Pre- and postsynaptic mechanisms underlying inhibition of hypoglossal motor neuron excitability by riluzole

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Bellingham MC. Pre- and postsynaptic mechanisms underlying inhibition of hypoglossal motor neuron excitability by riluzole. J Neurophysiol 110: 1047–1061, 2013. First published June 5, 2013; doi:10.1152/jn.00587.2012.—Riluzole is the sole treatment for amyotrophic lateral sclerosis (ALS), but its therapeutically relevant actions on motor neurons are not well defined. Whole cell patch-clamp recordings were made from hypoglossal motor neurons (HMs, n = 25) in brain stem slices from 10- to 23-day-old rats anesthetized with pentobarbital sodium to investigate the hypothesis that riluzole inhibits HMs by multiple mechanisms. Riluzole (20 μM) hyperpolarized HMs by decreasing an inward current, inhibited voltage-gated persistent Na⁺ and Ca²⁺ currents activated by slow voltage ramps, and negatively shifted activation of the hyperpolarization-activated cation current (Iₐ). Repetitive firing of HMs was strongly inhibited by riluzole, which also increased action potential threshold voltage and rheobase and decreased amplitude and maximum rise slope but did not alter the maximal afterhyperpolarization amplitude or decay time constant. HM rheobase was inversely correlated with persistent Na⁺ current density. Glutamatergic synaptic transmission was inhibited by riluzole by both pre- and postsynaptic effects. Riluzole decreased activity-dependent glutamate release, as shown by decreased amplitude of evoked and spontaneous excitatory postsynaptic currents (EPSCs), decreased paired-pulse ratio, and decreased spontaneous, but not miniature, EPSC frequency. However, riluzole also decreased miniature EPSC amplitude and the inward current evoked by local application of glutamate onto HMs, suggesting a reduction of postsynaptic glutamate receptor sensitivity. Riluzole thus has a marked inhibitory effect on HM activity by membrane hyperpolarization, decreasing firing and inhibiting glutamatergic excitation by both pre- and postsynaptic mechanisms. These results broaden the range of mechanisms controlling motor neuron inhibition by riluzole and are relevant to researchers and clinicians interested in understanding ALS pathogenesis and treatment.

persistent sodium current; excitatory synaptic transmission; hyperpolarization-activated cation current; ionotropic glutamate receptor; amyotrophic lateral sclerosis

The neuroprotective properties of riluzole in ALS were originally hypothesized to be due to its ability to reduce glutamatergic synaptic transmission (Benavides et al. 1985; Centonze et al. 1998; He et al. 2002; Lamanauskas and Nistri 2008; Prakriya and Mennerick 2000). However, other neuronal responses also occur at similar or lower concentrations of riluzole, including inhibition of the persistent Na⁺ current (Lamanauskas and Nistri 2008; Urbani and Belluzzi 2000; van Zundert et al. 2008), reduced action potential firing (Kuo et al. 2006; Urbani and Belluzzi 2000; van Zundert et al. 2008), potentiation of the Ca²⁺-dependent K⁺ current (Cao et al. 2002), and inhibition of voltage-gated Ca²⁺ channels (Huang et al. 1997; Hubert et al. 1998; Lamanauskas and Nistri 2008). Comparison of the possible therapeutic effects of riluzole on motor neurons thus remains fraught with difficulty, especially because the above studies have been carried out with a variety of experimental preparations (often not the motor neurons affected in ALS) from several species, making direct comparison of the different effects of riluzole problematic. To date, there has been no attempt to directly compare the diverse effects of riluzole at a clinically relevant concentration in a single type of motor neuron from the same species.

The tongue muscles participate in a wide range of motor activities, including swallowing, mastication, vocalization, and maintenance of a patent upper airway during respiration (Fregosi 2011; Sawczuk and Mosier 2001). The intrinsic and most extrinsic muscles of the tongue are controlled by hypoglossal motor neurons (HMs) (Fregosi 2011). Loss of HMs is common in the adult-onset neurodegenerative disease ALS (DePaul et al. 1988), causing deficits in tongue muscle activity, most commonly manifested as difficulty in swallowing or speech (Hillel and Miller 1989; Rowland and Schneider 2001). Indeed, in ∼30–40% of patients, these bulbar symptoms are the first seen during the inexorable progression of ALS toward respiratory paralysis and death (Fujimura-Kiyono et al. 2011; Hardiman et al. 2011; Weikamp et al. 2012; Zoccolella et al. 2006); bulbar symptoms at presentation are more common with increasing age, occurring in up to 60% of older patients (Zoccolella et al. 2006). Progressive bulbar impairment of speech and swallowing are also frequently co-occurrences in limb-onset ALS patients (Fujimura-Kiyono et al. 2011; Kühllein et al. 2008; Weikamp et al. 2012), and symptoms due to bulbar deficits are among the most distressing for ALS patients (Hillel and Miller 1989). Loss of HMs (Dal Canto and Gurney 1995; Ferrucci et al. 2010) and lingual movement deficits (Smittkamp et al. 2008, 2010) are also seen in transgenic animal models of ALS; HMs from transgenic models of ALS show an early onset of hyperexcitability due to an increased persistent Na⁺ current density (van Zundert et al. 2008). HMs

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are therefore an important motor neuron group known to be susceptible to early changes in excitability and adult demise in ALS and its transgenic models. While over 50 genetic mutations have been associated with ALS (Bruijn et al. 2004; Pasinelli and Brown 2006), it is not yet known why these mutations lead to motor neuron death. A number of nongenetic mechanisms have also been proposed, including glutamatergic excitotoxicity, growth factor deficiency, environmental factors, and autoimmune responses (Bruijn et al. 2004). The cause of motor neuron loss in ALS is thus still unknown.

HM activity is driven by the interchange between excitatory glutamatergic inputs from premotor neurons (Bellingham and Berger 1994, 1996) and a range of ionic currents that regulate HM excitability by electrophysiological recording from HMs. Electrophysiological recordings of membrane potential, resistance, holding current, repetitive firing and single action potential properties, voltage-sensitive persistent Na+ and Ca2+ currents, evoked, spontaneous, and miniature excitatory synaptic potentials, and postsynaptic responses to glutamate were made with whole cell patch-clamp techniques in rat HMs in in vitro brain stem slices from rats aged 10–23 days, a period when HM morphology (Núñez-Abades et al. 1994) and electrical excitability (Berger et al. 1995; Núñez-Abades et al. 1993) have matured sufficiently to assume or be close to their adult properties.

Therapeutic doses of riluzole (oral intake of 50 mg twice daily) produce a median peak serum concentration of 183 ng/ml, equivalent to 0.8 μM, in humans (Groeneveld et al. 2008). However, in humans, there is a large variability in pharmacokinetic clearance rate (Bruno et al. 1997) and in individual peak serum concentration, which ranges up to 1,552 ng/ml, equivalent to 6.6 μM (Groeneveld et al. 2008); while individuals with high serum levels showed a lower level of muscle fasciculation and cramping, high serum levels do not correlate with slower disease progression or longer survival time (Groeneveld et al. 2003, 2008). In addition, levels of riluzole are four- to sixfold higher than plasma/serum levels after oral dosing in mice, rats, and monkeys, and high CNS levels persist for many hours after a single dose of riluzole (Colovic et al. 2004; Maltese et al. 2005; Martinet et al. 1997; Milane et al. 2009; Wu et al. 2013). This high brain-to-blood concentration ratio is presumably due to the high lipid solubility of riluzole, although there is also evidence that riluzole is a substrate for blood-brain barrier transporters (Milane et al. 2007, 2009). Riluzole concentration in the human brain after oral administration is not known; however, cortical excitability is altered for up to 24 h after a single dose of riluzole, even though plasma levels have dropped to ~5% of peak levels at that time (Schwenkreis et al. 2000). Given the large variability in riluzole serum levels, conjoined with the higher concentration of riluzole achieved in the CNS, it is possible that neural actions of riluzole at 20 μM or less are likely to be the most relevant for its clinical effects. As shown by a survey of the literature (Bellingham 2011), all of the above neural responses are reported to be altered at or below this concentration, which also approximates the brain level achieved by the therapeutic use of riluzole in individuals with high serum levels.

This study therefore tested the hypothesis that a clinically maximal concentration of riluzole decreases the excitability of HMs by multiple pre- and postsynaptic mechanisms. These results show that riluzole decreases motor neuron excitability via multiple mechanisms, by decreasing synaptic glutamate release, depressing postsynaptic glutamate receptor responses, and inhibiting several postsynaptic ion currents that control resting membrane potential and action potential firing and threshold. These data should be of interest both to neurophysiologists investigating the control of motor neuron excitability and to researchers and clinicians interested in understanding the treatment of ALS.

MATERIALS AND METHODS

Anesthesia and brain slice preparation. All experimental procedures and animal experimentation were in accordance with institutional and national body guidelines for experimental animal use (National Health and Medical Research Council, Australia) and were submitted to and approved for use in this study by the University of Queensland Animal Ethics Committee. Animals were deeply anesthetized by intraperitoneal injection of pentobarbital sodium (100 mg/kg) and then killed by rapid decapitation. The brain stem was removed and placed in ice-cold Ringer solution (see below for composition) in which NaCl was isosmotically replaced with sucrose (Bellingham and Berger 1996). Transverse slices (thickness 300 μm) were cut with a vibratome (DSK Microslicer, Dosaka Instruments). Slices were incubated for 50–70 min in this sucrose-containing solution at 34–37°C and then maintained at room temperature (21–25°C) in a bicarbonate-buffered Ringer solution (see below) bubbled with carbogen.

Recording methods. Whole cell patch-clamp recordings (n = 25 HMs) were made with in vitro brain stem slices from rats (n = 16) of either sex (10–23 days old). Slices were submerged and continuously superfused with bicarbonate-buffered Ringer solution at a rate of 1.5–2 ml/min in a chamber (volume 0.2 ml) mounted on a fixed-stage microscope (Zeiss Axioscope or Nikon Eclipse) equipped with Nomarski optics, a ×40 or ×60 water-immersion objective (numerical aperture 0.75 and 0.9, respectively) and a 100-W halogen light source. HMs were visualized by infrared videomicroscopy using an infrared band-pass filter (Omega Optical, Brattleboro, MA; band pass 750–790 nm) placed in the light path and a video camera and controller (Newvicon 2400-07ER, Hamamatsu) to record the resulting image, displayed on a video monitor (Sony). HMs were identified visually by their location within the hypoglossal nucleus, their size and shape, and their whole cell capacitance (~20 pF). Whole cell patch-clamp recordings were obtained after formation of a membrane seal with a resistance of >2 GΩ.

Electrical recordings were performed at room temperature (21–25°C) with patch electrodes pulled from borosilicate glass capillaries (Vitrex Modulohn) on a two-stage puller (Narishige) to a DC resistance of 5–7 MΩ and connected to the headstage of an Axopatch 1D patch-clamp amplifier or a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 2 or 10 kHz with a four-pole, low-pass Bessel filter and digitized at a sampling rate of 5 or 40 kHz for storage in a PC (Dell, North Ryde, Australia). Action potentials were always recorded with a Multiclamp amplifier to avoid distortion of rapid potential changes and with the higher sampling rate and low-pass filter settings given above. Series resistance of 6–30 MΩ was compensated by 60–80%. The pCLAMP suite of programs (v 8–10, Axon Instruments) was used to apply voltage commands, record whole cell currents, and measure responses.

Excitatory postsynaptic currents (EPSCs) were evoked by electrical stimulation (5–120 V, 0.05–0.2 ms, 0.1 Hz) of the reticular formation (5–7 V, 0.05 ms) and then maintained at room temperature (21–25°C) in a bicarbonate-buffered Ringer solution (see below) bubbled with carbogen.

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or more negative, and the stimulus intensity was adjusted to give a first EPSC of consistent amplitude >150 pA (range −155 to −566 pA). In all HMs studied, stimulus intensity was greater than four times the stimulus threshold for a minimal response, which was typically between 1 and 4 V. Action potentials were evoked by depolarizing current steps from a subthreshold membrane potential, which was kept constant in control and drug conditions by manual adjustment of baseline current injection levels. The current response to a negative or positive voltage step of 5–10 mV was also recorded and displayed to monitor changes in series and input resistance.

**Solutions.** The external sucrose-substituted solution used for cutting and initial incubation of slices contained (in mM) 26 NaHCO₃, 3 KCl, 1 CaCl₂, 5 MgCl₂, 1.25 NaPO₄, 10 glucose, and 218 sucrose. Slices were maintained and recorded in a similar external solution, but with 130 mM NaCl added in place of sucrose and with 2 mM CaCl₂ and 1 mM MgCl₂. All solutions were continuously bubbled with carbogen gas to maintain pH at 7.2. The osmolarity of both external solutions was 310 mosM. The patch pipette internal solution for recording all EPSCs and for persistent Na⁺ currents was 310 mosM. The patch pipette internal solution for recording membrane potential/current, action potentials, and other voltage-gated currents contained (in mM) 135 K⁺, 8 NaCl, 10 HEPES, 2 Mg-ATP, 0.3 Na₂-GTP, and 0.3 EGTA; pH 7.2 with KOH. The osmolarity of both pipette solutions was adjusted to 290–300 mosM by addition of sucrose as required. Strychnine HCl (20 μM) was added to all external recording solutions to block inhibitory synaptic currents; at this concentration, strychnine blocks both glycine and GABAₐ receptors expressed by HMs (O’Brien and Berger 1999). Liquid junction potentials were not corrected for in the results.

**Drugs.** Drugs and chemicals were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) or ICN (K methyl sulfate) and were prepared as stock solutions and stored frozen at −20°C. Drug stock solutions were added to the external Ringer solution to give the final concentration desired to reduce or eliminate voltage-gated Ca²⁺ and K⁺ currents contained (in mM) 130 CsCl, 10 NaCl, 0.001 CaCl₂, 10 Cs-β-hydroxyethylpiperazine-N²-N⁴'-tetraacetic acid (HEPES), 10 Cs-ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 3 ATP-Mg, and 0.3 GTP-Tris; pH 7.2 with CsOH. The pipette solution for recording membrane potential/current, action potentials, and other voltage-gated currents contained (in mM) 135 K⁺ methyl sulfate, 8 NaCl, 10 HEPES, 2 Mg-ATP, 0.3 Na₂-GTP, and 0.3 EGTA; pH 7.2 with KOH. The osmolarity of both pipette solutions was adjusted to 290–300 mosM by addition of sucrose as required. Strychnine HCl (20 μM) was added to all external recording solutions to block inhibitory synaptic currents; at this concentration, strychnine blocks both glycine and GABAₐ receptors expressed by HMs (O’Brien and Berger 1999). Liquid junction potentials were <1 mV and were not corrected for in the results.

**Data analysis.** Evoked EPSC amplitude, holding current, and input resistance were measured for individual responses with Clampfit 10 software (Axon Instruments). These values were imported into Excel (Microsoft, Redmond, WA) for further analysis with custom-written Visual Basic for Applications routines. Drug-induced changes in evoked EPSC amplitude were determined by finding the maximal change of an average of 8–12 consecutive responses compared with a control average of the same number of consecutive responses immediately prior to the drug application. Paired-pulse ratio (PPR) of two evoked EPSCs at an interval of 60 ms was determined by averaging the evoked EPSCs in the control block and in the test block and then dividing the second averaged EPSC amplitude by the first averaged EPSC amplitude in each block; this method avoids spurious paired-pulse facilitation, which can be produced by calculating PPR as the average of individual EPSC amplitude ratios (Ireland et al. 2004; Kim and Alger 2001).

Spontaneous and miniature EPSCs were recorded in two or more blocks of continuously acquired data of 120-s duration at gains of 10–20× and then detected and analyzed off-line with Clampfit 10 (Axon Instruments). Parameters for the EPSC detection template were determined from EPSCs detected by eye from these records, and detection threshold was set at a level that resulted in rejection of >20% of detected events after visual inspection (Clements and Bekkers 1997). These detection parameters were kept constant for EPSC detection from all records for each HM but did vary between HMs; typical parameters for an EPSC detection template were a rise time of 1–2 ms, a decay time of 10–20 ms, and a nominal amplitude of −10 pA (the last parameter is optimally scaled as part of the detection algorithm). At least 50 EPSCs were analyzed for control and drug treatments in each HM. Data were only accepted for analysis when stationarity of EPSC parameters was confirmed by linear correlation, to ensure that small changes in recording conditions (Scanziani et al. 1992) or random fluctuations in event frequency (Fatt and Katz 1952) did not influence drug-induced changes in EPSC amplitude or frequency; the measured amplitudes and interevent intervals for episodes of EPSCs were plotted against time, a linear regression for the parameter against time was done, and the slope of the linear regression was tested for significant deviation from 0. A minimum of 5 min of drug treatment was allowed before EPSC recording commenced, to ensure equilibration of drug concentration.

Action potentials were detected off-line in Clampfit, and action potential threshold potential was determined as the potential at which the first derivative of membrane potential exceeded two times the standard deviation of baseline noise (Kole et al. 2008; Sekerli et al. 2004). To factor out the effects of differences in resting membrane potential and input resistance of individual HMs, measurement of action potential amplitude and half-width and afterhyperpolarization amplitude were made relative to action potential threshold in Clampfit.

**RESULTS**

**Riluzole hyperpolarizes hypoglossal motor neurons by decreasing an inward membrane current active at resting membrane potential.** In current-clamp recordings from nine HMs, bath application of riluzole (20 μM) caused a small but significant membrane hyperpolarization (Fig. 1, A and B) from −59.7 to −62.4 mV (mean change of −2.6 mV, 95% CI 0.3−5 mV; P = 0.03, paired t-test); one of nine HMs tested did not hyperpolarize with riluzole application. In six HMs voltage-clamped at −65 mV, riluzole caused a significant outward shift in holding current (Fig. 1B) from −268 to −159 pA (mean change +110 pA, 95% CI of +55 to +164 pA; P = 0.005, paired t-test). In these 15 HMs, the membrane hyperpolarization or outward current elicited by riluzole was associated with a small (+9%) but significant increase in neuronal input resistance (Fig. 1C) from 105 to 115 MΩ (mean change +10.6 MΩ, 95% CI 0.2–20.8 MΩ; P = 0.047, paired t-test).

Together, these changes show that riluzole hyperpolarized HMs by reducing an inward membrane current, which is active at the resting membrane potential.

**Riluzole suppresses an inward current similar to the persistent Na⁺ current, which is partially active at resting membrane potential.** In many neurons, a voltage-dependent persistent Na⁺ current is partially active at resting membrane potential levels, and this current is highly sensitive to riluzole (Bellingham 2011; Urbani and Belluzzi 2000). In 15 HMs, a slowly depolarizing and hyperpolarizing voltage ramp could selectively activate the persistent Na⁺ current, in contrast to the
predominant fast-inactivating voltage-dependent Na⁺ current. Figure 2A shows an example of the current evoked by a ramp protocol (Fig. 2A2, consisting of a 2-s ascending ramp from −70 to 0 mV and a 2-s descending ramp back to −70 mV) before and after bath application of riluzole (20 μM). In control conditions, an inward current was progressively activated during the ascending voltage ramp, from close to resting membrane potential to approximately −30 mV and then decreased again. One hallmark of the persistent Na⁺ current is that of hysteresis (Crill 1996), that is, the inward current exhibits different voltage-dependent characteristics during the ascending and descending phases of the voltage ramp; note that in Fig. 2A1, the inward current in control conditions was markedly less during the descending ramp. Riluzole markedly suppressed these inward currents, and the riluzole-sensitive current shown in Fig. 2A3 (produced by digital subtraction of the riluzole current from the control current) showed a marked hysteresis for the ascending and descending phases of the ramp, with a lower peak current during descent. In seven HMs recorded with a potassium methyl sulfate-based internal solution, the averaged riluzole-sensitive current (normalized to the whole cell membrane capacitance to factor out differences in current magnitude related to the membrane area of each HM) during the ascending voltage ramp [shown by the dark current-voltage (I-V) trace in Fig. 2B] showed voltage-dependent activation of an inward current from −70 onward, with peak inward current at approximately −30 mV.

As riluzole may also inhibit voltage-gated Ca²⁺ and some K⁺ currents active in this potential range (reviewed in Bellingham 2011), recordings were also made from eight HMs with a cesium chloride-based internal solution to decrease voltage-gated K⁺ currents and addition of 100 μM CdCl₂ to the external solution to block voltage-gated Ca²⁺ currents (Fig. 2B). Under these conditions, a voltage-dependent riluzole-sensitive inward current was still present and showed progressive activation from approximately −65 to −40 mV, but there was a large reduction in the riluzole-sensitive inward current from −40 to −10 mV under these recording conditions. A comparison of the normalized riluzole-sensitive mean current density for all HMs recorded at a ramp voltage of −40 mV and at −20 mV in the two recording conditions is shown in Fig. 2C; with no external Cd²⁺ present, HMs had a mean current density of −9.3 pA/pF (95% CI from −3.8 to −14.8 pA/pF) at −40 mV and −11.8 pA/pF (95% CI from −3.9 to −19.8) at −20 mV; with external Cd²⁺ present, the mean current density at −40 mV was not significantly altered (mean of −7.7 pA/pF, 95% CI from −3.7 to −11.7; unpaired t-test, P = 0.58), but the mean current density at −20 mV was significantly decreased (mean of −2.7 pA/pF, 95% CI of 1.3 to −6.8; unpaired t-test, P = 0.02). Comparison to the riluzole-sensitive current evoked without Cd²⁺ present suggests that high-voltage-gated Ca²⁺ channels sensitive to riluzole were also active in this voltage range (Lamanauskas and Nistri 2008; Umemiya and Berger 1994). Taken together, these results indicate that riluzole inhibits both voltage-dependent persistent Na⁺ and Ca²⁺ currents in rat HMs. As a riluzole-sensitive inward current, most likely the persistent Na⁺ current, was active at membrane potentials more positive than −65 mV, inhibition of this inward current will contribute to the observed membrane hyperpolarization in rat HMs.

Riluzole inhibits the hyperpolarization-activated cationic current by shifting its voltage-dependent activation to more hyperpolarized potentials. Resting membrane potential can also be partially regulated by the hyperpolarization-activated cationic current (Iₜₜ) in HMs (Bayliss et al. 1994; Chen et al. 2009). This inward current is slowly activated by hyperpolarization from levels close to rest and provides a depolarizing current that returns membrane potential to a resting level at which Iₜₜ activation is minimal. Effects of riluzole on Iₜₜ have not been
previously reported. $I_H$ was activated by a family of long (1 s) hyperpolarizing voltage steps from $-60$ to $-120$ mV; the voltage dependence of $I_H$ activation was determined by non-linear curve fitting of a Boltzmann equation to the peak amplitude of tail currents present during a constant step to linear curve fitting of a Boltzmann equation to the peak voltage dependence of $I$.

Figure 2 illustrates the inhibitory effects of riluzole on hypoglossal motor neurons and changes action potential shape. Riluzole can markedly inhibit neuronal activity by decreasing neuronal firing in a wide variety of neurons (reviewed in Bellingham 2011). Repetitive firing was evoked by the application of progressively larger depolarizing current steps to HMs recorded in current clamp. Repetitive firing during the current steps (Fig. 3A) increased in frequency rapidly up to $-40$ pA current injection and then increased more modestly up to $100$ pA current injection (Fig. 3B). Riluzole markedly reduced action potential firing over the range of injected currents (Fig. 3A and B; $n = 9$ HMs) and significantly reduced the maximum number of action potentials evoked by current injection (Fig. 3C) from 11 to 2 action potentials (mean

$P = 0.042$, paired 2-tailed t-test) without significant change in the Boltzmann curve slope ($-9.2$ to $-9.8$; $P = 0.19$).

Riluzole inhibits repetitive firing of hypoglossal motor neurons and changes action potential shape. Riluzole can markedly inhibit neuronal activity by decreasing neuronal firing in a wide variety of neurons (reviewed in Bellingham 2011). Repetitive firing was evoked by the application of progressively larger depolarizing current steps to HMs recorded in current clamp. Repetitive firing during the current steps (Fig. 3A) increased in frequency rapidly up to $-40$ pA current injection and then increased more modestly up to $100$ pA current injection (Fig. 3B). Riluzole markedly reduced action potential firing over the range of injected currents (Fig. 3A and B; $n = 9$ HMs) and significantly reduced the maximum number of action potentials evoked by current injection (Fig. 3C) from 11 to 2 action potentials (mean
difference of 9 action potentials, 95% CI 3 to 15; \( P = 0.01 \), paired 2-tailed \( t \)-test). Current injection in the subthreshold range also elicited a lower steady-state membrane potential in the presence of riluzole (Fig. 3A).

Riluzole also significantly changed action potential shape, reducing and slowing single action potentials (Fig. 3D). Mean action potential amplitude and rise slope was significantly decreased from 83 to 68 mV (Fig. 3E; mean change \( -15 \) mV, 95% CI \(-8 \) to \(-22 \) mV, \( n = 9 \); \( P = 0.0012 \), paired 2-tailed \( t \)-test), and maximal rise slope was also significantly decreased from 151 to 99 mV/ms (Fig. 3E; mean change \( -52 \) mV/ms, 95% CI \(-28 \) to \(-75 \) mV/ms; \( P = 0.0009 \), paired 2-tailed \( t \)-test). Riluzole significantly increased action potential half-width from 0.95 to 1.1 ms (Fig. 3F; mean change \(-0.15 \) ms, 95% CI \(-0.06 \) to \(-0.18 \) ms; \( P = 0.037 \), paired 2-tailed \( t \)-test).

Riluzole increases action potential threshold voltage and rheobase current but does not change afterhyperpolarization amplitude or decay time constant. Riluzole significantly increased the minimum current required to elicit an action potential (rheobase current) in these HMs (Fig. 4A), from a mean of 34 pA to 47 pA (mean change of \(+13 \) pA, 95% CI \(+2 \) to \(+25 \) pA; \( P = 0.029 \), paired 2-tailed \( t \)-test). This increase in rheobase current was due to a positive shift in action potential threshold, from \(-44 \) to \(-38 \) mV (Fig. 4A; mean change of \(+6 \) mV, 95% CI \(+3 \) to \(+9 \) mV; \( P = 0.0013 \), paired 2-tailed \( t \)-test). An example of increased threshold for firing is shown in Fig. 4B, where the action potential is initiated at a more depolarized membrane potential in the presence of riluzole compared with the level required in control conditions with the same current injected; the differentiated membrane potential trace in Fig. 4B, bottom, shows the method used for determining threshold voltage.

\( \text{Ca}^{2+} \)-dependent K\(^+\) currents underlying the afterhyperpolarization following action potentials (Viana et al. 1993) can be highly sensitive to riluzole (Beltran-Parrazal and Charles 2003; Cao et al. 2002), which enhances these currents and could therefore contribute to decreased repetitive firing. However, as previously reported for mouse HMs (van Zundert et al. 2008), riluzole did not significantly alter either the maximal amplitude (Fig. 4C; mean change of \(+2 \) mV, 95% CI \(-2 \) to \(+5 \) mV; \( P = 0.23 \)) or decay time constant of the action potential afterhyperpolarization (Fig. 4C; mean change of \(-7 \) ms, 95% CI \(-20 \) to \(+6 \) ms; \( P = 0.24 \)).

Rheobase current is inversely proportional to hypoglossal motor neuron persistent Na\(^+\) current density. In the course of analyzing the effects of riluzole on persistent Na\(^+\) current and firing threshold, a relationship between these two parameters was noticed. Figure 4D shows the persistent Na\(^+\) current density (measured at \(-40 \) mV, where the inward current is completely due to a riluzole-sensitive Na\(^+\) current, divided by the total cell capacitance value, in order to normalize membrane current for HMs of differing size) plotted against the rheobase current measured in the same seven HMs. A strong inverse correlation existed between these two values: HMs with smaller persistent Na\(^+\) current density had a larger rheobase current, while HMs with greater persistent Na\(^+\) current density had a smaller rheobase current. The Pearson correlation coefficient between these two measured parameters was 0.874.
(P = 0.01, 2-tailed t-test), giving an $R^2$ value of 0.76. This relationship suggests that, in addition to regulating repetitive firing, persistent Na$^+$ current density also contributes significantly to setting action potential threshold and therefore that inhibition of the persistent Na$^+$ current contributes to increasing action potential rheobase and threshold voltage.

Riluzole decreases evoked glutamatergic EPSCs by a combination of pre- and postsynaptic effects. Riluzole was originally suggested as a treatment for ALS because of its ability to reduce glutamatergic synaptic transmission (Benavides et al. 1985) and hence reduction of excitotoxic insult to motor neurons (Rothstein et al. 1993). In six HMs voltage-clamped at −65 mV, paired glutamatergic EPSCs (Fig. 5C) evoked by electrical stimulation (interstimulus interval of 60 ms) of the reticular formation lateral to the hypoglossal motor nucleus (Bellingham and Berger 1996) were significantly reduced by bath-applied riluzole (20 μM). As shown in Fig. 5A, the amplitude of the first evoked EPSC was significantly reduced by 28% (95% CI 17% to 39%; P = 0.012, paired 2-tailed t-test), while the amplitude of the second evoked EPSC was also significantly reduced by 42% (95% CI 25% to 59%; P = 0.0029, paired 2-tailed t-test). The PPR (2nd EPSC/1st EPSC amplitude) also decreased significantly (Fig. 5B; P = 0.02, paired 2-tailed t-test) with riluzole, from 1.80 (95% CI from 1.47 to 2.12) to 1.43 (95% CI from 1.06 to 1.81).

This reduction in PPR suggested that riluzole reduced the presynaptic probability of glutamate release (Bellingham and Walmsley 1999; Ireland et al. 2004). Two possible mechanisms could account for this—a decrease in the excitability of presynaptic elements (presynaptic terminals, axons, or somata) or a reduction in the probability of quantal release (Bellingham and Berger 1996; Doze et al. 1991; Scanziani et al. 1992). These two mechanisms can be differentiated by comparing the effects of riluzole on spontaneous EPSC activity and on miniature (quantal) EPSC activity. First, recordings of spontaneous glutamatergic EPSCs (Fig. 6A) consist of a mixture of presynaptic activity (action potential-dependent transmitter release and random activity-independent (quantal) transmitter release (Bellingham and Berger 1996; Scanziani et al. 1992). In eight HMs, the mean amplitude of spontaneous EPSCs (Fig. 6B) was significantly reduced by riluzole application (mean reduction of −3.6 pA, 95% CI −7.1 to −0.2 pA; P = 0.042, paired 2-tailed t-test). This reduction in spontaneous EPSC amplitude was due to a significant shift in EPSC amplitude distribution to a higher prevalence of low-amplitude EPSCs (Fig. 6C); the mean cumulative frequency distribution of spontaneous EPSC amplitudes was significantly different from the control spontaneous EPSC amplitude distribution (2-sample Kolmogorov-Smirnov test, P < 0.001). The instantaneous frequency (the reciprocal of the interevent interval) of spontaneous EPSCs was also significantly decreased by riluzole application (Fig. 6D; mean change of −2.1 Hz, 95% CI −4 to −0.3 Hz; P = 0.027, paired 2-tailed t-test), although the mean spontaneous EPSC frequency (the number of EPSCs divided by the recording time in seconds) was not significantly changed (Fig. 6D). The decrease in the instantaneous frequency of events was due to a lengthening of the interevent interval, as shown by a significant change in the cumulative frequency distribution of intervals to larger values (Fig. 6E; P < 0.001, 2-sample Kolmogorov-Smirnov test). These two changes in spontaneous EPSCs suggested that riluzole might act to decrease both activity-dependent glutamate release and/or the postsynaptic responses to synaptically released glutamate.

However, decreases in spontaneous EPSC amplitude and frequency could also be due to a decrease in frequency and amplitude of quantal miniature EPSCs, as these are mixed in with true spontaneous EPSCs. This second mechanism can be distinguished with recording of quantal miniature glutamat-
significant, with more small-amplitude events in the presence of riluzole (Fig. 7C; \( P = 0.003 \), 2-sample Kolmogorov-Smirnov test). The reduction in miniature EPSC amplitude, in combination with a reduction in spontaneous EPSC amplitude, supports the hypothesis that riluzole decreases postsynaptic responses to glutamate. In contrast, riluzole had no significant effect on either mean miniature EPSC frequency (Fig. 7D; \( P = 0.23 \)) or the mean cumulative frequency distribution of inter-event intervals (Fig. 7E; \( P = 0.37 \), 2-sample Kolmogorov-Smirnov test). This lack of effect on miniature EPSC frequency supports the hypothesis that riluzole does not change the quantal probability of transmitter release. Comparison with the depressive effects of riluzole on spontaneous EPSC frequency and on PPR of evoked EPSCs supports the hypothesis that riluzole acts to presynaptically decrease activity-dependent release.

As riluzole decreased both spontaneous EPSC and miniature EPSC amplitude, a further direct test of the postsynaptic effect of riluzole on glutamate receptor activation was made. A micropipette filled with \( t \)-glutamic acid (500 \( \mu \)M) dissolved in the external bathing solution was placed close to the soma of three different recorded HMs, and a puff of \( t \)-glutamate was directly applied to the soma by pressure injection (Fig. 8A). The inward current evoked by local glutamate application was significantly reduced by bath-applied riluzole (Fig. 8, A and B; 20 \( \mu \)M), which caused a mean reduction of 44\% (\( P = 0.046 \), paired 2-tailed \( t \)-test). The inward current evoked by local glutamate application was markedly reduced (mean of 92\% reduction, 95\% CI 85 to 94\% reduction; \( P = 0.038 \), paired 2-tailed \( t \)-test) by bath application of 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzof\[\text{1}\text{]quinoxaline-7-sulfonamide (NBQX) and \( dl \)-2-amino-5-phosphono-pentanoic acid (\( dl \)-APV) to block non-NMDA and NMDA glutamate receptors, respectively (Fig. 8, A and B), demonstrating that the current evoked by pressure application of \( t \)-glutamic acid was due to glutamate receptor activation.

**DISCUSSION**

Riluzole remains the only drug treatment for ALS with significant clinical effects, albeit a relatively modest extension of life span of 2–6 mo in humans (Miller et al. 2009) and several days in animal models of ALS (Bellingham 2011). Although loss of cranial and spinal motor neurons in ALS (Kier et al. 2011) and in animal models of ALS (Gurney et al. 1994) is a prominent feature of the disease, the effects of riluzole on the factors regulating activity and death of motor neurons are still not well understood. In part this is because riluzole has a wide range of possible neural actions at doses from \(<1\) up to 20–40 \( \mu \)M, which include inhibition of the persistent and fast-inactivating voltage-gated \( Na^+ \) currents, inhibition of repetitive firing, potentiation of \( Ca^{2+} \)-dependent \( K^+ \) currents, inhibition of neurotransmitter release, and inhibition of voltage-gated \( Ca^{2+} \) channels (Bellingham 2011). While many of these effects have been tested in motor neurons, others have not; even within the available data from motor neurons, the age, species, type of motor neuron, and experimental preparation have varied considerably, making a direct comparison of the effects of riluzole on factors that influence motor neuron activity fraught with difficulty. The purpose of this study was to address this difficulty, by determining which
of the several possible effects of riluzole at a single clinically relevant dose led to altered motor neuron activity. The findings show that riluzole acts to inhibit motor neurons by effects on multiple postsynaptic voltage-gated ion currents, including inhibition of Na\(^+\), Ca\(^{2+}\), and I\(_H\) ionic currents, with the last finding being the first report of the effects of riluzole on I\(_H\). Riluzole was first proposed to have its clinical effect in ALS by decreasing glutamatergic transmission (Doble 1996), and there is increasing use of riluzole for this purpose in treating other neurodegenerative diseases, mood and anxiety disorders, and neurotrauma. Despite this therapeutic use, only limited data from motor neurons are available (Lamanaukas and Nistri 2008; Tazerart et al. 2007), and no single study has previously measured the effect of riluzole on evoked glutamatergic EPSC amplitude and PPR, spontaneous and miniature EPSC amplitude and frequency, and postsynaptic ionotropic glutamate receptor currents. The present study uses these measures of neurotransmission to show that riluzole depresses glutamatergic transmission to motor neurons by a combination of decreased presynaptic release and reduced postsynaptic ionotropic glutamate receptor responses.

Hypoglossal MNs receive respiration-related synaptic inputs and could thus be considered a respiratory motor neuron pool (Fregosi 2011). However, deficits in tongue activity are the first symptoms to occur in 30–40% of all ALS patients (Fujimura-Kiyono et al. 2011; Hardiman 2011; Logroscino et al. 2010; O’Toole et al. 2008; Weikamp et al. 2012; Zoccolotella et al. 2006) and up to 60% of older ALS patients (Zoccolotella et al. 2006), while respiratory deficits due to loss of phrenic and intercostal spinal respiratory motor neurons almost uniformly occur late in ALS disease progression (Fujimura-Kiyono et al. 2011; Hardiman 2011). A longitudinal study of the progressive involvement of different motor neuron pools in 150 ALS patients showed that bulbar symptoms occurred before (21% of patients), concurrent with (6%), or after upper (8%) or lower (5%) limb symptoms (Fujimura-Kiyono et al. 2011). The loss of HMs in ALS is thus likely to occur relatively early in disease progression compared with other respiratory motor neurons, suggesting that the underlying pathogenesis of HM loss is more likely to be similar to that of limb motor neurons rather than spinal respiratory motor neurons.

While riluzole has a relatively limited range of actions at the blood plasma/serum concentrations commonly achieved (0.5–2 \(\mu\)M) in human ALS patients (Groeneveld et al. 2003), the variability in peak serum riluzole levels in humans following standard therapeutic dosing is quite high, with levels reaching as high as 6–7 \(\mu\)M in some subjects (Groeneveld et al. 2003). Furthermore, pharmacokinetic studies in mice, rats, and monkeys indicate that riluzole concentration in brain tissue is four to six times higher than peak plasma concentration after a single dose of riluzole (Colovic et al. 2004; Maltese et al. 2005; Martinet et al. 1997; Milane et al. 2009; Wu et al. 2013). In these species, CNS levels of riluzole remain high with a half-life of >9 h, and repeated dosing causes accumulation of
riluzole to ~60% greater concentration than is reached after a single dose (Milane et al. 2009; Wu et al. 2013). The factors causing a high brain-to-blood ratio of riluzole are unclear. Riluzole is highly lipid soluble, with an octanol:water partition coefficient of 3,000:1 (Chow et al. 2012), allowing it to readily enter brain tissue by passive diffusion; the relatively high lipid content of brain tissue, especially white matter, where riluzole is found in high concentration (Martinet et al. 1997), may then tend to retain riluzole until it is excreted. Excretion of xenobiotics from brain tissue is at least partially regulated by the P-glycoprotein, found in high concentration in the luminal membrane of brain capillaries (Miller et al. 2008). Inhibition of this transporter protein by minocycline or by gene knockout increases the brain concentration of riluzole severalfold (Milane et al. 2007, 2009). This excretion mechanism probably accounts for the relatively long persistence of high concentrations of riluzole in brain tissue (Chow et al. 2012; Milane et al. 2009). While the concentration of riluzole in human brain tissue achieved by clinical doses of riluzole remains unknown, the lipophilic permeation of riluzole through the blood-brain barrier is highly likely to be constant across all species, while the degree of riluzole efflux mediated by P-glycoprotein will then determine the steady-state brain concentration in humans (Miller et al. 2008). Indirect evidence suggests that a high and persistent brain-to-blood ratio also occurs in humans, as a single dose of riluzole produces changes in cortical excitability up to 24 h after administration, at a time point when plasma concentration has decreased to ~5% of peak plasma levels and is roughly 300 nM (Schwenkreis et al. 2000). Consideration of these factors suggests that neuronal responses to riluzole at concentrations ranging between 10 and 20 μM are thus likely to be most important in its therapeutic effects in ALS patients, and thus the upper limit of this range was used for comparison of effects in this study.

Riluzole does not usually cause changes in resting (i.e. subthreshold) membrane properties, such as resting membrane potential or input resistance (Centonze et al. 1998; Kuo et al. 2005; Tazerart et al. 2007; van Zundert et al. 2008), although membrane hyperpolarization has been reported in spontaneously active, but not nonspiking, spinal cord neurons in culture (Darbon et al. 2004) and in pacemaker neurons of the pre-Bötzinger complex (Del Negro et al. 2002). Riluzole caused a small but significant membrane hyperpolarization of 3 mV in rat HMs, which was associated with an outward current of ~100 pA and an increase in input resistance of ~10%. Taken together, these results show that riluzole inhibited an inward membrane current active at resting potentials in rat HMs. Further investigation of the effects of riluzole on specific ionic currents showed that it inhibited a persistent Na⁺ current, which was active at voltages positive to ~65 mV, thus potentially accounting for membrane hyperpolarization by riluzole, as inhibition of an active inward current will produce both hyperpolarization and an increase in steady-state input resistance, as seen here. The persistent Na⁺ current is mediated by voltage-gated Na⁺ channels that do not readily inactivate and is one of the few currents active in the subthreshold range from...
resting membrane potential to threshold (Alzheimer et al. 1993; Carter et al. 2012; Crill 1996). In this voltage range, the persistent Na\textsuperscript+ current can be activated by excitatory postsynaptic potentials (EPSPs) and inactivated by inhibitory postsynaptic potentials (IPSPs), with the functional effect of amplifying both types of synaptic input (Carter et al. 2012; Stuart and Sakmann 1995). The ongoing synaptic inputs received by HMs are thus likely to be enhanced and will also provide a tonic source of subthreshold voltage changes that will continually activate and inactivate Na\textsuperscript+ channels.

However, a novel effect of riluzole on \textit{I\textsubscript{H}} was also revealed, as riluzole shifted the voltage-dependent activation of \textit{I\textsubscript{H}} to more negative levels. \textit{I\textsubscript{H}} plays an important role in setting HM resting membrane potential, as transgenic deletion of the HCN1 gene encoding an \textit{I\textsubscript{H}} isoform strongly expressed in mouse HMs results in significant hyperpolarization of resting membrane potential (Chen et al. 2009), while positive shifts in \textit{I\textsubscript{H}} modulation cause membrane depolarization in adult rat HMs (Wenker et al. 2012) and juvenile mouse HMs (Ireland et al. 2012). Spontaneously active spinal neurons expressing both persistent Na\textsuperscript+ current and \textit{I\textsubscript{H}} were hyperpolarized by either riluzole or \textit{I\textsubscript{H}} blockade with ZD7288, but direct effects of riluzole on \textit{I\textsubscript{H}} were not tested (Darbon et al. 2004). The membrane hyperpolarization is certainly consistent with a negative shift in \textit{I\textsubscript{H}} activation; however, membrane hyperpolarization was associated with an increase in input resistance. As \textit{I\textsubscript{H}} is minimally active at resting membrane potential, a change in steady-state input resistance is less likely to occur with \textit{I\textsubscript{H}} modulation (Ireland et al. 2012). This suggests that membrane hyperpolarization by riluzole may thus be due to the combination of its effects on both persistent Na\textsuperscript+ current and \textit{I\textsubscript{H}} that contribute independently to regulation of resting membrane potential in rat HMs.

An increasing body of literature has consistently shown that riluzole inhibits cranial (Cramer et al. 2007; Lamanauskas and Nistri 2008; van Zundert et al. 2008) and spinal (Harvey et al. 2006a, 2006b; Kuo et al. 2006; Miles et al. 2005; Quinlan et al. 2011; Theiss et al. 2007) motor neuron firing in several species. The present study shows that riluzole greatly reduces repetitive firing in juvenile (P10–23) rat HMs, similar to the inhibition of repetitive firing seen in neonatal (P1–5) rat HMs (Lamanauskas and Nistri 2008) and mouse (P4–10) HMs (van Zundert et al. 2008). This inhibition of repetitive firing has been attributed to inhibition of the persistent Na\textsuperscript+ current (Cramer et al. 2007; Harvey et al. 2006a, 2006b; Kuo et al. 2006; Lamanauskas and Nistri 2008; Miles et al. 2005; Quinlan et al. 2011; Theiss et al. 2007; van Zundert et al. 2008), largely based on the potent and preferential inhibition of this current by riluzole at <10 \mu M (Urbani and Belluzzi 2000), combined with limited or no effects of riluzole on individual action potential and afterhyperpolarization amplitude (Kuo et al. 2006; Miles et al. 2005; van Zundert et al. 2008). The close correspondence in dose dependence between inhibition of repetitive firing (EC\textsubscript{50} of 0.5–1 \mu M) and of persistent Na\textsuperscript+ current (EC\textsubscript{50} of 1.8–2 \mu M) in cultured spinal neurons (Kuo et al. 2006) and cortical neurons in brain slices (Urbani and Belluzzi 2000) provides good evidence for this mechanism. The present results show that marked suppression of motor neuron repetitive firing by riluzole is associated with inhibition of the persistent Na\textsuperscript+ current, without any significant increase in the action potential afterhyperpolarization, thus reinforcing the conclusion that the persistent Na\textsuperscript+ current plays an important role in regulating repetitive firing of motor neurons.

In addition, detailed kinetic modeling of voltage-gated Na\textsuperscript+ channels has highlighted an important role for the persistent Na\textsuperscript+ current in lowering the threshold for action potential initiation (Kuo et al. 2006). These results clearly demonstrate a strong inverse correlation between the persistent Na\textsuperscript+ current and action potential initiation, in that action potential rheobase current decreases as persistent Na\textsuperscript+ current density increases in individual HMs. This relationship is also reflected in the increase in rheobase current and positive shift in action potential threshold voltage seen in response to riluzole application to HMs, as has also been reported for other neurons (Del Negro et al. 2002; Kuo et al. 2006). These results thus strengthen the conclusion that the persistent Na\textsuperscript+ current also plays an important role in lowering motor neuron action potential threshold.

Although riluzole did not prevent single action potential initiation in rat HMs, there were significant effects on single action potentials, as peak amplitude and maximal rate of rise were both significantly reduced and half-width was increased, as seen in some previous studies (Beltran-Parrazal and Charles 2003; Del Negro et al. 2002) but not others (van Zundert et al. 2008). Inhibition of the fast-inactivating Na\textsuperscript+ current by riluzole at <20 \mu M has been described in brain stem neurons.
(Ptak et al. 2005) and may contribute to these effects in HMs. The high affinity of riluzole for the inactivated state of voltage-gated Na⁺ channels (Benoit and Escande 1991; Urbani and Belluzzi 2000) may contribute to these effects, as the number of Na⁺ channels available for activation may be progressively decreased as riluzole binds to and inhibits Na⁺ channels entering the inactive state during previous action potentials. This is consistent with the marked decrease in action potential amplitude and maximal rise slope seen here, as these parameters are thought to be a function of the number of available Na⁺ channels (Kole et al. 2008; Raman and Bean 1999).

Previous work has established that the action potential in HMs is followed by a fast afterhyperpolarization mediated by a TEA-sensitive K⁺ channel and a medium afterhyperpolarization mediated by an apamin-sensitive (SK) Ca²⁺-dependent K⁺ current, while the great majority of HMs lack a slow afterhyperpolarization entirely (Viana et al. 1993). These experiments focused on the effects of riluzole on the medium afterhyperpolarization, as its inhibition strongly increases HM repetitive firing frequency (Viana et al. 1993). Despite reports that riluzole potently enhances SK Ca²⁺-dependent K⁺ current in cell expression systems (Cao et al. 2002; Sankaranarayanan et al. 2009), evidence that the action potential afterhyperpolarization in neurons is increased by riluzole is scant (Beltran-Parrazal and Charles 2003; Cao et al. 2002) and the lack of any effect of riluzole on the medium afterhyperpolarization seen here is in accord with other reports (Del Negro et al. 2008; Kuo et al. 2006; Miles et al. 2005; van Zundert et al. 2008). However, it should be noted that riluzole clearly inhibited a voltage-sensitive Ca²⁺ current in HMs, and this effect might be expected to reduce Ca²⁺ influx during the action potential and, in turn, reduce Ca²⁺-dependent K⁺ currents. Indeed, one effect of blocking Ca²⁺ influx is an increase in action potential half-width (Viana et al. 1993), similar to that seen here. The possibility that potentiation of Ca²⁺-dependent K⁺ current by riluzole is counterbalanced by decreased Ca²⁺ influx cannot be ruled out by the present results.

The excitotoxic hypothesis of motor neuron death in ALS (Rothstein et al. 1993) and several early studies that showed that riluzole inhibited a number of glutamate receptor-dependent neuronal responses (reviewed in Doble 1996) led to the hypothesis that inhibition of glutamatergic synaptic transmission by riluzole would reduce motor neuron death in ALS. Despite clear confirmation that riluzole treatment does extend survival in ALS patients, the mechanism(s) underlying the effect of riluzole on glutamatergic synaptic transmission has remained unclear.

As Doble (1996) pointed out, early studies did not distinguish between effects of riluzole on glutamate release and direct effects on glutamate receptors. Subsequent reports of the effects of riluzole on excitatory synaptic transmission have largely concluded that riluzole (at doses ranging from 0.5 to 10 μM) reduces presynaptic excitability (He et al. 2002; Maclver et al. 1996; Pace et al. 2007; Prakriya and Mennerick 2000; Rammes et al. 2008; Tazerart et al. 2007) and thus glutamate release but has little effect on postsynaptic glutamate receptor responses elicited by direct application of glutamate (He et al. 2002; Prakriya and Mennerick 2000), with the notable exception of Centonze et al. (1998), where responses to glutamate were decreased by riluzole. The tests used to support the hypothesis of reduced presynaptic excitability varied among these studies, and no single study has previously measured the effect of riluzole on evoked EPSC amplitude and PPR, spontaneous and miniature EPSC amplitude and frequency, and postsynaptic glutamate receptors. The PPR of evoked EPSCs was altered by riluzole in some studies (He et al. 2002; Prakriya and Mennerick 2000) but was unchanged in others (Centonze et al. 1998; Maclver et al. 1996), while spontaneous EPSC frequency was decreased by riluzole without change in amplitude (Pace et al. 2007). Two studies have examined the effects of riluzole on miniature EPSC frequency and amplitude: one found that riluzole had no effect on either parameter (Tazerart et al. 2007), while the other found that riluzole decreased both frequency and amplitude of miniature EPSCs only in HMs whose firing was also depressed by riluzole (Lamanaukas and Nistri 2008). In the latter study, as miniature EPSCs were recorded in TTX-containing solution, the effects of riluzole cannot be attributed to modulation of Na⁺ channels and were occluded by prior treatment with a protein kinase C inhibitor or with a NMDA glutamate receptor antagonist. In contrast to these previous studies, the present study clearly demonstrates depressive effects of riluzole on glutamatergic EPSCs recorded from rat HMs via a dual mechanism of decreased presynaptic excitability combined with postsynaptic reduction of ionotropic glutamate receptor responses.

Evidence supporting a mechanism of decreased presynaptic excitability consists of the alteration of PPR and the decreased frequency of spontaneous, but not miniature, EPSCs with riluzole application. As PPR is commonly held to be related to the release probability of activated synapses, with low release probability leading to paired-pulse facilitation and high release probability leading to paired-pulse depression (Bellingham and Walmsley 1999; Manabe et al. 1993), the present observation that riluzole both decreased evoked EPSC amplitude and reduced paired-pulse facilitation is paradoxical at first glance, as lowering release probability (and thus reducing EPSC amplitude) would be expected to cause increased paired-pulse facilitation. However, this finding is consistent with that of Prakriya and Mennerick (2000), who found that riluzole both reduced autaptic EPSCs and increased paired-pulse depression in cultured hippocampal neurons; they hypothesized that this was due to a selective inhibition of synapses with low release probability by riluzole, leaving transmission at high-release-probability synapses relatively intact, thus strengthening paired-pulse depression. This mechanism can also account for the responses reported here; if we assume that excitatory synapses onto HMs have a range of release probabilities giving rise to net paired-pulse facilitation, then inhibition of synapses with the lowest release probability will raise the average release probability and thus decrease paired-pulse facilitation.

However, it is also important to bear in mind that the stimulus used to evoke EPSCs does so by local excitation of multiple presynaptic axons and/or somata, followed by action potential invasion of the presynaptic terminals and Ca²⁺-dependent glutamate release. If riluzole increases the threshold for excitation of presynaptic axons or somata, this would decrease the number of stimulated terminals, and hence decrease activity-dependent glutamate release; changes in action potential amplitude and waveform could decrease presynaptic Ca²⁺ influx, altering PPR. Similarly, spontaneous EPSC frequency depends on spontaneous firing of presynaptic neurons, which could also be decreased by riluzole. While the experi-
mements carried out here do not distinguish between these mechanisms potentially causing reduction of presynaptic transmitter release, it is worth noting that the dose range for reduction of glutamatergic EPSCs in previous studies is relatively low, with effects being apparent at 0.5 μM and maximal at 10 μM (He et al. 2002; Prakriya and Mennerick 2000). This is consistent with inhibition of presynaptic voltage-gated Na⁺ channels (either persistent or fast inactivating) (Huang and Trussell 2008; Leao et al. 2005), although inhibition of presynaptic voltage-gated Ca²⁺ channels cannot be ruled out as a contributing factor.

Evidence supporting a mechanism of postsynaptic reduction of ionotropic glutamate receptor responses is the finding that the amplitudes of evoked, spontaneous, and miniature EPSCs and of currents evoked by direct activation of ionotropic glutamate receptors were all reduced by riluzole. While reduction of evoked and spontaneous EPSC amplitude could be accounted for by decreased synaptic release of glutamate, decreased amplitude of miniature EPSCs and glutamate receptor currents cannot. It is worth noting that miniature EPSC amplitude reduction by riluzole, although statistically significant, was small, suggesting that direct modulation of synaptically activated glutamate receptors by riluzole may be a minor contributor to reduction of synaptic transmission; in contrast, while the reduction of glutamate receptor currents by riluzole was greater, the method of application used here is more likely to activate predominantly extrasynaptic glutamate receptors (Townsend et al. 2003). In addition, although some studies have reported that electrophysiological responses to glutamate receptor ligands were inhibited by riluzole (Centonze et al. 1998; Debono et al. 1993; Zona et al. 2002), this inhibition may be via indirect modulation of receptor function, as a number of radioligand binding studies have consistently failed to find any interaction of riluzole with the NMDA, glycine, or phencyclidine binding sites of NMDA glutamate receptors or the ligand binding site of non-NMDA glutamate receptors (Benavides et al. 1985; Debono et al. 1993; He et al. 2002).

While the effects of riluzole on the full range of mechanisms examined in HMs has not yet been carried out in spinal motor neurons innervating respiratory or limb muscles, recent studies of lumbar motor neurons from transgenic animal models of ALS have shown that these limb motor neurons are hyperactive from an early age, firing action potentials at a higher frequency (Kuo et al. 2004, 2005; Pambo-Pambo et al. 2009; Quinlan et al. 2011), and express a larger persistent Na⁺ (Kuo et al. 2004, 2005; Pambo-Pambo et al. 2009; Quinlan et al. 2011). The changes seen in HMs from transgenic animal models of ALS, which also receive more frequent spontaneous synaptic activity (van Zundert et al. 2008). All of these changes in motor neurons can be inhibited by therapeutic levels of riluzole, as demonstrated in the results here. The use of riluzole to suppress motor neuron hyperactivity during neuromotor development may therefore be a useful “proof of concept” experiment to determine whether motor neuron hyperactivity is a factor driving adult motor neuron death in ALS.

In conclusion, riluzole can reduce HM activity by inhibiting a range of distinct postsynaptic mechanisms involving Na⁺, Ca²⁺, and Ih ionic currents, as well as by decreasing glutamatergic neurotransmission through reducing presynaptic excitability and postsynaptic glutamate receptor currents. These multiple effects widen the possible range of “targets” in the efforts to better understand the pathogenesis of motor neuron loss in ALS, and to devise more effective therapeutic strategies.

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Author contributions: M.C.B. conception and design of research; M.C.B. performed experiments; M.C.B. analyzed data; M.C.B. interpreted results of experiments; M.C.B. prepared figures; M.C.B. drafted manuscript; M.C.B. edited and revised manuscript; M.C.B. approved final version of manuscript.

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