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Therapeutic neuroprotective agents for amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal chronic neurodegenerative disease whose hallmark is proteinaceous, ubiquitinated, cytoplasmic inclusions in motor neurons and surrounding cells. Multiple mechanisms proposed as responsible for ALS pathogenesis include dysfunction of protein degradation, glutamate excitotoxicity, mitochondrial dysfunction, apoptosis, oxidative stress, and inflammation. It is therefore essential to gain a better understanding of the underlying disease etiology and search for neuroprotective agents that might delay disease onset, slow progression, prolong survival, and ultimately reduce the burden of disease. Because riluzole, the only Food and Drug Administration (FDA)-approved treatment, prolongs the ALS patient's life by only 3 months, new therapeutic agents are urgently needed. In this review, we focus on studies of various small pharmacological compounds targeting the proposed pathogenic mechanisms of ALS and discuss their impact on disease progression.

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Keywords

Neuroprotective agents; Motor neurons; Glial cells; Muscle; Amyotrophic lateral sclerosis pathogenesis

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS), a.k.a. Lou Gehrig's disease, is a chronic neurodegenerative disease with a prevalence of 1–2 per 100,000 [1] and an incidence of 6–8 per 100,000 people/year. More than 300,000 Americans will die from ALS over the next 10 years unless an effective cure or means of prevention is found. Approximately 90–95 % of ALS cases are sporadic ALS (sALS), with familial ALS (fALS) or inherited cases comprising the remaining 5–10 % of cases. The degeneration of motor neurons in primary motor cortex, corticospinal tracts, brain stem, and spinal cord is responsible for the muscle weakness that typifies ALS.

Skeletal muscle is considered a key target in the development of ALS. Mice expressing mutant G93A SOD1 (mSOD1^{G93A}) selectively in skeletal muscle demonstrate progressive muscle atrophy and spinal motor neuron degeneration [2, 3]. Because muscle cells can clear misfolded mSOD1 proteins more efficiently than motor neuron-like cells [4], several treatments have targeted skeletal muscle. Unfortunately, though these treatments help sustain muscle function, they do not significantly extend survival in ALS mice, which may imply that motor neuron-targeted treatment alone or combined with skeletal muscle-targeted treatment would be more effective in ALS treatment [5–8].

In ALS pathogenesis, it has been assumed that damage to a selective population of motor neurons leads to disease onset, duration, disease progression, and length of survival. Emerging evidence supports the involvement of neighboring non-motor neuron cell types including microglia, astrocytes, and other glial-type Schwann cells, oligodendrocytes, and NG2 cells, as well as targeted muscle cells in the process of ALS-related pathological development; astrogliosis and microgliosis are notable hallmarks of ALS disease. We will focus on therapeutic agents targeting motor neurons and summarize the effects of agents targeting astrocytes and microglia.

The neuroprotective agent riluzole, the only treatment currently approved by the US Food and Drug Administration (FDA), demonstrates only marginal efficacy, prolonging the patient's life by only a few months [9]; its proposed mechanism of action is thought to be anti-excitotoxicity. Hence there is hope that neuroprotective agents counteracting excitotoxicity or other pathogenic mechanisms might ameliorate the clinical symptoms of ALS. Below we review the current knowledge regarding mechanisms underlying the disease pathology with the goal of identifying neuroprotective agents for ALS. We highlight the single small-chemical compounds that affect one or more proposed pathogenic ALS pathways and show neuroprotective efficacy in ALS mouse models. Although one agent may target multiple action mechanisms, we have divided neuroprotective agents into categories based on whether they mainly affect protein-degradation-clearing pathways,

excitotoxicity, mitochondrial dysfunction, apoptosis, oxidative stress, or inflammation (Table 1; Fig. 1).

Genetic factors in the ALS and mutant SOD1^{G93A} mouse model

Amyotrophic lateral sclerosis is a heterogeneous disease. Though mutations in copper–zinc superoxide dismutase 1 (SOD1) have been linked to ~20 % of fALS cases [10], other genes have been implicated, including ubiquilin 2 [11] (a recent finding), TDP-43 [12], FUS/TLS [13, 14], TAF-15 [15], and the hexanucleotide-repeat expansion in the uncharacterized gene C9ORF72 [16, 17].

Transgenic mice overexpressing mutant G93A SOD1 constituted the first and most commonly used animal model to evaluate the efficacy of potential treatments including small chemical compounds for ALS [18, 19] although other animal models are available. The mSOD1 mice models (including G37R, G85R, G127X, D90A, and H46R) share similar pathological hallmarks but differ in disease onset and progression related to gender, generation, and copy number [20]. Like humans with ALS, mSOD1 mice exhibit symptoms such as muscle loss [3], respiratory distress, upper and lower motor neuron involvement, immune system activation, blood–brain barrier (BBB) disruption, inclusion bodies and protein aggregation, and neuromuscular junction disruption [18, 19, 21, 22]. Recent mouse studies began intervention at symptom onset [1, 23–25] or after onset [26, 27] to mimic the clinical treatment of ALS patients. However, most early studies treated mice before onset (preventively), which may have made that animal work less applicable to clinical studies, in that human ALS patients almost invariably receive treatment after diagnosis. Here we include mSOD1 mouse studies involving treatment before, at, or after disease onset.

Protein degradation pathways and SOD1-clearing agents

A significant amount of ALS research has been focused on understanding protein aggregation pathology. Protein degradation pathways play a crucial role in removing misfolded proteins and preventing protein aggregation; the ubiquitin proteasome system (UPS) and autophagy are the two major known protein degradation pathways [28]. Cytoplasmic inclusions in ALS are usually ubiquitin-positive [29–34], and alterations in ubiquitin and proteasome are found not only in motor neurons but also in astrocytes from ALS patients. That evidence supports the involvement of the ubiquitin–proteasome system in both motor neurons and astrocytes in the pathogenesis of ALS [32, 33]. However, because accumulation of ubiquitinated proteins in patient samples could reflect the failure of the UPS, autophagy, or both, the role of the UPS in ALS is ambiguous. Most studies have reported mild-to-severe proteasomal dysfunction in ALS [35], although upregulation has also been reported [36]. A recent study using an in vivo UPS activity reporter mouse model demonstrated a mild decrease in UPS activity in ALS mice [37]. Mutations in ubiquilin 2, a protein that regulates the degradation of ubiquitinated proteins, have recently been reported to cause dominantly inherited, chromosome-X-linked ALS and ALS/dementia [11]. Although the details of ubiquilin 2's role in ALS disease etiology remain to be determined, this new finding supports a significant relationship among proteasomal dysfunction, abnormal protein aggregation, and neurodegeneration.

It is critical to maintain autophagy activity in the central nervous system (CNS). Deletion of the key autophagy genes *atg5* or *atg7* in neurons in transgenic mice resulted in neurodegeneration and the accumulation of polyubiquitinated proteins and ubiquitin-immunoreactive inclusions [38]. The involvement of autophagy in ALS has been reported, but its functional role in disease etiology is less clear. Analysis of post-mortem spinal cord samples from sALS and fALS patients revealed elevated levels of poly-ubiquitin and autophagy markers Beclin-1 and LC3-II, suggesting autophagy [39]. The protein inclusions in ALS are often immunopositive for ubiquitin and p62, both of which are found in protein inclusions in *atg5* and *atg7* knockout mice. Moreover, alterations in autophagy caused by different gene mutations were reported in multiple fALS models.

p62 is an adaptor protein for autophagy substrates found in pathological inclusions in ALS. Ubiquitin is commonly found in p62-positive inclusions [40]. P62-immunopositive inclusions were reported in fALS caused by CHMP2B mutation [41], ANG/angiogenin mutation [42], FIG 4 mutation [43], and more recently in TDP-43 [44] and FUS inclusions [45]. p62 co-localized with SOD1- and ubiquitin-positive inclusions in G93A SOD1 mice and was co-immunoprecipitated with fALS mutants but not with wild-type SOD1 [30]. Moreover, p62 can function as an adaptor between mSOD1 and the autophagy machinery, potentially by a ubiquitin-independent mechanism [46]. P62 provides a potential target for novel therapeutic strategies that focus on clearing misfolded and aggregated proteins.

Several chemical compounds are capable of reducing SOD1 levels/aggregates in models of ALS. Arimoclomol, an amplifier of heat shock protein expression, delays disease progression and extends the lifespan of pre- or early symptomatic stages of $mSOD1^{G93A}$ mice [47]. Late-stage treatment improves muscle function [26]. Arimoclomol reduces ubiquitin aggregates in the spinal cord of G93A mice. Because the accumulation of ubiquitinated proteins reflects UPS failure and/or autophagy, these results may suggest a direct role of arimoclomol in protein aggregation [26] and effects on UPS and/or autophagy. Ongoing phase II/III clinical trials indicated arimoclomol's good safety and tolerability [48, 49].

Another SOD1-clearing agent is edaravone. As an antioxidant and a free-radical scavenger, it effectively slows symptom progression, body weight loss, and motor neuron degeneration, and decreases the mean area with SOD1 aggregates in $mSOD1^{G93A}$ mice; these effects may be attributed to an enhancement of proteasomal activity yet to be characterized [25]. Pyrimethamine (Daraprim), an FDA-approved medication for the treatment of malaria and toxoplasmosis, reduces SOD1 levels in cultured cells, mice, and ALS patients [50], although it cannot decrease SOD1 expression in PC12 cells [51]. Lithium also reduces ubiquitin and SOD1 aggregates in motor neurons [52], inhibits excitotoxic motor neuron death in organotypic spinal cord cultures [53], and provides neuroprotection in cerebellar granule cells [54]. Moreover, lithium significantly delayed disease onset and duration, augmented the lifespan of the G93A mouse, and reduced reactive astrogliosis [52]. The same report suggests that lithium combined with riluzole delayed disease progression in ALS patients [52]. However, another report on G93A mice failed to show neuroprotection [55]. An Italian study suggested that lithium combined with riluzole delayed disease progression in ALS

patients [52]. However, two larger multicenter trials failed to confirm these findings and were halted due to serious safety and efficacy concerns [56].

Excitotoxic mechanisms and anti-excitotoxic agents

Compared with other neurons, motor neurons are particularly vulnerable to excitotoxicity. In addition, astrocytes facilitate the removal of excessive glutamate and affect the calcium permeability of AMPA receptors of motor neurons [57]. Glutamate-induced excitotoxicity leading to motor neuron death is one pathogenic mechanism of ALS. NMDA and AMPA receptors are responsible for calcium entry. Under the pathological conditions of ALS, mSOD1 increases the sensitivity of the AMPA receptor to glutamatergic stimulation, disrupts mitochondrial function, and affects the surrounding astrocytes by interrupting their maintenance of extracellular glutamate levels. Astrocytes expressing mSOD1 are highly vulnerable to glutamate-induced excitotoxicity mediated by metabotropic glutamate receptor 5 (mGluR5) [32, 58].

As stated earlier, riluzole is the only FDA-approved treatment for ALS, but has only a modest effect on survival. Riluzole protects against motor neuron degeneration through excitotoxicity by interrupting glutamatergic transmission and lowering glutamate concentration, with effects on NMDA or AMPA receptors. This explains the strong interest in developing drug candidates that reduce glutamate-induced excitotoxicity. Riluzole significantly extended survival in ALS transgenic mice although it had no effect on disease onset. Riluzole also blocks Ca^{2+} and Na^{+} channels and modulates the GABAergic system, which suggests that it targets multiple pathogenic pathways [59, 60]. Combining riluzole with other drugs showing protective efficacy in ALS mice or patients may yield greater therapeutic potential for ALS. Ceftriaxone, a β-lactam antibiotic approved for treatment of bacterial infections, protected neurons against apoptosis and was identified from among 1,040 FDA-approved drugs as capable of increasing glutamate transporter gene expression [61]. Ceftriaxone prevents glutamate neurotoxicity, and when initiated at disease onset delays loss of muscle strength and body weight and prolongs survival [61]. Talampanel, a noncompetitive AMPA antagonist, reduces motorneuronal calcium levels if applied presymptomatically [62]. Memantine is an FDA-approved drug used for the treatment of Parkinsonism, vascular dementia, and Alzheimer's disease. It is a *N*-methyl-D-aspartate (NMDA) receptor blocker that attenuates excitotoxicity [63]. If administered orally to $mSOD1^{G93A}$ mice either at symptom onset or pre-symptomatically [64], it prolongs survival [23]. However, memantine did not elicit any reversal of motor deterioration [23].

The histone deacetylases (HDACs) play an important role in protein acetylation in histones and in regulation of transcription [65]. Along with anti-excitotoxic effects, HDAC inhibitors have anti-inflammatory and neurotrophic properties. The HDAC inhibitor valproic acid (VPA) protects against glutamate or kainite-induced excitotoxicity in cultured neurons [66, 67] and achieves neuroprotection by reducing the number of apoptotic cells [68] and upregulating Bcl-2 [69]. Combined valproate and lithium treatment delays disease onset, reduces neurological deficits, and prolongs survival in ALS mice [70]. Sodium phenylbutyrate, another HDAC inhibitor, protects against glutathione-induced oxidative stress in cultured cortical neurons [71]. Sodium phenylbutyrate, administered by injection to

mSOD1^{G93A} mice either before or after symptom onset, prolongs survival and reduces the severity of pathological phenotypes [72].

Mitochondrial dysfunction and mitochondrial protectants

Mitochondria, cellular organelles that generate energy, play a key role in the intrinsic apoptotic death pathway, cellular calcium homeostasis, and ALS pathogenesis. A tendency to accumulate and aggregate in mitochondria is common to all mSOD1 [73], which may disrupt the activity of complex IV and impair the association of cytochrome *c* with the inner membrane [74]. mSOD1 aggregation in the mitochondria is linked with spinal cord–specific dysfunction of mitochondria, suggesting an association between defective mitochondria due to the toxic function of mSOD1 and the pathogenesis of ALS, prompting the search for neuroprotective agents targeting mitochondria [75]. Below is a brief review of candidate compounds.

KNS-760704 (dexpramipexole) is the optical enantiomer of pramipexole. Dexpramipexole was developed to maintain the neuroprotective properties while reducing the dopaminergic side effects of pramipexole. Both drugs include the benzothiazole core also present in riluzole [76]. Dexpramipexole functions at the level of mitochondria to enhance ATP output, reduce the generation of reactive oxygen species, and suppress apoptosis. It shows neuroprotection in vitro and in vivo, including the G93A mouse model [76], and hence is a candidate agent against ALS. However, dexpramipexole recently failed in a phase III trail.

Olesoxime (TRO-19622 or mitotarget), a small molecule with a cholesterol-like structure, is neuroprotective in animal and cellular models of ALS. It acts on the mitochondrial permeability transition pore (mPTP) [77]. Mitochondrial swelling and vacuolization are early pathological features of ALS [18], and genetic deletion of a major regulator of the mPTP in ALS mice delays disease onset and extends survival [78]. Olesoxime protects against motor neuron death, increasing the lifespan of mSOD1^{G93A} mice via delaying the onset of motor dysfunction and weight loss rather than slowing disease progression [79]. Furthermore, olesoxime delays muscle denervation, astrogliosis, microglial activation, and motoneuron death in mSOD1^{G93A} mice [80]. Olesoxime directly binds TSPO and VDAC, two proteins of the outer mitochondrial membrane [79].

Creatine helps prevent bioenergetic dysfunction and mitochondrial impairment, producing a dose-dependent improvement in motor performance and extending survival in G93A mice [81]. Creatine supplementation also reduces oxidative damage and neuron loss in G93A mice [81]. The addition of other agents, including minocycline or CELE-BREX, produces additive effects in ALS mice [82, 83].

Induction of mPTP results in the release of mitochondrial proteins such as cytochrome *c* into the cytoplasm, which eventually induces cell-death pathways and leads to cell demise [84]. Inhibitors of mPT have been suggested to be neuroprotective [85]. Among these inhibitors, nortriptyline is an FDA-approved tricyclic antidepressant [85] that significantly delays disease onset and reduces motor neuron loss in ALS mice [86]. Caspase-3 activation and cytochrome *c* release in the lumbar spinal cord of ALS mice were inhibited by nortriptyline [86]. Another mPT inhibitor, the immunosuppressant cyclosporine, has mitochondrial

protective properties. It prevents the assembly of mPT and stabilizes mitochondrial membranes, thus preventing apoptosis [87]. Intrathecal injections of cyclosporine starting at disease onset extended the survival of ALS mice [87]. Taken together, these studies support the potential of mPT inhibitors in therapeutic applications for ALS.

P7C3 and its analog P7C3A20 are aminopropyl carbazoles shown to be neuroprotective and to encourage neurogenesis. P7C3 exhibits protection of mitochondrial member integrity against calcium in vitro [88]. Besides its neuroprotective effect in a mouse model of Parkinson disease [89], when administrated at onset, P7C3A20 was also neuroprotective in a G93A SOD1 mouse model of ALS, protecting lumbar spinal motor neurons and improving motor performance in accelerating rotarod test and walking gait [90].

Apoptotic pathways and anti-apoptotic agents

Though debatable, there is evidence that apoptosis, particularly the mitochondrial apoptotic pathway, has some involvement in ALS. An early event in the mechanism of toxicity of ALS is the activation of initiator caspase-1 [34, 91, 92]. The deletion of the Bax/Bak pathways of mitochondrial apoptosis in G93A mice provides strong support for the role of mitochondrially regulated apoptosis in ALS [93]. Understanding these apoptotic pathways is an active area for future ALS therapeutic studies.

Melatonin has an indole core skeleton, easily penetrates the BBB, and acts as an anti-apoptotic agent, an antioxidant, and a free radical scavenger [94]. Our own recent studies indicate that melatonin administrated by intraperitoneal injection not only delays disease onset and increases mortality in both $SOD1^{G93A}$ ALS mice and R6/2 Huntington's disease transgenic mice but also inhibits mutant huntingtin-induced cell death and caspase activation and preserves melatonin receptor 1A in mutant huntingtin ST14A cells [95–97]. In addition, melatonin reduces superoxide-induced cell death and attenuates glutamate excitotoxicity in NSC-34 cells [98]. Astrocyte activation with increased levels of GFAP (a marker for astrocytes) and microglial activation with increased levels of RCA-1 (a marker for microglia/macrophages) in the spinal cord are both correlated with motor-neuron degeneration. Interestingly, we found that melatonin treatment reduced the expression of GFAP and RCA-1 in $mSOD1^{G93A}$ ALS mice [98]. High oral doses of melatonin delayed disease progression and increased longevity in $SOD1^{G93A}$ mice [98]. In a clinical safety study, high-dose rectally administered melatonin was well tolerated in patients with sALS [98]. However, melatonin has two weaknesses: a relatively short half-life ($t½$ = 20–50 min in adults) and multiple binding sites. Future research should be directed at identifying analogs and further drug design to overcome melatonin's weaknesses and strengthen its neuroprotective effects in slowing disease progression in ALS.

The hematopoietic growth factor erythropoietin (EPO) inhibits apoptotic neuronal changes [99]. Non-hematopoietic erythropoietin derivatives, including asialo and carbamylated erythropoietin, improve motor behavior and reduce motor neuron loss. They reduce the activation of microglia and astrocytes in vivo and in vitro [100]. EPO prevented neuronal injury and early motor neuron degeneration and delayed the onset of motor deterioration in female animals without prolonging survival [101]. EPO levels in the CSF have been reported to be decreased in ALS patients [102].

Minocycline is an antibiotic approved by the FDA for the treatment of bacterial infection. As an anti-apoptotic agent, it inhibits the release of cytochrome *c*, the activation of caspase-1 and -3, reactive microgliosis, as well as p38 mitogen-activated protein kinase [103–105]. Minocycline improved muscle strength, increased longevity, and delayed the onset of motor neuron degeneration in G93A mice [103] and $SOD1^{G37R}$ mice [106]. It also reduced microglial activation in G93A mice [107]. Moreover, a cocktail of minocycline and creatine offered additive neuroprotection, improving motor performance and extending survival in $mSOD1^{G93A}$ mice [82]. TCH346 treatment at disease onset slowed disease progression, increased lifespan, and preserved both body weight and motor performance in a mouse model of mutant with progressive motor neuronopathy [108]. Additionally, TCH346 prevents p53-related neuronal apoptosis. However, chronic subcutaneous treatment with TCH346 offers no benefit in high-copy $SOD1^{G93A}$ mice [109].

zVAD-fmk is a broad caspase inhibitor that delays disease onset and mortality in $mSOD1^{G93A}$ mice. zVAD-fmk inhibits caspase-1 activity as well as caspase-1 and caspase-3 mRNA upregulation [92, 110]. However, because zVAD-fmk is too toxic for human use, development of safer versions is under way.

Oxidative damage and anti-oxidative agents

Several neuroprotective agents with antioxidant capabilities have been studied in relation to ALS. Here we summarize their neuroprotective effects and the success of some antioxidants that target the oxidative stress pathogenic pathway in ALS animals.

Manganese porphyrin (AEOL10150), an antioxidant and a free-radical scavenger, markedly extends survival of $mSOD1^{G93A}$ mice when administered at symptom onset [111] as well as improving motor neuron architecture and reducing astrogliosis. Manganese porphyrin also shows anti-inflammatory properties [112]. Rasagiline (AZI-LECT), a monoamine oxidase inhibitor used in the treatment of Parkinson's disease [113], has neuroprotective properties via its antioxidant activity and has demonstrated mitochondrial protection [114]. Rasagiline, either alone or in combination with riluzole, improves both motor performance and survival in $mSOD1^{G93A}$ mice [114].

Various metals are cytotoxic to motor neurons, leading to neurodegeneration. The lipophilic metal chelators DP-109 and DP-460 chelate calcium, copper, and zinc; both extended survival, improved motor performance, and reduced spinal cord cell loss and oxidative damage markers, as well as decreased reactive astrogliosis and microgliosis in ALS mice [115]. Iron dysregulation promotes oxidative damage, and altered iron homeostasis has been found in ALS patients [116]. The HFE gene is involved in iron regulation, and HFE polymorphism is increased in ALS patients [117]. The multifunctional iron-chelating drugs M30 and HLA20 reduce neurotoxicity induced by the peroxynitrite ion generator SIN-1 and H2O2, augment the expression of iron metabolism—related protein Tfr in NSC-34 cells [118], and prevent G93A SOD1-induced toxicity. M30 significantly delays disease onset and extends the survival of $mSOD1^{G93A}$ mice [119]. Thus, metal chelators may become a source for screening drugs against ALS.

H2O2 and UV light produce oxidative stress and cytotoxicity in SH-SY5Y cells. These effects are reversed by treatment with *N*-acetyl-L-cysteine (NAC), an antioxidant [120]. NAC improves survival and delays the onset of motor impairment in G93A mice [121], and the NAC precursor glutathione significantly reduced lower motor neuron degeneration in the wobbler mouse [122]. The antioxidant vitamin E (α-tocopherol) slows the onset and progression of paralysis in ALS mice, implying a possible future role in ALS prevention [123].

Inflammatory pathways and anti-inflammatory agents

Inflammation plays a major role in the pathogenesis of motor neuron death in ALS, and neuroinflammation accelerates disease progression.

Selective mutant expression of SOD1 in motor neurons did not progress to ALS disease [124, 125], while reduction of mSOD1 in motor neurons delayed disease onset and slowed early progression but had no benefit in terms of later disease progression and survival [126, 127]. A mixture of wild-type and mSOD1 in motor neurons was not sufficient to trigger disease onset [128, 129], and total ablation of mSOD1 expression (in either astrocytes or microglia) slowed disease progression and extended survival [126, 127, 130]. Together, these findings indicate that ALS is not motor–neuron autonomous and that glial cells play important roles in motor neuron degeneration.

Evidence further supports the contention that expression of mSOD1 in motor neurons dominantly and primarily initiates ALS pathogenesis (in other words, affects disease onset and early stages of the disease), whereas neighboring cell types other than motor neurons (including microglia, astrocytes, and Schwann cells) as well as other cells interact both with damaged motor neurons and each other to mediate and affect ALS disease progression and duration [131, 132].

Rather than playing simply a supportive role, astrocytes surrounding motor neurons provide nutrients, maintain the homeostatic environment, and carry out multiple functions. Impaired astrocytic functions including extracellular glutamate clearance and neurotrophic factors release have been implicated in ALS disease. Astrogliosis is present at symptomatic stages of ALS mice [97, 133] and may precede and drive the deterioration of motor activities in animal models of ALS [134, 135], while astrogliosis is detectable in post-mortem spinal cord tissue from fALS and sALS patients [136]. Accumulating evidence shows that astrocytes may modulate microglial activation and infiltration, speeding disease progression [127, 137, 138]. We and other researchers have reported that astrocytes carrying ALS-causing genes, especially mutated SOD1, as the non-cell autonomous components in ALS pathogenesis, may play a critical role in stimulating damage and degeneration of neighboring motor neurons [58, 137–143]. The selective toxicity induced by mSOD1 astrocytes is involved in multiple mechanisms including activation of oxidative stress, secretion of toxic factors, disruption of Ca^{2+} oscillations, Wnt signaling dysfunction, and glutamate- and mGluR5-mediated excitotoxicity [32, 58, 143–145]. Astrocytes derived from familiar ALS and sporadic ALS patients are similarly toxic to motor neurons [146]. On the other hand, wild-type glial cells extend the survival of mSOD1 motor neurons [144].

Microglial cells immigrate from the periphery and enter the brain and spinal cord through the BBB in response to damaged motor neurons. Indeed, activated microglia are implicated in the pathogenesis of ALS, and the intensity of microglial activation is correlated with the severity of motor neuron damage [131, 147, 148]. The fact that progression can be slowed by selectively decreasing mSOD1 in microglia or the addition of microglia expressing normal SOD1 suggests that mSOD1 affects disease progression more than disease initiation [126, 130]. In addition, by wrapping full-length axons of lower motor neurons in myelin, neighboring Schwann cells protect signal transduction. However, there are controversial reports that other glial-type oligodendrocytes and NG2 cells are involved in ALS pathogenesis [131]. Additionally, T lymphocytes also play a role in mSOD1-caused ALS [149, 150].

Below we summarize a number of protective agents targeting inflammatory pathways shown in animal models to demonstrate beneficial effects against ALS.

Cyclooxygenase-2 (COX-2) is a key molecule in the inflammatory pathogenic pathway in ALS. Expressed in spinal astrocytes and neurons, COX-2 catalyzes prostaglandin 2, which stimulates glutamate release from astrocytes via a calcium-dependent pathway [151–155].

Moreover, along with cytokines, reactive oxygen species, and free radicals, COX-2 is involved in the inflammatory process in the central nervous system [156]. The COX-2 inhibitor and anti-inflammatory agent CELEBREX (celecoxib) significantly delays the onset of weakness and weight loss and prolongs survival of ALS mice; the administration of CELEBREX in mice provides significant preservation of spinal neurons and reduced astrogliosis and microglial activation [157].

TNF-α activates microglia and introduces neuronal apoptosis, and elevated levels of TNF-α have been found in the spinal cords of G93A mice and serum of humans with ALS [158–160]. TNF-α in the lumbar spinal cord of G93A mice is increased before symptom onset and loss of motor neurons and is correlated with pathological progression, indicating that activation of inflammation (including TNF-α) plays a critical role in ALS pathogenesis [161, 162].

Both thalidomide and its analog lenalidomide are immunomodulatory agents that inhibit TNF-α production, attenuate weight loss, enhance motor performance, decrease motor neuron death, and significantly increase survival in $mSOD1^{G93A}$ mice when administered prior to onset of disease [133]. When administered after symptom onset, lenalidomide also provides neuroprotection in $mSOD1^{G93A}$ mice [1]. The anti-inflammatory and immunomodulatory properties of both agents play a role in their neuroprotection; both reduce the expression of proinflammatory cytokines [1, 133]. Microglial activation was ameliorated and neuronal loss was reduced in lenalidomide-treated mice [1].

Cannabinoids produce anti-inflammatory actions via cannabinoid receptor 2 (CB2) and cannabinoid receptor 1 (CB1). There is significant temporal elevation of CB2 mRNA and CB2-related activation of microglia/macrophages in the spinal cord of G93A mice [24] and in post-mortem human ALS samples [163]. Cannabinoids ameliorate disease progression in animal models of ALS. Δ^9-tetrahydrocannabinol (THC), the major effective active

ingredient of cannabis, is a CB2 antagonist. It delays motor impairment and prolongs survival when administered either before or after disease onset in ALS mice [164]. Δ^9-THC is also anti-excitotoxic, reducing oxidative damage in vitro [164]. AM-1241 is a CB2 selective agonist that prolongs survival in mSOD1^{G93A} mice when administered either after disease onset [165] or at symptom onset [24]. Cannabinoids may prove useful in the treatment of ALS.

Conclusions

Except for riluzole, promising results from animal models of ALS have not translated well into humans. For instance, talampanel, CELEBREX, and thalidomide did not succeed in phase II trials in ALS patients [166–168], while minocycline, olesoxime, and TCH346 failed in large phase III or phase II/III clinical trials among ALS patients [169, 170]. These results highlight the challenges in predicting human clinical trial success from animal models of disease [171]. Applying rational design to produce significant improvements over current ALS preclinical studies should help produce more reliable and translatable results.

The translational failures may be explained in part by the following: (i) though SOD1^{G93A} transgenic mice have been the most common model for therapeutic agent studies, there is genetic variation/mutation among ALS patients [172]. To what extent the SOD1 mouse model faithfully represents human ALS disease remains debatable; it may more accurately represent a small fraction of fALS rather than sALS patients [173, 174]. Unanswered questions include (1) how generalizable the drugs targeting pathogenic mechanisms of mSOD1^{G93A} mice are to other animal models of ALS, and whether similar events occur in human ALS. Given the difficulty of treating a progressive disease with symptoms appearing in late adulthood, besides the timing of drug treatment before or on onset, mice trials could be more carefully designed, controlled, and elaborated to make them more translatable to the clinic, (2) methodological flaws such as randomization, blinded outcome assessment, and sample size calculation should be corrected [175], and more accurate controls such as sibling matched controls and monitoring the copy number of transgenes would improve the design of pre-clinical trials, and (3) Clinical trials have been marked by the failure to determine the correct dosage and ideal duration, inappropriate sample sizes, and poor selection of timing factors to identify a person as eligible. It may be necessary to identify a primary outcome for measurement other than survival, with biomarkers providing information on target engagement and effects on disease progression [173, 176–178].

The neurobiology of ALS is complicated by multiple pathogenic mechanisms; which one(s) will predominate in any particular patient remains unpredictable. Drug candidates that ameliorate symptoms (including damage to muscle function) but provide no benefits in terms of survival or delayed onset are still valuable and informative for further investigation. A pleiotropic neuroprotective agent targeting multiple mechanisms may be most promising.

Of the current clinical trials, both melatonin [97, 98] and Δ^9-THC [179], though in the early stages of clinical study, have multiple modes of action, including anti-oxidant, anti-excitotoxity, and anti-inflammatory effects. Along with their protection against apoptosis and mitochondrial damage (in the case of melatonin), those qualities may predict strong

showings in further investigations. In addition, ceftriaxone's effects against excitotoxity and apoptosis imply great promise. Melatonin's indole core skeleton may facilitate further rational drug design and analog search, while the CB2 receptor's non-selective or selective agonist/antagonists may provide a novel source of candidate drugs for screening against ALS. On the other hand, finding a single "magic bullet" for this heterogeneous disease may be difficult, and it is likely that the most effective treatment will turn out to be a combination therapy involving drugs that target pathogenic pathways.

Further direction of basic research should focus on deepening our understanding of the mechanisms underlying ALS pathogenesis, highlighting first/early triggers of this disease. Given the common appearance of reactive microglial and astroglial cells in ALS, Alzheimer's disease, Parkinson's disease, and Huntington's disease, the notion of "non-cell autonomous" neurodegeneration has been applied to these neurodegenerative diseases. Further research on the biology, function, activation mechanisms, and regulation of astrocyte and microglial cell function is urgently needed and should make significant contributions to the discovery of therapeutic agents that will slow ALS disease progression, benefiting sALS and fALS patients, as well as those suffering from other neurodegenerative diseases. We anticipate that the accumulating knowledge of pathogenic mechanisms, drug discovery, and drug bioavailability, along with the evidence gathered in experimental preclinical studies and human trials, will soon yield more effective therapies for ALS.

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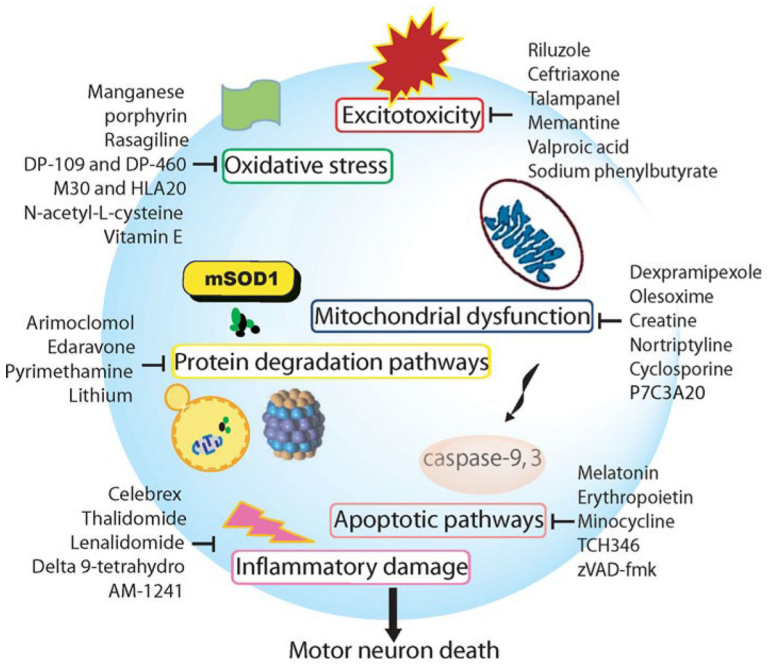

Fig. 1.

Neuroprotective agents target the pathogenic pathways in ALS

Table 1

Therapeutic neuroprotective agents and their effects on models of ALS

Agent	Mechanism	In vivo model	Treatment	Animal Number	In vitro/ex vivo model	Protection	References
Protein clearing agents for ALS							
Arimoclomol	Inducer of hsp70 Reduces aggregation	G93A mice	Preventively	10	–	Improves muscle function, delays disease progression and extends survival	[26, 47]
Edaravone	Anti-apoptosis Anti-oxidative stress Prevents aggregation	G93A mice	Therapeutically	15	SH-SY5Y cells	Slows symptom progression, body weight loss, and motor neuron degeneration	[25]
Pyrimethamine	Reduces SOD1 level	G93A mice	–	–	–	Improves muscle function, delays disease progression and extends survival	[50, 51]
Lithium	Reduces ubiquitin and SOD1 aggregates Anti-inflammatory	G93A mice	Therapeutically	20	Organotypic spinal cord slice culture Cerebellar granule cells	Delays disease onset and duration, reduces reactive astrogliosis, and augments the life span Prevents excitotoxic motoneuron cell death	[52–55]
Anti-excitotoxic agents for ALS							
Riluzole	Prevents excitotoxity blocks Ca^{2+} and Na^{+} channels modulates GABAergic	G93A mice system	Preventively	11	Cell based assays	Prolongs life span	[59, 60]
Ceftriaxone	Prevents glutamate neurotoxicity Anti-apoptosis	G93A mice	Preventively	20	Spinal cord organotypic cultures and primary cortical neurons	Delays loss of neurons and muscle strength, and increases mouse survival	[61]
Talampanel	A noncompetitive AMPA antagonist can reduce motoneuronal calcium	G93A mice	Preventively Therapeutically	12	–	No significant effect on the number of motorneurons in SOD1 mice	[62]
Memantine	Attenuates excitotoxicity as NMDA receptor blocker	G93A mice	Therapeutically	10	Cortical and retinal ganglion neuronal cultures	Prolongs life span	[23, 63, 64]
VPA	HDAC inhibitor Prevents excitotoxity	G93A mice	Preventively Therapeutically Preventively	9 11 6	Rat embryonic motoneurons Prolongs disease duration and offers neuroprotection Cerebral cortical neurons		[66–70]

Agent	Mechanism	In vivo model	Treatment	Animal Number	In vitro/ex vivo model	Protection	References
					Primary spinal cord neurons Primary hippocampus neurons		
Sodium phenylbutyrate	Anti-inflammatory Anti-oxidative stress	G93A mice	Preventively Therapeutically	20	Cortical neuron cultures	Prolongs survival and improves motor performance	[71, 72]
Mitochondrial protectants							
Dexpramipexole	Mitochondrial modulator Anti-oxidative stress anti-apoptosis	G93A mice	Preventively	15	SHSY-5Y cells H-22 hippocampal neuroblastoma cells	Neuroprotection	[76]
Olesoxime	Inhibition of mPTP Anti -inflammatory	G93A mice	Therapeutically	11	Rat embryonic motor neurons	Delays disease onset and weight loss, extends survival, prevents neuronal cell death, and reduces astrogliosis and microglial activation	[77, 79, 80]
Creatine	Prevents mitochondrial impairment Anti-oxidative stress	G93A mice	Therapeutically	7	–	Improves motor performance, and extends life span	[81–83]
Nortriptyline	Inhibition of mPT Anti-apoptosis	G93A mice	Preventively	12–29	Mutant Htt ST 14A cells	Delays disease onset and extends the lifespan	[86]
Cyclosporine	Mitochondrial protection	G93A mice	Therapeutically	7	–	Extends survival	[87]
P7C3A20	Mitochondrial protection	G93A mice	Preventively Therapeutically	20	–	Protects from cell death and preserves motor functions	[89]
Anti-apoptotic agents for ALS							
Melatonin	Anti- apoptosis Anti-oxidative stress Prevents excitotoxity Anti -inflammatory	G93A mice G93A mice	Preventively Preventively	25–29 15	NSC-34 motoneurons Mutant Htt ST 14A cells	Delays disease onset and extends the lifespan Delays disease progression Decreases astrogliosis and microglial activation	[95–98]
EPO	Anti- apoptosis Prevents glutamate excitotoxicity	G93A mice	Preventively	9–11	Rat organotypic spinal cord slice cultures	Inhibits early motor neuron degeneration Delays the onset of ALS mice without prolonging survival Reduces the astroglial and microglial activation	[99–101]

Agent	Mechanism	In vivo model	Treatment	Animal Number	In vitro/ex vivo model	Protection	References
Minocycline	Anti-apoptosis Anti-inflammatory	G93A mice G37R mice	Preventively Preventively	10 12–17	Rat primary cortical neurons SH-SY5Y cells	Improves muscle strength, increases longevity, delays disease onset and reduces microglial activation Inhibits cytochrome *c* release and caspase-l/-3 activation	[82, 103–107]
TCH346	Anti-apoptosis Mitochondrial protection	G93A mice Progressive motor neuron opathy mice	Preventively Preventively	– 9–12	– –	Slows disease progression, increases lifespan, and preserves body weight and motor performance in progressive motor neuronopathy mice, but no effect in G93 A mice	[108, 109]
zVAD-fmk	Anti-apoptosis	G93A mice	Preventively	7	–	Delays disease onset and mortality, inhibits caspase-1 activity, caspase-1 and -3 mRNA up-regulation	[92, 110]
Anti-oxidant agents for ALS							
Manganese porphyrin	Antioxidant Anti-inflammatory	G93A mice	Therapeutically	7–11	–	Extends survival, provides better architecture of motor neuron, less astrogliosis, nitrotyrosine and malondialdehyde	[111, 112]
Rasagiline	Antioxidant Mitochondrial protection	G93A mice	Therapeutically	15	–	Improves motor performance and survival	[114]
DP109 and DP460	Antioxidant Metal chelator Anti-inflammatory	G93A mice	Preventively	30	–	Extends survival, improves motor performance, reduces spinal cord cell loss and oxidative damage markers, and decreases reactive astrogliosis and microgliosis	[115]

Agent	Mechanism	In vivo model	Treatment	Animal Number	In vitro/ex vivo model	Protection	References
M30-HLA20	Antioxidant Iron chelation	G93A mice	Therapeutically	16	NSC-34 cells	Delays onset of disease and extends survival	[118, 119]
NAC	Antioxidant	G93A mice Wobbler mice	Preventively	6–12	SH-SY5Y neuroblastoma cells	Improves survival, delays onset of motor impairment and lowers motor neuron degeneration in wobbler mice	[121, 122]
Vitamin E	Antioxidant	G93A mice	Preventively	10–14	–	Delays onset and progression of paralysis	[123]
Anti-inflammatory agents for ALS							
Celebrex	Anti-inflammatory	G93A mice	Preventively	45	–	Delays onset of weakness and weight loss, prolongs survival, preserves spinal neurons, and diminishes astrogliosis and microglial activation	[157]
Thalidomide	Anti-inflammatory Immunomodulatory	G93A mice	Preventively	10–12	–	Attenuates weight loss, enhances motor performance, decreases motor neuron death, and increases survival	[133]
Lenalidomide	Anti-inflammatory Immunomodulatory	G93A mice	Preventively	12	–	Attenuates weight loss, enhances motor performance, decreases motor neuron death, increases survival, ameliorates microglial activation, and redues neuronal loss	[1, 133]
A9-THC	Anti-inflammatory Anti-oxidative stress Prevents excitotoxity Acts via cannabinoid receptor 2	G93A mice	Preventively Therapeutically	7–9	–	Delays disease progression and prolongs survival	[164]
AM-1241	Anti-inflammatory Acts via cannabinoid receptor 2	G93A mice	Therapeutically	7–9	–	Delays disease progression and prolongs survival	[24, 165]

The action mechanisms and neuroprotective effects of therapeutic agents in different models of ALS are tabulated

Preventively indicates that the intervention started before symptom onset [1, 23–25]; *therapeutically* indicates that the intervention began at or after symptom onset [1, 23–25]

Numbers of animals used in the studies are indicated

EPO Erythropoietin, *VPA* valproic acid, *NAC N*-acetyl-L-cysteine, *mPTP* mitochondrial permeability transition pore, *mPT* mitochondrial permeability transition, Δ^9-*THC* Δ^9-tetrahydrocannabinol