
Synaptic Transmission and Motoneuron Excitability Defects in Amyotrophic Lateral Sclerosis

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

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

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INTRODUCTION

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

ELECTROPHYSIOLOGICAL PROPERTIES OF MOTONEURONS IN ALS

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

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

Electrophysiological changes in motoneurons during embryonic and postnatal development

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

TABLE 1 Alterations of motoneuron electrophysiological features in ALS models

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

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

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

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

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

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

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

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

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

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

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

Intrinsic features of adult motoneurons in ALS experimental models

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

TABLE 2
Electrophysiological characteristics of motoneuron clusters in adult mice

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

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

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

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

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

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

ALS-ASSOCIATED INHIBITORY TRANSMISSION DEFECTS

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

GABAergic and glycinergic transmission

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

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

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

Postmortem histological studies of ALS tissues have mainly described a decrease in inhibitory GABAergic and glycinergic interneurons. A layer-specific reduction of calbindin (CB)⁺ neurons has been shown in cortical layers V and VI and in the ventral horn of spinal cords of patients with ALS (55–58). Analysis of the motor cortex of patients with ALS has also revealed a trend toward reduced calretinin (CR)⁺ cells and a reduction in parvalbumin (PV)⁺ cells (55, 59). Results from other studies also indicate that there are alterations in GABA homeostasis and transmission in cortical and spinal ALS inhibitory neurons. The motor cortices of patients with ALS exhibit a downregulation and an increase in the mRNA levels of the $\alpha 1$ -subunit and the $\beta 1$ -subunit of the GABA receptor, respectively, versus control motor cortices, which could indicate altered receptor function (60). This correlates with the reduced binding of flumazenil (an $\alpha 1$ -selective benzodiazepine antagonist) observed in positron emission tomography (PET) scanner studies (61) and the decrease in GABA levels observed by proton magnetic resonance spectroscopy (62) in the cortices of patients with ALS compared with controls.

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

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

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

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

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

Electrophysiological studies performed in spinal cords revealed more discrepancies in impaired inhibition transmission: no significant differences in GABAergic mIPSCs and GABAergic currents were observed between *SOD1^{G93A}* and control spinal cord cultured motoneurons (75, 76). However, GABA_A receptors had higher affinity and lower desensitization levels, and $\alpha 1$ subunit expression level were doubled in *SOD1^{G93A}* motoneurons. These differences could be the result of an adaptive process in response to reduced glycinergic inhibition but could also contribute to excitotoxic motoneuron death (75).

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

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

TABLE 3 ALS-associated features of inhibitory currents

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

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

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

Spinal embryonic neurons were isolated from E12–14 mice and cultured for 12–16 days (76), or from E15 and cultured for 8–10 days (75). Embryonic spinal cord preparations were obtained from E17.5 mice (25, 34). Brainstem slices were obtained from P4–P10 mice and midbrain slices obtained from P10–P12 mice (26). Cortical interneurons were isolated from E15.5 mice and cultured for 12 days (69). VP+ inhibitory interneurons (*Gad67-GFP*) were recorded in brain slices of *SOD1^{G93A}* (and control) at 6 (neonatal), 26–35 days (pre-symptomatic) and 90–101 days (symptomatic) of age (70). Brain slices were obtained from P3 *TDP-43^{93A}* mice (74), or from P9 *SOD1^{G93A}* mice (235). Layer 5 pyramidal neurons in brain slices from P15–P25 wobbler mice (76). Exposed spinal cord was prepared from 4 days post-fertilization larvae *Sod1^{G93A}* zebrafish (87). $[Cl^-]_i$, intracellular chloride concentration; E_{GABAAR} , reversal potential for $GABA_A$ R; mIPSC, miniature inhibitory postsynaptic current; PV, parvalbumin.

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

Recurrent and cortical inhibition

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Therapeutic approaches

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

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

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

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

In ALS, specific motoneurons are spared, such as the oculomotor and abducens populations (192), and gene expression studies have identified striking differences in genes responsible for the GABA and glutamate receptor subunits that may contribute to differential vulnerability. Indeed, in disease-resistant oculomotor neurons, $\alpha 1$, $\beta 1$, $\beta 2$, ϵ , $\gamma 1$, and θ GABA_A receptor subunits are upregulated, whereas the $\alpha 1$ subunit is consistently reduced in vulnerable spinal and cortical motoneurons in patients with ALS (60, 62, 193). In addition, the specific vulnerability of ALS-resistant and ALS-vulnerable motoneurons correlates with the subunit composition of GABA_A receptors, Gly/GABA_A receptor density ratios, and the incidence of synaptic versus extrasynaptic GABA_A receptors (194, 195). Considering that the subunit composition of GABA_A receptors determines the location of the receptor as well as its specific pharmacological and electrophysiological properties, differential GABA_A subunit expression will alter GABAergic receptor function (196–198). Thus, considering that an increase in GABA_A receptors could generate a better GABAergic influence and protection, the development of GABA receptor subtype-selective compounds to counteract reduced inhibitory activity and modulate inhibition may be another interesting future therapeutic approach.

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

ALTERATIONS IN THE MODULATORY TRANSMISSION IN ALS

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

Cholinergic transmission

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

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

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

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

Monoaminergic systems

The developmental assembly and function of the locomotor circuits is subject to neuromodulation to provide adaptive behaviors (214). The monoaminergic system that encompasses NA, 5-HT, and dopamine (DA) has been shown to influence the rhythmic firing pattern of motoneurons and contribute to the flexibility of locomotor functions with premotor inputs and sensory afferents. A reduction in descending serotonergic fibers, linked to reduced levels of 5-HT in the spinal cord of *SOD1^{G93A}* mice, has been reported as early as E17.5. 5-HT hyperpolarizes E_{GABAAR} through 5-HT₂ receptors in embryonic motoneurons; similar intensities are observed in wildtype and *SOD1^{G93A}* mice (215). During postnatal development, while the levels of DA, NA, and 5-HT in the lumbar spinal cord increase between P1 and P10, only DA is increased in *SOD1^{G93A}* mice compared with wildtype mice, although this difference is mainly due to changes in the dorsal part of the spinal cord (35). DA, NA, and 5-HT increase all exerts marked modulatory activity, by potentiating fictive locomotion in spinal cord preparations. However, NA is the only biogenic amine to differentially enhance motor burst in ALS mice, potentially through modulation of excitatory inputs (35).

In the adult spinal cord, DA levels in patients with ALS were shown to be similar to those in control individuals; NA levels were found to be increased, as was the ratio of 5-HT to its metabolite 5-hydroxyindole-3-acetic (5-HIAA) (216). However, another study in patients with ALS documented a loss of dopaminergic neurons in the substantia nigra (217), which is consistent with the reduced dopaminergic function and nigrostriatal DA deficits observed in patients (218, 219). A study in ALS mice showed that reduced numbers of dopaminergic neurons in the substantia nigra pars compacta and ventral tegmental area were associated with reduced levels of DA (220). PET analysis showed a decrease in the binding of a selective 5-HT_{1A} receptor in motor and extramotor regions of the brain in patients with ALS versus healthy volunteers (221). Another study reported that 5-HIAA/5-HT were elevated only in the lateral white matter of the cervical spinal cord of patients. A reduction in 5-HT₂ receptor binding, but not in the 5-HT_{1A} receptor, was also observed in the motor and premotor cortex (222). A more recent study revealed a loss of serotonergic neurons in the brainstem and their projections in the hippocampus and spinal cord of patients with ALS (223). This loss is also found in *SOD1^{G86R}* mice and correlates with reduced levels of 5-HT in the cortex, brainstem, and spinal cord, even at the non-symptomatic stage (223). In ALS mice, reduced serotonergic innervation is associated with upregulation of the 5-HT_{2B} receptor and development of spasticity, which can be abrogated by administration of a 5-HT_{2B/C} receptor antagonist (223, 224). Treatment of *SOD1^{G93A}* neonates with fluoxetine, a selective serotonin reuptake inhibitor that acts at presynaptic terminals to increase 5-HT levels, decreased motor performance and weight of adult mice, without affecting disease onset (225). Analysis of P10 spinal cord slices revealed that 5-HT depolarizes RMP, hyperpolarizes the persistent inward current peak and increases motoneuron excitability, despite wildtype and *SOD1^{G93A}* motoneuron showing similar responses (225). Of note, treatment of adult ALS mice with fluoxetine has no effect on disease course. This underlines the importance of the monoaminergic, and in particular the serotonergic, system during critical stages of development; it will have long-term effects on the motor system. Monoaminergic system changes, which have a critical influence

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

ALS-ASSOCIATED DEFECTS IN EXCITATORY TRANSMISSION

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

Glutamatergic inputs

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

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

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

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

interneurons (232). It was thus unexpected that, in symptomatic *SOD1^{G93A}* mice, inhibitory PV⁺ neurons became hyperexcitable, with a decrease in rheobase and a leftward shift in their output gain (without change in the maximal frequency of firing). However, this study did not address whether there was a partial loss of these inhibitory interneurons. This longitudinal analysis of cortical excitability highlights that neuronal plasticity occurs during disease progression, beginning with hyperexcitability at the neonatal stage, followed by normal excitability and a return to hyperexcitability at symptomatic stages of ALS. These results supporting an overall hyperexcitability of excitatory and inhibitory cortical neurons were further confirmed and suggested to involve compensatory mechanisms occurring all along disease progression in *SOD1^{G93A}* mice (70). It is interesting to note that spinal motoneurons also display hyperexcitability at embryonic and neonatal stages, which is followed by hypoexcitability in adults without reemergence of hyperexcitability. To further investigate the overall effects of neuronal hyperexcitability on the homeostasis of layer V neurons, intracellular Ca²⁺ levels were assessed using two-photon imaging of GCaMP6s-infected neurons (70). The main conclusion was that basal levels of intracellular Ca²⁺ are increased in *SOD1*-mutant layer V neurons, supporting a net hyperexcitability and/or an inability to maintain Ca²⁺ homeostasis, a factor that is known to be responsible for neuronal toxicity. It should be noted that spinal motoneurons have a poor capacity to buffer intracellular Ca²⁺ and are thus very sensitive to Ca²⁺-induced toxicity. Consequently, the hypoexcitability reported at symptomatic stages could be a compensatory mechanism to prevent Ca²⁺ overload.

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

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

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

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

Proprioceptive system

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

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

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

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

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

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

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

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

CONCLUSION

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

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