Chapter 2

Multiple Routes of Motor Neuron Degeneration in ALS

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurological disorder with higher selectivity in the degeneration of the upper and lower motor neurons, which leads to progressive paralysis of voluntary muscles. Although most cases fall under sporadic ALS (sALS), 10% of cases are inherited and known as familial ALS (fALS). The etiology of most ALS cases remains unknown, but mutations of ALS-linked Cu/Zn superoxide dismutase 1 (SOD1) are the most common causes of fALS and are responsible for its neurotoxicity and disease propagation due to the acquired toxic gain-of-function [1-2]. Studies in both human ALS patients and the transgenic ALS mouse model have delineated multiple pathological mechanisms of neuronal death that include genetic mutations, excitotoxicity, free radicals, apoptosis, inflammation, and protein aggregation. Targeting the multiple routes of the motor neuron degeneration is likely to contribute to the development of novel therapeutics for ALS patients.

2. Excitotoxicity

2.1. Glutamate neurotoxicity

Glutamate mediates excitatory synaptic transmission by activating the ionotropic glutamate receptors that are sensitive to N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), or kainate. While the ionotropic glutamate receptors constitute fast excitatory synapses in the brain and the spinal cord, the glutamate receptors are excessively activated under pathological conditions such as hypoxic ischemia, trauma, and epilepsy, which triggers degeneration of neurons and oligodendrocytes. Extensive evidence supports the causative role of Ca^{2+}-permeable ionotropic glutamate receptors in motor neuron degeneration in ALS patients. Intracellular Ca^{2+} overload causes catastrophic neuronal death

by impairing mitochondria or activating proteases, cytosolic phospholipase A2, kinases, endonucleases, and nuclear factor kappa B [3].

2.1.1. Abnormal glutamate re-uptake in ALS

Glutamate transporter 1 (GLT-1), also known as excitatory amino acid transporter 2 (EAAT2), and glutamate-aspartate transporter (GLAST), the primary transporters of glutamate into astrocytes, plays a central role in regulating the extracellular levels of glutamate [4-5]. The expression of GLT-1 was markedly reduced in the motor cortex and the spinal cord of sporadic and familial ALS patients [6]. In mutant SOD1 mice, the levels and the activity of EAAT2 were reduced in the spinal cord [7-8]. The levels of extracellular glutanmate increased in the plasma and the cerebrospinal fluid of ALS patients [9-10] and of mutant SOD1-expressing rodent models [7,11-12]. Reducing the expression of EAAT2 with antisense oligonucleotide reduced transporter activity induces neuronal death in vitro and in vivo [13]. Crossing transgenic mice that overexpress EAAT2 with SOD1G93A mice caused delayed motor deficit [14]. In addition, increasing the expression of GLT-1 significantly extended the survival of mutant SOD1 mice [15]. More recently, a sumoylated fragment of EAAT2 cleaved to by activating caspase-3 was shown to cause motor neuron death [16]. This implies that reduced glutamate uptake into astrocytes mediates degeneration of spinal motor neurons in ALS.

2.1.2. Mediation of motor neuron degeneration by the Ca^{2+} permeability of AMPA receptors

Ca^{2+}-permeable AMPA glutamate receptors appear to mediate chronic motor neuron degeneration in ALS. AMPA receptors consist of heteromeric combinations of four sub-units, GluR1-4 [17]. The glutamate (Q)/arginine (R)-editing of the GluR2 mRNA provides a positively charged form of GluR2 protein with arginine, which is responsible for Ca^{2+} impermeability [18]. When AMPA receptors contain reduced levels of Q/R-edited GluR2, the AMPA receptor complex becomes more permeable to Ca^{2+} [18]. The motor neuron of ALS patients showed evidence of defective editing of the pre-mRNA of GluR2 [19]. While lack of GluR2 accelerated motor neuron degeneration and shortened the life span of the SOD1 mice, overexpression of GluR2 delayed the disease onset and reduced the mortality of mutant SOD1 mice [20-21]. Moreover, the GluR2-N transgenic mice that expressed GluR2 gene encoding a asparagine at the Q/R site showed late-onset degeneration of the spinal motor neurons and motor function deficit [22]. Crossbreeding GluR2-N mice with mutant SOD1 mice aggravated motor neuron degeneration and shortened the survival time.

2.1.3. Therapies related to glutamate-mediated excitotoxicity

Although riluzole, the only approved disease-modifying therapy available to ALS patients since 1995, has been shown to inhibit glutamate release, subsequent studies demonstrated that riluzole inhibited AMPA receptors and presynaptic NMDA receptors [23-24]. Administration of riluzole significantly improved the motor neuron survival, motor function, and life expectancy of mutant SOD1 mice [25]. Similar beneficial effects of AMPA receptor antagonists such as memantine, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX), and talampanel have been verified in mutant SOD1 mice [26-28]. The B-lactam

antibiotic cefriaxone increased GLT-1 expression in spinal cord culture and in normal rats. The cefriaxone treatment delayed motor deficits with marginal survival in SOD1G93A mice [15]. An adaptive design Phase II/III study revealed good tolerability over 20 weeks [29]. The extened phase III of this study is ongoing.

3. Oxidative stress

3.1. Homeostasis and generation of free radicals in cells

Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), are characterized by unpaired electrons in their outer orbit. The most common cellular free radicals are hydroxyl (OH) radicals, superoxide (O_2^-) anions, and nitric monoxide (NO). Although hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO-) are literally not free radicals, they are deemed to generate free radicals through various chemical reactions in many cases. Free radicals are cleared through several defense mechanisms, as follows: (1) catalytic removal of reactive species by enzymes such as superoxide dismutase, catalase, and peroxidase; (2) scavenging of reactive species by low-molecular-weight agents that were either synthesized in vivo (including glutathione, α-keto acids, lipoic acid, and coenzyme Q) or obtained from the diet [including ascorbate (vitamin C) and α-tocopherol (vitamin E)]; and (3) minimization of the availability of pro-oxidants such as transition metals [30]. CNS, which is mainly composed of polyunsaturated fatty acids (PUFAs), is readily susceptible to oxidative damage because the system demands a high metabolic oxidative rate with limited anti-oxidants and has a high transition metal content that acts as a potent pro-oxidant through the Haber-Weiss reaction or the Fenton reaction [51]. Upon shifting to pro-oxidants, CNS is promptly attacked by ROS that includes H_2O_2, NO, O_2^- , and highly reactive OH and NO and undergoes serious functional abnormality that is directly related to the demise of the course of neurons.

3.2. Evidence of oxidative stress in ALS

There is extensive evidence of the causative role of oxidative stress in motor neuron degeneration in ALS. The 3-nitrotyrosine(3-NT) level was elevated in subjects with both sporadic and familial cases of ALS, and the immunoreactivity of 3-NT became more evident within large motor neurons in the ventral horn of the lumbar spinal cord [31-32]. Higher carbonylation of proteins with the use of 2,4-dinitrophenylhydrazine (DNPH) was detected in the spinal cord in sporadic ALS [33]. Elevation of 8-hydroxy-2-deoxyguanosine (8-OHdG) was found in the CSF, serum, and urine of ALS patients [34]. The 4-hydroxynonenal level increased in the serum of ALS patients [35]. Transgenic ALS mice overexpression of the human mutant SOD1 revealed oxidative damage to proteins, lipids, and DNA [36-37].

3.2.1. Role of mitochondria in oxidative stress

Mitochondria produce ATP using about 90% of the O_2 that is taken up by neurons. During electron transfer in the inner membrane of the organelle, electrons spontaneously leak from

the electron transport chain and react with available O_2 to produce superoxide, which makes mitochondria the major cellular sources of ROS. Mitochondria exist in the motor neurons due to the high rate of metabolic demand, which makes motor neurons more vulnerable to cumulative oxidative stress. Free radicals that accumulate over time decrease mitochondrial efficacy and increase the production of mutated mitochondrial DNA related to the aging process, although mitochondria have their own specific anti-oxidants that consist of SOD1, SOD2, glutathioneperoxidase, and peroxiredoxin 3 and can usually combat the high rate of ROS production [38]. Morphological abnormality in the organelle, which includes a fragmented network and swelling, and increased cristae have been observed in the soma and proximal axons of ventral motor neurons of sporadic ALS (sALS) patients [39]. In the axon and soma of motor neurons of mice that expressed $SOD1^{G93A}$ and $SOD1^{G37R}$ [40-41], membrane vacuoles derived from degenerating mitochondria were reported. Morphological alteration in mitochondria was also illustrated in NSC34 motor-neuron-like cells that expressed $SOD1^{G93A}$ [42-43]. Mutant SOD1 that was localized in mitochondria was associated with increased oxidative damage, decreased respiratory activity of the mitochondria, and architectural change. The interaction of mutant SOD1 and mitochondria was enough to result in motor neuron death in neuroblastoma cells [44]. Mitochondrial SOD1 and its chaperone protein named copper chaperone for SOD1 (CCS) are co-localized in the mitochondrial inter-membrane space [45]. The aggregates of mutant SOD1 were shown within the mitochondria in the spinal cord of $SOD1^{G93A}$ mice before the onset of the symptoms [46-47] and were implicated in increased oxidative damage, decreased respiratory activity of mitochondria [48], and mitochondrial swelling and vacuolization [47].

3.2.2. Role of transition metals in oxidative stress

Redox-active transition metals are useful but harmful trace elements. Copper and iron are abundant (~0.1-0.5 mM) in the brain and have been implicated in the generation of ROS in various neurodegenerative diseases that include Alzheimer's disease and Parkinson's disease [49-50]. These transition metals mediate the formation of a hydroxyl radical through the iron-catalyzed or copper-catalyzed Haber-Weiss reactions [51]. Once copper ions are transported into the cell, they must be delivered to specific targets (e.g., SOD1 and cytochrome c oxidase) or stored in copper scavenging systems (e.g., GSH and metallothioneins) [52-53]. When these events are out of control, the cells have an uncomfortable abundance of toxic and radical-generating metal ions. FALS-linked SOD1 mutation has weaker binding affinity to copper ions, which are readily libertated to increase oxidative stress in cells expressed with fALS-SOD1 [54]. The detrimental role of copper in fALS pathogenesis was supported by several experiments that used copper chelators, which delayed the disease onset and prolonged the survival of fALS-G93A mice [55], prevented peroxidase activity by expressing fALS-SOD1 A4V and G93A in vitro [56], and reduced elevated ROS production in the lymphoblasts of fALS patients [57]. Iron is vital for all living organisms because it has an essential role in oxygen transport and electron transfer, and is a cofactor in many enzyme systems that include DNA synthesis. Iron homeostasis and its regulatory system [58] was readily disrupted in the development and progress of neurodegenerative diseases such as AD or PD [59-60]. Recently, several pieces of evidence supported the concept that iron is dysregulated in ALS. An increased ferritin level

was observed in the serum of sporadic ALS patients, which suggests a possible risk factor and the disturbance of iron homeostasis [61-62]. Ferritin was upregulated just prior to the end-stage disease in SOD1-G93A mice, which supports increased Fe levels [63]. In the same animal model, increased iron was evident in the spinal cord at the ages of 90 and 120 days, with the onset of the symptoms and in the late stage, due to the disease progress. The increased iron levels were attenuated by iron chelators, which improved the motor function and the survival [64]. mRNAs associated with iron homeostasis (e.g., DMT1, TfR1, the iron exporter Fpn, and CP) also increased with a caudal-to-rostral gradient, with the highest levels rostrally in the cervical region in SOD1G37R [65]. HFE protein is a membrane protein that can influence cellular iron uptake, and mutated HFE is well recognized in haemochromatosis, a genetic disorder due to the irregular accumulation of free forms of Fe in parenchymal tissue. In studies of sporadic ALS patients, both the prevalence of HFE mutation and its polymorphisms (e.g., H63D) were evident [66-67]. Therefore, HFE polymorphisms in ALS may be associated with the altered Fe homeostasis and oxidative stress in this disease. Although abnormal iron homeostasis was evident, the iron regulation mechanisms for motor neuron death must be explained.

3.2.3. Possible mechanisms related to oxidative stress in ALS

Human SOD1 mutation has a toxic gain-of-function that may be due to loss of the active site of copper binding that converts the SOD1 itself to pro-oxidant proteins and participates in ROS generation [68]. Several pieces of evidence have been suggested to show that higher interaction of mutant SOD1 with mitochondria may induce mitochondrial dysfunction and selectively lead to excessive oxidative stress in motor neurons [46]. Reduced transcription factor nuclear erythroid 2-related factor 2 (Nrf2) mRNA and protein expression has been reported in the spinal cord of ALS patients [69]. Crossbreeding SOD1G93A mice with overexpressed Nrf2 extended their survival [70], which suggests that increasing the Nef2 activity may be a novel therapeutic target. Nrf2 activation increases the expression of anti-oxidant proteins due to its interaction with the anti-oxidant-response element (ARE) after its translocation to the nucleus. In another reported mechanism of oxidative stress, the activity of NADPH oxidase (Nox) increased in both sALS patients and mutant SOD1 mice. Expressed Nox in activated microglia may influence motor neuron death. Deletion of either Nox1 or Nox2 prolonged the survival of mutant SOD1G93A mice [71-72]. Protein aggregation is a common pathological feature in ALS patients and animal ALS models. TAR DNA-binding protein-43 (TDP-43) or mutant SOD1 is a constituent of inclusions in ALS patients and mutant SOD1 mice [73-74]. Mutant SOD1 itself caused oxidative damage of proteins in mutant SOD1 mice [37].

3.2.4. Therapeutic drugs for oxidative stress in ALS

Several anti-oxidants have been tested using animal ALS models (Table 1). Completed, ongoing, or planned trials explored, are exploring, or will explore the value of anti-oxidants. Vitamin E, the most potent natural scavenger of ROS and RNS, delayed their clinical onset and slowed the disease progression in mutant SOD1 mice [25]. Long-term vitamin E supplements reduced the risk of death from ALS in ALS-free subjects [75-76]. Unfortunately, two vitamin

E clinical trials failed to show the vitamin's efficacy in ALS patients due to impermeable BBB penetration [77]. Creatine, N-acetylcysteine, AEOL-10150, and edarabone have successfully improved the motor function and survival of mutant SOD1 mice [78-81]. Creatine and N-acetylcystein were not effective in the clinical trial phase II.

4. Apoptosis

4.1. Evidence of apoptosis in ALS

Kerr et al. (1972)[82] reported electron microscopic features of shrinkage necrosis or apoptosis that are expected to play a role in the regulation of the number of cells under physiological and pathological conditions. The apoptotic cells were accompanied by condensation of the nucleus and the cytoplasm, nuclear fragmentation, and aggregated condensation of nuclear chromatin. Interestingly, apoptosis is prevented by inhibitors of protein and mRNA synthesis, and thus, appears to require the expression and activation of death-regulating proteins in neurons and non-neuronal cells [83-84]. The morphological and molecular features of apoptosis have been reported in the nervous system during the development of various neurological diseases. Apoptosis is probably correlated with the demise of motor neurons in ALS. Degenerating motor neurons in the spinal cord and the motor cortex are illustrated by the dark and shrunken cytoplasm and nuclei, chromatin condensation, and apoptotic bodies in the cells. Various pro-apoptosis proteins are activated in the ALS-injured area, and protein synthesis inhibitors attenuate ALS-related neuronal death.

4.1.1. Death receptor Fas

The death receptor Fas (CD95 or APO-1) belongs to the tumor necrosis factor (TNF) receptor superfamily and functions as a key determinant of cell fate under physiological and pathological conditions [86-87]. The Fas ligand (Fas-L) activates Fas in an autocrine or paracrine manner, which leads to the trimerization of Fas with Fas-associating protein within the death domain (FADD) and procaspase-8. Fas activation has been shown as an obligatory step in apoptosis in neurons deprived of trophic factors [88-90]. Fas antibodies were more frequently found in the serum of sporadic or familial ALS patients than in that of the normal controls [91], which also induced apoptosis in the human neuroblastoma cell line and in neuron-glia co-cultured cells of the spinal cord of rat embryos [92]. Primary motor neurons of mouse embryos that expressed mutant SOD1 were susceptible to Fas-induced death [93]. Continuous silencing of the Fas receptor on the motor-neuron-ameliorated motor function and survival of SOD1G93A mice using small interfering RNA-mediated interference supported the role of Fas-linked motor neuron degeneration in ALS [94]. In SOD1G93A mice, a Fas pathway is required to allow Fas interaction with FADD, which in turn recruits caspase-8 as one of the downstream effectors. In addition, TIMP-3 controls Fas-mediated apoptosis by inhibiting the MMP-3-mediated shedding activity in the Fas ligand on the cell surface [95]. The FASS/FADD-mediated motor neuron degeneration was attenuated by Lithium treatment in SOD1G93A

mice [96]. A Fas/NO feedback loop with downstream Daxx and P38 was proposed as another Fas pathway of motor neuron death in mutant SOD1 mice [97].

4.1.2. Pro-apoptotic family of Bcl-2

The physiological and pathological roles of the Bcl-2 family have been extensively reviewed [98-99]. The physical balance between anti-apoptotic and pro-apoptotic members of the Bcl-2 family generally appears to determine the fate of developing and mature cells. Anti- and pro-apoptotic proteins are separated by the presence or absence of Bcl-2 homology (BH) domains. There are four domains: BH1-BH4. Bcl-2 and Bcl-xL contain all four domains and are anti-apoptotic. The pro-apoptotic Bcl-2 family includes Bax, Bcl-x_s, Bak, Bad, and Bid and participates in the neuronal death process. Unbalanced pro- or anti-apoptotic proteins activate caspase-realted apoptosis by releasing cytochrome c into cytosol. Bax is oligomerized, inserted into the outer membrane of mitochondria, and shown to induce cytochrome c release [100-101]. The ratio of the apoptotic cell death genes Bax to Bcl-2 increases at both the mRNA and protein levels in the spinal motor neurons of ALS patients and SOD1G93A mice [102-104]. Interestingly, mutant SOD1 was highly associated with Bcl-2 in the mitochondria, which resulted in conformational or phenotypic change of Bcl-2 that weakened the mitochondria in the spinal cord [105]. Blunt Bcl-2 may contribute to the activation of the mitochondrial apoptosis machinery such as caspase-9, caspase 3, and cytochrome c in the spinal motor neurons of ALS transgenic mice and humans with ALS [106-107]. To support this idea, Bcl-2 overexpression or Bax depletion crossbred with SOD1G93A mice delayed the onset of symptoms and extended the life expectancy [108-109].

4.1.3. Caspase cascade

Caspases, a family of cysteine-dependent aspartate-directed proteases, mediate the propagation and execution of apoptosis. They can be classified into initiator caspases and effector caspases [110]. Caspase-9 is an initiator caspase and is proteolytically activated by apaf-1, a cytoplasmic protein that is homologous to ced-4, and by cytochrome c. The latter is located in the intermembrane space of the mitochondria and released into the cytoplasm by the pro-apoptotic Bcl-2 (e.g., Bax) that is transported from the cytoplasm into the mitochondria in the early phase of apoptosis. Caspase-8, which is known as another initiator caspase, is activated through the interaction of procaspase-9 with the Fas receptor and the FADD adapter. Activated caspase-8 and caspase-9 can activate downstream caspases such as caspase-3, 6, and 7 that can cleave to a number of proteins that are essential to the structure, signal transduction, and cell cycle and terminate the overall apoptosis process. Under the ER (endoplasmic reticulum) stress, caspase-12 is activated with the cleavage (activation) of caspase-9 and caspase-3, regardless of the release of cytochrome c. Marginally, ER stress triggers caspase-8 activation, which results in a mitochondria-mediated pathway via Bid cleavage. The caspase-1, -3, and -9 activities were higher in the motor neurons of the spinal cord or the motor cortex of ALS patients than in those of the control [107,111]. Caspase-1 truncated Bid to be highly reactive [106]. The orderly activation of caspase-1 and -3 was evident, and their mRNAs were abundant in animal ALS models [111-112]. The sequential activation of caspase-9 to caspase-7 was

required for the mitochondria-dependent apoptosis pathway in a rodent ALS model [107]. Moreover, caspase-9 was simultaneously activated with a death receptor pathway that contained Fas, FADD, caspase-8, and caspase-3 in the ALS mice after their motor neuron death began [95-96]. Cleaved forms of caspase-12 were expressed presymptomatically in animal models, which shows evidence of ER stress [113]. A more advanced mechanism than that with caspases revealed that caspases such as caspase-3 or caspase-7 mediated TDP-43 cleavage [114], which was observed immunologically in an aggregated form in the cytoplasmic inclusions in ALS. Intraventricular administration of zVAD-fmk, a broad-spectrum caspase inhibitor, prolonged the survival of G93ASOD1 mice [111], which supports the causative role of caspase cascade in motor neuron death.

4.1.4. Anti-apoptotic drugs served as therapy for ALS

Even though minocycline has anti-inflammatory effects that prevent microglia proliferation, the drug prevented apoptotic motor neuron death by inhibiting cytochrome c release in mutant SOD1 mice [115]. The beneficial effects were proven in several studies to prolong survival and ameliorate the motor function [115-117]. Minocycline accelerated disease progression in a clinical trial, though [118]. TCH-346, a molecule that binds to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used in small samples in a Phase II/III randomized trial, but it did not show beneficial effects [119].

5. Inflammation

5.1. Microglia and astrocyte

Microglia activation is an early event in all forms of pathology. Thus, activated microglia was initially considered a sensitive marker to identify sites that were predestined for tissue. The classical bone-marrow-derived microglial cells dwell in the gray matter and have ramified (highly branched) structures with a small portion of perinuclear cytoplasm and a small, dense, and heterochromatic nucleus. In many CNS pathologies, the cells increase, and this may arise from either local proliferation or recruitment from the blood, or both. The morphology of microglia becomes reactive under pathological conditions that were determined as infiltration of blood-derived cells, local BBB [120], or presence of damaged neurons. Microglia near areas of neuronal injury tend to have more amoeboid features with intense cell bodies and reduced numbers of shortened and thick processes [121] that lead to a structural morphology similar to that of macrophages. A shift in the active style of microglia affects neural, vascular, and blood-borne cells due to several secretions that include pro-inflammatory cytokines and chemokines, nitric oxide, and reactive oxygen intermediates. Astrocytes have many essential physiological functions in the CNS such as provision of trophic support for neurons, conduct of synaptic formation and plasticity, and regulation of the cerebral blood flow. Due to their strategic structure, they are in close contact with CNS resident cells and blood vessels [122-123]. An inflammatory insult causes proliferation of astrocytes and morphological changes. Astroglial activation is recognized via increased expression of the intermediate

filament glial fibrillary acidic protein (GFAP) and the marker aldehyde dehydrogenase 1 family, member L1 (ALDH1L1). Although astrocytes are not immune cells, they can contribute to the immune response in pathological conditions. Microgliosis and astrocytosis are promient features of neurodegenerative diseases that include AD, PD, and ALS.

5.2. Evidence of inflammation in ALS

Several studies have shown the possibility that glial cells adjacent to degenerating motor neurons, mainly primed microglia and astrocytes, have causative roles in the course of disease propagation in ALS. Massive gliosis is apparent in pathologically vulnerable departments of CNS in both human ALS patients and ALS animal models [124-125]. Microglia antibodies have also been found in the CSF of an ALS patient [126]. Recently, the presence of activated microglia was visualized via positron emission tomography (PET), using [^{11}C](R)-PK11195, in the motor cortex, dorsolateral prefrontal cortex, thalamus, and pos of living patients [127]. In the presymptomatic stage of the disease, TNF-α and M-CSF expression increased in a transgenic ALS model. Interestingly, the increase in the expressed TNF-α was found to be correlated to the severity of motor neuron loss [128]. The elevation of TNF-α and of its two receptors [TNFRI (p55TNF) and TNFRII (p75TNF)] was observed in the serum of ALS patients, unlike in those of healthy controls [129]. To date, primed microglia-sensitive intracellular signaling that affectas ALS is authorized by the activation of p38 mitogen-activated protein kinase (p38MAPK), the translocation of the transcription factor NF-κB into the nucleus, and the upregulation of COX-2. The activation of NF-κB regulates the transcription of a wide range of inflammation-related genes that include inducible nitric oxide synthesis (iNOS), COX-2, MCP-1, MMP-9, IL-2, IL-6, IL-8, IL-12p40, IL-2 receptor, ICAM-1, TNF-α, and IFN-γ [130], which leads to the secretion of many inflammatory mediators. The aforementioned genes were shown to have changed in the tissues of ALS patients and hSOD1 transgenic mice [128,131-133]. COX-2 is inducible and is a rate-limiting enzyme of the synthesis pathways of the prostaglandins (PG) PGD_2, PGE_2, PGF_2a, and PGI_2 and thromboxane (TXA_2). Prostaglandins play a role in various cellular effectors that include the instigation of inflammatory responses, the re-arrangement of cytoskeletons, and gene transcription changes [134]. COX-2 expression was significantly elevated in motor neuron and glial cells in the spinal cord of ALS patients [135-136], and the COX-2 activity increased in the spinal cord of ALS patients [137]. In addition, the PGE_2 levels jumped up in the CSF of ALS patients by two to 10 times, compared with the controls [137]. The deletion of the prostaglandin E(2) EP2 receptor in SOD1G93A mice improved their motor function and prolonged their survival, which suggests that PGE2 signalling via the EP2 receptor acts as an inflammatory mediator in motor neuron degeneration [138].

5.2.1. Non-cell-autonomous neurotoxicity in ALS

Aside from degenerating motor neurons, microglia and astrocytes concomitantly play a role in disease progression in ALS model mice. Recent reports emphasized the potential role of non-cell-autonomous mechanisms, which are harmonious with and critical in SOD1G93A-induced cell-autonomous death signals [139-140]. Either neuron-specific or glia-specific

expression of SOD1 mutation in mice led to the ALS phenotype, with marginal effects [141-142]. Specific expression of mutant SOD1 within neurons using *Nefl* (neurofilament light chain) promoters did not cause motor neuron degeneration in transgenic mice [142]. Consistently, selective expression of mutant SOD1 in microglia or astrocytes did not kill motor neurons [141,143].These non-cell-autonomous deaths of motor neurons were supported by an analysis of chimeric mice that had mixed populations of normal cells and cells that expressed mutant SOD1 [144]. Conditional knockout of mutant SOD1 in motor neurons using an *Isl1* promoter-driven *Cre* transgene that is expressed in the spinal cord delayed the disease onset in and prolonged the survival of mutant SOD1 transgenic mice. On the other hand, however, selective removal from cells of the myeloid lineage that included microglia using a *Cd11b* promoter-driven *Cre* transgene did not delay the disease onset but extended its progress [139]. In the same lineages, selective viral vector-mediated delivery of small interfering RNAs against human SOD1 in motor neurons delayed the disease onset but did not modify the disease progression once it started [145], whereas silencing of mutant SOD1 within myeloid cells or astrocytes slowed the disease progression rather than the disease onset [139-140]. After all the bone marrow of mutant-SOD1-expressing $PU^{-/-}$ mice, which lacked myeloid and lymphoid cells, were replaced with wild-type-SOD1 bone marrow, their disease progression and survival improved [143], which suggests that microglia and astrocytes were not sufficient for the initiation of motor neuron death, but hastened the disease progression.

5.2.2. Systemic inflammation

Damaged or aged brains continuously suffer from systemic inflammation connected with peripheral factors, regardless of the presence of innate inflammation in the CNS [146-147]. Three critical components are directly correlated with the synthesis of cytokines and inflammatory mediators in the brain parenchyma to communicate an inflammatory signal to the brain and to trigger tissue injury. First, inflammatory responses in the thoracic-abdominal cavity are transduced into the brain via vagal-nerve sensory afferents, and then the outflow of a vagal efferent seems to manipulate these events through acetylcholine secretion, which acts on alpha 7 nicotinic receptors of macrophages [148]. Second, cytokines and inflammatory mediators from the specific area of the inflammation are put into the blood and communicate with macrophages and other cells in the circumventricular organs, which lack a patent blood-brain barrier [149]. Third, the cytokines or inflammatory mediators themselves might directly communicate with the brain endothelium via receptors expressed on the endothelium [150]. Several pieces of evidence showed that a systemic immune response is related to a clinically symptomatic feature of a neurodegenerative disease such as AD. In accordance with frequently circulating cytokines in the blood or CSF of AD patients, the abundance of pro-inflammatory factors preceded the clinical onset of dementia in the subjects [151]. Aged people with systemic infections have a double risk of developing AD. Similarly, the correlation of clinical events with systemic immunity was experimentally evaluated in an animal that was challenged with systemic stimulation. Infection of aged rats with LPS revealed neuronal loss in the brain and the memory deficits [152]. Thus, it can be said that systemic inflammation contributes to the onset and progression of neurodegenerative diseases. In recent clinical and pathological studies, ALS patients revealed dysregulation of their systemic inflammatory components,

which belonged to alterations in their microglia/macrophage activation profiles [153]; elevated levels of complementary proteins in their sera [154]; increased IL-13-producing T cells and circulating neutrophils [155-156]; and higher production of $CD8^+$ T cells in the lymphocytes [157]. Monocyte chemoattractant protein (MCP)-1 and RANTES were abundant in the cerebrospinal fluid and sera of ALS patients [158-161]. Increased MCP-1 was shown in the microglia of mutant SOD1 mice [162-163]. Moreover, the higher LPS level in the plasma of ALS patients was proportional to the total abnormally activated monocyte/macrophage contents of the peripheral blood [164]. Long-term exposure to LPS also furthered the disease progression in animal ALS models, which implies that systemic inflammation connected to peripheral factors and innate immunity in the CNS concurrently influences the disease course [165]. With aging, the blood-brain barrier (BBB) is less tight and thus, more vulnerable to systemic inflammation. The collapse of BBB or of the blood-spinal cord barrier (BSCB) was shown in animal ALS models or human ALS patients using evans blue leakage and immunohistochemistry against the anti-CD44 antibody, respectively [166-167]. Under these conditions, peripheral-inflammation-inducing factors were very apparent in the CNS and thereby affected the neurodegeneration.

5.2.3. Therapies for inflammation in ALS

Minocycline, which is believed to attenuate microglia activation, or celecoxib, a cox-2 inhibitor, showed beneficial effects in mutant SOD1 mice [115-117,168-169]. Clinical studies on the two drugs did not disprove, however, their therapeutic property in ALS patients. Thalidomide, glatiramer acetate, and ONO-2506 also supported the causative role of the inflammation in the pathology in ALS mice that showed improved motor function and survival [170-171], but their beneficial effects were not linked to the ALS patients.

6. Mitochondrial pathology in ALS

Mitochondria constitute approximately 25% of the cytoplasmic volume in most eukaryotic cells and produce cellular energy in the form of ATP via electron transport and oxidative phosphorylation. During electron transfer in the inner membrane of the organelle, electrons spontaneously leak from the electron transport chain and react with available O_2 to produce superoxide, which makes mitochondria the major cellular sources of ROS. Mitochondria have been recognized as target organelles for the regulation and execution of cell death under pathological conditions [172-173]. There are many mitochondria in the motor neurons because of the high rate of metabolic demand therein, which implies that motor neurons are susceptible to functional or morphological alteration in mitochondria. Mtochondrial abnormality may play a crucial role in the pathologic mechanism of motor neuron diseases and of ALS. Studies with ALS patients and animal ALS models have been performed to examine both the morphologic and functional abnormalities of the mitochondria [174]. Morphological abnormality in the organelle that includes a fragmented network, swelling, and increased cristae has been observed in the soma and proximal axons of ventral motor neurons of sporadic ALS (sALS) patients [39]. In ALS patients, a reduction in complex IV of the electron transport chain activity

was evident and has been associated with mutations in mitochondrial DNA [175-176]. Although SOD1 is mainly localized in cytosols, it is also resilient in other subcellular compartments such as the mitochondria [45,177-178] and even the endoplasmic reticulum [182]. The aggregates of mutant SOD1 were shown within the mitochondria of the spinal cord of $SOD1^{G93A}$ mice before the onset of symptoms [46-47] and were implicated in increased oxidative damage, decreased respiratory activity of mitochondria [48], and the appearance of mitochondrial swelling and vacuolization [47]. Dissociated cytochrome c from the interaction of mitochondria with mutant SOD1 activates apoptosis [44]. Mitochondria function as reservoirs of intracellular Ca^{2+}, as ER. Once overloaded in cytosol, the accumulated Ca^{2+} in the mitochondria prepares the organelle to undergo permeability transition, and then swells and ruptures in their outermembrane, which in turn produces free radicals from them and oxidizes their lipids and DNA [179-180]. Ca^{2+}-induced mitochondrial damage can also result in mitochondrial release of cytotoxic substances such as cytochrome c [181] and can affect caspase cascade. The homeostasis at the intracellular Ca^{2+} level was also disturbed in motor neurons of SOD1G93A mice [182]. Moreover, increased Ca^{2+} uptake into the mitochondria of motor neurons easily occurred after exposure to the glutamate agonist AMPA or kinate, and triggered increased ROS generation [183]. ALS-linked SOD1 has been shown to slow down fast axonal transport of mitochondria. The axonal mitochondria transport was primarily reduced in the anterograde direction, which suggests that the energy supply in the presynaptic terminals of the motor endplates is compromised [184]. Multiple functions of the mitochondria over cellular injury and the apearance of mitochondrial dysfunction in the presymptomatic stage may contribute to various routes of neuronal death in ALS. More recently, in mice that expressed human TDP-43 only in neurons that included motor neurons, massive accumulation of mitochondria in TDP-43-negative cytoplasmic inclusions in the motor neurons were reported and the lack of mitochondria in the motor axon terminal was observed [185]. In addition, the transgenic mice that overexpressed human TDP-43 driven by the mouse prion promoter demonstrated motor deficits, early mortality, and mitochondrial aggregation [186]. These results imply that TDP-43 is indirectly involved in mitochondrial dysfunction in neurodegenerative diseases such as ALS.

7. Autophagy in ALS

Autophagy is a degradative mechanism that is involved in the recycling and turnover of long-life proteins and organelles [187]. Autophagy is basically induced by lack of nutrients and energy or by various toxicants. Although its primary role is adaptation to scarcity, this degradative process is also critical for the normal turnover of cytoplasmic contents that include neurons. Genetic ablation of autophagy-related genes provokes neurodeneration even with lack of disease-like mutant proteins [188]. Recent studies verified the importance of the autophagy pathway in various pathological conditions that include neurodegenerative diseases [189]. Interestingly, the catabolic process is both beneficial and detrimental to cells, depending on its context and specific stimuli. The lethality of mutated SOD1 is the result of abnormal protein aggregates, which impair the degradation machinery such as the ubiqutin-

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proteasome system and the autophay-lysosome pathway [190-191]. Enhancing the latter with physiological characteristics prevents motor neuron dysfunction in vivo [192-193]. Defects in the autophagy pathway have a principal disease-causing role in human pathologies that include neurodegeneration [189,194]. Studies of the spinal motor neurons of ALS patients [195] and ALS transgenic mice [196] have delineated the abnormality in autophagy, which is probably correlated with the pathogenesis of the disease [192-193,197]. A growing number of studies support the concept that autophagy makes diseased motor neurons healthy by clearing the aggregated mutant SOD1, which was accomplished by inducing autophagy, as illustrated by the increased number of autophagosomes and the higher level of autophagy markers such as Beclin-1, ATG5-ATG12 complex, and LC3-II [192-193]. It is also possible, however, that blunt autophagy in neurodegenerative conditions was accompanied by the abnormal accumulation of autophagosomes and excessive markers, which might have killed the neurons [197-198] and which indicates the compensatory role of autophagy in inherited ALS. Thus, the detailed molecular mechanism of the development of autophagy-mediated diseases must be explained.

8. Therapeutic strategy for ALS

8.1. Separate routes of motor neuron degeneration in ALS

The parallel pathway of oxidative stress and Fas-mediated apoptosis in motor neuron death in SOD1G93A mice was previously focused on [96]. This study provided the first evidence that combination therapy that targets oxidative stress and apoptosis together also delays the onset and progression of motor dysfunction and extends the survival time of ALS transgenic mice. Evidence was accumulated that shows that oxidative stress and apoptotic insults cause neuronal death through distinctive pathways and with unique morphological changes. The neurotrophins' nerve growth factor, the brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and NT-4/5, and the insulin-like growth factors IGF-I and IGF-II, promote neuronal survival by preventing programmed cell death or apoptosis, but they significantly enhance necrotic degeneration of neurons exposed to oxidative stress or deprived of oxygen and glucose [199-200]. Neurotrophins can induce oxidative stress by upregulating NADPH oxidase, which leads to neuronal cell necrosis [201]. Surprisingly, the insulin-like growth factor 1 (IGF-1) prevented neuronal cell apoptosis and protected spinal motor neurons in ALS mice [199,202], but markedly potentiated neuronal cell necrosis induced by hydroxyl radicals or glutathione depletion [203]. Given that oxidative stress and apoptosis play a central role in motor neuron degeneration and can contribute to neuronal death through distinctive routes in ALS, it was hypothesized that a therapeutic approach that targets both oxidative stress and apoptosis would have additive effects on neuronal survival and the motor function. To pharmacologically prevent oxidative stress and apoptosis, Neu2000, a novel anti-oxidant, and Li^+, a well-known anti-apoptosis agent, were used. The former, a chemical derivative of aspirin and sulfasalazine, was developed to protect neurons from oxidative stress with greater potency and safety, and has been shown to be a potent and secure anti-oxidant in vitro and in animal models of hypoxic ischemia [204]. Li^+ has been shown to prevent apoptosis through mecha-

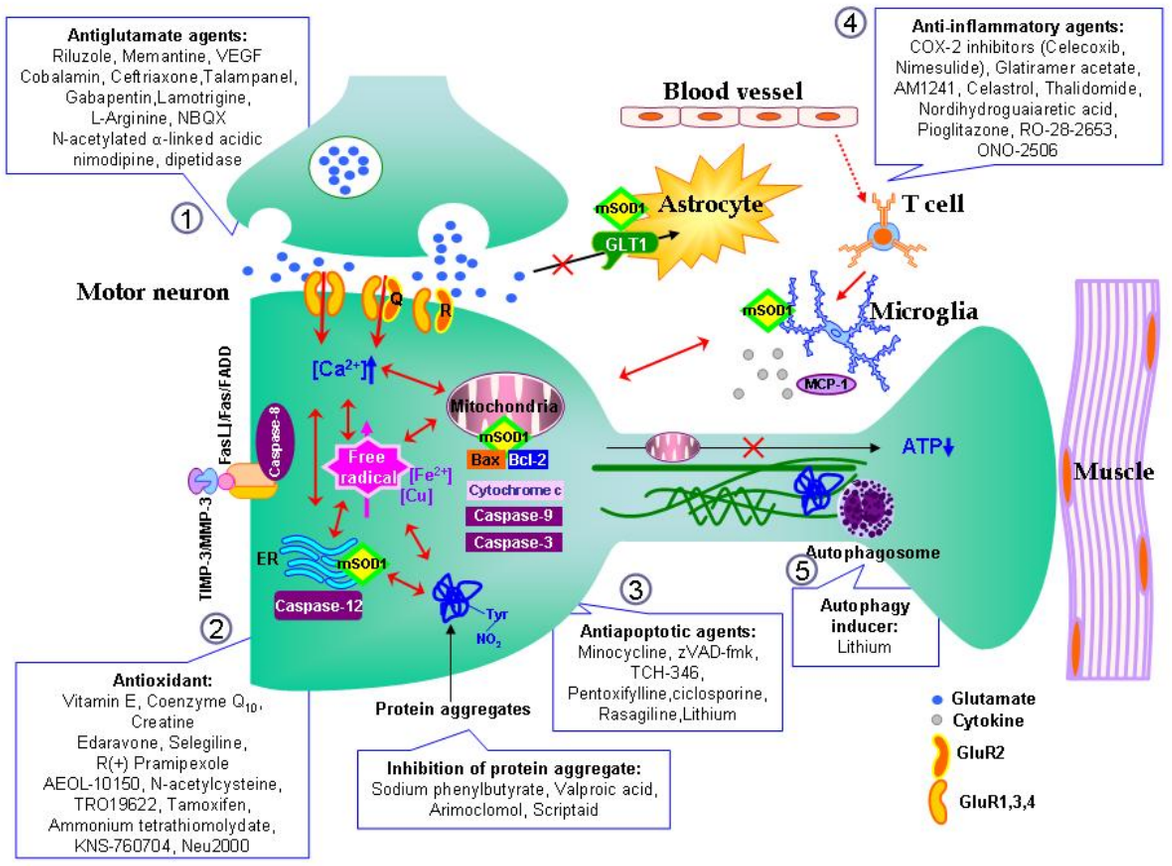

Figure 1. Multiple pathways of motor neuron degeneration and their therapeutic drugs in ALS: (1) increased Ca^{2+} in the motor neuron: dysfunction or downregulation of glutamate transporters such as GLT1 on the astrocytes, elevation of the Ca^{2+} permeable AMPA receptor via downregulation of or a deficit in the post-transcriptional edition of GluR2 sub-units, and mitochondrial dysfunction; (2) oxidative damage of the motor neuron: increased intracellular Ca^{2+} contents, high levels of mitochondria due to high energy demand, and increase in free metal ions such as copper and iron; (3) apoptosis in the motor neuron: activation of the Fas-mediated pathway, alteration of Bcl-2 family proteins via mitochondrial interaction with mSOD1, and initiation, propagation, or execution of caspase cascade; (4) inflammation: non-cell-autonomous motor neuron death (the disease progression is coordinated by mSOD1 expression in all neuronal and non-neuronal cells) and concurrent activation of the innate immune system and systemic inflammation (BBB breakdown may induce a vicious cycle of inflammation); and (5) autophagy: increased autophagosome formation.Current therapeutic drugs were developed basically against a specific route of ALS disease progression.

nisms that involve Bcl-2 upregulation, glycogen synthase kinase-3 beta inhibition, and activation of phosphatidylinositol 3-kinase that activates serine/threonine kinase Akt-1 and phospholipase C gamma [205-206]. An additional benefit of Li+ was recently demonstrated the induction of an autophagy pathway at a low dose, clears altered mitochondria and protein aggregates [192]. In the results of this study, the concurrent administration of Neu2000 and Li^+, which block free-radical-mediated necrosis and Fas-mediated apoptosis, respectively, significantly delayed the onset and progression of motor neuron degeneration and motor function deficits. Thus, targeting both oxidative stress and the Fas apoptosis pathway with concurrent treatment with Neu2000 and Li^+ may further improve the neurological function

Compound	Dose	Administration route	Hypothetical mechanism	Survival	Reference
Creatine	1%	diet	Antioxidant	9%	Klivenyi P et al., 1999 [78]
	2%	diet	Antioxidant	17%	
Creatine	2%	diet	Antioxidant	20%	Klivenyi P et al., 2004 [169]
creatine	2%	diet	Antioxidant	12%	Zhang W et al., 2003 [117]
Vitamin E	200 IU	chow	Antioxidant	No effect	Gurney ME et al., 1996 [125]
Edaravone	5 mg/kg	ip	Antioxidant	12.4%	Ito H et al., 2008 [81]
	15 mg/kg	ip		17%	
AEOL-10150	2.5 mg/kg	ip	Antioxidant	26%	Crow JP et al., 2005 [80]
	2.5 mg/kg	sc		22%	
N-acetylcysteine	2 mg/kg/d	drinking water	Antioxidant	7%	Andreassen OA et al., 2000 [79]
TRO19622 (Olesoxime)	3 mg/kg	sc	Antioxidant	10%	Bordet T et al., 2007 [209]
	30 mg/kg	sc		8%	
Ammonium tetrathiomolybdate	5 mg/kg	not described	Antioxidant	25%	Tokuda E et al., 2008 [210]
Neu2000	30 mg/kg	diet	Antioxidant	15%	Shin et al., 2007 [96]
zVAD			Antiapoptotic	22%	Li et al., 2000 [111]
Cyclosporin A	18mg/kg	intrathecal	Antiapoptotic	12%	Keep M et al., 2001 [211]
Minocycline	25 mg/kg	ip	Antiapoptotic/Anti-inflammator	10%	Van Den Bosch L et al., 2002 [116]
	50 mg/kg	ip		15.8%	
Minocycline	11 mg/kg		Antiapoptotic/Anti-inflammator	9%	Zhu S et al., 2002 [115]
Minocycline	22mg/kg/d	ip	Antiapoptotic/Anti-inflammator	13%	Zhang W et al., 2003 [117]
Lithium	1 mEq/kg	ip	Antiapoptotic/Autophagy induc	36%	Fornai F et al., 2007 [192]
Lithium	60 mg/kg	ip	Antiapoptotic/Autophagy induc	8%	Feng H et al., 2008 [212]
Lithium	2%	diet	Antiapoptotic/Autophagy induc	10%	Shin et al., 2007 [96]
Celecoxib	1500ppm	chow	Anti-inflammatory	25%	Drachman DB et al., 2002 [168]
Celecoxib	0.012%	diet	Anti-inflammatory	21%	Klivenyi P et al., 2004 [169]
Thalidomide	50 mg/kg		Anti-inflammatory	12%	Kiaei M et al., 2006 [213]
	100 mg/kg			16%	
Glatiramer acetate	7ug/0.1 ml PBS	immunization	Anti-inflammatory	1.4%	Banerjee R et al., 2008 [171]
AM1241	1 mg/kg	ip	Anti-inflammatory	3%	
Celastrol	8 mg/kg	diet	Anti-inflammatory	13%	Kiaei M et al., 2005 [213]
	2 mg/kg			9.4%	
Nordihydroguaiaretic acid	2500ppm	po	Anti-inflammatory	10%	West M et al., 2004 [214]
RO-28-2653	100 mg/kg	po	Anti-inflammatory	11%	Lorenzl S et al., 2006 [215]
Riluzole	100ug/ml	drinking water	Antiglutamatergic	10%	Gurney ME et al., 1996 [117]
Riluzol	30 mg/kg	drinking water	Antiglutamatergic	11%	Waibel et al., 2004 [224]
Gabapentin	3%	chow	Antiglutamatergic	5%	Gurney ME et al., 1996 [117]
Memantine	10 mg/kg	subcutaneus	Antiglutamatergic	7%	Wang et al., 2005 [216]
Memantine	30 mg/kg	drinking water	Antiglutamatergic	5%	Joo IS et al., 2007 [26]
	90 mg/kg	drinking water		1%	
vegf	1.0 ug/kg	ip	Antiglutamatergic	8%	Zheng C et al., 2004 [217]
		Lentiviral vecor	Antiglutamatergic	30%	Azzouz M et al., 2004 [218]
Ceftriaxone	200 mg/kg	ip	Antiglutamatergic	10%	Rothstein JD et al., 2005 [219]
L-Arginine	6%	drinking water	Antiglutamatergic	20%	Lee J et al., 2009 [220]
N-acetylated a-linked acidic dipetidase	30 mg/kg	po	Antiglutamatergic	15%	Ghadge GD et al., [221]

Table 1. List of drugs tested with ALS mice

and neuronal survival in ALS and possibly other neurological diseases such as stroke, Alzheimer's disease, and Parkinson's disease. The authors' hypothesis was supported by other experiments in which a cocktail of neuroprotective drugs with different modes of action more significantly improved survival and the motor function than did monotherapy in transgenic mouse ALS models [117,207].

8.2. Current treatment and new approach of ALS medications

Riluzole, the only therapeutic drug approved for ALS, extends life expectancy to up to 3 months in human patients. The symptomatic drug potentially targets gluatamate- or oxidative-stress-induced neurodegeneration with marginal apoptosis effects [25]. As mentioned,

Compound	Dose	Survival	Reference
Creatine	2%	12%	Zhang W et al., 2003 [117]
Minocycline	22mg/kg	13%	
Creatine/Minocycline		25%	
Creatine	2%	20%	Klivenyi P et al., 2004 [169]
Celecoxib	0.012%	21%	
Rofecoxib	0.005%	19%	
Creatine/Celecoxib		29%	
Creatine/Rofecoxib		31%	
Rasagiline	2 mg/kg	14%	Waibel et al., 2004 [224]
Riluzol	30 mg/kg	11%	
Rasagiline/Riluzol		20%	
Neu2000	30 mg/kg	15%	Shin et al., 2007 [96]
Lithium	2%	10%	
Neu2000/Lithium	2%	22%	
Lithium	60 mg/kg	8%	Feng H et al., 2008 [212]
Valproic acid	300 mg/kg	10%	
Lithium/ VPA		15%	
Riluzole		7.5%	Del Signore Sj et al., 2009 [222]
Sodium phenylbutyrate		12.8%	
Riluzole/Sodium phenylbutyrate		21.5%	
Minocycline/ Riluzole/ Nimodipine	80 + 40 +30 (mg/kg)	13%	Kriz et al., 2003 [223]

Table 2. Additive effect of combination therapy in ALS mice

therapeutic strategies and drugs developed based on them, as shown in Figure 1, explain the multiple-disease-causing process of ALS. As shown in Table 1, many drugs were evaluated in mice that expressed mutant SOD1. Most of the drugs were beneficial to the motor function and survival in the tests with the mice. Several drugs (such as creatine, celecoxib, gabapentin, topiramate, lamotrigine, minocycline, thalidomide, valproate, vitamin E, and even lithium) showed beneficial effects in animal ALS models, but none of them significantly prolonged the survival or improved the quality of life of human ALS patients. The therapeutic effects on the animal models and the human patients significantly differed due to the following translational mismatch issues: first, the methological inappropriateness of the drug screening with the use of animals that had biological confounding variables such as sex and differences in the treatment initiation time point; second, the lack of correct pharmacokinetics, which were considered in a dose-ranging study of safety/toxicity and BBB penetration; and finally, the methodological pitfall of ALS clinical trials due to the insufficiency of the number of patients, the inclusion of heterogeneous populations, the short duration of the trial, and the inadequate analysis of the efficacy. It should be noted that the combination of creatine and celecoxib improved the motor function in a randomized clinical phase II trial of ALS patients and SOD1G93A mice, although single treatment with either creatine or celecoxib failed to show beneficial effects in human ALS trials [208], which suggests the greater efficacy of combined anti-oxidant and NSAID therapy than those of monotherapy. Several pieces of evidence support the notion that therapeutic combinations are more effective than individual agents in animal ALS models (Table2). More recently, the authors reported that a single agent named

AAD-2004, which has a dual mode of action as an anti-oxidant and an mPGES-1 inhibitor, had better efficacy on the motor function and survival than those of riluzole and ibuprofen.

In support of such a notion, a phase II clinical trial was recently conducted, which showed that the suggested strategy may be feasible and efficient.

9. Conclusion

In ALS, knowledge of the contribution of multiple pathways to the degeneration of motor neurons has expanded greatly and has challenged clinical trials of drugs that target the processes. Better understanding of the detrimental processes that cause neurodegeneration will help define its medical importance and clarify the therapeutic potential of interfering with them.

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