

Riluzole effects on behavioral sensitivity and the development of axonal damage and spinal modifications that occur after painful nerve root compression

Laboratory investigation

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Object. Cervical radiculopathy is often attributed to cervical nerve root injury, which induces extensive degeneration and reduced axonal flow in primary afferents. Riluzole inhibits neuro-excitotoxicity in animal models of neural injury. The authors undertook this study to evaluate the antinociceptive and neuroprotective properties of riluzole in a rat model of painful nerve root compression.

Methods. A single dose of riluzole (3 mg/kg) was administered intraperitoneally at Day 1 after a painful nerve root injury. Mechanical allodynia and thermal hyperalgesia were evaluated for 7 days after injury. At Day 7, the spinal cord at the C-7 level and the adjacent nerve roots were harvested from a subgroup of rats for immunohistochemical evaluation. Nerve roots were labeled for NF200, CGRP, and IB4 to assess the morphology of myelinated, peptidergic, and nonpeptidergic axons, respectively. Spinal cord sections were labeled for the neuropeptide CGRP and the glutamate transporter GLT-1 to evaluate their expression in the dorsal horn. In a separate group of rats, electrophysiological recordings were made in the dorsal horn. Evoked action potentials were identified by recording extracellular potentials while applying mechanical stimuli to the forepaw.

Results. Even though riluzole was administered after the onset of behavioral sensitivity at Day 1, its administration resulted in immediate resolution of mechanical allodynia and thermal hyperalgesia ($p < 0.045$), and these effects were maintained for the study duration. At Day 7, axons labeled for NF200, CGRP, and IB4 in the compressed roots of animals that received riluzole treatment exhibited fewer axonal swellings than those from untreated animals. Riluzole also mitigated changes in the spinal distribution of CGRP and GLT-1 expression that is induced by a painful root compression, returning the spinal expression of both to sham levels. Riluzole also reduced neuronal excitability in the dorsal horn that normally develops by Day 7. The frequency of neuronal firing significantly increased ($p < 0.045$) after painful root compression, but riluzole treatment maintained neuronal firing at sham levels.

Conclusions. These findings suggest that early administration of riluzole is sufficient to mitigate nerve root-mediated pain by preventing development of neuronal dysfunction in the nerve root and the spinal cord.

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KEY WORDS • nerve root • neuronal hyperexcitability • injury •
riluzole • radiculopathy • GLT-1 • spinal cord injury • pain

INJURY to the cervical nerve root is a common source of radicular pain and can result from disc herniation, spinal stenosis, or neck trauma.^{2,10,18,30,43} In animal models of radiculopathy from nerve root compression, axonal degeneration develops in the root and extends toward the synapses in the dorsal horn, where axon terminals become enlarged and neurotransmitter levels are altered.^{16,32-34,41,52,60} Although these spinal modifications after painful root

compression suggest that injury may mediate afferent signaling, such effects have not been evaluated after a painful nerve root compression. Furthermore, the presence of degeneration of the primary afferents at their spinal synapses suggests that downregulation of the astrocytic glutamate transporter GLT-1 may contribute to the spinal neuronal excitability that also develops.^{35,77}

Neuronal hyperexcitability is associated with neural tissue damage, including axonal injury—a hallmark of nerve root compression injuries—and altered phenotypic

Abbreviations used in this paper: CGRP = calcitonin gene-related peptide; HSD = honestly significant difference; IB4 = isolectin-B4; LTM = low-threshold mechanoreceptive; NF200 = neurofilament-200; ROI = region of interest; WDR = wide dynamic range.

This article contains some figures that are displayed in color online but in black-and-white in the print edition.

behavior in the spinal cord.^{33,36,41,51} Sensitization of wide dynamic range (WDR) neurons, in particular, is thought to drive neuronal hyperexcitability and behavioral sensitivity after spinal cord ischemia and spinal nerve ligation.^{28,48} Although increased calcitonin gene-related peptide (CGRP) and associated signaling contribute to neuronal hyperexcitability after painful neural trauma,^{7,51} painful root compression decreases CGRP in the superficial dorsal horn.^{32,40} No study has evaluated CGRP expression in the deep laminae, despite the known involvement of neurons in that region of the dorsal horn in pain from peripheral nerve injuries.^{13,39}

Riluzole is an anticonvulsant that has neuroprotective properties and has been used clinically for a number of neurodegenerative diseases, most recently for spinal cord injury.²⁴ Riluzole has also been used effectively to reduce symptoms in animal models of neurodegenerative disease and neural tissue injury, which is attributed to its inhibition of presynaptic glutamate release by blocking voltage-gated sodium channels.^{5,15,21,36,67} Riluzole has been shown to decrease the size of spinal cord lesions; it promotes motor function recovery and restores the electrophysiological properties of spinal neurons after spinal cord injury.^{62,68,76} It also has been shown to mitigate axonal degeneration and promote axonal regeneration after nerve injury.^{17,54,73} These results suggest that riluzole may inhibit the axonal degeneration that is induced after a painful root compression.^{32,52} Further, although regeneration of dorsal roots after injury restores normal sensation⁵⁷ and riluzole is anti-allodynic for neuropathic pain,^{15,69} it is not known if, and to what extent, riluzole may preserve the normal axonal structure and function and alleviate that pain after compression of the nerve root. It is also unclear whether a single dose of riluzole is sufficient to provide sustained pain relief when administered after pain has developed.²⁶

This study investigates whether a single dose of riluzole can eliminate behavioral sensitivity and axonal damage in the injured root as well as prevent the spinal changes that develop after root compression in a rat pain model. We hypothesized that riluzole would abolish behavioral sensitivity by preserving axonal morphology and would abate spinal modifications of CGRP and neuronal excitability that are observed after painful root compression. We further hypothesized that riluzole can preserve the expression of the spinal glutamate transporter GLT-1, which is known to be reduced in painful neuropathies but is expected to be restored by preserving axonal health via riluzole treatment. Mechanical allodynia and thermal hyperalgesia were assessed after injury to evaluate pain symptoms, with and without riluzole treatment, or sham surgery. Axonal morphology in the root and spinal CGRP were evaluated at Day 7 using immunohistochemistry in one group of rats (Table 1). At that same time point in a separate group of rats (Table 1), electrophysiological recordings were made in the spinal cord to evaluate the effect of riluzole on neuronal firing after a painful root compression.

Methods

All studies used male Holtzman rats (300–400 g, Harlan Sprague-Dawley). Rats were housed in a 12-/12-

hour light-dark cycle and given free access to food and water. Studies were approved by our Institutional Animal Care and Use Committee and carried out under the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.⁷⁸

The C-7 dorsal nerve root was compressed under isoflurane inhalation anesthesia (4% for induction, 2% for maintenance). The rat was placed in a prone position and an incision was made along the midline over the cervical spine from the base of the skull to the T-2 spinous process.^{34,52,59,60} The C-6 and C-7 vertebrae were exposed by carefully separating the overlying musculature, and a right hemilaminectomy and partial facetectomy were performed at C6–7 to expose the right C-7 nerve root. A small incision was made in the dura mater over the C-7 nerve root, and a 10-gf microvascular clip (World Precision Instruments) was placed on the dorsal root for 15 minutes and then removed. A surgical control group received sham procedures, which included all of the same procedures as the surgical manipulation and nerve root exposures, but without any compression applied. Wounds were closed using 3-0 polyester sutures and surgical staples, and the rats were monitored while they recovered in room air.

On Day 1 after compression, rats were randomly assigned to receive either riluzole or its vehicle carrier (Table 1). The treatment group (inj+ril) received a 1-ml intraperitoneal injection of 3 mg/kg riluzole (Sigma-Aldrich) dissolved in 10% β -cyclodextrin (vehicle, Sigma-Aldrich). In the group receiving vehicle treatment (inj+veh), a 1-ml injection of 10% β -cyclodextrin dissolved in saline was administered. The same vehicle treatment was also given on Day 1 to the rats that had undergone sham surgery (sham+veh). All injections were administered after the behavioral assessments were performed on Day 1.

Mechanical allodynia and thermal hyperalgesia were assessed in the ipsilateral forepaw as measures of behavioral sensitivity. Mechanical allodynia was measured prior to injury (at baseline) and on postinjury Days 1, 2, 3, 5, and 7.^{31,32,59,60} After a 20-minute period of acclimation, a 4-gf von Frey filament (Stoelting Co.) was applied to the plantar surface of the forepaw 10 times, in 3 rounds, with 10 minutes of rest between rounds. The total number of paw withdrawals for each rat was summed for the 3 rounds on each day and averaged across groups. Thermal hyperalgesia was measured at baseline and on Days 1 and 7 using established methods.^{20,29} After a 20-minute acclimation period, the thermal stimulus was focused on the plantar surface of the forepaw using a radiant heat source until a withdrawal response was provoked. If the withdrawal was a sudden and quick movement and/or the rat licked, shook, curled or looked at the paw, the response was considered positive. The time period during which the thermal stimulus was applied to the forepaw until observation of a positive response was recorded as the withdrawal latency. On each testing day, the withdrawal latency time was measured 3 times, with 10 minutes of rest between assessments. The average latency across the rounds for each day was recorded for each rat and averaged for each group. For each behavioral assessment, a 2-way repeated measures ANOVA tested for differences between groups over time. To determine differences between groups at each day, separate

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TABLE 1: Study design

Characteristic	Description
treatment groups	injury+riluzole, injury+vehicle, sham+vehicle
measurement type	immunohistochemistry (n = 7 per treatment group); spinal electrophysiology (n = 7 per treatment group)
Day 7 outcome	C-7 spinal cord (CGRP, GLT-1), C-7 nerve root (NF200, IB4, CGRP), evoked action potentials, neuron phenotype

1-way ANOVAs with post hoc Bonferroni correction were performed, with significance for all tests at $p < 0.05$.

The dorsal nerve root and spinal cord at C-7 were harvested from 7 animals in each group on Day 7 after behavioral testing to assess axonal morphology in the nerve root and spinal expression of CGRP and GLT-1 (Table 1). Rats were anesthetized with an intraperitoneal injection of 65 mg/kg pentobarbital and transcardially perfused with 200 ml of Dulbecco's phosphate-buffered saline followed by 300 ml of 4% paraformaldehyde. The C-7 cervical spinal cord and adjacent nerve roots were harvested, postfixed overnight, transferred to 30% sucrose for cryoprotection and then embedded in optimal cutting temperature (OCT) medium (Sakura Finetek USA, Inc.). Samples were cryosectioned at 14 μm , such that the spinal cord tissue was sectioned axially and the adjacent nerve roots were sectioned along their longitudinal axis and thaw-mounted onto slides. Each slide contained 6 nonadjacent sections spanning a region at the centerline of the nerve root. For comparison, matched tissue samples also were harvested from 2 normal, naïve rats and included in tissue processing.

Sections were co-labeled for neurofilament-200 (NF200), CGRP, and isolectin-B4 (IB4) to label myelinated, peptidergic, and nonpeptidergic neurons, respectively. Sections were blocked in 10% normal goat serum (Vector Laboratories) with 0.3% Triton-X100 (Bio-Rad Laboratories) then incubated overnight at 4°C in mouse anti-NF200 (1:500, Sigma-Aldrich), rabbit anti-CGRP (1:1000, Peninsula Laboratories), and biotinylated IB4 (5 $\mu\text{g}/\text{ml}$, Sigma-Aldrich). Sections were then fluorescently labeled with secondary antibodies for goat anti-mouse Marina Blue (1:200, Invitrogen), goat anti-rabbit Alexa Fluor 546 (1:1000, Invitrogen), and fluorescein (DTAF)-conjugated streptavidin (1:500, Jackson ImmunoResearch, Inc.), respectively. Separately, spinal sections were labeled for GLT-1 by blocking in 5% normal goat serum with 0.3% Triton-X100. Sections were then incubated overnight at 4°C in rabbit anti-GLT-1 (1:1000, Abcam) and fluorescently labeled with secondary antibody for goat anti-rabbit Alexa Fluor 488 (1:1000, Invitrogen).

Axonal morphology in the affected nerve root was evaluated by digitally imaging NF200, CGRP, and IB4 in the root at 200 \times (3–6 sections per sample). Two independent reviewers who were blinded to the sample groups assessed the extent of axonal abnormalities for each of the types of labeled axons, separately, using customary methods.^{33,52,63,66} Nerve roots that did not differ from normal uncompressed roots were assigned a negative score (–), indicating the absence of axonal pathology. Nerve roots that contained any evidence of axonal swelling or dis-

continuous labeling were assigned a positive score (+). If the abnormalities extended across the entire length of the root, that section was assigned a positive score of “++,” indicating extensive damage. For each rat, the ratings were averaged across the tissue sections, and between both reviewers, such that each nerve root was assigned a single score on a 5-point scale: –, –/+, +, +/++, or ++.

Axonal transport to the spinal cord was evaluated by quantifying CGRP labeling in uniformly sized regions of interest (ROIs) in the superficial and deep laminae of the dorsal horn. In addition, expression of GLT-1 was quantified in the superficial laminae of the dorsal horn as well. The dorsal horn ipsilateral to the injured side was imaged at 200 \times (ROI 1360 \times 1024 pixels) and images were separately cropped over the superficial laminae (I–II, ROI 750 \times 150 pixels) and deeper laminae (IV–V, ROI 696 \times 380 pixels). GLT-1 expression and CGRP expression were each measured in at least 3 sections from each rat using quantitative densitometry and were reported as a percent of the expression of each protein in normal tissue.^{1,34,58,60} Differences in the expression of GLT-1 and CGRP between groups in the superficial and deep laminae were tested by separate mixed-effect ANOVAs with sections nested by rat and rats nested within groups. Differences between the groups were determined by post hoc Tukey honestly significant difference (HSD) tests.

In a separate group of rats, neuronal hyperexcitability was measured in the deep laminae of the ipsilateral spinal cord at Day 7 (Table 1). Rats underwent a C-7 nerve root compression or sham procedure and were assigned to the same treatment paradigms as described above (7 rats per group): inj+ril, inj+veh, or sham+veh. Mechanical allodynia was measured in the ipsilateral forepaw before injury (baseline) and on Day 7, using stimulation by 1.4-, 4.0-, and 10.0-gf von Frey filaments, as described above. A 1-way ANOVA with post hoc Bonferroni correction was used to test for differences in response between groups for each filament, separately. A t-test was used to compare the number of paw withdrawals elicited by the 4.0-gf filament between each group used for the electrophysiological study and the matched group in the immunohistochemistry study to test that the studies used comparable conditions.

Following behavioral testing on Day 7, rats were anesthetized with 45 mg/kg pentobarbital via intraperitoneal injection. Adequate anesthesia was confirmed by a hind paw pinch and was maintained with an additional dose of pentobarbital (1–5 mg/kg) administered intraperitoneally approximately every 40–50 minutes, or as needed. The cervical spine was re-exposed via a dorsal, midline incision and any scar tissue that formed over the right

C6–7 spinal cord from the initial surgery was carefully removed. A laminectomy removed any remaining bone at C-6 and C-7 on the left side to fully expose the spinal cord at those levels, and the dura was then removed. The rat was placed on a stereotactic frame using bilateral ear bars and a clamp on the spinous process of T-2. Mineral oil was applied to the spinal cord to maintain hydration. A thoracotomy was performed to minimize spinal cord motion associated with normal breathing and respiration was maintained by mechanical ventilation via a midcervical tracheotomy (40–50 cycles/minute, Harvard Small Animal Ventilator Model 683, Harvard Apparatus).¹⁹ The expired CO₂ concentration was continuously monitored (Capnogard, Novamatrix Medical Systems), and the core body temperature was maintained between 35° and 37°C using a heat plate and a rectal probe (TCAT-2DF, Physi-temp Instruments Inc.).

Extracellular spinal cord recordings were acquired using a glass-insulated tungsten probe (< 1 μm tip; FHC) inserted vertically into the dorsal spinal cord on the side ipsilateral to the affected nerve root, proximal to the site where the C-7 nerve root exits the spinal cord. The signal was amplified with a gain of 3000 (ExAmp-20KB, Kation Scientific, Inc.), processed with a 60-Hz noise eliminator (Hum Bug, Quest Scientific), and digitally stored at 25 kHz (MK1401, CED). Mechanoreceptive neurons innervating the forepaw were searched for by lightly brushing the plantar surface of the forepaw and slowly advancing the probe through the deep laminae (400–1000 μm below the pial surface) until a neuron responsive to the light brushing was found.^{19,25,56} Once a neuron was identified, a sequence of 6 mechanical stimuli was applied to the forepaw: 1) 10 light brush strokes with a brush applied over 10 seconds; 2–5) a series of 4 von Frey filaments (1.4, 4.0, 10.0, 26.0 gf), each applied 5 times for 1 second with a 1-second rest between applications; and 6) a 10-second, 60-gf pinch by a microvascular clip (Roboz, Inc.).⁵⁶ There was a 60-second rest between applications of each of the different stimuli.

Voltage recordings were spike-sorted in Spike2 (CED) to count the number of action potentials evoked by each stimulus for individual neurons. For the brush stimulus, the number of action potentials was summed over the period of light brushing. For each von Frey filament application, the number of action potentials was summed over both the stimulation period and the rest period that immediately followed. For both the brush and the von Frey filament stimuli, the baseline number of spikes occurring in the 10-second period prior to the first stimulation was subtracted from the spike counts to identify only the spikes evoked by those stimuli.²⁵ For the 60-gf pinch, the number of spikes was summed over the 5-second period between 3 and 8 seconds after the clip was applied in order to consider only those spikes evoked by the pinch and to exclude the spikes evoked by the application and removal of the clip. The number of spikes evoked by the clip stimulus was determined by subtracting the baseline number of spikes that occurred in the 5-second window prior to the first stimulation from the spike count. For statistical analysis, the spike count was log-transformed because of a positive skew in the data distribution.⁵⁶ Separate mixed-effect 1-way ANOVAs with Tukey HSD

post hoc tests were used to compare the differences in the number of action potentials that were evoked by each filament between groups; neurons were nested within rats and rats were nested in groups. A mixed-effect 1-way ANOVA with the same levels of nesting was used to test for differences between groups for the depth at which the neurons were recorded.

Neurons were classified as either wide dynamic range (WDR) or low-threshold mechanoreceptive (LTM) neurons by comparing the spike rate (spikes/second) evoked by the light brushing and the 60-gf clip stimuli.^{25,45,61} Neurons that responded maximally to the light brush were identified as LTM and those that responded in a graded manner were identified as WDR.^{25,75} The distribution of WDR and LTM neurons between groups was compared using Pearson chi-square tests. All electrophysiology data are expressed as the mean ± SEM.

Results

Sustained behavioral sensitivity was attenuated within 1 day after a single intraperitoneal injection of riluzole (Fig. 1). There was a significant difference ($p < 0.001$) in the number of paw withdrawals in response to mechanical stimulation between the groups over time (Fig. 1A). At Day 1, the number of paw withdrawals in the ipsilateral forepaw was significantly ($p < 0.002$) elevated over sham responses for both groups undergoing a nerve root compression (inj+veh, inj+ril). However, at Day 2 after compression, the number of paw withdrawals was reduced to sham levels with riluzole treatment (Fig. 1A), which represents a significant decrease compared with injury and vehicle treatment ($p = 0.014$). The number of paw withdrawals in the inj+veh group remained significantly greater than the number in either the inj+ril or the sham+veh groups at all time points after Day 1 ($p < 0.003$) and there were no significant differences between the sham+veh and inj+ril groups at any time point after treatment on Day 1 (Fig. 1A). Similarly, riluzole also attenuated the thermal hyperalgesia that is evident after a nerve root compression (Fig. 1B). At Day 1, the withdrawal latency in the ipsilateral forepaw for both of the nerve root compression (inj+Ril and inj+veh) groups was significantly ($p < 0.025$) shorter than that of the sham procedure group (Fig. 1B). After riluzole was given, however, the withdrawal latency at Day 7 in the injury group (6.6 ± 0.9 seconds, inj+veh) was significantly shorter than the withdrawal latency for either the riluzole treatment (8.3 ± 1.2 seconds, inj+Ril, $p = 0.044$) or the sham (9.6 ± 1.3 seconds, sham+veh, $p = 0.001$) groups (Fig. 1B). There was no difference in latency between the inj+ril and sham+veh groups at Day 7 (Fig. 1B).

Riluzole treatment after a painful nerve root compression partially prevented the axonal swelling, thinning of the myelinated axons, and axon disorganization in the nerve root that is typically evident at Day 7 after a painful root compression (Fig. 2 and Table 2). The nerve root for one sample from the inj+veh group (from Rat 157) was damaged at the time of harvest so it was not evaluated (Table 2). After the nerve root compression procedure, there were regions of discontinuous NF200 immunoreac-

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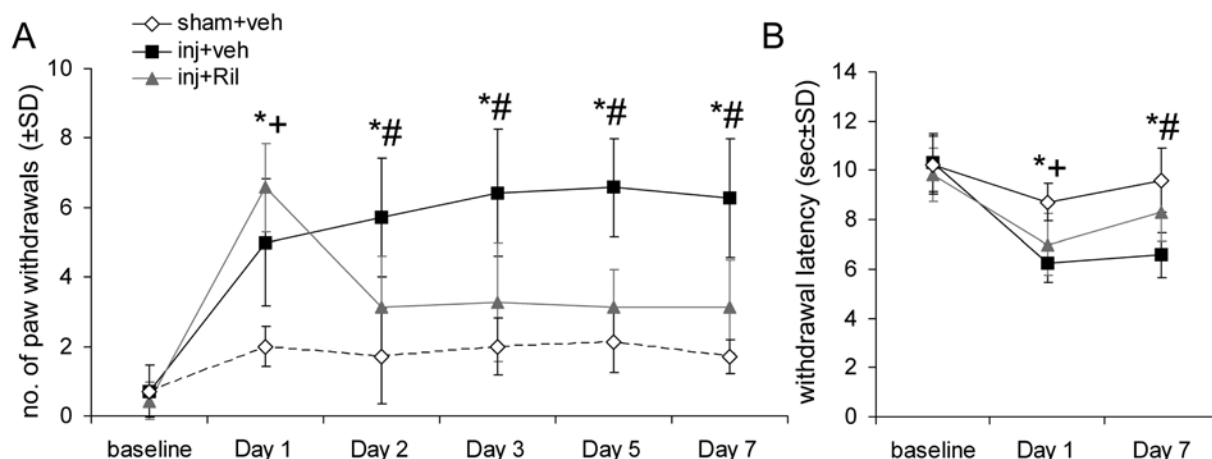


Fig. 1. Mechanical allodynia and thermal hyperalgesia after nerve root compression with and without riluzole treatment. **A:** Prior to treatment (Day 1), mechanical allodynia (as indicated by number of paw withdrawals) was significantly greater in both the vehicle-treated (inj+veh) and riluzole-treated (inj+ril) root compression groups than in the sham surgery (sham+veh) group. Mechanical allodynia remained significantly greater in the inj+veh group compared with the sham+veh group but was also increased over the treatment group (inj+ril) for all subsequent testing days. **B:** At Day 1, the withdrawal latency to a thermal stimulus significantly decreases in both compression groups (inj+veh, inj+ril) compared with sham+veh. After treatment, the withdrawal latency at Day 7 is significantly less for the inj+veh than both inj+ril and sham+veh. *Significant difference for inj+veh vs sham+veh. +Significant difference for inj+ril vs sham+veh. #Significant difference for inj+veh vs inj+ril.

tivity along the length of the nerve root (Fig. 2). Axonal swelling was also evident for all 3 types of labeled axons (Fig. 2), such that the diameter of the axons labeled for NF200, CGRP, and IB4 varied along the length of the root (Fig. 2). In the nerve roots harvested from 3 rats in

the vehicle-treated injury group (Rats 156, 163, and 198), axonal injury extended across the greater part of the root in at least 2 of the 3 types of labeled axons (Table 2). In contrast, the NF200-labeled axons remained uniform in diameter along the length of the root and were evenly

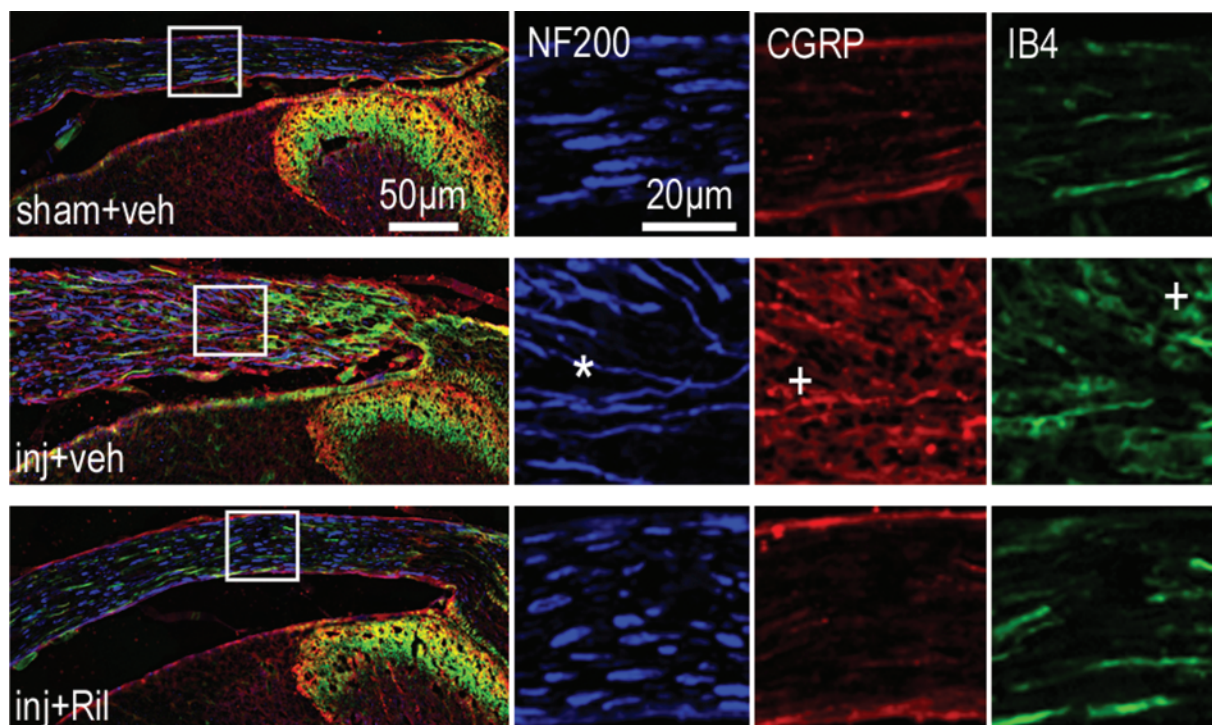


Fig. 2. Representative images of C-7 nerve roots from animals in the sham+veh, inj+veh, or inj+ril groups obtained at Day 7 and labeled for myelinated (NF200), peptidergic (CGRP), and nonpeptidergic (IB4) axons. The region within the box is enlarged to show each of the 3 labels separately for clarity. In the sham+veh specimen, axons are evenly labeled for NF200, CGRP, and IB4 and are uniformly distributed throughout the root. After a painful root compression (inj+veh), axons exhibit regions of swelling (+) and the NF200-labeled axons, in particular, appear to thin (asterisk). These hallmarks are not evident in the specimen from the inj+ril group, which exhibits characteristics similar to the specimen from the sham+veh group.

TABLE 2: Summary of the NF200, CGRP, and IB4 ratings*

Group	Rat ID No.	NF200	CGRP	IB4
sham+veh	162	-	-	-
	193	-	-	-
	195	-	-	-/+
	196	-	-	-
	200	-	-	-
	211	-	-	-
	212	-	-	-
inj+veh	156	+ / ++	+	+ / ++
	157	ND	ND	ND
	163	+ / ++	+	++
	194	+	+	+
	198	++	+ / ++	+ / ++
	199	- / +	+	- / +
	210	- / +	- / +	-
inj+ril	147	- / +	+	- / +
	148	+	+	+
	149	-	-	-
	158	- / +	- / +	-
	159	+	- / +	+
	160	-	-	-
	161	+ / ++	+	+

* ND = no data due to specimen damage; - = no abnormality; + = abnormalities in regions of axons; ++ = abnormalities across most of the root.

distributed along the width of the root in the sham+veh group (Fig. 2). The unmyelinated axons (CGRP and IB4 positive-labeled) were also uniform in their distribution within the root and were noticeably smaller in diameter than the myelinated axons (NF200). Although there was some evidence of axonal swelling in some of the axons of some of the rats in the inj+ril group, the diameter of the axons remained relatively consistent across the length of the root (Fig. 2). Furthermore, only one root (from Rat 161) exhibited substantial axonal damage across its entire length, and this was evident only in the NF200-labeled axons (Table 2). In two of the nerve roots from animals in the inj+ril group (Rats 149 and 160) there was no indication of injury in any of the NF200-, CGRP-, or IB4-labeled neurons (Table 2).

Similar to the neuroprotective effects of riluzole on nerve root morphology that were observed at Day 7 (Fig. 2 and Table 2), the expression of CGRP in the superficial laminae was increased and the expression of this neurotransmitter in the deep laminae of the dorsal horn was reduced with riluzole treatment (Fig. 3). CGRP expression in the dorsal horn of the inj+veh group was significantly decreased ($p = 0.009$) in the superficial laminae compared with the sham group (Fig. 3). In the deep laminae, CGRP expression was significantly increased ($p = 0.003$) after compression (inj+veh) compared with the sham+veh group (Fig. 3). With riluzole treatment, however, there were no differences in the expression of CGRP

in either the superficial or deep laminae compared with either the sham+veh or the inj+veh groups (Fig. 3).

Riluzole also restored the expression of GLT-1 in the superficial laminae at Day 7 (Fig. 4). Similar to the expression of CGRP in this same region of the spinal dorsal horn (Fig. 3), there was a significant decrease ($p = 0.031$) in spinal GLT-1 following a painful nerve root compression treated with the vehicle (inj+veh) compared with sham+veh (Fig. 4). However, following riluzole treatment given after the painful root compression, the expression of GLT-1 in the superficial dorsal horn was unchanged from sham and was significantly elevated over the expression in the inj+veh group ($p = 0.027$, Fig. 4).

Riluzole also abated the hyperexcitability of the deep dorsal horn neurons that is observed following a painful nerve root compression. Similar to the lack of mechanical allodynia observed at Day 7 after riluzole treatment (Fig. 1), the number of paw withdrawals elicited by the 4.0-, 10.0-, and 26.0-gf filaments was also decreased compared with vehicle treatment (Fig. 5A). No significant differences were observed between any groups for testing with the 1.4-gf filament. Extracellular recordings were made from 110 neurons at an average depth of 629 ± 143 μm , and no significant differences were detected in the recording depth between groups. The number of spikes evoked by each of the 4.0-, 10.0-, and 26.0-gf filaments in the inj+veh group significantly increased ($p < 0.045$) by nearly 2-fold over the number of spikes evoked for the sham+veh group (Fig. 5B). After riluzole treatment, the spike counts decreased to sham group levels for each filament (Fig. 5B). Specifically, for stimulation with the 4.0-gf filament, the number of spikes in the inj+veh group (19 ± 4 spikes) was significantly greater than the number evoked in the sham+veh group (11 ± 2 , $p = 0.0337$) and the inj+ril group (12 ± 2 , $p = 0.0421$). No between-group differences were detected in the number of spikes evoked by the 1.4-gf filament (Fig. 5B).

Riluzole treatment reduces the number of WDR neurons in the deep dorsal horn (Fig. 6). A total of 75 WDR neurons and 35 LTM neurons were identified in all groups. Specifically, there was a significantly greater proportion of WDR neurons (83%) in the inj+veh group than the sham+veh (61%, $p = 0.030$) and inj+ril groups (61%, $p = 0.042$) (Fig. 6B). In general, the WDR neurons responded to the light brush and noxious pinch in a graded manner. The frequency of evoked firing in response to the noxious pinch in WDR neurons was significantly greater ($p = 0.048$) than the frequency measured in LTM neurons (Fig. 6); the spike rate in WDR neurons during the pinch was double the rate measured in LTM neurons.

Discussion

This is the first study to demonstrate that a single dose of riluzole given early after the onset of pain from radicular injury is sufficient to immediately abolish the mechanical and thermal sensitivity that develop otherwise, along with preventing the development of neuronal pathology and spinal hyperexcitability (Figs. 1, 2, 5, and 6). Riluzole mitigated the axonal damage of primary afferents that is normally observed after painful root com-

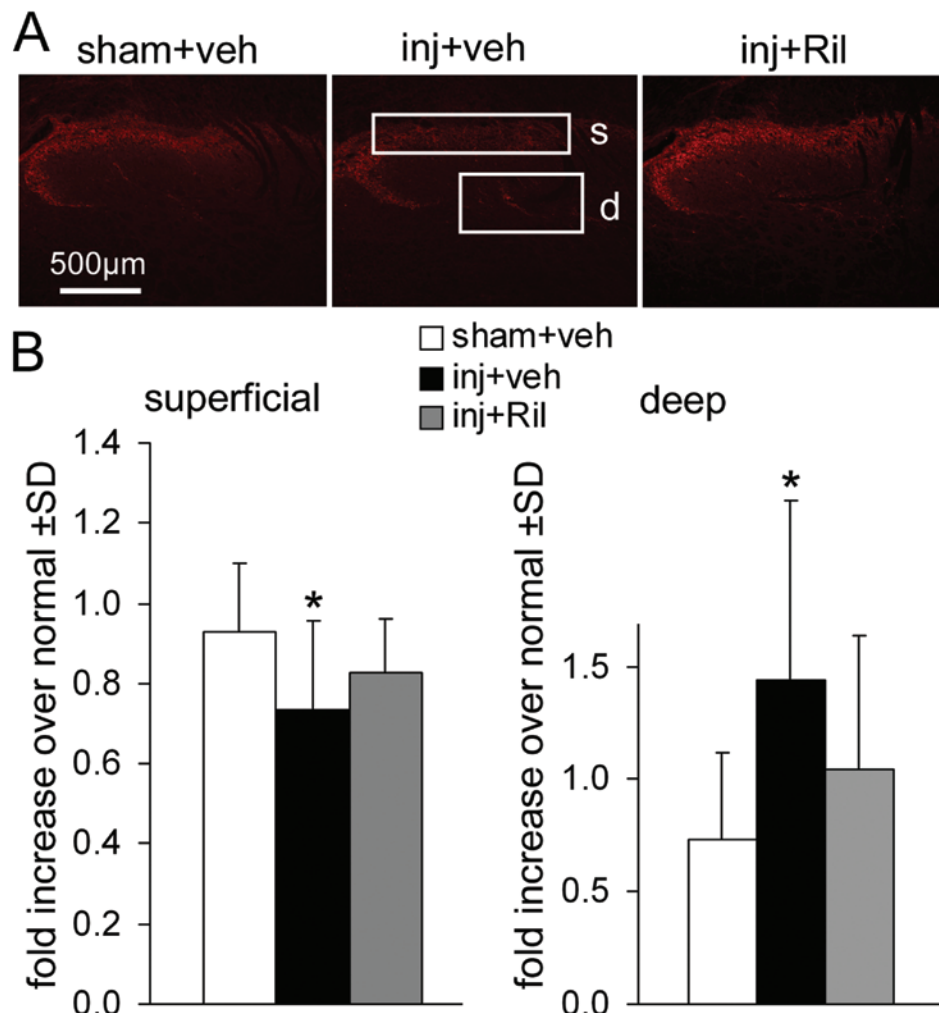


Fig. 3. CGRP expression in the dorsal horn at Day 7. **A:** Representative images of CGRP-labeled dorsal horns in the superficial (s) and deep (d) laminae after sham injury (sham+veh), and after nerve root compression with vehicle (inj+veh) and with riluzole (inj+ril). **B:** Quantification of CGRP labeling demonstrates that nerve root compression significantly decreases in the superficial dorsal horn and increases in the deep dorsal horn after a painful nerve root compression compared with sham, but is not changed from sham+veh values with riluzole treatment. *Significant difference vs sham+veh.

pression (Fig. 2) and restored the normal expression of both CGRP and GLT-1 in the dorsal horn, where many of those afferent neurons synapse (Figs. 3 and 4). Further, the frequency of neuronal firing in the deeper laminae ($640 \pm 140 \mu\text{m}$) and the proportion of WDR neurons were also attenuated after riluzole treatment (Figs. 5 and 6). Given that riluzole abolished both behavioral and spinal neuronal sensitivity to the 4.0- and 10.0-gf stimuli applied to the forepaw (Fig. 5), this study provides the first evidence that dorsal horn neuron sensitization contributes to forepaw sensitivity in this model of cervical radicular pain. Interestingly, neuronal hyperexcitability, down-regulation of GLT-1, and behavioral sensitivity were each completely abolished by riluzole (Figs. 1, 4, and 5), even though axonal swelling was still evident in some samples (Table 2 and Fig. 2) and spinal CGRP expression did not completely return to sham levels (Fig. 3).

Inhibiting neuronal signaling in the brain may be essential to riluzole's antinociceptive properties²⁶ and may augment its effects to produce the pronounced reduction

in behavioral sensitivity and neuronal signaling despite the presence of axonal swelling and discontinuities in neurofilament labeling (Figs. 1, 2, and 5). Riluzole has been shown previously to cross the blood-brain barrier rapidly and to reduce spinal glutamate and mechanical hyperalgesia within 1 hour after an intraperitoneal injection.^{15,26,76} After spinal cord contusion, riluzole alleviates pain when it is systemically administered or by an intracerebroventricular injection; yet, it is ineffective in alleviating behavioral sensitivity when it is directly administered into the intrathecal space of the spinal cord.²⁶ Therefore, the effects of riluzole on supraspinal glutamate appear to play a critical role in reducing pain associated with spinal cord injury. Since supraspinal signaling after radicular pain has not been well defined, it is unclear whether such effects also contribute to the reduced behavioral sensitivity that was observed in this study (Fig. 1).

Systemic delivery of riluzole mitigated mechanical sensitivity within 1 day and maintained its analgesic effect on both mechanical and thermal sensitivity for at

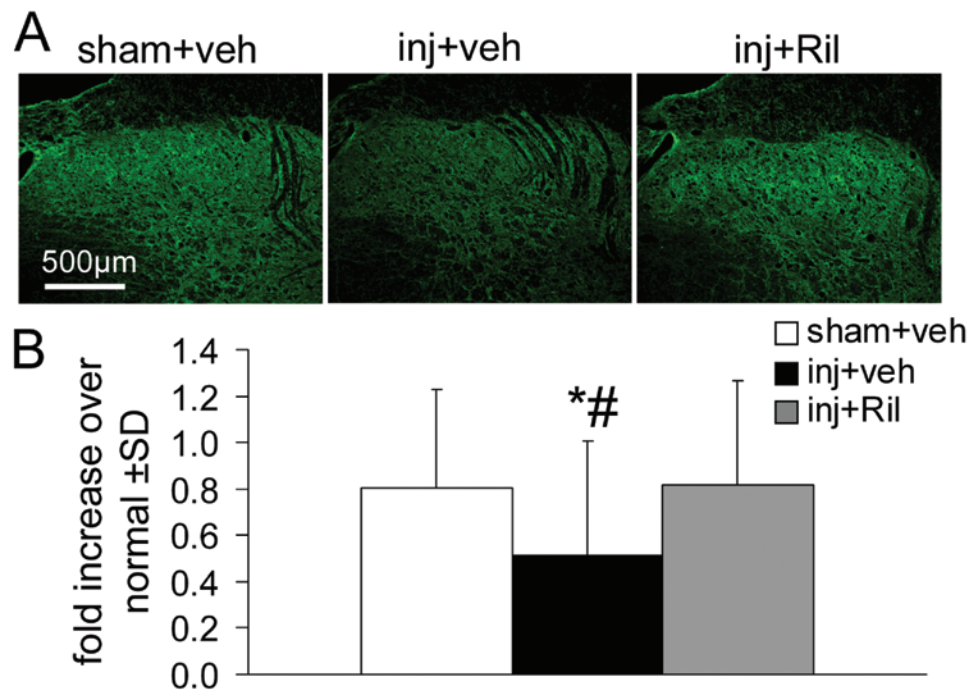


FIG. 4. Expression of GLT-1 in the superficial laminae at Day 7. **A:** Representative images of spinal GLT-1 after a painful nerve root compression treated with vehicle (inj+veh) and with riluzole (inj+Ril) or sham procedures (sham+veh). **B:** Quantification demonstrates that GLT-1 expression in the inj+veh group is significantly reduced compared with values in animals that underwent the sham procedure (sham+veh) and those that received riluzole treatment (inj+ril). *Significant difference vs sham+veh. #Significant difference vs inj+ril.

least 6 days after that single injection (Fig. 1). To date, riluzole's effects on behavior have been evaluated only for 2 hours after a single treatment and for 4 days after the final dose of daily repeated injections.^{26,76} Accordingly, this is the first study to demonstrate that a single dose, given after the development of pain has long-lasting effectiveness on behavioral sensitivity and nociceptive responses. Although our study evaluated riluzole as a therapy for radicular pain from nerve root compression in the rat, a recent clinical trial evaluated the therapeutic effects of riluzole for spinal cord injury.²⁴ In that clinical trial, a 50-mg dose of riluzole was given twice daily for 2 weeks after cervical spinal trauma significantly improved motor outcomes for 90 days after injury.²⁴ However, unlike spinal cord trauma, radicular pain can have delayed onset and diagnosis,^{2,53,74} which has complicated finding effective potential therapies for pain relief. Further studies investigating the effectiveness of riluzole for delayed administration at later time points would provide added utility for its potential use clinically for radicular pain.

Although riluzole promotes cell survival and neurite outgrowth *in vitro*,⁶⁵ this is the first *in vivo* study demonstrating that riluzole reduces damage to primary afferents after a root injury (Fig. 2). Riluzole not only mitigated axonal damage in the compressed nerve root, but also restored CGRP transport to the superficial dorsal horn (Figs. 2 and 3). In neuropathic pain, spinal CGRP expression and behavioral sensitivity have been shown to be positively correlated.^{39,51} Nevertheless, riluzole reduced behavioral sensitivity while also increasing superficial dorsal horn expression of CGRP (Figs. 1 and 3); yet, CGRP transport is

only one indicator of neuronal function. In addition to reduced CGRP transport, myelin degeneration and reduced axonal conduction have also been reported.^{12,33,34,52,53} Riluzole has been shown to inhibit the development of both of these injury markers after nerve and spinal cord injury.^{17,68} Therefore, in addition to preserving axonal morphology and CGRP expression in the superficial dorsal horn in this study (Figs. 2 and 3), riluzole likely also inhibited the development of myelin degeneration and changes to the conduction properties of the axons that normally develop after painful radiculopathy.^{12,33,41,53}

The normal morphology of and axonal transport through the primary afferents that are maintained after riluzole treatment (Figs. 2 and 3) may maintain the normal expression of spinal GLT-1 after a painful root compression (Fig. 4). In this manner, riluzole may increase spinal glutamate uptake after a painful root injury.^{11,35,38,69} GLT-1 is only expressed by glial cells in the rat spinal cord,⁷⁰ and astrocytes require neuronal signaling to express GLT-1.^{6,23,77} The downregulation of GLT-1 after a painful nerve root compression may be due to impaired afferent neurotransmission in the superficial laminae that is associated with the overall decrease in neuropeptides in this region (Figs. 3 and 4).^{4,31,40} Even though studies have demonstrated that riluzole can upregulate GLT-1 on cultured astrocytes,^{3,22} even in the absence of neurons,⁸ riluzole does not alter the expression of striatal GLT-1 in a rodent model of Parkinson's disease when given at a dosage (4 mg/kg) comparable to that used here.⁹ Therefore, it can be hypothesized that upregulation of spinal GLT-1 with riluzole treatment after a painful nerve root com-

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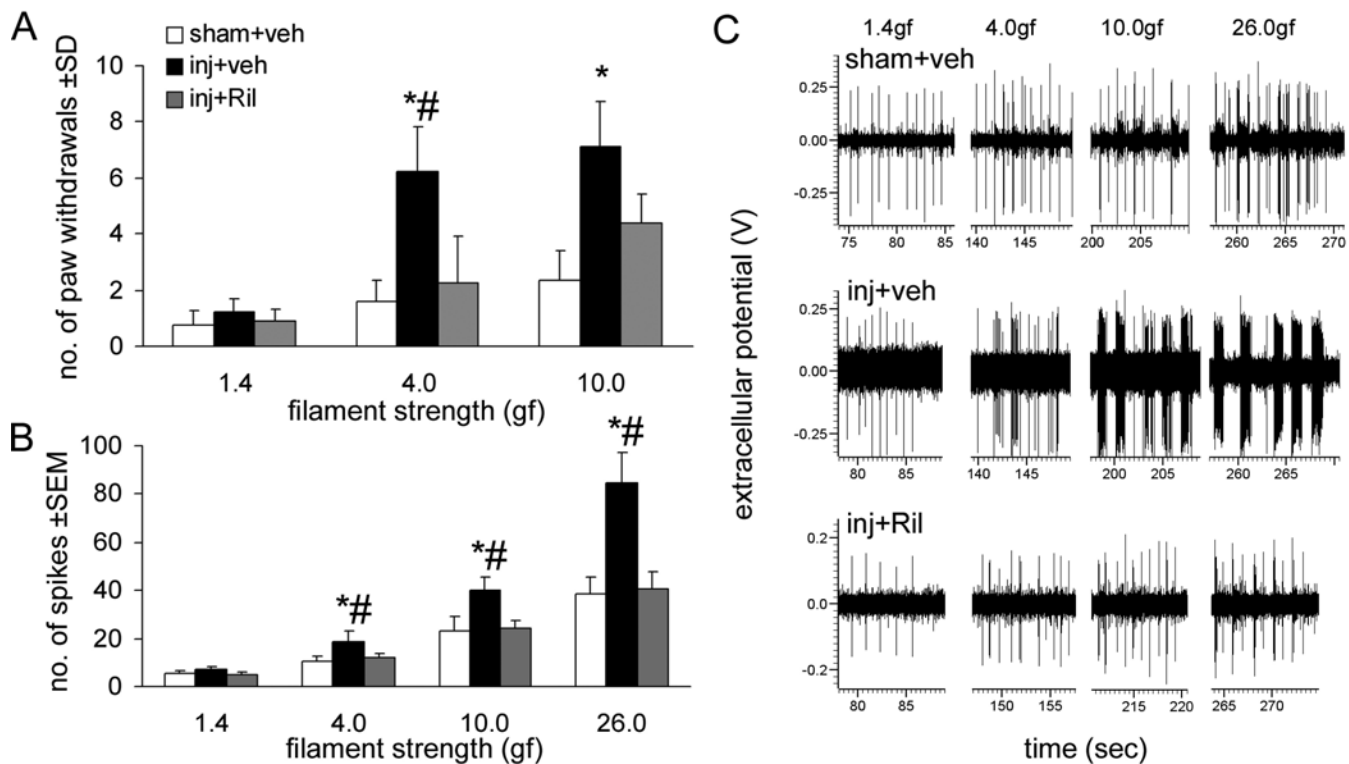


Fig. 5. Behavioral sensitivity in the forepaw and neuronal excitability in the spinal cord at Day 7. **A:** The number of paw withdrawals is significantly increased in the inj+veh group compared with sham+veh (4.0- and 10.0-gf filaments) and inj+ril (4.0-gf filament). **B:** Neuronal excitability is significantly elevated in the inj+veh group compared with both the sham+veh and inj+ril groups in response to paw stimulation by the 4.0-, 10.0-, and 26.0-gf filaments. **C:** Representative extracellular recordings for each injury group during the 1.4-, 4.0-, 10.0-, and 26.0-gf filament stimuli applied to the forepaw. *Significant difference vs sham+veh. #Significant difference vs inj+ril.

pression is likely a secondary mechanism of its neuroprotective properties (Figs. 2 and 4).^{5,23,55,76,77} Downregulation of GLT-1 expression in the superficial laminae suggests that glutamate uptake is impaired (Fig. 4),^{11,70} which has been associated with increased spinal glutamate concentration,^{11,35,38} and may contribute to the increase in the amplitude of excitatory currents on postsynaptic neurons in the superficial laminae that are reported after a sustained painful root constriction.⁷¹ By maintaining normal spinal GLT-1 expression in the superficial dorsal horn, riluzole may alleviate radicular pain via modulating excitatory neurotransmission in that region (Fig. 4).^{11,35,38,69,71}

Like the increase in spinal GLT-1 after riluzole administration (Fig. 4), the reduced expression of CGRP that was observed in the deep dorsal horn after riluzole treatment (Fig. 3) may be secondary to the improved neuronal health that is also maintained with riluzole (Fig. 2). In vitro studies demonstrate that riluzole has the opposite effect of increasing neuronal CGRP expression, together with promoting neurite growth.^{47,65} Therefore, it is likely that the reduced CGRP expression in the deep dorsal horn is an indirect consequence of riluzole administration. Aberrant fiber sprouting from the superficial dorsal horn is thought to increase CGRP expression in the deeper laminae.^{39,42} Extending those findings to our study, the increased expression of CGRP after a painful nerve root compression (Fig. 3) may indicate an increase in the number of CGRP-labeled fibers that do not normally extend

into the deeper laminae. In fact, aberrant sprouting in the dorsal horn occurs when the nerve root is transected and its primary afferents degenerate,⁵⁰ suggesting that compression to the root may also induce similar sprouting. By preserving axonal morphology in the root, concurrent axonal degeneration is mitigated and aberrant sprouting of neurons into the deep dorsal horn is also prevented after riluzole treatment.

In the spinal cord, neurotransmission of CGRP is essential for enhancing WDR neuron excitability in models of painful knee joint inflammation and spinal cord injury.^{7,14,28,48,51} Therefore, the elevated expression of CGRP in the deep dorsal horn after a painful nerve root compression likely contributed to the neuronal hyperexcitability and the shift in the phenotypic properties observed in this same region (Figs. 3, 5, and 6). Consistent with the current study, hyperexcitability of WDR neurons, in particular, is thought to underlie pain after neural injury.^{25,28} It has been suggested that a painful nerve root ligation amplifies input from primary afferents by increasing the amplitude of postsynaptic excitatory currents in the superficial dorsal horn.⁷¹ This amplification may be attributed to the increase in the number of WDR neurons observed in our study (Fig. 6), reflecting an increase in the number of neurons that respond to noxious stimuli.^{25,28,37,55,56,64,72} It is possible that the increased number of WDR neurons together with the elevated CGRP expression in the deep laminae may act to enhance the neuronal excitability that

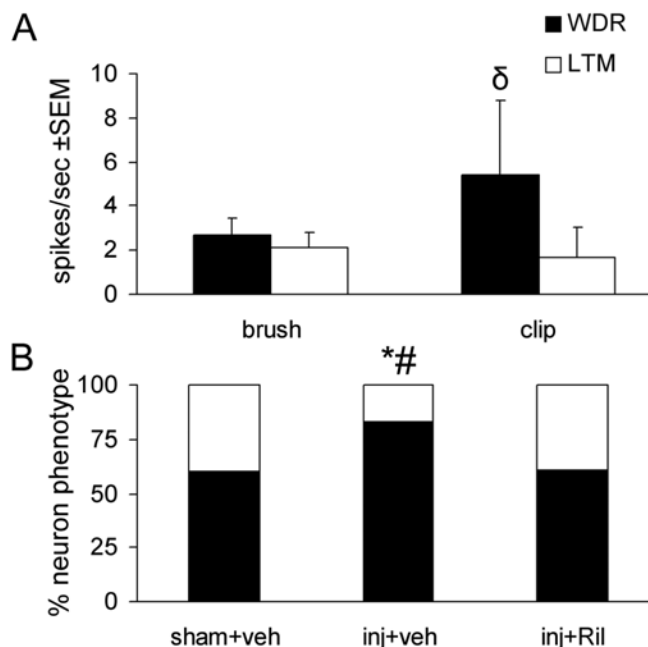


FIG. 6. **A:** The response of WDR and LTM neurons in the spinal cord to light brushing and a noxious clip applied to the forepaw on Day 7. WDR neurons exhibited a graded response, while the LTM neurons primarily responded to the light brushing. During the noxious clip application, the frequency of neuronal firing in WDR neurons was significantly greater than that observed in LTM neurons. **B:** The proportion of neurons classified as WDR was significantly greater in animals in the inj+veh group than in the sham+veh and inj+ril groups. δ indicates significant difference between WDR and LTM neurons. *Significant difference vs sham+veh. #Significant difference vs inj+ril.

develops after a painful root compression (Figs. 3, 5, and 6). Riluzole may have reduced neuronal hyperexcitability in the current study by reducing the expression of CGRP in the deep laminae (Figs. 3 and 5). Riluzole has been reported to bind to voltage-gated sodium channels and inhibit the persistent sodium current, reducing the frequency of repetitive firing of neurons.^{5,36,67} This is consistent with the lower frequency of firing that was observed after its administration in this study (Fig. 5). However, the effects of a single dose of riluzole on the temporal response of neuronal signaling was not investigated here, nor were specific relationships determined between axonal morphology, CGRP expression, GLT-1 expression, and the frequency of neuronal firing. Considering that an intraperitoneal injection of riluzole in the rat has a half-life up to 25–31 hours and, at high doses has sedative effects that last for only 4 hours,^{46,49,76} it is likely that riluzole had peak effects on glutamate signaling in the present study within the first day after its injection. Administering riluzole at Day 1 may have prevented the development of tissue pathology in the root and spinal cord, thereby also preventing the development of heightened spinal neuronal firing even after the effects of riluzole had worn off (Figs. 1 and 5).^{46,76} At Day 1 after the same painful compression, CGRP in the superficial dorsal horn has been previously shown to be not modified from sham controls and axonal injury in the root is absent.^{31,33,40,59} Administering riluzole at this time point is likely to have inhibited, but not re-

versed, the development of axonal pathology in the root and loss of CGRP transport to the spinal cord. Additional studies measuring the temporal responses in the glutamatergic system for nerve root-mediated pain are needed to fully understand the mechanism(s) by which riluzole may be acting.

Conclusions

This study establishes that even a single dose of riluzole given after the onset of behavioral sensitivity can inhibit the evoked neuronal signaling in the spinal cord in association with attenuation of thermal and mechanical behavioral sensitivity. Furthermore, the spinal expression of CGRP and GLT-1 were both restored by Day 7 after riluzole treatment. Unlike the wealth of animal studies demonstrating riluzole's ability to prevent motor impairment and to improve motor function after injury,^{5,17,21,44,54,62,68,76} the current study demonstrates that a single injection of riluzole also improves sensory function after a painful radicular injury. Contrary to the present findings, however, riluzole did not alleviate hyperalgesia in a clinical study of lower limb burn injury,²⁷ suggesting that it may only be effective in treating pain associated with direct trauma to neural tissues.^{15,26,69} The current study provides new insight into how riluzole may alleviate radiculopathy by protecting the morphology of injured afferents, limiting redistribution of spinal neurotransmitters, and abolishing afferent hyperexcitability in the spinal cord.

Disclosure

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Author contributions to the study and manuscript preparation include the following. **Conception and design:** Winkelstein, Nicholson. **Acquisition of data:** Nicholson, Zhang. **Analysis and interpretation of data:** all authors. **Drafting the article:** Winkelstein, Nicholson. **Critically revising the article:** all authors. **Reviewed submitted version of manuscript:** all authors. **Approved the final version of the manuscript on behalf of all authors:** Winkelstein. **Statistical analysis:** Winkelstein, Nicholson. **Study supervision:** Winkelstein.

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