoter, an ALS phenotype was observed only after 400 days [60]. It was next observed that expression of mSOD1 with a MN-specific promoter does not produce any ALS phenotype [61, 62]. Similarly, expression of mSOD1 only in astrocytes or microglia failed to produce any pathology [63, 64]. Next, several studies used fALS mice carrying a floxed mSOD1 transgene to selectively excise the transgene by expressing the Cre recombinase only in a given cell types. Using this approach, they could demonstrate that mSOD1 expression in MNs determines the time of disease onset. Expression of mSOD1 in either astrocytes or oligodendrocytes affects both disease onset and progression [65, 66], whereas the pathogenic contribution of mSOD1 in microglial cells is mainly observed during disease progression [67]. Similar effects have been observed with a different approach, using an AAV-based system to selectively express an artificial microRNA to target mSOD1 either in MNs or in astrocytes [68].

The majority of the vulnerable MNs are already degenerating before the clinical onset of the disease (**Figure 2**). When the resistant pool of MNs undergoes degenerative changes, they are typically exposed to a local environment containing reactive glial cells. Indeed, astrocytes and microglia become activated mainly in the late phase of the pathology. Several studies have shown their implication in the progression of the disease after the onset of the symptoms, most likely via their pathogenic interaction with the remaining MNs.

These findings point out the importance to investigate the role of broad range of cell types in the ALS pathology. Sun *et al*. [69] have recently addressed the temporal sequence of gene expression changes occurring in glial cells in the $SOD1^{G37R}$ mice. They applied the translating ribosome affinity purification (TRAP) technique [70], coupled with high-throughput RNA

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sequencing, to investigate the gene expression changes in MNs, astrocytes and oligodendrocytes. Just before disease onset, they observed the most prominent gene expression changes in MNs, mainly affecting the ER stress pathways. Later, astrocytes show changes in the expression of genes mostly involved in inflammation and metabolism. In oligodendrocytes, gene expression is most significantly changed at an early symptomatic stage. The observed dysregulation affects genes implicated in the myelination and lipid signaling pathways. This result somewhat contrasts with the delay in disease onset, which has been previously observed following the selective excision of mSOD1 in oligodendrocytes [65].

Oligodendrocytes are known to support the metabolic activity and the myelination of axons in the CNS (reviewed by [71]). Degenerating oligodendrocytes have been observed in ALS mice and patient tissues. Moreover, the pool of oligodendrocyte progenitors, identified by the expression of NG2, fails to properly differentiate, and they generate oligodendrocytes lacking expression of the myelin basic protein (MBP) and monocarboxylate transporter 1 (MCT1) [65, 72]. The loss of MCT1 may contribute to MN vulnerability, as this transporter is normally required for the supply of lactate, which is an important energy substrate for axonal support [73]. Remarkably, MCT4—another lactate transporter— is preferentially expressed in astrocytes and is reduced in ALS [74].

Although the role of astrocytes in ALS has raised a lot of interest, the mechanisms underlying astrocyte-mediated toxicity toward MNs are still incompletely resolved. The interaction between astrocytes and MNs has been mainly explored *in vitro*, using coculture systems. Recently, Meyer *et al*. [75] were able to differentiate "induced astrocytes" (i-astrocytes) from neuronal progenitor cells derived from the fibroblasts of ALS patients. In cocultures of MNs and i-astrocytes derived from SOD1 ALS, C9ORF72 ALS and sALS patients, the astrocytes are toxic to MNs, similarly to cocultures using astrocytes derived from the ALS spinal cord [9, 76]. This study suggests that astrocytes may convey toxic effects toward MNs, regardless of their tissue of origin. One of the first mechanisms proposed for astrocyte toxicity is the potential excitotoxicity caused by glutamate mishandling (reviewed by [77]). In ALS patients, there is reduced expression of the excitatory amino acid transporter 2 (EAAT2), which is the main glutamate transporter in astrocytes [78–80]. Inefficient removal of glutamate from the synaptic cleft may lead to MN hyperactivation and to a massive entry of calcium. Calcium influx into MNs can overload the storage capability of the ER and mitochondria, particularly in MNs where calcium storage could be already impaired [57, 81]. Several studies have highlighted the secretion of toxic factors by glial cells expressing mSOD1. Indeed, *in vitro* experiments have shown that activated astrocytes and microglia expressing mSOD1 secrete toxic factors such as FasL, nitric oxide (NO) and the IFN-γ cytokine. These factors can induce the death of MNs expressing mSOD1 (reviewed by [82]). In particular, the coculture with mSOD1 astrocytes leads to the selective death of MNs mediated through the LIGHT-lymphotoxin-β receptor death pathway [83].

Therefore, although glial cells are essential to support the function and metabolism of MNs in the healthy CNS, they are likely to play an active role in the disease process. Astrocytes, oligodendrocytes and microglial cells carrying ALS-causing mutations appear to malfunction, leading to the selective death of MNs.

The release of misfSOD1 is another major component, which may contribute to MN degeneration via extrinsic mechanisms. As mentioned before, misfSOD1 plays an important role in the degeneration of MNs and its toxicity has been implicated in many cellular dysfunctions (review by [58]). Most importantly, recent evidence suggests a role of misfSOD1 also in some sALS cases that are not related to SOD1 mutations. It has been demonstrated that misfSOD1 can convert WT SOD1 [84, 85] leading to the formation of fibrils and aggregates *in vitro* [86]. High expression of WT SOD1 can lead to an ALS-like phenotype in mice [87]. A prion-like mechanism of propagation of misfSOD1 from cell to cell has been highlighted in the past few years (reviewed by [88]). In ALS, exogenous mSOD1 protein [85], as well as the WT SOD1 protein [89], has been shown to penetrate the cell membrane of neuron-like cells by mechanisms related to macropinocytosis. Cell-to-cell transfer of misfSOD1 may also be mediated by the ER chaperone chromogranin or through exosomes [26, 90]. Importantly, disease onset occurs earlier in mSOD1 mice crossed with mice overexpressing chromogranin A, demonstrating that this mechanism may have an important role *in vivo* [91]. Basso *et al*. [90] highlight a potential role for astrocytes in mSOD1 propagation. Indeed, they compared *in vitro* the proteome and secretome of WT SOD1 and $SOD1^{G93A}$ astrocytes. Interestingly, these latter produced less protein than the WT SOD1 astrocytes. However, although fewer proteins are secreted by astrocytes expressing mSOD1, the amount of proteins shed via exosomes is increased. Furthermore, exosomes derived from astrocytes can transfer mSOD1 to MNs and induce cell death. It is proposed that astrocytes may secrete mSOD1 to limit the intracellular deposition of SOD1 aggregates. In turn, the released mSOD1 may exert toxic effects on neighboring cells. For example, exogenous forms of mSOD1 can be toxic to MNs *in vitro* via microglial activation [92].

Although experimental evidence for the propagation of misfSOD1 is still lacking *in vivo*, these results suggest that several cell types, including neurons, astrocytes and microglia, may contribute to the transfer of the protein and lead to toxic effects throughout the CNS. This pathogenic mechanism may participate in the cascade of events leading to MN degeneration, while the disease is progressing. With high level of SOD1 protein expression, and low levels of ER chaperones, MNs may be particularly vulnerable in case of exposure to misfSOD1.

3.2.2. PNS compartment

On top of the CNS components, it is also important to investigate the role of cells that may control the function of specific subtypes of MNs in the periphery, especially the Schwann cells and the skeletal muscle.

3.2.2.1. Schwann cells

The Schwann cells are the counterparts of oligodendrocytes in the peripheral nervous system (PNS), as they are the primary supporting and myelinating cells for the neurons in the PNS. Surprisingly, the suppression of mSOD1 in Schwann cells accelerates disease progression [93]. The terminal Schwann cells (TSCs) are also of particular interest as they play an important role in the maintenance, plasticity and regeneration of the NMJs (reviewed by [94]). Semaphorin 3A (Sema3a), a chemorepellent expressed by TSC, is involved in the repulsion of motor axons

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away from the end plate, leading to the denervation of the NMJ [95]. Remarkably, during reinnervation or toxin-induced paralysis of the *gastrocnemius* muscle, Sema3a is abundantly expressed by TSC located at the NMJ of type IIb/x muscle fibers, which are known to have low sprouting capacity [3, 96]. Even more intriguing is the upregulation of Sema3a in the TSC covering the motor nerve terminals innervating the type IIb/x muscle fibers in a mouse model of ALS [97]. Blocking the Sema3a receptor NRP1 was found to delay NMJ denervation in ALS mice, extending their lifespan [98], thus suggesting that Sema3a could contribute to the early loss of NMJ in these specific muscle fibers in ALS, and be implicated in their low sprouting capacity [3]. Moreover, a recent study has highlighted an increase of Sema3a levels in the motor cortex of ALS patients [99].

3.2.2.2. Muscle

One of the first changes observed in ALS patients and mouse models is the denervation of the NMJ, often long before the death of MNs. However, the role of the muscle in ALS is still debated (reviewed by [100]). Dobrowolny *et al*. [51] showed that overexpression of mSOD1 specifically in the muscle tissue leads to muscle atrophy and a loss of muscle strength. However, the shRNA-mediated silencing of mSOD1 in the muscle of SOD1 ALS mice, using either an AAV vector [101] or a lentiviral vector [102], does not provide any beneficial effect. The absence of any protective effect was confirmed using mice with Cre expression restricted to the skeletal muscle to suppress floxed mSOD1 in a tissue-specific manner [102]. Although these studies indicate that the skeletal muscle is not a primary site for the SOD1 pathology, it may have an important role in the maintenance of the neuromuscular connections. The neurite outgrowth inhibitor A (Nogo-A) is upregulated in the skeletal muscle of the mSOD1 mouse model and in ALS patients and this upregulation seems to occur specifically in the slow twitch muscle fibers [103]. Moreover, Nogo-A expression correlates with the severity of the clinical symptoms [104]. Overexpression of Nogo-A in the mouse muscle induces NMJ denervation, whereas crossing mSOD1 mice with Nogo-A KO mice protects the NMJ [105, 106]. Therapeutic approaches to block the action of Nogo-A have been proposed. Injection of an anti-Nogo-A antibody in $SOD1^{G93A}$ mice from the age of 70 days onward protects motor units and increases muscle strength in 90-day-old mice, although this protective effect seems to be lost at 120 days [107]. Ozanezumab, a humanized version of the anti-Nogo-A antibody, has been tested in ALS patients in a phase I clinical trial [108]. Overall, it appears that pathogenic processes taking place in the skeletal musculature can impact on the neuromuscular function, via mechanisms that may be specific to motor unit subtypes.

4. Identification and validation of therapeutic targets

Despite years of research and clinical testing, Riluzole remains the only FDA approved drug for ALS. It is therefore urgent to identify novel targets for therapeutic intervention against MN degeneration. The study of the different types of MNs highlights the fact that subpopulations of MNs can survive for long term and function in the context of the disease, which provides novel molecular targets for neuroprotective treatments.

One possibility is to identify factors that are active in the most vulnerable neurons, and design approaches to reduce their activity, with the hope to obtain neuroprotective effects in ALS. In SOD1 mice, MMP-9 has recently been shown to cause deleterious effects in the FF MNs, where it is preferentially expressed [38]. Edaravone is a free radical scavenger that inhibits MMP-9 upregulation [109]. This compound has been used since many years ago for the treatment of cerebral infarction or ischemic stroke. Edaravone has demonstrated therapeutic efficiency in the $SOD1^{G93A}$ mouse model [110] as well as in the $SOD1^{H46R}$ rat model [111], and more recently in the wobbler mice, which is often considered as a model for sALS [112]. A phase II clinical trial has shown that Edaravone can slow down the progression of the motor impairments in ALS patients, although this effect could not be statistically confirmed in a recent phase III trial [113]. Nevertheless, further analysis of the results has revealed the beneficial effects of the compound in a subgroup of ALS patients, according to the revised El Escorial diagnostic criteria, prompting the initiation of a new trial (http://www.alzforum.org/news/conference-coverage/does-free-radical-scavenger-edavarone-slow-als).(http://www.alzforum.org/news/conference-coverage/does-free-radical-scavenger-edavarone-slow-als). Of note, Edaravone is already approved in Japan for the treatment of ALS.

Another possibility is to identify proteins that are expressed only in the disease-resistant MNs. Here, factors implicated in the control of ER stress may play an important role in MNs (reviewed by [114]). Possible therapeutic approaches to relieve ER stress have been tested in the context of the ALS mice. For instance, Salubrinal has been shown to reduce ER stress [115]. In SOD1 mice, Salubrinal administration alleviates disease manifestation and slows down the progression of the disease [13]. However, Salubrinal as such cannot be used for treating ALS patients as it has been shown to affect long-term memory in mice [116]. Guanabenz is another FDA approved antihypertensive drug known to reduce ER stress. Its efficacy in ALS mice is however still controversial [117, 118]. It is therefore important to unravel targets that may be more specific to ALS. The discovery that the ER chaperones SIL1 and CRT are centrally involved in the most resistant populations of MNs has raised attention to these factors as potential specific targets [49, 50]. In particular, AAV-mediated overexpression of SIL1 in the MNs of ALS mice dramatically increases innervation of the NMJs and prolongs animal survival by 25—30%. However, it remains to be determined how to therapeutically target these factors in ALS patients, perhaps using adapted pharmacological approaches.

As the vulnerability of MNs could be caused by the accumulation of misfSOD1 in these cells, one potential therapeutic strategy is to prevent SOD1 toxicity by targeting the partially unfolded intermediates of the SOD1 protein that can later form aggregates. Israelson *et al*. [43] recently identified an ATP-independent protein chaperone called multifunctional macrophage migration inhibitory factor (MIF). This factor prevents the misfolding of SOD1, and decreases cell death in MNs expressing $SOD1^{G93A}$ *in vitro*. Moreover, this chaperone is expressed only at low levels in MNs, which may contribute to their selective vulnerability.

Another approach to prevent SOD1 misfolding is to provide the metal cofactors that are critical for the proper folding and stability of the functional Cu/Zn SOD1 dimer (reviewed by [119, 120]). CuATSM is a chelator widely used for PET-imaging as it rapidly carries copper across the blood-brain barrier into the CNS. Preclinical studies in SOD1 mouse models have reported

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beneficial effects of CuATSM, including on animal survival [121, 122]. Recently, Williams *et al*. [123] used transgenic SOD1^{G93A} mice coexpressing the Copper-Chaperone-for-SOD (CCS) protein, which is normally expressed in humans but not in mice. Although these mice do not live longer than 2 weeks, the treatment with CuATSM delayed onset and slowed down the progression of the disease, dramatically extending the lifespan of these animals to 18 months [123]. A phase I clinical trial will be soon initiated to test the effects of CuATSM in ALS patients.

Other strategies directly target SOD1 to prevent its deleterious effects. The use of antibodies specific for misfSOD1 has been proposed [47, 124, 125]. On the other hand, gene therapy techniques can be used to reduce the overall level of SOD1 expression. This approach can be considered as SOD1 null mice are viable and do not show any obvious motor dysfunction (reviewed by [126]). Viral vectors delivering artificial shRNA or microRNA for RNA interference against SOD1 [68, 127–130], as well as antisense oligonucleotides targeting the SOD1 mRNA [131], are currently being investigated to suppress the expression of this protein. It remains however debated whether these techniques could be effective in patients other than those carrying SOD1 mutations, as the role of SOD1 in sALS remains unclear [132]. Nevertheless, some mechanisms contributing to SOD1 toxicity, such as ER stress and UPR, are likely to be implicated in a broad range of ALS cases, providing opportunities for largely applicable treatments.

5. Selective neuronal vulnerability in non-SOD1 ALS and other motoneuron diseases

Most of the studies described in this chapter have used the SOD1 mouse model to explore the selective vulnerability of MNs in ALS [13]. The pattern on MN vulnerability in ALS patients is still a matter of speculation. At the late stage of the disease, there is a dramatic degeneration of the MNs throughout the spinal cord. However, as the access to *post-mortem* tissue at various stages of the disease is obviously limited, it has not been possible to precisely determine how the ALS pathology affects the different subtypes of MNs in patients. Nevertheless, the preservation of the oculomotor and Onuf's nuclei in ALS patients has been confirmed by histological analysis [133].

It is also likely that differences exist between familial and sALS, as well as between the different genetic forms of ALS. This may also reduce the efficacy of therapies tested in the SOD1 model. SOD1 mutations are the cause of ALS only in a small fraction of the patients. In addition, the SOD1 pathology does not appear to be associated with Frontotemporal-Lobar degeneration, which is linked with other forms of ALS (FTLD-ALS) [134]. Therefore, there is an urgent need for other models of ALS to develop therapies better adapted to the different forms of the disease. During the past 10 years, genetic studies have identified ALS-causing mutations in several genes mainly involved in RNA metabolism, such as FUS, TARDBP and C9ORF72. FUS and TARDBP represent around 3 and 5% of fALS, respectively, whereas C9ORF72 cases are more prevalent and cover 30—50% of fALS as well as 5—7% of sALS [134]. Moreover, these genes have been found mutated in both ALS and FTLD-ALS patients, and TDP-43 positive

inclusions are observed in non-SOD1 ALS patients [134]. Several research groups have generated rodents either overexpressing mutated forms of these genes or carrying gene deletions, with the objective to model the ALS pathology. Overall, the current rodent models for TDP-43-ALS or FUS-ALS display relatively mild MN degeneration. Axonal degeneration has been seen in the transgenic TDP-43 rat model, which seems to selectively affect the largest MNs [135]. This observation suggests that selective vulnerability is also likely to occur in the TDP-43 pathology.

Recently, more emphasis has been placed on modeling C9ORF72-ALS. Mouse lines generated with a bacterial artificial chromosome carrying a pathogenic hexanucleotide expansion in the full human C9ORF72 gene did not develop any behavioral symptoms in two independent studies [136, 137]. By contrast, using AAV vectors to express the G4C2 repeat expansion in the mouse CNS led to both histological and behavioral defects similar to the pathology observed in C9ORF72-ALS/FTLD patients [138]. Although 20% of neuron death was measured in the cortex, motor cortex and cerebellum of these mice, the number of spinal cord MNs was not reported. With the continuous development of ALS models, it will hopefully be possible to more accurately design therapeutic approaches against pathogenic pathways that may be common to different forms of ALS.

The recent development of mouse and human ES and iPS cells could provide an alternative to *in vivo* models. Several hundred lines with various mutations have been generated and are publicly available. In the context of SOD1, these models have been used to explore the non-cell autonomous pathogenic mechanisms. Survival of mES- or hES-derived MNs is decreased when the cells are grown on astrocytes differentiated from SOD1 or sporadic-ALS induced neural progenitors [76, 132]. MNs derived from patients carrying the C9ORF72 repeats display typical histopathological features, such as nuclear foci and the non-ATG translation of peptides encoded by the hexanucleotide repeats [139]. Remarkably, these neurons have an increased susceptibility to glutamate excitotoxicity. FUS, SOD1 and C9ORF72 ALS-derived MNs show electrophysiological abnormalities [140]. This technology presents the advantage to model a larger portion of ALS cases, allowing for comparative gene expression profiling experiments and testing of therapeutic compounds. However, it remains difficult to study the selective vulnerability of MN subtypes in such *in vitro* models, as they fail to replicate the differentiation into cellular subtypes and the precise architecture of the CNS tissue.

Lately, the transcriptome of the cerebellum and frontal cortex was analyzed *post mortem* in samples from C9ORF72-ALS and sALS patients [141]. The results show a prominent dysregulation of genes involved in the UPR and intracellular protein transport machinery in C9ORF72-ALS. In sALS, it is the genes involved in cytoskeleton organization and synaptic transmission, which are most affected. One should keep in mind that these molecular changes reflect only the late stage of the disease and in the future, it will be important to also access spinal cord tissue to perform similar transcriptome analysis.

More generally, it is tempting to speculate that the selective MN vulnerability observed in ALS may also apply to other MN disorders, such as spinal muscular atrophy (SMA) and Charcot-Marie-Tooth (CMT) diseases. In SMA patients, muscle biopsies show atrophy of the type II muscle fibers, with a compensatory hypertrophy of the type I fibers [142]. Moreover, the Onuf's

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nucleus is preserved, even in the most severely affected SMA type I patients. Even though atrophy is restricted to certain muscles in mouse models of the disease, it does not seem to be related to muscle fiber type or NMJ size [143]. However, the large size MNs innervating the proximal forelimb and axial muscles are specifically lost in presymptomatic Δ7 SMN mice [144]. This last observation could indicate a preferential vulnerability of some MN subtypes, although this will need to be confirmed with more specific markers to identify the FF or S MNs.

CMT is a heterogeneous group of genetic diseases affecting motor and/or sensory functions. Several genes have been identified linked to demyelinating CMT (CMT1) or axonal CMT (CMT2). Among the CMT2 cases, 20% are caused by mutations in the MFN2 gene encoding mitofusin 2 and are referred as CMT2A [145]. Several observations indicate a pattern of neurodegeneration, which may be very different from ALS. Biopsies of the *tibialis anterior* in CMT1 and CMT2 patients show atrophy of the type IIa muscle fibers and hypertrophy of the type I fibers [146]. Furthermore, in the late-onset CMT2A patients, the *soleus* muscle is affected before, and more severely than other leg muscles, including the *gastrocnemius* [147]. Few mouse models of CMT2 have been generated and are currently being studied. CMT2A mice based on the overexpression of $MFN2^{R94Q}$ develop motor deficits correlating with an overabundance of small axonal fibers in the sciatic nerve [148]. Further studies will be needed to characterize how CMT2A affects different subtypes of muscle fibers and MN populations, and leads to the observed neuromuscular impairments.

6. Conclusions

The identification of ALS-causing mutations in the SOD1 gene in 1993 has raised hopes in the ALS scientific community [10]. One year later, Gurney *et al*. [8] generated the well-known $SOD1^{G93A}$ mouse model, which reproduced most of the ALS features, and opened the way for exploring the causes of the disease and developing therapies. However, the challenge turned out to be more difficult than expected. Indeed, more than 20 years later, the identification of therapeutic targets in the mSOD1 mouse model has failed to bring any effective treatment to ALS patients.

Nevertheless, the characterization of mouse models overexpressing mutant forms of SOD1 has dramatically improved our understanding of the cellular processes leading to ALS. In particular, the very reproducible course of the disease observed in these mice has been instrumental to study the different stages of the disease, highlighting the fact that not all MNs are equally affected, and that glial cells are important actors in the pathogenic process. Recently, several research groups have identified critical factors, which determine MN vulnerability, providing novel targets for therapeutic intervention. One obvious difficulty in designing treatments for ALS is that at a given stage of the disease, the various pools of MNs or glial cells may face different toxic mechanisms, depending on their intrinsic vulnerability. This may limit the therapeutic efficacy of a single drug.

It remains unknown whether the observed MN vulnerability pattern is specific to mSOD1, and if the identified molecular mechanisms can be applied to other forms of ALS. To address this

question, it is critical to further develop animal and cellular models of ALS, based on the genes, which have recently been linked to the disease. By comparing these models, it will be possible to pinpoint common pathogenic pathways, and with the development of more specific biomarkers, apply therapies when and where they are the most likely to succeed.

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Author details

Cylia Rochat, Nathalie Bernard-Marissal and Bernard L. Schneider*

*Address all correspondence to: bernard.schneider@epfl.ch

Brain Mind Institute, Ecole polytechnique fédérale de Lausanne (EPFL), Lausanne, Switzerland

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