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Research report

Riluzole suppresses experimental autoimmune encephalomyelitis: implications for the treatment of multiple sclerosis

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Abstract

Recent studies suggest that glutamate neurotoxicity is involved in the pathogenesis of multiple sclerosis (MS), and that treatment with glutamate receptor (AMPA/kainate) antagonists inhibits experimental autoimmune encephalomyelitis (EAE), the conventional model of MS. Therefore, we examined whether riluzole, an inhibitor of glutamate transmission, affects the pathogenesis and clinical features of MS-like disease in myelin oligodendrocyte glycoprotein (MOG)-induced EAE in mice. Here we report that riluzole (10 mg/kg×2/day, i.p.), administered before and even after the appearance of clinical symptoms, dramatically reduced the clinical severity of MOG-induced EAE, while all the MOG-immunized control mice developed significant clinical manifestations. Moreover, the riluzole-treated mice demonstrated only mild focal inflammation, and less demyelination, compared to MOG-treated mice, using histological methods. Furthermore, riluzole markedly reduced axonal disruption, as assessed by Bielshowesky's silver staining and by antibodies against non-phosphorylated neurofilaments (SMI-32). No difference was detected in the immune system potency, as T-cell proliferative responses to MOG were similar in both groups. In conclusion, our study demonstrates, for the first time, that riluzole can reduce inflammation, demyelination and axonal damage in the CNS and attenuate the clinical severity of MOG-induced EAE. These results suggest that riluzole, a drug used in amyotrophic lateral sclerosis (ALS), might be beneficial for the treatment of MS.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Multiple sclerosis; Experimental autoimmune encephalomyelitis; Myelin oligodendrocyte glycoprotein; Glutamate; Riluzole

1. Introduction

Multiple sclerosis (MS) is a chronic disabling autoimmune neurological disorder targeting the white matter of the central nervous system (CNS). It often has an initial relapsing–remitting course, which later changes into a secondary progressive mode. The etiology of MS has not yet been fully elucidated, but it is believed that immunological mechanisms operate in disease initiation and progression [45]. In addition to the autoimmune attack, there is also a local inflammatory response and demyelination, oligodendrocyte death, axonal damage and even neuronal loss in the CNS [17,48]. Recent experimental evidence implicates glutamate, the major excitatory neurotransmitter in the mammalian brain, as an important contributing factor in MS pathogenesis. Oligodendrocytes, the myelin-producing cells of the CNS, are highly vulnerable to glutamate excitotoxicity, mainly via the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)/kainate receptors [34,35]. Demyelinating lesions caused by excitotoxins are histologically similar to those observed in MS [34,35]. In addition, treatment with AMPA/kainate antagonists was shown to ameliorate experimental autoimmune encephalomyelitis (EAE), which is the commonly used experimental model of MS in mice. AMPA/kainate antagonists were also shown to increase oligodendrocyte survival and reduce dephosphorylation of neurofilament H, an indicator of axonal damage [37,42]. Moreover, Stover et al. [46] demonstrated an increase in CSF glutamate levels in MS patients which correlated with the

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severity of the disease [2]. It was suggested that glutamate production by macrophages might be involved in axonal damage and oligodendrocyte pathology in MS lesions [52].

The excitotoxic hypothesis in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) suggests that anti-glutamatergic agents might be neuroprotective. Indeed, riluzole (2-amino-6-[trifluoromethoxy] benzothiazole; 6-trifluoromethoxy-1,3-benzothiazolde-2-ylamine), an anti-glutamatergic agent, was found to be protective in several models of neurodegenerative diseases including ALS [19,20], Parkinson's [1,3,5,9,10] and ischemia [31,39].

Riluzole was demonstrated to modulate the anti-glutamatergic activity through glutamate and sodium receptors. Studies on neuronal cultures and brain slices showed that riluzole inhibits the release of glutamate and L-aspartate from nerve terminals [11,32], modulates the *N*-methyl-D-aspartate (NMDA) ionotropic receptors [14] and stabilizes the voltage-dependent sodium channels in myelinated fibers [7,47]. In addition, riluzole was shown to modulate sodium and kainate-induced currents in mammalian cortical neurons [50,55,56] and to reduce the tonic firing of neocortical neurons in brain slices [41]. Due to its safety and neuroprotective properties, riluzole was approved by the US Food and Drug Administration for ALS therapy [8]. We therefore investigated the clinical effects of riluzole in reducing the insult to neurons and axons in myelin oligodendrocyte glycoprotein (MOG)-induced EAE in C3H.SW mice, a chronic model which appears to resemble the clinical course of progressive MS better than the other disorders induced by auto-antigens [27].

2. Materials and methods

2.1. Animals

Six- to eight-week-old C3H.SW/C57/bl female mice weighing 20 g were obtained from Harlan Laboratories (Rehovot, Israel). The animals were housed in standard conditions: constant temperature (22±1 °C), humidity (relative, 30%) and a 12-h light/dark cycle, and were allowed free access to food and water. The animals and protocol procedures were approved and supervised by the Animal Care Committee of the Rabin Medical Center.

2.2. Induction of EAE

EAE was induced by immunization with the peptide encompassing amino acids 35–55 of rat MOG. Synthesis was carried out by the Weizmann Institute Synthesis Unit, using a solid-phase technique on a peptide synthesizer (Applied Biosystems, Foster City, CA, USA). Mice were injected subcutaneously at one site in the flank with a 200 μl emulsion containing 75 μg MOG peptide (300 μg in the case of C57/bl) in complete Freund's adjuvant (CFA) and 200 μg Mycobacterium tuberculosis (Sigma, Israel). An identical booster was injected at one site in the other flank 1 week later.

2.3. Neurological evaluation of EAE

Following the encephalitogenic challenge, mice were observed daily and clinical manifestations of EAE were scored as follows: 0, no clinical symptoms; 1, loss of tail tonicity; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, paralysis of four limbs; 5, total paralysis; 6, death [36].

2.4. Treatment

Riluzole (Sigma, Israel) was stored at room temperature until use and was prepared fresh each day prior to injection. Riluzole was dissolved in DMSO and diluted in saline to the desired concentration.

2.5. Histopathology

Spinal cords from riluzole-treated and MOG mice were dissected 28 days after immunization with pMOG 35–55, fixed in 10% buffered formalin and embedded in paraffin. Five-micron-thick sections were stained with Hematoxylin and Eosin (H&E) to assess inflammation, and Luxol Fast Blue (LFB) to assess myelin loss [29]. Bielshowesky's silver impregnation was used to evaluate axonal integrity [30].

2.6. Immunohistochemistry

Paraffin sections were exposed to SMI 32 antibodies against mouse non-phosphorylated neurofilament H (Sternberger Monoclonals, Baltimore, USA) to assess the amount of axonal loss. Briefly, sections were deparaffinized, blocked with 3% normal goat serum, and incubated with SMI-32 at a dilution of 1:1000 at room temperature for 30 min. They were then washed and incubated with goat anti-mouse IgG at room temperature for 30 min and then washed and incubated with ColonoPAP for 30 min. Neurofilaments were visualized with DAB [48].

2.7. Microscopes

All slides for histopathology and immunocytochemistry were analyzed under an Olympus BX52TF microscope (Olympus, Tokyo, Japan). A DP50-CU microscope digital camera system (Olympus) was used to photograph all pictures. ViewfinderLite™ software (Olympus), with the digital camera attached to the microscopes, was used to acquire images. StudioLite™ software (Olympus) was used to edit and analyze the images recorded.

2.8. Image analysis technique

Image Pro Plus software (MediaCybernetics, USA) was used in order to quantify areas stained by Bielshowesky's silver staining, H&E and by immunohistochemistry (for anti-SMI-32). Spinal cords of five animals taken from each experimental group were used for the analysis. The spinal cord sections were photographed in a series of six frames (a total of 30 pictures for each group), and were assessed by two examiners in a double blind assessment.

2.9. T-cell proliferation assay

The proliferation response of spleen cells was tested 28 days after EAE induction. Three animals from each group were sacrificed by cervical dislocation, the spleens removed and placed in RPMI 1640 medium supplemented with 2 mM glutamine, 5×10^{-5} M 2-ME, antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin) and 10% heat-inactivated fetal calf serum (all from Beit Haemek, Israel). Splenocytes were then plated at a concentration of 3×10^{5} cells/well. Cultures were maintained in microtiter plates as previously described [6]. The relevant peptide was added (2, 10 and 25 μg/well) to triplicate wells. They were then incubated for 72 h at 37 °C in humidified air containing 5% CO_2. [^{3}H]-Thymidine (1 μCi/well) was added for the last 16 h of incubation and the cultures were then harvested and counted using a Matrix 96 Direct beta counter (Packard Instruments, Meridien, CT, USA). The proliferative response was measured using [^{3}H]-thymidine incorporation expressed as mean counts per minute (CPM) of triplicate wells.

2.10. Statistical analysis

The statistical significance of differences in the clinical severity of EAE following pMOG 35–55 induction between the MOG- and riluzole-treated mice groups was evaluated using Student's *t*-test. The statistical significance of differences in disease-free animals was evaluated using the χ^2 test.

3. Results

In five separate experiments, we tested the ability of riluzole (10 mg/kg, twice a day, i.p.) to suppress EAE (see Table 1). In all experiments, riluzole, when it was administered together with the first injection of MOG, was shown to be beneficial in reducing clinical symptoms (Table 1, experiments I, II and III). In experiment IV, riluzole (10 mg/kg, i.p., twice a day) was given 14 days after the first MOG injection, at the appearance of symptoms. The mean score of the riluzole-treated mice ($n=10$), as measured 30 days after MOG injection, was much lower than for the MOG-treated mice (1.18 ± 0.47 vs. 2.31 ± 0.23, $P<0.03$).

We further examined whether continuous riluzole administration is needed to prevent the development of disease. Therefore, we divided 40 mice into four groups, 10 mice each. The first group (CC) was treated with saline; the second group (RC) was treated with riluzole from day 12 (when the first clinical symptoms appeared) to 18; the third group (CR) was treated with riluzole from day 18 to 27; group 4 (RR) was treated with riluzole from day 12 to 27. We found that, after 18 days, all the mice (20/20) in

Table 1
Riluzole suppresses EAE in C3H.SW and C57/bl mice

	Disease-free mice	Mortality incidence	Mean EAE score[a] (*P*-value)[b] at endpoint	Mouse strain
Experiment I				C3H.SW
Control	6/10	0/10	1.3±0.58	
Riluzole (10 mg/kg×2/d, i.p.)	9/10	0/10	0.1±0.4 (0.01)	
Experiment II				C3H.SW
Control	0/10	6/10	4.5±0.63	
Riluzole (10 mg/kg×2/d, i.p.)	0/10	1/10	2.85±0.38 (0.03)	
Experiment III				C57/bl
Control	2/10	0/10	1.35±0.3	
Riluzole (10 mg/kg×2/d, i.p.)	10/10	0/10	0±0 (0.0006)	
Experiment IV				C3H.SW
Control	1/11	0/11	2.31±0.23	
Riluzole (10 mg/kg×2/d, i.p.)	3/8	0/8	1.18±0.47 (0.03)	
Experiment V				C3H.SW
Control (CC)	0/10	2/10	3.25±0.48	
Riluzole (RR)	4/10	0/10	0.9±0.26 (0.0005)[c]	
Control–Riluzole (CR)	1/10	0/10	1.7±0.22 (0.006)[c]	
Riluzole–Control (RC)	0/10	1/10	2.6±0.47 (0.32)[c]	

[a] Calculated from the mean of the peak clinical score for each group of animals.
[b] *P*-values were calculated using the two-tailed Student *t*-test; $P<0.05$ was defined as significant.
[c] Compared to control (represents mice that received MOG only).

the groups that were treated with saline (CC and CR) developed severe EAE characterized by complete hind limb paralysis (mean total clinical score 2.85±0.21). In contrast, mice treated with riluzole from day 12 (the RR and RC groups) showed a much lower mean clinical score (1.8±0.34, $P=0.012$), and only 14 out of 20 were affected ($P=0.001$ using χ^2 values). Twenty-seven days after MOG injection, there were marked differences between the control (CC) and the long-term riluzole (RR) mice groups; eight of the 10 control mice developed severe EAE characterized by complete hind limb paralysis (mean total score 3.25±0.48). In contrast, four of the 10 riluzole-treated (RR) mice remained resistant to MOG-induced EAE and remained disease free ($P=0.001$ using χ^2 values), and the others (6/10) exhibited a milder form of illness (mean total score 0.9±0.26, $P=0.0001$) (Fig. 1, Table 1, experiment V).

Long-term riluzole treatment (day 12 to 27) demonstrated greater protection than the day 12–18 group (RR vs. RC, mean total score 0.9±0.26 vs. 2.6±0.47, $P=0.02$). In addition, short-term riluzole treatment started in the late onset of disease, from day 18, was also effective and markedly reduced the clinical score compared to saline-treated mice (CR vs. CC, mean total score 1.7±0.22 vs. 3.25±0.48, $P=0.007$) (Fig. 1, see also Table 1, experiment V).

Thus, riluzole markedly reduced both the incidence and clinical severity of the disease when administered not only at the onset of the disease, but even after the clinical symptoms were already fully established. However, riluzole achieves the best protection by its continuous administration.

To further evaluate the effects of riluzole treatment, spinal cords were examined by light microscopy for inflammation and demyelination. Representative H&E staining of spinal cords from five immunized MOG mice, 28 days after immunization, revealed marked multifocal lymphohistiocytic inflammation in the white matter which was both perivascular and diffuse (Fig. 2A). In contrast, considerably reduced inflammation was observed in sections taken from five riluzole-treated mice with focal perivascular lymphohistiocytic inflammation (Fig. 2B). To quantify the lymphocyte count in spinal cords, we used Image Pro Plus analysis. We found a marked increase in the number of lymphocytes in the spinal cord of MOG-treated mice compared to naive mice as indicated by arbitrary units representing the pixel area (0.072±0.01 vs. 0.005±0.001, $P<0.02$), while their number was dramatically reduced in riluzole-treated mice as compared to the MOG-treated group (0.036±0.007 vs. 0.072±0.01, $P<0.01$, Fig. 3A). Inflammation in the MOG-treated mice was associated with myelin loss, as indicated by LFB staining (Fig. 2C). In contrast, one out of five of the riluzole-treated mice exhibited a normal myelin pattern, while the other four showed only mild myelin loss (Fig. 2D). Axonal damage is another important feature of MS lesions and has

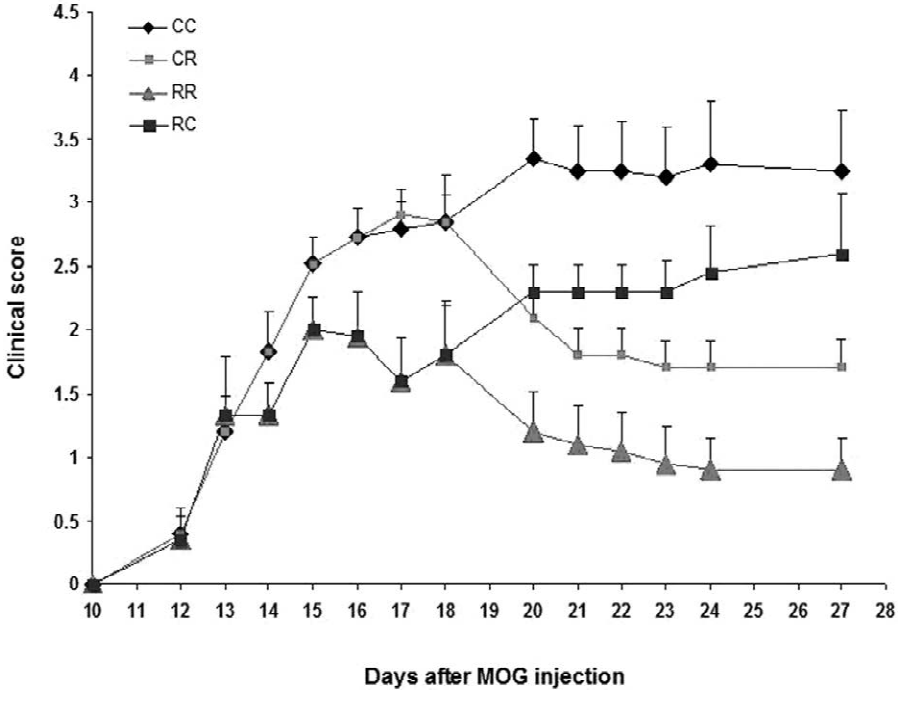

Fig. 1. Induction of EAE in C3H.SW mice with pMOG 35–55. All mice (40) were injected with pMOG 35–55 in CFA supplemented with *Mycobacterium tuberculosis* and divided into four groups of 10 mice each. The first group (CC) was treated with saline; the second group (RC) was treated with riluzole from day 12 to 18; the third group (CR) was treated with riluzole from day 18 to 27; group 4 (RR) was treated with riluzole from day 12 to 27. The mean daily clinical score±S.D. is shown for each group of mice (0, no clinical symptoms; 1, loss of tail tonicity; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, paralysis of four limbs; 5, total paralysis; 6, death).

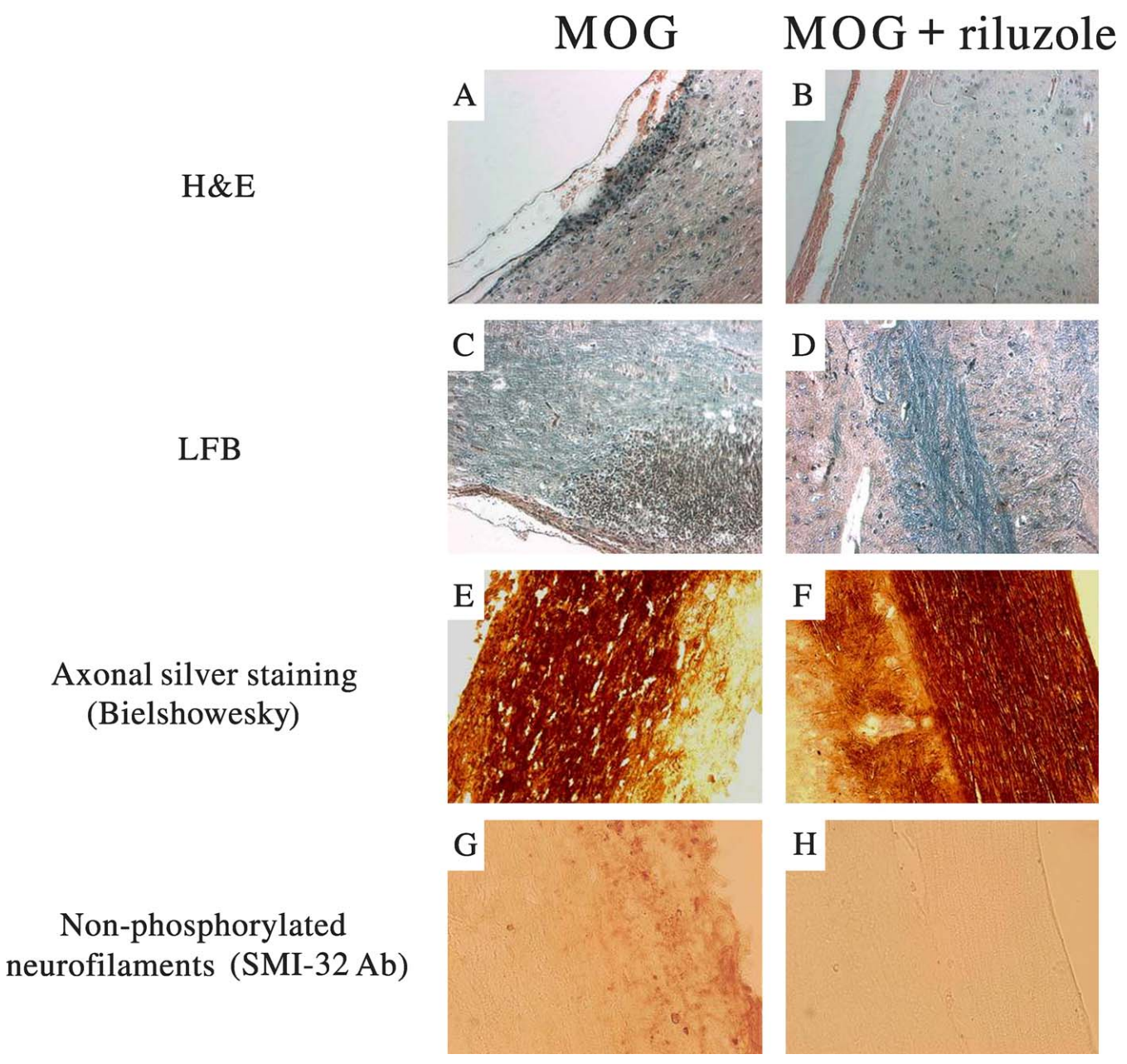

Fig. 2. Spinal cord longitudinal sections from MOG-induced EAE mice treated with riluzole were stained with Hematoxylin and Eosin (H&E) for lymphocyte infiltration (A, B), Luxol Fast Blue (LFB) for myelin (C, D) and Bielshowesky's silver staining for axons (E, F). Abnormally dephosphorylated neurofilament H was visualized using SMI-32 antibodies (G, H). Original magnification (A–H): ×100.

been shown to increase with lesion activity [29]. Axonal staining of spinal cord sections from MOG-treated mice showed severe axonal damage, using Bielshowesky's method (Fig. 2E), whereas those from the riluzole-treated mice demonstrated minimal axonal impairment occurring only in regions surrounding foci of inflammation (Fig. 2F). When we analyzed the intensity of staining using Image Pro Plus analysis we found marked axonal loss in the spinal cords of MOG-treated mice compared to naive mice (0.44 ± 0.03 vs. 0.65 ± 0.03, $P<0.0001$), while in the riluzole-treated mice, axonal integrity was preserved compared to the MOG-treated group (0.54 ± 0.02 vs. 0.44 ± 0.03, $P<0.02$, Fig. 3B). We also used antibodies against non-phosphorylated neurofilament H (SMI-32), a recently developed immunohistochemical marker of axonal damage in MS [48,52]. Based on such testing, the spinal cords of MOG-treated mice showed an increase of abnormal dephosphorylated NF-H (Fig. 2G), while minimal abnormal dephosphorylation was observed in the riluzole-treated mice (Fig. 2H). When we quantified axonal damage in spinal cords using Image Pro Plus analysis, we found massive axonal damage in the MOG-treated mice compared to naive mice (0.03 ± 0.003 vs. 0.0005 ± 0.0002, $P<0.0001$), while in the riluzole-treated mice there was a

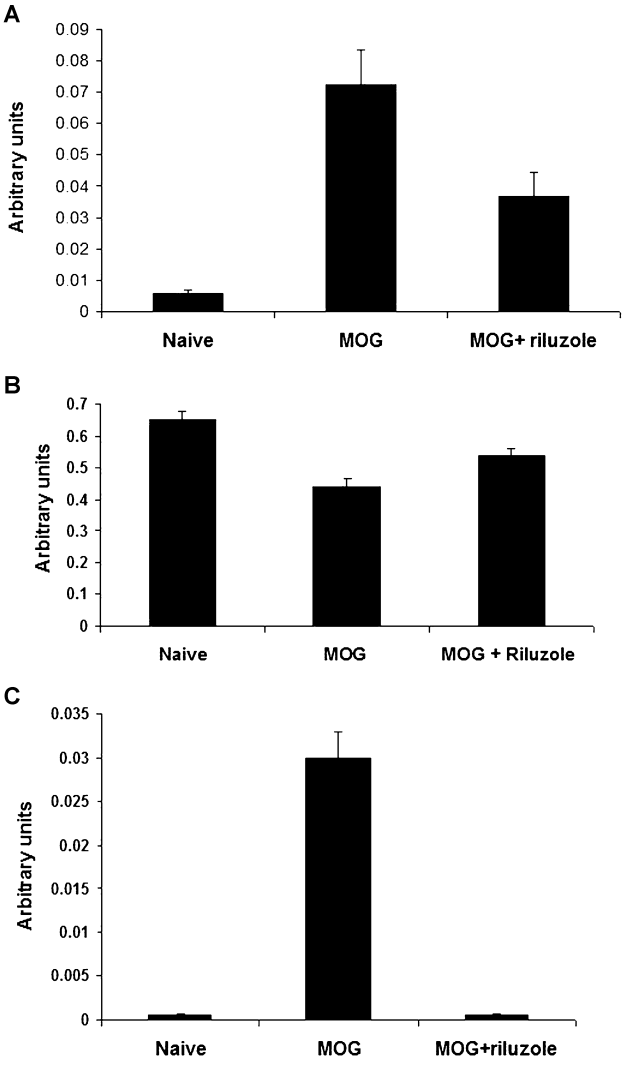

Fig. 3. Quantitative assessment of lymphocyte infiltration (A), axonal damage, as assessed by Bielshowesky's silver staining (B), and abnormally dephosphorylated neurofilament H (SMI-32 antibodies, C) was processed by Image Pro Plus analysis. Results are shown in arbitrary units, representing the pixel area.

marked reduction in the area stained by SMI-32 as compared to the MOG-treated group (0.0006 ± 0.0001 vs. 0.03 ± 0.003, $P < 0.0001$, Fig. 3C).

We compared the T-cell response in the riluzole- and MOG-treated mice groups to rule out the possibility that the observed differences in clinical and histological manifestations of the disease were due to a generalized immune effect of the drug. Riluzole-treated and MOG-treated mice (three in each group) were immunized with pMOG 35–55 and their recall T-cell proliferative response against the antigen was assessed. As can be seen in Fig. 4, the in vitro primary proliferative response of both MOG and riluzole-treated mice against pMOG 35–55 was similar. This suggests that riluzole treatment did not affect the proliferative capacity of auto-reactive T-cells.

4. Discussion

Our study shows that riluzole-treated C3H.SW mice are highly resistant to MOG-induced chronic EAE. The incidence of disease development and its clinical severity were markedly reduced in the riluzole-treated mice. Furthermore, improvement in motor function was demonstrated not only when riluzole was given immediately after the appearance of the first symptoms, but even when it was initiated a week later, when the mice were already severely affected. Histological examination of spinal cord tissues demonstrated reduced levels of inflammation, demyelination, and axonal damage in the riluzole-treated mice. Furthermore, while immunohistochemical examination with SMI-32 antibodies demonstrated axonal disruption, indicating a large increase of abnormally dephosphorylated NF-H in MOG-immunized spinal cords, in the riluzole-treated mice there was minimal abnormal dephosphorylation, suggesting that riluzole was effectively protective against axonal damage. The in-vitro response of T-cells specific for pMOG 35–55 was similar in the riluzole-treated and MOG-immunized mice, suggesting that the

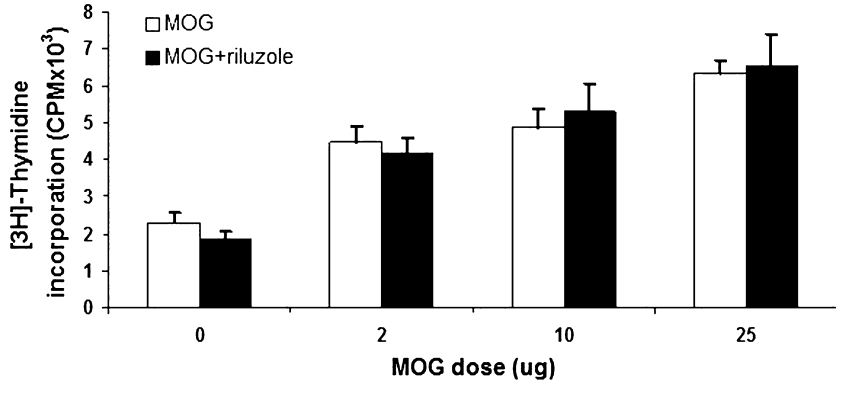

Fig. 4. Proliferation assay in the presence of pMOG 35–55. Splenocytes were isolated from mice 28 days following pMOG 35–55 immunization. MOG and riluzole-treated mice (three mice each) were examined for their responses to different concentrations (2, 10 and 25 μg) of pMOG 35–55. Each histogram represents the mean CPM$\times 10^3 \pm$S.E.M. of triplicate cultures.

beneficial effect of riluzole is probably not mediated via interference with immunological mechanisms.

Glutamate receptor-mediated toxicity has been observed in an oligodendroglial cell line [28] and in cultures of differentiated oligodendrocytes [26,42] and in an animal model for MS [44]. This toxicity was triggered by over-activation of AMPA and kainate receptors and caused by an increase in Ca^{2+} influx subsequent to receptor activation [34,40,54]. Hardin et al. [21] showed that expression of glutamine synthetase and glutamate dehydrogenase (the enzymes that are responsible for glutamate degradation) was dramatically reduced in astrocytes during the course of EAE, affecting the reuptake of glutamate. Moreover, Matute et al. [33] demonstrated that microinfusions of kainate into the optic tract in rodents can cause demyelination. In previous studies the neurological symptoms in several forms of EAE were ameliorated by AMPA and kainate receptor antagonists [38,43]. However, their main problem is that they have toxic effects, and therefore are not approved for clinical use.

Riluzole is a neuroprotective agent with several pharmacological properties. Previous studies have shown that it works by suppressing glutamatergic neurotransmission in the CNS achieved by inhibiting the release of glutamate from nerve terminals [11,24,32]. It has also been demonstrated that glutamatergic transmission, pre- and post-synaptically, is altered by riluzole [12,23], although radioligand binding studies have not demonstrated that riluzole interacts directly with glutamate [4,14,28].

It is believed that this effect may be partly due to the inactivation of voltage-gated sodium channels [7,49,50,55] and activation of a G-protein-dependent signal transduction processes [16]. Moreover, experiments on currents carried by cloned rat brain sodium channel alpha subunits expressed in Xenopus oocytes have shown that riluzole binds selectively to the alpha subunit of the sodium channel, suggesting that stabilization of the inactivated state may underlie the neuroprotective properties of riluzole [22]. It has also been shown to stimulate the large-conductance calcium-activated potassium channels in rat GH3 cells [53] and to block the effects of NMDA and kainate in the mouse [15] and rat striatum [25]. Moreover, previous work showed that riluzole is able to interact with ionotropic glutamate receptors expressed in Xenopus oocytes [14].

In addition, riluzole was shown to exert neuroprotective properties in several experimental models associated with excitotoxic injury, such as cerebral ischemia [39,42,51], Parkinson's disease [1,3,5,9,10] and ALS [19,20].

The exact mechanism by which riluzole exhibits its observed beneficial effect in EAE is still unknown. However, based on several previous studies showing that the CSF glutamate levels are increased in MS patients [2] and that glutamate receptor antagonists ameliorate neurological symptoms in several forms of EAE [38,43], it is tempting to suggest that it works by the suppression of glutamatergic neurotransmission in the CNS, achieved by inhibiting the release of glutamate from nerve terminals [11,24,32]. This inhibition might prevent the glutamate-induced toxicity of oligodendrocytes seen in the spinal cord of EAE mice [38,43]. Moreover, we found far fewer T-cells in the spinal cord of riluzole-treated mice. This observation might be explained by the reduction of inflammation, and, as a result, the decrease in recruitment of T-cells entering the brain. A recent study found high expression of the glutamate ion channel receptor (GluR3) on normal human T-cells, human T leukemia cells, and mouse anti-myelin basic protein T-cells [18]. It was also found that glutamate itself, or its agonists, triggered T-cell functions: integrin-mediated T-cell adhesion to laminin and fibronectin, and that glutamate antagonist blocked this effect [18]. Thus, the ability of glutamate to directly activate T-cell function might explain the beneficial effect of riluzole in our experimental model by inhibiting T-cell transmigration to the CNS.

Although there has been progress in the treatment of MS, therapeutic options are still limited and only partly effective [13]. Corticosteroids are usually given during acute exacerbation with variable success [37]. Chronic administration of various interferon-beta preparations and glatiramer acetate (Copaxone) can reduce the number of relapses, but their long-term effect on the neurological outcome and particularly progressive disability has not yet been established [37]. Clearly, more treatment alternatives are urgently needed. Our study shows, for the first time, that riluzole reduces inflammation, demyelination and axonal damage and attenuates the severity of chronic manifestations in EAE. Indeed, in a small non-controlled pilot study, Kalkers et al. have shown that riluzole reduced the rate of cervical cord atrophy and the development of hypointense T1 brain lesions on magnetic resonance imaging of 13 patients with primary progressive MS [25]. These findings stress the possible importance of neuronal excitotoxicity in the pathogenesis of EAE and, by inference, human MS. Therefore, riluzole might prove valuable in the future treatment of MS both during acute relapse and in preventing disease progression.

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