Brief Communications

The Neuroprotective Drug Riluzole Acts via Small Conductance Ca^{2+}-Activated K^+ Channels to Ameliorate Defects in Spinal Muscular Atrophy Models

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Spinal muscular atrophy (SMA), a recessive neuromuscular disorder, is caused by diminished function of the Survival Motor Neuron (SMN) protein. To define the cellular processes pertinent to SMA, parallel genetic screens were undertaken in *Drosophila* and *Caenorhabditis elegans* SMA models to identify modifiers of the SMN loss of function phenotypes. One class of such genetic modifiers was the small conductance, Ca^{2+}-activated K^+ (SK) channels. SK channels allow efflux of potassium ions when intracellular calcium increases and can be activated by the neuroprotective drug riluzole. The latter is the only drug with proven, albeit modest, efficacy in the treatment of amyotrophic lateral sclerosis. It is unclear if riluzole can extend life span or ameliorate symptoms in SMA patients as previous studies were limited and of insufficient power to draw any conclusions. The critical biochemical target of riluzole in motor neuron disease is not known, but the pharmacological targets of riluzole include SK channels. We examine here the impact of riluzole in two different SMA models. In vertebrate neurons, riluzole treatment restored axon outgrowth caused by diminished SMN. Additionally, riluzole ameliorated the neuromuscular defects in a *C. elegans* SMA model and SK channel function was required for this beneficial effect. We propose that riluzole improves motor neuron function by acting on SK channels and suggest that SK channels may be important therapeutic targets for SMA patients.

Introduction

Spinal muscular atrophy (SMA), an autosomal recessive neuromuscular disorder, is the leading genetic cause of infant death in the United States (Pearn, 1978; Crawford and Pardo, 1996). SMA primarily affects the α-motor neurons in the anterior horn of the spinal cord and is characterized by progressive muscle degeneration, loss of neuromuscular function, paralysis, and/or death. SMA is caused by *Survival Motor Neuron 1* (*SMN1*) homozygous loss of function mutations that lead to decreased Survival Motor Neuron (SMN) protein levels (Lefebvre et al., 1995). Amyotrophic lateral sclerosis (ALS) has commonalities with SMA as both target spinal cord motor neurons and share phenotypic, genetic, and molecular similarities. For example, SMN gene variants have been associated with sporadic ALS (Veldink et al., 2001; Corcia et al., 2009; Blauw et al., 2012), overexpression of TDP-43, an ALS protein, increases SMN mRNA levels (Bose et al., 2008), and mutation of the vesicle-trafficking protein VAPB can cause late-onset SMA and ALS (Nishimura et al., 2004). Therefore, these neuromuscular disorders may share a common neurodegenerative pathway and respond to similar treatments.

We have previously reported that small conductance Ca^{2+}-activated K^+ (SK) channels are cross-species invertebrate SMN modifiers (Chang et al., 2008; Dimitriadi et al., 2010). SK channels are activated by intracellular calcium; are potassium selective; have been implicated in epilepsy, ataxias, and other disorders (Pedarzani and Stocker, 2008); and play roles in after-hyperpolarization, repetitive firing, dendritic integration, synaptic transmission, and synaptic plasticity (Xia et al., 1998; Keen et al., 1999; Schumacher et al., 2001). In neurons, SK channel activity is regulated by calcium entry through voltage-gated calcium channels. The overall impact of SK channels on neuronal activity can be difficult to predict. Increased potassium efflux can reduce excitability, but when coupled with depolarization-induced calcium influx, SK channels can increase firing rates by accelerating repolarization. SK2 channels are often found in a complex with L-type Ca^{2+} channels and α-actinin, an actin-binding protein (Lu et al., 2007, 2009). α-actinin interacts directly with SMN in *Drosophila* adult muscle (Rajendra et al., 2007), and α-actinin

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orthologs are invertebrate SMN modifier genes (Chang et al., 2008; Dimitriadi et al., 2010).

SK channels are among the pharmacological targets of riluzole. Riluzole ameliorates the aberrant cytoskeletal organization of synaptic terminals in mice lacking SMN exon 7 (Haddad et al., 2003). Riluzole had no impact on severely affected SMA patients in a short duration study with a limited number of subjects (Russman et al., 2003). This study was of insufficient power to determine whether riluzole might benefit other SMA patient populations. Elucidating the biochemical target of riluzole may increase our understanding of ALS and SMA pathogenesis. Here, we test the hypothesis that riluzole can ameliorate SMN loss-of-function defects across species.

Materials and Methods

Caenorhabditis elegans strains. LM99 *smn-1(ok355)I/hT2 (I;III)* (Briese et al., 2009), HA2207 *kcnl-2(tm1885) smn-1(ok355)/hT2*, *HA2415 kcnl-2(ok2818) smn-1(ok355)/hT2*, HA2400 *smn-1(ok355)/hT2; slo-1(js118)V*, HA2402 *smn-1(ok355)/hT2; slo-2(nf100)X*, and HA2404 *smn-1(ok355)/hT2; sup-9(n180)II* strains were cultivated at 20°C under standard conditions (Brenner, 1974). *kcnl-2* alleles *tm1885* and *ok2818* were backcrossed six and three times, respectively. *tm1885* removes three transmembrane domains; *ok2818* perturbs transmembrane domains and the calmodulin-binding domain. *kcnl-2(tm1885)* pumping rates are slightly lower than *kcnl-2(ok2818)* ($p = 3 \times 10^{-4}$). All assays used the progeny of *hT2* parents to control genetic background.

C. elegans assays. The pharyngeal pumping assay was performed as previously described (Dimitriadi et al., 2010). Eggs hatched on L4440 control vector (Kamath and Ahringer, 2003) were reared at 2 d at 25°C and 1 d at 20°C. Pumping rates were determined at the last larval stage. Average pumping rates (±SEM) were derived from at least three independent trials ($n \geq 25$ animals in total). Experimenters were blinded to genotype/treatment for at least one trial. For Figure 2, *A* and *C*, more than three independent trials were performed and were pooled together for the final figure. Unpooled results Figure 2*A*: Trials 1–3 Control/DMSO 302 ± 7, Control/3 μM 266 ± 17, Control/33 μM 242 ± 12, *Cesmn-1*/DMSO 23 ± 5, *Cesmn-1*/3 μM 59 ± 14, *Cesmn-1*/33 μM 70 ± 13; *Cesmn-1*/DMSO vs *Cesmn-1*/3 μM, $p = 0.04$ and *Cesmn-1*/DMSO vs *Cesmn-1*/33 μM, $p = 0.007$; Trials 4–5 Control/DMSO 268 ± 9, Control/1 μM 257 ± 9, *Cesmn-1*/DMSO 48 ± 11, *Cesmn-1*/1 μM 38 ± 11. Unpooled results Figure 2*C*: Trials 1–4 Control 244 ± 6, *kcnl-2(tm1885)* 194 ± 13, *Cesmn-1* 57 ± 10, *kcnl-2(tm1885) Cesmn-1* 24 ± 8; $p = 0.01$ *Cesmn-1* vs *kcnl-2(tm1885) Cesmn-1*; Trials 5–7 Control 238 ± 7, *kcnl-2(ok2818)* 210 ± 12, *Cesmn-1* 74 ± 14, *kcnl-2(ok2818) Cesmn-1* 36 ± 10; $p = 0.01$ *Cesmn-1* vs *kcnl-2(ok2818) Cesmn-1*; Trials 8–10 Control 224 ± 9, *slo-1* 195 ± 13, *slo-2* 217 ± 11, *sup-9* 238 ± 11, *Cesmn-1* 47 ± 11, *Cesmn-1; slo-1* 51 ± 8, *Cesmn-1;slo-2* 74 ± 11, *Cesmn-1;sup-9* 62 ± 11; $p = 0.04$ *Cesmn-1* vs *Cesmn-1;slo-2*.

The motility assay was described previously (Briese et al., 2009). Here, *C. elegans* was reared on plates for 2 d at 25°C and 1 d at 20°C. Motility was assessed manually after 2 min in M9 buffer at day 3 post hatching regardless of developmental stage. A complete bend at mid-body was scored as a beat. At least three independent trials were performed.

Compounds. Riluzole (R116) and apamin (A9459) were purchased from Sigma-Aldrich. Riluzole dramatically decreased egg laying (data not shown).

Hippocampal cell culture. Experimental procedures were performed in compliance with animal protocols approved by Children's Hospital Institutional Animal Care and Use Committee, Boston. Hippocampi were dissected from E18 Sprague Dawley rat embryos (Charles River). Neurons were dissociated with papain, triturated, and plated onto poly-D-lysine/laminin (Sigma/Invitrogen)-coated plates at 250,000 cells/6-well culture plates for Western blotting and 20,000 cells/24-well culture plates for immunostaining. Neurons were cultured in Neurobasal medium with B27 supplement (Invitrogen), 500 μM L-glutamine (Invitrogen), and 1× penicillin/streptomycin (Invitrogen) at 37°C in a humidified incubator with 5% CO_2.

Inhibition of SMN, riluzole treatment, and measurement of neuronal morphology. SMN was knocked down using conventional siRNA technique (Applied Biosystems), Lipofectamine 2000 (Invitrogen), and Opti-MEM (Invitrogen). Briefly, neurons were transiently transfected with siRNA and treated with riluzole or DMSO after 24 h. Four days post-transfection, protein lysates were collected; SMN (BD Biosciences) and synaptophysin (Cell Signaling Technology) protein levels were measured by Western blot using GAPDH (Life Technologies) as a control. For immunohistology, neurons were fixed with 4% paraformaldehyde and stained with Tau antibody (Millipore) (Choi et al., 2008). Length of Tau-positive axons was measured using ImageJ.

Statistical analysis. Significance was determined with Mann–Whitney *U* (two-tailed) or one-way ANOVA. After ANOVA, paired *t*-tests were used to identify significantly different pairs. Corresponding *p* and *F* values are reported.

Results

Riluzole prevents axonal defects in vertebrate neurons

We examined the impact of riluzole on rat embryonic hippocampal neurons with reduced SMN levels. SMN knockdown reduced axon outgrowth in these neurons based on overall process length ($p = 6.7 \times 10^{-5}$). Treatment with 10 and 50 nM riluzole increased control and restored SMN-deficient axons to comparable levels (Fig. 1*A*,*B*). Longest axon length was also significantly reduced upon SMN knockdown ($p = 1.4 \times 10^{-5}$), but restored to wild-type levels after riluzole treatment (Fig. 1*C*). Riluzole does not ameliorate SMN knockdown defects by increasing SMN protein levels; riluzole treatment actually decreased SMN protein levels in both control and SMN-deficient neurons (Fig. 1*D*). SMN protein levels are tightly regulated during development; expression is high in embryonic tissues, but the concentration of SMN decreases as cells differentiate (La Bella et al., 1998; Grice and Liu, 2011). To address the possibility that riluzole might accelerate hippocampal neuron maturation, thereby lowering SMN, we examined synaptophysin levels (which normally increase as hippocampal neurons mature in culture) (Daly and Ziff, 1997). Riluzole raised synaptophysin levels consistent with accelerated maturation (Fig. 1*E*). Therefore, the riluzole neuroprotection is not due to increased SMN levels, and riluzole may accelerate neuronal maturation.

Riluzole ameliorates SMN loss of function defects in *C. elegans*

To address the mechanism of riluzole protection, we turned to a *C. elegans* SMA model. The *C. elegans* genome harbors a single ortholog of SMN, *smn-1*, referred to here as *Cesmn-1* for clarity. Complete loss of *Cesmn-1* causes slow growth, larval lethality, and impairs neuromuscular function in locomotion and in pharyngeal pumping during feeding (Briese et al., 2009; Dimitriadi et al., 2010). *C. elegans* feed on microorganisms using a discrete subset of muscles and neurons in the pharynx (Avery, 1993). Animals pump continuously at >250 beats per minute on food. The pumping rates of SMN loss-of-function animals (*Cesmn-1(lf)*) are significantly reduced; these defects are progressive and not a developmental defect. *Cesmn-1(lf)* is recessive and heterozygous animals are overtly normal (Briese et al., 2009; Dimitriadi et al., 2010). To assess the impact of riluzole, control and *Cesmn-1(lf)* animals were reared on plates containing the drug. Riluzole partially rescued SMN loss-of-function defects as treatment increased *Cesmn-1(lf)* pharyngeal pumping rates ($p = 0.044$ for 3 μM, $p = 0.004$ for 33 μM; Fig. 2*A*). In contrast, riluzole lowered the pumping rates in controls ($p = 0.027$ for 1 μM, $p = 0.001$ for 33 μM) suggesting that riluzole is only beneficial when neuromuscular function is perturbed. The efficacy of riluzole was

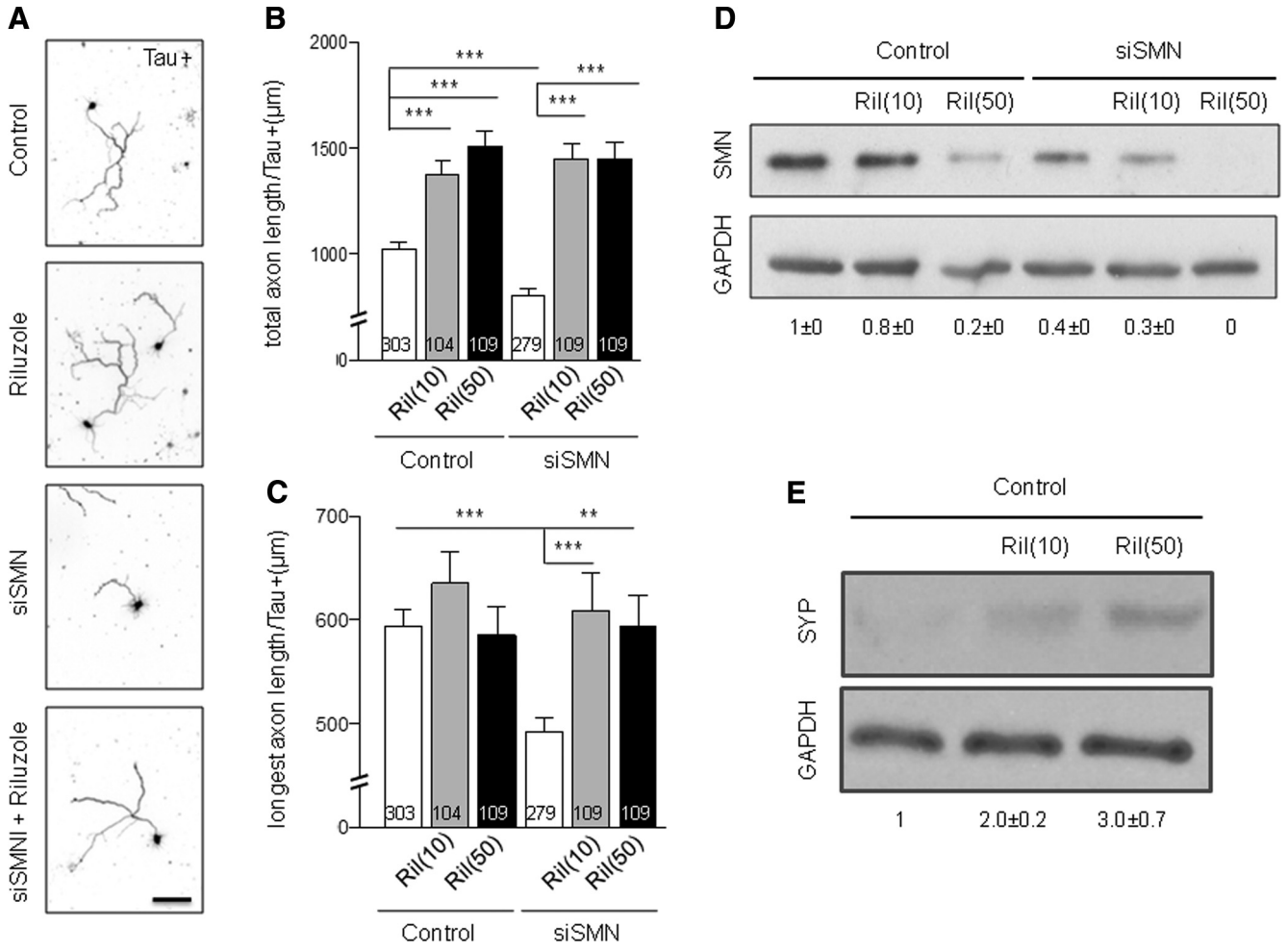

Figure 1. Riluzole treatment rescues axonal outgrowth defects in SMN-deficient vertebrate neurons. ***A***, Representative images of rat hippocampal neurons (days *in vitro* 5, Tau staining, 50 nM riluzole). Scale bar, 100 μm. ***B***, Analysis of total axon length for SMN-deficient and control rat hippocampal neurons after riluzole treatment. SMN siRNA was followed by treatment with 10 nM or 50 nM riluzole. Number of neurons scored is reported in each bar; $p = 2.2 \times 10^{-16}$, $F = 36.6$; one-way ANOVA. ***C***, Analysis of longest axon length for SMN-deficient and control rat hippocampal neurons after riluzole treatment; $p = 6.75 \times 10^{-6}$, $F = 6.4$; one-way ANOVA. Four independent experiments; >50 neurons each condition. Error bars indicate SEM, $^{**}p < 0.01$, $^{***}p < 0.001$ (paired *t*-test). ***D***, Treatment with riluzole decreases SMN protein levels. Representative Western blot comparing SMN and GAPDH protein levels in SMN-deficient and control rat hippocampal neurons in the presence of riluzole. ***E***, Riluzole treatment (10 nM or 50 nM) increases levels of the maturation marker synaptophysin (SYP) in rat hippocampal neurons. Representative Western blot. For two independent biological samples, fold change of SMN and SYP levels normalized to GAPDH by densitometry are shown ± SD.

also tested using motility, a neuromuscular assay that measures the frequency of body bends during swimming (Briese et al., 2009). Riluzole significantly increased the *Cesmn-1*(*lf*) motility ($p = 0.04$ for 33 μM; Fig. 2*D*) consistent with riluzole ameliorating *Cesmn-1* loss of function neuromuscular defects.

Riluzole likely acts via SK channels

We tested the hypothesis that riluzole acts via SK channels to ameliorate defects using *C. elegans*. First, SK channels were blocked pharmacologically. Application of the SK2/SK3 channel blocker apamin exacerbated *Cesmn-1*(*lf*) pumping defects, suggesting that blocking SK channels impairs the *Cesmn-1*(*lf*) neuromuscular function (Fig. 2*B*). Second, SK channels were tested using genetic tools. Previously, RNAi knockdown of the *C. elegans* SK channel ortholog (*kcnl-2*) enhanced *Cesmn-1*(*lf*) growth defects and ameliorated their pumping defects (Dimitriadi et al., 2010). As RNAi can have off-target effects and RNAi by feeding is inefficient in *C. elegans* neurons, we used two *C. elegans kcnl-2* alleles (*kcnl-2*(*tm1885*) and *kcnl-2*(*ok2818*)) that likely cause complete loss of function, to accurately assess the impact of *kcnl-2* loss on *Cesmn-1*. Pumping rates of *kcnl-2*(*tm1885*) *Cesmn-1*(*lf*) and *kcnl-2*(*ok2818*) *Cesmn-1*(*lf*) double mutant animals were both significantly decreased ($p = 0.003$ and $p = 0.023$, respectively; Fig. 2*C*). If riluzole activates SK channels to ameliorate SMN loss-of-function defects, then loss of *kcnl-2* should abrogate the beneficial effects of riluzole. Consistent with this hypothesis, riluzole treatment did not increase the pumping rates of *kcnl-2 Cesmn-1*(*lf*) mutant animals (Fig. 2*C*, *tm1885*; data not shown *ok2818*). Therefore, riluzole requires *kcnl-2* SK channel function to ameliorate the SMN loss of function neuromuscular defects. We confirmed this in another assay. *kcnl-2*(*tm1885*) and *Cesmn-1*(*lf*) animals show decreased motility when swimming compared with controls ($p = 0.029$ and $p = 0.007$, respectively). Loss of *kcnl-2* exacerbated the motility defects of *Cesmn-1*(*lf*) ($p = 0.001$), and riluzole treatment did not benefit *kcnl-2 Cesmn-1*(*lf*) mutant animals (Fig. 2*D*, *tm1885*; data not shown *ok2818*). To address the specificity of *kcnl-2*, other potassium channels were examined. Loss of *slo-1*(*js118*), *slo-2*(*nf100*), or *sup-9*(*n180*) did not exacerbate *Cesmn-1*(*lf*) pumping defects, suggesting that riluzole requires *kcnl-2* SK channels to ameliorate SMN loss-of-function neuromuscular defects.

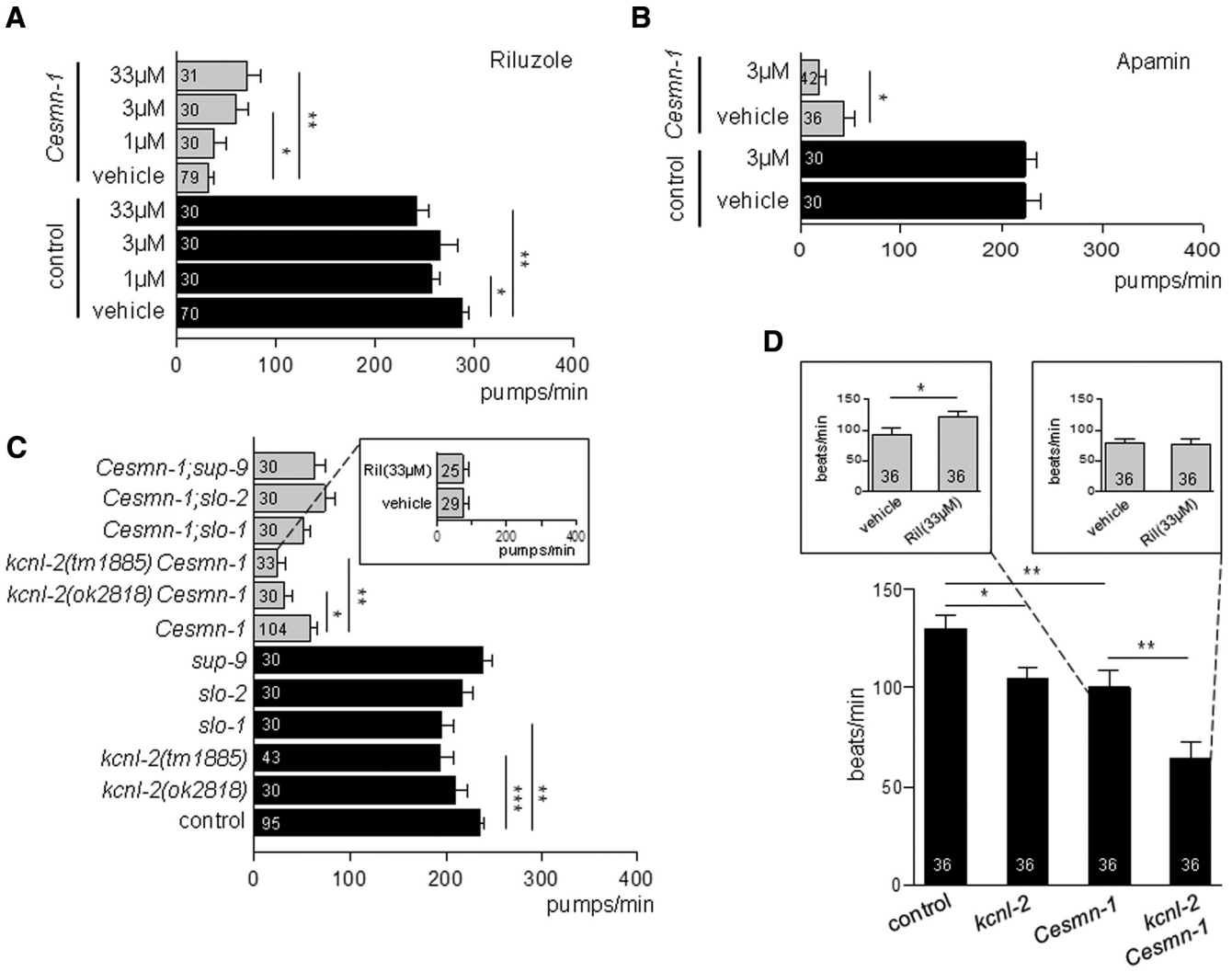

Figure 2. Riluzole improves the neuromuscular function in *Cesmn-1*(*lf*) animals via SK channels. ***A***, Riluzole, an SK channel activator, increases *Cesmn-1*(*lf*) pumping rates. *Cesmn-1*(*lf*) and control animals were reared on riluzole (1, 3, and 33 μM) and pumping rates were scored at day 3, post hatching. Control: $p = 0.007$, $F = 4.2$; *Cesmn-1*(*lf*): $p = 0.02$, $F = 3.5$; one-way ANOVA. ***B***, Apamin, an SK2 and SK3 channel blocker, exacerbates *Cesmn-1*(*lf*) pumping defects. Animals were reared on apamin (3 μM) and pumping rates were scored at day 3, post hatching. ***C***, Loss of the *C. elegans* SK gene ortholog *kcnl-2* enhanced *Cesmn-1*(*lf*) pumping defects and blocked the beneficial effect of riluzole (inset, *kcnl-2*(*tm1885*) *Cesmn-1*(*lf*) double mutant animals). Loss of other potassium channel genes (*slo-1*, *slo-2*, *sup-9*) did not exacerbate *Cesmn-1*(*lf*) defects. Control: $p = 9 \times 10^{-4}$, $F = 4.3$; *Cesmn-1*(*lf*): $p = 0.005$, $F = 3.5$; one-way ANOVA. For presentation purposes, ***A*** and ***C*** combine results from independent experiments; see Materials and Methods for details and results of independent experiments. ***D***, Loss of *kcnl-2*(*tm1885*) exacerbated *Cesmn-1*(*lf*) locomotion defects; riluzole significantly improved *Cesmn-1*(*lf*) motility (left inset, *Cesmn-1*(*lf*) animals). *kcnl-2* function is required for riluzole to improve *Cesmn-1*(*lf*) performance (right inset, *kcnl-2*(*tm1885*) *Cesmn-1*(*lf*) animals) $p = 3.5 \times 10^{-7}$, $F = 12.3$; one-way ANOVA. SEM is shown; paired *t*-test or Mann–Whitney *U* test: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Discussion

The early observations of abnormal glutamate metabolism and decreased glutamate transport in the brain and spinal cord of ALS patients led to the hypothesis that the excitatory amino acid neurotransmitter glutamate may be involved in the ALS pathogenesis (Plaitakis, 1991; Rothstein et al., 1992). Hence, drugs affecting the glutamatergic system were suggested as putative therapeutic agents. Riluzole was initially identified as a paralytic agent (Domino et al., 1952) and was later shown to indirectly modulate glutamatergic transmission (Benavides et al., 1985; Doble et al., 1992; Debono et al., 1993; Albo et al., 2004). It was subsequently found to significantly improve muscle strength and disease progression in ALS patients (Bensimon et al., 1994; Lacomblez et al., 1996). The mechanism of riluzole protection remains unclear (Kuo et al., 2005; Bellingham, 2011; Schuster et al., 2012), as riluzole has diverse direct targets. These include potassium channels: SK channels (Grunnet et al., 2001; Cao et al., 2002), large conductance Ca^{2+}-activated BK channels (Wu and Li, 1999; Wang et al., 2008), or TREK-1 and TRAAK two-pore-domain channels (Fink et al., 1998; Duprat et al., 2000). Also, riluzole blocks voltage-dependent sodium channels (Benoit and Escande, 1991; Song et al., 1997; Zona et al., 1998) and voltage-gated N- and P/Q-type calcium channels (Huang et al., 1997). Additionally, riluzole may inhibit cholinergic receptors (Deflorio et al., 2012) and decrease protein kinase C (PKC) activity (Noh et al., 2000).

SK channels were identified previously as cross-species genetic modifiers in invertebrate SMA models (Chang et al., 2008; Dimitriadi et al., 2010). Here, we provide evidence that riluzole has beneficial effects in two SMA models and may act via SK channels. Loss of the *C. elegans* SK channel ortholog *kcnl-2* exacerbated *Cesmn-1*(*lf*) neuromuscular defects. Apamin, which blocks SK2 and SK3 channels, also exacerbated *Cesmn-1* loss-of-function defects. Treatment with riluzole, whose actions include

SK channel activation, improved the neuromuscular function of *Cesmn-1*(*lf*) animals and the axon outgrowth of SMN deprived rat hippocampal neurons.

Although Franks et al. (2002) identified a sodium current in pharyngeal muscles that is sensitive to sodium channel drugs, neither genes nor mRNAs encoding classical voltage-gated sodium channels have been found in *C. elegans*, suggesting that riluzole likely does not act via these channels to ameliorate *Cesmn-1*(*lf*) defects. Riluzole also restored axon outgrowth caused by diminished SMN in vertebrate neurons. Apamin, which blocks some classes of SK channels, had no impact on hippocampal neurons (data not shown), suggesting that either apamin-sensitive SK channels are not expressed at this stage or the beneficial effects of riluzole are not solely through SK channels in these mammalian neurons.

The present study reports that riluzole, while beneficial, does not increase SMN protein levels. However, riluzole treatment increased levels of a hippocampal neuron maturation marker, synaptophysin, suggesting that riluzole may accelerate maturation with consequent decreases in SMN levels. Therefore, it might be worthwhile to investigate the synergistic effects of riluzole in combination with drugs that directly increase SMN levels (Wadman et al., 2011a, b). In summary, our studies demonstrate the beneficial impact of riluzole in SMA models and suggest that riluzole acts via SK channels to ameliorate SMN loss-of-function defects, delineating an important therapeutic pathway for neuromuscular disease patients.

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