

Riluzole, Neuroprotection and Amyotrophic Lateral Sclerosis

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Abstract: Amyotrophic lateral sclerosis (ALS) is a universally fatal neurodegenerative disease of the human motor system. Aetiological mechanisms implicated in the development of ALS have been linked to the glutamatergic neurotransmitter system, with destruction of motor neurons triggered through excessive activation of glutamate receptors at the synaptic cleft. This 'excitotoxicity' theory of ALS gave rise to the development of therapeutic approaches and ultimately clinical trials involving riluzole, initially thought to act solely as an inhibitor of glutamate release. Subsequent effects of riluzole have been postulated to include indirect antagonism of glutamate receptors, in addition to inactivation of neuronal voltage-gated Na⁺ channels. Riluzole remains the only disease-modifying therapy available to patients with ALS. Despite having been clinically available since the mid-1990s, the *in vivo* pharmacological targets of riluzole have been poorly defined. An improved understanding concerning the potential neuroprotective mechanisms of riluzole may unearth pathophysiological processes that mediate neurodegeneration in ALS. The present review summarises the known chemical and pharmacological properties of riluzole. The failure of other putative neuroprotective therapies to demonstrate positive treatment outcomes in this intractable disease will be reviewed. Finally, the hypothesis that Na⁺ conductances may be involved in the processes of neuronal and axonal degeneration in ALS will be explored.

Keywords: Amyotrophic lateral sclerosis, motor neuron disease, motor neuron, clinical trial, glutamate, excitotoxicity, riluzole.

INTRODUCTION

First described by Jean-Martin Charcot in 1869 [1], amyotrophic lateral sclerosis (ALS) encompasses a group of neurodegenerative disorders characterised pathologically by motor neuron loss in the motor cortex, brainstem and spinal cord. Amyotrophic lateral sclerosis is universally fatal, with a median survival of three years from symptom onset [2]. Clinically, ALS manifests as progressive weakness of voluntary muscles, with relative sparing of muscles responsible for eye movements and control of the pelvic floor [3]. The disease most typically begins in the limbs, but in 20% of cases, weakness is first evident in the oropharyngeal muscles (bulbar onset) [4]. A minority of ALS patients may initially complain of shortness of breath, signifying respiratory muscle involvement as the sole presenting symptom [5]. Irrespective of the site of disease onset, weakness of respiratory muscles culminates in respiratory failure [6], the principal cause of ALS mortality.

The pathophysiological mechanisms underlying ALS appear multifactorial, with evidence emerging of complex interactions between genetic factors and molecular pathways including oxidative stress, glutamate-mediated excitotoxicity, mitochondrial dysfunction, impaired axonal transport, glial cell dysfunction and the precipitation of cytoplasmic aggregates [7-12]. Riluzole, a 2-aminobenzothiazole, is the only disease-modifying therapy available for ALS [13]. Although riluzole is known to modulate excitatory neurotransmission, the precise neuroprotective mechanisms remain largely speculative. An improved understanding of riluzole and its pharmacological targets may provide further insight into the pathophysiology of ALS and potentially enable identification of novel therapeutic strategies.

PATHOPHYSIOLOGY OF NEURODEGENERATION IN ALS

The processes implicated in motor neuron degeneration in ALS are complex. Of central importance is the susceptibility of motor neurons to the excitatory properties of glutamate. Excessive activation of post-synaptic ionotropic receptors appears to result in a flood of Ca²⁺ entry into motor neurons, leading to the activation of free-radical generating enzyme systems (including nitric oxide synthase, phospholipase A₂ and xanthine oxidase) and activation of degradative and apoptotic enzymes such as proteases. This detrimental influence of glutamate on motor neurons in ALS has been termed the 'excitotoxicity hypothesis' (Fig. (1)).

Glutamate and Excitotoxicity

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) [14]. It is synthesized from reductive deamination of α -ketoglutarate by glutamate dehydrogenase and from the action of aminotransferases on amino acids. Approximately 20% of the total glutamate pool is stored in pre-synaptic nerve terminals [14]. During neurotransmission, glutamate is released from pre-synaptic neurons by means of depolarisation of the pre-synaptic neuronal endplate, and then diffuses across the synaptic cleft to activate post-synaptic glutamatergic receptors. Such receptors are ionotropic (i.e. they allow the passage of ionic species), and are classified according to their affinity to exogenous agonists, N-methyl-D-aspartate (NMDA), α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainate [14]. The excitatory signal is terminated with removal of glutamate from the synaptic cleft by the Na⁺-dependent glutamate transporter [known as excitatory amino acid transporter-2 (EAAT-2) in humans, and glutamate transporter-1 in rodents (GLT-1)], present on astrocytic processes that envelop the synapse [15]. This transporter is responsible for removing up to 94% of glutamate from the

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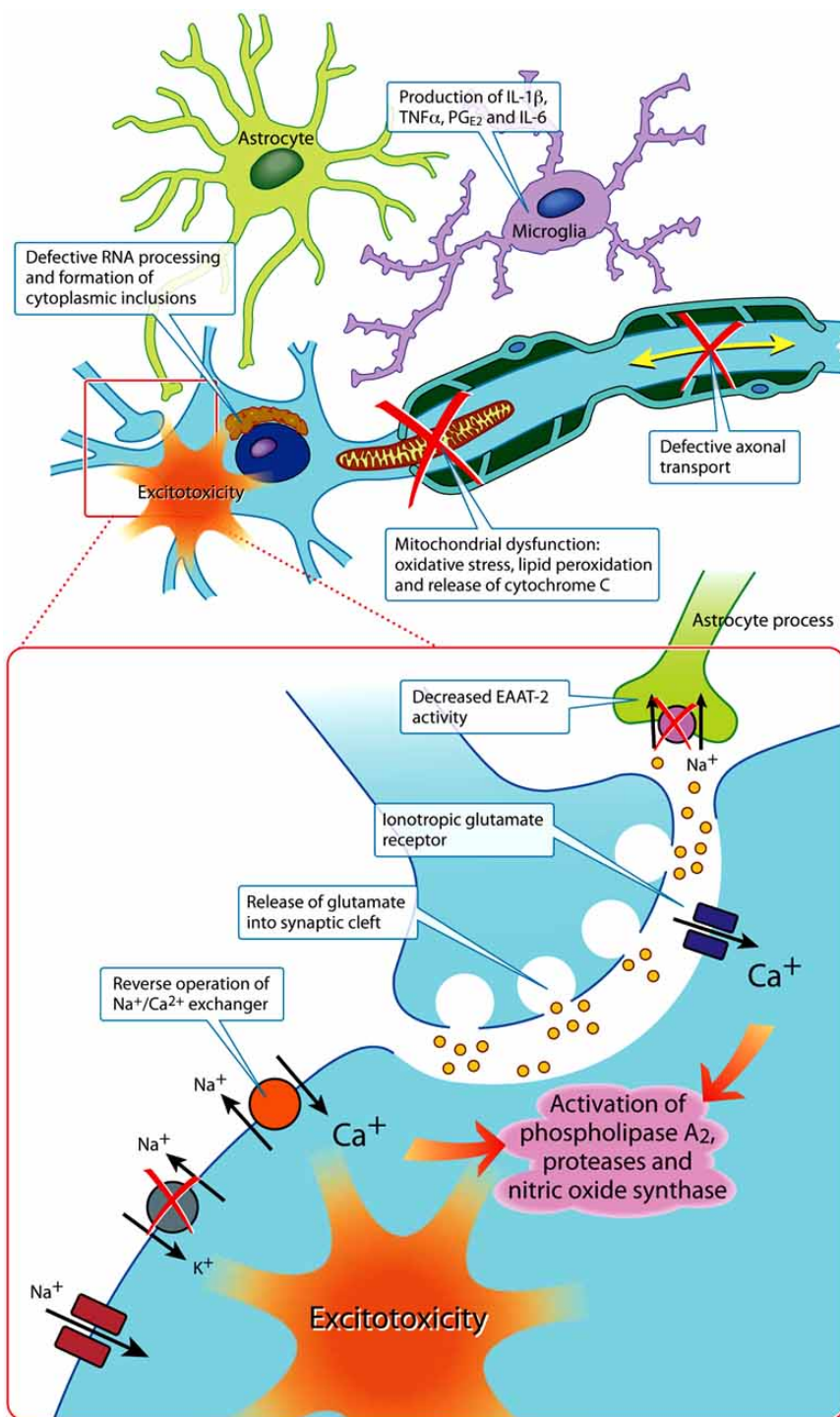


Fig. (1). The pathogenic processes that trigger motor neuron degeneration in ALS. At the core of motor neuron degeneration is overactivation of glutamatergic receptors located on the post-synaptic neuron. Pathological activation of post-synaptic glutamate receptors may be attributed to decreased glutamate uptake by astrocytic processes. The passage of excessive Ca^{2+} through ionotropic receptors increases intracellular Ca^{2+} levels, leading to the activation of degradative enzymes, including phospholipase A_2 , proteases and nitric oxide synthase. Elevations in intracellular Ca^{2+} also cause perturbations in mitochondrial function, which results in the production of free radicals, and as well as impaired production of adenosine triphosphate (i.e. (ATP)). Combined with ATP depletion, the production of nitric oxide and other free radical species results in the inactivation of the Na^+/K^+ pump, raising intracellular Na^+ concentrations, which leads to neuronal depolarisation (i.e. 'hyperexcitable'). As a consequence, reverse operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger may occur in an attempt to normalise intracellular Na^+ levels, but the increased entry of Ca^{2+} may exacerbate the already elevated intracellular Ca^{2+} levels. Axonal transport may become impaired secondary to excitotoxicity. Furthermore, the formation of cytoplasmic aggregates (TDP-43 in sporadic ALS and a minority of familial cases; and SOD-1 and FUS in their respective familial counterparts) may incite neurodegeneration through undefined mechanisms. Microglial infiltration marks the development of neuroinflammatory processes, which may exacerbate excitotoxicity through the production of inflammatory cytokines.

synaptic cleft [16]. Within astrocytes, glutamate is converted to glutamine by glutamine synthetase. Glutamine is subsequently returned to the neuron for glutamate re-synthesis [17].

Crucial towards forging the connection between excitotoxicity and ALS was the discovery that cerebrospinal fluid (CSF) in ALS patients contained three-fold higher concentrations of glutamate, aspartate, N-acetyl-aspartyl glutamate and N-acetylaspartate compared to healthy controls [18]. By contrast, other amino acid levels were unchanged. Furthermore, raised glutamate levels were not been observed in patients with other CNS disorders (including progressive supranuclear palsy, pseudotumour cerebri), suggesting that elevated CSF glutamate appeared specific for ALS. It was later demonstrated that CSF extracted from ALS patients was toxic to motor neurons in culture [20, 21], and normal motor neurons exposed to high extracellular levels of glutamate underwent degeneration [20, 22]. Additional exposure to AMPA receptor antagonists and glutamate inhibitors protected against glutamate-induced motor neuron loss [19]. Furthermore, the expression of various glutamate transporter subtypes were significantly decreased in motor regions of the CNS in ALS patients [20, 21]. The pathogenic role for glutamate may be further substantiated by subsequent studies involving riluzole, with a principal action to augment glutamatergic activity in the CNS.

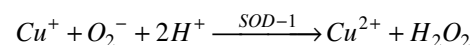
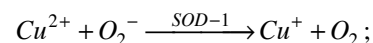
Reduction in astrocytic GLT-1 activity with impaired glutamate uptake from the synaptic cleft was subsequently established in rat models, with neuronal death linked to over stimulation of post-synaptic glutamate receptors. In the same study, reduced GLT-1 activity resulted in a progressive paralytic syndrome in rodents [22]. However, growing evidence has suggested that diminished astrocytic EAAT-2 activity may indeed represent an epiphenomenon of motor neuron loss, given that activity is down-regulated in primary astrocytic cultures where motor neuronal influences are absent [23]. Alternatively, reactive oxygen species produced by excitotoxic motor neurons may diffuse locally to decrease astrocytic EAAT-2 activity [24]. Such a process could result in a vicious cycle, whereby further excitotoxic activation of motor neurons by glutamate results in even greater production of reactive oxygen species.

Alterations in cytoplasmic Ca^{2+} have also been implicated in motor neuronal susceptibility to excitotoxicity. Incorporation of the GluR2 sub-unit into AMPA/kainate glutamate receptors diminishes Ca^{2+} -permeability due to the presence of a positively charged arginine residue inside the channel pore [25]. In comparison to other neuronal populations, greater expression of AMPA and kainate glutamate receptors lacking the GluR2 sub-unit have been identified in α -motor neurons [26]. Injection of AMPA into the ventral horn of rodent spinal cord produced widespread loss of motor neurons and resultant paralysis [27, 28]. Conversely, blockade of NMDA glutamate receptors with resultant sequestration of cytoplasmic Ca^{2+} may avert neuronal fall-out and thereby the development of muscle weakness [28]. Furthermore, it appears that motor neurons have lower cytoplasmic Ca^{2+} buffering capacity, owing to the absence of Ca^{2+} binding proteins parvalbumin and calbindin D-28k [29, 30], rendering them more vulnerable to deleterious influxes of Ca^{2+} . Indeed, increased calbindin expression in mutant SOD-1 positive mo-

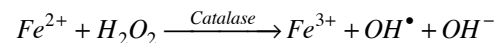
tor neurons enhanced neuronal survival [31]. Therefore, strong evidence exists for a relationship between Ca^{2+} -mediated neurotoxicity arising from excessive glutamatergic activity in ALS.

Oxidative Stress and Mitochondrial Dysfunction

Free radicals are molecules containing unpaired electrons in their outer orbit. In humans, the principal source of free radicals relates to mitochondrial respiration. Given its high level of oxygen utilisation and dependence on adenosine triphosphate (ATP), the brain produces more free radicals than any other organ in the body. Pivotal to unravelling putative disease mechanisms underlying ALS was the discovery of mutations in the Cu^{2+} - Zn^{2+} -binding superoxide dismutase-1 (SOD-1) gene on chromosome 21q22.1 in patients with familial ALS [32]. The SOD-1 enzyme is a ubiquitous, cytosolic protein that catalyses the conversion of superoxide anion to hydrogen peroxide and oxygen:



Hydrogen peroxide is then converted to water by glutathione peroxidase, or to the hydroxyl free radical by catalase in the Fenton reaction [33]:



Deletion of the SOD-1 gene does not result in disease, suggesting that overproduction of free radicals ('toxic gain of function') causes the deleterious effects [34]. Mutations in SOD-1 account for approximately 20% of familial ALS cases (or 2% of all ALS cases) [32]. More than 150 point mutations have been documented (visit <http://alsod.iop.kcl.ac.uk/als/Index.aspx> for a complete listing of all known mutations), and all except one are associated with autosomal dominant inheritance.

The discovery of mutations in SOD-1 heralded the development of transgenic SOD-1 mouse models, which in some respects resemble human ALS [35]. The commonest genotypic variant is G93A, whereby glycine is substituted with alanine at position 93. From a therapeutic perspective, the transgenic mouse model has represented a platform for testing potential therapies. Unfortunately, translational research has failed to deliver meaningful results in trials on human disease (discussed later). Riluzole, which was tested in the mouse model after its effectiveness had been proven in human disease, afforded a survival benefit of 11% over control mice [36]. Riluzole also reduced the rate of decline in muscle function in transgenic mice, though therapy was initiated before disease onset [37, 38].

Free radical injury to motor neurons may compromise cellular homeostasis through oxidation and nitration of membrane lipids and neuronal proteins, thereby further lowering the tolerance of motor neurons to the damaging effects of glutamate. Although SOD-1 is ubiquitously expressed, it is possible that motor neurons are particularly vulnerable to the toxicity of free radicals, due to increased membrane permeability to Ca^{2+} . Free radicals inhibit the activity of astrocytic EAAT-2 [39] through oxidation of thiol groups and formation of disulfide bonds on cysteine residues [40].

Neuronal homeostasis is critically dependent on energy consumption and oxygen utilisation. Mitochondria are the key energy-producing organelles present in all neurons. The corollary, however, is that mitochondria represent important sources of free radicals. Mitochondrial abnormalities, in the form of morphological changes, were first reported in the skeletal muscle of ALS patients [41]. Mitochondrial dysfunction may also develop as an early feature in ALS, when motor neurons are structurally normal [42].

Synthesis of ATP depends on maintenance of the mitochondrial membrane potential. Mitochondrial permeability transition is a state wherein this membrane potential is lost and may be induced through free radical injury and high mitochondrial Ca^{2+} concentrations. The mitochondrial membrane potential appears to become disturbed in G93A SOD-1 transgenic mice [43], and may thus contribute to neuronal dysfunction in the transgenic model. Furthermore, mitochondrial permeability transition enables increased entry of small molecules and water into mitochondria, leading to mitochondrial swelling [44]. A potential consequence of this is rupture of the outer mitochondrial membrane, releasing cytochrome C into the neuronal cytoplasm [45]. Cytochrome C is involved in mitochondrial respiration and typically located in the mitochondrial intermembrane space. Its displacement into the cytosolic space propagates a chain of caspases that initiate apoptosis [46]. In support of the deleterious role of mitochondrial permeability transition, deletion of the gene encoding a protein involved in this pathogenic state delayed disease onset and improved transgenic SOD-1 mouse survival [47].

Mitochondria are also capable of sequestering cytoplasmic Ca^{2+} [48]. Mitochondrial Ca^{2+} -buffering appears impaired in transgenic SOD-1 mice motor neurons [49], potentially exacerbating the toxic effects of excessive cytosolic Ca^{2+} . Free radicals, especially nitric oxide and its derivative, peroxynitrite are known to further inhibit mitochondrial respiration. *In vivo* co-injection of pyruvate, a substrate of the Krebs's cycle, and AMPA into rodent α -motor neurons prevented deleterious increases in cytoplasmic Ca^{2+} , as well as the development of motor disturbances [26], suggesting that pyruvate may afford protection against Ca^{2+} -induced mitochondrial dysfunction (and neurodegeneration). In total, such findings suggested that dysregulation of mitochondrial Ca^{2+} homeostasis, in addition to energy depletion, may serve to trigger and perpetuate neurodegeneration in ALS.

Involvement of Non-Neuronal Cells

Growing evidence supports a role for non-neuronal cells in the pathophysiology of ALS, highlighting that motor neuron degeneration in ALS is 'non-cell autonomous'. Pathological changes include microglial infiltration (cells of monocytic origin; equivalent to macrophages) into affected areas of brain parenchyma in ALS patients, consistent with the occurrence of neuroinflammation [50]. Microglia may be toxic to motor neurons through the production of free radicals and inflammatory cytokines, including interleukin-1 β , interleukin-6, nitric oxide (from inducible nitric oxide synthase) and tumor necrosis factor- α (TNF- α) [50]. Transgenic studies have suggested that microglia may become more important once motor neuron death has developed into an established process [51].

In further support of an inflammatory contribution to neurodegeneration in ALS, CSF obtained from patients contained significantly greater levels of inflammatory markers than neurological disease control subjects [52]. In addition, marked elevations (up to seven-fold) in cyclooxygenase-2 (COX-2) levels and COX-2 messenger ribonucleic acid (RNA) in affected regions of the CNS have also been documented in ALS patients [53]. Consistent with these findings, prostaglandin E_2 levels were increased in CSF collected from a separate group of ALS patients.

The importance of astrocytes in ALS pathogenesis was underscored by the observation that exclusive expression of mutant SOD-1 in motor neurons did not manifest disease in transgenic mouse models [54]. Astrocytes are capable of regulating motor neuronal expression of the GluR2 sub-unit of the AMPA/kainate glutamate receptor, suggesting they play a determining role over the susceptibility of motor neurons to glutamate-mediated excitotoxicity. Moreover, astrocytic expression of mutant G93A SOD-1 resulted in abolishment of their capacity to regulate GluR2 sub-unit expression in motor neurons [55]. The effect of astrocyte-derived oxidative stress on motor neuronal death was further supported through the observation that neuroprotection and delayed disease onset in transgenic mice occurred in G93A SOD-1 mice with up-regulated astrocytic synthesis of the endogenous anti-oxidant, glutathione [56]. The combined findings highlighted the complex interplay between motor neurons and astrocytes in the pathogenesis of ALS.

Axonal Transport

Given the long distances that corticomotoneurons and α -motor neurons project within the CNS and into the peripheral nervous system respectively, defects in axonal transport have been explored as a potential pathogenic process in ALS. Successful movement of cargo along an axon is dependent on kinesin-1 and cytoplasmic dynein, which serve as 'molecular motors', and microtubule neurofilaments on which these motors run. Molecular motors consume ATP in order to drive the movement of vesicles along axons. Given that ATP production occurs in mitochondria, and that mitochondrial disturbances are well characterised in ALS, molecular motor proteins are likely to be starved of ATP, resulting in a breakdown of vesicular transport [43]. Conversely, the expression of mutant G93A SOD-1 resulted in impaired anterograde migration of mitochondria, resulting in the proximal accumulation of mitochondria in motor neuronal soma, depleting axons of mitochondria and hence ATP production [43].

Following axonal conduction of electrical impulses, restoration of the axonal membrane potential is critically dependent on adequate ATP supplies to drive axonal $\text{Na}^+\text{-K}^+$ ATPase. Failure of this electrogenic pump, due to ATP depletion secondary to deficient numbers of mitochondria in the axon, may result in the toxic accumulation of Na^+ within the axoplasm (the potential consequences of this are discussed later). Furthermore, mitochondria-depleted axons may be more susceptible to the toxic effects of raised intraxonal Ca^{2+} , given the Ca^{2+} -sequestering role of mitochondria [48].

Finally, defective axonal transport has also been linked to excitotoxicity through demonstration of glutamate-induced slowing of axonal transport, which may be mediated through increased phosphorylation of neurofilament side-arm domains [57]. Additional support for the pathogenic role of defective axonal transport in ALS was the discovery of a point mutation in the p150 sub-unit of the dynactin-1 protein, encoded on chromosome 2p13. Dynactin-1 forms a complex with microtubule neurofilaments and is involved in retrograde axonal transport [58]. Therefore, it appears that abnormalities in a whole host of different systems converge upon motor neuronal axonal transport systems, rendering this aspect of motor neurons particularly susceptible to toxic processes occurring upstream.

Defective RNA Processing and Formation of Cytoplasmic Inclusions

The pathological hallmark of sporadic ALS is the presence of cytoplasmic inclusions, comprising phosphorylated TAR DNA binding protein-43 (TDP-43) [12]. TAR DNA binding protein-43 is a 414 amino acid, 43KDa nuclear protein, encoded by the TARDBP gene on chromosome 1p36.2. The expression of TDP-43 is ubiquitous, though its precise role in the human body is unknown. The formation of TDP-43 aggregates in ALS may be attributed to disturbances in protein trafficking between the neuronal nucleus and cytoplasm [59] and its phosphorylated state may prevent its entry into the nucleus. Pivotal to appreciating the importance of TDP-43 in the pathophysiology of sporadic ALS was the discovery of familial ALS kindreds with mutations in the TARDBP gene [60]. Unlike familial disease attributed to SOD-1 mutations, familial ALS patients with TARDBP gene mutations pathologically resembled sporadic ALS patients owing to the presence of cytoplasmic accumulations of phosphorylated TDP-43 [61]. Given that TDP-43 inclusions were once strictly considered a pathological feature of sporadic disease, the recognition that mutations in the gene encoding this protein could underlie a minority of familial ALS kindreds was perhaps the first discovery to unify sporadic and familial disease.

The importance of cytoplasmic aggregates in ALS pathogenesis is reinforced by the occurrence of different inclusions in other forms of familial disease. Patients with familial ALS attributed to mutations in the SOD-1 gene contained neuronal and astrocytic SOD-1 aggregates [62]. Similarly, different genotypic variants of transgenic SOD-1 mice also contained such aggregates [31]. Mutations in the fusion in sarcoma (FUS) gene, located on chromosome 16p11.2, were recently found to be responsible for disease in approximately 3% of SOD-1 negative familial ALS kindreds [63, 64]. The FUS gene encodes a nuclear protein involved in the regulation of nucleic acid processing, including transport of messenger RNA from the nucleus to other neuronal structures. Immunohistochemical studies revealed the presence of cytoplasmic inclusions, which stained for FUS protein in motor neurons from FUS-positive familial ALS patients [63]. By contrast, TDP-43 inclusions were absent in such patients. Whether the formation of cytoplasmic inclusions caused motor neuron degeneration in ALS or was consequent to this process remains unanswered. However, defective RNA processing is surely of pathogenic importance in ALS, given that TDP-43 and FUS proteins are

that TDP-43 and FUS proteins are both involved in nucleic acid handling.

The formation of cytoplasmic aggregates within motor neurons may also represent the aftermath of motor neuronal deficiency of molecular chaperones. Molecular chaperones promote the natural folding of newly synthesised proteins, as well as the re-folding of damaged proteins [65]. Cellular stress activates the 'heat shock response', which comprises an increase in the expression of various heat shock proteins (HSP). Almost all HSPs function as molecular chaperones. Heat shock proteins are classified according to six variants, based on their molecular weight (in KDa): HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (weighing less than 40KDa) [66]. A progressive reduction in the expression of the HSP70 in both central and peripheral nervous tissue in transgenic G93A SOD-1 mice [67]. At least in transgenic SOD-1 mice, HSPs may have been depleted by excessive formation of SOD-1 aggregates, preventing HSPs from interfering with other deleterious processes co-occurring within motor neurons [68].

NEUROPROTECTIVE THERAPY IN ALS

Background of Riluzole

Riluzole (6-(Trifluoromethoxy)-2-aminobenzothiazole; PK 26124; RP 54274; registered under 1744-22-5; Fig. (2A)), is a derivative of benzothiazole [69], a bicyclic ring compound, which comprises a benzene ring, fused to a five-membered ring containing one nitrogen and one sulfur atom (Fig. (2B)). Benzothiazole derivatives were first identified as potential muscle relaxants in clinical medicine during the 1950s [70]. Research into benzothiazole derivatives subsequently subsided, although interest was re-ignited with identification of the anti-convulsant properties of riluzole during the 1980s [71, 72]. In addition to exhibiting anti-convulsant activities, benzothiazole derivatives have demonstrated anti-tumour, anti-microbial, anti-helminthic, anti-leishmanial, anti-inflammatory and psychotropic properties [73]. More specifically, 2-aminobenzothiazoles, the subclass of which riluzole is a derivative, have been used in the treatment of diabetes, epilepsy, inflammation, analgesia, tuberculosis and viral infections [74].

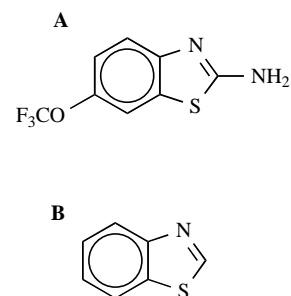


Fig. (2). A. Chemical structure of riluzole; molecular mass: 237.23g/mol. B. Chemical structure of benzothiazole.

The techniques used to synthesise riluzole were originally described by Yagupol'skii and Gandel'sman in the 1960s [75], and later patented by Mizoule and Gandel'sman

at Rhône-Poulenc Rorer (now Sanofi Aventis; Paris; France) [71]. In brief, the original methods and commercial production incorporated potassium thiocyanate and trifluoromethoxy-4-aniline as the principal reagents. More recently, new one-pot methods of preparing riluzole that did not incorporate the use of toxic and potentially corrosive bromine were identified. The first reaction involves condensing equimolar amounts of 6-(trifluoromethoxy)-aniline with tetrabutylammonium thiocyanate in dichloromethane at room temperature to yield 61% riluzole (Fig. (3A)) [74]. Alternatively, riluzole may be prepared through reacting equimolar amounts of 6-(trifluoromethoxy)-aniline with ammonium thiocyanate in an acetonitrile solvent, with an increased yield of 71% (Fig. (3B)) [74].

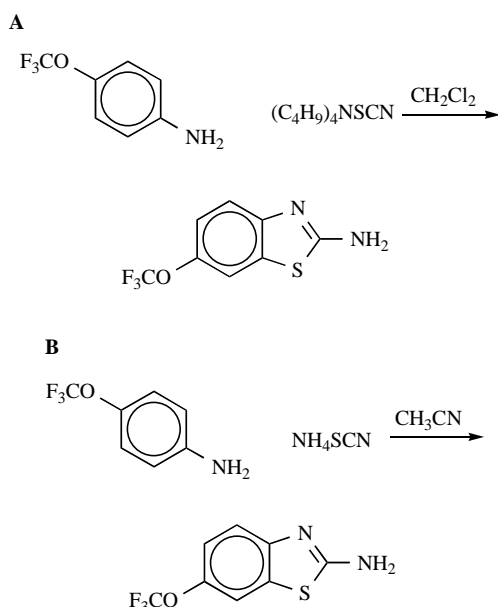


Fig. (3). One-pot methods for synthesising riluzole. **A.** Equimolar amounts of 6-(trifluoromethoxy)-aniline and tetrabutylammonium thiocyanate are reacted in dichloromethane at room temperature [74]. **B.** Equimolar amounts of 6-(trifluoromethoxy)-aniline and ammonium thiocyanate are reacted in an acetonitrile solvent [74].

After discovery of its neuroprotective effects in initial laboratory studies, riluzole was subjected to phase II testing undertaken in 155 ALS patients in France and Belgium [76]. On 12th December 1994, riluzole was approved by the U.S. Food and Drug Administration, and was subsequently marketed under the trade name, Rilutek® [77].

Chemical Properties of Riluzole

In a landmark study, the chemical properties of riluzole were interrogated by comparing the anti-convulsant and neuroprotective properties across 94 of its derivatives [78]. The principal finding was that anti-convulsant activity was endowed by the lipophilic properties of riluzole, given that 6-substituted-2-aminobenzothiazole derivatives bearing sufficiently large alkyl constituents, or polyfluoroalkyl or polyfluoroalkoxy constituents possessed more potent anti-convulsant properties (i.e. they had lower ED₅₀, defined as the dose required to suppress glutamate-induced seizures in 50% of rats). Furthermore, the 6-position of the trifluoro-

methoxy group appeared critically important, as moving it to other carbon atoms along the benzene ring diminished the potency of riluzole isomers (i.e. ED₅₀ >10mg/Kg i.p.; compared to ED₅₀ of riluzole =3.2mg/Kg i.p.).

When evaluating the chemical series in animals exposed to an hypoxic environment, the ED₅₀ of riluzole was 4.0mg/Kg i.p., while the values for the majority of its derivatives ranged from 2.5 to 10mg/Kg, indicating that all were similar to riluzole in the magnitude of their neuroprotective properties. Given the nature of this *in vivo* study, it was not possible to determine whether changes in the potency of each derivative reflected differences in pharmacokinetic or pharmacodynamic properties. Further assessment of its anticonvulsant activity determined that the inhibition of Na⁺ flux was primarily dictated by increased lipophilicity, decreasing molecular size, increasing electron withdrawal in a resonance sense and the absence of a 4- or 5-substitution of the benzothiazole ring system [79]. As a result, it appeared that the chemical structure of riluzole may already have been optimised for Na⁺ channel inhibition.

Pharmacological Properties of Riluzole

Recent evidence suggests that the neuroprotective effects of riluzole extend beyond the realm of slowing disease progression in ALS. Indeed, the neuroprotective properties of riluzole have been illustrated in models of anoxic and traumatic CNS injury [80-94]. Of relevance to Huntington's disease, riluzole suppressed excitatory activity of striatal spiny neurons elicited by exogenous glutamate [95]. Moreover, riluzole decreased glutamate release from caudate nucleus neurons [96]. Similarly in relation to Parkinson's disease, riluzole curbed the development of movement disorders in various animal models [97, 98]. In the clinical setting, however, the effectiveness of riluzole appears confined to ALS, given that it demonstrated no overall clinical benefit in patients with Huntington's disease [99] and Parkinsonian-plus disorders [100].

Unlike most drugs, which have single molecular targets, riluzole may exert its neuroprotective effects through a number of interdependent pathways. As it stands, the precise mechanisms behind the neuroprotective properties in human ALS have not been definitively elucidated and include:

1. Anti-Glutamatergic Actions

Riluzole may dampen excitotoxicity through interruption of glutamatergic transmission in the CNS [72], and has been demonstrated to prevent motor and cortical neuron degeneration induced by glutamate and NMDA *in vitro* [101-103]. However, electrophysiological and molecular studies have yielded conflicting results in relation to anti-glutamatergic properties of riluzole. Electrophysiologically, riluzole may capably inhibit responses evoked by activation of NMDA glutamate receptors, confirming a direct effect on this receptor [104]. Furthermore, riluzole (10μM) depressed glutamatergic transmission through antagonism of presynaptic NMDA glutamate receptors [105]. Interference with glutamatergic transmission only occurred in cells with high firing frequencies, suggesting a frequency-dependent antagonistic effect. However, more recent electrophysiological studies have not supported the finding that riluzole suppresses

NMDA-evoked responses [106], as well as glutamate-induced membrane depolarisation [107]. Furthermore, radioligand studies failed to show any adherence to known ligand binding sites on NMDA glutamate receptors: the glutamate site [72]; the phencyclidine site [104]; the glutamate-like compound, cysteine sulphinate [72]; the antagonist, 3-(2-carboxypiperazim-4-yl)-propyl-1-phosphonic acid; the binding site of the dissociative anaesthetic, 1-(1-(2-thienyl)-cyclohexyl)-phosphonic acid; or the glycine binding site [72].

In addition to its possible effects on the NMDA glutamate receptors, riluzole may act on AMPA/kainate glutamate receptors. Riluzole exhibited non-competitive antagonism of the AMPA glutamate receptor in cultured α -motor neurons [108] and inhibited electrophysiological responses evoked by kainate acid [104]. Radioligand studies, however, again failed to demonstrate that riluzole displaced kainate or AMPA from AMPA/kainate receptors [104]. The findings of such studies were again supported by electrophysiological studies conducted by Prakriya and Mennerick [106]. Therefore, pharmacologists remain divided as to whether riluzole exerts any direct inhibitory effects on NMDA and AMPA/kainate glutamate receptors.

Riluzole also lowers the concentrations of glutamate and other excitatory neurotransmitters in the synaptic cleft. At concentrations achievable in human plasma, riluzole increased glutamate uptake into rat cortical synaptosomes through increasing its affinity with GLT-1 [109]. Riluzole (up to 20 μ M) also inhibited glutamate and aspartate release from cortical neurons, possibly through K^+ channel blockade [110, 111].

2. Ca^{2+} Channel Blockade

Calcium ions are downstream mediators of neuronal death following excitotoxic activation of glutamatergic receptors, through activation of Ca^{2+} -dependent phospholipases and proteases, activation of nitric oxide synthase, disruption of mitochondrial oxidative phosphorylation and generation of reactive oxygen species [112]. Riluzole may inhibit glutamate release into the synaptic cleft through blockade of voltage-gated Ca^{2+} channels [107, 113, 114]. Consistent with such findings was the observation that riluzole prevented deleterious increases in cytoplasmic Ca^{2+} , thereby inhibiting the release of glutamate from synaptosomes [115]. Inhibition of persistent Ca^{2+} currents may also account for the ability of riluzole to dampen glutamatergic release [105] through reducing basal Ca^{2+} entry into neurons.

3. Na^+ Channel Blockade

Whilst Ca^{2+} channel blockade may contribute towards safeguarding motor neurons against excitotoxic death, Ca^{2+} channel blockade alone may be insufficient in protecting against neuronal and axonal fallout [31, 116-118]. Riluzole may also reversibly block Na^+ channels, with its specific target being the α -sub-unit [119]. At low concentrations (i.e. up to 10 μ M), riluzole may antagonise persistent Na^+ currents [120, 121]. In the SOD-1 G93A transgenic mouse model, riluzole reduced the persistent Na^+ current by 46%. Higher concentrations (>10 μ M) resulted in complete suppression of the current [122]. Blockade of the persistent Na^+ currents may afford neuroprotection through reducing intrinsic motor

neuronal excitability [105], in addition to dampening the susceptibility of motor neurons to potential Na^+ -induced insults (discussed later).

Of further relevance, the pharmacology of riluzole is reminiscent of local anaesthetics and various anti-convulsant agents, which are characterised by their ability to stabilise the transient Na^+ channel in its inactivated state [106, 119, 122-124]. In fact, the affinity of riluzole for the transient Na^+ channel was approximately 300 times stronger when the Na^+ channel was in the inactivated state than in the resting state [122], and occurred in a dose-dependent manner ($ED_{50} = 2.3 \mu$ M) [107, 125].

Some have proposed that the observed anti-glutamatergic properties of riluzole were merely downstream manifestations of its Na^+ channel-blocking properties [106, 126]. Riluzole-induced blockade of Na^+ channels has been associated with reduced glutamate release from presynaptic nerve terminals. Furthermore, the riluzole derivative, RP 66055 exemplified that blockade of voltage-gated Na^+ channels was fundamental to the anti-excitotoxic effects of riluzole [127]. RP 66055 possessed 100-fold greater affinity towards voltage-gated Na^+ channels than riluzole ($ED_{50} = 0.4 \mu$ M; riluzole, $ED_{50} = 60 \mu$ M). RP 66055 did not bind to any known excitatory amino acid receptor, yet it cushioned the impact of ischaemia on neuronal survival from carotid (4x 8mg/Kg i.p.) and middle cerebral arterial (2x 8mg/Kg i.p.) occlusion in animal models, suggesting that riluzole may confer neuroprotection through Na^+ channel blockade.

4. GABAergic Mechanisms

In contrast to glutamate, γ -amino-butyric acid (GABA) is the major inhibitory neurotransmitter throughout the CNS. Early studies failed to demonstrate interaction of riluzole with known GABA receptor binding sites, including those targeted by benzodiazepines and baclofen on $GABA_A$ and $GABA_B$ receptors respectively [72, 128]. However, growing evidence points to some degree of modulation of the GABAergic systems by riluzole. At concentrations that may be unattainable in human subjects ($ED_{50} = 43 \mu$ M), riluzole blocked the uptake of GABA into striatal synaptosomes [129]. Riluzole may also prolong the decay of inhibitory post-synaptic currents through potentiation of $GABA_A$ receptor affinity for GABA (at concentrations of at least 20 μ M) [128].

Murine studies have demonstrated potential GABAergic properties of riluzole. Riluzole enhanced slow wave and rapid eye movement sleep (0.5-8mg/Kg) [130]. Although it is of direct relevance to suppressing the glutamatergic system, the demonstration of anaesthetic properties at very high doses (i.e. 20-43mg/Kg i.p. in rats) by riluzole also suggested that GABAergic pathways may be involved as pharmacological targets [149-151]. Perhaps most importantly from a clinical perspective relevant to ALS, riluzole restored cortical inhibition in ALS patients, a measurable parameter of cortical excitability [131].

5. Miscellaneous Actions

In addition to effects on neuronal membrane receptors, riluzole may interact with various intracellular proteins involved in second messenger systems. At therapeutic concentrations, riluzole exerted non-competitive antagonism of pro-

tein kinase C [113, 132], and at higher concentrations (100 μ M), it inhibited phosphorylation of neurofilaments, improving the rate of axonal transport [114]. In addition, inhibition of pertussis-toxin and cholera-toxin sensitive G-proteins may contribute to preventing the release of excitatory amino acids from presynaptic nerve terminals [115, 133, 134].

Pharmacokinetic Properties of Riluzole

Riluzole is orally administered, and due to its lipid-solubility, is absorbed within one hour of ingestion [135] resulting in high bioavailability (approximately 91%) [136]. Ingestion of riluzole following consumption of a high-fat meal impedes absorption from the gastrointestinal system, resulting in lower plasma concentrations [136], and patients should be properly informed about this.

Riluzole was metabolised principally by the phase I hepatic microsomal enzyme, cytochrome P450 1A2 (CYP1A2), in an NADPH-dependent reaction. This reaction resulted in the formation of the N-hydroxylated derivative [137], which may be pharmacologically active [138]. Patients with hepatic impairment thus had reduced riluzole clearance [135]. In addition, tobacco smoking induced CYP1A2 and thereby hastened riluzole clearance [135, 138], potentially diminishing the effectiveness of riluzole. Riluzole itself also demonstrated weak inhibitory effects on CYP1A2. Riluzole undergoes biotransformation through glucuronidation by UDP-glucuronosyltransferase to a lesser extent [137]. Long-term changes did not occur in riluzole clearance [135]. Males tended to clear riluzole faster than females and overall renal clearance appeared negligible [135].

Steady state plasma concentrations of riluzole are typically achieved after five days of multiple-dose administration [139], with a plasma half-life of approximately 12 hours [140]. Plasma concentration of riluzole was highly variable, with the mean peak serum concentration in ALS patients reported as 0.432mg/L [or 1.82 μ M; range: 0.034-1.61mg/L (or 0.143-6.79 μ M)] [141]. In two separate studies conducted in healthy volunteers, mean peak plasma riluzole concentration ranged between 0.173-0.296mg/L (or 0.73-1.25 μ M) [139, 142]. Therefore, the presence of ALS itself seemingly does not interfere with riluzole metabolism. Although it is unknown how well riluzole penetrates the blood-brain barrier in humans, animal studies have suggested that brain riluzole concentrations are five-fold higher than plasma levels [143].

Despite the presence of a dose-dependent effect in the phase III trial of riluzole [144], ALS patients with higher serum riluzole concentrations did not have a survival advantage over those with lower serum riluzole concentrations [138]. However, that study may have been underpowered to detect differences in survival, and unlike a clinical trial, strict eligibility criteria were not established *a priori*.

Clinical Trials of Riluzole

Riluzole was evaluated in two trials for regulatory approval. At the end of 12 months follow-up in the initial phase II trial [76], 74% of patients allocated to riluzole (100mg/day) were still alive, compared to 58% of the placebo group ($P = 0.014$). Functional decline was slower in the

riluzole-treated group, and those with bulbar-onset disease responded to therapy better than patients with limb-onset disease. The survival benefits of riluzole were confirmed in a subsequent dose-ranging, phase III trial, though the treatment effect was similar in all patient groups, irrespective of site of disease onset [144]. Post hoc analyses of phase III data suggested that riluzole was effective at maintaining patients in the milder stages of disease for longer periods of time [145]. The phase III study concluded that a dose of 100mg/day offered the best benefit-to-risk ratio.

Even after evaluation in over 1000 clinical trial patients, it remained to be clarified whether riluzole was effective in treating elderly patients or those with advanced disease. Therefore, a 'compassionate' study of riluzole was undertaken that recruited such patients [146]. There was limited evidence to suggest any benefit of riluzole in patients with advanced ALS, though this trial was underpowered to detect a survival benefit, as it failed to recruit enough patients to fill its target sample size. A Cochrane meta-analysis, combining data from all three trials, concluded that riluzole had an overall survival benefit of approximately three months [147].

Adverse effects and toxicity

Riluzole tends to be well tolerated by ALS patients. Adverse events included asthenia, nausea, headache, rhinitis, abdominal pain, hypertension, diarrhoea, somnolence and circumoral paraesthesia. The most alarming adverse effects were elevations in alanine aminotransferase and aspartate aminotransferase liver enzymes. Elevated liver function tests usually resolved spontaneously, and were an uncommon reason for discontinuing therapy in the phase III trial. Idiosyncratic events reported in the literature include acute hepatitis and jaundice [148, 149]. Importantly both toxicities resolved completely following withdrawal of riluzole therapy.

CLINICAL TRIALS IN ALS

Advances in statistical tools and increased interest in the development of disease-modifying therapies for neurodegenerative diseases have triggered an evolution in ALS clinical trial design. Although phase III ALS trials have retained the randomised-controlled design, phase II trials have made a departure from such conventions [150]. The focus of phase II trials is to quickly and efficiently (i.e. using a small sample size) identify compounds with a high likelihood of succeeding during phase III testing, so that time and resources are not invested into compounds that are unlikely to be efficacious.

Historical control data has been used for comparison in the evaluation of recently tested therapies in phase II trials [151]. The use of such data, however, has received mixed reviews amongst the scientific community [152], given the evolving standards of ALS patient care, which have resulted in improved patient survival [153], as well as regional differences in clinical practice.

Other developments in phase II trial design for ALS include the futility or non-superiority design, whereby the goal is to identify compounds that demonstrate little promise over the control group [154]. In stark contrast to conventional statistical methodology, the null hypothesis of a futility study

states that a therapeutic benefit exists. Therefore, substantial evidence needs to be demonstrated in a futility study before a medication is deemed inefficacious. Another novel design uses a selection paradigm, whereby the object is to identify the best neuroprotective agent amongst a number of different putative therapies [155]. The actual effectiveness of the superior treatment may be later evaluated in a phase III trial. Both novel phase II trial designs have resulted in substantial reductions in sample size required to address the respective study questions.

The assumption that putative neuroprotective therapies may not accelerate ALS disease progression remains controversial. Whilst the vast majority of clinical trials have yielded equivocal findings in this respect, a small number of clinical trials have suggested that certain medications hasten ALS disease progression [156-158]. Such trials have highlighted the importance of two-sided comparison tests when performing *a priori* sample size calculations and on trial completion. The potential for harm also further highlighted the dangers associated with off-label use of medications outside the setting of clinical trials, so-called 'compassionate use of medications' [159].

The Potential Need for Combination Therapies

Given that multiple pathogenic processes likely underlie motor neuron degeneration in ALS [51], more effective disease-modifying strategies are likely to arise from the combined use of other pharmacological agents alongside riluzole. In transgenic murine studies exploring the efficacy of neuroprotectants, some medications delayed disease onset without impacting on survival. Conversely, some medications actually increased mouse survival, without influencing disease onset [160]. This suggested that factors responsible for initiating motor neuron degeneration were different to those that sustained neurodegeneration. In support of this theory, combinations of riluzole, nimodipine and minocycline [161], minocycline and creatine [162], and valproic acid and lithium [163] resulted in significant improvements in transgenic mouse survival and motor function, over and above that demonstrated with monotherapy, although this has not translated to clinical practice.

Combination therapy, involving medications in addition to riluzole, has been evaluated in a single clinical trial comparing minocycline-creatine to celecoxib-creatine [155]. Consistent with the findings of an earlier phase III minocycline trial [157], patients allocated to the celecoxib-creatine group performed marginally better than those in the minocycline-creatine group. No phase III trials of combination therapy in ALS were registered at the time of preparing this manuscript.

Increasing the number of medications in any patient population introduces the possibility of pharmacokinetic drug interactions, which may obscure treatment effects. For example, some investigators have attributed the faster decline in ALS FRS-r experienced by patients randomised to active treatment in the phase III minocycline trial to riluzole toxicity [157]. Riluzole toxicity may have resulted from increased intracerebral riluzole concentrations, secondary to a reduction in the removal of riluzole from the CSF. P-glycoprotein is a ubiquitous transporter protein, which facili-

tates the movement of amphipathic molecules across luminal membranes, and normally serves to remove xenobiotic compounds (such as riluzole) from the intracerebral compartment. *In vitro* studies have demonstrated that minocycline inhibits p-glycoprotein activity, thereby potentially leading to raised riluzole concentrations in the intracerebral compartment [164, 165]. However, despite these possible drug interactions, dissecting the phase III trial data further did not indicate that the concurrent use of riluzole negatively impacted on rate of decline in ALS FRS-r in patients who received minocycline. Regardless, conflicting results from pre-clinical and clinical studies serve to further highlight the need for careful consideration of pharmacokinetic issues prior to embarking on clinical trials in neurodegenerative disorders.

Reasons for Treatment Failure

Choice of Primary Outcome Measure

The failure of ALS clinical trials to demonstrate a positive treatment effect may relate to selection of the primary outcome measure. In contradistinction to secondary outcome measures, the primary outcome measures remain important in clinical trials, as the trial sample size is calculated *a priori* based on pre-existing data concerning the primary outcome measure. Moreover, the 'success' of a trial is largely determined by whether a treatment effect is demonstrated in the primary outcome measure.

Survival became less popular as a primary outcome measure, replaced by functional scales in recent clinical trials [166-168]. Validated functional scales are predictors of patient survival in clinical trials [169], and changes reflected in such measures are easy to interpret and relevant to patients. Although once considered an unambiguous outcome, death in the context of ALS is blurred by the use of ventilatory assistance and nutritional interventions [168, 170]. The use of non-invasive ventilatory support for long periods may not be equivalent to death [171]. In addition to requiring a large number of patients, death as a primary endpoint dictates that trials be of long duration to ensure that enough events are accrued [172]. Given that neuroprotective strategies are most warranted in the 'early' stages of disease when more motor neurons are still intact, and median patient survival is 3-5 years from symptom onset, survival as a primary outcome measure may be judged by most trial investigators as an outmoded measure.

On the other hand, survival may be the only definitive measure of treatment efficacy in ALS trials. By the time ALS patients develop clinically-detectable weakness, at least 50% of motor neurons have been lost [173]. As a result, it may be over-optimistic to expect that the introduction of a disease-modifying therapy after disease onset may slow the rate of functional decline, given the extent of existing neurodegeneration. It was only through combining patient numbers from all three riluzole trials that a small yet statistically significant effect on muscle strength, bulbar and limb function could be demonstrated [147]. As such, one may argue that the sample size required to detect a small positive effect on disease progression (as measured using muscle strength and functional scales) could be similar, if not greater than that required to demonstrate an improvement in patient sur-

vival [147]. Therefore, despite the methodological challenges conventionally associated with survival analysis techniques, survival may ultimately represent the most robust outcome measure for recognising effective disease modifying therapy in ALS, potentially requiring a smaller sample size than when functional scales (with perhaps suboptimal effect sizes) are used as primary outcome measures.

Dosing Issues

Perhaps due to limited patient availability and the intuitive assumption that higher doses of neuroprotective agents are more likely to result in demonstrable treatment effects, few ALS trial investigators have endeavoured to conduct dose-ranging studies. Not only do dose-ranging studies enable exploration of drug toxicity, a dose-dependent disease-modifying effect provides compelling evidence that such an effect actually exists [144]. Following completion of the pentoxifylline [156], minocycline [157] and topiramate [158] phase III trials, which used the highest tolerable doses of medication and showed exacerbation of the rate of disease progression, clinical trial investigators warned against testing the maximum tolerated dose. Reversal of treatment efficacy at high doses was exemplified in several pre-clinical studies that examined the pharmacological properties of riluzole. These studies demonstrated that very high concentrations (i.e. in excess of 30 μ M) may have lead to deleterious outcomes, including raised intracellular Ca²⁺ concentrations [126, 174-176], and neuronal and glial death [102, 177]. Therefore, unless there is strong reason to suggest otherwise (e.g. clinical experience with the medication in other diseases) [178], it is recommended that dose-ranging studies are implemented into clinical trial protocols, patient numbers and resources permitting.

Relevance of Animal Models to Human Disease

The development of the SOD-1 transgenic ALS mouse model appeared seminal in understanding the role of oxidative stress in the pathogenesis of ALS. However, the model has been disappointing from a trial investigator's perspective. Although differences in pharmacokinetics and dosing regimens between murine and human studies may have confounded the efficacy of medications tested in the transgenic mouse model, there is increasing agreement that the mouse model poorly represents the sporadic form of disease from a pathogenic perspective, given its inability to predict drug efficacy in human disease [179, 180]. Unlike human disease, which is variable in clinical presentation and rate of disease progression, the transgenic mouse undergoes a series of stereotypic changes [35]: weakness begins in the hind legs at approximately 104 days of life, with mortality occurring approximately 30 days later [160]. From a statistical perspective, Benatar concluded that the quality of therapeutic trials involving ALS mouse models may be plagued by inadequate sample size, as well as other methodological rigours employed in clinical trials [160, 181]. To complicate matters further, the G93A model is more commonly used than other genotypic variants in murine studies, and whether treatment effects observed in the G93A SOD-1 model generalise across to mice (and humans) bearing different SOD-1 mutations remains to be elucidated.

Of the many medications tested in SOD-1 transgenic mice, only riluzole has produced consistent results between

human and animal studies. Minocycline is an example of a medication that demonstrated great promise in transgenic studies but failed at the clinical level. Minocycline delayed disease onset to 109 \pm 1.5 days of life (control, 90.3 \pm 2.2 days) and extended survival to 136.8 \pm 1.2 days (control, 125.6 \pm 3.5 days), when the medication was administered at five weeks of age [182], but accelerated the rate of disease progression in human ALS [157]. Although there was no evidence of a harmful effect on human disease, creatine monohydrate was another compound that improved SOD-1 transgenic mouse survival and afforded neuroprotection (determined histologically) [183], but phase III clinical trials failed to reveal any clinical benefit [184, 185].

Testing of medications often involves the administration of ultra-high doses, and this may have confounded the apparent benefits observed in such studies. The administration of such high doses to ALS patients would inevitably produce untoward side effects, which even in the presence of a disease-modifying effect, may warrant treatment discontinuation. Adverse effects are not typically under close scrutiny in murine interventional studies. Timing of the introduction of medications into mouse models has also been heavily criticised, given that pre-symptomatic administration of drug therapy is, in reality, of little relevance to sporadic disease in humans [181]. As it currently stands, given that a whole host of factors could have produced discrepancies between human and murine interventional studies, it is clear that further work is required to dissect the precise pharmacokinetic and pharmacodynamic mechanisms that distinguish human disease from ALS in transgenic SOD-1 mice.

Na⁺ CHANNEL BLOCKADE – A ‘NOVEL’ NEURO-PROTECTIVE STRATEGY

The corticomotorneuronal or ‘dying-forward’ hypothesis suggested that ALS is a primary disorder of the motor cortex, with α -motor neuron loss occurring secondary to reduced descending inhibitory input [3]. This hypothesis was recently investigated using threshold-tracking transcranial magnetic stimulation (TMS), a research technique that enables non-invasive stimulation of the brain to explore *in vivo* functional properties [186]. Studies using TMS have revealed that the motor cortex is more excitable earlier (i.e. cortical hyperexcitability) in the disease course [187], with cortical inexcitability the ensuing manifestation [188]. Furthermore, longitudinal studies on asymptomatic individuals who bear SOD-1 mutations have demonstrated progressive increase in cortical excitability prior to the appearance of clinical disease [189]. Cortical hyperexcitability is thought to reflect: 1). increased intrinsic corticomotorneuronal excitability (due to increased Na⁺ conductances and excessive glutamate neurotransmission); and 2). loss of inhibitory interneuronal mechanisms (due to a reduction in GABA-mediated processes) [190].

Relevance of Na⁺ Conductances to ALS

The pathogenicity of Na⁺ channels is implicated at all levels of the human motor system in ALS. An increase in the excitability of individual corticomotorneurons, may be attributed to increased excitability intrinsic to corticomotorneurons themselves. Electrophysiological studies on corticomoto-

torneurons extracted from the G93A SOD-1 transgenic mouse model of ALS have demonstrated increased persistent Na^+ conductance [191]. In human disease, increased corticomotoneuronal excitability may reflect an up-regulation in corticomotoneuronal persistent Na^+ conductance [187]. Furthermore, increased cytoplasmic Ca^{2+} levels in the motor neurons of ALS patients may exacerbate the increase in neuronal membrane Na^+ conductance [192], perpetuating a vicious cycle that leads to even greater amounts of Na^+ and Ca^{2+} entering motor neurons. Finally, pharmacological support for the involvement of Na^+ channels in the pathogenesis of ALS is the use of riluzole in the treatment of this disease.

The contribution of Na^+ channels in increased excitability of nervous tissue has also been demonstrated downstream in peripheral motor nerves of ALS patients. Axonal excitability testing, an electrophysiological measure that examines axonal ion channels *in vivo*, has revealed increased persistent Na^+ conductance in the peripheral motor axons of ALS patients [193-197]. A persistent elevation in Na^+ entry into motor axons may enable the membrane potential of motor axons to reach threshold potential more frequently than otherwise expected at rest, resulting in spontaneous depolarisation [195]. Such depolarisation is likely involved in the genesis of fasciculations (involuntary muscle twitching), which are an inevitable feature of ALS and reflect spontaneous firing of intact motor units. Of further relevance, voltage-dependent Na^+ channels from G93A SOD-1 transgenic mouse α -motor neurons demonstrated faster recovery from inactivation [198]. Such a characteristic may enable motor neurons to activate more rapidly, contributing to neuronal hyperexcitability and excitotoxicity.

The Pathogenicity of Na^+ Currents in Neurological Disease

The hypothesis that increased Na^+ entry into neural structures may result in degeneration was first proposed by Stys and colleagues in 1992 [199]. Elevations of intraneuronal Na^+ concentrations beyond 20mM may result in compensatory reverse operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which under normal conditions, serves to extrude Ca^{2+} from the intracellular space, whilst facilitating Na^+ entry. Reverse operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger may thus produce deleterious elevations in intra-axonal Ca^{2+} concentrations, possibly resulting in the initiation of degradative cascades [117, 200].

Given the potential pathogenic role of Na^+ currents in neurological disease, Na^+ channel blockade has received increasing attention as a neuroprotective strategy [201]. Neuroprotection conferred by Na^+ channel-blocking agents have been demonstrated across a broad range of paradigms of Na^+ -induced neurotoxicity, including neuroinflammatory models of disease and *ex vivo* electrical nerve stimulation with concomitant exposure to nitric oxide, using a whole host of different Na^+ channel-blocking agents. The beneficial effects of Na^+ channel-blocking agents in models of Na^+ -induced neurotoxicity were demonstrated electrophysiologically, histologically, as well as clinically [202-206]. Inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger resulted in similar neuroprotective effects [117, 203]. Importantly, Na^+ channel blockade did not interrupt impulse conduction along axons, suggesting that interfering with persistent Na^+ currents may

selectively confer neuroprotection. In support of the injurious role of Na^+ in axonal degeneration, the enhancement of Na^+ entry into mammalian white matter with a potentiator of Na^+ channels exacerbated white matter destruction, whilst an environment devoid of Na^+ was protective against anoxic damage [199].

Raised cytoplasmic Na^+ concentrations may lead to neurodegeneration through other means. Increased Na^+ entry into the neuron or axon may increase energy demands, through activity of the Na^+/K^+ ATPase [207]. Moreover, the progressive loss of motor units in ALS may result in compensatory overuse of surviving motor units, thus increasing their demand for ATP [208]. Na^+/K^+ ATPase pump activity may appear extinguished in α -motor neurons from transgenic SOD-1 mice [209], although seems abnormal in patients with sporadic disease [208]. Furthermore, the dependence of EAAT-2 on the electrochemical gradient of Na^+ may translate to symport failure in an environment intoxicated with Na^+ , increasing synaptic glutamate concentrations, hence exacerbating excitotoxicity [207].

FUTURE DIRECTIONS

Instead of focusing on the modest benefits conferred by riluzole in ALS, more efforts need to be directed towards further understanding its neuroprotective effects so that new drugs with similar properties may be synthesised, as well as yielding further insights into the pathophysiology of this elusive disease. Since many anti-glutamatergic effects of riluzole have already been investigated in the form of other clinical trials, Na^+ channel blockade, as an avenue for drug development in ALS needs to be pursued. This is especially given the plethora of evidence that supports the use of commercially available Na^+ channel blocking agents in a variety of neurological disorders. Given the potential for neuroprotection in ALS, our group has commenced a phase II clinical trial of a Na^+ channel-blocking agent in ALS (registered under ANZCTR12608000338369). In addition to measuring clinical efficacy, this trial also incorporates the use of electrophysiological biomarkers as secondary outcome measures.

High throughput screening is standard practice in the pharmaceutical industry for identifying lead compounds, which may be subjected to further laboratory and clinical testing. Using automated assay systems, high throughput screening is capable of efficiently examining thousands of small molecules *in vitro* in the hope of detecting biologically active compounds [210]. For example, the effects of ceftriaxone on EAAT-2 were stumbled upon by applying high-throughput screening to over 1000 FDA-approved chemical substances [211]. In such an instance, studying medications already approved by drug regulatory authorities obviated the need for conducting extensive laboratory testing and phase I clinical trials [179]. In ALS, assays that have been used in high throughput screening for identifying lead compounds have centred around models of excitotoxicity, apoptosis and mutant SOD-1 [210].

Whilst the discovery of SOD-1 mutations has made an everlasting impact on understanding ALS pathophysiology, the failure of transgenic mice bearing the aberrant mutation to mimic human disease, as well in predicting the utility of

Table 1. Summary of the Known Mechanisms of Action of Riluzole

Mechanisms of Action	References
Inhibiting glutamatergic neurotransmission: <ul style="list-style-type: none"> Potential interactions with the NMDA glutamate receptors (pre- and post-synaptic) Potential interactions with AMPA/kainate glutamate receptors Enhance glutamate uptake from synaptic cleft Inhibit glutamate and aspartate release 	[117] [104, 108] [109] [120, 121, 130]
Ca ²⁺ channel blockade	[105, 115, 213, 214]
Na ⁺ channel blockade: <ul style="list-style-type: none"> Site of action: α-sub-unit Antagonist of persistent Na⁺ current Blockade of transient Na⁺ channel 	[119] [117, 122, 135, 141, 143] [121, 123, 126, 191, 213, 215-218]
GABAergic mechanisms: <ul style="list-style-type: none"> Reduced uptake of GABA from neuronal synapse Potential of GABA_A receptor affinity for GABA Demonstration of general anaesthetic properties at high doses Restoration of cortical inhibitory patterns in ALS patients 	[129] [128] [219, 220] [131]
Miscellaneous actions: <ul style="list-style-type: none"> Non-competitive antagonism of protein kinase C Inhibition of pertussis toxin-sensitive and cholera toxin-sensitive G-proteins Antagonism of neuronal nitric oxide synthase 	[132] [133] [219]

disease-modifying therapies in clinical trials have been disappointing. Given that TDP-43 may bridge the gap between sporadic and familial disease, it is plausible that a transgenic mouse model bearing a mutation in the TARDBP gene would produce a phenotype that more closely resembles sporadic disease [212]. Similarly, it is hoped that testing new therapies on such a model may overcome the poor validity of the SOD-1 transgenic mouse in predicting success in clinical trials [181].

CONCLUSION

The introduction of riluzole in the 1990s was a source of significant hope for patients with ALS. The success of riluzole encouraged the establishment of ALS clinical trials consortia worldwide. Although the magnitude of a disease-modifying effect may remain modest, riluzole is a testament to the fact that the natural history of neurodegenerative diseases, once refractory to all pharmacotherapies, may be altered. Encased within the pharmacology of riluzole are clues to the mechanisms of motor neuron degeneration in ALS. As such, a deeper understanding of how riluzole affords neuroprotection in ALS may promote a greater understanding of this disease, which would facilitate the identification of more pharmacological targets.

Clinical trials in ALS have undergone an evolution since the initial trials. An improvement in statistical methodology and the establishment of clinical trials consortia have ensured that these resource-intensive studies are adequately powered to detect clinically meaningful treatment effects. However, there is a greater need to develop more sophisticated techniques to identify ineffective compounds at the phase II stage, so that resources are not spent on evaluating

futile compounds in expensive phase III trials. With respect to phase III trials, the choice of the most appropriate primary outcome measure remains a source of great debate. While there remains no definitive answer, the polar ends of the spectrum seem to range from searching for drugs that are effective in slowing disease progression (with small sample sizes) to conducting large studies, potentially incorporating over 1000 patients, to identify drugs with survival benefits, but with 'just noticeable' effects on disease progression. Furthermore, at the pre-clinical level, the development of new models of disease that more closely resemble sporadic ALS in humans clinically, as well as pathophysiologically, would expedite the laboratory testing of new candidate compounds. Only once these important issues have been addressed should a revolution in ALS drug development occur.

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