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Inhibitory Synaptic Regulation of Motoneurons: A New Target of Disease Mechanisms in Amyotrophic Lateral Sclerosis

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Abstract Amyotrophic lateral sclerosis (ALS) is the third most common adult-onset neurodegenerative disease. It causes the degeneration of motoneurons and is fatal due to paralysis, particularly of respiratory muscles. ALS can be inherited, and specific disease-causing genes have been identified, but the mechanisms causing motoneuron death in ALS are not understood. No effective treatments exist for ALS. One well-studied theory of ALS pathogenesis involves faulty RNA editing and abnormal activation of specific glutamate receptors as well as failure of glutamate transport resulting in glutamate excitotoxicity; however, the excitotoxicity theory is challenged by the inability of anti-glutamate drugs to have major disease-modifying effects clinically. Nevertheless, hyperexcitability of upper and lower motoneurons is a feature of human ALS and transgenic (tg) mouse models of ALS. Motoneuron excitability is strongly modulated by synaptic inhibition mediated by presynaptic glycinergic and GABAergic innervations and postsynaptic glycine receptors (GlyR) and $GABA_A$ receptors; yet, the integrity of inhibitory systems regulating motoneurons has been understudied in experimental models, despite findings in human ALS suggesting that they may be affected. We have found in tg mice expressing a mutant form of human superoxide dismutase-1 (hSOD1) with a Gly93 → Ala substitution (G93A-hSOD1), causing familial ALS, that subsets of spinal interneurons degenerate. Inhibitory glycinergic innervation of spinal motoneurons becomes deficient before motoneuron degeneration is evident in G93A-hSOD1 mice. Motoneurons in these ALS mice also have insufficient synaptic inhibition as reflected by smaller GlyR currents, smaller GlyR clusters on their plasma membrane, and lower expression of GlyR1α mRNA compared to wild-type motoneurons. In contrast, GABAergic innervation of ALS mouse motoneurons and $GABA_A$ receptor function appear normal. Abnormal synaptic inhibition resulting from dysfunction of interneurons and motoneuron GlyRs is a new direction for unveiling mechanisms of ALS pathogenesis that could be relevant to new therapies for ALS.

Keywords Chloride channel · Glutamate receptor · Glycine receptor · Hb9-eGFP · Excitotoxicity · Hyperexcitability · Interneuron · Renshaw cell

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Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and severely disabling fatal adult-onset neurological disease characterized by initial muscle spasticity, cramps, and fasciculations, and then muscle weakness, atrophy, and eventual paralysis and death typically 3 to 5 years after symptoms begin [1, 2]. More than 5,000 people in the USA are diagnosed with ALS each year (ALS Association, http://www.alsa.org), and the global incidence of ALS is about two per 100,000 persons [3]. The cause of the spasticity,

paralysis, and death is believed to be progressive degeneration and elimination of upper motoneurons in cerebral cortex and lower motoneurons in brainstem and spinal cord [1, 4, 5]. Other than life support management, no effective treatments exist for ALS [2, 3, 6]. The molecular pathogenesis of ALS is understood poorly [5, 7, 8], contributing to the lack of appropriate target identification and effective mechanism-based therapies to treat this disease. The majority of ALS cases are sporadic with few known genetic contributions [1, 5, 7].

Familial forms of ALS are autosomal dominant or recessive and make up ~5% of all ALS cases [7]. ALS-linked mutations occur in the genes encoding Cu/Zn superoxide dismutase-1 (SOD1, *ALS1*), Alsin (*ALS2*), senataxin (*ALS4*), fused in sarcoma (FUS, *ALS6*), vesicle associated membrane protein (VAMP/synaptobrevin)-associated protein B (VAPB, *ALS8*), p150 dynactin (DCTN1), TAR-DNA binding protein (TADBP or TDP43, *ALS10*), and optineurin (*ALS12*) [7, 9–12]. Variations in the phosphoinositide phosphatase *FIG4* gene cause *ALS11* [13]. Several other genes are believed to be susceptibility factors for ALS.

Mouse Models of SOD1-Linked ALS

Autosomal dominant mutations in the *SOD1* gene occur in ~20% of familial ALS cases [9, 14]. SOD1 is a metalloenzyme comprising 153 amino acids (≈16 kDa) that binds one copper ion and one zinc ion per subunit and is active as a non-covalently linked homodimer [15]. SOD1 is responsible, through catalytic dismutation, for the detoxification and maintenance of intracellular $O_2^{\bullet-}$ concentration in the low femtomolar range [15]. SOD1 is ubiquitous (intracellular SOD concentrations are typically ≈ 10–40 μM) in most tissues, possibly with highest levels in neurons [16]. Mutated human SOD1 (hSOD1) proteins appear to acquire a toxic property or function, rather than having diminished $O_2^{\bullet-}$ scavenging activity [14, 17, 18]. Wild-type SOD1 can gain toxic properties through loss of Zn [19] and oxidative modification [20, 21]. G93A-hSOD1 has enhanced free radical-generating capacity compared to wild-type enzyme [18] and can catalyze protein oxidation by hydroxyl-like intermediates and carbonate radical [22]. Toxic properties of mutant hSOD1 might also be mediated through protein binding or aggregation [23].

Transgenic (tg) mice expressing different mutated *hSOD1* genes (G93A, G37R, G85R) develop fatal motoneuron disease resembling ALS in humans [24]. In the original lines of mice, hSOD1 is expressed ubiquitously, driven by its endogenous promoter in a tissue/cell non-selective pattern, on a background of normal wild-type mouse SOD1 [24]. Effects of this human mutant gene in mice are profound. Hemizygous tg mice expressing a high copy number of the G93A-hSOD1 mutant become completely paralyzed and die at ≈ 16–18 weeks of age [24]. hSOD1 mice with reduced transgene copy number have a much slower disease progression and die at ≈ 7–9 months of age [24, 25].

The extent to which specific intrinsic abnormalities within motoneurons and other cells in spinal cord contribute to the pathogenesis in models of ALS is discrepant. In tg mice expressing mutant hSOD1 selectively in neurons driven by a Thy1 promoter, motoneuron degeneration was absent [26, 27]; however, other studies have shown that neuron-specific expression of mutant hSOD1 is sufficient to induce motoneuron degeneration in mice [28, 29]. Tg mice with astrocyte-specific expression of mutant hSOD1 did not develop disease [30], but cell culture studies reveal that mutant hSOD1-expressing astrocytes can cause degenerative changes in wild-type motoneurons and worsen toxicity of mutant hSOD1 in motoneurons [31]. Mutant hSOD1 expressed in microglia might also be toxic to motoneurons in mice [32, 33] and in cell culture [34]. Furthermore, degeneration of mutant hSOD1-expressing motoneurons that are surrounded by wild-type astrocytes or microglia appears delayed or prevented in chimeric mice [35], and transplantation of wild-type neural progenitor cells in spinal cord of mutant hSOD1 tg mice delays disease and extends survival, possibly by differentiating into glial cells [36]. However, elimination of proliferating microglia-expressing mutant hSOD1 in mice did not affect motoneuron degeneration [37]. More recently, a new tg mouse expressing mutant and wild-type hSOD1 only in skeletal muscle has been shown to develop motoneuron disease phenotype with cytopathological and biochemical changes very similar to those seen in human ALS motoneurons, demonstrating clearly a motoneuron-non-autonomous process for the degeneration of motoneurons in SOD1-linked ALS [38].

Disease Mechanisms in ALS

Many theories implicate perturbations in glutamatergic neurotransmission, axonal transport, proteasome and protein integrity, mitochondria, oxidative stress, copper chemistry, apoptosis, and inflammation in the mechanisms of ALS pathogenesis [4, 5, 39–42]. Some theories have been springboards for drug trials, but all therapeutic trials, except two with Riluzole, have failed.

Glutamate Excitotoxicity

One leading theory of ALS involves glutamate excitotoxicity [4, 42]. Reductions in the activity of glutamate transport occur in human sporadic ALS spinal cord [43]

due to loss of astroglial glutamate transporter [44]. This abnormality could theoretically increase the extracellular concentrations of glutamate at synapses on motoneurons, leading to glutamate receptor-mediated excitotoxicity [4, 45]. However, reduced glutamate transport occurs in several other neurodegenerative settings, including cerebral hypoxia–ischemia, Alzheimer's disease, and Parkinson's disease [46, 47], and sometimes in the absence of neurodegeneration [48] and thus is not specific to ALS etiology.

Interestingly, disease-specific changes in RNA editing of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptor subunit 2 (GluR2) have been found in spinal motoneurons in human sporadic ALS autopsy cases [49]. These data show that, in normal motoneurons, the genomic DNA codon for glutamine (CAG) transcribed in the GluR2 pre-mRNA is converted post-translationally by an RNA-acting adenosine deaminase (ADAR) to an arginine-encoding codon (CGG) and that this RNA editing is faulty in sporadic ALS [49]. Subunits derived from unedited GluR2-mRNA translated with a glutamine at the Q/R site are Ca^{2+}-permeable [50–52]. In postmortem human ALS spinal motoneurons, analyzed at a single-cell level acquired by laser capture microdissection, AMPA receptors have reduced GluR2 subunit editing by ADAR, but RNA editing is normal in cerebellar Purkinje cells [49] and in another motor neuron disease called spinal bulbar muscular atrophy [53]. Genetically engineered mice without ADAR develop pathological changes suggestive of motoneuron degeneration [54]. Moreover, tg mice expressing a mutated *GluR2* gene that encodes asparagine at the Q/R site, causing GluR2 to be unedited, develop late-onset motoneuron disease [55]. In contrast, G93A-hSOD1 mice with enforced expression of normal editable GluR2 have delayed disease onset and decreased mortality [56]. G93A-hSOD1 mice that lack GluR2 entirely develop worse disease [57].

Hyperexcitability

Intrinsic motoneuron hyperexcitability can also be a contributor to excitotoxic vulnerability. The excitability of a neuron is reflected by the cells ability to generate action potentials per unit of input [58]. Hyperexcitability of upper and lower motoneurons is a characteristic feature of human ALS and tg mouse models of ALS. Electrophysiological studies of live ALS patients reveal signs of hyperexcitability in motor cortex and surrounding areas [127]; however, such clinical studies lack cellular resolution and it is difficult to determine if the changes are intrinsic to specific cell populations. In tg mice, cultured embryonic spinal cord neurons expressing mutated hSOD1 have AMPA receptor channels with modified permeability, altered agonist cooperativity between the sites involved in the process of channel opening, and slower spontaneous synaptic events [59]. Intrinsic hyperexcitability of motoneurons is observed in neonatal (P7) spinal cord organotypic slices [60], neonatal (P4–P10) acute brainstem slices [61], dissociated embryonic spinal cord cell cultures [60, 62], and in embryonic cortical neuron cultures and acute slices of P29–P31 cerebral cortex [63] from high expressing G93A-hSOD1 mice. In these experiments, the passive membrane properties of motoneurons were unaltered, but the relationship between current injected and frequency of motoneuron firing was altered in mutant hSOD1-expressing cells, suggesting a change in membrane excitability. Motoneuron excitability abnormalities have also been reported in neonatal (P6–P10) acute spinal cord slices of G85R-hSOD1 mice and G93A-hSOD1 low-expressing mice [64]. In acute spinal cord slices from G85R-hSOD1 mice, lumbar motoneurons show reduced input resistance and altered frequency–intensity relationships in the second week of life, and these changes have been interpreted as structural changes indicative of larger motoneurons in mutant mice [65]. The signs of motoneuron hyperexcitability are thus occurring very early in disease progression. Even though the hyperexcitability abnormalities appear intrinsic to the motoneurons in ALS mice, it is currently not known if the changes are pathological or adaptive and if they are caused by direct actions of the mutant hSOD1 protein in motoneurons or indirectly by actions of the mutant protein in other cells such as skeletal myofibers, interneurons, or glial cells. Enhanced excitability of motoneurons in ALS mice does not appear to be due to increased monosynaptic input from group I muscle afferents, but might be related to increased excitability and bursting of spinal excitatory interneurons [66].

Abnormal motoneuron excitability in ALS could be due to involvement of Na^+ channels [60, 67, 68]. Tetrodotoxin can protect against excitotoxicity in cultured primary embryonic motoneurons from G93A-hSOD1 mice [68]. Voltage-dependent Na^+ channels in cultured primary embryonic motoneurons from G93A-hSOD1 mice also show enhanced recovery from fast inactivation, but voltage dependency of activation and steady-state inactivation appear normal [67]. A contributor to neuronal hyperexcitability is the persistent inward current generated by Na^+ channels [69]. Persistent Na^+ currents have been shown to be increased in spinal cord and cerebral cortex neurons in mutant hSOD1 tg mice in several different preparations, including dissociated embryonic spinal cord culture and neonatal spinal cord slices [60, 61, 64, 69] and dissociated embryonic cortical neuron culture [70]. The finding of increased persistent Na^+ current in cultured cortical neurons from mutant hSOD1 tg mice [70] is interesting because these mice generally do not develop overt cortical pathology [71], but subtle dendritic abnormalities have been found

on prefrontal cortex pyramidal neurons [72] which could be evidence for aberrant neuroplasticity.

Motoneurons in ALS have Deficient Inhibition

The glutamate excitotoxicity and hyperexcitability theories of ALS emphasize the contribution of excessive synaptic excitation in part through AMPA receptors [4, 42, 73] and Na^+ channels [60, 67–69], while the possibility of insufficient synaptic inhibition has been theorized based on clinical data [127] but largely ignored experimentally. Abnormal GABA and glycine levels are observed in ALS patients [74, 75]. In human ALS autopsy spinal cord, glycine binding sites are reduced in anterior horn [76, 77], and in human ALS autopsy motor cortex, some $GABA_A$ receptor subunits show reduced levels of mRNA expression [78]. Adult tg G93A-hSOD1 mouse spinal cord [79] and organotypic spinal cord-slice cultures from embryonic G93A-hSOD1 tg mice [80] show evidence for imbalanced excitatory and inhibitory innervations, possibly indicative of aberrant or failed neuroplasticity. Based on these findings, aberrant inhibition could also have a role in ALS mechanisms of disease.

Spinal cord motoneurons receive extensive glycinergic and GABAergic innervations from vast numbers of spinal interneurons [81–83], and they express glycine receptor (GlyR) and $GABA_A$ receptor chloride-conducting ion channels which regulate motoneuron excitability through various mechanisms (Fig. 1) [84]. GlyRs isolated from mammalian spinal cord are pentameric membrane proteins composed of α and β subunits (Table 1) [85]. GlyRs exhibit subtype heterogeneity due to four isoforms of α subunit (α1–α4) [86, 87]. GlyRs in immature neurons contain the abundantly expressed α2 subunit, while GlyRs in mature neurons contain predominantly the α1 subunit within 2 weeks after birth [88]. The α3 subunit is mainly expressed in the cerebellum [88] and the α4 subunit is a murine gene that is not expressed in humans [89]. The majority of glycinergic neurotransmission in adults is mediated by α1β GlyRs (Table 1). $GABA_A$ receptors are also pentameric pore-forming membrane proteins and can be assembled from six different α subunits, three different β subunits, three different γ subunits, as well as δ, ε, π, and θ subunits (Table 2) [90]. α and β subunits are both required to generate a functional GABA-gated chloride ion channel with the most common composition being $\alpha_1\beta_2\gamma_2$, but this varies depending on CNS regions and cell type (Table 2) [90]. In adult rat brain, facial, hypoglossal, and trigeminal motoneurons differ from oculomotor, trochlear, and abducens motoneurons in their expression of $GABA_A$ receptor subunits, GlyR/$GABA_A$ receptor density ratios, and presence of synaptic versus extrasynaptic $GABA_A$ receptors [91]. These findings might be important because, in human ALS, brainstem and spinal motoneurons are differentially affected; motoneurons in anterior horn and the facial, hypoglossal, and trigeminal cranial nerve nuclei are more vulnerable than motoneurons in Onuf's nucleus and the oculomotor, trochlear, and abducens cranial nerve nuclei [92].

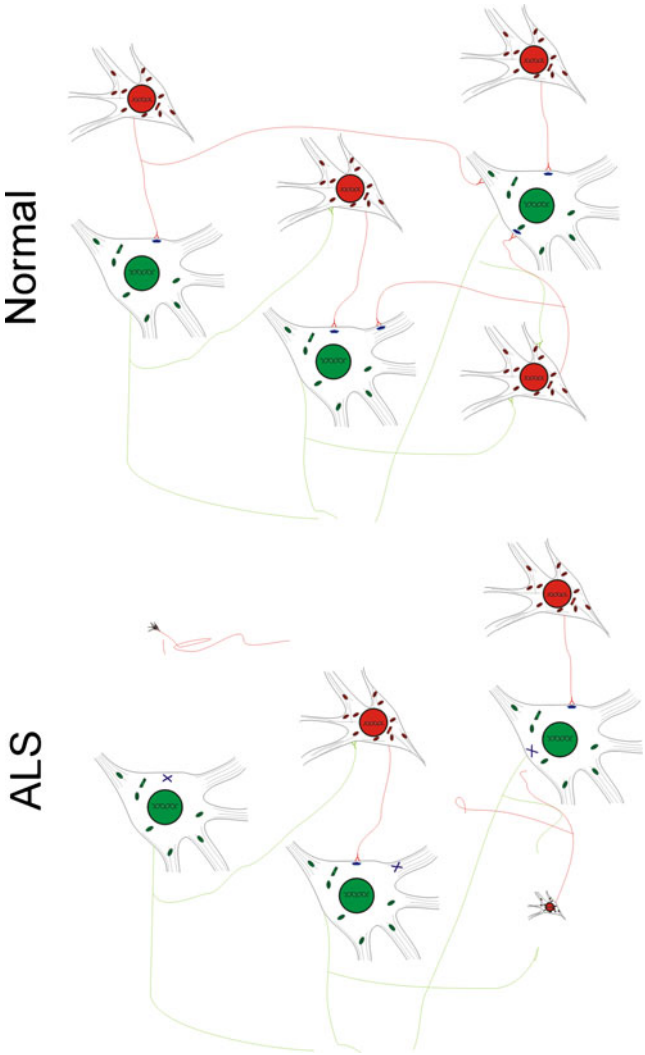

Fig. 1 Schematic drawing of inhibitory insufficiency of motoneurons in ALS spinal cord. Shown is a very simplified network of cells in ventral horn comprised of motoneurons (cells with green nucleus and green axon) and interneurons (cells with red nucleus and red axon). The green axons are exiting the spinal cord from the ventral root exit zone. In the normal network, motoneurons issue axons with collaterals that contact glycinergic inhibitory interneurons (Renshaw cells) whose activation in turn silences the activity of motoneurons that express glycine receptor clusters (blue postsynaptic complex). In ALS transgenic mice, the spinal cord undergoes network failure early in disease resulting from degeneration and loss of interneurons and abnormalities in motoneuron glycine receptor complex formation (*blue x*), possibly leading to hyperexcitability and excitotoxicity

Table 1 Glycine receptor composition

Isoforms	Subunit size (kDa)	Endogenous assembly (adult)	Comment
α1, α2, α3, α4	48	$\alpha1_3\ \beta_2$, $\alpha1_4\ \beta_1$	α4 is murine, not found in human
β	58		Binds scaffold protein gephyrin

Primary Cell Culture as a Model for Studying Motoneuron Function in ALS

In vitro models, including primary cell culture and slice preparations, offer several advantages in studying the function of individual neurons as well as neuronal circuits. First, these isolated preparations represent simplified systems that allow study of neuronal properties and cellular mechanisms without confounding by uncontrolled "outside" modifiers of synaptic and channel function such as steroids, hormones, and plasma proteins. Second, the internal and external solution of the neuron can be controlled, thus providing opportunities for testing the influence of drugs or mimicking the physiological conditions, but this can also cause "run-down." Finally, it is feasible to combine electrophysiological and powerful single-cell molecular or genetic techniques on these preparations, such as RT–PCR and genotyping, thus allowing for direct correlation between structure and function at the ion channel level. Compared to acute slice preparations, neurons maintained in dissociated primary cultures have better stability for patch-clamp recordings. Although dissociated embryonic spinal cord cultures have disrupted anatomic architecture, they can develop spontaneously synaptic connections mimicking the spinal cord architecture [93] and network synaptic activity, including bursts with oscillations as demonstrated with multi-electrode arrays [94]. Organotypic slice cultures of embryonic spinal cord have better maintained architecture [95]. Nevertheless, in dissociated embryonic spinal cord cultures, the neuronal surface is better exposed to allow more optimal access of solutions and pharmacological agents to neurons, and, since the neurons are in a monolayer with modest cellular vestments, the intracellular contents can be harvested more cleanly for single-cell RT–PCR (Fig. 2) [93].

Although primary cell culture systems are well suited for studying neuronal functions, the information on neuron physiology in models of ALS is sparse, in part, because of difficulties in unequivocally identifying motoneurons in culture. Previously, the identities of motoneurons in culture were confirmed using immunohistochemistry. Motoneurons in embryonic spinal cord cultures were fixed and identified using antibodies to non-phosphorylated neurofilament SMI-32 and the acetylcholine synthesizing enzyme choline acetyltransferase [96, 97]. We use tg mice that express enhanced green fluorescent protein (eGFP) driven by the Hb9 promoter [98–100] to identify motoneurons [83, 93]. Hb9-eGFP labeling reveals good morphological details (Fig. 2a), and, more importantly, it labels living motoneurons essential for electrophysiological studies. In these mice eGFP is also expressed in a small subset of spinal interneurons, but these cells are distinctly different morphologically from motoneurons in vivo and in culture [93, 101].

We developed using Hb9-eGFP tg mice a dissociated mixed total spinal cord culture that includes all neighboring cells in spinal cord capable of supporting the survival of cultured motoneurons. Motoneurons in our cultures stay healthy for at least 4 weeks without deterioration of morphology and physiological functions [93]. Our motoneuron cell culture approach is distinctly different from motoneuron enrichment techniques, utilizing density gradients [102], retrograde labeling [103, 104], and immunopanning [105], which give low yield of motoneurons or lack complete specificity for motoneurons. In addition, removal of other spinal cord neurons and glial cells that interact with motoneurons can alter their survival or

Table 2 $GABA_A$ receptor composition

Isoforms	Subunit size (kDa)	Endogenous assembly (adult)	Comment
α1, α2, α3, α4, α5, α6	52	$\alpha_1\ \beta_2\gamma_2$ (most common), but varies depending on CNS region and cell type	Must include both α and β subunits to form GABA-gated ion channel; α1 subunit binds gephyrin
β1, β2, β3	54		Interacts with phosphoinositide-3 kinase
γ1, γ2, γ3	53		Important for trafficking; has the benzodiazepine binding site
δ	51		Forms extrasynaptic receptors
ε	58		–
π	51		–
θ	72		–
ρ1, ρ2, ρ3	56		Do not co-assemble with other subunits but homooligomerize in retina

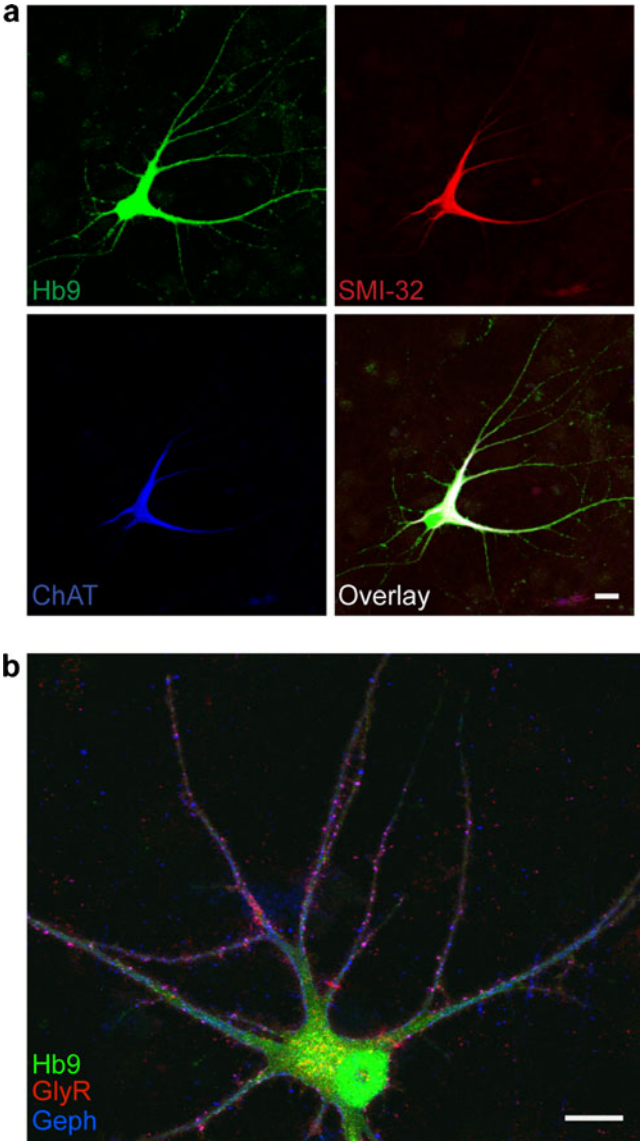

Fig. 2 Identification and receptor characterization of motoneurons in Hb9-eGFP mouse spinal cord cultures. **a** Confocal microscopy showing a representative example of an Hb9-eGFP (*green*) expressing motoneuron in dissociated spinal cord cultures co-labeled with neurofilament SMI-32 antibody (*red*) and choline acetyltransferase (ChAT, *blue*). *Scale bar*=20 μm. **b** Confocal image showing the localization of glycine receptors (GlyR, *red*) in close apposition to postsynaptic scaffold protein gephyrin (Geph, *blue*) on the surface of the soma and proximal dendrites of an Hb9-eGFP (*green*) expressing motoneuron. *Scale bar*=20 μm

phenotypes [106]. In previous electrophysiology studies, putative motoneurons in mixed spinal cord culture were visually identified by their morphological appearance and threshold diameter criteria (>28 μm) [62, 107]. Because the amount of motoneurons relative to all other cells in the total spinal cord culture is very low (<1%), and there are other cells that are large (>28 μm), this method does not reveal sufficient morphological detail and is not specific for motoneurons; thus, the number of motoneurons is prone to overestimation and the recordings could be from heterogeneous cells. Our culture model using genetic labeling of motoneurons solves these problems (Fig. 2) [93].

We used this motoneuron cell culture as a model to study physiological consequences of a genetic mutation causing ALS. Studies have shown that primary spinal cord neuron cultures express functional GlyRs forming postsynaptic receptor aggregates [108–110] and that adult GlyRα1 subunit was detected within the second week of culture [111, 112]. Using immunocytochemistry and confocal microscopy, we have demonstrated robust localization of the GlyRα1 on cultured motoneurons at times when recordings were made (Fig. 2b). Furthermore, we confirmed the postsynaptic localization of GlyRs and their associations with presynaptic glycinergic terminals by their colocalization with gephyrin (Fig. 2b), synaptophysin, and glycine transporter-2 (GlyT2) [93].

GlyRs are Abnormal in Spinal Motoneurons in ALS Mice

We are examining the consequences of ALS-causing mutant genes on neuron synaptic function because little is known about motoneuron functional changes in ALS and how mutant proteins cause functional abnormalities and disease. Using whole-cell patch clamp recordings, we have shown that glycine-evoked current density is significantly smaller in large-sized (diameter >28 μm) G93A-hSOD1 motoneurons compared with control. Furthermore, the averaged current densities of spontaneous glycinergic miniature inhibitory postsynaptic currents (mIPSCs) are significantly smaller in the large-sized G93A-hSOD1 motoneurons than in control motoneurons. In contrast, glycine-evoked currents and glycinergic mIPSCs in a morphologically distinct subgroup of medium-sized (diameter 10–28 μm) Hb9-eGFP^{+} motoneurons, presumably gamma or slow-type alpha motoneurons, are not different from control [101]. These results indicate that GlyR inhibitory insufficiency in motoneuron pools is specific for large α-motoneurons [101].

$GABA_A$ Receptor Currents are not Affected in G93A-hSOD1 Mouse Motoneurons

To determine if the functional abnormalities in synaptic inhibition of ALS mouse motoneurons were general or specific to GlyRs, we studied $GABA_AR$ currents. We did not find significant changes in the current density and kinetics of $GABA_ARs$ in G93A-hSOD1 motoneurons [93]. Thus, of the two major ligand-gated chloride ion channels in spinal cord, the functional alteration appears to be selective for GlyRs.

Single-Cell Analysis of Motoneuron Gene Expression Using Real-Time RT–PCR

Reduced glycine-evoked currents and glycinergic mIPSCs in G93A-hSOD1 motoneurons could be caused by decreased expression of GlyRs. We have analyzed motoneuron gene expression by single-cell quantitative reverse transcription-PCR (qRT–PCR) following patch-clamp recordings on Hb9-eGFP$^+$ motoneurons [93]. This methodology allows us to record specific properties of living neurons, to identify their ion channel functioning, and expression of their constituent channel subunits in the presence of human transgene expression. This approach offers a unique opportunity to study diseased motoneurons in direct relation to electrophysiology, molecular biology, and structure. Using single-cell qRT–PCR, we found decreased GlyRα1 mRNA expression in G93A-hSOD1 motoneurons. By immunofluorescence (Fig. 2b), this finding was substantiated at the protein level by diminished expression of surface GlyRs on G93A-hSOD1 motoneurons [93].

Interneuron Networks in ALS

Interneuronal networks in spinal cord modulate motoneuron activity (Fig. 1). Spinal interneurons sculpt patterns of motoneuron activity critical for limb movements [82, 84, 113], but the involvement of interneurons in shaping motoneuron activity in non-locomotor activity, such as breathing, is defined less clearly [114–116]. Four classes of genetically distinct interneuron pools (V0–V3) are derived from the ventral embryonic spinal cord, with each class exhibiting unique features [82]. V0 interneurons are commissural. V1 interneurons are inhibitory with axons issuing rostroipsilaterally. V1 cells include the Renshaw cell and Ia inhibitory interneurons. V2 neurons project ipsilaterally and can be glutamatergic (V2a neurons) or inhibitory. V3 interneurons are excitatory commissural cells.

Glycinergic interneurons and synapses are abundant in adult mammalian spinal cord and brainstem [81, 83, 117, 118], regions vulnerable in ALS [92]. Renshaw cells, a type of glycinergic neuron, account for 2–3% of all ventral interneurons in mouse spinal cord [119]. They can be identified unambiguously by their size (mostly medium to large relative to other ventral interneurons), morphology, glycinergic (GlyT2$^+$) and other markers (calbindin and nicotinic acetylcholine receptor α2), large gephyrin clusters, location, and physiology (high postsynaptic sensitivity to acetylcholine and large glycine- and GABA-evoked currents) [120–122]. Renshaw cells receive extensive cholinergic, glutamatergic (mediated by AMPA receptors), GABAergic, and glycinergic inputs. They form synapses directly on α-motoneurons (Fig. 1), Ia inhibitory interneurons, and other Renshaw cells [122, 123]. The Renshaw cell to motoneuron ratio is estimated to be 1:5 [119]. Renshaw cells mediate recurrent inhibition of motoneurons (Fig. 1) (modulating recruitment and firing rate) and can modulate the inhibition produced by Ia inhibitory interneurons [119, 123, 124]. Renshaw cells exhibit vigorous, long-lasting EPSCs that evoke high-frequency burst discharges with initial instantaneous frequencies of ~1,500 Hz [123, 125]. Thus, Renshaw cells require an effective inhibitory synaptic input to modulate their excitatory responses [123]. Renshaw cell inhibitory synaptic properties develop during the first three postnatal weeks [126]. Ia inhibitory interneurons receive extensive convergent inputs from skeletal muscle Ia proprioceptive afferents (glutamatergic) and Renshaw cells.

Little is known about the roles that interneurons have in the disease mechanisms of human ALS and mouse models of ALS. Spasticity, as well as muscle cramps and fasciculations, are early clinical features of human ALS [127, 128] and are often treated by intrathecal baclofen (a GABA receptor agonist) [2, 3]. Clinical studies suggest that spinal cord recurrent inhibition is abnormally reduced and related to the spasticity [129]. Transcranial magnetic stimulation studies showing increased corticomotor hyperexcitability in ALS patients have been interpreted as being suggestive of cortical interneuron degeneration [130, 131]. However, because distal musculature has a relative lack of recurrent inhibition in humans and undergoes prominent early wasting in ALS, the involvement of Renshaw cells in the disease mechanisms has been questioned [132]. Nevertheless, loss of putative interneurons has been reported in human ALS primary motor cortex [133] and spinal cord [134–137]. In G93A-hSOD1 tg mice parvalbumin-containing cortical interneurons were found to be increased in motor and somatosensory cortex, possibly reflecting a compensatory adaptation [138]. In contrast, a loss of putative spinal interneurons has been reported in tg mice expressing mutated mouse SOD1 (instead of human mutant SOD1) [139].

Our first glimpse of spinal interneuron degeneration in ALS mice came from ubiquitin immunostaining, a marker for neural degeneration [140]. In G93A-hSOD1 mice at presymptomatic stages of the disease, ubiquitin$^+$ neuritic abnormalities were found in the neuropil of the ventral horn, while motoneuron cell bodies did not display ubiquitin-containing inclusions. In early symptomatic mice, ubiquitin accumulated in smaller neurons with a fusiform or polygonal morphology but not yet in motoneuron cell bodies [140]. These ubiquitin-accumulating neurons were observed in the ventral horn and intermediate zone and were parvalbumin$^+$, suggesting that that they could be GABAergic or glycinergic spinal interneurons [126]. In

G93A-hSOD1 mice, some spinal interneurons in laminae VII, VIII, and IX were reduced in number (Fig. 1), determined by counting cell bodies positive for calcium-binding proteins [140, 141]. This loss occurs long before the degeneration of motoneurons (Fig. 1), as assessed by mitochondrial swelling and cell body numbers [140, 141]. Motoneurons in these tg mice also lose glycinergic innervation, but not GABAergic or cholinergic innervation, at presymptomatic stages of disease [141]. A loss of inhibitory innervation of motoneurons as a feature of early disease in G93A-hSOD1 mice has been confirmed by electron microscopy [142]. The preservation of cholinergic C-boutons on motoneurons of G93A-hSOD1 mice has been confirmed by electron microscopy as well [143]. Some lost interneurons are parvalbumin$^+$ [140] and others cells are calbindin$^+$ [141]. The loss of parvalbumin in G93A-hSOD1 mouse spinal cord has also been shown by western blotting [144]. Some of the lost cells could be Renshaw cells because of their size, location, and calbindin calcium-binding protein signature [126, 145]. However, these interneurons could also be GABAergic, glutamatergic, or other glycinergic interneurons such as the Ia inhibitory interneurons [82, 126]. Renshaw cells have been implicated in the disease mechanisms in mouse and human ALS and have been identified as a target of neuroprotection mediated by lithium [146]. Another piece of evidence leading to our ALS-interneuron theory [140] was the finding that a subset of spinal cord-transplanted neuroprogenitors differentiates into interneurons in ALS mouse spinal cord, suggesting neuroplasticity response driven by a need for replacement or reconstitution of inhibitory circuits due to aberrant loss of interneurons [36]. Although motoneurons are a major target of spinal interneurons [119, 123, 124], we postulate that the loss of interneurons is a primary mechanism of disease and is not secondary to motoneuron loss because it precedes motoneuron loss, and complete elimination of somatic motoneurons by target deprivation or neonatal axotomy does not affect interneuron number [147–149].

An early disease-initiating vulnerability of interneurons in ALS could be related to the intrinsic properties of interneurons compared to motoneurons, including high excitability with fast, repetitive, and prolonged action potential bursts, increased reliability of firing, high expression of Na^+ channels, high expression of Ca^{2+}-permeable AMPA receptors, spontaneous Ca^{2+}oscillations regulated by mitochondria, high oxidative metabolism, and substantial nitric oxide input [122, 150, 151]. This abnormality is likely to be incomplete because complete failure of glycinergic neurotransmission causes hyperekplexia [152], although ALS patients have some clinical features that can be interpreted as partially mimicking hyperekplexia, such as exaggerated reflexes (positive Babinski sign) and spasticity [1, 127]. Interestingly, a report on *spastic* mice which can present phenotypically with spasticity and then limb weakness and paralysis, caused by a mutant gene for GlyR β subunit, has shown early loss of ventral horn interneurons followed by motoneuron loss [153]. The participation of interneuron-related and GlyR-related early-onset pathology in ALS would be another example of motoneuron non-autonomous mechanisms of disease, but more importantly, it would identify new cellular (interneurons) and molecular (GlyRs) targets for disease therapy.

Opening New Therapeutic Doors to ALS Treatment

The only drug approved by the US Food and Drug Administration for the treatment of ALS is Riluzole, which blocks tetrodotoxin-sensitive Na^+ channels and has anti-glutamate actions [2]. However, the efficacy of this drug in the treatment of ALS has been debated because its effects are marginal [2].

New clinically relevant molecular targets and drug developments and applications are necessary to move ALS therapy in new meaningful directions [2, 6]. Motoneurons in ALS mice have insufficient synaptic inhibition as reflected by reduced glycinergic innervation [141], smaller GlyR currents, smaller GlyR clusters on their plasma membrane, and lower expression of GlyR1α mRNA compared to wild-type motoneurons [93]. These findings are important because glycinergic synaptic transmission is the main inhibitory neurotransmission system in spinal cord and modulates the excitability of motoneurons and interneurons [84, 87, 154]. It seems possible that inhibitory neural systems are disrupted as part of the pathogenesis in ALS (Fig. 1) and that this process might be engaged long before adulthood, thus the timing of disease could have much more protracted duration than generally appreciated. This work lays a foundation for the novel idea that motoneurons in ALS might undergo chronic stress due to inhibitory insufficiency. This inhibitory insufficiency could be a motoneuron autonomous process or secondary to glycinergic interneuron disease or dysfunction [141] and thus is a motoneuron non-autonomous process of degeneration involving Renshaw cells and neural networks (Fig. 1).

If our hypothesis is correct regarding glycinergic inhibitory insufficiency as a mechanism of disease, then potentially new avenues for therapeutics become available to ALS. Positive modulators of GlyRs and antagonists to glycine transporters (GlyT1 and GlyT2) are available for testing in preclinical models. Positive modulators of GlyRs are neurosteroids (progesterone and allopregnanolone), estrogen receptor modulators (tamoxifen), anesthetics (isoflurane, halothane, propofol, and nonanesthetic analogs of propofol), and cannabinoids [87, 155–158]. Tamoxifen,

progesterone, and allopregnanolone are of particular interest because they increase spontaneous glycinergic mIPSCs in motoneurons [157, 159]. Extracellular concentrations of glycine at glycinergic synapses are regulated by Na^+/Cl^--dependent GlyTs [118, 152, 154, 160]. GlyT1 is widely expressed in glial cells and functions in lowering extracellular glycine concentration at the glycinergic synaptic cleft [154, 160]. GlyT2 is largely localized only to presynaptic terminals of glycinergic neurons found mostly in spinal cord and brainstem [117, 161]. Antagonists to both GlyT1 and GlyT2 are available that can increase extracellular concentrations of glycine in CNS in vivo [158]. This idea can open motoneuron disease to an entirely new, rational, mechanism-based treatment strategy utilizing GlyT and GlyR pharmacologies.

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