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

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Inhibitory effects of riluzole on hypoglossal motor neurons

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Abstract (250 words)

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 from hypoglossal motor neurons (HMs, n=25) in brainstem slices from 10-23 day old rats anesthetised with sodium pentobarbitone, to investigate the hypothesis that riluzole inhibited 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 I₉. 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 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.
Keywords

Persistent sodium current, excitatory synaptic transmission, hyperpolarization-activated cation current, ionotropic glutamate receptor, amyotrophic lateral sclerosis
Introduction

The only approved treatment for the fatal adult onset neurodegenerative disease, amyotrophic lateral sclerosis (ALS) is riluzole, which produces a small (2-4 months) but significant extension of life in ALS patients (Miller et al. 2007; Orrell 2010) and small increases in survival time and motor function in transgenic animal models (Bellingham 2011; Gurney et al. 1996; Gurney et al. 1998). However, the mechanism(s) by which riluzole has its therapeutic effect in ALS remains elusive, as riluzole has a wide spectrum of effects on factors controlling neuronal excitability (Bellingham 2011).

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 using 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,
amyotrophic lateral sclerosis (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 approximately 30-
40% of patients, these bulbar symptoms are the first seen during the inexorable
progression of ALS towards 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; Kühnlein 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; Smittkamp et al. 2010)
are also seen in transgenic animal models of ALS; HMs from transgenic models of
ALS show an early onset of hyper-excitability due to an increased persistent Na+
current density (van Zundert et al. 2008). HMs 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
It is not yet known why these mutations lead to motor neuron death. A number of non-genetic 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 (Berger et al. 1995; Rekling et al. 2000) (Ireland et al. 2012). The aim of this study was to directly compare effects of riluzole at a maximal clinically relevant dose on the range of factors controlling motor neuron excitability by electrophysiological recording from hypoglossal motor neurons.

Electrophysiological recordings of membrane potential, resistance, holding current, repetitive firing and single action potential properties, voltage-sensitive persistent Na\(^+\) and Ca\(^{2+}\) currents, evoked, spontaneous and miniature excitatory synaptic potentials and postsynaptic responses to glutamate were made using whole cell patch clamp techniques in rat hypoglossal motor neurons in *in vitro* brainstem slices from rats aged 10-23 days old, 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 \(\mu\)M, 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 1552 ng/ml, equivalent to 6.6 \(\mu\)M (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
progress or longer survival time (Groeneveld et al. 2003; Groeneveld et al. 2008). In
addition, CNS levels of riluzole are 4-6 fold higher than plasma/serum levels after
oral dosing in mice, rats and monkeys, and high CNS levels persist for many hours
following 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: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; Milane et al. 2009). Riluzole concentration in the human brain following oral
administration is not known; however, cortical excitability is altered for up to 24
hours after a single dose of riluzole, even though plasma levels have dropped to
approximately 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. Based on 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 hypoglossal motor neurons 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 which control resting membrane potential,
action potential firing and threshold. These data should be of interest to both to
neurophysiologists investigating the control of motor neuron excitability as well as to researchers and clinicians interested in understanding the treatment of ALS.
Materials and Methods

Anaesthesia 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 sodium pentobarbitone (100 mg/kg) and then killed by rapid decapitation. The brainstem was removed and placed in ice-cold Ringer solution (see below for composition) in which NaCl was iso-osmotically replaced with sucrose (Bellingham and Berger 1996). Transverse slices (thickness 300 µM) were cut with a vibratome (DSK Microslicer, Dosaka Instruments, Japan). Slices were incubated for 50-70 minutes in this sucrose-containing solution at 34-37°C, 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 using in vitro brainstem 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/minute 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 100W 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, displaying it on a video monitor (Sony). HMs were identified visually by their location within the hypoglossal nucleus, their size, shape and by 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), using patch electrodes pulled from borosilicate glass capillaries (Vitrex Modulohm, Denmark) on a two-stage puller (Narishige, Japan) 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 using a Multiclamp amplifier to avoid distortion of rapid potential changes and using 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, Foster City, CA) was used to apply voltage commands, record whole-cell currents, and to measure responses.

Excitatory postsynaptic potentials (EPSCs) were evoked by electrical stimulation (5-120 V, 0.05-0.2 ms, 0.1 Hz) of the reticular formation lateral to the border of the ipsilateral hypoglossal nucleus with an insulated bipolar platinum wire electrode (Frederik Haer, Bowdoin ME). HMs were voltage-clamped at membrane potentials of -60 mV or more negative, and the stimulus intensity adjusted to give a first EPSC of consistent amplitude >150 pA (range -155 to -566 pA). In all HMs studied, stimulus intensity was >4× 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 sub-threshold 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+ current recordings in conditions designed to reduce or eliminate voltage-gated
Ca2+ and K+ currents contained (in mM): 130 CsCl; 10 NaCl; 0.001 CaCl2; 10 Cs 4-
(2-hydroxyethyl)-1-piperazineethanesulfonic 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 sulphate; 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) were
added to all external recording solutions to block inhibitory synaptic currents; at this
concentration, strychnine blocks both glycine and GABA_A receptors expressed by
HMs (O'Brien and Berger 1999). Liquid junction potentials were <1 mV and was not
corrected for in the results.
Drugs and chemicals were obtained from Sigma Aldrich (Castle Hill NSW Australia) or ICN (K methyl sulphate) 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 concentrations given in the text; vehicle concentrations did not exceed 0.1% and application of maximal vehicle concentration alone did not alter HM responses. Bath application of drugs was always for >2 minutes; the time taken to completely exchange the recording chamber solution was <40 seconds. Drugs were applied to only one HM per brainstem slice, as prolonged (40-60 minutes) washing after riluzole application did not return measured parameters to control levels.

Data Analysis

Evoked EPSC amplitude, holding current and input resistance were measured for individual responses, using 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 to 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, 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 ≥2 blocks of continuously acquired data of 120 seconds duration at gains of 10-20x, then detected and analysed 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 which 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 analysed 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 inter-event 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 minutes 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 2x 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 are expressed as mean ± 95% confidence intervals (CI) unless otherwise stated, and changes in measured parameters were determined to be statistically significant at P < 0.05 by a paired two tailed t test, except where indicated, using Prism 5 (Graphpad, Sorrento CA). Changes in the shape of spontaneous and miniature EPSC cumulative frequency distributions were tested for significance by the two sample Kolmogorov-Smirnov test (Press et al. 2007).
Results

Riluzole hyperpolarizes hypoglossal motor neurons by decreasing an inward membrane current active at resting membrane potential

In current clamp recordings from 9 HMs, bath application of riluzole (20 μM) caused a small but significant membrane hyperpolarization (Figure 1A 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); 1 of 9 HMs tested did not hyperpolarize with riluzole application. In 6 HMs voltage clamped at -65 mV, riluzole caused a significant outward shift in holding current (Figure 1B) from -268 to -159 pA (mean change +110 pA, 95% CI of +55 to +164 pA, P=0.0035, 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 (Figure 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 2A1 shows an example of the current evoked by a ramp protocol (Figure 2A2, consisting of a 2 second ascending ramp from -70 to 0 mV and a 2 second 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 figure 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 Figure 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 7 HMs recorded with a potassium methyl sulphate-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 I-V trace
Figure 2B) showed voltage-dependent activation of an inward current from -70
onwards, with peak inward current at approximately -30 mV.

As riluzole may inhibit also voltage-gated Ca²⁺ and some K⁺ currents active in
this potential range (reviewed in Bellingham 2011), recordings were also made from 8
HMs using a caesium 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 (Figure 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 Figure 2C; with no external Cd\textsuperscript{2+} 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 of -11.8 pA/pF (95% CI from -3.9 to -19.8) at -20 mV; with external Cd\textsuperscript{2+} present, the mean current density at -40 mV was not significantly altered (mean of -7.7 pA/pF, 95% CI -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 Cd\textsuperscript{2+} present suggests that high voltage-gated Ca\textsuperscript{2+} channels sensitive to riluzole were also active in this voltage range (Lamanaukas and Nistri 2008; Umemiya and Berger 1994). Taken together, these results indicate that riluzole inhibits both voltage-dependent persistent Na\textsuperscript{+} and Ca\textsuperscript{2+} currents in rat HMs. As a riluzole-sensitive inward current, most likely the persistent Na\textsuperscript{+} 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 (I\textsubscript{H}) 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 which returns membrane potential to a resting level at which I\textsubscript{H} activation is minimal. Effects of riluzole on I\textsubscript{H} have not been previously reported. I\textsubscript{H} was activated by a family of long (1 second) hyperpolarizing voltage steps from -60 mV to -120; the voltage dependence of I\textsubscript{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 -85 mV following the hyperpolarizing voltage step (Bayliss et al. 1994). Figure 2C shows an example of the slowly activating inward current evoked by a voltage step to -120 mV in control conditions and after bath application of riluzole. Riluzole decreased $I_{H}$ amplitude; this was due to a shift in the $I_{H}$ voltage activation curve for this HM to more negative voltages (Figure 2D; the voltage for 50% activation ($V_{50}$) of $I_{H}$ decreased from -96 to -104 mV, without change in the slope value). In 6 HMs, riluzole significantly decreased mean $I_{H}$ $V_{50}$ (from -100.5 to -105.2 mV; mean change of -4.7 mV, 95% CI 0.2 to 9.1 mV; $P=0.042$, paired two 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 (Figure 3A) increased in frequency rapidly up to approximately 40 pA current injection and then increased more modestly up to 100 pA current injection (Figure 3B). Riluzole markedly reduced action potential firing over the range of injected currents (Figure 3A and B, $n=9$ HMs) and significantly reduced the maximum number of action potentials evoked by current injection (Figure 3C) from 11 to 2 action potentials (mean difference of 9 action potentials, 95% CI 3 to 15, $P=0.01$, paired two tail t test).

Current injection in the sub-threshold range also elicited a lower steady state membrane potential in the presence of riluzole (Figure 3A).
Riluzole also significantly changed action potential shape, reducing and slowing single action potentials (Figure 3D). Mean action potential amplitude was significantly decreased from 83 to 68 mV (Figure 3E, mean change -15 mV, 95% CI -8 to -22 mV, n=9, P=0.0012, paired two tail t test) and maximal rise slope was also significantly decreased from 151 to 99 mV/ms (Figure 3E, mean change -52 mV/ms, 95% CI -28 to -75 mV/ms, P= 0.0009, paired two tail t test). Riluzole significantly increased action potential half-width from 0.95 to 1.1 ms (Figure 3F, mean change -0.15 ms, 95% CI -0.06 to -0.18 ms, P=0.037, paired two tail 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 9 HMs (Figure 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 two tail t test). This increase in rheobase current was due to a positive shift in action potential threshold, from -44 to -38 mV (Figure 4A, mean change of +6 mV, 95% CI +3 to +9 mV, P=0.0013, paired two tail t test). An example of increased threshold for firing is shown in Figure 4B, where the action potential is initiated at a more depolarized membrane potential in the presence of riluzole, compared to the level required in control conditions with the same current injected; the differentiated membrane potential trace below shows the method used for determining threshold voltage.

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 (Figure 4 C, mean change of +2 mV, 95% CI -2 to +5 mV, P=0.23) or decay time constant of the action potential afterhyperpolarization (Figure 4 C, 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 analysing 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 normalise membrane current for HMs of differing size) plotted against the rheobase current measured in the same 7 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, two 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 due to 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 6 HMs voltage-clamped at -65 mV, paired glutamatergic EPSCs (Figure 5C) evoked by
electrical stimulation (inter-stimulus 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 Figure 5A, the amplitude of the first evoked EPSC was significantly reduced by 28% (95% CI 17 to 39%, P=0.012, paired two tail 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 two tail t test). The paired pulse ratio (2nd EPSC/1st EPSC amplitude) also decreased significantly (Figure 5B, P=0.02, paired two tail 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 paired pulse ratio 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. Firstly, recordings of spontaneous glutamatergic EPSCs (Figure 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 8 HMs, the mean amplitude of spontaneous EPSCs (Figure 6B) was significantly reduced by riluzole (mean reduction of -3.6 pA, 95% CI -7.1 to -0.2 pA, P=0.042, paired two tail 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 (Figure 6C); the mean cumulative frequency distribution of spontaneous EPSC amplitudes was
significantly different to the control spontaneous EPSC amplitude distribution (two sample Kolmogorov-Smirnov test, \( P<0.001 \)). The instantaneous frequency (the reciprocal of the inter-event interval) of spontaneous EPSCs was also significantly decreased by riluzole application (Figure 6D, mean change of -2.1 Hz, 95% CI -4 to -0.3 Hz, \( P=0.027 \), paired two tail t test), although the mean spontaneous EPSC frequency (the number of EPSCs divided by the recording time in seconds) was not significantly changed (Figure 6D). The decrease in the instantaneous frequency of events was due to a lengthening of the inter-event interval, as shown by a significant change in the cumulative frequency distribution of intervals to larger values (Figure 6E, \( P<0.001 \), two 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 using recording of quantal miniature glutamatergic EPSCs in the presence of external Cd\(^{2+} \) (100 \( \mu M \)) to block presynaptic Ca\(^{2+} \) influx (Doze et al. 1991; Scanziani et al. 1992) and hence block presynaptic activity-dependent glutamate release (Figure 7A). If riluzole reduced the probability of quantal transmitter release, this should be evident as a decrease in the frequency of miniature EPSCs, while a reduction in postsynaptic response to glutamate should be evident as a decrease in miniature EPSC amplitude. In 6 HMs, riluzole caused a small but significant reduction in miniature EPSC amplitude; while mean miniature EPSC amplitude was not significantly changed (Figure 7B, \( P=0.78 \)), the mean cumulative
frequency distribution of miniature EPSC amplitude changed significantly, with more small amplitude events in the presence of riluzole (Figure 7C, P=0.003, two 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 (Figure D, P=0.23) or on the mean cumulative frequency distribution of inter-event intervals (Figure 7E, P=0.37, two 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 paired pulse ratio of evoked EPSCs supports the hypothesis that riluzole acts to pre-synaptically 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 L-glutamic acid (500 μM) dissolved in the external bathing solution was placed close to the soma of 3 different recorded HM, and a puff of L-glutamate was directly applied to the soma by pressure injection (Figure 8A). The inward current evoked by local glutamate application was significantly reduced by bath-applied riluzole (Figure 8A and B, 20 μM), which caused a mean reduction of 44% (P=0.046, paired two tail 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 two tail t test) by bath application of
NBQX and DL-APV to block non-NMDA and NMDA glutamate receptors respectively (Figure 8A and B), demonstrating that the current evoked by pressure application of L-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 months 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 (Kiernan 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 μM, which include inhibition of the persistent and fast inactivating voltage-gated Na⁺ currents, inhibition of repetitive firing, potentiation of Ca²⁺-dependent K⁺ currents, inhibition of neurotransmitter release and inhibition of voltage-gated Ca²⁺ 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 which 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⁺, Ca²⁺ and I₇₅ ionic currents, with the last finding being the first report of the effects of riluzole on I₇₅. 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 is 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 paired pulse ratio, 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; Zoccolella et al. 2006), and up to 60% of older ALS patients (Zoccolella et al. 2006), while respiratory deficits due to loss of phrenic and intercostal spinal respiratory motor neurons almost uniformly occurs 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 limb (5%) symptoms (Fujimura-Kiyono et al. 2011). The loss of HMs in ALS is thus likely to occur relatively early in disease progression compared to other respiratory MNs, 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 μM) 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 μM in some subjects (Groeneveld et al. 2003). Furthermore, pharmacokinetic studies in mice, rats and monkeys indicate that riluzole concentration in brain tissue is 4-6 times higher fold higher than peak plasma concentration following a single dose of riluzole (Colovic et al. 2004; Martinet et al. 1997) (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 hours, and repeated dosing causes accumulation of riluzole to approximately 60% greater concentration than is reached following a single dose (Milane et al. 2009; Wu et al. 2013). The factors causing a high brain:血 ratio of riluzole are unclear. Riluzole is highly lipid soluble, with an octanol:water partition coefficient of 3000:1 (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; Milane et al. 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 a high and persistent brain:blood ratio also occurs in humans, as a single dose of riluzole produces changes in cortical excitability up to 24 hours after administration, as a time point when plasma concentration has decreased to approximately 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 10-20 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 non-spiking, 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 approximately 100 pA and an increase in input resistance of approximately 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 which do not readily inactivate and is one of
the few currents active in the sub-threshold range from resting membrane potential to threshold (Alzheimer et al. 1993; Carter et al. 2012; Crill 1996). In this voltage range, the persistent Na⁺ current can be activated by EPSPs and inactivated by IPSPs, with the functional effect of amplifying both types of synaptic input (Carter et al. 2012; Stuart 1999; 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 sub-threshold voltage changes which will continually activate and inactivate Na⁺ channels.

However, a novel effect of riluzole on the Iᵢᴴ current was also revealed, as riluzole shifted the voltage-dependent activation of the Iᵢᴴ current to more negative levels. Iᵢᴴ plays an important role in setting HM resting membrane potential, as transgenic deletion of the HCN1 gene encoding an Iᵢᴴ isoform strongly expressed in mouse HMs results in significant hyperpolarization of resting membrane potential (Chen et al. 2009), while positive shifts in Iᵢᴴ activation causes 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⁺ and Iᵢᴴ currents were hyperpolarized by either riluzole or Iᵢᴴ blockade with ZD7288, but direct effects of riluzole on Iᵢᴴ were not tested (Darbon et al. 2004). The membrane hyperpolarization is certainly consistent with a negative shift in Iᵢᴴ activation; however, membrane hyperpolarization was associated with an increase in input resistance. As Iᵢᴴ is minimally active at resting membrane potential, a change in steady state input resistance is less likely to occur with Iᵢᴴ 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⁺ and Iᵢᴴ currents 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 motor neuron firing (Harvey et al. 2006a; Harvey et al. 2006b; Kuo et al. 2006; Miles et al. 2005; Quinlan et al. 2011; Theiss et al. 2007) 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$^+$ current (Cramer et al. 2007; Harvey et al. 2006a; Harvey et al. 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 μM (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$_{50}$ of 0.5-1 μM) and of persistent Na$^+$ current (EC$_{50}$ of 1.8-2 μM) 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 current, without any significant increase in the action potential afterhyperpolarization, thus reinforcing the conclusion that the persistent Na$^+$ current plays an important role in regulating repetitive firing of motor neurons.

In addition, detailed kinetic modelling of voltage-gated Na$^+$ channels has highlighted an important role for the persistent Na$^+$ 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\(^+\) current and action potential initiation, in that action potential rheobase current decreases as persistent Na\(^+\) 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\(^+\) 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\(^+\) current by riluzole at <20 \(\mu\)M has been described in brainstem 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) Ca\(^{2+}\)-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 reduces HM repetitive firing frequency (Viana et al. 1993). Despite reports that riluzole potently enhances SK Ca\(^{2+}\)-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 Ca\(^{2+}\) current in HMs, and this effect might be expected to reduce Ca\(^{2+}\) influx during the action potential and, in turn, reduce Ca\(^{2+}\)-dependent K\(^+\) currents. Indeed, one effect of blocking Ca\(^{2+}\) influx is an increase in action potential half-width (Viana et al. 1993), similar to that seen here. The possibility that potentiation of Ca\(^{2+}\)-dependent K\(^+\) current by riluzole is counterbalanced by decreased Ca\(^{2+}\) 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 which showed that riluzole inhibited an number of glutamate receptor-dependent neural 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 have 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 0.5-10 μM) reduces presynaptic excitability (He et al. 2002; MacIver 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 between these studies, and no single study has previously measured the effect of riluzole on evoked EPSC amplitude and paired pulse ratio, spontaneous and miniature EPSC amplitude and frequency, and postsynaptic glutamate receptors. The paired pulse ratio 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; MacIver 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 (Lamanauskas 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 glutamate 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 paired pulse ratio and the decreased frequency of spontaneous, but not miniature, EPSCs with riluzole application. As paired pulse ratio 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 hypothesised 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 Ca^{2+}-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 Ca\(^{2+}\) influx, altering paired pulse ratio. Similarly, spontaneous EPSC frequency depends on spontaneous firing of presynaptic neurons, which could also be decreased by riluzole. While the experiments 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 Ca\(^{2+}\) channels cannot be ruled out as a contributing factor.

Evidence supporting a mechanism of postsynaptic reduction of ionotropic glutamate receptor responses is 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 extra-synaptic 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 hyper-active from an early age,
firing action potentials at a higher frequency (Kuo et al. 2004; Kuo et al. 2005;
Pambo-Pambo et al. 2009; Quinlan et al. 2011) and express a larger persistent Na⁺
current (Kuo et al. 2004; Kuo et al. 2005; Quinlan et al. 2011). These changes are
also 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 Iᵢᵣ ionic currents, as well as by
decreasing glutamatergic neurotransmission by 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.
Figure legends

Figure 1. Hyperpolarization of HMs by riluzole is due to a decrease in an inward current active at resting membrane potential. A. Example traces of HM resting membrane potential before and after riluzole (20 μM, at arrow); the frequent upward deflections before riluzole are spontaneous EPSPs, which are markedly suppressed by riluzole. B. Resting membrane potential was significantly hyperpolarized (left axis, scatter plot of resting membrane potential for 9 HMs before (filled circles) and after riluzole (20 μM; open circles) was bath applied; mean of all cells ± 95% confidence interval is shown superimposed on this and all other scatterplots of individual cell data) and an outward membrane current was elicited (right axis, holding current at -65 mV before and after riluzole application for 6 HMs, as for left axis). C. HM input resistance, measured from a small hyperpolarizing voltage or current step in 15 HMs, is increased by riluzole (20 μM). All changes were significant (paired two-tailed t test). Significance is shown as *P<0.05, **P<0.01, ***P<0.001 in this and all other graphs; see text for exact P values.
Figure 2. Riluzole inhibits inward currents due to voltage-dependent persistent Na\(^+\) and Ca\(^{2+}\) currents, and the hyperpolarization-activated cationic current (I\(_{\text{H}}\)).

A. Currents activated by a voltage protocol with a slow (2 seconds) ascending and descending voltage ramp from -70 mV to 0 and back (trace 2); trace 1 shows currents before (gray) and after (black) riluzole (20 \(\mu M\)) and the riluzole-sensitive current (trace 3; digital difference between control and riluzole traces for this HM) shows marked hysteresis for the current in the ascending and descending ramp phases. The current trace in riluzole has been digitally scaled to account for changes in neuronal input resistance (see figure 1B).

B. Mean riluzole-sensitive current density recorded during the ascending voltage ramp from -70 to 0 mV, with a potassium methyl sulphate electrode solution (black trace, average of 7 HMs) in the absence of external Cd\(^{2+}\), or with a caesium chloride electrode solution with 100 \(\mu M\) CdCl\(_2\) in the bathing solution (gray trace, average of 5 HMs); the current density normalizes for HM membrane area by dividing the recorded current (pA) by the HM whole cell capacitance (pF). Note that a riluzole-sensitive inward current began activation from -65 mV onward, but that there was a marked reduction in riluzole-sensitive inward current from -40 onwards in the presence of external Cd\(^{2+}\) to block voltage-gated Ca\(^{2+}\) currents.

C. The mean normalized current density at an ascending ramp voltage of -40 mV (circles) and -20 mV (triangles) for 7 HMs (open symbols) recorded with potassium methyl sulphate electrode solution in the absence of external Cd\(^{2+}\) and 8 HMs (filled symbols) recorded with a caesium chloride electrode solution with 100 \(\mu M\) CdCl\(_2\) in the bathing solution; the current density at -40 mV was not significantly different for the two recording situations, but was significantly decreased at -20 mV in the presence of external Cd\(^{2+}\), indicating that riluzole inhibited voltage-gated Ca\(^{2+}\) channels.

D. Example traces showing I\(_{\text{H}}\) current evoked by a voltage step from -60 to
-120 mV (lower trace) before (gray trace) and after (black trace) riluzole (20 μM).  E. Example of voltage-dependent activation curves for $I_{\text{H}}$ in a single HM before (circles) and after (open circles) riluzole, with Boltzmann curves fitted to data; $V_{50}$ (horizontal dashed line) has shifted to a more negative voltage after riluzole (compare two vertical dashed lines).  F. $V_{50}$ (left axis) and slope (right axis) values for 6 HMs, calculated from fitted Boltzmann curves before and after riluzole, showing a significant (P<0.05) negative shift in $V_{50}$, and no significant change in slope.
Figure 3. Riluzole inhibits repetitive action potential firing, decreases action potential amplitude and rise slope and increases half width. A. Depolarizing current steps (upper traces) elicited steady state membrane depolarization in the sub-threshold range and repetitive action potential firing in the supra-threshold range (gray lower traces). Riluzole (20 μM) decreased steady state sub-threshold membrane depolarization and markedly decreased repetitive firing, but did not abolish action potential generation at the current step onset (black traces). B. The number of action potentials generated with different current injections increased rapidly up to 40 pA, then increased more modestly up to 100 pA; riluzole reduced the number of action potentials for all current injections (n=10 HMs up to 70 pA, then 5 to 7 HMs for greater current injections). C. The maximum number of action potentials elicited by current injection markedly and significantly decreased with riluzole application in all HMs tested (n=9). D. An example of single action potentials before (gray trace) and after (black trace) riluzole (same HM shown in A). E. Action potential amplitude and maximum rise slope before and after riluzole, showing a significant decrease in both parameters (P<0.01 and <0.001 respectively, n = 9 HMs). F. Action potential half-width significantly increased after riluzole (P<0.05, n = 9 HMs)
Figure 4. Riluzole (20 μM) depolarizes action potential threshold and increases rheobase current, but does not alter the medium after-hyperpolarization. A. The threshold potential for triggering an action potential (left axis) and the minimal current (rheobase) injection required for firing (right axis) were both significantly increased (P<0.01 and < 0.05 respectively, n=9) by riluzole. B. Example traces, showing that the threshold potential (dashed gray line) for the first action potential (upper gray trace) elicited by current injection in control conditions has shifted to a more positive level (dashed black line) for the action potential (upper black trace) elicited by the same current injection with riluzole. Lower traces show the differentiated membrane potential, as used to determine threshold potential. C. Riluzole does not significantly change the amplitude or decay time constant of the medium after-hyperpolarisation following an action potential. D. A strong inverse correlation between rheobase current and persistent Na⁺ current density exists in HMs. Rheobase current in control condition is plotted against the peak persistent Na⁺ current density (the peak current/divided by the whole cell membrane capacitance) for 7 HMs in which both parameters were measured. Linear regression (line ± 95% confidence intervals) found a significant correlation (R² = 0.76) between the parameters.
Figure 5. Evoked EPSC amplitude and paired pulse ratio is decreased by riluzole (20 μM). A. Mean amplitude of the first and second EPSCs evoked by electrical stimulation was significantly decreased by riluzole. B. Paired pulse ratio for the evoked EPSCs was significantly decreased by riluzole (P<0.05, n = 6 HMs). C. Example traces of paired evoked EPSCs before (gray trace) and after (black trace) riluzole.
Figure 6. Spontaneous EPSC amplitude and instantaneous frequency were reduced by riluzole. A. Example traces of spontaneous EPSCs before (left) and after (right) riluzole; an open circle marks each EPSC. B. Mean spontaneous EPSC amplitude was significantly reduced by riluzole (P<0.05, n = 6 HMs). C. Riluzole significantly shifted the mean cumulative distribution curve for spontaneous EPSC amplitude to lower amplitudes. D. Riluzole decreased mean instantaneous frequency significantly (P<0.01, but mean frequency was not significantly altered. E. Riluzole significantly shifted the mean cumulative distribution curve for spontaneous EPSC inter-event interval (the reciprocal of instantaneous frequency) to longer durations.
Figure 7. Riluzole reduced miniature (quantal) EPSC amplitude but did not alter miniature EPSC frequency. A. Example traces of miniature EPSCs before (left) and after (right) riluzole; an open circle marks each EPSC. B. Mean miniature EPSC amplitude was not significantly changed by riluzole (P<0.05, n = 6 HMs). C. Riluzole significantly shifted the mean cumulative distribution curve for miniature EPSC amplitude to lower amplitudes. D. Riluzole did not change mean or instantaneous miniature EPSC frequency significantly. E. Riluzole did not significantly shift the mean cumulative distribution curve for miniature EPSC inter-event interval to longer durations.
Figure 8. Riluzole decreased currents evoked by brief direct application of glutamate
to HMs to activate postsynaptic ionotropic glutamate receptors. A. Example traces
showing inward current evoked by brief pressure (dark bar) injection of glutamate
onto a single HM soma in control conditions (light gray trace), after addition of
riluzole (black trace) and after addition of NBQX and DL-APV (dark gray trace) to
block non-NMDA and NMDA glutamate receptors; note that riluzole reduced peak
current amplitude, and NBQX/DL-APV abolished the current (cf. steady state
baseline current indicated by dashed line). B. Mean peak glutamate receptor current
was reduced significantly by riluzole (P<0.05) and by NBQX/DL-APV (P<0.05, n=3
HMs).
Appendix

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Author Contributions

MCB designed and carried out experiments, analyzed data and wrote the manuscript.
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