Effect of prolonged riluzole exposure on cultured motoneurons in a mouse model of ALS

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Schuster JE, Fu R, Siddique T, Heckman CJ. Effect of prolonged riluzole exposure on cultured motoneurons in a mouse model of ALS. J Neurophysiol 107: 484–492, 2012. First published October 19, 2011; doi:10.1152/jn.00714.2011.—Riluzole is the only FDA-approved drug to treat amyotrophic lateral sclerosis, but its long-term effects on motoneurons are unknown. Therefore, we treated primary mouse spinal cord cultures with 2 μM riluzole for 4–9 days and then used whole cell patch clamp to record the passive and active properties of both wild-type and SOD1G93A motoneurons. At this concentration, riluzole blocks >50% of the sodium component of a persistent inward current that plays a major role in determining motoneuron excitability. Prolonged riluzole treatment significantly decreased the amplitude of the persistent inward current. This effect was specific for SOD1G93A motoneurons, where the amplitude decreased by 55.4%. In addition, prolonged treatment hyperpolarized the resting membrane potential as well as the voltage onset and voltage maximum of the persistent inward current (~2–3 mV in each case). These effects appeared to offset one another and resulted in no change in the firing properties. In a subset of cells, acute reapplication of 2 μM riluzole during the recording decreased repetitive firing and the persistent inward current, which is consistent with the normal effects of riluzole. The downregulation of the persistent inward current in response to prolonged riluzole administration is in contrast to the strong upregulation of this same current after descending neuromodulatory drive to the cord is lost following spinal injury. This dichotomy suggests that decreased activation of G protein-coupled pathways can induce upregulation of the persistent inward current that plays a major role in determining motoneuron excitability; amyotrophic lateral sclerosis; superoxide dismutase 1

AMYOTROPHIC LATERAL SCLEROSIS (ALS) is a multifactorial disease that prematurely kills motoneurons by both cell autonomous and nonautonomous mechanisms (Boilée et al. 2006; Ilieva et al. 2009). Riluzole, the only FDA-approved drug to treat ALS, extends survival by 2–3 mo but does not confer lasting protection (Bensimon et al. 1994; Lacomblez et al. 1996; Traynor et al. 2003). The effects of acute riluzole administration in animal preparations at therapeutically relevant doses include decreasing persistent voltage-gated sodium currents, potentiation of a calcium-dependent potassium current, and inhibition of neurotransmitter release (Bellingham 2011). These effects all act to decrease motoneuron excitability, and thus riluzole may act by limiting excitotoxicity (Bellingham 2011; Van Damme et al. 2005; Van Den Bosch et al. 2006).

It is unclear, however, how or if motoneurons adapt when riluzole is present for days or weeks during treatment. In other dissociated culture systems, decreases in network excitability lead to compensatory synaptic scaling (Ehlers 2003; Turrigiano et al. 1998) or the appearance of intrinsic bursting (Turrigiano et al. 1995). Furthermore, in spinal cord-injured (SCI) rats, motoneurons initially have diminished excitability due to loss of the descending monoaminergic input that is essential for facilitating the motoneuron persistent inward current (PIC) (Harvey et al. 2006b). With time, however, both the sodium and calcium components of the PIC (NaPIC and CaPIC, respectively) are reestablished and motoneuron excitability increases (Harvey et al. 2006b; Li and Bennett 2003). Therefore, we hypothesized that, although acute application of riluzole decreases motoneuron excitability, prolonged treatment with 2 μM riluzole may lead to compensatory mechanisms that reestablish motoneuron excitability. An important consideration, however, is riluzole’s mechanism of action. Riluzole suppresses the NaPIC by binding directly to the sodium channel (Benoit and Escande 1991; Hebert et al. 1994), whereas the loss of monoaminergic input stops PIC facilitation via a failure of the monoamines to activate G protein-coupled receptors. The ability for the PIC to adapt during direct channel block versus loss of monoaminergic input may therefore be very different.

We investigated how prolonged exposure to riluzole affects motoneuron NaPICs and other intrinsic properties. If the PIC is very plastic and adapts whenever it is inhibited, we would expect to see an upregulation of the current after prolonged riluzole exposure, which could be responsible for the loss of riluzole’s therapeutic action in ALS. Our results however, showed that the PIC amplitude remained the same [wild type (wt)] or decreased (SOD1G93A) after prolonged riluzole administration, suggesting that the NaPIC’s capacity for adaptation may be largely confined to its neuromodulatory control system via G protein-coupled receptors and that direct channel block is not effective.

MATERIALS AND METHODS

All procedures were approved by the Northwestern University Animal Care and Use Committee.

Mice. All mice were bred and maintained in barrier facilities. Nontransgenic female mice were mated with transgenic males expressing the human superoxide dismutase 1 protein with a glycine to alanine mutation at codon 93 (SOD1) (high expressor line). Embryos
were genotyped for SOD1 expression with standard PCR protocols after data collection and analysis (Rosen et al. 1993). Briefly, 20–25 mg of tissue was used for the DNA extraction. The primers for amplification were SOD1P7: CAT CAG CCC TAA TCC ATC TGA and SOD1P8: GCC GAC TAA CCA TCA AAG TGA. All breeding, maintenance, and genotyping were done by the Sidiqline laboratory.

**Cell culture.** Spinal cords were removed from embryonic day 12–14 mice and dissociated as previously described (Kuo et al. 2005). Dissociated cells were plated on glass coverslips coated with poly-D-lysine (Sigma, St. Louis, MO). The basic medium contained Neurobasal A medium (Invitrogen, Carlsbad, CA), 2 ml of B27 supplement (Invitrogen), 1 mM L-glutamine (Sigma), 1 mM of penicillin/streptomycin (Invitrogen), and 2 mg/ml glucose (Sigma). The plating medium contained the basic medium plus 15% heat-inactivated horse serum. The plating medium was changed after 2 days to the maintenance medium, which contained the basic medium plus 20 ng/ml nerve growth factor (Invitrogen). The maintenance medium was changed every 2–3 days. In the riluzole drug condition, 2 μM riluzole, dissolved in DMSO (final concentration 0.005%), was added to the maintenance medium after 7 days in culture. Riluzole is resistant to degradation in extreme heat and changes in pH. It is susceptible to oxidation and was degraded by 20% after 12 h in 1% H2O2, but we view this as a very extreme condition not encountered during this study (Kumari et al. 2009). Cells were recorded from between 12 and 16 days in culture. Vehicle control cells treated only with DMSO (N = 16) showed no significant differences and were included in the control group.

**Recording solutions.** Electrodes were between 3 and 5 MΩ in resistance and contained (mM) 145 K-gluconate, 0.1 CaCl2, 1.1 EGTA, 5.0 HEPES, 2.0 MgCl2, and 5.0 ATP-Mg2+ with a pH of 7.3 (all from Sigma). Artificial cerebrospinal fluid (aCSF) contained (mM) 126 NaCl, 26.2 NaHCO3, 1.0 NaH2PO4, 3.0 KCl, 1.5 MgSO4, 2.5 CaCl2, and 10 glucose. In all experiments 0.1 M picrotoxin (Sigma), 0.01 mM strychnine (Sigma), and either 0.01 mM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzof[f]isoquinoline-7-sulfonamide disodium salt (NBQX) (Tocris, Ellisville, MO) and 0.05 mM t-(-)-2-amino-5-phosphonomopentanoic acid (AP5) (Tocris) or 1.0 mM kynurenic acid (Sigma) were added to block synaptic currents. The aCSF was adjusted to a pH of 7.4 when bubbled with 95% O2-5% CO2. To block sodium channels, TTX was added to the aCSF as a 1 μM solution. In most experiments the aCSF did not contain riluzole. However, in some experiments riluzole was added to the aCSF as a 2 μM solution after all other measurements were recorded. All aCSF solutions were bath applied.

**Whole cell recordings.** Cells were transfected from the incubator to a recording chamber, and residual maintenance medium was washed out with riluzole-free aCSF for a minimum of 15 min before recording started. Selected neurons were large (20-μm diameter), multipolar, and had triangular cell bodies. Neurons identified by these selection criteria are consistently labeled by SM1-32, peripherin, acetylcholine, choline acetyltransferase, and calcitonin gene-related peptide, all markers of motoneurons in vivo (Carriedo et al. 1995, 1996). These criteria preferentially include large motoneurons while excluding small motoneurons. In the spinal cord, motoneurons are by far the largest neurons in vivo; nevertheless, it is possible that a small percentage of large interneurons are included in the sample. The exact developmental stage of these neurons is not known; however, they are thought to be relatively immature since they have not yet developed the CaPIC present in the late neonatal stage (Quinlan et al. 2011). Recordings were made at room temperature with the Multiclamp 700B amplifier (Axon Instruments, Union City, CA). In voltage-clamp mode, fast, slow, and whole cell capacitance transients were compensated for with the capacitance compensation in Multiclamp. Cells were discarded if the initial action potential (AP) overshoot was less than 20 mV, if the resting membrane potential (RMP) was greater than −40 mV or changed more than 5 mV, if access resistance was greater than 30 MΩ, or if input conductance changed more than 30%.

For riluzole to affect the cultured neurons, spontaneous activity must be present in the culture system. Therefore, in a number of experiments, spontaneous firing was recorded in aCSF without synaptic blockers. aCSF with synaptic blockers was used for all subsequent measures. In current-clamp mode, triangular current ramps were used to elicit repetitive firing (10 s total duration) and to produce firing frequency-current (FI) relationships. Depolarizing current steps were also used in some cells to determine the effects on firing behavior when riluzole was added to the aCSF. In voltage-clamp mode, current-voltage (IV) relationships were assessed with very slow triangular voltage ramps (9.4 mV/s) from −90 to −10 mV.

At the end of some whole cell recordings (N = 2), it was verified that riluzole could be washed out during the initial 15-min washout period at the beginning of the experiment. First, 2 μM riluzole was added to the aCSF, and firing was abolished or decreased to steady levels. The riluzole was then washed out of the recording chamber with normal aCSF, and the time until normal firing returned was recorded.

**Data analysis.** All data were collected at 20 kHz with a Power1401 (Cambridge Electronics Design). Cell capacitance was measured with the Multiclamp automatic whole cell capacitance compensation. All other data were analyzed off-line with Signal 4.01 software (Cambridge Electronics Design) with custom macros. Measurements were determined from the average of four traces. In current-clamp mode, AP overshoot (above 0 mV) and voltage and current recruitment thresholds were measured for the first AP elicited during a triangular current ramp. Voltage threshold was defined as the point when the voltage rate of rise exceeded 10 V/s. Current threshold was defined as the current input at the voltage threshold.

The FI relationship was obtained by plotting the instantaneous firing frequencies versus the intensity of the injected current during the ascending and descending portions of the current ramp. The ascending and descending FI gain were determined from the slope of the FI relationship. The difference between the current at AP recruitment (Irecr) and derecruitment (Idecr) (ΔI = Irecr − Idecr) was also measured.

In voltage-clamp mode, the input (leak) conductance was determined from the slope of the linear portion of the IV relationship taken between −80 and −70 mV. All other measurements were taken after leak subtraction. The PIC voltage onset, amplitude, and max voltage were measured on the ascending phase of the voltage ramp. Voltage onset was defined as the first negative current deflection. PIC amplitude was the maximum negative current, and the PIC max voltage was the voltage at this point.

The PIC is the result of an interaction between a voltage-gated sodium-based persistent inward current and TTX-insensitive outward currents. To determine how the sodium persistent inward current (NaPIC) and the TTX-insensitive current contribute to the PIC amplitude, 1 μM TTX was added to block voltage-gated sodium channels. The resulting IV relationship was labeled TTX insensitive (TTX-ins). After leak subtraction, the voltage onset of the TTX-ins outward current as well as its amplitude at the PIC max voltage were measured. The TTX-ins amplitude was measured at the PIC max voltage to determine how the TTX-ins current affected the PIC amplitude. The NaPIC voltage onset, amplitude, and max voltage could then be determined after subtracting the TTX-ins trace from the original PIC trace.

To determine whether riluzole retained the ability to decrease spontaneous repetitive firing and NaPIC amplitude after prolonged riluzole exposure, 2 μM riluzole was bath applied. The ability of the motoneuron to fire during triangular current ramps as well as during current steps was assessed. Because 2 μM riluzole has been shown to block ~50% of the NaPIC amplitude (Urbani and Belluzzi 2000), we wanted to compare the NaPIC amplitude before and after 2 μM riluzole was applied. After the initial measurements, 2 μM riluzole was applied and the resulting current trace was labeled +2 Ril. TTX (1 μM) was subsequently added to the aCSF to completely block the
sodium component of the PIC (see Fig. 4C). The NaPIC amplitude remaining after 2 μM riluzole was applied (+2Ril NaPIC) could then be determined by subtracting the TTX-ins current trace from the +2Ril current trace (see Fig. 4, C and D).

Statistics. All statistics were performed with SPSS 17.0 (SPSS, Chicago, IL). For all measures, 2 (drug: control, 2 μM riluzole) × 2 (genotype: wt, SOD1) factorial ANOVAs were used to determine significance (P > 0.05). Two measures, PIC amplitude and NaPIC onset, were nonparametric. For these measures, the natural log was taken, followed by an ANOVA. Since the statistical results were nearly identical to the nonnormalized data, the nonnormalized means and standard deviations are listed in Table 2. Individual t-tests with Bonferroni adjustments were used to determine pairwise differences if interactions were significant.

RESULTS

Wild type versus SOD1. Previous studies using a similar preparation have shown that SOD1 motoneurons are hyperexcitable compared with wt cells (Kuo et al. 2005; Pieri et al. 2009; van Zundert et al. 2008). We also saw signs of increased excitability. For instance, the AP current threshold was significantly decreased in SOD1 neurons (wt 27.5 ± 48.4 pA vs. SOD1 13.7 ± 34.2 pA), indicating that less excitatory input was needed to initiate AP firing in SOD1 neurons. There was also a significant decrease in input conductance (wt 2.8 ± 1.6 nS vs. SOD1 2.3 ± 1.3 nS) and hyperpolarization of the PIC onset (wt −44.3 ± 5.3 mV vs. SOD1 −45.9 ± 3.5 mV) for SOD1 neurons.

Both PIC onset and input conductance can affect AP current threshold. To determine whether the difference in AP current threshold between wt and SOD1 neurons was due to the difference in one or both of these parameters, AP current threshold was first normalized by PIC onset and input conductance separately and reanalyzed. A significant difference in AP current threshold remained between wt and SOD1 neurons when normalized by either parameter. We then normalized AP current threshold by both PIC onset and input conductance and reanalyzed. After normalization by both measures, AP current threshold was no longer significantly different between wt and SOD1 neurons. Therefore the difference in AP current threshold was dependent on both measures.

We also observed a novel difference between wt and SOD1 motoneurons in the remaining outward current after TTX application (hereafter referred to as TTX-ins). In SOD1 neurons, the TTX-ins outward current had a more depolarized voltage onset and smaller amplitude compared with wt neurons (for example, Fig. 1A). The onset of the TTX-ins outward current was nearly 7 mV more depolarized in SOD1 neurons (wt −53.7 ± 11.8 mV vs. SOD1 −46.9 ± 11.4 mV), and the TTX-ins amplitude measured at the PIC max voltage was significantly decreased by 13 pA. The difference in the TTX-ins amplitude, however, was not significant when normalized by conductance. This indicates that the smaller TTX-ins amplitude was a result of the lower SOD1 input conductance.

The genotype differences in the AP current threshold, PIC onset, input conductance, and TTX-ins current indicate an increase in the excitability of SOD1 motoneurons. In particular, these changes would increase the likelihood for a motoneuron to reach firing threshold during excitatory synaptic input, consistent with other studies showing an increase in neuron excitability (Kuo et al. 2005; Pieri et al. 2009; van Zundert et al. 2008). However, other measures of excitability such as the NaPIC and FI gain were unchanged (Table 1 and Table 2).

Influence of TTX-ins current on PIC. In cultured motoneurons, the PIC is a reflection of both the NaPIC and outward TTX-ins current. When the NaPIC is larger than the TTX-ins current, a downward current deflection is evident in the IV trace and the PIC can be measured. The amplitude of the PIC is therefore dependent on the balance between the NaPIC and TTX-ins amplitudes. In control conditions, the SOD1 motoneurons had a greater mean PIC amplitude than wt neurons, although this was not quite significant after Bonferroni adjustment (wt, control −36.8 ± 28.6 pA vs. SOD1, control −57.9 ± 58.7 pA) (for example, Fig. 1B, traces 1 and 2). Nearly two-thirds of this difference, however, could be accounted for by the smaller TTX-ins current in SOD1 motoneurons, indicating that the NaPIC was unchanged in SOD1 motoneurons.
As expected, the NaPIC amplitude was nearly identical in wt and SOD1 motoneurons (wt, control −74.7 ± 45.3 pA vs. SOD1, control −73.9 ± 41.8 pA) (for example, Fig. 1C). In conclusion, the TTX-ins current had an impact on the negative slope area of the PIC, but the total sodium component of the PIC was not altered in SOD1 versus wt motoneurons.

Control versus riluzole treatment. To test whether chronic riluzole treatment paradoxically increases intrinsic motoneuron excitability in response to prolonged hypocapitability, we treated our cells with 2 μM riluzole for 4–9 days before recording their electrical properties. For riluzole to have an effect, it was necessary for our cell culture system to be spontaneously active. To ensure that spontaneous firing was present in our cell culture system, aCSF without synaptic blockers was bath applied for a subset of neurons (N = 54) and spontaneous activity was recorded. Spontaneous firing was present in 41 of 54 neurons, and excitatory and/or inhibitory postsynaptic currents were visible in 11 of 13 of the neurons in which no APs were present. There was no difference in spontaneous activity between wt and SOD1 or between drug-treated and untreated neurons (Table 1).

Prolonged treatment with 2 μM riluzole had several main effects on both the passive and active properties of motoneurons, but these changes did not result in intrinsic hyperexcitability as measured by FI gain, FI ∆f, or AP threshold measures (Table 1). The RMP, PIC and NaPIC onset voltage, as well as PIC and NaPIC max voltage were all significantly hyperpolarized by 2–3 mV in the 2 μM riluzole condition (Fig. 2, A and B, respectively). Hyperpolarization of the RMP would indicate hypoexcitability, while hyperpolarization of the PIC and NaPIC onsets and max voltage would lead to hyperexcitability. Unexpectedly, the PIC amplitude (control −39.8 ± 35 pA vs. 2 μM −28.9 ± 19 pA), and there was a nonsignificant 13% decrease in the NaPIC amplitude. Therefore, chronic exposure to riluzole did not induce a compensatory upregulation of the PIC or NaPIC, contrary to our predictions (Fig. 3, A and B, respectively).

Although not the main aim of this study, extrinsic excitability did not appear to increase in the riluzole-treated cells. In the absence of synaptic blockers, spontaneous firing was not increased (Table 1) and there was no evidence for the development of bursting behavior. Synaptic scaling, however, was not directly tested and remains a possibility.

Interactions between genotype and drug treatment. Although acute riluzole application has similar effects on wt and SOD1 neurons (Cao et al. 2002; Kuo et al. 2005), prolonged riluzole treatment produced differential effects. In addition to the main effect of drug treatment on PIC amplitude, there was a significant interaction between genotype and drug treatment. Post hoc analysis revealed that the PIC amplitude between control and riluzole-treated SOD1 neurons was significantly decreased by 55.4%, but there was no change between control and riluzole-treated wt neurons (Fig. 3A; Table 2). Likewise, the NaPIC amplitude between control and riluzole-treated SOD1 neurons was decreased 23%, although this was not significant (Fig. 3B; Table 2). The decrease in PIC amplitude did not, however, lead to decreases in FI gain or changes in other firing parameters.

Reapplication of riluzole. Although chronic riluzole treatment did not have major effects on motoneuron output, it was important to test whether riluzole could still decrease motoneuron excitability or if neurons had become desensitized to it. For 11 neurons chronically treated with riluzole, 2 μM riluzole was added to the aCSF after initial measurements were taken. Subsequently, TTX (1 μM) was added to the external solution, allowing the comparison between the NaPIC amplitude before and after reapplication of 2 μM riluzole (+2Ril NaPIC) (Fig. 4, C and D). For two neurons, riluzole was washed out of the aCSF before TTX was added.

The reapplication of 2 μM riluzole either decreased or stopped AP firing during current ramps for both wt and SOD1 neurons, but there was no difference in FI gain or changes in other firing parameters.

Table 1. Passive and firing properties

<table>
<thead>
<tr>
<th>Measure</th>
<th>Significance</th>
<th>Direction</th>
<th>wt 0 μM</th>
<th>SOD1 0 μM</th>
<th>wt 2 μM</th>
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<td>N = 59</td>
<td>N = 40</td>
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<tr>
<td>Diameter, μm</td>
<td>ns</td>
<td></td>
<td>25.7 ± 4.9</td>
<td>25.2 ± 3.5</td>
<td>26.0 ± 4.3</td>
<td>26.6 ± 4.4</td>
</tr>
<tr>
<td>Passive properties</td>
<td></td>
<td></td>
<td>N = 57</td>
<td>N = 57</td>
<td>N = 59</td>
<td>N = 40</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>Drug*</td>
<td>0 &gt; 2</td>
<td>−50.5 ± 6.4</td>
<td>−49.2 ± 5.5</td>
<td>−52.7 ± 7.0</td>
<td>−52.0 ± 7.4</td>
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<tr>
<td>Capacitance, pF</td>
<td>Ns</td>
<td></td>
<td>30.4 ± 17.5</td>
<td>34.0 ± 16.6</td>
<td>33.5 ± 14.3</td>
<td>31.2 ± 10.6</td>
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<tr>
<td>Leak conductance, nS</td>
<td>Genotype*</td>
<td>WT &gt; SOD1</td>
<td>2.8 ± 1.3</td>
<td>2.6 ± 1.3</td>
<td>2.9 ± 1.6</td>
<td>2.1 ± 1.0</td>
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<td></td>
<td></td>
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<td>N = 57</td>
<td>N = 59</td>
<td>N = 38</td>
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<tr>
<td>Height (&gt;0 mV)</td>
<td>Ns</td>
<td></td>
<td>32.4 ± 6.8</td>
<td>32.8 ± 6.7</td>
<td>32.9 ± 8.0</td>
<td>32.2 ± 7.0</td>
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<td>Voltage threshold, mV</td>
<td>Ns</td>
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<td>Current threshold, pA</td>
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<td>26.8 ± 41.6</td>
<td>34.2 ± 32.0</td>
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<td>∆I, pA</td>
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<td>−27.7 ± 13.9</td>
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<td>Ascending gain, Hz/pA</td>
<td>Ns</td>
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<td>0.233 ± 0.12</td>
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<td>Descending gain, Hz/pA</td>
<td>Ns</td>
<td>0.382 ± 0.15</td>
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<td>0.346 ± 0.15</td>
<td>0.370 ± 0.13</td>
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<td>Spont. activity, AP/min</td>
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<td>71.5 ± 152</td>
<td>176.6 ± 197</td>
<td>139.5 ± 193</td>
<td>71.2 ± 152</td>
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</tbody>
</table>

Means ± SD for each of the 4 individual groups are presented separately (genotype:drug): wild type (wt) control (0 μM), wt riluzole (2 μM), and SOD1 riluzole (2 μM). The number of cells per group (N) is above each series of measurements. RMP, resting membrane potential; AP, action potential; FI, frequency-current; ∆f, difference between current at AP recruitment and derecruitment; Spont., spontaneous. Significant differences for drug, genotype, and interactions are listed in a separate column. Significance: not significant (ns); *0.05 ≤ P > 0.01. The direction of each significant difference is listed in a separate column. The direction is based on actual, not absolute, values.
neurons (N = 9/9; wt N = 5, SOD1 N = 4) (Fig. 4A). During 1-s current steps (N = 8/8; wt N = 4, SOD1 N = 4), the neurons fired repetitive APs but at a slower rate and AP failure occurred before the end of the step (Fig. 4B). However, there was little to no change in the initial AP height during the current step (Fig. 4B, inset). The NaPIC amplitude was decreased an average of 61.5% when 2 μM riluzole was reapplied (wt N = 7, SOD1 N = 4) (Fig. 4D), which is similar to the acute effects of 2 μM riluzole in other studies (Beltran-Parrazal and Charles 2003; Del Negro et al. 2005; Kononenko et al. 2004; Kuo et al. 2006; Ptak et al. 2005; Urbani and Belluzzi 2000; Wu et al. 2004; Zhong et al. 2007). Therefore neurons did not become desensitized to riluzole after prolonged exposure.

It is unlikely that the initial reduction in PIC amplitude for chronically drug-treated SOD1 neurons was due to residual effects of riluzole in the aCSF. First, in aCSF without synaptic blockers, there was no significant difference between spontaneous firing rates for drug-treated and untreated neurons (Table 1). Second, in two of two neurons (wt N = 1, SOD1 N = 1), riluzole was quickly washed out of the bath (within 7 min) after reapplication and repetitive firing returned to normal (data not shown). Both findings indicate that riluzole is easily washed out of the external solution. Although a number of explanations may explain a decrease in PIC amplitude, it is not clear why SOD1 neurons were more affected by prolonged riluzole treatment than wt neurons.

**DISCUSSION**

**Comparisons to past studies: SOD1 versus wt.** Early electrical abnormalities are apparent in SOD1 motoneurons. Previous studies have shown that the NaPIC amplitude was increased in SOD1 spinal (G93A, high expressor line) (Kuo et al. 2005; Quinlan et al. 2011), hypoglossal (G93A, high expressor line) (van Zundert et al. 2008), and corticospinal (G93A, high expressor line) (Pieri et al. 2009) motoneurons. In some of these studies there was also an increase in FI gain (G93A, high expressor line) (Kuo et al. 2005; Pieri et al. 2009; van Zundert et al. 2008). Other studies, however, found either no change in firing behavior (Quinlan et al. 2011) or a decrease in FI gain (G85R and G93A, low expressor line) (Bories et al. 2007; Pambo-Pambo et al. 2009) coupled with changes in motoneuron conductance (G85R and G93A, high expressor line) (Bories et al. 2007; Quinlan et al. 2011) and size (G85R) (Amendola and Durand 2008).

In this study a number of measures (AP current threshold, PIC onset, conductance, and TTX-ins current) indicated an increase in SOD1 motoneuron excitability. We did not, however, see an increase in NaPIC amplitude or FI gain. In control cells, the PIC amplitude appeared larger in SOD1 motoneurons.
but the pairwise difference between wt control and SOD1 control cells did not quite reach significance ($P = 0.069$). Some of the diversity between studies is likely due to differences in motoneuron type, preparation, age, and SOD1 mouse model, but all of the studies support the presence of early electrical abnormalities in SOD1 motoneurons. The most direct comparison to this work is the study by Kuo et al. (2005), which came from our laboratory and used similar cell culture procedures. In Kuo et al. (2005), the NaPIC was increased and an increase in the TTX-ins current was present in low-input conductance neurons (cutoff: $<3.25$ nS). These results are in contrast to this work, which found no change in the NaPIC and a decrease in the TTX-ins current. The techniques for measuring the amplitude of the NaPIC and TTX-ins current in the present study were very different from those in Kuo et al. (2005). Here we used much slower voltage ramps to decrease the occurrence of breakthrough spikes, which occur because of loss of clamp control over the rapidly activating sodium channels. The slower voltage ramp, however, also increases the effects of channel inactivation. These slower ramps enabled us to directly measure the NaPIC peak amplitude. The TTX-ins amplitude was then measured at the PIC

### Table 2. PIC, NaPIC, and TTX-ins measures

<table>
<thead>
<tr>
<th>Measure</th>
<th>Significance</th>
<th>Direction</th>
<th>wt 0 μM</th>
<th>SOD1 0 μM</th>
<th>wt 2 μM</th>
<th>SOD1 2 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIC</td>
<td>Drug**</td>
<td>0 &gt; 2</td>
<td>$N = 46$</td>
<td>$N = 25$</td>
<td>$N = 51$</td>
<td>$N = 30$</td>
</tr>
<tr>
<td></td>
<td>Genotype*</td>
<td></td>
<td>$N = 35$</td>
<td>$N = 20$</td>
<td>$N = 39$</td>
<td>$N = 22$</td>
</tr>
<tr>
<td>Max voltage, mV</td>
<td>Drug****</td>
<td>0 &gt; 2</td>
<td>-42.5 ± 5.0</td>
<td>-45.5 ± 3.0</td>
<td>-46.1 ± 5.1</td>
<td>-46.2 ± 3.9</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>Drug**</td>
<td>0 &lt; 2</td>
<td>-28.5 ± 4.7</td>
<td>-29.0 ± 2.6</td>
<td>-32.0 ± 4.1</td>
<td>-32.1 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>Interaction**</td>
<td>SOD1: 0 &lt; wt 2</td>
<td>-36.8 ± 28.6</td>
<td>-57.9 ± 58.7</td>
<td>-33.8 ± 25.3</td>
<td>-25.8 ± 18.9</td>
</tr>
<tr>
<td>NaPIC</td>
<td>Drug*</td>
<td>0 &gt; 2</td>
<td>-48.6 ± 6.6</td>
<td>-49.2 ± 3.9</td>
<td>-51.5 ± 3.5</td>
<td>-50.4 ± 4.4</td>
</tr>
<tr>
<td>Max voltage, mV</td>
<td>Drug*</td>
<td>0 &lt; 2</td>
<td>-24.5 ± 4.8</td>
<td>-26.0 ± 3.8</td>
<td>-26.7 ± 4.4</td>
<td>-28.0 ± 5.4</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>Genotype**</td>
<td>wt &lt; SOD1</td>
<td>-53.4 ± 12.9</td>
<td>-45.7 ± 8.8</td>
<td>-54.0 ± 11.0</td>
<td>-48.1 ± 13.4</td>
</tr>
<tr>
<td>TTX-ins</td>
<td>Drug****</td>
<td>0 &gt; 2</td>
<td>-43.9 ± 30.3</td>
<td>-28.7 ± 14.5</td>
<td>-45.1 ± 49.2</td>
<td>-32.1 ± 23.0</td>
</tr>
</tbody>
</table>

Means ± SD for each of the 4 individual groups are presented separately (genotype:drug): wt control (0 μM), SOD1 control (0 μN), wt riluzole (2 μM), and SOD1 riluzole (2 μM). The number of cells per group ($N$) is above each series of measurements. PIC, persistent inward current; NaPIC, sodium PIC; TTX-ins, TTX-insensitive current; Amp, amplitude. Significant differences for drug, genotype and interactions are listed in a separate column. Significance: not significant (ns), *$0.05 \leq P < 0.01$, **$0.01 \leq P < 0.001$, ****$P \leq 0.0001$. The direction of each significant difference is listed in a separate column. The direction is based on actual, not absolute, values.
In contrast, Kuo et al. (2005) measured the NaPIC and TTX-ins amplitude as the integral of the curve in a voltage range near firing threshold. Together, the different voltage ramp speeds and measuring techniques likely account for the variation in the NaPIC and TTX-ins current between the two studies. The very slow voltage ramps and smaller dendritic tree are also likely responsible for the lower overall NaPIC amplitudes in this study compared with Quinlan et al. (2011).

Intrinsic motoneuron excitability and riluzole. In ALS patients, riluzole decreases the rate of disease progression, but this effect is temporary even with continued use (Bensimon et al. 1994; Traynor et al. 2003). The purpose of this study was to determine whether prolonged riluzole treatment caused compensatory upregulation of the NaPIC and subsequently reestablished motoneuron excitability. Instead, the results showed that the NaPIC amplitude was decreased, although not significantly, and that motoneuron intrinsic excitability did not change. Furthermore, motoneurons did not become desensitized to riluzole. It is therefore unlikely that compensatory increases in motoneuron intrinsic excitability cause riluzole to lose its therapeutic value. These results are in sharp contrast to the work of the Bennett lab, which showed compensatory increases in both the NaPIC and the CaPIC after monoaminergic drive to the cord is lost and the PIC is diminished (Harvey et al. 2006c; Li and Bennett 2003).

In the normal state, the NaPIC and the CaPIC can be modulated by affecting the channels directly or by activating G protein-coupled 5-HT2 and NE α1 receptors. Riluzole directly inhibits the NaPIC by blocking inactivated sodium channels (Benoit and Escande 1991; Hebert et al. 1994). In contrast, the loss of monoaminergic drive decreases the PIC because the 5-HT2 and NE α1 receptors are no longer activated. However, when monoaminergic drive is lost, the PIC amplitude gradually increases because the 5-HT2, and possibly NE α1 receptors become constitutively active (Murray et al. 2010; Rank et al. 2011) and supersensitive (Harvey et al. 2006a; Li et al. 2007; Rank et al. 2007). This suggests an interesting speculation, that the loss of monoamines but not direct channel block can result in strong compensatory increases in the PIC amplitude. However, compensatory mechanisms may be impaired or altered during disease and injury and are therefore difficult to compare.

Riluzole produces a stronger effect on the SOD1 PIC. It was somewhat unexpected that riluzole treatment would have differential effects on wt versus SOD1 motoneurons. Prolonged riluzole treatment, however, decreased the PIC amplitude in SOD1 but not wt motoneurons. This result remained even when conductance was controlled for and cannot be attributed to changes in the TTX-ins current. Although the decrease in PIC amplitude did not change motoneuron firing behavior to injected current, it may have more pronounced effects on synaptic input because of the dendritic location of many PIC channels (Bennett et al. 1998; Lee and Heckman 2000). The specificity of the result suggests that the sodium channels underlying the PIC in SOD1 motoneurons have an altered response to riluzole that could result in greater channel internalization or stabilization of the channel in an inactive state.

Riluzole’s transience: alternative hypotheses. In this study the NaPIC was not upregulated after prolonged (5–9 days) riluzole exposure. In ALS patients, however, riluzole treatment is assessed over months, not days. Therefore it is possible that the exposure to riluzole was too short to produce compensatory changes in excitability. In a number of studies, however, compensatory changes in excitability have been reported after 48 h (Koch et al. 2010) and 3–4 days (Schonfeld-Dado et al. 2009) of exposure to TTX (1 μM) and after 48 h of exposure to prostaglandin E2 (Koch et al. 2010).

An alternative hypothesis is that although prolonged riluzole exposure does not increase intrinsic excitability, it may increase extrinsic motoneuron excitability through synaptic scaling. We saw no increase in spontaneous firing when synaptic blockers were absent, but this study did not directly test synaptic scaling, which can occur during periods of hypoxia-

citability. It has been repeatedly demonstrated that activity-deprived neurons die off (Baker and Ruijter 1991; Ramakers and Boer 1991; Ruijter et al. 1991) because of an increased sensitivity to glutamate-mediated postsynaptic responses (Fishbein and Segal 2007) and/or increased calcium entry through GluR2-lacking AMPA receptors (Schonfeld-Dado et al. 2009). In these studies, however, synaptic activity was completely blocked through chronic application of TTX (1 μM or higher). In this study, 2 μM riluzole was used, which does not completely block APs. Therefore its capacity to produce synaptic scaling is unknown; however, there was no sign of obvious cellular death between control and cells treated with 2 μM riluzole.

Finally, riluzole may be unable to permanently slow motoneuron death regardless of its effect on motoneuron excitability. Clinical intervention for ALS patients begins well after disease onset, and because of the array of cell autonomous and nonautonomous dysfunctions at this point, it is unlikely that one drug will affect enough pathways to produce lasting results. Drug combinations that include riluzole but affect additional pathways may therefore provide greater protection (Del Signore et al. 2009; Kriz et al. 2003; Wielbel et al. 2004).

Conclusion. Prolonged riluzole treatment had minimal effects on motoneuron firing behavior and remained a potent inhibitor of the PIC and repetitive firing when reapplied. It therefore seems possible that riluzole continues to decrease motoneuron excitability but its therapeutic effects are eventually overwhelmed by other pathologies associated with ALS.
REFERENCES


