The effects of Riluzole on sensory and motor nerve function

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The only Food and Drug Administration approved drug for amyotrophic lateral sclerosis (ALS) is Riluzole (Rilutek). However, the diverse mechanisms of how Riluzole functions physiologically are still being discovered. ALS is characterized by the progressive degeneration of motor neurons in the central nervous system. One approach in treating ALS is to reduce the glutamatergic excitotoxicity of the postsynaptic motor neurons. This is achieved by decreasing the activity of these motor neurons, reducing the influx of calcium into the neuron. Riluzole has been shown to antagonize presynaptic NMDA receptors mediated responses as well as directly block neuronal voltage gated Na^+ and Ca^{2+} channels. Glutamate release inhibitors, such as Riluzole, which act as therapeutics for ALS and Huntington's may have both pre-and post-synaptic mechanisms which are still being investigated. Here, experimental models included both glutamatergic synapses at the crayfish neuromuscular junctions (NMJs) and sensory neurons of crabs. The goal of this study was to determine if Riluzole had a direct effect on sensory neurons independent of synaptic properties and to determine if synaptic transmission is altered at glutamatergic synapses. The exposure of 1 mM Riluzole to the crayfish NMJs, at first, promotes synaptic transmission and then depresses synaptic transmission by blocking presynaptic function. The effects did not reverse readily with removal of Riluzole. We expected Riluzole to decreased the action potential amplitude in the motor neurons by blocking voltage gated Na^+ and Ca^{2+} channels. Proprioceptive sensory neurons in the crab were not affected by 1 mM Riluzole over the same time frame. Reproducibility in analysis was accomplished by using given data sets and having various participants analyze the responses. The trends reported were similar among different participants, but absolute values were different depending on the amplitude of the extracellular signals chosen as events.

Abbreviations: ACURE – authentic course-based undergraduate research experience; ALS – amyotrophic lateral sclerosis; PD – propodite-dactylopodite; EJP – excitatory junction potential; NMJ – neuromuscular junction; ROS – reactive oxygen species; STF – Short-term facilitation

Keywords: invertebrate; crustacean; neuromuscular

Introduction

Various medications are used in health care of humans and other animals without knowing the precise mechanism of action to produce the desirable effects (Lu and Wang, 2018). However, the use of therapeutics, which provide beneficial outcomes, continues to be utilized while further research is needed to understand the mechanism of action and potential side effects. One such compound is Riluzole (Rilutek). This is the only drug approved at present by the Food and Drug Administration for amyotrophic lateral sclerosis

(ALS) (U.S. Food and Drug Administration, 2019). However, Riluzole is also used to reduce the effects of other diseases (i.e., Parkinson's disease, atypical parkinsonism, Huntington disease, and hereditary ataxia) related to glutamate excitotoxity (Doble 1996; Douhou et al., 2002; Huntington Study Group, 2003; Liu and Wang, 2018). Riluzole is sometimes used for other off-label applications such as traumatic spinal cord injury (Wilson and Fehlings, 2014). The goal with the use of Riluzole is to reduce excessive glutamate release leading to over excitation of neurons and triggering a cellular cascade inducing excitotoxity (Nicotera and Orrenius, 1998).

The proposed mechanisms of action are broad from antagonistic action on N-Methyl-D-aspartate (NMDA) glutamatergic receptors to blocking voltage gated sodium and calcium channels as well as potentiation of calcium-dependent potassium (K^+) current (Bellingham, 2011). There is also potential that Riluzole inhibits cellular function as it can even inhibit cancer cell proliferation in cell culture conditions (Lemieszek et al., 2018; Seol et al., 2016). Additional effects in protecting cells from injury is a decrease in glutathione levels leading to a reduction in reactive oxygen species (ROS) production. Cells which express a form of ALS (familial ALS-related G93A-SOD1 mutation) have an increased ROS level with respect to wild-type cells (Sala et al., 2019). Thus, it is thought that Riluzole may help aid in damping glutamate cellular toxicity by reducing ROS production (Sala et al., 2019).

In this study, we examined potential acute mechanisms of action in altering primary sensory neuron function without the effects of synaptic properties. In addition, we used a glutamatergic synaptic model to address the acute effect on synaptic transmission. The use of primary sensory neurons eliminates the variables involved with pre- and post-synaptic communication and can focus on mechano-sensory transduction and relaying the electrical activity. Whereas the examination of the glutamatergic synaptic transmission at the crayfish neuromuscular junction allows one to determine potential pre- and post-synaptic actions to learn if glutamate receptors are blocked and/or if evoked transmission is reduced due to pre-synaptic actions on the motor neuron. We also chose these preparations because of their experimentation durability and ease in analysis since this was neurophysiological student driven class research project.

The neuromuscular junction of the crayfish opener muscle in the walking leg has been a common model for investigating synaptic transmission as it continues to provide a strong foundation when comparing results of various compounds. The motor nerve axons are large allowing for ease in addressing how compounds alter the shape of action potentials (Cooper AS, Cooper RL (2009). A readily accessible proprioceptive sensory organ in crabs (i.e., chordotonal organ) within the limbs has also been intensively studied anatomically and physiologically for many years (Alexandrowicz, 1972; Hartman and Boettiger, 1967, Dayaram et al., 2017; Malloy et al., 2017). In terms of ACURE (authentic course-based undergraduate research experiences) projects, the propodite-dactylopodite (PD) chordotonal organ has also been used for past studies in neurophysiology courses at the university level (Dayaram et al., 2017; Malloy et al., 2017; Stanback et al., 2019; Wycoff et al., 2018). In this study, the ability to reproduce the analysis of sensory nerve activity was addressed as this can aid future studies where data is supplied as an open resource.

The purpose of this study was to determine if Riluzole had direct effects on sensory neurons independent of synaptic properties and to determine if synaptic transmission is altered at glutamatergic synapses. This investigation further expands our understanding of the potential mechanism of action of Riluzole in well-established physiological invertebrate models, which serve to provide insights for mammals including humans.

Material and Methods

Animals

The maintenance and animals used were the same as mentioned in previous reports (Dayaram et al., 2017; Malloy et al., 2017; Stanback et al., 2019). In brief, blue crab

(*Callinectes sapidus*) and red swamp crayfish (*Procambarus clarkii*) were obtained from a distribution center in Atlanta, GA, and delivered to and bought from a local supermarket in Lexington, KY, USA.

The crayfish (6-10 cm in body length and 12.5-25 g in body weight) were housed in individual standardized plastic containers with weekly exchanged dry fish food and oxygenated water (20-21°C). The blue crabs were maintained in a seawater aquarium prior to use for three to five days. All experiments were implemented in female adults with a carapace width (from point to point) of 10-15 cm. The crabs were fed with frozen squid and the water temperature was maintained between 14-16°C. The crabs and crayfish were caught from the wild and were most likely two to three years old based on their size (Cooper and Cooper, 2004; Hartman and Cooper, 1994). Juveniles and older organisms may respond differently to compounds and are harder to obtain consistently from suppliers. Similar dissection procedures and electrophysiological measures for these preparations are described in detail with text and video format. They are described in brief below.

The crayfish walking leg opener neuromuscular preparation

The dissection and recording procedures are described in Cooper & Cooper (2009) and Stanley et al., (2020). In brief, the ventral cuticle of the propodite and the closer muscle is removed to expose the ventral surface of the opener muscle in the propodite cavity (Figure 1).

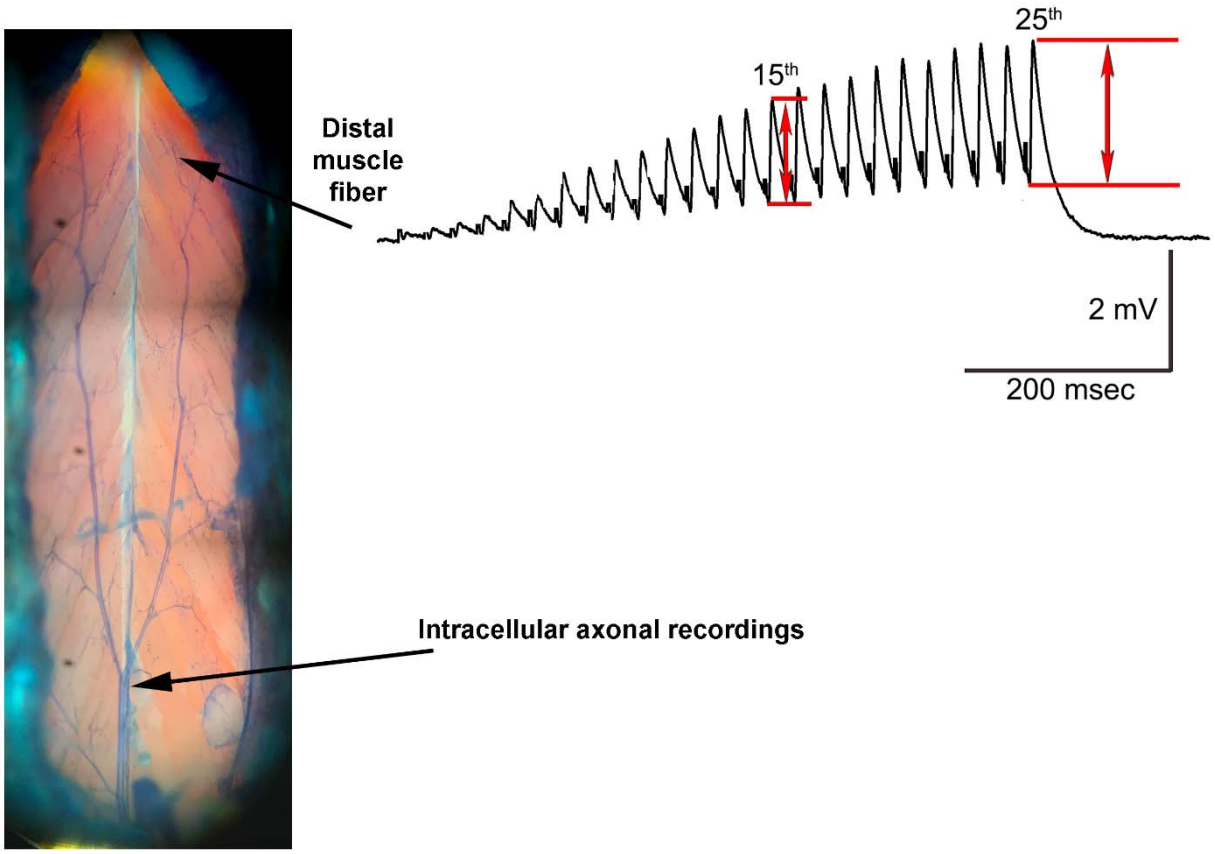

Figure 1: The opener muscle of a crayfish walking leg and electrophysiological recording locations. Representative trace of the excitatory junction potentials (EJPs) recorded with an intracellular electrode from the distal muscle fibers in opener muscle of a crayfish walking leg. The responses show a marked facilitation that occurs throughout the stimulation train delivered at 40Hz for 25 stimuli. The amplitude of the 25th EJP is used for indexing the effect of riluzole. The bifurcation of the nerve in the proximal region of the opener muscle is the location used to obtain intracellular recordings within the motor nerve axon.

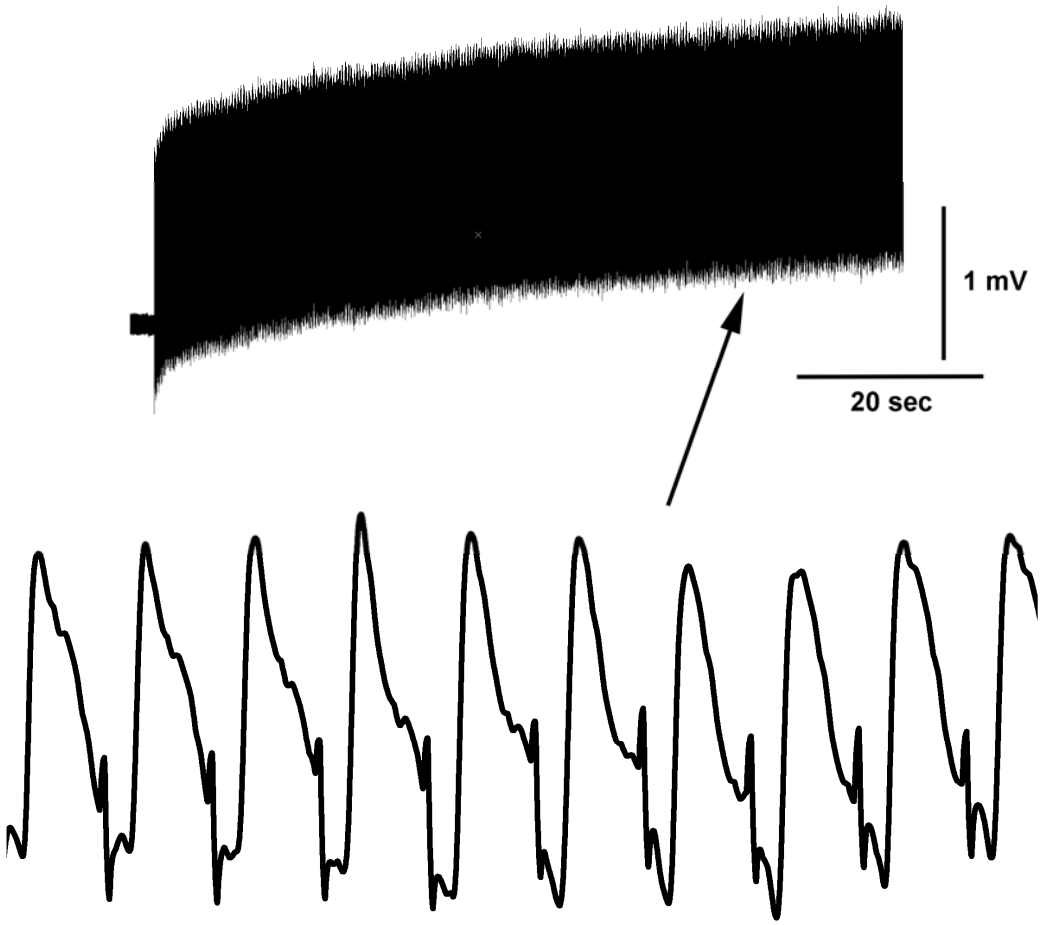

Figure 2: The continuous stimulation paradigm for examining the effect of riluzole. The motor nerve to the opener muscle is stimulated for one minute in saline and then stimulation is stopped and incubated in riluzole for 10 min. The preparation is again stimulated for 1 min. The amplitude of five EJPs at the end of the minute in the initial saline and at the end of the minute in the exchanged solution. The amplitude of the EJPs is obtained from measures at the trough from the start of the EJP to the peak of the EJP.

To evoke action potentials in the excitatory axon, it is selectively stimulated by a Grass stimulator. The distal muscle bundles (Figure 1) were impaled with a sharp intracellular electrode (20 to 30 MOhm resistance) filled with 3 M KCl. The excitatory junction potentials (EJPs) were recorded from the muscle fiber of interest with two different paradigms. One paradigm was inducing short-term facilitation (STF) in the EJPs by stimulating at 40 Hz for 25 stimuli within a train and repeating a train every 10 s. The second paradigm consisted of continuous stimulation at 20 Hz or a higher frequency, up to 60 Hz, to facilitate detectable evoked responses. The continuous stimulation occurred for 1 min to obtain a plateau in the amplitude of the EJPs (Crider and Cooper, 2000) (Figure 2).

The amplitude of the 25th EJP within the pulse trains was used for measures over time and effects due to Riluzole exposure. The presence or absence of EJP was used as an index for the effect of Riluzole with the continuous stimulation paradigm.

To record action potentials within the excitatory motor neuron, a microelectrode was placed into the excitatory axon of the opener muscle close to the axon bifurcation (Figure 1) (He et al., 1999). A standard head stage and amplifier for intracellular recording of the muscle and axon was used (Axonclamp 2B, and

1 X LU head stage, Molecular Devices, Sunnyvale, CA, USA). The crayfish saline used was a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 CaCl2·2H2O, 2.45 MgCl2·6H2O, and 5 HEPES adjusted to pH 7.4).

Crab chordotonal organ (PD)

The dissection and recording procedures are described in Majeed et al., (2013). In brief, the animal was induced to autotomize the first or second walking leg by lightly pinching at the base of the leg with pliers. The propodite-dactylopodite (PD) chordotonal organ spans the last segment of the leg (Figure 3). The PD organ was exposed by cutting a window in the cuticle on both sides of the leg in the propodite segment. The leg was pinned in a Sylgard-lined dish and submerged in crab saline. The PD nerve was then exposed and pulled into a suction electrode for recording. During the experiment, the dactyl was moved from a flexed position to an open position in a one-second time frame, held for 10 s, and then moved back to the starting position (Figure 3). An insect dissecting pin was used to mark the displacement range, and each displacement was marked on the computer recording file. The crab saline used during recordings of the sensory nerves consisted of (in mM) 470 NaCl, 7.9 KCl, 15.0 CaCl2·2H2O, 6.98 MgCl2·6H2O, 11.0 dextrose, 5 HEPES acid and 5 HEPES base adjusted to pH 7.4. One set of experiments was conducted at 1 mM, and a second group at 10 mM Riluzole.

The analysis of the electrical signals from the PD was processed by measuring the number of spikes for 10 s, which covered the dynamic movement of the joint to the stretched position (Figure 3). Three trials were performed for saline and Riluzole for each preparation. The exposure to Riluzole was static for 10 min prior to moving the joint again. The activity from the set of three trials was averaged for each condition and a percent change determined for each preparation (Stanback et al., 2019).

Data analysis

The rank sum pairwise test or a sign test was used to compare the differences in responses before and after exchanging solutions. When the assumption of normality held, a paired t-test was conducted to analyze the data. In some cases, synaptic responses were non-existent with exposure to Riluzole, which did not allow for parametric analysis. The analysis was performed with Sigma Stat software. A p-value < 0.05 was considered statistically significant for determining changes for exposure to Riluzole. To examine the consistency and reproducibility of the data, groups of participants blinded to the specific settings of the analysis software were asked to supply their interpretations of the number of spikes for some of the same data sets.

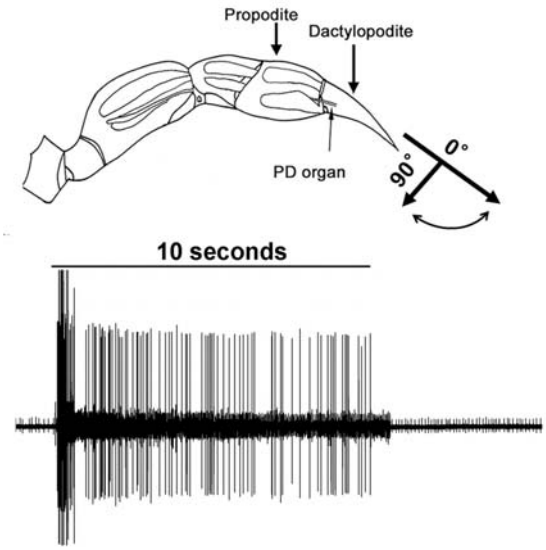

Figure 3: A schematic diagram of the first walking leg of the blue crab, *Callinectes sapidus*, containing the propodite-dactylopodite (PD) chordotonal organ used to assess the action of riluzole on primary sensory neurons. The joint containing the PD organ is initially bent at 90 degrees and extended to zero degree while obtaining extracellular recordings from the nerve of the PD organ with a suction electrode. The joint is rapidly moved within 1 s to the extended position and held for at least 10 s. The number of extracellular spikes measured over the 10 s is used a quantification of neural activity.

RESULTS

The crayfish walking leg opener neuromuscular preparation

The evoked EJP responses on the opener muscle rapidly facilitated with repetitive stimulation as illustrated (Figure 1). The amplitudes of the EJPs tend to reach a plateau

by the 25th stimuli within a train when the nerve is stimulated at 40 Hz (Crider & Cooper, 2000; Desai-Shah et al., 2008). Thus, the amplitude of the 25th EJP within the stimulus train is used for assessment for the effect of Riluzole on synaptic responses (Figure 4). In this stimulation paradigm, some preparations had an immediate response in decreasing evoked EJPs while others took up to 10 min to show failures in evoking EJPs. Out of the six preparations, four produced intermediate failures in evoking an EJP while two preparations completely shut down for an extended time. When increasing the stimulation voltage to the nerve, four of the six preparations regained some evoked response, but would also fail in time (Figure 4). In rapidly exchanging the bath to saline without Riluzole, four of the six were able to regain some evoked responses. This paradigm resulted in six out of six preparations showing failure in evoking a response with exposure to Riluzole (1 mM) (N=6, $p<0.05$, non-parametric Sign-test).

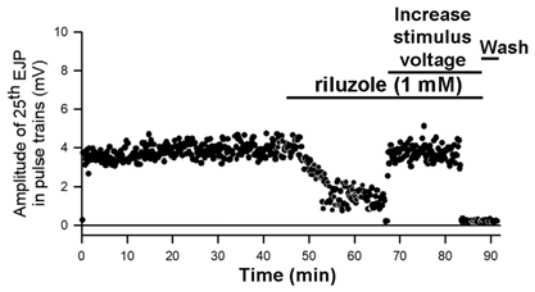

Figure 4: Indexing the effect of riluzole on evoked synaptic transmission. The amplitude of the 25th EJP from the 25th pulse stimulus train is measured over time in saline and during exposure to riluzole. When the EJPs showed failures the stimulus voltage is increased to the opener nerve. In some cases, the evoked responses return but will fail again in time. The bath saline was exchanged with fresh saline without riluzole to determine if the evoked EJPs would return.

The second paradigm with one minute of continuous minimal voltage stimulation to induce evoked EJPs was followed by 10 minutes of incubation without stimulation. In this paradigm all six preparations failed in evoking a response at the same voltage (Figure 2). One of the six preparations was able to regain some evoked responses by returning the stimulation voltage to the initial level with removal of Riluzole upon exchanging the bath with fresh saline. This stimulation paradigm also revealed that in all six preparations Riluzole (1 mM) was able to block evoked responses (N=6, $p<0.05$, non-parametric Sign-test).

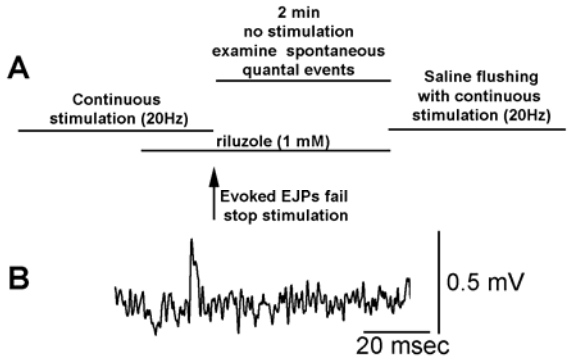

Figure 5: The stimulation paradigm of continuous stimulation at 20Hz. (A) Stimulation was continuous while applying riluzole until evoked stimulation failed, due to riluzole, then the stimulation was stopped, but the recordings continued to determine if quantal responses occurred. After 2 min of no stimulation, the nerve was again stimulated at 20Hz while flushing the preparation with fresh saline without riluzole. (B) While exposed to riluzole when evoked responses failed, spontaneous quantal events continued to occur. Thus, riluzole does not block the postsynaptic glutamate receptors on the opener muscle.

A separate set of six preparations were examined with continuous stimulation at a minimal voltage to induce evoked EJPs while applying Riluzole (1 mM). Once the preparations began showing failures in evoked responses, the stimulation was stopped to examine if spontaneous quantal events were present within a two minute period (Figure 5A). After the two minutes, the bathing media was extensively flushed five times with fresh saline and the nerve was returned to the same stimulation voltage as during the initial conditions to examine if the evoked responses returned. With this more intense flushing five of the six preparations were able to regain synaptic responses. In all six preparations, as with the previous simulation conditions, all six preparations shut down with Riluzole (1 mM) (N=6, $p<0.05$, non-parametric Sign-test). Spontaneous quantal events were observed during the time evoked responses failed and during the presence of Riluzole (Figure 5B).

Effect of Riluzole on the shape of the action potential

When Riluzole (1 mM) had an effect on the evoked EJPs, the amplitude depressed rapidly. Given that the spontaneous quantal events were still present indicates that Riluzole was not blocking the postsynaptic glutamate receptors, but rather having an effect on the ability of the motor nerve to induce an evoked response. To address if Riluzole (1 mM) had an effect on the induction of an action potential in the motor nerve, intracellular axon recordings were performed while exposing the preparation to Riluzole (1 mM). The action potential within

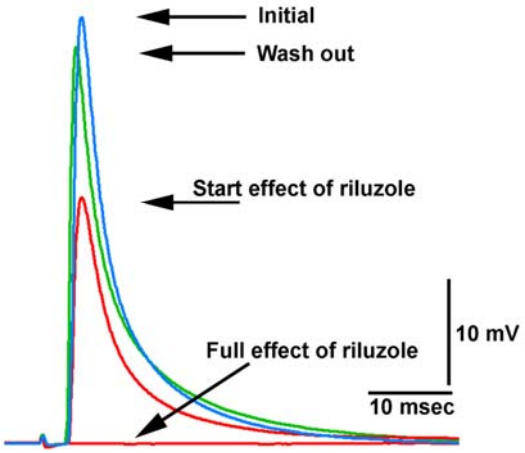

Figure 6: Representative traces of action potentials from the excitatory motor neuron to the opener muscle in saline and during incubation in riluzole. The amplitude is depressed and recovers upon extensive flushing out the riluzole.

the excitatory motor neuron innervating the opener muscle was depressed to being undetectable; however, the amplitude returned after removal of Riluzole with extensive flushing with fresh saline (Figure 6). This same trend occurred for all 6 preparations (Figure 6; N=6, $p<0.05$, sign test).

Effect of Riluzole on the PD organ

The primary sensory activity of the PD organ did not show a consistent trend in altering activity upon exposure to Riluzole (1 mM). The preparations were exposed for 90 minutes in a static bath to Riluzole after being examined in saline only. All the preparations produced a high

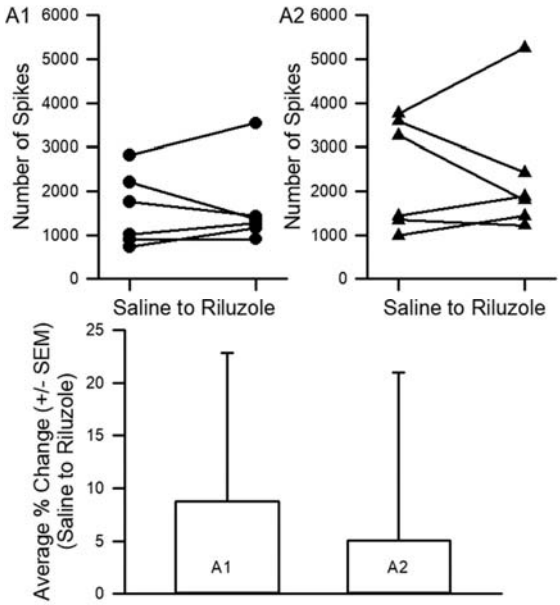

Figure 7: Analysis of a given set of recordings by two different participants with unbiased training in what spike amplitudes to measure for the crab PD organ before and during exposure to riluzole (1mM) for 10 min prior to moving the joint again. Two participants (A1 and A2) analyzed all six preparations. It is illustrated that one participant (A2) determined to choose small amplitude spikes in the analysis and a total number of spikes was higher for each preparation as compared to participant A1. The mean (+/- SEM) percent change from saline to riluzole is shown. Note the general trends in analysis by both participants is the same and there is no significant difference due to the effect of riluzole (1mM).

sensitivity to movement of the joint. All six preparations were analyzed by two independent students to determine what was considered activity of interest. One individual used a threshold of the standard deviation of the mean values to measure the small responses observed when the limb was bent at 90 degrees. Another individual used a set of measurements off the baseline which detected the smaller amplitude spikes, that occurred during the static bent position as well as during the extended movement and held position at 0 degrees. Even though the absolute values vary between the two analyses, the trends are similar. There is no significant effect by Riluzole (1 mM) for either analysis (Figure 7; N=6, $p>0.05$ paired T-test).

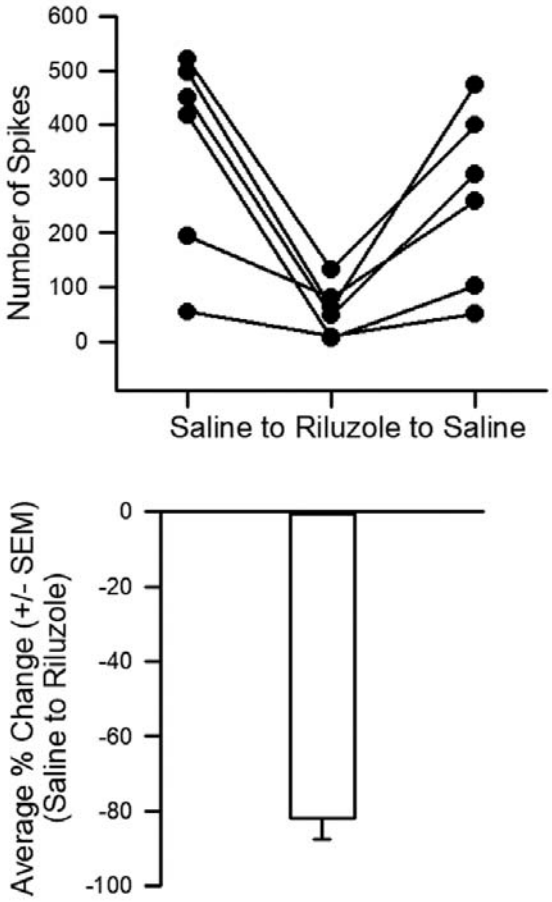

Figure 8: The effect of 10mM riluzole on the activity of the crab PD organ. Six out of six preparations drastically reduced in activity due to a 10 min exposure in 10mM riluzole. All six preparations showed some return in activity with extensive flushing out of the riluzole and exchange to fresh saline. The mean (+/-SEM) percent change in activity from saline to riluzole is approximately 80% with 10mM riluzole.

Reproducibility in analysis of sensory activity

Having participants in a college course re-examine given data sets from recordings of the crab leg PD organ resulted in different absolute values in the number of spikes but with the same general trends. By only providing a movie in how to use automated software to analyze events in a recording, students had different impressions in what were considered important to measure. When the amplitude of responses varies among preparations, due to the seal on the suction electrode around a nerve, even with automated software one can obtain different outcomes. Figure 9-A illustrates differences between the analyses of the participants. The standard deviation from the baseline, using a sine wave to follow the baseline, was set (i.e. 2.42) to detect events not observed prior to moving the crab leg. Using the same trail in the data set, but setting the standard deviation off the baseline to detect the events (i.e. 1.6529), provided a large number of spikes to measure during the 10 s of movements and holding the joint in an extended position (Figure 9-B). The events measured with the lower standard deviation are indicated with a pentagram outline. Note the event with a rectangle during the joint movement is detected with the lower standard deviation. Comparing Figures 9-A and B illustrates what is detected during the movement. The signal measured while the leg is bent at 90 degrees is likely from a position sensitive neuron, which increases in firing rate while extending the joint to 180 degrees (Cooper and Hartman, 1999; Cooper, 2008). Thus, the absolute differences is in part due to the increased firing rate of this neuron and potentially additional neurons being recruited during the movement not observed while the joint is held stationary in the 90 degree angle. There are likely about 80 neurons within the PD nerve being recorded and many with similar amplitude in the generated extracellular spikes making it impossible to separate the responses from individual neurons during the movement of the joint with whole nerve recordings (Hartman and Cooper, 1999).

A computer screen capture in two regions of a file where one has a prominent number of spikes (Figure 9A) and a second where there are no spikes (Figure 9B) illustrates the baseline differences. Each panel (Figure 9A&B) has a top region showing the raw data, along with a superimposed screen of the window to determine the parameters for counting the number of spikes. Note the analysis is set at the same standard deviation of the mean (i.e., 1.2396) for both regions of the file. The bottom aspect of each panel (Figure 9A&B) depicts the continuous running value in the number of counts. The number of spikes counted is noted by the open circles above the trace in the green boxes of the highlighted analysis windows

superimposed on panels for both A and B. In B the open circles are so close together it appears as a line to be counted

In comparing the analysis of the two participants with more training in data analysis of PD organ responses and subsets of additional participants for a single data set, one can see the absolute values did vary among the six preparations. However, the trends are again similar (Figure 10). The lines shown in red and blue are the two participants who analyzed all six data sets as in Figure 8.

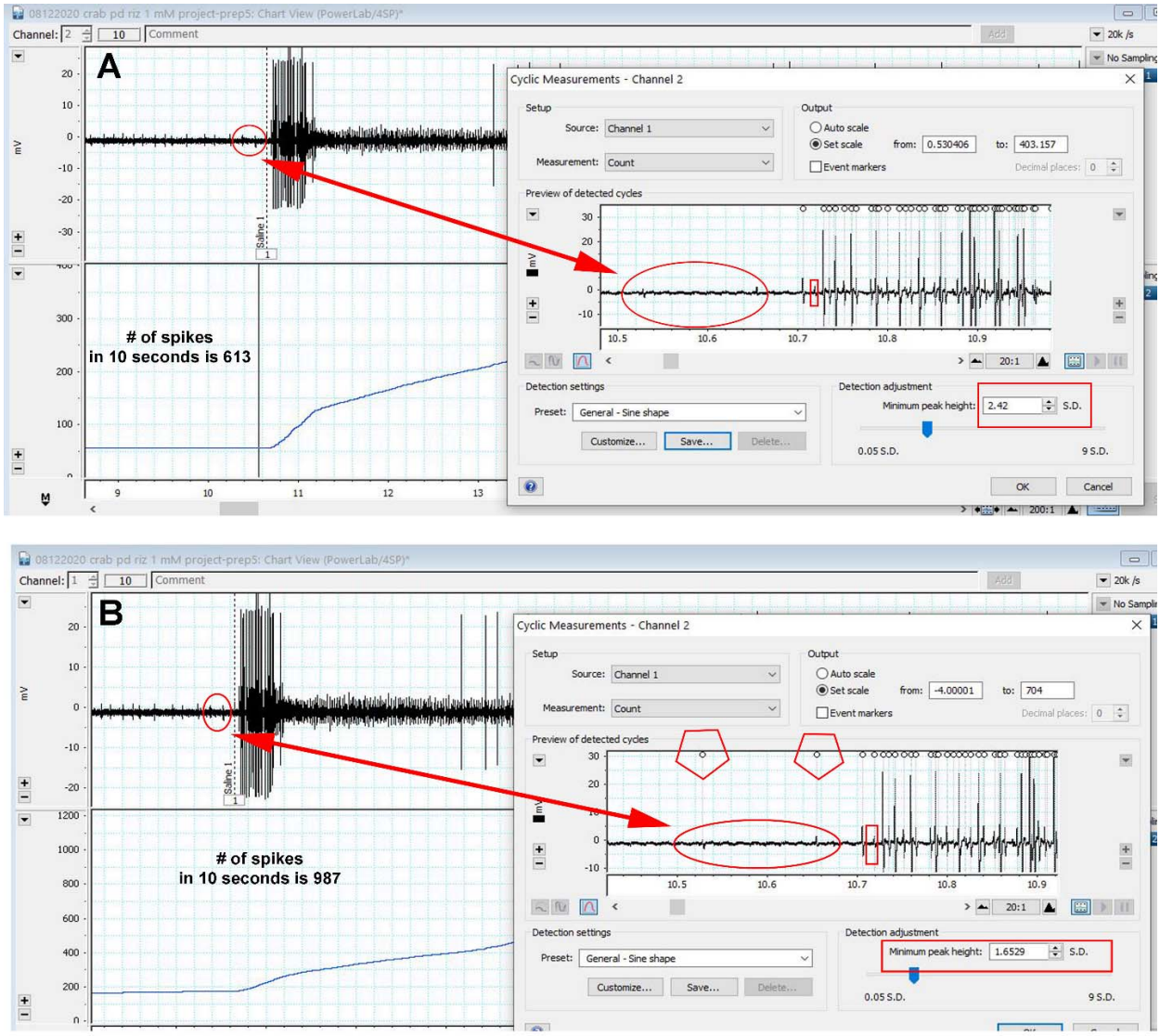

Figure 9: Analysis of a given data set from a recording of the crab PD organ with different thresholds of spike detection. (A) The mean sine shape baseline is used for a basal measure and a standard deviation of 2.42 is used to detect the extracellular spikes. Within 10 seconds 613 counts are measured. (B) The mean sine shape baseline is used for a basal measure and a standard deviation of 1.6529 is used to detect the extracellular spikes. Within 10 s 987 counts are measured. Note the small spikes observed prior to the movements are the sizes which would be detected within the responses during the 10 s. Shown as a polygram outlining the detection of small responses. Note these are not measured as these are prior to the 10 s movement. Note the rectangle within the time frame which would be used as a measure if activity.

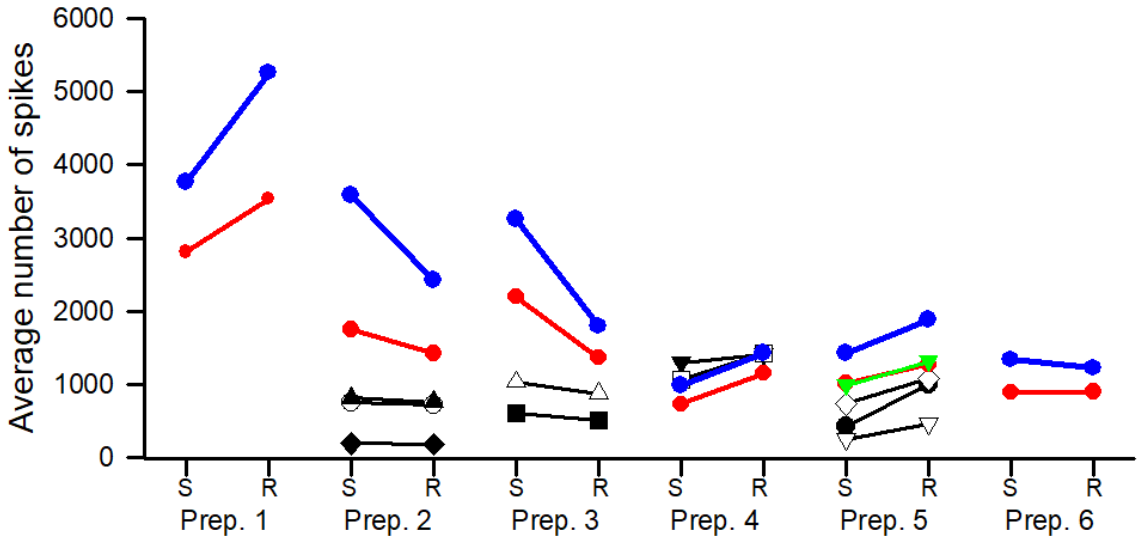

Figure 10: Varied analysis in the activity of given data sets by multiple participants. The red and blue lines are analysis from two participants who measured all the preparations. The additional lines are from different participants for each preparation. Note most all participants chose different levels of threshold from the baseline to detect the extracellular spikes from the activity recording of the crab PD organ in saline (S) and during exposure to 1 mM of riluzole (R). Note the trends are similar in the effect of riluzole either increasing activity or decreasing activity.

Discussion

In brief, this study demonstrated that Riluzole depressed evoked synaptic transmission at the crayfish NMJ but did not block the postsynaptic glutamate receptors since spontaneous quantal responses occurred while evoked responses were blocked. The amplitude of the action potential in the motor neuron was depressed but could be regained with extensive removal of Riluzole. In addition, primary sensory neurons in the crab model were suppressed in a dose dependent manner. These results indicate that the neural activity of sensory and motor neurons is reduced due to blocking or reducing the electrical excitability of the neurons. This is likely due to blocking of voltage -gated Na+ channels.

In mammals it appears that Riluzole blocks the NMDA glutamate receptors (Bellingham, 2011). The crayfish NMJ is glutamatergic but are not responsive to NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainate, which are agonists to subtypes of glutamate receptors. The quisqualate receptor subtype is the postsynaptic receptor subtype at the crayfish and *Drosophila* NMJs (Lee et al., 2009; Titlow and Cooper, 2018), which may explain why Riluzole did not block the quantal spontaneous events. These events occurred in the absence of evoking synaptic transmission by nerve stimulation. Even if pre-synaptic voltage-gated calcium channels are blocked, spontaneous quantal events still occur. Considering that Riluzole may block voltage-gated calcium or sodium channels (Bellingham, 2011; Lamanauskas and Nistri, 2008), this would not affect the occurrences of spontaneous quantal events. However, if action potentials are not able to be delivered to the presynaptic terminals, then the voltage-gated calcium channels will not be recruited. Considering the amplitude of the action potentials in the motor neurons are reduced may explain why synaptic transmission is reduced at the crayfish NMJ. Increasing the stimulation voltage was able to transiently re-establish synaptic transmission indicating a recruitment of more voltage-gated sodium

channels or potentially the enhanced field potential around the nerve transiently recruited the voltage-gated calcium channels. Given that the sensory activity of the crab is dampened suggests that the voltage-gated sodium channels is a target of Riluzole as the mechanism of action on these primary sensory neurons.

The lower concentration of Riluzole at 100 µM in an earlier study did not have an effect on the crab PD organ (Kallik et al., 2017), and in the study herein, 1 mM was shown not to have a significant effect, but 10 mM drastically reduced the activity of the crab PD organ. At the crayfish NMJ a 1 mM concentration was able to block the action potential relatively quickly after exposure. This potentially indicates differences in the affinity of Riluzole on the voltage-gated sodium channels between crayfish and crab neurons (Catterall et al., 2007; Zakon, 2012). However, other factors may be responsible for this observation, such as the composition or osmolarity differences in the saline solutions. The difference in saline may have an effect, as the physiological saline for the marine crustacean has a much higher osmolarity than the freshwater crayfish saline. In addition, the neuronal sheath wrapping between the species may be quite different, directly altering accessibility of compounds to the ion channels on the neuronal membranes.

Considering it took extensive flushing of fresh saline for the crayfish NMJ, as well as the crab PD organ, to obtain recovery of neuronal function indicates that Riluzole is not tightly bound to the targets. Riluzole dissolved readily in both saline solutions which may also indicate the reason for a relative short half-life in humans with it being excreted through the urine (FDA.gov, 2016). From results of this study the effect may be to reduce excitability of motor neurons and reducing electrical activity and thus ROS production as well as potentially reducing Ca^{2+} induced cellular effects related with excitotoxicity.

Future investigation could focus on if Riluzole blocks voltage-gated calcium channels in the presynaptic terminals by use of calcium sensitive indicators at the crayfish NMJ, in addition for other model experimental preparations such as the frog and rodent NMJs. Given that Riluzole has other potential actions on cellular function besides ion channels and glutamate receptors, there are many avenues remaining to be investigated. Even though Riluzole is not a cure for ALS or other potential glutamatergic excitatory toxic conditions, the better one understands the various effects of Riluzole may be enhanced treatments will be forthcoming. The new drug combination of sodium phenylbutyrate and tauroursodeoxycholic acid (i.e., AMX0035), which helps to minimize cellular damages, is opening novel approaches to treating ALS, as well as other diseases (Paganoni et al., 2020).

Another aspect of this study addresses the analysis of sensory activity in the whole nerve recordings. One must physically set a threshold level for each preparation to detect the signals in the recording activity. There is no standard value of the standard deviation from baseline to use for different preparations with the software commonly used in teaching laboratories. In addition, by setting a standard deviation of the signals to detect events in the automated software, if the activity becomes drastically increased or decreased by the application of a compound, then within a continuous recording the standard deviation of the signal will vary. Thus, a different threshold may be required to measure responses within a given trial. In addition, if the baseline wavers due to movement of the preparation while recording, it is not feasible to use a standard window discriminator at a set level. Even using a sine wave to follow the baseline trace, events observed by eye are noted to be missed or what is determined as noise during a stationary time in the recording may be counted as an event. One can use more comprehensive customized programs and software, but this may not be practical in a course setting for a class using educational level instrumentation and software. Using the software as used in this study, one can expand the time scale and count by eye the events to be considered, but this is time consuming and defeats the purpose of automated analysis. This is important to note when providing raw data with publications one may need to detail analysis for each preparation or possible even provided detailed videos of the analysis of each preparation to obtain the reproducible outcomes. This is also a concern if

one wanted to use a citizen science approach to help in data analysis (Clare et al., 2019; Lea, et al., 2017). In this study, the trends in the outcomes were similar among different participants despite the variation in measures of the sensory activity.

This ACURE (authentic course-based undergraduate research experiences; Malloy et al., 2017; Stanback et al., 2019; Wycoff et al., 2018) approach builds on the CURE (course-based undergraduate research experiences) philosophy (Bakshi et al., 2016; Linnet al., 2015); however, it aids students in undergoing a more complete research experience. The authentic scientific investigations with participating students in a classroom setting is a trend which is being promoted to expose students to research. In addition, utilizing participants within a course setting to analyze data sets blind to the experimental conditions provides an additional level for interpretation of the findings.

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