Neuro–degenerative Diseases

Neurodegener Dis
DOI: 10.1159/000357281

Received: September 9, 2013
Accepted after revision: November 13, 2013
Published online: December 20, 2013

The Neuroprotection Exerted by Memantine, Minocycline and Lithium, against Neurotoxicity of CSF from Patients with Amyotrophic Lateral Sclerosis, Is Antagonized by Riluzole

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Key Words
Memantine · Minocycline · Lithium · Amyotrophic lateral sclerosis · Neurotoxicity · Riluzole

Abstract
In a recent study we found that cerebrospinal fluids (CSFs) from amyotrophic lateral sclerosis (ALS) patients caused 20–30% loss of cell viability in primary cultures of rat embryo motor cortex neurons. We also found that the antioxidant resveratrol protected against such damaging effects and that, surprisingly, riluzole antagonized its protecting effects. Here we have extended this study to the interactions of riluzole with 3 other recognized neuroprotective agents, namely memantine, minocycline and lithium. We found: (1) by itself riluzole exerted neurotoxic effects at concentrations of 3–30 μM; this cell damage was similar to that elicited by 30 μM glutamate and a 10% dilution of ALS/CSF; (2) memantine (0.1–30 μM), minocycline (0.03–1 μM) and lithium (1–80 μg/ml) afforded 10–30% protection against ALS/CSF-elicited neurotoxicity, and (3) at 1–10 μM, riluzole antagonized the protection afforded by the 3 agents. These results strongly support the view that at the riluzole concentrations reached in the brain of patients, the neurotoxic effects of this drug could be masking the potential neuroprotective actions of new compounds being tested in clinical trials. Therefore, in the light of the present results, the inclusion of a group of patients free of riluzole treatment may be mandatory in future clinical trials performed in ALS patients with novel neuroprotective compounds.
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Introduction

Twenty years ago riluzole was introduced in the clinic to treat patients with amyotrophic lateral sclerosis (ALS). This indication was based on clinical trials showing that riluzole confers a modest beneficial effect on survival [1, 2]. In spite of much effort and investment made since then, today riluzole remains as the sole available medicine to combat this appalling disease.

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A few examples of failure in clinical trials of 3 compounds that showed neuroprotective effects in animal disease models illustrate some of the frustrating efforts made. Thus, the non-competitive N-methyl-D-aspartate receptor antagonist memantine prolongs survival in mouse ALS models [3, 4]; however, its co-administration with riluzole to ALS patients provided negative functional disability outcomes [5]. Also, minocycline slows disease onset and delays mortality of ALS mouse models [6–9] even when co-administered with riluzole [10]. Nevertheless, minocycline had no efficacy in clinical trials [11–13]. The third example concerns lithium that delays disease onset, reduces neurological deficits and prolongs survival in ALS mouse models [14–16]; however, two more studies showed no effect [17, 18]. Once more, lithium did not improve neurological outcomes in clinical trials [19, 20].

A recent finding from our laboratory can shed light on the frustrating issue of the poor translation of the positive neuroprotective results obtained in preclinical ALS models into clinical settings. As previously found by several authors [21–27] we corroborated that the cerebrospinal fluid (CSF) from ALS patients (ALS/CSF) impairs cell viability (for online suppl. fig. S1 and S2, see www.karger.com/doi/10.1159/000357281) in primary cultures from rat embryo motor cortex neurons (MCNs). We also found that the polyphenolic anti-oxidant resveratrol [28] afforded protection against ALS/CSF toxicity but riluzole did not. Surprisingly, we also observed that when MCNs were incubated with both compounds, riluzole antagonized the neuroprotective effects of resveratrol [29]. Thus, the question arises whether this negative interaction is specific for riluzole and resveratrol or if it extends to other neuroprotective compounds such as memantine, minocycline and lithium, referred to above. We show here that indeed, the 3 compounds afforded protection against ALS/CSF-elicited neurotoxicity, and that riluzole antagonized the neuroprotection effect of the 3 compounds. This could be explained by the fact that riluzole itself impaired MCN viability, an effect that could be masking the neuroprotective actions of memantine, minocycline and lithium upon MCN co-incubation of each of these compounds with riluzole and ALS/CSF. These findings strongly suggest that co-administration of riluzole with novel neuroprotective agents in ALS clinical trials could be a confounding factor in the expected outcomes, thus explaining the poor translation of neuroprotective compound efficacy from preclinical to clinical settings [30, 31].

Methods

Subjects and CSF Collection

Subjects were enrolled through the ALS unit in the Neurology Service following the clinical pathway established to diagnose and treat the disease [32] that includes lumbar puncture performance. Informed consent was obtained from all participants. All procedures were performed under hospital Ethic Committee guidelines for research studies involving human subjects. In subjects suspected of having ALS, evaluation included history from the subject, neurological examination, electromyography, neuroimaging study, screening blood tests and determination of forced vital capacity using spirometry when required; patients had documented treatment with riluzole at the moment of lumbar puncture performance. For the present study, CSFs from 17 patients with ALS were collected. Patients presented a mean age of 66.1 (range 40–77) years. Five patients presented a bulbar-onset form and 12 suffered a spinal-onset form. The mean time from the beginning of the symptoms until the extraction of CSF was 8.94 months (range 4–18). At the moment of the lumbar puncture performance, 7 patients were not taking riluzole; in 5 cases, treatment was initiated in the same week of the lumbar puncture and the duration of treatment was greater than 3 months in a single case.

Culture of Rat Cortical Neurons

Embryos were selected from 19- to 20-day pregnant rats, which were decapitated, and embryos were extracted from the womb by caesarean section. Meninges were removed and a portion of motor cortex was isolated after the dissection of the brain. Fragments obtained from several embryos were subjected to mechanical digestion, and cells were resuspended in neurobasal medium with 2% B-27 and seeded in 48-well plates at a density of 100,000 cells/ml. Neuronal cultures were allowed to grow for 8–10 days (DIV), and when the microscope showed the existence of a dense neuronal network, incubations with different CSFs were done. We performed selective labelling with conjugated Milli-Mark FluoroPan Neuronal Marker-Alexa 488 and with choline acetyltransferase antibody, thus confirming that our cultures contain populations of motor neurons.

Measurement of Neuronal Viability

To monitor neuronal viability we used the dimethylthiazolyl diphenyltetrazolium bromide (MTT) reduction assay [33]. After the appropriate incubations with the CSF, 0.5 mg/ml MTT was added to each well, and incubation was performed at 37°C for 2 h. The formazan salt formed was dissolved in dimethylsulphoxide, and colorimetric determination was performed at 540 nm. Control cells without CSF were considered to have 100% viability. Neuronal viability after exposure to CSF or different treatments was expressed as percent of control within each individual experiment. We have previously observed that CSF-elicited cytotoxicity measured through MTT reduction correlates with lactate dehydrogenase release, increment in caspase 3 expression and to cellular changes such as retraction of the cellular projections, reduction of the cellular volume, chromatin condensation and nuclear fragmentation.

Statistics

Graph Pad Prism Software (Graph Pad Software, San Diego, Calif., USA) was used to perform statistical analyses and graphi-

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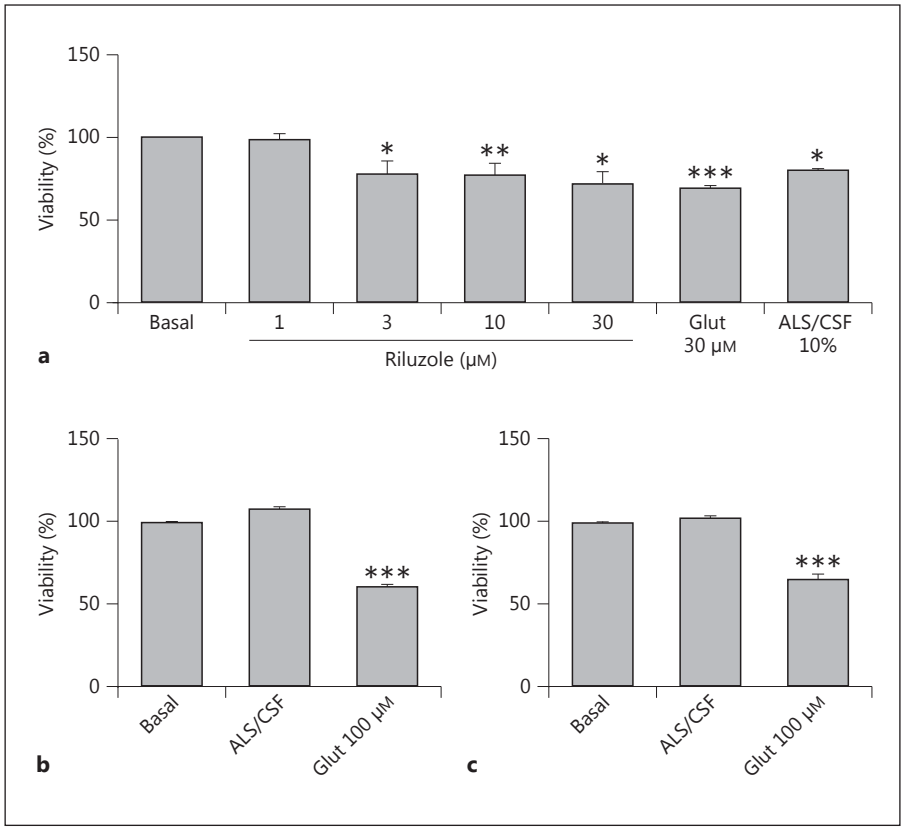

Fig. 1. Riluzole had concentration-dependent neurotoxic effects in rat embryo MCNs (**a**). Glutamate (Glut) elicited neurotoxicity in MCNs, in hippocampal neurons (**b**) and in sensory cortex neurons (**c**). **a** Rat embryo cortical motor neurons (8 DIV) were incubated for 24 h with neurobasal medium (basal) or with increasing concentrations of riluzole, glutamate or ALS/CSF at the concentrations indicated at the bottom of each column. At the end of this incubation period, neuronal viability was monitored with the mitochondrial MTT probe. Neuronal viability after exposure to CSF or different treatments was expressed as percent of control within each individual experiment. **b**, **c** Hippocampal neurons and sensory cortex neurons were incubated with 10% ALS/CSF or 100 μM glutamate, and after 24 h their viability was estimated as in **a**. Viability was normalized as percent of the control non-treated neurons (100% ordinate), within each individual experiment performed in triplicate. Data are means ± SEM of 5 different cultures. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ with respect to basal as determined by ANOVA/Dunnett's test.

cal presentation. Experiments were reproduced at least 3 times. Data were expressed as means ± SEM. Groups were compared by ANOVA/Dunnett's test. To compare the difference between two means, the t test was performed. A p value ≤0.05 was accepted as the limit of statistical significance.

Results

The Neurotoxic Effects of Riluzole

After 24 h incubation of MCNs with increasing concentrations of riluzole (1–30 μM) we found a loss of cell viability (fig. 1a). The threshold concentration for its neurotoxic effect was between 1 and 3 μM. At 3 μM the loss of neuronal viability was 22 ± 10.7%, at 10 μM 24.2 ± 14.2% and at 30 μM 28 ± 7.6%, showing a concentration-dependent effect in the range used. An ANOVA analysis of the 4 concentrations used showed significant differences between them. As positive neurotoxic comparators, glutamate and ALS/CSF were also assayed. Thus, at 30 μM, glutamate produced 41.4 ± 3.7% loss of neuronal viability (fig. 1a) that compares well with the neuronal loss elicited by riluzole also at 30 μM (28 ± 7.6%). At the 10% dilution used in our previous study [29], ALS/CSF produced 19.8 ± 2.6% neuronal loss (fig. 1a). This toxic effect was selective for motor neurons, since it did not appear in hippocampal neurons or in neurons from the sensorial cortex (fig. 1b, c).

Memantine, Lithium and Minocycline Provided Neuroprotection against the Neurotoxic Effects of ALS/CSF

As noted in the Introduction, memantine, lithium and minocycline exert neuroprotection in different in vitro models of neurotoxicity. Whether they have the potential to elicit neuroprotection against toxicity elicited by ALS/CSF is unknown. Therefore, we first tried to answer this question in our primary culture of MCNs.

We followed the optimized protocol used to monitor the neurotoxic effects of individual ALS/CSF from each patient, diluted in neurobasal medium to the final concentration of 10% [29]. After 24 h incubation in this medium, the neuronal viability decreased in a range of 15–30% with ALS/CSF from different patients, a figure similar to that recently reported by our laboratories [29]. Because of this variability in the extent of ALS/CSF-elic-

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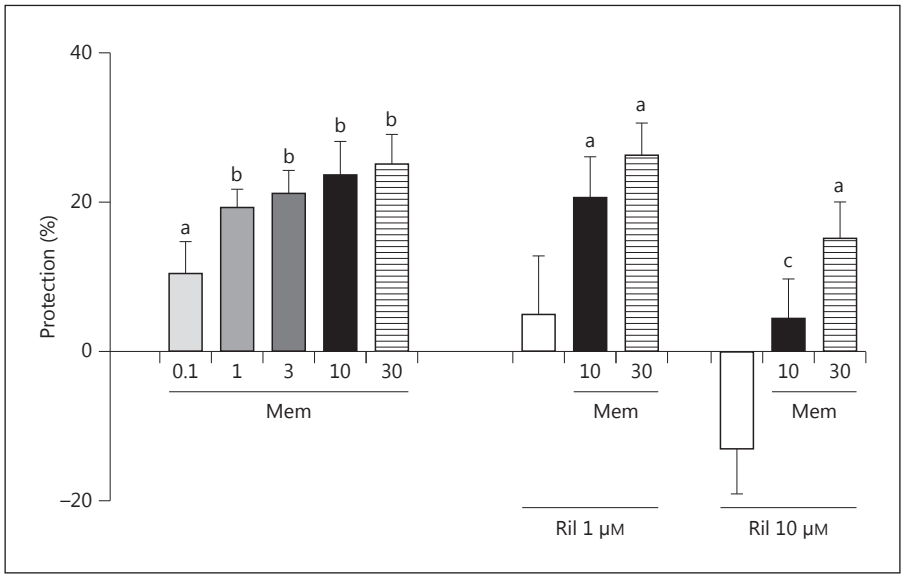

Fig. 2. Memantine (Mem) afforded neuroprotection against ALS/CSF neurotoxicity, and riluzole (Ril) antagonized those neuroprotective effects. Cortical motor neurons at 8 DIV were incubated for 24 h with 10% ALS/CSF in the absence and the presence of memantine or riluzole 1 and 10 μM plus memantine, at the concentrations indicated at the bottom of each column. In each individual experiment, the percentage of neuronal loss elicited by ALS/CSF (range: 17–24%) was subtracted from those achieved in the presence of memantine and expressed as percentage of protection (ordinate). Pooled data are expressed as means ± SEM of 3 individual experiments. [a] $p < 0.01$ and [b] $p < 0.001$ with respect to basal as determined by ANOVA/Dunnett's test; [c] $p < 0.05$ with respect to 10 μM memantine without riluzole as determined by the t test.

ited toxicity, in each individual experiment we normalized this value to 100% and calculated the protection exerted by a given compound as the fraction of recovery of neuronal viability (percent protection in the ordinates of fig. 2–4). Compounds were dissolved in neurobasal medium at the required final concentrations in the absence (100% viability) or the presence of 10% ALS/CSF. Then, 8–10 DIV MCNs were incubated for 24 h with each solution, and their viability was monitored using MTT.

Figure 2 shows that memantine alone afforded partial protection against ALS/CSF toxicity in a concentration-dependent manner. Threshold protection was at 0.1 μM (10.3 ± 5.8%) and maximum protection was at 30 μM (25 ± 5.4%); a graph-estimated EC_{50} of 0.15 ± 0.01 μM was obtained.

Figure 3 shows that lithium alone also afforded partial protection against ALS/CSF toxicity. However, the concentration-response curve was steeper with lithium as compared with memantine; threshold protection was at 10 μg/ml ($p < 0.05$ with respect to control; 17.75 ± 11.70%), and maximum protection was achieved at 80 μg/ml; a graph-estimated EC_{50} of 15.12 ± 1.41 μg/ml was obtained.

Figure 4 shows that minocycline alone afforded a clear-cut protection against ALS/CSF toxicity with high potency (submicromolar concentrations). Thus, at only 30 nM minocycline elicited 12.25 ± 5.32% protection; maximum protection achieved was at 1 μM (19 ± 4.57%). An EC_{50} could not be estimated but, apparently, it must be below 30 nM. It should be noted that because of scarcity of available ALS/CSF, the concentration range used for the compounds had to be necessarily limited.

Riluzole Antagonized the Neuroprotective Effects of Memantine, Lithium and Minocycline in ALS/CSF-Treated MCNs

Whether riluzole interfered with the neuroprotective effects of memantine, lithium and minocycline was explored in experiments with protocols similar to those described above. These experiments were run in parallel with those of neuroprotection (fig. 2–4, left). The incubation solutions contained the neuroprotective agent at different concentrations, in the absence and the presence of riluzole at 1 or 10 μM. MCNs were incubated with ALS/CSF, in the presence of the different treatments, for 24 h and then cell viability was monitored with MTT. The simultaneous run of the different experimental conditions narrowed the statistical variation, making the results more meaningful.

Figure 2 shows that at 1 μM, riluzole did not alter by itself the viability of MCNs. Neither riluzole concentration interfered with the protection afforded by 10 and 30 μM memantine. However, at the higher concentration of 10 μM, riluzole decreased cell viability by 12 ± 6.78%, practically abolished the protection provided by 10 μM memantine, and reduced by 40% the protection offered by 30 μM memantine (fig. 2, right). The greater effect of riluzole at the higher concentration suggests a competitive antagonism between riluzole and memantine.

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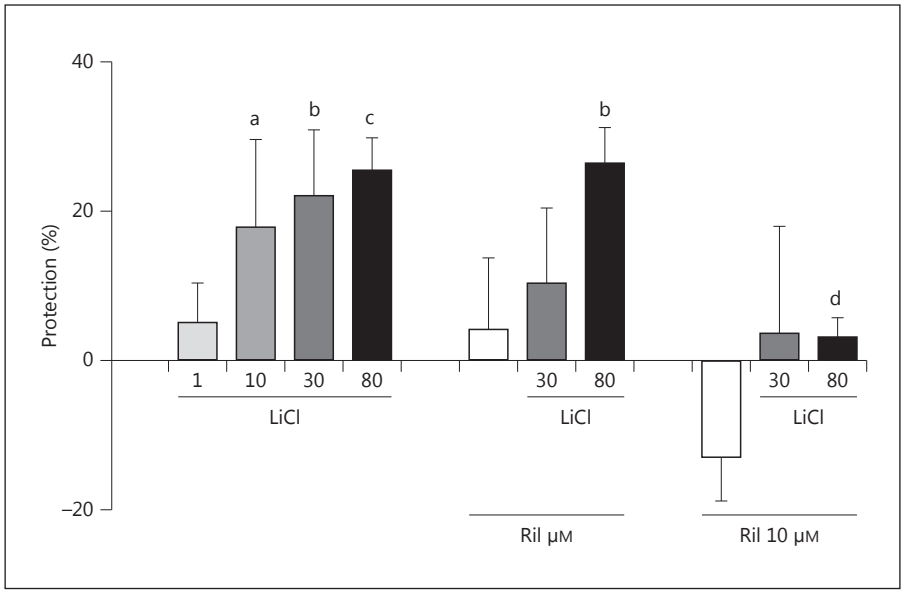

Fig. 3. Lithium (LiCl) afforded neuroprotection against ALS/CSF neurotoxicity, and riluzole (Ril) antagonized those neuroprotective effects. Cortical motor neurons at 8 DIV were incubated for 24 h with 10% ALS/CSF in the absence and the presence of LiCl or riluzole 1 and 10 μM plus LiCl, at the concentrations indicated at the bottom of each column in micrograms per millilitre. In each individual experiment, the percentage of neuronal loss elicited by ALS/CSF (range: 17–24%) was subtracted from that achieved in the presence of LiCl and expressed as percentage of protection (ordinate). Pooled data are expressed as means ± SEM of 3 individual experiments. [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$ with respect to basal as determined by ANOVA/Dunnett's test; [d] $p < 0.01$ with respect to 80 μg/ml LiCl without riluzole as determined by the t test.

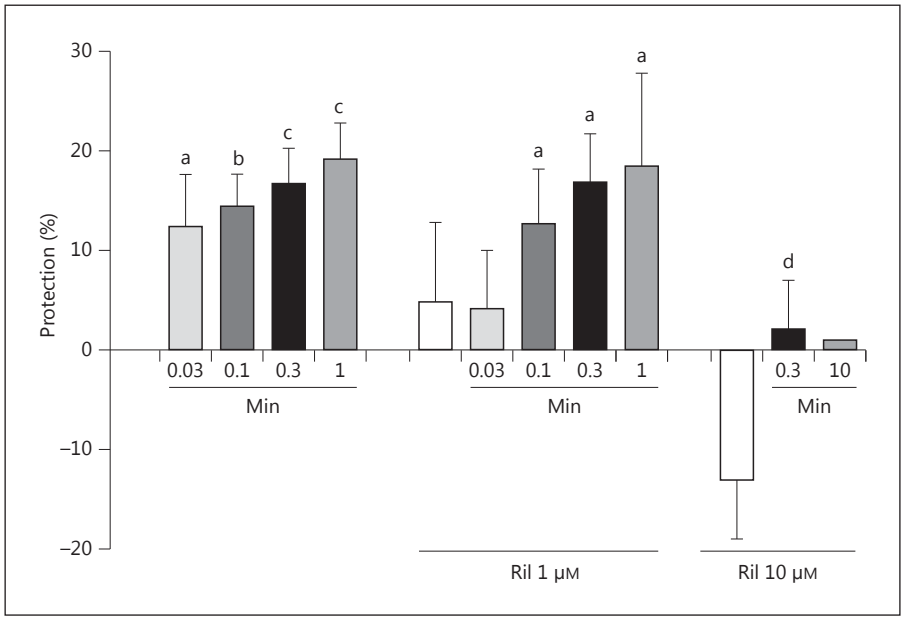

Fig. 4. Minocycline (Min) afforded neuroprotection against ALS/CSF neurotoxicity, and riluzole (Ril) antagonized those neuroprotective effects. Cortical motor neurons at 8 DIV were incubated for 24 h with 10% ALS/CSF in the absence and the presence of minocycline or riluzole 1 and 10 μM plus minocycline, at the concentrations indicated at the bottom of each column. In each individual experiment, the percentage of neuronal loss elicited by ALS/CSF (range: 17–24%) was subtracted from that achieved in the presence of minocycline and expressed as percentage of protection (ordinate). Pooled data are expressed as means ± SEM of 5 individual experiments. [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$ with respect to basal as determined by ANOVA/Dunnett's test; [d] $p < 0.05$ with respect to 0.3 μM minocycline without riluzole as determined by the t test.

The interaction lithium-riluzole seemed to be of a different nature. For instance, 1 μM riluzole diminished by 53% the neuroprotective action of 30 μg/ml lithium, although it did not interfere with the effect of 80 μg/ml lithium (fig. 3, middle). Once more, at the higher concentration of 10 μM, riluzole caused by itself a reduction of 12 ± 6.78% in neuronal viability. At this higher concentration, riluzole abolished the neuroprotective effects of 30 and 80 μg/ml lithium, indicating a non-competitive interaction between the two drugs (fig. 3, right).

Finally, we studied the interaction riluzole-minocycline. At 1 μM, riluzole reduced by 61.5% the neuroprotective actions of 30 nM minocycline; however, riluzole did not affect the protection afforded by 0.1, 0.3 and 1 μM minocycline (fig. 4, middle). This was not the case for the higher 10 μM riluzole concentration that once more abolished the neuroprotective effects of 0.3 and even 10 μM minocycline, again indicating a non-competitive interaction.

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Discussion

In discussing our data we should first consider a relevant methodological issue that concerns the nature of the cell types present in our primary cultures of rat embryo MCNs. That they were enriched in cortical motor neurons was demonstrated by selective staining with conjugated Milli-Mark FluoroPan Neuronal Marker-Alexa 488, as well as with choline acetyltransferase antibody and Alexa Fluor 488 secondary antibody. Additional functional experiments confirmed the immunofluorescence results. For instance, ALS/CSF did not exert toxicity in cortical sensory neurons, hippocampal neurons (fig. 1b, c), mixed global populations of cortical neurons, and neurospheres (data not shown). In contrast, as previously shown [29] and in the present study (see below), ALS/CSF indeed impaired cell viability in MCN cultures. Also, it has been shown that ALS/CSFs exert neurotoxic changes in motor neurons from rat cortex and spinal cord [34]. Additionally, ALS/CSF only increases the cytosolic calcium concentration in motor neurons [24]. Furthermore, in NSC34, a hybrid cell line of neuroblastoma and motor neuron, ALS/CSF also exerts cytotoxic actions; however, this effect is only seen in differentiated cells [35, 36]. Thus, it seems reasonable to conclude that the cytotoxic effect elicited by ALS/CSF on our MCN cultures is mostly exerted on cortical motor neurons. This view is strengthened by the observation of a selective loss of motor neurons from the cerebral cortex of ALS patients [37, 38], thus reinforcing the validity of our model for the neurotoxic studies here performed.

We found in this investigation that ALS/CSF exhibited neurotoxic effects on primary cultures of rat embryo MCNs. This observation corroborates recent [29] and earlier findings [21–27] on the neurotoxicity elicited by ALS/CSF on neuronal cultures as well as after its in vivo intracerebroventricular or intrathecal administration [39–41].

We believe that our second finding that memantine, minocycline and lithium afford clear-cut neuroprotection against ALS/CSF neurotoxicity, is of interest and compatible with the in vivo neuroprotective properties of these compounds in animal models of ALS [3, 4, 6–10, 14–16]. Their neuroprotective properties have also been demonstrated in primary cultures of neurons [42–44]. The fact that they also afford protection against the neurotoxic effects of ALS/CSF on MCN cultures (fig. 2–4, left) adds clinical relevance to their neuroprotective effects. Unfortunately, as mentioned in the Introduction, these compounds gave negative neurological outcomes in clinical trials performed on ALS patients [5, 11–13, 19, 20].

This frustrating failure to translate the positive neuroprotective effects shown preclinically with memantine, minocycline and lithium into a clinical set-up could find an explanation in the observation that riluzole antagonized the neuroprotective effects of resveratrol [29] as well as those of memantine, lithium and minocycline (this study, fig. 2, 3, 4, right). This could simply be due to the fact that riluzole itself has neurotoxic effects as reported earlier [21–27]. In this study, we also found that riluzole itself exerts a neurotoxic activity comparable to that produced by glutamate or ALS/CSF (fig. 1).

In its dual neuroprotective [45] and neurotoxic actions (this study), riluzole seems to belong to a class of compounds that elicit neuroprotection at their lower concentrations; however, at their higher concentrations the neuroprotection diminishes or even disappears, as the case is for compounds derived from the glutamic acid moiety [46]. This dual action has also been found with galantamine and donepezil [47, 48], two drugs that are being clinically used to treat Alzheimer's disease [49]. In line with this concentration-dependent dual behaviour is riluzole that at 1 μM scarcely affected the neuroprotective effects of memantine (fig. 2, middle), lithium (fig. 3, middle) and minocycline (fig. 4, middle); however, at the higher 10 μM concentration, riluzole almost fully counteracted the neuroprotective effects of memantine (fig. 2, right), lithium (fig. 3, right) and minocycline (fig. 4, right).

The reason for this dual behaviour could be linked to the activation by riluzole of different survival and/or apoptotic signalling pathways at its lower and higher concentrations, respectively. For instance, riluzole has been shown to distort the intracellular Ca^{2+} homeostasis [50–52]; this may be linked to its reported apoptotic and cytotoxic effects in human prostate cancer cells [53] and in Madin-Darby canine kidney cells [52]. On the opposite side are the findings that riluzole regulates the expression of neurotrophic factor genes in C6 glioma cells [54], stimulates the synthesis of nerve growth factor and brain-derived neurotrophic factor in cultured mouse astrocytes [55], and augments brain-derived neurotrophic factor production with consequent proliferation of granule precursor cells in the rat hippocampus [56] and promotion of survival of rat MCNs through stimulation of trophic activity produced by spinal astrocyte monolayers [45]. These paradoxical and complex responses suggest that riluzole could exert different neuroprotective or neurotoxic actions by activating different signalling pathways at lower or higher concentrations.

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The antagonism by riluzole of the neuroprotective properties of memantine, minocycline and lithium has certainly some relevant clinical implications. The first issue to consider concerns whether in ALS patients riluzole reaches brain concentrations in the range of those used in the present study. The answer is yes because those concentrations are in the range of 1–10 μM [57, 58]. The second issue concerns the design of clinical trials performed in ALS patients with novel neuroprotective compounds, as mentioned in the Introduction. Because of ethical issues, all patients included in clinical trials were being treated with riluzole. However, we suggest that a treatment arm without riluzole should be included in future clinical trials because of the following reasons: (1) the study with xaliproden was the only clinical trial that included a group of patients not treated with riluzole; this group showed a respiratory improvement that was absent in the groups treated with riluzole [59]; (2) although done on in vitro primary cultures of rat embryo MCNs, our early [29] and present studies convincingly suggest that riluzole could be masking the neuroprotective effects of other compounds being tested in ALS patients simultaneously treated with the drug and the novel compound; (3) originally, the clinical indication of riluzole was established on the basis of clinical trials performed more than 20 years ago showing modest beneficial effects on ALS patient survival [1, 2, 60, 61]; because of poor design and bias, these studies may not have been accepted with today's standards for clinical trials. Thus, the ethical restriction that ALS patients should be maintained under riluzole treatment is weakened in the light of current data; furthermore, we may conclude that its administration to all group treatments during clinical trials could be a confounding factor in the expected outcomes [30, 31, 62].

In conclusion, in this study we found that by itself, riluzole exhibited neurotoxic effects in primary cultures of rat embryo MCNs. We also found that memantine, minocycline and lithium exerted neuroprotective effects against ALS/CSF-elicited neurotoxicity on MCNs. Finally, we found that riluzole antagonized the neuroprotective actions of the three compounds. These results suggest that the routine treatment with riluzole of ALS patients enrolled in clinical trials could be a confounding factor in the neurological outcomes attributed to the novel compound being tested.

Acknowledgements

This study has been partially supported by a grant to A.G.G. from the Fundación Eugenio Rodríguez Pascual, Madrid, Spain, as well as by grants to J.M.-G. from Mutua Madrileña, Spain, in 2008 and 2009. Support to A.G.G. from the following institutions is also acknowledged: (1) RD06/0026, Retics Renevas, I.S. Carlos III, (2) PI016/09 Fundación CIEN. I.S. Carlos III and (3) SAF 2010-21795, Ministry of Economy and Competitivity, Spain. We thank the continued support of Fundación Teófilo Hernando.

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