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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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Abstract

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 *C. 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 (ALS). It is unclear if riluzole can extend lifespan 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 motorneuron 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 motorneuron 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 (Crawford & Pardo, 1996; Pearn, 1978). SMA primarily affects the α-motorneurons 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 motorneurons and share phenotypic, genetic and molecular similarities. For example, *SMN* gene variants have been associated with sporadic ALS (Blauw *et al.*, 2012; Corcia *et al.*, 2009; Veldink *et al.*, 2001), over-expression of TDP-43, an ALS protein, increases *SMN* mRNA levels (Bose *et al.*, 2008), and mutation of the vesicle-

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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 & Stocker, 2008), and play roles in after-hyperpolarization, repetitive firing, dendritic integration, synaptic transmission and synaptic plasticity (Keen *et al.*, 1999; Schumacher *et al.*, 2001; Xia *et al.*, 1998). 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.*, 2009; Lu *et al.*, 2007). α-actinin interacts directly with SMN in *Drosophila* adult muscle (Rajendra *et al.*, 2007), and α-actinin 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 limited number of subjects (Russman *et al.*, 2003). This study was of insufficient power to determine if 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

C. 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, 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)* ($p=3\times10^{-4}$). 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 & Ahringer, 2003) were reared at two days at 25°C and one day at 20°C. Pumping rates were determined at the last larval stage. Average pumping rates (± standard error of the mean, SEM) were derived from at least three independent trials (n≥ 25 animals in total). Experimenters were blinded to genotype/treatment for at least one trial. For Figure 2A and 2C, more than three independent trials were performed and were pooled together for the final figure. Unpooled results Figure 2A: Trials 1–3 Control/DMSO 302±7, Control/3μM 266±17, Control/33μM 242±12, *Cesmn-1*/DMSO 23±5, *Cesmn-1*/3μM 59±14, *Cesmn-1*/33μM 70±13; *Cesmn-1*/DMSO vs *Cesmn-1*/3μM p=0.04 & *Cesmn-1*/DMSO vs *Cesmn-1*/33uM p=0.007; Trials 4–5 Control/

DMSO 268±9, Control/1μM 257±9, *Cesmn-1*/DMSO 48±11, *Cesmn-1*/1μM 38±11. Unpooled results Figure 2C: 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* were reared on plates for two days at 25°C and one day 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 IACUC, 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), 500μM L-glutamine (Invitrogen), 1x 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 hours. Four days post-transfection, protein lysates were collected; SMN (BD Biosciences) and synaptophysin (Cell Signaling) protein levels were measured by Western 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 ($p=6.7\times10^{-5}$). Treatment with 10nM and 50nM riluzole increased control and restored SMN-deficient axons to comparable levels (Figure 1A & 1B). Longest axon length was also significantly reduced upon SMN knockdown ($p=1.4\times10^{-5}$), but restored to wild type levels after riluzole treatment (Figure 1C). 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 (Figure 1D). SMN protein levels are tightly regulated during development; expression is high in embryonic tissues, but the concentration of SMN decreases as cells differentiate (Grice & Liu, 2011; La Bella *et al.*, 1998). 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 & Ziff, 1997). Riluzole raised synaptophysin levels consistent with accelerated maturation (Figure 1E). 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 *Caenorhabditis 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 over 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 3μM, $p=0.004$ for 33μM, Figure 2A). By contrast, riluzole lowered the pumping rates in controls, ($p=0.027$ for 1μM, $p=0.001$ for 33μM) suggesting that riluzole is only beneficial when neuromuscular function is perturbed. The efficacy of riluzole was 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 333M, Figure 2D) consistent with riluzole ameliorating *Cesmn-1* loss of function neuromuscular defects.

Riluzole likely acts through 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 (Figure 2B). 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 ($p=0.003$ & $p=0.023$, respectively, Figure 2C). 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 (Figure 2C *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 to controls ($p=0.029$ & $p=0.007$, 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 (Figure 2D *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.

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 (Albo *et al.*, 2004; Benavides *et al.*, 1985; Debono *et al.*, 1993; Doble *et al.*, 1992). 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 (Bellingham, 2011; Kuo *et al.*, 2005; Schuster *et al.,* 2012), as riluzole has diverse direct targets. These include potassium channels: SK channels (Cao *et al.*, 2002; Grunnet *et al.*, 2001), large conductance Ca^{2+}-activated BK channels (Wang *et al.*, 2008; Wu & Li, 1999), or TREK-1 and TRAAK two-pore-domain channels (Duprat *et al.*, 2000; Fink *et al.*, 1998). Also, riluzole blocks voltage-dependent sodium channels (Benoit & 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 and co-workers identified a sodium current in pharyngeal muscles that is sensitive to sodium channel drugs (Franks *et al.*, 2002), 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; Wadman *et al.,* 2011b). 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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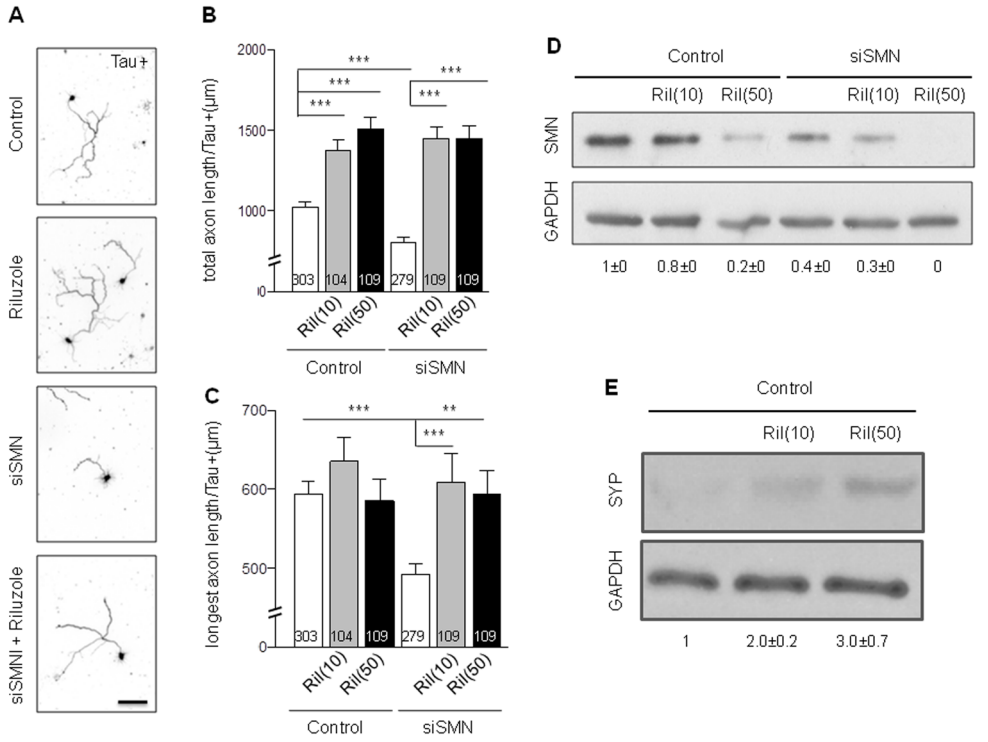

Figure 1.

Riluzole treatment rescues axonal outgrowth defects in SMN deficient vertebrate neurons. A) Representative images of rat hippocampal neurons (DIV 5, Tau staining, 50nM 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 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: standard error of the mean (SEM), **<0.01, ***<0.001 (paired *t*-test) D) Treatment with riluzole decreases SMN protein levels. Representative Western blot comparing SMN & GAPDH protein levels in SMN deficient and control rat hippocampal neurons in the presence of riluzole. E) Riluzole treatment (10 or 50nM) 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 ± standard deviation.

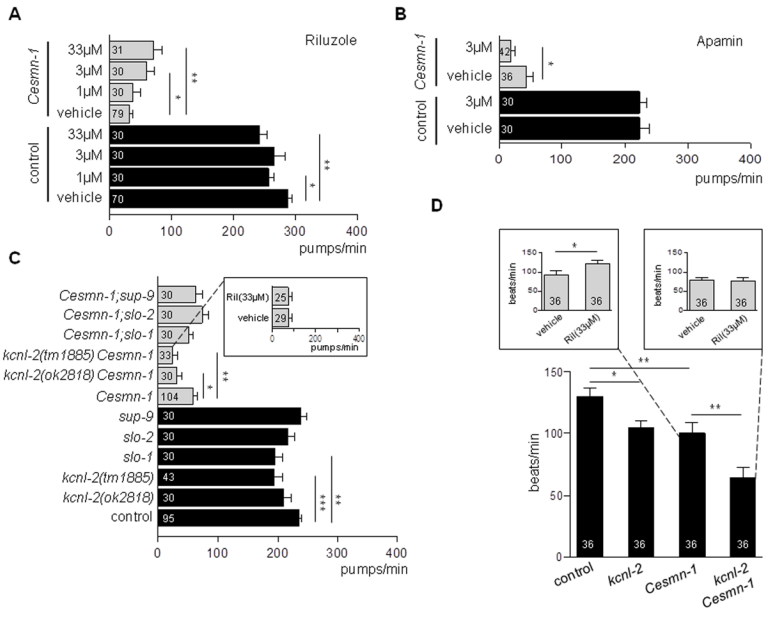

Figure 2.

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 (1μM, 3μM and 33μM) 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, a SK2 and SK3 channel blocker, exacerbates *Cesmn-1(lf)* pumping defects. Animals were reared on apamin (3μM) 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: $p=9\times10^{-4}$, F=4.3; *Cesmn-1(lf)*: p=0.005, F=3.5; one-way ANOVA. For presentation purposes, 2A and 2C 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) $p=3.5\ x10^{-7}$, F=12.3; one-way ANOVA. Standard error of the mean (SEM) is shown; paired *t*-test or Mann-Whitney *U* test: *< 0.05, **<0.01, ***<0.001.