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Abstract

Amyotrophic lateral sclerosis (Lou Gehrig's disease) is a devastating neurodegenerative disorder for which the only licensed treatment is riluzole. Although riluzole clinical efficacy is rather limited, its use has important implications for identifying those parameters that might improve its clinical benefits (dose, timing, disease stage) and for its off-label administration in other neurodegenerative diseases, such as spinal cord injury. Studies of riluzole also have an intrinsically heuristic value to unveil mechanisms regulating the excitability of brain and spinal neurons because this drug is a pharmacological tool to probe the function of certain ion channels, or to study neurotransmitter release processes, and intracellular neuroprotective pathways. The present review focuses on how riluzole acts on brain and spinal neurons within motor networks, what mechanisms can be deduced from its effects, and what conditions may favor its use to contrast neurodegeneration or to ameliorate late symptoms like spasticity. Taking as an example the experimental neurodegeneration caused by overactivation of glutamatergic synapses (excitotoxicity), it seems likely that protection of motor networks by riluzole involves selected administration timing and dosing to target processes for releasing glutamate from very active synapses or for dampening repetitive firing by hyperfunctional motor cells.

Keywords

spinal cord injury, amyotrophic lateral sclerosis, glutamate uptake, persistent inward current, motoneuron, excitotoxicity

How Riluzole Can Help to Devise New Strategies and Better Understanding of Pathological Processes Underlying Neurodegenerative Diseases

Riluzole (2-amino-6-trifluoromethoxybenzothiazole; Rilutek®; Fig. 1) is one of the few drugs licensed to treat a neurodegenerative disease, and, as such, it has attracted considerable interest for its potentially wider therapeutic applications. Originally synthesized by researchers at Rhône-Poulenc Rorer (Antony, France), riluzole was first described for its anticonvulsant, anxiolytic, and anesthetic properties (Wokke 1996). Clinical interest in this drug dates back to the mid-1990s when, in the United States, it became available for treating motor neurone disease (MND) (Miller and others 1996). MDN (which includes amyotrophic lateral sclerosis [ALS], or Lou Gehrig's disease) causes progressive damage and cell death of lower motor neurons in the spinal cord and brain stem and upper motor neurons in the motor cortex (Shaw 1999). Riluzole is the only drug currently licensed for the symptomatic treatment of ALS, even though clinical benefits are modest (Miller and others 1996). The ability to counteract at least some symptoms of neurodegeneration has led to studies of riluzole as an agent against a host of CNS diseases affecting motor networks. One major clinical trial for acute human spinal cord injury (SCI) is currently in progress through the North American Clinical Trials Network (Kwon and others 2011). While experimental and preclinical data have helped in clarifying some important aspects of riluzole action, its therapeutic effects and exact mechanism of action on different cell types and disease models remain

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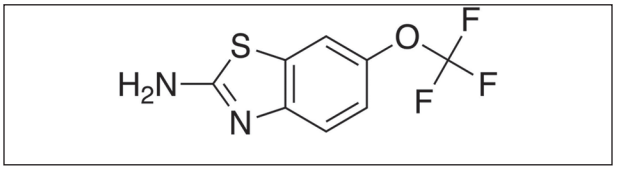

Figure 1. Chemical structure of riluzole.

incompletely understood. Thus, the current prospects for exploitation of the use of riluzole remain limited.

The therapeutic potential of riluzole is believed to be mainly related to its well-known antiglutamatergic action (Wokke 1996); in fact, although the exact cause for motoneuron degeneration in ALS remains unclear, one major factor in MND pathogenesis is excitotoxicity (namely, neuronal death due to overactivation of glutamate receptors; Choi 1992) caused by dysregulation of glutamate neurotransmission (Shaw and Ince 1997). Likewise, excitotoxicity is one important factor to induce neurodegeneration following SCI, characterized by primary (at the site of the lesion) and secondary (spreading to uninjured areas) damage to spinal networks (Dumont and others 2001). Hence, to explore and exploit the action of riluzole as a neuroprotective agent, several experimental models have been implemented to mimic the basic processes underlying MND and SCI (Cifra and others 2011; Guzmán-Lenis and others 2009; Mazzone and Nistri 2011a; Rothstein and Kuncl 1995).

It is likely that a key property in observing any riluzole-mediated neuroprotection is its ability to reduce glutamate extracellular concentration via a mechanism that, in early studies, was shown to be due to inhibition of glutamate release (Cheramy and others 1992; Coderre and others 2007). This effect, attributed to depression of presynaptic calcium influx and block of autoreceptors controlling transmitter release (Lamanauskas and Nistri 2008; Wang and others 2004), should dampen neuronal excitability and prevent spreading of excitation evolving into excitotoxicity. Nevertheless, apart from its interaction with the glutamatergic system, riluzole can act on other cellular targets; in fact, as recently reviewed by Bellingham (2011), riluzole is reported to inhibit persistent inward currents, ion channels, neurotransmitter receptors, and protein kinases and to enhance the recently discovered two-pore domain potassium channels (TREK-1) and intracellular heat shock proteins (HSP), whose roles are supposed to be neuroprotective.

All these targets may contribute to the therapeutic potential of riluzole but, in addition, may represent useful tools to unveil important mechanisms regulating neuronal excitability and network behaviors.

The focus of the present review is to summarize the principal findings concerning the effects of riluzole on mechanisms of rhythmic activities generated by spinal and brainstem motor networks particularly in relation to models of SCI and MND and their neuroprotection. Although the predictive value of laboratory data for clinical use might be questioned in general, it is important to emphasize that discrepancies between experimental and clinical studies are attributable to different protocols for drug regimens and diversity of pathological conditions (Faden and Stoica 2007; Savitz and Fisher 2007). In this framework, it is useful to note that, in humans, the plasma therapeutic concentration of free riluzole is 1–5 μM (Cheah and others 2010). Even if riluzole is extensively bound to plasma proteins, its liposolubility ensures high bioavailability (about 90%) after a single dose administration (Cheah and others 2010; Wokke 1996). These pharmacokinetic properties have implications to understand laboratory experiments with different drug application times and protocols.

It is helpful to consider what goals should be attained with the experimental administration of riluzole. On the one hand, one aim might be to hinder neuronal death by depressing glutamate-mediated excitotoxicity developing within a short time frame. On the other hand, riluzole can be administered to control late symptoms associated with a variety of chronic neurodegenerative conditions, when additional mechanisms of riluzole action are perhaps necessary to account for its activity. Even with relatively simple experimental models it is not always straightforward to distinguish between these objectives.

Changes in Extracellular Glutamate Concentrations Evoked by Riluzole: An Approach to Prevent Early Neuronal Loss in Spinal Networks

Chronic diseases of the nervous system have a slow progression that can be difficult to mimic with in vivo animal experiments especially when the research focus is on disease mechanisms at molecular and cellular level. For these reasons, many in vitro studies have been performed with organotypic spinal cord cultures that have the advantage to replicate the morphology and local connectivity together with long-term survival of the spinal cord networks. For example, using rat spinal cord organotypic cultures, Rothstein and Kuncl (1995) have modeled chronic glutamate neurotoxicity with the glutamate uptake inhibitor THA (threo-hydroxyaspartate) applied for two (or more) weeks either alone or together with neuroprotective drugs including riluzole. In this study, the large loss of lumbar motoneurons was halved by 100 μM riluzole, yet lower doses were ineffective. The neuroprotective action of riluzole observed by Rothstein and

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Kuncl (1995) was likely due to inhibition of glutamate release, as glutamate uptake was chronically blocked. More recent data have shown that long-lasting (3 days) application of 100 μM riluzole actually upregulates glutamate transporters in purified glial cell preparations (Carbone and others 2012), adding a new mechanism to the action of riluzole.

Several investigations have used short-term applications (a few minutes) of riluzole to study how it may modulate glutamate uptake. For example, using HEK cells to express glial transporters, Fumagalli and others (2008) observed that the most effective concentration of riluzole to increase glutamate uptake is 10 μM, a result confirmed with purified astrocytes (Frizzo and others 2004). Studies of synaptosomal uptake of glutamate have indicated that even lower (0.1-1 μM) doses of riluzole rapidly (10 min) enhance glutamate uptake by increasing the glutamate affinity for the carrier and the transport efficacy (via G-protein mediated phosphorylation), while higher doses (100 μM) become ineffective (Azbill and others 2000) or even toxic (Frizzo and others 2004). Because synaptosomal uptake is primarily via neuronal rather than glial transporters, these data outline a subtle effect by riluzole on glutamate uptake by presynaptic nerve endings and confirm that the enhancement of distinct glutamate transport systems is dose dependent.

Using organotypic spinal cultures, Guzmán-Lenis and others (2009) have shown that an acute (30 min) application of glutamate (50 μM) induces strong motoneuron damage largely prevented by 1 μM riluzole. Hence, the simplest conclusion is that rapid excitotoxic damage caused by exogenous glutamate application can be inhibited by low doses of riluzole, whereas a slowly developing, insidious damage caused by raising ambient glutamate when uptake is chronically blocked is much less susceptible to riluzole protection unless very high doses of this drug are employed with associated side effects.

Our laboratory has developed a test to mimic the excitotoxic process occurring acutely after an impact injury: this is achieved by 1 h application of the glutamate agonist kainate, which is not transported by glutamate carriers and not subjected to cell metabolism (Taccola and others 2008). This protocol evokes excitotoxicity via direct activation of glutamate receptors plus depolarization-dependent glutamate release (Fig. 2A) and examines the histological, biochemical, and functional outcome 24 h later (Mazzone and others 2010). Kainate primarily damages neurons rather than glia via a large depolarizing action associated with a strong release of endogenous glutamate demonstrated with a focal biosensor (Mazzone and Nistri 2011b). Coapplication of riluzole (5 μM) significantly inhibits kainate-induced calcium dependent (neuronal) and independent (glial) release of endogenous

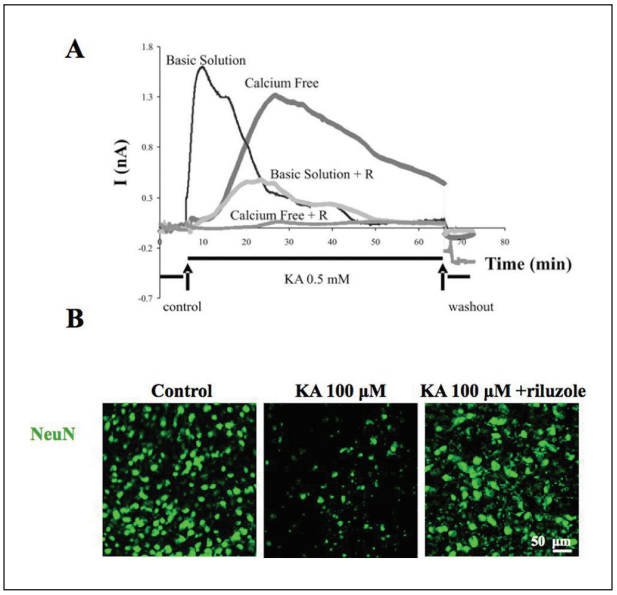

Figure 2. Riluzole suppresses kainate-evoked glutamate release and induces delayed neuroprotection on spinal cord organotypic cultures. (A) Time course of endogenous glutamate release induced by kainate (0.5 mM, applied for 1 h, see arrows) alone or together with riluzole (5 μM). The ordinate shows the real-time redox current monitored with a glutamate biosensor in basic solution (fast time course, black trace) or in Ca^{2+} free solution (slow time course, gray trace). Note that, regardless of the medium composition, riluzole strongly blocks glutamate release. (B) Example of neuronal (NeuN positive) staining of the central region of a representative spinal cord culture at 24 h in control solution, after 1 h application of kainate (0.1 mM), or after 1 h of kainate followed by 24 h of riluzole (5 μM), demonstrating that riluzole is able to protect neurons when its administration is delayed with respect to the start of the insult. Figure reprinted from Neuroscience 190:318–27, Mazzone GL, Nistri A, Delayed neuroprotection by riluzole against excitotoxic damage evoked by kainate on rat organotypic spinal cord cultures, Copywright (2011), with permission from Elsevier.

glutamate (see Fig. 2A) and suppresses levels of lactate dehydrogenase (LDH; a hallmark for necrosis), but, unexpectedly, it cannot prevent neuronal cell death (Mazzone and Nistri 2011a). Curiously, when riluzole is applied later (after kainate washout), a degree of neuroprotection is observed (Fig. 2B) in the dorsal and central regions but not in the ventral one (Mazzone and Nistri 2011a). These results highlight the neuron-selective ability of riluzole to induce neuroprotection probably in relation to the different vulnerability of neurons within the spinal cord (see also Mazzone and others 2010, Taccola and others 2008) in accordance with previous finding by Van Westerlaak and others (2001) in the cerebral cortex. Since the action of riluzole is time dependent and accompanied by strong depression of glutamate release (and

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likely facilitation of glutamate uptake), the simplest interpretation is that, at a rather early stage of excitotoxic stimulation, there are glutamate-mediated endogenous mechanisms that are coactivated to counteract the initial damage and yet remain insufficient to prevent it. Although the identification of these mechanisms requires future studies, one can hypothesize a role for metabotropic glutamate receptors (Niswender and Conn 2010) or release of endocannabinoids (Alger and Kim 2011). Notwithstanding the resolution of this question, it is interesting that former studies have reported opposing effects of excitatory amino acids on spinal motoneurons depending on the length of application (Lladó and others 1999) and that, according to the extent and nature (excitotoxic versus mechanical damage) of SCI in vivo, the extracellular glutamate rise may not be the primary mechanism to induce cell loss (McAdoo and others 2005).

The quest for the mechanism of action of riluzole must therefore be extended also to other targets controlling neuronal excitability in physiological and pathological conditions.

Riluzole suppression of neuronal excitability by targeting the persistent sodium current (I_{NaP})

Riluzole is known to be an inhibitor of the I_{NaP} (Urbani and Belluzzi 2000), namely a voltage-activated Na^+ conductance with low activation threshold and very slow inactivation, properties that confer it the ability to support sustained spike firing. In fact, in locomotor spinal networks, persistent currents mediate locomotor-related rhythmic activities expressed by interneurons (Tazerart and others 2007) and motoneurons (Harvey and others 2006). While I_{NaP} is essential for repetitive firing by motoneurons under physiological conditions, in chronic spinal rats this current becomes much larger and is believed to be a major contributor to the onset of spasticity (Harvey and others 2006). Indeed, the electrophysiological signature of I_{NaP} upregulation is the genesis of very large depolarizations with all-or-none characteristics (plateau potentials) that ultimately may be major contributors to the genesis of spasticity (Li and Bennet 2003). Thus, the inhibition of I_{NaP} by riluzole may explain its usefulness to decrease human spasticity, a distressing symptom frequently appearing after SCI (Theiss and others 2011) even though the final clinical benefits are modest. Likewise, daily treatment by riluzole of rats after chronic SCI produces only a moderate improvement in locomotor scores (reviewed by Kwon and others 2011). While riluzole can be viewed as a useful pharmacological tool to study I_{NaP}, it seems unlikely that its block of I_{NaP} is the main mechanism for any early neuroprotection. In fact, the sodium channel blocker tetrodotoxin fails to produce neuroprotection against excitotoxicity in vitro (Mazzone and Nistri 2011a) and cannot contrast the homeostatic plasticity developing in spinal circuits after chronic block of neurotransmission (Ballerini and others 1999).

The beneficial effect of riluzole on spasticity is probably best observed at later times when I_{NaP} becomes upregulated by the constitutive hyperactivity of sublesional serotonin receptors and thus can play a proportionally more important role in sustained neuronal firing (Murray and others 2011). This action may be functionally more important than depression of extracellular glutamate concentrations because in chronic SCI monosynaptic excitatory potentials are not augmented (Murray and others 2011). Plateau potentials supported by I_{NaP} in chronic SCI are likely triggered by slow polysynaptic potentials (Murray and others 2011) that comprise reversed inhibitory potentials due to the positive shift in the Cl^- equilibrium potential with consequent attenuation of the role of GABA and glycine (Boulenguez and others 2010).

Upregulation of I_{NaP} is also detected in cultured spinal motoneurons from $SOD1^{G93A}$ mice (a transgenic ALS model) with associated excitability increase (Kuo and others 2005) that is reversed by riluzole (0.5 μM). In spinal cord culture, riluzole (5–20 μM) blocks bursting by silencing intrinsically spiking neurons and suppressing network recruitment, indicating that the blocking action by riluzole is largely dependent on the activation state of the cell (Darbon and others 2004). On the same preparation, lower doses of riluzole (1-2 μM) convert slow bursts into fast oscillations probably because of an additional effect on fast sodium current inactivation (Yvon and others 2007). Taken together, these mechanisms would concur to tune down network hyperexcitability, a desirable goal when the aim is to stifle overexcitable networks in chronic CNS diseases.

Riluzole Effects on Brainstem Neurons: Implications for Respiration and Rhythmic Motor Patterns

The regular rhythm of respiration is produced by a pontine-medullary network that generates alternating patterns of inspiratory and expiratory activities relying on neurons endowed with voltage-dependent bursting properties, including I_{NaP} (Pace and others 2007; Paton and others 2006; Smith and others 2007). As the role of I_{NaP}-dependent bursting in respiratory rhythmogenesis has been debated, clarification of this issue has been investigated by using riluzole as a pharmacological tool to block I_{NaP}. In the in vitro brainstem slice preparation, microinjection of riluzole (10 μM) into the pre-Bötzinger

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rhythmogenic area does not attenuate the inspiratory drive potential nor does it block the respiratory rhythm (Pace and others 2007). However, during hypoxia (Paton and others 2006; Peña and others 2004) or in reduced pontomedullary preparations (Smith and others 2007), riluzole-sensitive pacemaker neurons become the essential drivers for respiratory bursts. Thus, while riluzole-sensitive I_{NaP} does not play an essential role for rhythmogenesis (Pace and others 2007) in normoxic rhythm (eupnea), it becomes increasingly important with changes in metabolic conditions when certain network elements are turned off (Smith and others 2007) and gasping mechanisms of autoresuscitation are switched on (Paton and others 2006).

Riluzole effects become even more important for certain forms of MND in which brainstem motoneurons are particularly affected (Kühnlein and others 2008). Among brainstem motoneurons, hypoglossal motoneurons (HMs) provide the exclusive innervation to tongue muscles, thus controlling rhythmic contractions of the tongue in concert with the respiratory drive and playing a fundamental role in respiration-related functions. When these cells are damaged, especially in the bulbar form of ALS, distressing symptoms such as dysarthria, dysphagia, and breathing difficulty appear (Kühnlein and others 2008). Thus, experimental studies of HMs in vitro can help to understand the effects of ALS-related etiopathological factors on brainstem motoneurons (Cifra and others 2011; van Zundert and others 2008). Our laboratory has developed an excitotoxic model of HMs caused by the glutamate uptake inhibitor threo-β-benzyloxyaspartate (TBOA). This drug triggers long-lasting HM bursting (see Fig. 3A) accompanied by intracellular calcium waves with delayed HM loss preceded by the strong expression of the ATF-3 transcription factor, a distress marker indicative of impending motoneuron death (Fig. 3B; Cifra and others 2011; Sharifullina and Nistri 2006). As shown in Figure 3, when riluzole (5 μM) is applied early after the start of the excitotoxic insult, it not only blocks TBOA-induced bursting but also prevents ATF-3 upregulation and preserves HM numbers (Cifra and others 2011). It is likely that such a riluzole protection is due to the block of glutamate release as demonstrated by the riluzole-induced decrease in glutamatergic synaptic currents (Cifra and others 2011). In particular, riluzole preferentially blocks glutamate release from very active synapses through a PKC-dependent mechanism (Lamanauskas and Nistri 2008). These phenomena have been observed with a dose of riluzole below threshold for inhibiting I_{NaP} and repetitive firings (Lamanauskas and Nistri 2008). Hence, as described for spinal cord motoneurons, acute neuroprotection by riluzole seems to be exerted through inhibition of glutamate release rather than suppression of I_{NaP}. Nonetheless, this current is considered to be an important contributor to enhanced motoneuron excitability in genetic models of ALS in both hypoglossal (van Zundert and others 2008) and spinal (Kuo and others 2005) motoneurons, indicating that riluzole may have an important role in controlling early abnormal neuronal activity emerging in certain genetic ALS forms (Kuo and others 2005; van Zundert and others 2008).

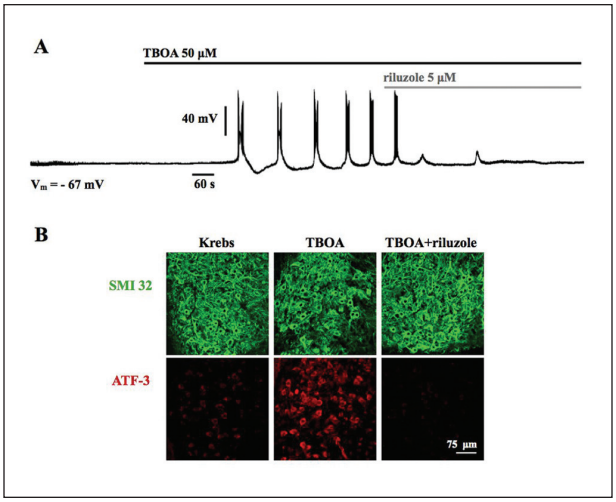

Figure 3. Riluzole counteracts the effects of the TBOA-induced block of glutamate uptake on brainstem hypoglossal motoneurons. (A) Sample record of a representative hypoglossal motoneuron under current clamp configuration (Vm = −67 mV resting potential) showing bursting activity elicited by 50 μM TBOA blocked by subsequent application of riluzole (5 μM). (B) From left to right (top row), examples of confocal images of hypoglossal motoneurons stained with the motoneuronal marker SMI 32 (green) after 4 h treatment in control (Krebs), TBOA (50 μM), or TBOA+riluzole (5 μM, started 15 min after TBOA and continuously coapplied up to 4 h). Bottom row shows corresponding confocal images with the ATF-3 distress marker (red). Note that application of riluzole not only protects hypoglossal motoneurons but also counteracts the TBOA-induced increase in ATF-3 signal. Vm, membrane voltage at rest.

Conclusions

Riluzole is a drug of interest to basic scientists as it enables them to investigate molecular mechanisms regulating synaptic transmission and neuronal excitability and their relative contribution to experimental models of neurodegeneration monitored at various stages of evolution. Figure 4 shows an idealized diagram summarizing the principal actions by riluzole on such processes that comprise uptake (A) and release (B) of glutamate, and block of I_{NaP} (C). From future studies of riluzole, more clinical benefit might ensue if the time and length of this drug administration during neurodegenerative diseases are optimized. More important, studies with riluzole might provide new clues to the design of mechanism-based, disease-modifying novel agents.

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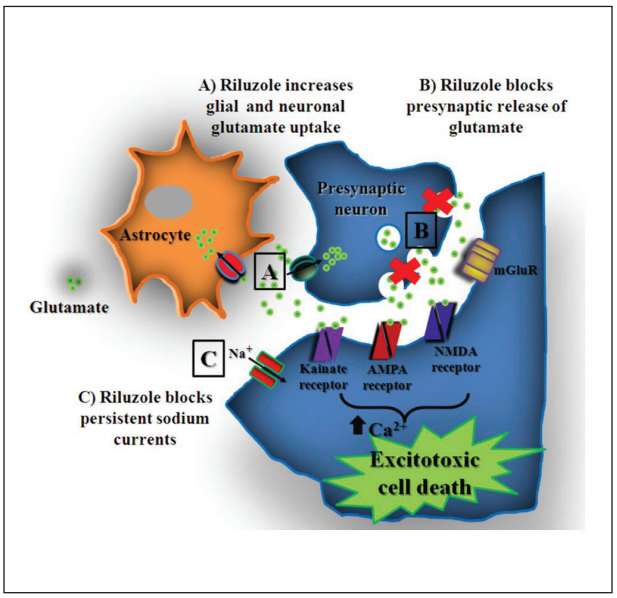

Figure 4. Schematic diagram summarizing the main effects of low doses of riluzole on glutamatergic transmission and motoneuron excitability. (A) In the low micromolar concentration range, riluzole enhances the uptake of ambient glutamate by astrocytes as well as presynaptic glutamatergic nerve terminals, and it also reduces the endogenous release of glutamate particularly from very active synapses (B). These phenomena would therefore limit the absolute level of extracellular glutamate binding to various classes of ionotropic (kainate, AMPA, and NMDA) and metabotropic (mGluR) receptors. Strong and/or persistent activation of ionotropic receptors is thought to raise intracellular Ca^{2+} that triggers a cascade of intracellular signal pathways leading to cell death by excitotoxicity. (C) Riluzole can also inhibit a persistent Na^{+} current that, in view of its slow kinetics of inactivation, can support long-lasting firing of action potentials by motoneurons. This depressant effect by riluzole is expected to limit neuronal excitability and restrict the spread of network overactivity that perpetuates a vicious circle of further excessive release of glutamate and increased neuronal damage.

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Declaration of Conflicting Interests

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