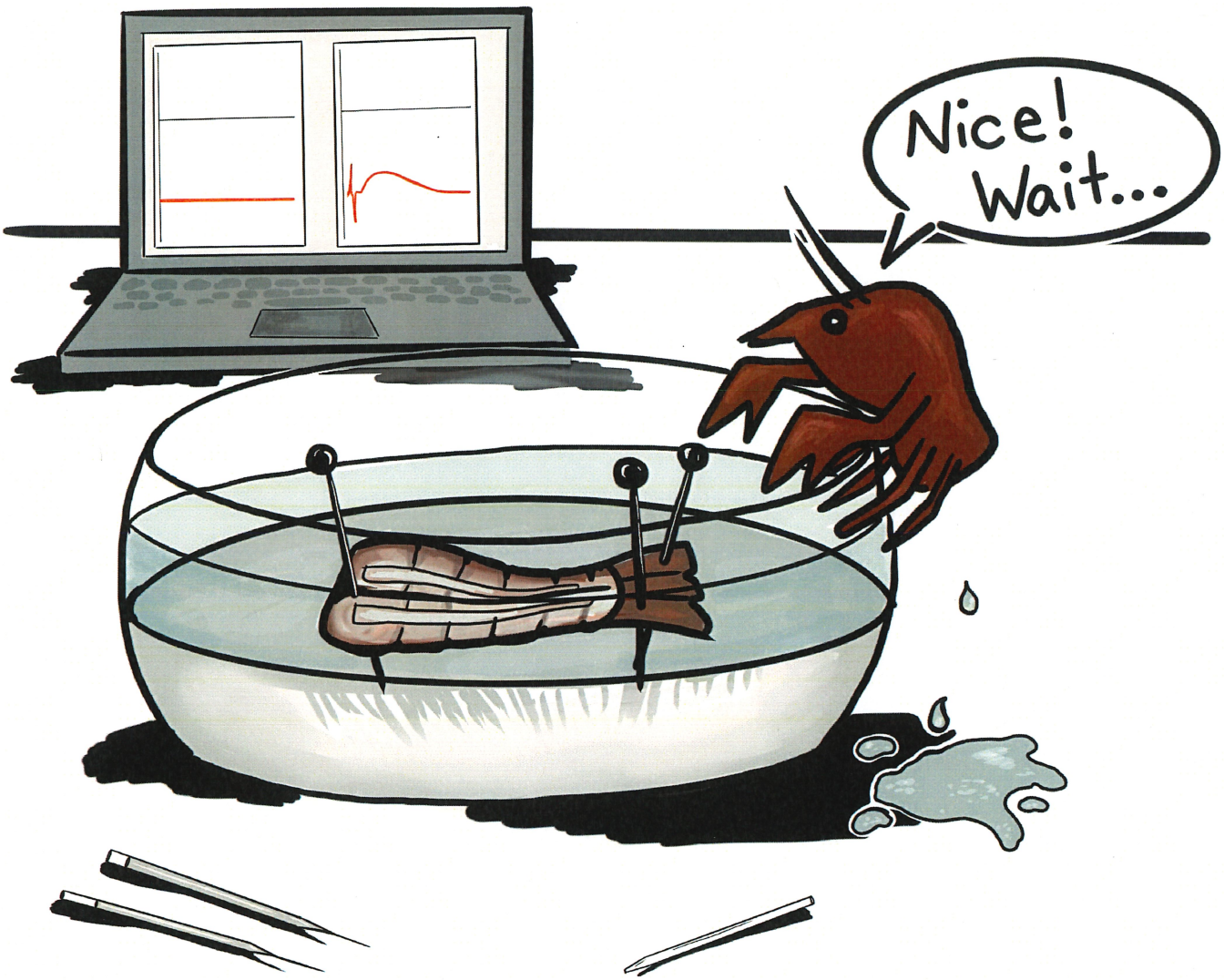
Pioneering Neuroscience



Volume 18; December 2019

PIONEERING NEUROSCIENCE

VOLUME 18, DECEMBER 2019

Contents

Information on this journal can be accessed at:
https://ojs.grinnell.edu/index.php/pnsj

Typeset, printed and bound by Grinnell College, Grinnell, IA 50112, USA.

It is my pleasure to present the eighteenth volume of *Pioneering Neuroscience: The Grinnell Journal of Neurophysiology.* The articles collected in this volume represent original contributions to the field of neuroscience offered by students *in the seventeenth offering* of Biology 150: Introduction to Biological Inquiry - the Language of Neurons. As has been true for sixteen previous classes of Bio 150 (one of the volumes was created by students in an advanced class), this course was taken by most of the students during their first semester in college. For all of the students, this was their first college-level biology course!

I wish to thank the students of Biology 150 for their hard work and collegiality. None of this would have been possible without the contributions of Ashley Wolterstorff, the lab instructor, and the excellent work of mentor/lab assistant Emily Kozik ’19. The cover art was created by Aubrie Torhorst ’23. Other artwork credits include: Hyejun Jung ’23 (p. 6) and Nate L’esperance ’23 (p. 20). Last, but not least, I wish to recognize Dr. Steve Ryan for creating and Jamie Lee ’23 for naming the wonderful SLURP (Simultaneous Liquid Uptake and Replacement Pump).

Clark Lindgren, Editor
December, 2019
Grinnell, Iowa

Pioneering Neuroscience, 2019, **18**, 1-5.

Temperature Dependence of DF_2's Effectiveness is Due to Temperature Sensitivity of Phosphatases

CHUNKAI JIN, HYEYUN JUNG, and MINH G. PHAM
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

The neuropeptide DRNFLRFamide (DF_2) increases synaptic transmission at the crayfish neuromuscular junction, but its function depends on temperature. The difference in DF_2's effectiveness in synaptic modulation may be due to different degree of dephosphorylation by protein phosphatases. Motivated by previous studies on temperature dependence of synaptic enhancement induced by DF_2, we hypothesized that (1) DF_2's function depends on phosphorylation and inhibition of phosphatases would increase DF_2's effectiveness. (2) At lower temperature phosphatases will be less active. Therefore, increase in DF_2's function at low temperatures is due to more phosphorylated proteins induced by inactiveness of phosphatases. We experimented with DF_2 and phosphatase inhibitors at both low and room temperatures. We determined the effectiveness of DF_2 by measuring Excitatory Postsynaptic Potential (EPSP) of extensor muscle cells in different solutions with five minutes of adjustment period using intracellular recording and repeated stimulation of every five seconds. Our data shows that DF_2's effectiveness increases at low temperature, and the effect of phosphatase inhibitors at low temperatures was not as prominent as that at room temperature. The results indicate that at low temperature, phosphatase has already been inhibited, and further inhibition with phosphatase inhibitors has no notable effect. This result supports our hypotheses, suggesting that the effectiveness of DF_2 increases in low temperature due to minimized function of phosphatase.

INTRODUCTION

DF_2 (DRNFLRFamide) is an FMRFamide-like peptide and a neuromodulator. DF_2 binds to a receptor and activates a G-protein cascade (guanine nucleotide-binding protein cascade). This cascade leads to the activation of protein kinases which phosphorylate their target proteins. Protein phosphorylation, in turn, potentiates the effect of DF_2 on synaptic transmission (Badhwar, Weston, Murray, & Mercier, 2006). As a result, DF_2 enhances Excitatory Postsynaptic Potential (EPSP) by increasing the amount of transmitter released from axon terminals (Badhwar, et al., 2006).

Dunn & Mercier (2003) demonstrated the close relationship between the efficiency of neuropeptides and temperature. DF_2 is more effective at 7-9 °C than at 15-17 °C (Friedrich, Quigley, Srivastava, Skerrett, & Mercier, 1994). However, little is known about the mechanism behind the temperature dependence of DF_2. Friedrich et. al (1994) suggested that DF_2's effects on synaptic transmission is enhanced because phosphatases are less functional at low temperatures.

Phosphorylation and dephosphorylation are processes that modulate the function of DF_2 (Abdul-Ghani, Kravitz, Meiri, & Rahamimoff, 1991). Phosphatases negatively modulate the degree of phosphorylated proteins in the cell. It has been shown to promote the degradation of organic phosphorous compounds (Pant, 2014) They reverse the function of the protein kinases, which add a phosphate group to their target proteins and lead to DF_2's long-lasting functions (Badhwar et al., 2006).

The effect of DF2 requires phosphorylation of the proteins, enhancing the amount of neurotransmitter released by changing the currents of potassium and calcium (Friedrich et al., 1998). However, phosphatases reverse the phosphorylated proteins to the initial state and thus, minimize the effect of DF_2.

According to Elias, Wieczorek, Rosenne, & Tawfik (2014), enzymes have universal enzymatic rate-temperature dependency. Every enzyme functions at its ideal temperature. Phosphatase, an enzyme, is thus affected by the temperature. At low temperatures, phosphatases' ability to remove the phosphate group from target proteins is inhibited. As a result, the abundance of phosphorylated proteins would further enhance DF_2's effect on synaptic transmission.

We hypothesized that phosphatases are less functional at low temperatures, and it potentiates the effect of DF_2 on synaptic enhancement by slowing down the decay of phosphorylated target proteins. We measured DF_2's effectiveness by observing the change of EPSP amplitude. DF_2 increased the EPSP amplitude at both room and low temperature. To confirm that phosphatases are less functional at low temperatures, we compared the EPSP amplitudes with and without phosphatase inhibitors at low temperatures. Our data suggested that at low

temperature, phosphatases are less functional, and further inhibition of phosphatases does not affect the activity of DF_2. Although our data is not statistically significant, it tentatively supports our hypothesis. This research will provide a better understanding of the role of phosphatases in synaptic enhancement and the mechanism behind the increase in DF_2's effectiveness at low temperatures.

MATERIALS AND METHODS

Preparation

Crayfish were maintained and provided by Grinnell College. The crayfish were first anesthetized by being placed in an ice bath for 15 minutes. We experimented on extensor muscle cells and the nerves in the dorsal part of the crayfish tail. To isolate the muscles, we separated the tail from the crayfish by cutting the crayfish between the tail and thorax using scissors. We removed the ventral surface of the tail. To make the extensor muscles and the nerves more visible, we scooped out excess muscles and gut. We pinned the dorsal surface of the tail facing down in the dish. We submerged the tail with normal crayfish Ringers' solution. The crayfish tail always rested in the normal crayfish Ringers' solution unless we changed to different solutions.

Solutions

We used normal crayfish Ringers' solution (5.4mM KCl, 196mM NaCl, 2.6mM $MgCl_2$-$6H_2O$, 10mM HEPES, 13.5 mM $CaCl_2$-$2H_2O$, pH 7.4) to bath the muscle cells and maintain their normal function. The preparation was bathed in normal crayfish Ringers' unless switched to a different solution. We applied 50nM DF_2 in Ringers' solution as suggested by Weiss, Kreissl, & Rathmayer (2003) and 100µL of phosphatase inhibitors cocktail solution (2.5µM Bromotetramisde, 0.5µM Cantharidin, 1µM Calyculin A) at room temperature (RT) (19-20°C) and at low temperature (LT) (7-10°C). We adjusted the temperature by putting the solution in the freezer 15 minutes prior to the experiment. We put cubes of ice-Ringers' inside the solutions to maintain the temperature and measured the temperature near the muscle cell with a digital thermometer.

Intracellular EPSP Recording

We made suction electrodes and recording electrodes using glass capillary tubes pulled with PUL-1 World Precision Instruments puller. We filled the recording electrode with 3M KCl. The acceptable resistance for the recording electrodes was between 4 and 25 MΩ. For the suction electrode, we sanded the tip of microelectrodes. After sucking up the nerve, we stimulated the nerve with a suprathreshold stimulus using a Grass stimulator (f=0.2Hz). We measured the membrane potential using an amplifier for 10 minutes and calculated the EPSP by subtracting the minimum from the maximum value following the stimulus artifact. The data was collected with Powerlab and the lab chart software with mains filter on.

Data Analysis and Statistical Test

We calculated percentage change for each EPSP amplitude using the formula:

$$\% \text{ Change} = \frac{(EPSP\ treatment)}{EPSP_{control}} \times 100\%$$

RESULTS

We studied the effect of DF_2 at different temperatures with phosphatase inhibitors. For each chemical, we recorded EPSPs using intracellular recording for 10 minutes after adding the chemical for 5 minutes and calculated the percentage change using the formula. (See *Methods*)

To confirm that DF_2 increases synaptic efficacy (Skerrett, Peaire, Quigley, & Mercier, 1995), we compared EPSP percentage change in control experiments (n=6) to those treated with DF_2 (n=5) at room temperature.

For our control experiment, we measured EPSP amplitude in normal crayfish Ringers' solution. We calculated our baseline data to compare with other sets of experiments. We used the baseline data of control experiment as the benchmark for all graphs we show.

On the same crayfish, we changed the solution to that with addition of DF_2. We let the solution infiltrate the cells for five minutes. On average, EPSP amplitude increased by 141% under the presence of DF_2 at room temperatures. The EPSP percentage with DF_2 at room temperatures were also higher than that of control until 535 seconds (Fig.1). The result reconfirmed the potentiating role of DF_2 in synaptic transmission.

To examine DF_2's temperature sensitivity, we compared percentage of EPSP change induced by DF_2 at room temperature (n=5) and low temperature (n=2) over time.

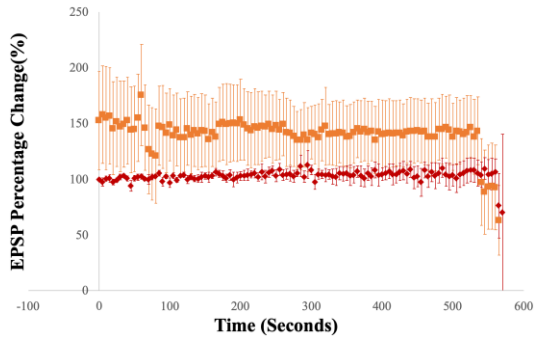


Fig.1*:* The orange squares represent percentage change of EPSP amplitude in DF_2 solution at room temperatures over time (n=5), and the red diamonds represent percentage of EPSP change for control experiment (n=6). Each marker represents mean of two to five data. Error bars are the standard errors of the mean.

We used the data from the previous experiment on EPSP amplitude induced by DF_2 at room temperature. On the same crayfish, we switched the DF_2 solution into that of low temperature (with the average of 10.2°C for first trial and 11.2°C for second trial). We modulated the temperature using Ringers' ice cubes for the first trial and we put the DF_2 solution beaker in the freezer for the second trial. We maintained the temperature for both trials by using Ringers' ice cubes and measured the temperature near the muscle cell throughout the trials. The results suggest that compared to baseline data, the effect of DF_2 at low temperature is not as prominent as that of room temperature. While at room temperature, the mean value of percentage change was 141% (Fig.1), that of low temperature was 120% (Fig.2). However, if we consider the initial drop of EPSP at low temperature by 88% (Friedrich et al., 1994), through calculation, DF_2's effectiveness actually increases by 450% at low temperature. Therefore, although our data seems to contradict Friedrich et al.'s (1994) finding that the effect of DF_2 is enhanced at low temperature, our results still support the same finding. (See *Discussion*)

To determine the role of phosphatases in the synaptic enhancement induced by DF_2, we compared percentage change of EPSP amplitude induced by phosphatase inhibitors and DF_2 (n=2) to that of DF_2 only (n=5) both at room temperature.

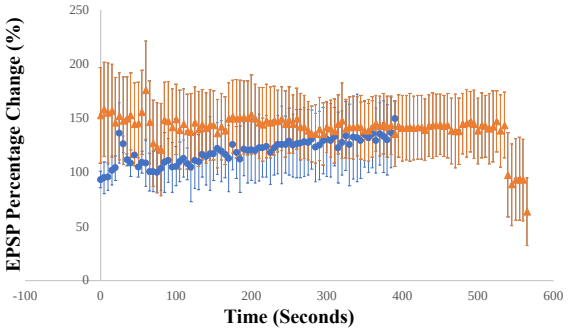


Fig.2: The orange triangles represent the percent change of EPSP amplitude under the condition of DF_2 at room temperature over time (n=5), and the blue circles represent the percent change of EPSP amplitude induced by DF_2 at low temperature over time (n=2). Each marker represents mean of each data set. Error bars are the standard errors of the mean.

We used data from the experiment on EPSP amplitude induced by DF_2 at room temperature. We used the same crayfish and added phosphatase inhibitors cocktail using a micropipette into the DF_2 solution. We had two trials with different methods of mixing chemical for this experiment. For the first trial of the experiment, we injected the phosphatase inhibitors cocktail with a micropipette and dispersed the cocktail by pumping repeatedly. For the second trial of the experiment, we followed the same procedure to add phosphatase inhibitors but mixed the solution by stirring with forceps. For this trial, we let the solution infiltrate the muscle cells for five minutes. Under the treatment with the combination of phosphatase inhibitors and DF_2, the EPSP amplitude increased by 260%. This was a greater change than when treated with only DF_2 up to 170 seconds (Fig.3). After 170 seconds, the percentage increase of EPSP amplitude dropped abruptly to average of 84% (Fig.3).

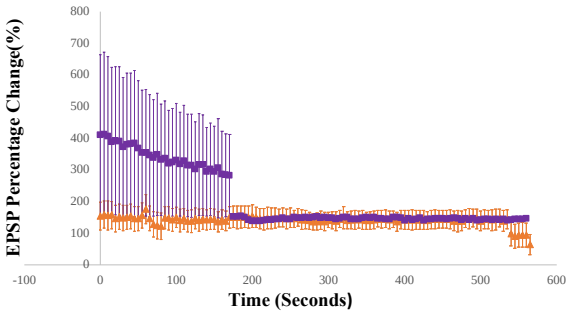


Fig.3*:* The purple squares refer to the percentage change of EPSP amplitude at room temperature induced by phosphatase inhibitors cocktail and DF2 solution over time (n=2). The orange triangles represent the percentage change of the EPSP amplitude in the solution treated with DF_2 at room temperature over time (n=5). Each marker represents mean of two to five data. Error bars are the standard errors of the mean.

To verify the change in effectiveness of phosphatases at low temperature, we compared the percentage change of EPSPs in DF_2 with (n=1) and without (n=2) phosphatase inhibitors at low temperature.

We used the same data on DF_2 low temperature in Fig.2 to compare. After measuring the EPSP at low temperature, we used a micropipette to pump the phosphatase inhibitors into the solution. The solution's temperature at the time of injection was 9.7°C and it was maintained throughout the trial with cubes of iced-Ringers'. We let the solution sit and kept stimulating to make sure we did not miss out any possible change in EPSP. The data shows that the sudden jump in EPSP amplitude is relatively big, up to 153% but it can only maintain for 50 seconds before dropping back to normal. Afterwards, the EPSP amplitude constantly increased until the end of the trial, averaging 132% (Fig.4)

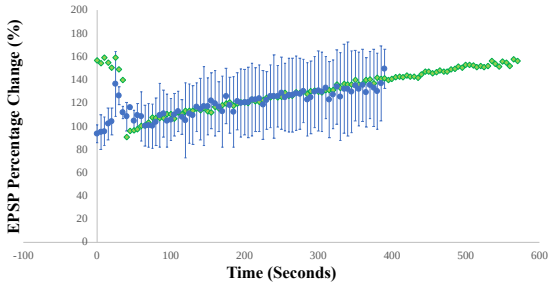


Fig.4*:* The green diamonds represent change of EPSP amplitude in DF_2 and phosphatase inhibitor cocktail solution at low temperature (n=1), and the blue circles represent change of EPSP amplitude in DF_2 solution at low temperatures (n=2). Each marker represents mean of two to five data. Error bars are the standard errors of the mean.

Compared to the control data, all of the experiments show an increase in EPSP amplitude (Fig.5). Since the extreme change by phosphatase inhibitors lasted a short amount of time, we separated data into two groups: change before the sudden drop and total change over time. The data of cocktail inhibitor and DF_2 before dropping had the highest average EPSP percentage change (260%), and the second highest group was the data of cocktail inhibitor and DF_2 induced group before drop at low temperature (153%). This data indicates that at experimental temperatures, the effect of phosphatase inhibitors is prominent.

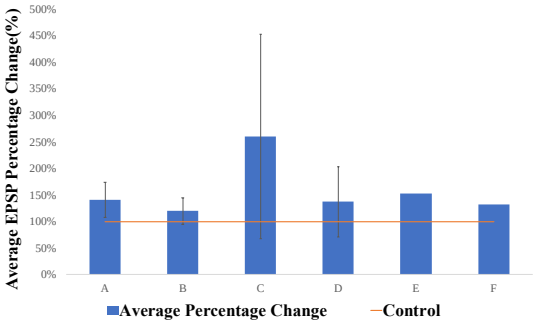


Fig5*:* The bar graph illustrates average change of EPSP amplitude of all experiments we measured. (A) DF_2 induced EPSP at room temperature (n=5); (B) DF_2 induced EPSP at low temperature (10-12°C) (n=2); (C) DF2 induced EPSP with phosphatase inhibitors at room temperature before the drop (n=2); (D) DF2 induced EPSP with phosphatase inhibitors overall (n=2); (E) DF2 induced EPSP with phosphatase inhibitors at low temperature before the drop (9.7°C) (n=1); (F) DF_2 induced EPSP with phosphatase inhibitors at low temperature overall (n=1). The orange line at 100% is the control data baseline (n=6).

DISCUSSION

Our data tentatively support our hypothesis that DF_2's activity is more profound at low temperature because phosphatases are less active. We confirm that (1) DF_2 increases synaptic activity at room temperature and low temperature. (2) EPSP amplitude induced by DF_2 at low temperature is enhanced by roughly 450% compared to that of room temperature. (3) At low temperature, phosphatases are less effective in negatively modulating synaptic transmission induced by DF_2. Therefore, our result explains the conclusion on the study of temperature-dependence of synaptic modulation by DF_2 on crayfish (Friedrich et al., 1994).

Our results reaffirm the potentiating effects of DF_2 in both room temperature and low temperature. However, for the sets of experiments to compare DF_2's effectiveness at low temperature and room temperature, our data contradicts Friedrich et al. (1994). We expected that at low temperature, EPSP amplitude would be higher than that of room temperature, but our results show that the average EPSP amplitude is decreased by 19%. However, at low temperature, EPSP amplitude is decreased by 88% (Friedrich et al., 1994) and DF_2 helps compensate for the decreased synaptic efficacy. Our calculation confirms that although the initial results might seem counterintuitive, the effect of DF_2 on synaptic transmission is 450% higher at low temperature than that at room temperature.

We also determined the modulating role of phosphatase and its temperature dependency by comparing the EPSP amplitude after adding the phosphatase inhibitors cocktail at room temperature and at low temperature. While for room temperature, the effects on EPSP amplitude induced by DF_2 is prominent as the percentage change in EPSP amplitude increases by

260% and is maintained by around three minutes. At low temperature, the effect of the inhibiting cocktail is reduced, as EPSP amplitude only increases by 153% for 30 seconds before dropping back. The results indicate that before adding the cocktail, phosphatases have already been inhibited by the drop in temperature.

Another interpretation of this data is that the abrupt changes in EPSP with phosphatase inhibitors were due to the stimulation becoming subthreshold for one or more axons in the nerves. This could reflect the unbeknownst effect of phosphatase inhibitors on the threshold in the nerve. Further research can look into other possible effects of the phosphatase on synaptic efficacy other than inhibiting phosphatase.

However, a variety of limitations affect the reliability and validity of this study. Firstly, our lab data is inconsistent because of the lack of lab sessions and hours and as a result, our result is not statistically significant. Secondly, temperature is a hard variable to maintain, so the margin of error for low temperature in our experiment is sometimes out of the pre-determined parameter. Since phosphatase enzyme is very sensitive to change in temperature, the inconsistency in temperature between experiments might hinder our final results. Thirdly, we were unable to gather enough data points for some experiment sets. For example, we only have one suitable data points for the effecs of DF_2 after adding the cocktail at low temperature. Future research can conduct more experiments to provide more reliability to the final data. Finally, because we use normal crayfish Ringers' with Ca^{2+}, the muscle usually twitched a lot and electrodes were frequently broken or slipped out of the targeted cell. Future research can consider using lower concentration of Ca^{2+} crayfish Ringers' to prevent or reduce muscle twitching and therefore, maintain a consistent result throughout the experiment.

ACKNOWLEDGEMENTS

We would like to thank our professor Clark Lindgren, our lab assistant Ashley Wolterstorff, and our mentor Emily Kozik for their instructions and assistances throughout the semester.

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Ketamine reduces synaptic transmission and DF2 enhances recovery from ketamine-induced synaptic depression at crayfish neuromuscular junctions.

KOFFI AMEGBLE, RIANA MULLIGAN, and RACHEL WOOCK
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Ketamine is a chemical that blocks N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-isoxazole propionic acid (AMPA) receptors and reduces excitatory postsynaptic potential (EPSP) amplitude. Due to this capability, ketamine is commonly used as an anesthetic; however, recent studies are showing its potential use as an antidepressant. DF2, an FMRFamide-related neuropeptide, creates a long-lasting enhancement of EPSPs, which may be able to counter the effects of ketamine. To see if DF2 enhances recovery from ketamine-induced EPSP depression, we induced EPSPs in crayfish tail extensor muscles. We stimulated the muscle in a normal physiological saline solution (Ringer's solution), a ketamine solution, a Ringer's solution with DF2 added, and finally, a ketamine solution with DF2 added. Our results show that ketamine reduced EPSP amplitude, and DF2 was not able to successfully recover the EPSP back to normal ranges after ketamine. Thus, although ketamine can decrease EPSP, DF2 was able to minimize ketamine's effects.

INTRODUCTION

As depression continues to plague the world, researchers are striving to find effective treatments for combating mental illness, an example being ketamine (Meisner, 2019). This mental disorder may require long-term pharmacological treatment, to which the body may become resistant over time (Chen, 2019). Additionally, it may take days or even weeks for the patient to begin seeing a significant change. For some individuals, this may be incredibly harmful, which is why it would be beneficial to introduce faster-acting medications (Chen, 2019). Traditionally, ketamine was a medication used as an anesthetic that can provide pain relief and sedation, among other uses. Ketamine effectively and rapidly blocks the glutamate receptors N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and beta-adrenoceptor receptors. Glutamate blockage reduces the EPSP amplitude found in postsynaptic cells. The reduced EPSP has drawn many researchers' attention to utilizing it as an antidepressant once more (Mayer & Westbrook, 1987).

EPSPs, or excitatory postsynaptic potentials, occur within postsynaptic neurons (Shepherd, G. M., & Hanson, P. I., 2014). A change in the postsynaptic membrane creates EPSPs. Change in the postsynaptic membrane encourages action potentials to fire by bringing the membrane potential closer to the threshold potential (Matthews, 2001). Generally, the average resting membrane potential is around -70 mV and occurs from having the intracellular space more negatively charged than its surrounding environment (Chrysafides, 2019).

DF2, an FMRFamide-related neuropeptide, creates a long-lasting enhancement of synaptic transmission (Friedrich, R. W., Molnar, G. F., Schiebe, M., & Mercier, A. J., 1998). Neuropeptides such as DF2 activates protein kinases, which causes more neurotransmitters to spill into the synaptic gap. These solutions have not been tested together extensively, so DF2 may be able to counter the effects of ketamine.

There is not extensive research on the effects of ketamine on neurons. Although this research is missing on humans, it should be tested extensively on other organisms first to evaluate its impact on humans for later use. By analyzing EPSPs in the presence of ketamine and DF2, we can study how ketamine could be used to alter long-term synaptic plasticity and its strength as a possible long-term antidepressant.

We tested the effect of ketamine on EPSPs in the crayfish neuromuscular junction and how DF2 impacts the long-term effects of ketamine. The overarching question we tested was, does DF2 enhance recovery from ketamine-induced EPSP depression? By looking at the synaptic plasticity of ketamine on crayfish ventral abdominal extensor muscles, we can gain insight into its possible effects on humans as an antidepressant (Hoang, Slattery, & Park, 2015). In addition to finding its potential impact on humans, we may gain an understanding of possible conditions needed to override its receptor blocking capabilities. We hypothesized that ketamine would decrease EPSP function, and DF2 will make minimal impact on the recovery of ketamine-induced EPSP depression. Our results showed that ketamine

decreased EPSP amplitude, and DF2 was unable to restore EPSP amplitude.

MATERIALS AND METHODS

Preparation of the Crayfish

Our instructor supplied us with the crayfish *Procambarus clarkii*. The crayfish were anesthetized in a container of ice water before dissection. We removed the tail of the crayfish with a pair of scissors. We then dissected the crayfish by cutting the lateral sides of the tail with scissors and peeling off the outer shell and muscles. We used our thumb to remove the remaining muscle mass in the dorsal portion of the tail, comprising the swimmerets, flexor muscles, and the digestive tract. Removing this muscle mass exposed the superficial extensor muscles and nerves. We pinned the tail with its exposed extensor muscles ventral side up in a sylgard-coated dish and covered it with 100 mL of Ringer's solution. Ringer's solution simulates the extracellular fluids in a crayfish that prolongs the lifespan of the exposed extensor muscles. The solution contained 5.4 mM of potassium chloride (KCl), 196 mM of sodium chloride (NaCl), 2.6 mM MgCl2-6 H2O, 10 mM HEPES, and 13.5 mM CaCl2-2 H2O at a pH of 7.4.

Solution Preparation

We acquired baseline data from each crayfish prior to collecting any data with the DF2 and ketamine solutions. The Ringer's solution comprised the baseline data for each crayfish. Finding this baseline data for each crayfish allowed us to later normalize the data. We prepared a DF2 solution with a 50 nM concentration to observe the effects of the enhancer on EPSP generation. We added the DF2 solution to the ketamine solution or ringer's solution with a micropipette when appropriate. We then prepared 100 mL of 50 μM ketamine solution by using 10 mL of 500 mM stock ketamine solution and diluting it with 90 mL of Ringer's solution. Once we collected data on each solution's individual effects on EPSP generation, we made the 50 μM ketamine solution the base solution in the dish and let the crayfish abdominal extensor muscles bathe in the base solution for 15 minutes.

From there, we generated EPSPs. Once the EPSPs were stable we added the DF2 solution and continued data collection. The duration for each experiment permits a 15-minute bathing time and another 30 minutes of data recording. After bathing in the ketamine solution for 15 minutes, we added DF2 to the sylgard-coated dish at the 10-minute mark of the 30-minute recording period, leaving 20 minutes for measuring the effects of the DF2 on the neuromuscular junction of the abdominal extensor muscles.

Arrangement of Laboratory Equipment

We pulled borosilicate glass capillary tubes with a 1.2-millimeter diameter with a microelectrode puller (World Precision Instruments, Pul-1). We filled the microelectrodes with 3 M KCl solution using a syringe with a microfil head attachment. After removing the air bubbles by flicking the microelectrode body, we placed the filled microelectrode into a microelectrode holder (E Series with Straight Body, Warner Instruments), which was inserted into a micromanipulator. We connected the microelectrode to an intracellular amplifier and placed a wire connected to the electrometer's negative terminal in the Ringer's solution.

Additionally, we created suction electrodes from microelectrodes by filing down the tip against sandpaper. These microelectrodes were filled with Ringer's solution and put on a separate micromanipulator. We connected the electrode to a plastic syringe that was later used to suck in a nerve for the duration of the experiment. With the nerve sucked up, we used a stimulator (Grass SD9) to apply an electrical pulse.

Intracellular Recording and Nerve Stimulation

Using the micromanipulator, we inserted the recording microelectrode into crayfish extensor muscles to measure the cell membrane potential in millivolts (mV) with microelectrodes connected to a DC amplifier (Model 1600 DC Neuro Amplifier, AM Systems). The output from the amplifier was fed into a Power Lab (AD Instruments, driven by Lab Chart software). To test the microelectrode, we inserted the tip of the electrode into the solution, zeroed the amplifier, and ran an electrode test to determine whether the resistance fell within the acceptable 5-20 M ohms (**Ω**). If the resistance was in an acceptable range, it permitted us to proceed with the experiment. For each solution, we recorded EPSPs for 30 minutes after the abdominal extensor muscles soaked in the designated solution for 15 minutes. We held the stimulus frequency at 0.4 hertz (Hz) and the duration at 3.5 milliseconds (ms). We tapped the intracellular recording electrode to maintain the resting membrane potential in the -50 to -70 mV range. We analyzed our data at a later time and displayed graphs using a spreadsheet program (Excel 2019, Microsoft).

RESULTS

The goal of our research was to see whether ketamine reduces the amplitude of EPSPs and if DF2 can revert the amplitude of the EPSPs to its prior range in the crayfish extensor muscles. The results of this experiment show if ketamine is capable of long-term potentiation at crayfish neuromuscular junctions. Our process consisted of inducing EPSPs in crayfish extensor muscles using Ringer's solution, then adding either a ketamine solution or DF2 activator to see how the EPSPs changed with the new substances.

We gave each of the substances an introductory period of 5-10 minutes before recording any data. This introductory period ensured that the solution was affecting the crayfish extensor muscles. After the introductory period ended, we stimulated the extensor muscles for 30 minutes. The substances we used were Ringer's solution, ketamine, DF2, and a combination of ketamine and DF2. We used the Ringer's solution as the baseline of our data, which allowed for us to determine the average amplitude of EPSPs for the duration of the experiment.

Our first trials involved the ketamine only solution. The solution consisted of 100 mL of 50 μM ketamine solution, which we created by using 10 mL of 500 mM stock ketamine solution and diluting it with 90 mL of Ringer's solution. The average percent change of all four trials was -39.1% however the error bar ranges from -60.5% to -17.7% to so it is possible that -39.1% doesn't represent the true average of the results of the ketamine solution.

The next step in our experiment was testing the effect of DF2 only solution on EPSP. We added 50μM of DF2 to Ringer's solution. The graph shows that the average percent change of EPSP using DF2 compared to the original baseline EPSP using Ringer's solution was 168.5%. However, with only 2 trials, the error bar was very large ranging from 14.3% to 322.7%, so the results are uncertain.

The last set of trials involved a ketamine solution with drops of DF2 activator added. We conducted 3 trials. The solution consisted of 100 mL of 50 μM ketamine solution and 50μM of DF2. Results show that the average percent change from baseline was -60.2%, however standard error suggests that the average percent change from the baseline ranges from -80.4% to -40.0% during the 30-minute trial period.

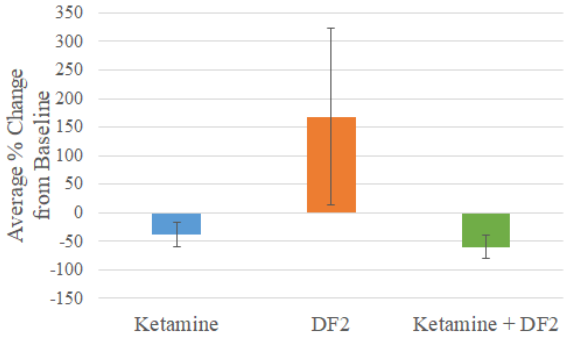

Figure 1. The average percent change compiled from the experiments conducted on ketamine, DF2, and ketamine and DF2 combined. Ketamine is seen to consistently decrease the amplitude of EPSPs throughout all of its experiments. DF2 is shown to have a very large error bar, indicating that the effects of DF2 are uncertain. p-value = 0.3741588

DISCUSSION

We hypothesized that exposure to ketamine would reduce EPSP amplitude and that DF2 would not significantly increase the EPSP to its prior range. Ketamine did in fact reduce the amplitude of EPSP and we found that DF2 was unable to increase the amplitude of the crayfish EPSPs, therefore didn't fully override the effects of ketamine.

Furthermore, due to the high variability of the DF2 efect, it's difficult to fully determine the effects of DF2 on the EPSP. Although DF2 did have a statistically higher average percent changes from the baseline, the error bar was so large that these results are uncertain. Granted the ketamine and DF2 solution caused the average percent change from the baseline to increase more than that of the ketamine solution alone.

Because DF2, an artificial neuromodulator, was unable to override the effects of ketamine in crayfish, it may be possible that natural neuromodulators in humans would be unable to override the effects of ketamine as well. These results may support research on the long-term effects of ketamine on depression. An experiment conducted by Hoang, Slattery, and Park supports our findings that ketamine has long lasting effects on the neuromuscular junction (2014). In their experiment, they examined the effect of ketamine on EPSP of crayfish, similar to our study. They, however, applied paired-pulse stimulation on crayfish tails for different concentrations of ketamine solution. Their results showed that ketamine generally decreased EPSP amplitude, which directly supports our trials involving the ketamine only solution.

If a follow-up experiment was conducted, we would record significantly more trials with the solutions used, especially DF2. Additionally, we would test the effects of the solutions as they are being introduced to the crayfish extensor muscles in order to more precisely see how these solutions affect the amplitude of the EPSPs over time. We would also test the effects of the solutions for a more

extended period of time to determine if the effects of ketamine or DF2 diminish after a certain number of hours. With these modifications to the experiment, it would help determine if these solutions are metabolized by the crayfish tail. The trends shown by ketamine could show its potential use as medicine.

ACKNOWLEDGEMENTS

We thank our professor Clark Lindgren, our lab assistant Ashley Wolterstorff, and our class mentor, Emily Kozik for their help throughout this project.

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Pioneering Neuroscience, 2019, **18**, 11-14

Amiloride Inhibits Synaptic Transmission at Crayfish Neuromuscular Junctions, but no Evidence Suggests Its Interaction with DRNFLRFamide.

CAMILA HASSLER, JOSIE NOLAND, and SOPHIE SMITH
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

FMRFamide-activated sodium channels (FaNaCs) are similar to mammalian acid-sensing ion channels (ASICs). We analyzed the reaction of FaNaCs exposed to DRNFLRFamide (DF2) at the crayfish neuromuscular junction. We measured excitatory postsynaptic potentials (EPSPs) after exposing crayfish extensor muscle cells first to DF2, then DF2 and amiloride, and finally, amiloride alone. Contrary to previous studies, DF2 inhibited synaptic transmission. Amiloride also inhibited synaptic transmission. Therefore, FaNaCs are similar to ASICs in regard to their response to amiloride. The results were inclusive in determining the interaction between amiloride and DF2.

INTRODUCTION

FMRFamide-activated sodium channels (FaNaCs) are sodium-selective ion channels found in invertebrates. In order for a FaNaC to open, a natural or synthetic FMRFamide must be present. FaNaCs respond quickly to the presence of a FMRFamide. We used crayfish as our model organism because they are inexpensive and have nerves that are easy to find under a microscope.

DRNFLRFamide (DF2) is a peptide that activates FaNaC channels (Cottrell, 2005). DF2 prompts depolarization, along with decreased input resistance. It also increases Ca^{2+} current, which then activates muscle contraction in crustacean ventral muscles. The increased Ca^{2+} current results in an increase of neurotransmitter release from the presynaptic cell (Weiss, 2003). Amiloride is a small molecule diuretic, that has been used to dissect sodium transport pathways in many different systems. It interacts with the epithelial sodium channel and acid-sensing ion channel proteins. In the medical field, amiloride is frequently used to treat high blood pressure.

DF2 is a known activator of FaNaCs, and amiloride is known to block the effects of DF2 in mammalian acid-sensing ion channels (ASICs). We wanted to better understand the relationship between ASICs and FaNaCs—especially if amiloride has the same inhibitory effects of FaNaCs in both channels. We tested if amiloride inhibits the effect of the FMRFamide DF2 on crayfish neuromuscular junctions. We selected this question to explore FaNaCs and their correlation to excitatory postsynaptic potentials (EPSPs). FaNaC channels are similar to ASIC channels, so we hypothesized amiloride would inhibit the FaNaC that becomes activated by DF2.

Our study investigated the following:

1. Are FaNaCs similar to ASICs in how they react to amiloride? Will EPSP amplitude be reduced after crayfish extensor muscles have been exposed to amiloride?
2. How significant of a change in EPSP amplitude will occur when DF2 and amiloride are used in combination?

We expected DF2 alone would increase EPSPs, as it is a known activator of FaNaCs. However, amiloride alone should not impact the EPSPs at all, because the FaNaCs have not yet been activated (unless there are naturally occurring FMFRamides in the crayfish). We anticipated the trial in which the crayfish is exposed to DF2, followed by amiloride would show the inhibitory effects of amiloride.

Our results show amiloride depressed synaptic potential at crayfish neuromuscular junctions. Surprisingly, DF2 also depressed synaptic potential at crayfish neuromuscular junctions. Lastly, DF2 combined with amiloride had a large margin of error, so conclusions cannot be drawn. However, the large range of EPSP values suggest there was interaction between the chemicals.

MATERIALS AND METHODS

Dissection and Preparation

To prepare for dissection, we anesthetized a crayfish with an ice bath. We began the dissection by removing the tail using scissors. Then, we made two lateral cuts on each side of the tail and removed the exoskeleton on the ventral side to reveal the underlying muscle. To reveal the desired dorsal extensor muscles, we carefully used our

thumbs to scrape off the interior crayfish muscles and fat stores—being sure to remove all of the gastrointestinal tract to prevent contamination. Once the dissection was complete, we placed the specimen in 100mL of crayfish Ringer's solution. After completing a control reading, we also added DF2 and/or amiloride to the solution depending on the particular trial being completed.

Condition	Average EPSP
Control	25.3 mV
Amiloride	2.4 mV
DF2	13.7 mV
Both	11.4 mV

Table 1. This table shows the average EPSP for each condition. Percent change values were comparisons between average EPSP of the control and other conditions.

Preparing Micro-electrodes

We created two different types of electrodes using glass capillary tubes and a micro-electrode puller. We first made a micro-electrode with which we measured intra-cellular EPSPs. We filled the electrode with three molar potassium chloride (KCl). The measuring electrode cannot contain any bubbles (otherwise the resistance would be off), so we inverted the electrode and used light tapping to remove any potential bubbles. Then we placed the electrode on a micro-manipulator that allowed us precision entering cells and protection from large, uncontrolled movements that would likely break the electrode tip.

We also created suction electrodes which were used to gather and stimulate individual neurons. First, we pulled a standard electrode on the micro-electrode puller. Then, we gently filed the tip to produce an opening large enough to admit a nerve axon.

Intracellular Recording and Electrical Stimulation

We first measured EPSPs of crayfish extensor muscles exposed to only normal Ringer's solution (5.4 mM KCl, 196 mM NaCl, 2.6 mM MgCl2 x 6H2O, 10 mM HEPES, and 13.5 mM CaCl2 x 2H2O). The suction electrode stimulated a neuron by applying a supra-threshold stimulus, while the micro-electrode measured EPSPs in corresponding muscle cells. Stimulation was applied using a Grass SD9 Stimulator. We used LabChart programming to record our data.

RESULTS

We explored how amiloride and DF2 impacted synaptic transmission at crayfish neuromuscular junctions. We ran four trials in which we recorded EPSPs. The control trial featured physiological crayfish Ringer's solution. Then, we added 300 micro-molar amiloride to the 100mL Ringer's solution. After five minutes, we recorded EPSPs for the amiloride trial. Then, we disposed of the amiloride solution and filled the dish with 100mL Ringer's solution and fifty nano-molar DF2. We repeated the process of recording EPSPs after DF2 exposure. Our last trial combined the DF2 solution with 300 micro-molar amiloride. Then we recorded EPSPs after exposure to both chemicals simultaneously. The average EPSP amplitude for each condition is shown in Table 1.

We hypothesized adding amiloride and DF2 would result in a decrease in EPSP amplitude. Our raw data supported our hypothesis because the EPSP amplitude of the trial with amiloride and DF2 was 11.4 mV, 13.9 mV less than the control. However, we also predicted the application of DF2 would increase EPSP amplitude because it is a known activator of ASICs. Our results reject our hypothesis regarding DF2 exposure increasing

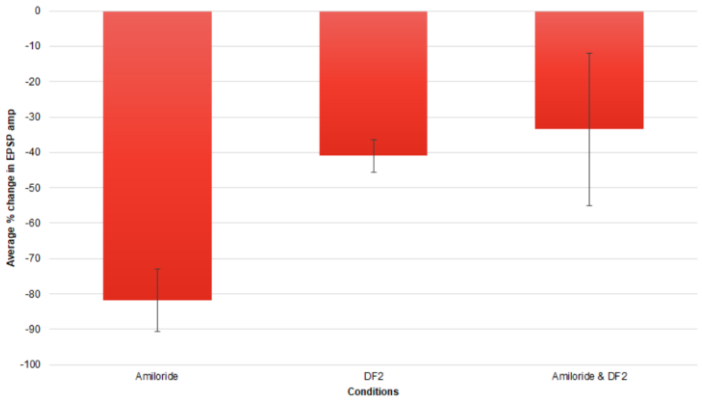

Figure 1. This figure shows the average percent change in EPSP amplitude of the variable conditions compared to the control. The average percent change in EPSP amplitude for only amiloride was -81.8%. The average percent change in EPSP amplitude for only DF2 was -41.0%. The average percent change in EPSP amplitude for both DF2 and amiloride was -33.5%.

FaNaC activation. We found DF2 had the opposite—lowering EPSPs.

Condition	P-Value
Both	0.787
Amiloride	0.068
DF2	0.072

Table 2. This table shows the p-values for three conditions.

According to our p-values as shown in Table 2, our results were inconclusive in finding significant changes in synaptic transmission after exposure to amiloride or DF2 at crayfish neuromuscular junctions. However, we cannot ignore the difference of average EPSP (shown in Figure 1) between amiloride alone versus amiloride and DF2. We collected marginally significant data suggesting a relationship between amiloride and DF2. However, our large margin of error makes our results more difficult to analyze.

DISCUSSION

We expected DF2 alone would increase EPSPs and amiloride alone would have no effect. We anticipated a trial with both DF2 and amiloride together would be the only way to witness the inhibitory effects of amiloride. We expected these results because DF2 is a known activator, amiloride is an inhibitor, and FaNaCs must be open for amiloride to demonstrate inhibitory effects.

The data we collected is inconclusive in determining the interaction between amiloride and DF2. Our margin of error is too large to assign significant value to our data. While Figure 1 shows a slight difference between DF2 alone and DF2 with amiloride, our statistical evaluation of the conditions only shows marginal significance.

However, amiloride alone created a large depression in synaptic transmission. This was expected because amiloride is a known inhibitor of ASICs, and we expected FaNaCs to respond similarly. Our results with DF2 were surprising—having the opposite effect than we expected. Previous research has shown DF2 activates crayfish synapses, but our data shows DF2 inhibiting them. While our results prove inconclusive in determining a relationship between DF2 and amiloride, it is possible DF2 prevented an even larger inhibition of synapses. The large margin of error associated with our data could signify an interaction between DF2 and amiloride over time. Regardless, the data is minimally helpful in forming conclusions.

In another study, DF2 was tested in crayfish with several enhancers and inhibitors. The inhibitors used in this experiment were Rp-cAMPS (which inhibits PKA), and Rp-8-pCPT-cGMPS (which inhibits PKG). The inhibitors alone barely altered EPSPs and barely inhibited the response to DF2. However, used together, the two inhibitors totally blocked the effect of DF2. (Badhwar, 2006)

We found a similar result in our experiment. DF2 combined with amiloride did not inhibit FaNaCs. We were interested in exploring FaNaCs because of their relevancy to understanding ASICs. Both FaNaCs and ASICs contain fundamental functions to pathological and physiological processes, including synaptic transmissions (Yang, 2017). They are also are connected to inflamed tissue (Poet, 2001). Our work inhibiting FaNaCs further explores ligand-gated channels, as well as their involvement in tissue damage. We hope our research will prompt more experiments with DF2 in relation to the FaNaC channel. Future work could include testing DF2 in combination with additional activators to determine the response of multiple FMRFamides on synaptic transmission.

ACKNOWLEDGEMENTS

We would like to thank our professor, Clark Lindgren, for helping us learn about neurophysiology and gain a better understanding of what it means to be a biologist. We would also like to thank our neuroscience lab technician, Ashley Wolterstorff, who has tirelessly prepared our chemical solutions and helped us maintain a safe lab environment. Emily Kozik also played in instrumental role in our research by providing support during testing and helping us learn the analytical software. Lastly, we would like to thank Grinnell College for providing us with the opportunity to conduct neuroscience research.

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Pioneering Neuroscience, 2019, **18**, 15-19

Protein Kinase C is Partially responsible for the Effects of NF_1 in Sustaining EPSP Amplitude Increase

CHARLES LU AND KAITLIN MICHAELS
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

We used crayfish neuromuscular preps to examine the role of protein kinases in general and Protein Kinase C (PKC) in particular in the effects of sustaining EPSP amplitude increases induced by NF_1. We used intracellular recordings to measure changes in EPSP amplitudes over time under the effects of different protein kinase inhibitors and NF_1 as compared to baseline measurements taken before the exposure to drugs/NF_1. We found that preps exposed to NF_1 experienced a sustained increase in EPSP amplitude relative to their baseline measurements. We also found that while preps exposed to NF_1 with a PKC inhibitor experienced an initial increase in EPSP amplitude similar to that of preps exposed to NF_1 alone, they experienced a subsequent drop in EPSP amplitude relative to baseline levels, indicating that PKC is involved in the effects of NF_1 in that it plays a role in sustaining EPSP amplitude. Preps exposed to NF_1 and a general protein kinase inhibitor experienced a greater drop in EPSP amplitude than those exposed to NF_1 and a PKC inhibitor, indicating that PKC is not the only protein kinase involved in the effects of NF_1, inviting further research into the roles of other protein kinases in NF_1's effects. Our research fills a gap in the current literature, as although previous studies indicated the role of PKC in the effects of DF_2, a similar FMRFamide, we did not find any existing literature on the role of PKC in the effects of NF_1.

INTRODUCTION

Neuromodulators are substances which affect communication at the synapse (Nadim & Butcher, 2014). We know from previous studies that two neuromodulators, FMRF-amide-like peptides NF_1 and DF_2 have both been isolated in crayfish (Mercier et. al, 1993). Skerrett et al (1995) found that both DF_2 and NF_1 cause synaptic enhancement and EPSP amplitude increase in crayfish deep extensor muscles.

Protein Kinase C is an enzyme family. They respond to DAG (diacylglycerol), and Ca^{2+} (Huang,1989). Protein Kinase C (aka PKC) mediates the phosphorylation of certain cellular proteins. It plays a role in many signal transduction events in cells and is a member of the serine threonine kinase family (Mochly-Rosen & Kauvar, 1998). In the nervous system, the activation of Protein Kinase C relates to the regulation of neurotransmitter release, ion channels, and neuroplasticity (Huang, 1989). The binding of DF_2 to a GPCR (G-coupled protein receptor) leads to a signal cascade which activates PKC (Newton & Gold, 2008).

Research has demonstrated that PKC plays a role in synaptic enhancement by DF_2 (Freidrich et al, 1998). Researchers studying the role of PKC in synaptic enhancement by DF_2 previously concluded that PKC is responsible for the effects of DF_2 in that PKC limits the initial amplitude of EPSPs, but allows EPSPs to last longer (Freidrich et al, 1998). However, there appears to be a gap in the literature regarding the role of protein kinases and protein kinase C in the effects of NF_1. Thus, we intend to provide insight into the role protein kinases, especially protein kinase C in particular, play in causing the effects of the FMRF-amide like neuromodulator NF_1.

As NF1 and DF2 are members of the same family of peptides, and research has shown them to have similar effects on EPSPs in crayfish (Skerrett, et al. 1995), we predict that PKC plays a similar role in NF1 as it does in DF2. Therefore, we predicted that PKC will also sustain the EPSP amplitude enhancement by NF1 in the crayfish deep extensor muscles.

Our results supported our hypothesis that PKC does play a role in sustaining the prolonged increasing effect of NF_1 on EPSP amplitude$_1$. However, PKC is not the only protein kinase that helps sustaining the increasing effect of NF_1 on EPSP amplitude.

MATERIALS AND METHODS

Organisms and Preparation

We performed experiments on nerve muscle preps taken from adult red crayfish (*Procambarus clarkii*) obtained from Carolina Labs. Prior to dissection, crayfish were kept in an ice bath. To expose the extensor muscles, we made a diagonal cut through the connection between the crayfish's thorax and its tail, separating the thorax and

the tail. We then turned the crayfish abdomen dorsal side up and separated the shells of the ventral abdomen from the dorsal shells by using a pair of scissors to cut along the side of its abdomen. After lifting up the abdominal shell, which was still connected to the tail, we cut it off the tail completely, exposing the muscles underneath. We used a thumb to scrape off the muscle mass and expose the extensor muscles we were testing.

After dissecting the crayfish, we pinned the prep ventral side up in a glass dissection dish; one needle on the posterior side of the tail and the other two on two separate sides of the anterior part of the tail.

Equipment and Setup

To record EPSPs, our team used borosilicate glass recording microelectrodes with an outside diameter of 1.2 mm and a tip diameter of <1μm filled with 3mol 1^-1 potassium chloride with a resistance of 5~20 MΩ. They were connected to a microelectrode holder attached to a microelectrode manipulator. We used a 1600 A-M Systems DC amplifier, a Power Lab, and a computer running Lab Chart by AD Instruments to record the membrane potentials. We used a suction microelectrode made from borosilicate glass with an outside diameter of 1.2 mm connected to syringe to apply suction to isolate and stimulate crayfish nerves. We used the DS9 Simulator by Astro-Med Inc to stimulate the axon sucked in by the suction electrode.

Intracellular Recording and Data Collection

To take baseline measurement, we placed each prep in normal crayfish physiological saline (5.4μM KCl,196 μM NaCl, 26μM $MgCl_2 \bullet 6H_2O$, 10 μM HEPES, 13.5 μM $CaCl_2$,pH=7.4) prepared by our lab. After isolating a nerve using a suction microelectrode, and inserting the recording microelectrode into the appropriate muscle, we stimulated the nerve, and recorded baseline EPSPs.

After recording baseline EPSPs for each crayfish, we used a solution switching device to replace the normal crayfish physiological saline for crayfish physiological saline containing NF1, a LY 333 531, or staurosporine or NF1 with staurosporine or LY 333 531(which were prepared by our lab with the concentrations noted above). EPSPs we recorded using the same procedures used when taking baseline EPSPs. We began recording 1-5 minutes after switching solutions.

We used the data we collected of EPSP measured in crayfish physiological saline with no additional chemicals as our control group and everything else as the comparison group. For the data we collected, we compared EPSP measured in crayfish physiological saline with NF1 alone, staurosporine alone, LY 333 531 alone, NF1 with staurosporine, and NF1 with LY 333 531 with EPSP measured in crayfish physiological saline with no additional chemicals. We presented the comparison groups data as percentage in respect to the control group data.

We measured the EPSPs for 10-20 minutes with 5 seconds interval for each control group for each crayfish. For the comparison groups, we measured the EPSP with 5 seconds intervals for 20 minutes of NF1 alone, Staurosporine alone, LY 333 531 alone, NF1 combined with Staurosporine, NF1 combined with LY 333 531. The concentration for NF1 was 50 nM, for Staurosporine was 2.15 μM, and for the PKC inhibitor was 10 μM.

For the raw data we collected, we took only the difference between the maximum and minimum membrane potential without the artifact using Microsoft Excel to get the EPSP amplitude. Then we treated the control group data as 100%, or 1. After that, we compared the comparison group data to the control groups data and present as the percentage in respect to the control group data. We graphed the data using the percentage as the vertical axis and time as the horizontal axis.

RESULTS

To examine the role of PKC on the effects of NF_1, we exposed crayfish neuromuscular preps to crayfish physiological saline containing NF_1 alone, NF_1 and staurosporine together, staurosporine alone, LY 333 531 alone, and NF_1 and LY 333 531 together. We used intracellular recording to record EPSPs. For each prep, we recorded EPSPs stimulating every 5 seconds for 10-20 minutes prior to exposure to Staurosporine, LY 333 531, and NF1 as the baseline, and for an additional 10-20 min stimulating every 5 seconds following the exposure to the three substances mentioned to observe their effects on EPSPs over time. We calculated the mean value of our baseline EPSP since it was mostly the same number and the mean value eliminated variability in the data collecting process. We compared the EPSPs with the influence of substances to the mean value of the baseline in percentage. Then we computed and compared these data with respect to time and came up with the graphes being shown below.

The Effects of Staurosporine on EPSPs

We found that when we added Staurosporine, a protein kinase inhibitor, into the crayfish, the EPSPs immediately started to drop (figure 1). The EPSPs dropped at a relatively rapid rate in the first ten minutes when staurosporine was added, then the EPSPs stayed at a relatively steady level and dropped at a much slower rate at the second ten minutes. As shown in Fig. 1, in the first ten minute period, the amplitude of the EPSPs affected by

Staurosporine compared to the baseline EPSP dropped from about 75% to 15%. In the second ten minutes period, the EPSP affected by Staurosporine compared to the baseline EPSP dropped from 15% to 10%.

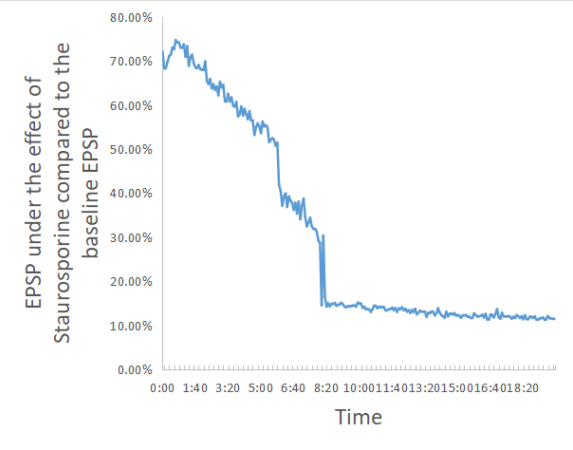

Fig. 1. EPSP under the effect of Staurosporine compared to the baseline EPSP in percentage with respect to time.

The Effects of LY 333 531 on EPSP

We found that when we added LY 333 531, the protein kinases C inhibitor, into the crayfish, the EPSP immediately experienced a slight drop. As shown in Fig. 2, the EPSP dropped to around 80% compared to the baseline EPSP and stayed around this value for the ten minutes period. The EPSP stayed steady after the initial drop.

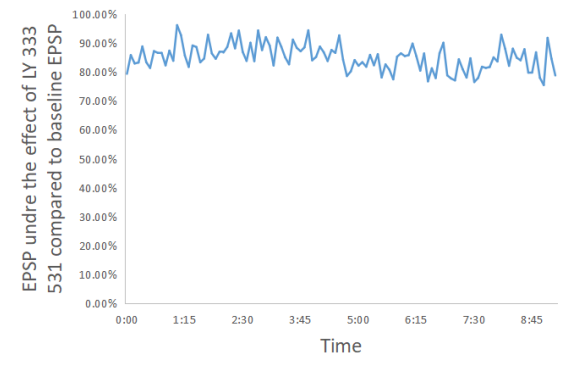

Fig. 2. EPSP under the effect of LY 333 531 compared to baseline EPSP in percentage with respect to time.

The Effects of NF1 on EPSP

We found that when we added NF1, a neuromodulator from the FMRFamide family, the EPSP did not immediately started to raise. As shown in Fig. 3, in the first five minutes, the EPSP stayed fairly steady at around 120 to 140%. However, after the first five minutes, the EPSP started increasing very fast. It went from 140% to almost 200%.

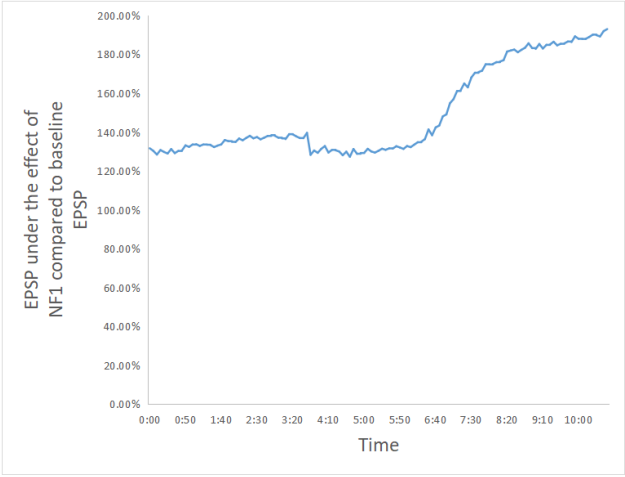

Fig. 3. EPSP under the effect of NF1 compared to baseline EPSP in percentage with respect to time.

The Effects of Staurosporine Combined with NF1

We found that when we added Staurosporine combined with NF1, the EPSP experienced an increase from 100% to 140% in the first five minutes (Fig. 4.). Then it started dropping from 140% to 20%. We believed that the abrupt rise and drop of EPSP at around 1:40, 4:10, and 8:20 was due to the fact that we sucked in two axons in the suction electrode at the same time. The suction electrode stimulated one axon before 1:40 but started stimulating two axons at the same time from 1:40 to 4:10, then the second axon dropped out after 4:10, and got sucked in again at 8:20. These stimulation and dropping out of the second axon caused the rise and drop of the EPSP. However, if we removed these distractions, we could still see a gradual drop of EPSP. Therefore, we believed that the effects of Staurosporine combined with NF1was causing the EPSP to drop.

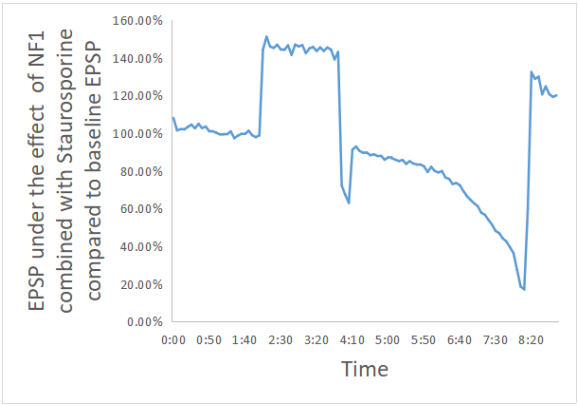

Fig. 4. EPSP under the effect of NF1 combined with Staurosporine compared to baseline EPSP in percentage with respect to time.

The Effects of LY 333 531 Combined with NF1

We found that when we added LY 333 531 combined with NF1, the EPSP immediately went to 200% (Fig. 5). As soon as it reached 200%, it started dropping to baseline level in ten minutes.

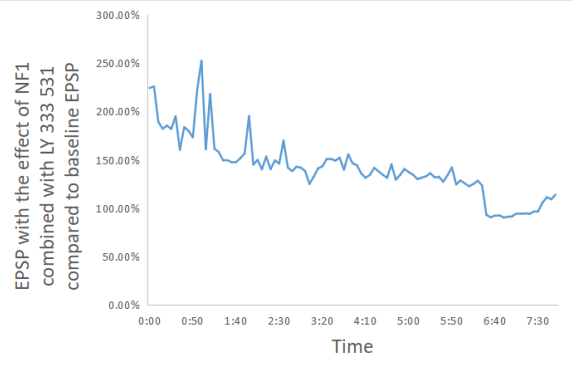

Fig. 5. EPSP under the effect of NF1 combined with LY 333 531 compared to baseline EPSP in percentage with respect to time.

Our data supported our hypothesis that protein kinase C does play a role in sustaining NF1's increasing effect on EPSP amplitude. When protein kinase C is inhibited, although EPSP amplitude did experience an initial increase like it did with solely NF1, this increasing effect did not sustain over time and the EPSP amplitude dropped back to baseline level instead of staying at a higher level. However, we found that compared to inhibiting protein kinase C alone, inhibiting all protein kinases made the EPSP amplitude drop below the baseline a lot when NF1 was induced.protein kinase C. Therefore, protein kinase C is not the only protein kinase that help sustain the increased EPSP amplitude when NF1 was induced. Other protein kinases also play a role in this sustaining effect.

DISCUSSION

The results of our study supported our hypothesis that PKC would play a similar role in the effects of NF_1 as it does in the effects of DF_2 in that it sustains the increasing effect of NF1 on EPSP amplitude over time. Our data revealed that preps exposed to NF_1 experienced about a 200% increase in EPSP amplitude. However, the preps exposed to NF_1 & LY 333 531 experienced a subsequent drop in EPSP amplitude (in which amplitudes dropped to baseline levels) while the preps exposed to NF_1 alone continued to demonstrate an increase in EPSP amplitudes. The data is consistent with our hypothesis in that preps in which PKC was inhibited were unable to sustain EPSP amplitude increases induced by NF_1, indicating that PKC plays a role in this effect over time.

Our data also indicated that although PKC plays a role in causing the effects of NF_1, it is not the only protein kinase that is responsible for NF_1 's effects. Preps exposed to NF_1 and staurosporine, a broad spectrum protein kinase inhibitor, demonstrated a greater decrease in amplitude than preps exposed to NF_1 and LY 333 531. The fact that nonspecific inhibition of protein kinases caused a greater decrease in EPSP amplitude than specific inhibition of PKC indicates that other protein kinases also play a key role in the effects of NF_1. This invites further research into the roles other protein kinases may play in the effects of NF_1, as well as research comparing the significance of said proteins in relation to NF_1 and DF_2.

Our study yielded similar results to previous research on NF_1 in crayfish neuromuscular preps, in that it indicates that NF_1 exposure leads to an increase in EPSP amplitude over time (Skerret et al, 1999). Our results are similar to those of previous investigations into the role of PKC in the effects of DF_2 in crayfish, in that researchers studying DF_2 also found that PKC played a role in causing a sustained increase in EPSP amplitude (Freidrich et al, 1998). Our findings are significant, however, in that they fill a gap in the literature, providing insight into the role of PKC and in causing the effects of NF_1, a topic on which we did not find any previous research.

ACKNOWLEDGEMENTS

We thank our Professor Clark Lindgren, biology mentor Emily Kozik, and lab instructor Ashley Wolterstorff for their great support and help with our experiment.

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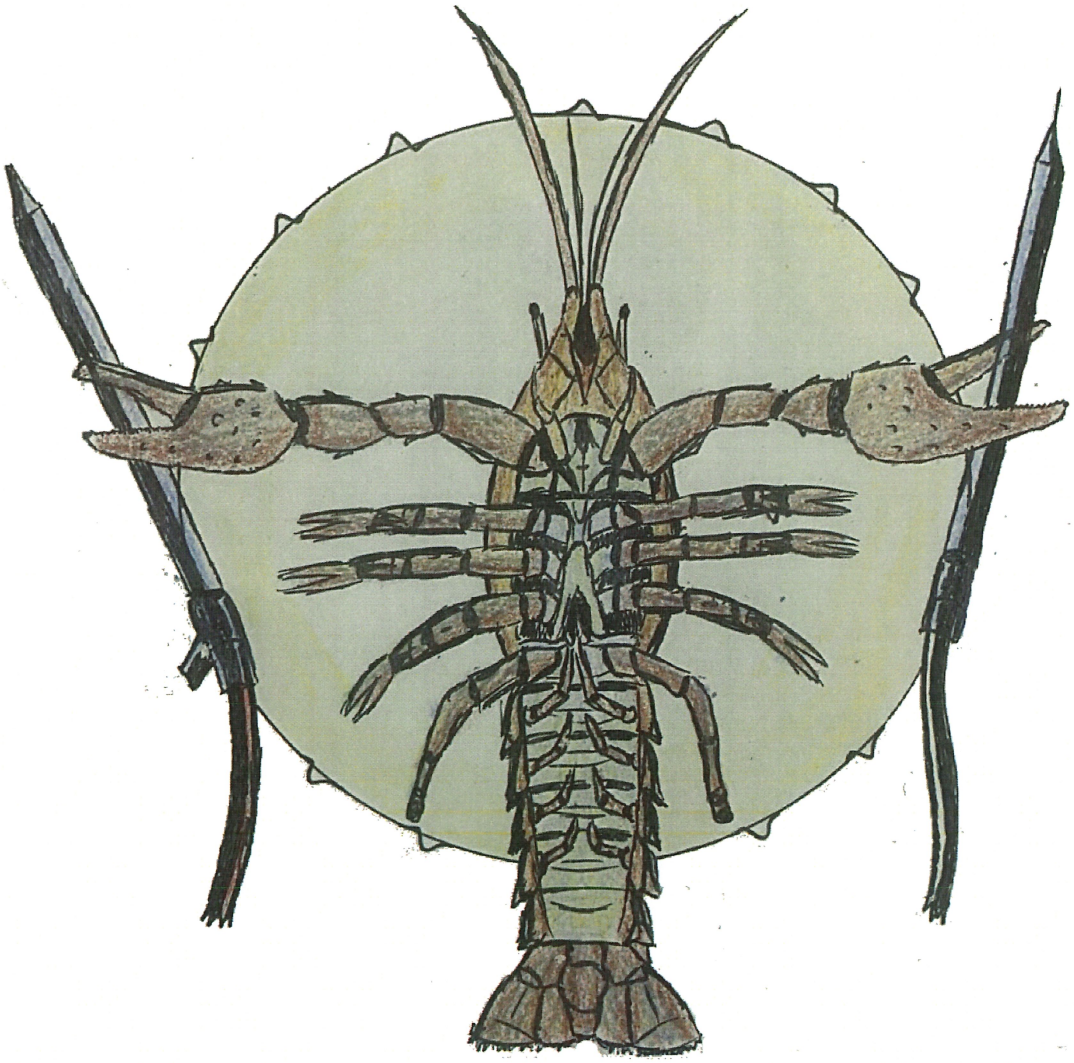

Pioneering Neuroscience, 2019, **18**, 21-26

The FMRFamide-Gated Sodium Channel's Effects on the EPSP Amplitude at the Neuromuscular Junction are Inconclusive

OLIVIA CARR, JAMIE LEE, and NATHAN L'ESPERANCE
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Within the nervous system, neurons communicate at synapses via neurotransmitters transported between neurons until reaching the postsynaptic cell. They then bind to and activate specific proteins in the postsynaptic cell. Ion channels in the postsynaptic cell open and allow an influx of positive ions, depolarizing the cell, leading to an EPSP (Excitatory Postsynaptic Potential). FaNaC is a sodium-gated ion channel found in crayfish muscle cells that is activated by FMRFamide peptides. Since FaNaC allows positive ions into the postsynaptic cell, we sought to understand how it alters the strength of an EPSP by isolating the effects of FaNaC alone. We used NF1, a FMRFamide, to activate FaNaC. Additionally, we used a known FaNaC inhibitor, Amiloride, to see how EPSPs changed when FaNaC was not active. Protein kinases have also been implicated in the response to FMRFamide, so we utilized Staurosporine, a protein kinase inhibitor to isolate FaNaC and better understand its involvement in synaptic transmission. Our research potentially indicates that FaNaC decreases EPSP amplitude, but due to control discrepancies, it requires further research.

INTRODUCTION

To communicate within itself and alongside other systems of the body, the nervous system must use a combination of "all or none" electrical transmission and graded chemical transmission. Electrical signals occur within a single neuron in the form of action potentials, while chemical transmission occurs in the synaptic cleft, allowing communication from one neuron to another neuron or muscle fiber. Excitatory Postsynaptic Potentials (EPSPs) are a fundamental part of this communication process between two cells. EPSPs continue communication from one neuron to another by depolarizing the resting membrane potential of the postsynaptic cell enough to trigger the opening of voltage-gated sodium channels, starting a new action potential which will travel along the axon to a new cell (Pieroni, Byrne 1992). EPSPs occur in the neuromuscular junction, which increases the likelihood of an action potential in the muscle cell (Purves, Augustine, Fitzpatrick 2001). We experimented on crayfish extensor muscle cells because they do not have voltage-gated ion channels, and will not trigger an action potential, whereas other species would require toxins to stop action potentials. Also, the axons and extensor muscles are easily accessible, and crayfish are inexpensive.

Both presynaptic and postsynaptic cells in the neuromuscular junction require neurotransmitters to transport and transduce signals. Often, these come in the forms of neuropeptides. Neuropeptides are small chains of amino acids held together through peptide bonds. One group of neuropeptides are the FMRFamides. FMRFamide peptides are cardioexcitatory tetrapeptides and are essential to EPSP amplitude control (Duttlinger, 2003). NF1 is one of the FMRFamide-type neuropeptides and is the focus of our experiment. NF1 plays a role in the presynaptic cell in an EPSP, as it is the tetrapeptide that activates FaNaC, or FMRFamide-gated ion channels. These channels are gated to keep Ca^{2+} outside the presynaptic cell, which allows for a stronger EPSP when the cell is stimulated.

Previous research shows that the strength of an EPSP, measured by its amplitude, can be enhanced or suppressed using a variety of natural and synthetic chemicals (Pieroni, Byrne 1992). In particular, invertebrate FMRFamides significantly strengthen EPSPs (Friedrich, Molnar, Schiebe, Mercier 1998). The majority of research on NF1's effects on EPSPs has centered around its activation of kinases, which enhance EPSPs (Friedrich, Molnar, Schiebe, Mercier 1998; Papp 1996). However, NF1 also activates another more recently discovered protein, the positive ion channel FaNaC (Lingueglia 2006). Since both FaNaC and NF1 naturally occur in crayfish muscle cells, we ask whether FMRFamide's activation of FaNaC has any effect on the EPSP of crayfish muscle cells (Zhuang, Ahearn 1996). We want our research to connect the ideas presented in these various research articles. We also want these lab results to serve as a foundation for more in-depth experiments to advance the current knowledge of FaNaC's function in crayfish neuromuscular junctions.

We tested whether FaNaC affects the EPSP of crayfish extensor muscles. We hypothesized that FaNaC would increase the amplitude of the EPSP due to the

activation of FaNaC increasing the influx of positive ions into the cell, depolarizing the cell, activating voltage-gated calcium channels which would increase the amount of calcium entering the cell, therefore increasing the amount of neurotransmitter released and accepted by postsynaptic cell membrane protein channels.

We tested our hypothesis by submerging crayfish muscles in solutions, each containing amiloride or staurosporine with or without the presence of FMRFamide. We stimulated the extensor muscles in the crayfish tail and took EPSP recordings. Compiling this data, we were able to isolate FaNaC's effects on the EPSPs. Overall, our data suggests that there is an entity outside of kinases that, when activated by NF1, affects the EPSP amplitude; however, due to control data revealing unanticipated side effects of Amiloride, we cannot definitively conclude whether this effect is from FaNaC, only speculate that activated FaNaC decreases EPSP amplitude.

MATERIALS AND METHODS

Materials

We submerged our crayfish muscles in 100mL physiological saline containing 5.4 mM KCL, 196 mM NaCl, 2.6 mM $MqCl_2$-$6H_2O$, 10mM HEPES, 13.5 mM $CaCl_2$-$2H_2O$, and unique chemical inhibitors depending on the experimental trial. We used kinase inhibitor staurosporine at a concentration of 100 nM, FaNaC inhibitor amiloride at a concentration of 100 nM, and FMRFamide NF1 at a concentration of 10 and 50 nM in our experimental trials. We used a DC amplifier (A-M Systems 1600), a PowerLab (AD Instruments), an SD9 Square Pulse Stimulator (Grass Instruments), suction electrodes made of 1.2 mm heated and pulled borosilicate ground with sandpaper at the tip, and glass electrodes made of heated and pulled borosilicate capillary tubes with 1.2 mm diameter filled with 3M KCL (the highest possible concentration without precipitate used to lower resistance) to record data. We used a computer and the software LabChart, Datapad, and Microsoft Excel to store, process our data.

Dissection

We began by placing the crayfish in an ice bath. This action anesthetized the crayfish by drastically slowing its metabolism. After removing the crayfish from the ice bath, we used scissors to sever the tail from the rest of the body. We then used smaller scissors to cut along the border of the ventral and dorsal surfaces, separating the two at the base of the telson. Using our thumbs, we removed the bulk of the tail's muscle mass and intestines, leaving the dorsal extensor muscles and the connected axons. We then removed any remaining muscle mass, obscuring the extensor muscles using forceps. After completing our dissection, we used pins to secure the specimen to the dish, filling the dish with 100mL of physiological saline to simulate the extracellular fluid of the crayfish.

Maintaining axons and extensor muscles

Our primary method of maintaining our axons and muscles was to submerge the tail in physiological saline. The physiological saline is crucial because it simulates the extracellular fluids of a living crayfish and has the concentrations of ions necessary to allow for the continuation of normal physiological processes. However, the physiological saline must be changed consistently as the dissected tail continues to pollute the physiological saline throughout testing with metabolic waste products. To counteract this, we replaced the saline every 30 minutes with a new 100mL to maintain a consistent, accurate resting potential and EPSP. To replace the saline, we used the Simultaneous Liquid Uptake and Replacement Pump, removing the old solution with one side and replacing it with the other syringe.

Administering chemicals

After recording a successful EPSP, we administered chemicals by pipetting them into the physiological saline submerging the crayfish extensor muscles. We stirred the chemicals to disperse them into the physiological saline evenly, allowing them to permeate and affect the crayfish muscle. Recordings were taken immediately after introducing the chemical.

Recording data

We used two separate microelectrodes to produce and record EPSPs. We filled the first microelectrode with 3M KCl, leaving no air pockets (Dulhunty, 1977). We inserted the electrode into the micromanipulator, zeroed its voltage in the physiological saline. We checked the resistance of the electrode to see how much our current was disturbed. A high resistance could signal a broken electrode, which we would replace. We found between 5 and 15 megaohms an acceptable resistance. We looked through the microscope to observe where the tip punctured the muscle cell; a successful penetration of the muscle fiber showed a negative number on the DC amplifier after running through the PowerLab. We sanded the tip of our second microelectrode to create a wider-mouthed suction electrode. We attached the electrode to a syringe to suction the axons and to an SD9 Square Pulse Stimulator (Grass Instruments) to stimulate the axons and produce an EPSP in the muscle fiber. We used LabChart and DataPad software to record our results. EPSPs were recorded once every 30 seconds for 20 minutes.

Processing and presenting data

We chose to analyze each data point as a percentage of the first recording taken in each trial. This allowed us to compare trials despite differences in EPSP size. We presented data two ways to show the effects of FaNaC on overall EPSP strength and the prolonged effects of FaNaC on the EPSP. We used line graphs with time as the dependent variable in 30-second intervals and percent of the initial EPSP recording. This presentation showed the effects of FaNaC over time. We also presented our data in a bar graph, presenting the most significant percentage in each experiment to illustrate the effects of FaNaC on the EPSP overall.

In tests comprised of more than three trials, we used student's test, accepting p-values<.05 as statistically significant and p-values< .1 as marginally significant. We only used error bars represent standard error in graphs using three or more trials.

RESULTS

To determine whether FaNaC has any significant effect on EPSP amplitude, we ran a multitude of control and experimental tests using either Staurosporine, a kinase inhibitor, Amiloride, an FaNaC inhibitor, NF1, a neuropeptide that activates both kinases and FaNaC or a combination of the drugs. We compared the results from our trials to specific baselines to reach our conclusion.

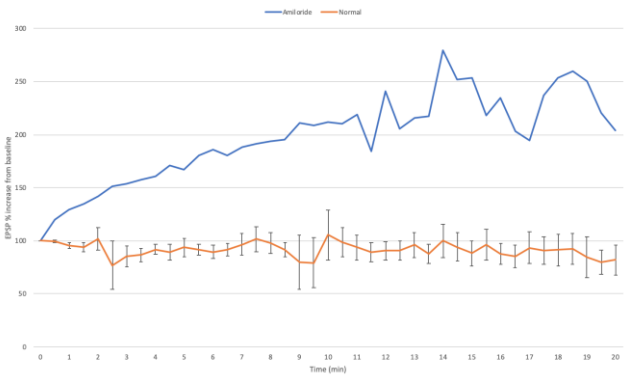

Fig. 1 shows the % change compared to the initial EPSP of crayfish extensor muscles submerged in saline solution containing Amiloride (blue) and unaltered physiological saline (orange). The Amiloride data represents the average of two trials and the unaltered data represent four trials. Bars represent the standard error of the average of EPSPs taken at the same time.

The Effects of Amiloride

As a control test to determine if Amiloride produced any unexpected effects on EPSP amplitude, we compared trials using just Amiloride to EPSPs from crayfish muscles in an unaltered solution. We submerged the crayfish extensor muscles in a saline solution with a 300 microMolar concentration of Amiloride and began recording EPSPs. We proposed that Amiloride alone would alter the EPSPs of the crayfish extensor muscles in a meaningful manner. The graph (fig. 1) indicates that Amiloride increases EPSP amplitude compared to EPSPs of crayfish muscles in unaltered physiological saline over time.

The Effects of Staurosporine

To determine whether Staurosporine exhibited any unexpected effect, we compared the EPSP amplitudes of trials using just Staurosporine to trials using crayfish muscle cells in unaltered physiological saline. In this test, we submerged the crayfish tail in the physiological saline and added 2.15μM Staurosporine. Once the Staurosporine was administered, we began recording EPSPs. Our null hypothesis stated that there would be no statistically significant difference between EPSP amplitudes in our unaltered trials and our Staurosporine trials. Figure 2 visualizes this data. From 0 minutes to 2 minutes, we observed a spike in the EPSP amplitude, which sank back down after minute 2. For the remaining time, the EPSP amplitudes were generally below the amplitude of the EPSP with no chemicals added. Consistent overlapping error bars and a p-test of .2 and above when comparing all data at a specific time represent no statistically significant data and supports the null hypothesis, indicating that Staurosporine has no unanticipated effects.

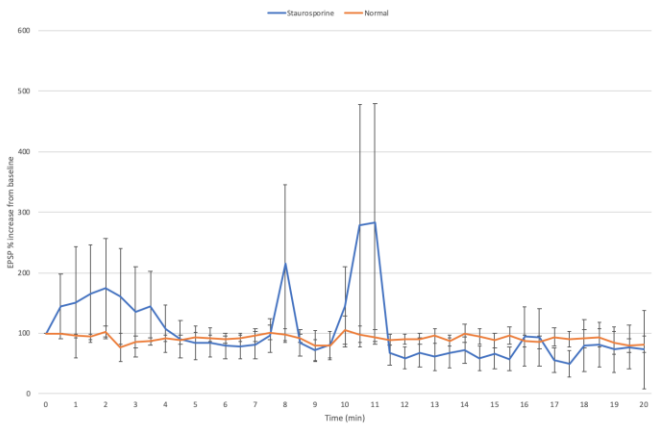

Fig. 2 shows the % change compared to the initial EPSP of crayfish extensor muscles submerged in saline solution containing Staurosporine (blue) and unaltered physiological saline (orange). The Staurosporine data represents the average of four trials and the unaltered data represent four trials. Bars represent the standard error of each EPSP taken.

The Effects of NF1

We conducted this control test to test for any unexpected effects of Staurosporine on the EPSP amplitudes by comparing it to a baseline of EPSP from crayfish muscles in an unaltered solution. We submerged our crayfish tail in a physiological saline solution with

either 10 or 50nM of NF1. After we added NF1, we began recording EPSPs. Our null hypothesis stated that there would be no statistically significant difference in EPSP amplitudes between trials using NF1 and trials using unaltered physiological saline. Figure 3 shows that crayfish muscle cells exposed to NF produced EPSP amplitudes that generally overlapped and remained close to one another.

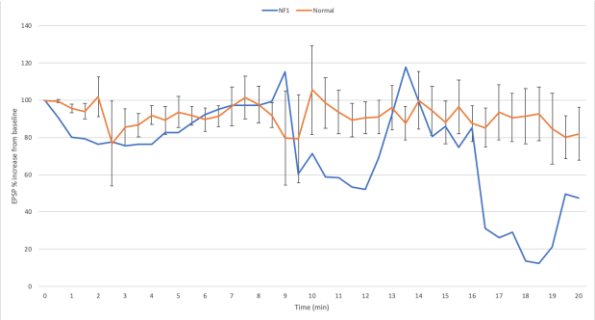

Fig. 3 shows the % change compared to the initial EPSP of crayfish extensor muscles submerged in saline solution containing NF1 (blue) and unaltered physiological saline (orange). The NF1 data represents 1 trial, and the unaltered data represents four trials. Bars represent the standard error of each EPSP taken.

The Effects of Staurosporine and NF1 over Time

In this experiment, we sought to determine whether anything other than kinases contributed to the amplification of EPSPs when NF1 is applied. To do this, we submerged crayfish extensor muscles in a 2.15 microMolar concentration saline solution and began to record EPSPs, which are displayed below (fig.4). Our null hypothesis stated that there would be no statistically significant difference between this data and our baseline data, EPSP amplitudes recorded from crayfish muscle cells submerged in unaltered physiological saline. The graph shows overlapping standard error bars from times t=0-13, from then on the EPSPs amplitudes of Staurosporine and NF1 increasing dramatically. Student's test gave p-values of around .09, one p-value taken using all data at one specific time. This semi-refutes our null hypothesis and gives us strong reason to believe that Staurosporine and NF1 EPSP amplitudes measured are different enough from EPSP recordings from muscle cells in unaltered physiological saline to indicate a potential additional NF1 activated entity amplifying EPSPs.

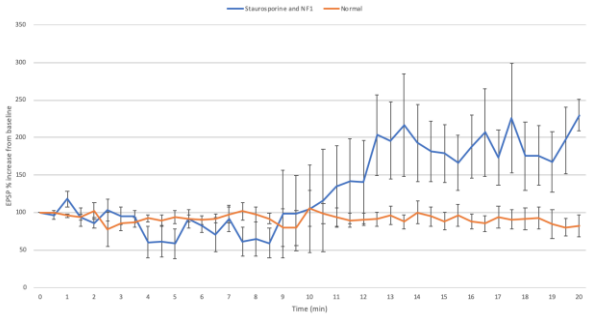

Fig.4 compares the effects of Staurosporine and NF1 on the EPSP of the crayfish extensor muscles (blue) to EPSPs of unaltered crayfish muscle cells (orange). EPSPs altered with Staurosporine and NF1 show a gradual increase over time as compared to the relatively unchanging, unaltered EPSPs. Bars represent standard error of the average of all the EPSPs taken at that specific time. We conducted four trials with staurosporine and NF1 and four trials using unaltered physiological saline.

The Effects of Amiloride and NF1 over Time

In this experiment, we looked at the effects of NF1 and Amiloride, a FaNaC blocker, on the EPSP amplitude of crayfish extensor muscles to look for any potential effects of FaNaC. We submerged the crayfish muscle cells in physiological saline and added NF1 and Amiloride until a 300 microMolar concentration of Amiloride and either a 10 or 50 microMolar concentration of NF1 was achieved. Looking at the graph (fig. 5), the two trials produce similar EPSP amplitudes for the first 9 minutes before the trials using Amiloride and NF1 quickly increased while trials using just NF1 remained steady. This indicates that the inhibition of FaNAC increase EPSP amplitude over time.

Fig. 5 compares the effects of Amiloride and NF1 on the EPSPs of crayfish extensor muscles (blue) to the effects of solely NF1 (orange). During times 18-19.5, we lost the EPSP and recorded from a different cell, accounting for the sudden drop in EPSP % change. The overall trend indicates that the addition of amiloride and NF1 increases the EPSP over time. Bars represent the standard error of the average EPSP at a given time. Amiloride and NF1 represents two trials while solely NF1 represents one trial.

The Effects of Amiloride, Staurosporine, and NF1

In this experimental test, we studied the effects to look for any effects recorded in previous data not contributed by kinases or FaNaC. To accomplish this, we added all chemicals at their prior concentrations to our

physiological saline and recorded EPSPs. The graph (fig. 6), when excluding points where EPSPs were unable to be recorded, shows the experimental trials are relatively close to the baseline of EPSPs from crayfish muscles in unaltered solution, only slightly higher over time. This indicates minimal effects outside of those caused by kinases and FaNaC: however, the lack of trials run for this experimental test makes the results less valid.

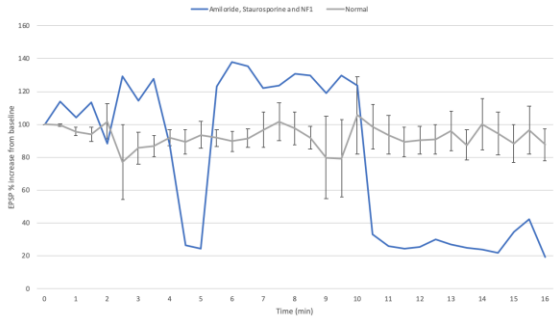

Fig. 6 compares the effects of all chemicals combined on the EPSP of the crayfish extensor muscles (blue) to EPSPs of unaltered crayfish muscle cells (grey). Bars represent standard error of the average of all the EPSPs taken at that specific time. We conducted one trial with the combination of inhibitors and NF1 and four trials using nothing.

Maximum Overall Changes in Control and Experimental Trials

Finally, we wanted to display the overall strengthening or weakening effects on EPSPs found within all of our experiments side by side. Figure 7 illustrates the compared average maximum increase from the initial EPSP of all tests. The trial "Normal" acts as a baseline for all other tests since it provided information on EPSP amplitudes without the influence of drugs. This graph serves as a summary of our individual data trials, showing them compared to one another to depict which inhibitor or combination of inhibitors caused the overall most substantial change in EPSP amplitude. This graph further displays the findings of our previous experiments in a more concise manner and allows all data to be compared.

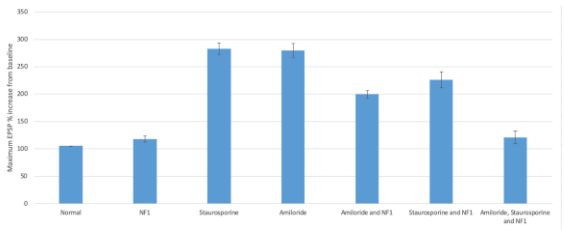

Fig. 7 showcases the effects of all of our experimental trials on the EPSP amplitude of our crayfish extensor muscles. Each bar represents the highest % change from initial EPSP amplitude from the averaged data of all trials. We arranged our trials in the following order. We ran four trials collecting data on the "Nothing" column, one trial for the NF1 column, four trials for the Staurosporine column, two trials for the Amiloride column, two trials for the Amiloride and NF1 column, four trials for the Staurosporine and NF1 column, and one trial for the Staurosporine, Amiloride and NF1 column.

DISCUSSION

Interpretation of Data

Our tests indicate that FaNaC may augment the EPSP amplitude, but the results remain inconclusive due to unanticipated side effects of the inhibitor Amiloride. Our test comparing EPSPs from crayfish muscles submerged in saline containing Staurosporine and NF1 versus muscles submerged in unaltered physiological saline indicate that something other than kinases contribute to the amplification of EPSPs. We drew this conclusion as the data reveals that to a significant degree, EPSP % increase from the initial reading is much higher over time in crayfish muscles submerged in the Staurosporine NF1 saline than the unaltered physiological saline. By using Staurosporine, an inhibitor that works by blocking the kinase function, halting their ability to change protein function via structural alterations, we isolate any remaining EPSP enhancement from unknown entities activated by FMRFamides. This initial research led us to believe that FaNaC potentially augmented the EPSP amplitude tested in our following experiment. Since our control comparing the effects of Staurosporine on crayfish muscle EPSPs and unaltered EPSPs showed no significant difference in % increase from the initial EPSP, we can assume with reasonable confidence that the results concluded from this experiment were caused by the blocking of kinases, not by any unintended effects of Staurosporine on the EPSP amplitude.

Our experiment comparing EPSPs from crayfish muscles submerged in saline containing Amiloride and NF1, versus muscles submerged in saline containing just NF1 initially showed with marginal significance that blocking FaNaC increased EPSP amplitude over time. This would potentially indicate that FaNaC channels, when activated, lower EPSP amplitudes. However, the control data comparing EPSPs from crayfish muscles submerged in saline and Amiloride compared to EPSPs from muscles submerged in unaltered physiological saline showed that with marginal significance, Amiloride increased the EPSP amplitude over time. This data conflicts with data collected from our control. As a result, we cannot confirm whether the EPSP amplitude augmentation in our experimental trial resulted from the inhibition of FaNaC, the unintended side effects of Amiloride, or a combination of both factors.

Figure 7 continues to support the evidence we found in figures 1-6. As shown, our highest EPSPs amplitudes recorded came from Amiloride and NF1, as well as Staurosporine and NF1. The patterns observed in our data

provide a potential answer that contradicts our hypothesis, in which we stated that activated FaNaC contributes to the amplification of EPSP amplitude in the presence of an FMRFamide. However, we would encourage more research using a different FaNaC inhibitor that has more limited excess effects on EPSPs' amplitude. Our data neither contradicts nor refutes any claims made by previous research as none exists in this exact niche.

Sources of Error

Throughout this experiment, we encountered multiple unforeseen difficulties, which likely affected our data in an unanticipated manner.

For some of our experiments and controls, we were unable to run a sufficient amount of trials, which makes our data less reliable as it represents a more limited number of repetitions. This issue stems from a lack of time and resources available to complete a desired number of runs and can be avoided in the future with additional funding and time.

During our recordings, due to muscle spasms and any additional movement of the lab table or pumping the electrode holder with the SLURP when replacing or removing the solution, sometimes electrodes slid out of cells, and EPSPs were "lost." This is reflected in our graphs by large, sudden drops. This primarily occurs due to the prolonged period and the fragility and general difficulty of working with organisms. We counteracted this by readjusting our electrodes and positioning them into new cells. However, this led to a different EPSP amplitude reading, which, when concerning the initial reading, sometimes created data discrepancies or data that was not accurate to what happened. In the future, we advise extreme caution when moving around the lab table and altering the setup of the experiment in any way to minimize these errors.

ACKNOWLEDGEMENTS

We would like to thank Dr. Clark Lindgren, Ashley Wolterstorff, and Emily Kozik for providing us with resources and both their time and knowledge to assist us in furthering our education in and out of the lab.

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Pioneering Neuroscience, 2019, **18**, 27-30

Amiloride hydrochloride does not have a significant effect on crayfish muscle extensor cells pH_i

ANNABELLA MATHEUS, LUCIA PASQUARIELLO, and ALIYA SINGLETON
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Amiloride is a diuretic drug known for its ability to regulate K^+ levels and prevent the body from absorbing excessive levels of Na^+ by inhibiting FMRF-amide gated Na^+ channels (FaNaC channels). Amiloride has the ability to reduce the effect of FMRF-amide peptides on the synapses in the crayfish neuromuscular junction. However, in addition to its ability to block FaNaC channels, it is possible that Amiloride may have offset effects that inhibit normal functioning within the cell. We therefore hypothesize that the addition of Amiloride will make the intracellular pH more acidic. We used fluorescence microscopy to quantify changes in intracellular pH in crayfish extensor muscle cells by comparing the changes in fluorescence between a Ringer's solution and a solution containing Ringer's and Amiloride. Through doing this, we found that while the data was not statistically significant, it did tentatively support our hypothesis.

INTRODUCTION

Voltage-gated ion channels play a significant role in synaptic transmission at the neuromuscular junction. Voltage-gated sodium channels in particular regulate the influx of Na^+ in response to membrane potential changes. This regulation of the amount of Na^+ ions inside and outside of the cell directly affects synaptic transmission through the normal depolarization of the cell to fire an action potential. A lower presence of Na^+ inside the cell lowers the amount of neurotransmitters released, leading to weaker excitatory postsynaptic potential measurements (EPSPs).

Amiloride prevents excessive influx of Na^+ by inhibiting FaNaC channels (Schaffhauser et Al 2016). FaNaC belongs to the larger ion channel family Deg/ENaC. ENaC allows Na^+ to enter the cell and contributes to ion transport across epithelial barriers. FaNaCs can be activated by external protons or the cell's pH level. Amiloride is known to block ENaCs and DEGs; this is because when amiloride is in an environment with a high pH, it breaks apart and neutralizes the pH levels in the environment. Because FaNaCs react to the pH levels, Amiloride reduces their activity.

Amiloride is known to inhibit plasma membrane-bound transport systems by binding to sodium channels in cellular tubules to block sodium reabsorption. Amiloride acts presynaptically to inhibit an induction of neurotransmitter release; however, it does not prohibit action potential propagation in nerve terminals (Smith 2000). Although Amiloride is known to prohibit normal functioning of FaNaC voltage gated sodium channels, it is possible that an additional offset reaction can inhibit normal functioning of the Na^+/H^+ exchanger, the H^+ pump, or the HCO_3/Na^+ exchanger, leading to a change in pH within the cell (pH_i). Further research is necessary to better understand the linkage between Amiloride and its effects on intracellular function.

We predict that by blocking the normal functioning of the voltage gated FaNaC sodium channels, Amiloride will have an offset effect of additionally interfering with the active extrusion of H^+ from cells. We hypothesize that Amiloride will lead to a buildup of H^+ ions within the cell, thus decreasing pH_i. Because intracellular pH is a vital aspect of all cellular processes (i.e. membrane potential, transportation across the cellular membrane, and ability of muscle cells to contract), we hope to better understand the secondary effects of Amiloride in invertebrates by specifically examining the role it may play in maintaining pH_i. We ultimately found that intracellular pH decreased with the addition of Amiloride although this effect was not statistically significant.

MATERIALS AND METHODS

Crayfish Dissection

Crayfish specimens were first anesthetized in an ice bath for 15 minutes, and then dissected according to a procedure described by Atwood and Parnas (1968). Following this procedure, we used scissors to remove the organism's abdomen and make cuts along the edge of the ventral side of the crayfish. We removed the ventral section of the exoskeleton and the swimmerets and scraped off the flexor muscles and digestive tract with a thumb, leaving the dorsal

extensor muscles attached to the exoskeleton. These are the muscles that we examined for changes in fluorescence. We used a new crayfish for each day of experimentation.

Solutions

We used a normal Ringer's solution composed of 5.4 mM KCl, 196 mM NaCl, 2.6 mM MgC•6H2O, 10mM Hepes Buffer, and 13.5 mM CaCl2•2H2O. For the positive control solution, we replaced the 196 mM NaCl with 196 mM Propionic acid. We also used 300 µM Amiloride which was added to Ringer's.

pH_i Measurement

To test our hypothesis that the addition of Amiloride makes the intracellular pH more acidic, we completed a series of tests. We used BCECF-AM and fluorescence microscopy to measure the changes in fluorescence (which correspond to changes in pH_i) in crayfish extensor muscle cells. To do this we followed the procedure described by Lindgren, Emery, and Haydon (1997). BCECF-AM is designed to act hydrophobically so that it can move across the phospholipid bilayer into the cell. Esther groups then cut off the AM group, trapping the BCECF dye in the cell.

The BCECF-AM was put in Ringer's solution and allowed to set for one hour, then it was rinsed with ringers and allowed to sit for another hour. We then took 10 images of the crayfish extensor muscle cells in the Ringer's. Then an Amiloride (or Propionic Acid) and Ringer's solution was put on the crayfish and allowed to sit for 20 minutes before we took 10 more images. We finished the process with a wash of normal Ringer's and waited another 20 mins before taking the final 10 images. This was repeated for each crayfish used.

We used a Nikon Eclipse 50i fluorescence microscope and collected images using Nikon Elements software and a Nikon DS-Ri1 camera. The wavelength emitted by BCECF-AM was greater than 515nm and the wavelength of the light used to excite it was less than 488nm. We ran 3 positive control experiments with 196 mM Propionic acid to show that the method using BCECF-AM could detect a change in pH. Propionic acid is a relatively strong acid with a pH of 2.94 that has few secondary effects on the cell. We used fluorescence microscopy and image processing to measure quantifiable changes in pH_i from the measured changes in fluorescence. We then used the same process mentioned above to run a series of tests using both BCECF-AM and Amiloride and observed any changes in fluorescence/pH_i.

Changes in pH_i were quantified from our images using ImageJ (NIH). While taking our measurements and images, we recorded all changes in fluorescence emission intensity in Excel by calculating $\mathbf{\Delta}F/F_R$ (the change in fluorescence emission intensity from the control that correspond to changes in pH_i). We also took the average fluorescence measurements from all solutions and ran t-tests to determine if the changes we observed were significant.

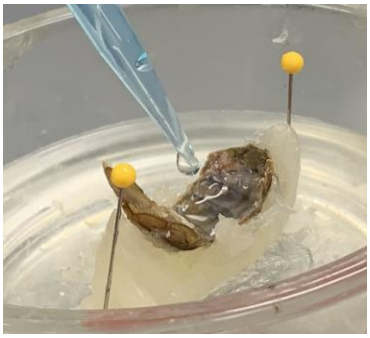

Figure 1. Crayfish boat made of wax and vaseline used to hold BCECF-AM solution.

Because BCECF-AM is an expensive chemical, we made a boat out of wax and Vaseline to hold the crayfish tail and keep the solution sealed inside. This allowed us to fit 1 ml of the solution inside the crayfish tail so that there was limited waste (Fig. 1). This posed a possible difficulty at the start, but through trial and error the boat proved to be an adequate solution and allowed us to carry out the experiment in an effective manner.

RESULTS

To analyze the effect of Amiloride on pH_i, we first loaded the crayfish muscle extensor cells with the pH indicator BCECF-AM. We then measured the fluorescence emission of the cells while also suspended in Propionic acid. The positive control Propionic acid showed high levels of fluorescence in comparison to normal Ringers, with similarly high levels seen after a wash step (Fig.2).

A fluorescence test quantified the difference in fluorescence due to changes in pH_i between crayfish muscle cells exposed to normal Ringer's solution and Amiloride. We calculated fluorescence levels by comparing the variable solution mean fluorescence levels to normal Ringers F/F_R.

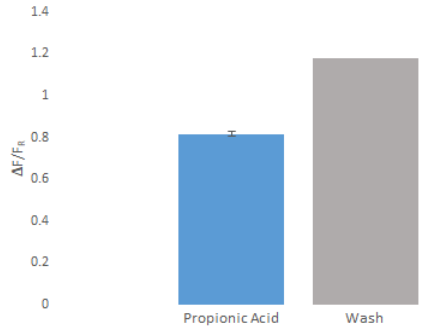

Figure 2. The average F/F_R of $[pH]_i$ of Propionic acid and wash. Error bars indicate the standard error and range of values of the recordings taken for each solution. Propionic Acid: n=3 Wash: n=1

Figure 3 shows the results of an individual experiment where Amiloride was shown to lower fluorescence, then after a wash step fluorescence was shown to increase again. This was consistent with our other experiments (Fig. 4), with the exception of our first trial.

In the first experiment, the fluorescence increased, which is consistent with an increase in intracellular pH. In the remaining five experiments, fluorescence was shown to decrease. A typical example of this trend is shown in Figure 3. Thus, the data plotted represents the latter five data points. This was done to correct and account for any error due to a lack of technique or experience with the fluorescence microscopy method.

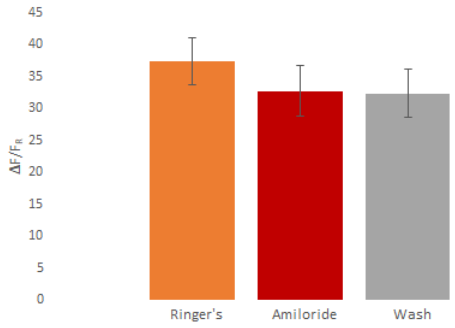

Figure 3. The fluorescence of crayfish extensor muscle cells with Ringer's, Amiloride, and wash solutions. Error bars indicate the standard error and range of values of the recordings taken for each solution. n=1

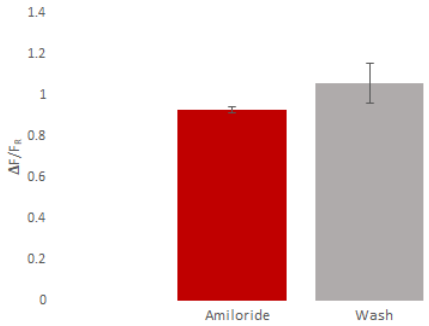

Figure 4. The average F/F_R of Amiloride and wash. Error bars indicate the standard error and range of values of the recordings taken for each solution. n=5

DISCUSSION

To test the effects of Amiloride on intracellular pH, we used fluorescence microscopy to measure quantified changes between Ringer's and Amiloride solutions. As a positive control, we used Propionic acid to demonstrate that the methods of measuring fluorescence were effective. Before testing amiloride we wanted to test our ability to measure pH with Propionate. After proving BCECF's effectiveness with Propionate (Fig.2) we continued our experimentation by testing Amiloride. We conducted five experiments where we applied amiloride; the first experiment showed an increase in overall fluorescence while the remaining four showed an overall decrease. A typical example of the latter four experiments is shown in Fig.3.

Our positive control experiment was successful, but we found that the wash did not return to fluorescence emission levels similar to those in the Ringer's solution. This is presumably because there was not a strong enough gradient for the Propionic acid to diffuse out of the cell. This experiment demonstrated that Propionic acid diffuses into the cell and dissociates to H^+ and propionate, leading to its inability to be simply washed out of the cell. The gradient driving the hydrogen propionate into the cell diffuses very slowly and the wash step starts a reversal; however, there is not a large enough gradient to make a difference. Therefore, the BCECF-AM indicator detects a drop in pH yet cannot be fully washed out.

Although our other data was not statistically significant, our experiment supported the hypothesis that pH_i would become more acidic with the addition of Amiloride.

While our first set of data showed the pH_i becoming less acidic, the remaining five tests became more acidic with the addition of Amiloride. This is why we disregarded the first set of data in our final

data analysis. By repeating these tests, and therefore gaining more data points, the results could become statistically significant, and might further affirm that the first test was an outlier in the data set.

In most cells pH_i is maintained at around 7.0, making it relatively acidic compared to the extracellular fluid. Free-floating H^+ ions can increase the acidity of their surrounding solution. When these H^+ ions enter into the cell the pH_i becomes increasingly acidic. Oftentimes changes in pH_i are in response to externally applied inhibitory/regulatory substances, like the Amiloride used in the experiment. Thus, cells must regulate pH_i with Na^+/H^+ exchangers, H^+ pumps, and HCO_3 /Na^+ exchangers (Putnam 2016). The Na^+/H^+ exchanger is a membrane protein that facilitates movement of Na^+ into the cell and H^+ out of the cell. This is important to the functioning of the cell because changes in pH can alter the chemical structures of proteins and thus their function and overall functionality of the cell. To regulate pH_i, there is an H^+ pump that solely moves H^+ out of the cell through the use of ATP (Demaurex et al., 1997). Both the Na^+/H^+ exchanger and the H^+ pump are responsible for raising the pH_i, but the HCO_3 /Na^+ exchanger lowers the $[pH]_i$ (Wang et al., 2000). The blockage of any of the first two systems and/or activation of the third could be responsible for the change in pH_i that we saw in the crayfish extensor muscle cells.

Although we measured pH_i in muscle cells, these changes would also likely be occurring in other places, such as nerve terminals. Further experimentation is needed to see which of the aforementioned pumps could be the cause for the measured changes in pH_i. A separate experiment could include individually blocking each of the systems that regulate pH_i to see which results are consistent with those of Amiloride.

ACKNOWLEDGEMENTS

We thank Professor Lindgren for providing assistance throughout the designing and carrying out of our experiment and for introducing us to methods used to measure the changes in $[pH]_i$. We'd also like to thank our wonderful lab assistants Emily Kozik and Ashley Wolterstorff for helping us safely handle chemicals, teaching us to operate the fluorescence microscope, and providing guidance throughout the research process.

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Pioneering Neuroscience, 2019, **18**, 31-38

Ruthenium red causes reversible synaptic depression in crayfish extensor muscles.

AUBRIE TORHORST, DAVID ZHANG, and MARIA ZHU
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Cells, especially those involved in synaptic transmission, keep intracellular calcium concentration low for proper functioning. In addition to pumping calcium out via ATP-powered pumps, the cell also stores calcium within organelles such as the endoplasmic reticulum and mitochondria to remove it from the cytoplasm. Mitochondria specifically use the mitochondrial uniporter to take up calcium from the cell. We used ruthenium red, a mitochondrial uniporter channel inhibitor, to test how the inhibition of the uniporter channel affects synaptic transmission. We found that under low and high frequency stimulation, the application of ruthenium red depressed excitatory postsynaptic potentials (EPSPs) over time. Additionally, the presence of the potassium channel inhibitor 4-AP with ruthenium red under high frequency stimulation did not influence the effectiveness of ruthenium red's inhibition. Compared to 4-AP alone, a combination of both chemicals produced a faster and stronger depression of EPSPs. After a test including both chemicals, removing ruthenium red while leaving 4-AP allowed some recovery from the inhibitory effect of ruthenium red, as EPSPs remained partially depressed under 4-AP alone. In all experiments, the depression resulting from either chemical was reversible. The same EPSPs recovered to their previous EPSPs after a wash with normal crayfish Ringer's solution. From our findings, we can conclude that ruthenium red produces reversible synaptic depression in crayfish extensor muscles.

INTRODUCTION

Mitochondria are critical to cell functioning. Like other excitable cells, neurons have a high energy demand; hence, mitochondria are abundant in the cytoplasm. Mitochondria create ATP, regulate Ca^{2+}, and have roles in lipid production and apoptosis, among other functions in the cell (Lee et al., 2018; Tong, 2007). Additionally, their delivery to the synapse proves critical to synaptic potentiation. Blocking the movement of mitochondria to the nerve terminal reduces synaptic potentiation while enhancing movement promotes potentiation, proving the importance of mitochondrial delivery to the synapse for synaptic plasticity (Tong, 2007).

Additionally, ATP from mitochondria is necessary for cAMP-dependent pathways in the presynaptic cells of the nervous system. cAMP, a small molecule made from ATP, acts as a secondary messenger that directly activates cyclic nucleotide-gated ion channels and protein kinase A (PKA). When activated, PKA phosphorylates target proteins responsible for increasing neurotransmitter release by decreasing potassium currents, increasing calcium currents, or increasing the store of vesicles available for exocytosis (Kandel et al., 1982).

Mitochondria also take up calcium from the cytoplasm to maintain a low intracellular calcium concentration. They take up calcium through the mitochondrial calcium uniporter (MCU) located in the inner membrane of mitochondria. Keeping calcium concentrations low prevents cell death in all cells. Calcium homeostasis disruption and mitochondrial dysfunction are observed in many neurodegenerative disorders (Liao et al., 2017). Thus, a more comprehensive understanding of the mitochondrial calcium uniporter would provide new insights into treating many neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis which result from the dysfunction of mitochondria and mitochondrial homeostasis (Liao et al., 2017). In neurons, a low calcium concentration not only prevents cell death but also establishes a steep concentration gradient across the cell membrane. Mitochondria aid the establishment of this gradient by storing calcium.

Within mitochondria, the calcium gradient enables them to decode Ca^{2+} signals and thus tune ATP synthesis to the energetic requirements of the cell (Santo-Domingo et al., 2010). Calcium also serves as a coupler for increased ATP synthesis in mitochondria by activating enzymes that fuel the electron transport chain (Finkel et al., 2015). However, Finkel et al. (2015) found that decreased expression of the uniporter channel did not affect mitochondrial respiration rates and thus metabolism, indicating that calcium enhances ATP synthesis but is not required for it. Therefore, inhibiting the influx of calcium into mitochondria will not affect normal ATP production.

ATP itself also plays a critical role in maintaining low calcium in the cell. In the outer cell membrane, a

pump continuously removes intracellular calcium from the cytoplasm and transports it outside of the cell. Because calcium ions travel against their concentration gradient, the pump requires ATP.

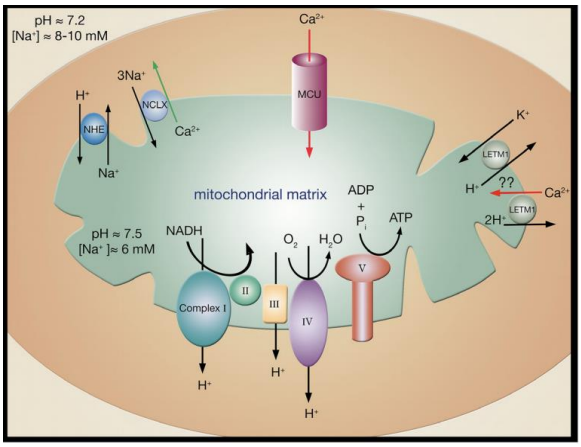

Figure 1. Mitochondrial Ion Transport Mechanisms (Finkel et al., 2015). Calcium enters through mitochondrial calcium uniporter (MCU). Calcium uptake enhances ATP production and regulates the internal calcium concentration. By blocking MCU with ruthenium red, the intracellular calcium concentration increases without preventing ATP production.

Ruthenium red is an inhibitor of the mitochondrial calcium uniporter, and its effects are comparable to Ru360 (Ying et al., 1991). While ruthenium red is less potent and pure than Ru360, its inhibition is stronger (Ying et al., 1991). Before studies confirmed its role as a uniporter inhibitor, scientists suspected that blocking the increase of calcium in the cell prevented the release of neurotransmitters from nerve terminals (Taipale, 1988), implying that an increase of intracellular calcium is involved in neurotransmitter release and thus the resulting strength of the synapse. However, an increase in intracellular calcium can also become toxic to the cell, weaken the concentration gradient, or affect other processes that weaken synaptic strength. How the calcium concentration in the cell is affected by ruthenium red will determine the resulting strength of synapses.

To specifically investigate the role of the mitochondrial uniporter during synaptic transmission, we could not block mitochondria entirely. Entirely stopping the function of mitochondria would stop ATP production, and thus most processes in the cell since ATP fuels many of them. If we inhibited mitochondria entirely, our data could not be specifically attributed to the disruption of calcium regulation. Using ruthenium red allows us to inhibit the mitochondrial uniporter channel without disrupting other mitochondrial functions, which allows ATP to execute its roles related and unrelated to calcium regulation during our tests.

We used crayfish in our experiment because the preparation to access nerves and crayfish extensor muscles is fast and simple and the nerve-to-muscle relationship is easy to identify under the microscope. Compared to more complex organisms such as mammals, crayfish nervous systems are simpler because they do not have any voltage-gated ion channels. Voltage-gated channels complicate experiments with mammals, so by using crayfish, we eliminated extra costs and complications that voltage-gated channels add to experiments in the neuromuscular junction. Crayfish are a model organism for the nervous system and allowed us to test a universal question without the added complexity that larger, mammalian organisms add.

In our experiment, we tested crayfish extensor muscles in the presence and absence of ruthenium red to investigate the its effect on synaptic transmission. Nerves were stimulated in three conditions: low frequency stimulation, high frequency stimulation, and high frequency stimulation plus blocked potassium channels. High frequency stimulation delivers mitochondria to the synapse quickly (Tong, 2007); therefore, mitochondria were highly present and active under these conditions. Adding on the inhibition of potassium channels to high frequency stimulation increases the influx of calcium during synaptic transmission by prolonging the action potential in the nerve. With a slower removal of potassium ions from the cell because fewer channels are open, the cell cannot repolarize as quickly, leaving the membrane potential depolarized longer, which keeps voltage-gated calcium channels open longer. Hence, more calcium entered the cell under these conditions, heightening the interaction of mitochondria and calcium ions in our experiment.

The goal of our experiment was to further understand the mitochondrial calcium uniporter and its effects on synaptic transmission. Specifically, we aimed to find a significant effect of ruthenium red on synaptic transmission. We found that under low and high frequency stimulation, EPSPs decreased over time. The addition of the potassium channel inhibitor 4-AP did not affect the level of inhibition ruthenium red caused. While both ruthenium red and 4-AP depressed EPSPs, ruthenium red's inhibition onset faster and reached a more potent level than the application of 4-AP alone. Regardless, a wash with regular crayfish Ringer's solution reversed the effects of all experiments we ran. Therefore, we concluded that ruthenium red causes reversible synaptic depression in crayfish extensor muscles.

MATERIALS AND METHODS

Crayfish Preparation

Crayfish were chilled over ice to anesthetize them before dissection. After they chilled in ice for enough time, we removed the tail and exposed the extensor muscles on the dorsal surface of the tail by removing the muscle and gut on the ventral part of the abdomen. After pinning the resulting dissection into a tray, we submerged it in 100 mL of normal crayfish Ringer's solution, which contains 5.4 mM KCl, 196 mM NaCl, 2.6 mM $MgCl_2$-$6H_2O$, 10 mM HEPES, and 13.5 mM $CaCl_2$-$2H_2O$ at a pH of 7.4.

Microelectrode Preparation

We measured the resting membrane potential with microelectrodes pulled from borosilicate glass in an electrode puller (Pul-1, World Precision Instruments) to a diameter of < 1μm. These measuring electrodes were filled with 3 M KCl. When the electrode tips were placed in the Ringer's solution, their resistance fell approximately between 5 MΩ to 20 MΩ during all recordings.

Our suction electrodes were made from the same glass and pulling apparatus but were left unfilled. In order to suck up the nerves, we increased the pulled electrode's diameter by slowly dragging its tip across sandpaper. Using a syringe attached to the electrode holder, we sucked crayfish saline into the microelectrode before sucking up the nerve for stimulation.

Equipment and Recording

The cells and electrodes were visualized using a dissecting microscope. We used a stimulator (SD9, Grass Instument Company) to apply a suprathreshold stimulus. Across our experiments, stimulus voltage varied depending on each nerve's threshold. The stimulator also allowed us to manipulate the stimulus frequencies for our specific testing conditions. Our low frequency tests ran at a frequency of 0.2 Hz while our high frequency tests ran at a frequency of 10 Hz. An amplifier measured the membrane potential of muscle cells detected by the measuring microelectrodes. Membrane potentials of the muscle cells fell between -45 and -70 mV.

Chemical Application and Washout

100 μL of 12.7μM ruthenium red, a mitochondrial calcium uniporter inhibitor, was added to 100 mL of normal Ringer's solution in all experimental tests. Before beginning recording, we thoroughly mixed the chemical into the normal Ringer's solution for 30 seconds. Each test ran between 5 and 15 minutes. After each test, we rinsed the crayfish muscles thoroughly with regular crayfish Ringer's solution before resetting the dish with 100 mL of normal Ringer's solution.

For the high frequency test involving the potassium inhibitor 4-aminopyridine, we added 100 μL of 50 mM of 4-AP to 100 mL normal Ringer's solution, resulting in a final concentration of 0.5 mM. When ruthenium red was added with 4-AP, the same concentration and amount of ruthenium red as mentioned previously was added.

Calculations

LabChart software allowed us to simultaneously view the membrane potential and synaptic responses. We obtained the maximum and minimum values of each synapse with DataLab on LabChart, and we used these values to calculate EPSP amplitudes. We measured the percent EPSP change versus time in each test. Because EPSPs varied between different nerves and crayfish, all were normalized to the initial EPSP of that respective experiment. The percent change for each EPSP was calculated using this equation:

$$\%\, Change = \left[\frac{EPSP - EPSP_{initial}}{EPSP_{initial}}\right] \times 100\%$$

RESULTS

To investigate the effects of ruthenium red on synaptic transmission, we measured the resting membrane potential and the excitatory postsynaptic potential (EPSP) of a muscle cell that is innervated by a nerve. First, we searched the dissection under the microscope and found a viable nerve for testing. Using the suction electrode, we sucked the nerve up for stimulation. To measure the muscle membrane potential, we inserted the measuring microelectrode into a muscle cell in the same region as the acquired nerve in the suction electrode. The measuring microelectrode measured the difference in voltage across the membrane to determine the cell's membrane potential. After both electrodes were set-up with viable nerves and cells, we stimulated the nerve at varying voltages until the LabChart software indicated the firing of an EPSP. If the reading of the resting potential of the cell remained stable after several test stimulations, we proceeded to test the muscles under constant low or high frequency, depending on our intended test, for 5 to 15 minutes.

First, we investigated EPSPs under low frequency stimulation. Before applying ruthenium red, we ran a 5-minute long test without any chemicals to see how constant low frequency stimulation affected EPSPs under normal conditions. This test ran at a frequency of 0.2 Hz. Without ruthenium red, EPSPs under low frequency

stimulation remained stable at an amplitude of 8 to 10 mV over a 5-minute time period (Fig 2).

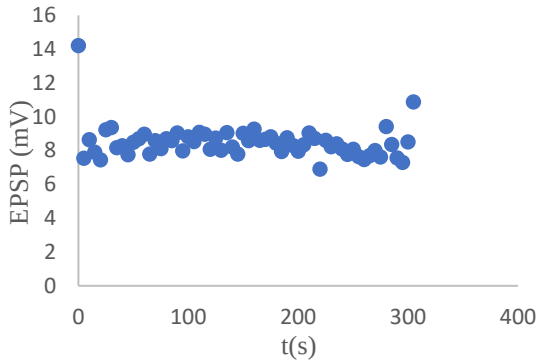

Figure 2. The baseline reading of EPSPs under low frequency stimulation. n=1.

Next, to investigate the effect of ruthenium red on EPSPs under low frequency stimulation, we applied ruthenium red to the Ringer's solution, mixed it in for 30 seconds, then ran another 5-minute long test and compared it to our baseline results. Figure 2 shows EPSPs from another muscle than the muscle used in this experiment, so before adding ruthenium red, we obtained baseline EPSPs from the muscle under experimentation to track its changes during this experiment. Baseline data is not included in the figure. In the presence of ruthenium red, EPSP decreased as time increased (Fig 3). Ruthenium red reached a maximum inhibitory effect at -86% (Fig 4). While EPSPs decreased for the first 2 minutes of the experiment, once the chemical reached its maximum level of inhibition, EPSPs remained constant at this inhibited value for the remaining time of the experiment. We repeated this experiment twice and found similar results in both runs. Under low frequency, the presence of ruthenium red caused EPSPs to decrease as time increased.

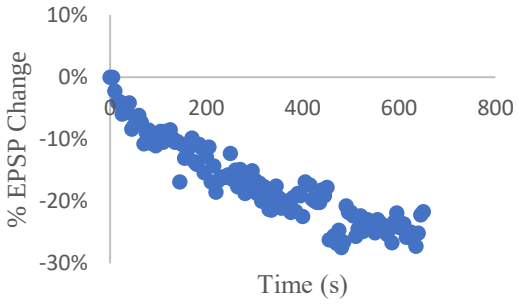

Figure 3. The effect of ruthenium red on EPSPs during low frequency stimulation. Ruthenium red was applied at t=-30. n=1.

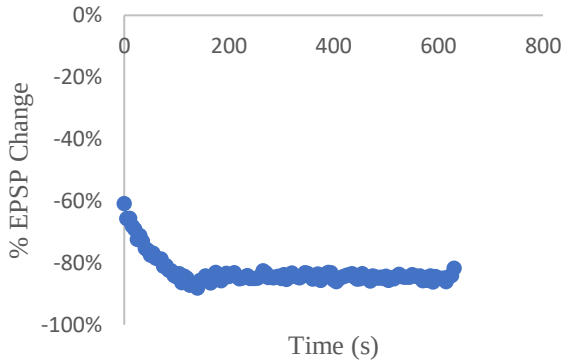

Figure 4. The maximum inhibitory effect of ruthenium red on EPSPs under low frequency stimulation. Ruthenium red was applied at t=-30. n=1.

Next, we stimulated nerves under high frequency stimulation. Each high frequency test ran at a frequency of 10 Hz. First, we ran a test to see how EPSPs reacted to constant high frequency stimulation under normal conditions. This baseline test indicated a slight increase followed by a slight decrease in EPSPs over time (Fig 5). It is likely that the decrease is due to fatigue, as amplitudes recovered to their original, pre-testing value 30 seconds after continuous high frequency stimulation stopped.

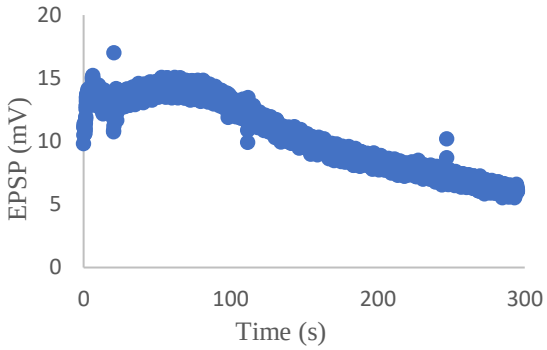

Figure 5. The baseline reading of EPSP during high frequency stimulation. No ruthenium red was added. n=1.

To test how ruthenium red affects EPSPs under high frequency stimulation, we added it to the normal Ringer's solution and stimulated the nerves at 10 Hz. Like in the low frequency experiments, ruthenium red was mixed into the Ringer's solution 30 seconds before we began recording. Additionally, we gathered baseline readings for this particular muscle before testing with ruthenium red to see how this particular EPSP changed over time. This baseline data is not included the figure. Our data shows that in the presence of ruthenium red, EPSPs initially increase for a short amount of time but then decrease for the remainder of the experiment (Fig 6). We ran the test twice, and the data produced similar results both times. Although this initial EPSP increase is uniform throughout

all of our high frequency tests, some later tests had a more gradual increase in percent EPSP change (Fig 7). Regardless of time under high frequency stimulation, ruthenium red reaches a maximum inhibitory effect of -97% in most experiments. Additionally, our data shows that under high frequency stimulation, the inhibitory effect of ruthenium red onsets faster than the inhibition seen under low frequency stimulation.

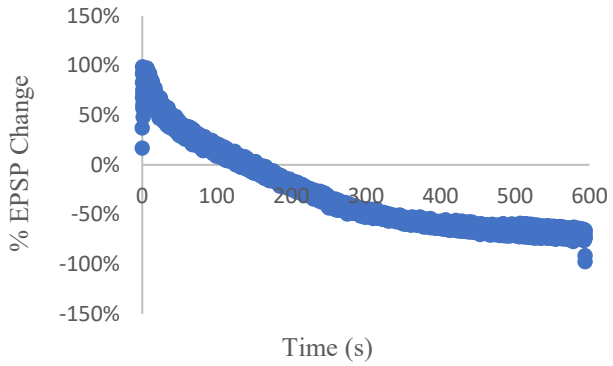

Figure 6. The inhibitory effect of ruthenium red on EPSPs under high frequency stimulation. Ruthenium red was applied at t=-30. n=1.

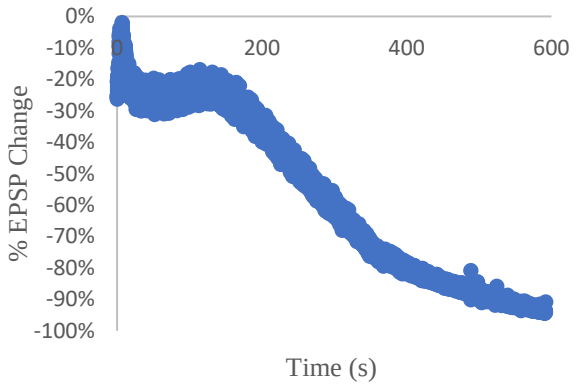

Figure 7. The inhibitory effect of ruthenium red on EPSPs under high frequency stimulation (continued). Ruthenium red was applied at t=-30. n=1.

During our high frequency tests, we had difficulties acquiring stable data when the experiment reached its halfway point at roughly 5 minutes, making data analysis difficult and inconclusive. To combat the fluctuations, which we speculated was a result of muscle fatigue or overload, we ran an experiment with multiple intervals of stimulation rather than one constant period of stimulation (Fig 8). We stimulated the nerve in three, 2-minute intervals with 1-minute break periods in between. Within each interval, EPSPs decreased over time. However, after the 1-minute break, the EPSPs showed some recovery, as the initial EPSPs in the new interval were slightly higher than the final EPSPs in the previous interval. Still, in each interval, EPSPs decreased more than the previous interval, and the interval experiment showed results consistent with the other experiments under high frequency stimulation.

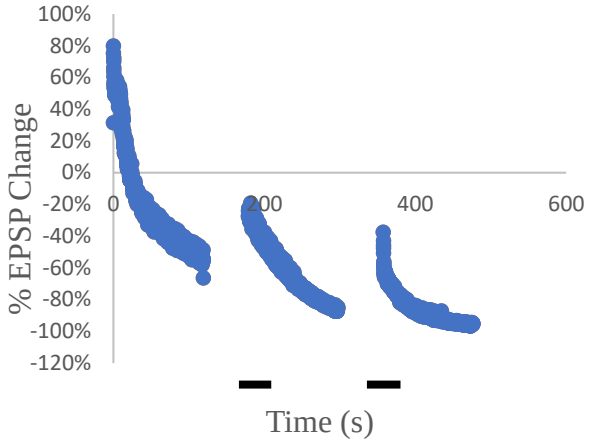

Figure 8. The inhibitory effect of ruthenium red during high frequency stimulation intervals. The black bars represent a 1-minute period of no stimulation. n=1.

Like the experiment in Figure 8, the we ran another test in intervals with 1-minute breaks between each interval. However, in the break periods of this experiment, we altered the chemicals present, so each interval had a different testing solution to investigate how 4-AP alters the effects of ruthenium red in our experiment. The first section of the graph in Figure 9 is a baseline reading of EPSP during high frequency stimulation without any chemicals. After the first 1-minute break, we applied ruthenium red and 4-AP together. Compared to the first section, the percent EPSP change decreased faster but reached the same amount of inhibition. In the final interval of the graph, ruthenium red and 4-AP were removed and replaced with new crayfish Ringer's solution containing only 4-AP. In this interval, percent EPSP change increased from the values at the end of the second interval. Figure 9 shows that EPSP partly recovered during the application of 4-AP alone under high frequency stimulation. After the increase, EPSPs remained near -60±5% for the duration of the experiment, still depressed from the original value but much higher than the -97% value than previous intervals trended towards. The maximum inhibitory effect in the test with 4-AP alone was -68%, which is 29% higher than the maximum inhibitory effect of -97% when ruthenium red was present.

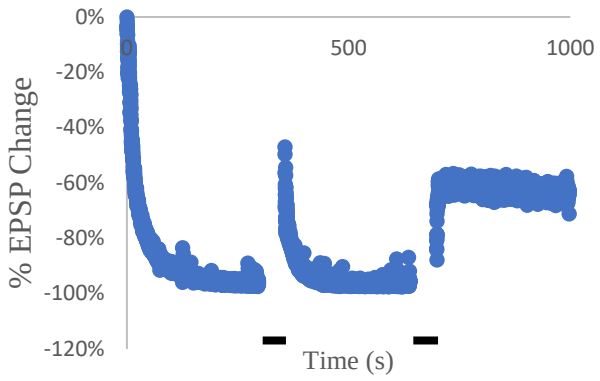

Figure 9. The removal of ruthenium red increases EPSP. The black bars indicate 1-minute breaks from stimulation. The first interval contains no chemicals. The second interval includes both 4-AP and ruthenium red. The last interval includes only 4-AP. n=1.

Because both 4-AP and ruthenium red depressed EPSPs under high frequency stimulation, we ran another test to see how ruthenium red and 4-AP differ in their speed of inhibition. In this test, we added 4-AP alone and measured EPSPs for 5 minutes. After a 1-minute break, we added ruthenium red to the preexisting solution and measured EPSPs for another 5 minutes. The results are shown in Figure 10. 4-AP alone takes over 30 seconds to reach the half of the maximum inhibitory effect on EPSPs. However, when we added ruthenium red, the time taken to reach this same value decreases to under 5 seconds.

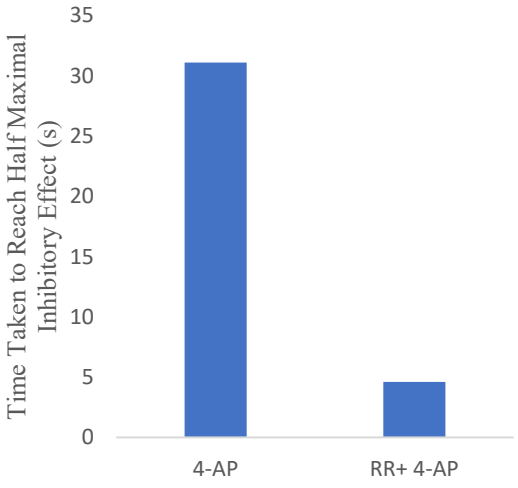

Figure 10. The time taken in seconds to reach half of the maximum inhibitory effect.

DISCUSSION

We investigated the effects of ruthenium red on synaptic transmission in crayfish extensor muscles. Under both low and high frequency stimulation, ruthenium red decreased EPSPs. Under high frequency stimulation, depression was achieved faster, but in many of our trials, the depression was preceded by a slight increase in amplitude. 4-AP also depressed EPSPs on its own. However, it appears to have no significant impact on the maximum inhibitory effect of ruthenium red when in solution together. When 4-AP and ruthenium red were combined together in solution, the time taken to reach half of the maximum inhibition was greatly decreased from the time taken by 4-AP alone, proving the potency of ruthenium red in synaptic depression. After rinsing the muscles with normal Ringer's solution, the EPSPs under testing returned to their previous strength, indicating that the depression caused by ruthenium red is reversible.

Without ruthenium red, high frequency stimulation alone depresses synaptic transmission. Our baseline data shows that EPSPs decrease over time without ruthenium red, which we speculated was due to fatigue. This fatigue can be caused by a number of factors: the decrease of releasable vesicles, inactivated presynaptic calcium currents, a depleted amount of neurotransmitter in each vesicle, postsynaptic desensitization, a failure of presynaptic action potentials, and more (Kim et al., 2012). Streit et al. (1992) noticed similar effects in their testing of nerves under various frequencies in embryonic spinal cord tissue cultures from rats. Without any other independent variables other than stimulus frequency, they noticed transmission failures at higher frequencies that were not present in the results at lower frequencies. They concluded that these failures were a result of presynaptic conduction failures (Streit et al., 1992). Adding ruthenium red to our experiment likely further complicated the presynaptic failures already occurring under high frequency, making synaptic depression much more evident.

In the high frequency tests, EPSPs quickly increased before decreasing for the remainder of the time. The initial increase in amplitude can be explained by the initial increase in internal calcium concentration caused by the blockage of the MCU. Blocking these channels eliminates mitochondria as a store for intracellular calcium, which keeps more calcium in the cytoplasm. Temporarily, this extra calcium binds to more synaptotagmin, which activates more vesicles to release neurotransmitters into the synaptic cleft. An increase in neurotransmitter release increases the strength of the synapse. Eventually, however, the extra calcium may become toxic to the cell or use up releasable vesicles. The blockage of calcium uptake by mitochondria limits the amount of intracellular calcium storage space, and as calcium continues to enter the cell upon depolarization

and the opening of voltage-gated channels, the calcium concentration inside the cell continues to grow. A greater number of vesicles are released to a certain extent, but soon the supply of releasable vesicles diminishes and cannot provide enough for the greater amount of calcium available to activate them. With the calcium concentration reaching toxic or overwhelming levels, the cell reduces its functioning, including synaptic transmission, resulting in a decreased EPSP.

Another explanation may be that the increased intracellular concentration of calcium becomes large enough to affect the immense gradient that exists across the membrane. With intracellular and extracellular concentrations more similar in value, the gradient is weaker, causing less calcium to flow into the cell than normal and diminishing how much synaptotagmin is activated to spur neurotransmitter release. Bernath & Vizi (1986) theorized the same possibility for the inhibitory effect of ruthenium red on neurotransmitter release. However, Bernath and Vizi (1986) also speculated that the increase in intracellular calcium by ruthenium red may affect membrane excitability and K-selective channels, which increases the membrane's K^+ permeability. Increasing K^+ permeability hyperpolarizes the cell, resulting in less calcium influx. Several possibilities can explain the decrease in amplitude over time from ruthenium red's disruption of calcium homeostasis inside the cell, but we cannot conclude which mechanism specifically depresses synaptic transmission based on the results of our experiment.

Regardless, after tests where EPSPs decreased significantly, a washout with normal Ringer's solution revived the synaptic strength of the muscles under testing. However, all of our tests added only 100 μL of 12.7μM of ruthenium red. It is possible that increasing the concentration of the chemical may produce stronger, more evident inhibitory effects. Additionally, Ru360, a chemical similar to ruthenium red, may show stronger or irreversible inhibition. Only further testing can determine these possibilities.

Our data does not specifically identify what the disruption by ruthenium red ultimately causes in the cell. We named several possibilities for what may have occurred, but our results cannot pinpoint a specific cause for synaptic depression. Our results simply identify that the application of ruthenium red depresses synaptic transmission and that this depression can be reversed. Future research may extend inquiry into what the increase in intracellular calcium caused by ruthenium red ultimately causes in the cell to spur synaptic depression.

ACKNOWLEDGEMENTS

We thank Professor Clark Lindgren for first introducing us to neuroscience concepts such as action potentials and EPSPs, which inspired us to investigate deeper questions in respect to synaptic transmission and the role of calcium in it. He also taught us how to prepare and utilize the tools in lab and helped problem-solve any issues that occurred during our experiment. We also thank our lab instructor Ashley Wolterstorff for obtaining and preparing our chemicals for experiments and working with us daily in lab so that our experiments had the proper chemicals. Additionally, we also thank our class mentor Emily Kozik for answering content and lab-related questions over the course of our research.

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Pioneering Neuroscience, 2019, **18**, 39-44

Preliminary evidence that riluzole protects against glutamate-induced excitotoxicity at the crayfish neuromuscular junction

ATHENA FRASCA, SAKURA ISHIZAKI, and LILY KIRSCH
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

We investigated whether the drug riluzole, a glutamate antagonist, protects against glutamate-induced excitotoxicity at the crayfish neuromuscular junction. We measured the excitatory postsynaptic potential (EPSP) amplitudes of crayfish abdominal extensor muscles with intracellular microelectrodes while stimulating the motor neuron through suction micropipettes. We compared the EPSP amplitudes of crayfish muscle cells exposed to glutamate only with those exposed to both riluzole and glutamate, using EPSP amplitude as an indicator of the level of excitotoxicity. We did not observe a significant difference in percent change of EPSP amplitude between trials conducted with 10mM glutamate only and both 10mM glutamate and 50µM riluzole; however, we believe that this is because 10mM glutamate was insufficient to induce excitotoxicity. We observed a loss of EPSPs across three trials soon after the application of 20mM glutamate, which suggested that 20mM glutamate induced excitotoxicity. In trials with 20mM glutamate and 50µM riluzole we did not lose the EPSP and observed a strong EPSP amplitude for the duration of the trial, suggesting that riluzole likely protected against glutamate-induced excitotoxicity.

INTRODUCTION

Riluzole, a chemical with the formula $C_8H_5F_3N_2OS$, is the first drug to be approved by the Food and Drug Administration (FDA) for the treatment of the neurological disease amyotrophic lateral sclerosis (ALS) (Dharmadasa & Kiernan, 2018). While the exact mechanism of riluzole's ability to treat ALS is unconfirmed, one of the proposed pathways is that it is able to reduce excitotoxicity. Excitotoxicity is a type of cell damage and death brought on by the overactivation of glutamate receptors (Dharmadasa & Kiernan, 2018).

Riluzole may extend the amount of time ALS patients can live without requiring a respirator once they have been diagnosed (Lacomblez *et al*, 1996). However, researchers are still uncertain whether or not riluzole prolongs life (Logroscino *et al*, 2007). Currently, there are no adequate treatments for ALS. The cause of the disease is unknown; however, one hypothesized mechanism is excitotoxicity. Glutamate, the primary excitatory neurotransmitter in the central nervous system, causes excitotoxicity. Excitotoxicity occurs when glutamate accumulates at high concentrations. Cells responsive to glutamate become overstimulated and allows toxic concentrations of calcium ions into the cell. The resulting nerve damage and death can be indicated by diminished and disappearing electric post-synaptic potentials, or EPSPs (Van Den Bosch *et al*, 2006). The first reason researchers suspect that excitotoxicity plays a role in ALS is because motor neurons, which are affected by ALS, are especially sensitive to excitotoxicity. The second reason is because the only drug shown to slow ALS is riluzole, which has a primary effect of working against excitotoxicity (Van Den Bosch *et al*, 2006).

The aim of this experiment is to explore the mechanisms of action of riluzole. The current mechanisms thought to be relevant to riluzole's ability to block excitotoxicity are that it increases astrocytic uptake of glutamate in the central nervous system (CNS) and that it inhibits glutamate release pre-synaptically (Pittenger *et al*, 2008). Because we are using exogenous glutamate, inhibition of glutamate release cannot explain the effects we see. We are experimenting on the crayfish neuromuscular system, meaning our EPSP measurements will come from the peripheral nervous system (PNS) rather than the CNS. Crayfish have glial cells which are similar to astrocytes in the CNS, and which riluzole may act on to increase glutamate uptake. This experimental design will demonstrate that riluzole's protective effects are not only relevant in the CNS, but in the PNS as well.

We did not observe a significant difference in percent change of EPSP amplitude between trials conducted with 10mM glutamate only and both 10mM glutamate and 50µM riluzole. However, once we applied 20mM glutamate, we observed a complete loss of EPSP in all trials. When we conducted trials using 20mM glutamate after exposure to 5µM riluzole, we observed strong EPSP amplitudes for the duration of the trials. This suggests that riluzole protected against glutamate-induced excitotoxicity.

MATERIALS AND METHODS

Preparation

We prepared each preparation by cutting the tail off a crayfish that was submerged in ice water for at least 20 minutes and separating the ventral surface of the tail from the rest of the abdomen. We removed the main muscles from the abdomen until two strands of extensor muscles attached to the dorsal exoskeleton were left. We then placed the preparation on a dish with Sylgard, pinned it on three corners, and covered it immediately with crayfish Ringer's solution.

We used five solutions: normal crayfish Ringer's solution, normal crayfish Ringer's solution mixed with 50µM riluzole, normal crayfish Ringer's solution mixed with 10mM glutamate, normal crayfish Ringer's solution mixed with 20mM glutamate, and normal crayfish Ringer's solution mixed with 50µM riluzole and 20mM glutamate. The normal crayfish Ringer's solution was comprised of 202.4mM NaCl, 2.6mM $MgCl_2.6H_2O$, 10mM HEPES, 13.5 mM $CaCl_2.2\ H_2O$, and a pH of 7.4. We made the solution containing 50µM riluzole, the solutions containing 10mM and 20mM glutamate respectively, and the solution containing both 50µM riluzole and 20mM glutamate by dissolving the substances in the Ringer's solution before pouring each solution into their respective dishes.

We exchanged solutions using a Simultaneous Liquid Uptake and Replacement Pump (SLURP) and left the recording microelectrode in the same muscle cell while switching solutions. We left the stimulus running throughout the solution swap in order to monitor any change in EPSP amplitude.

Electrodes

We measured EPSP amplitudes using a microelectrode and an amplifier. We used glass capillary tubes and created the electrode tips using a device that heats the glass capillaries and pulls each end, yielding two micropipettes. We injected 3M of KCl solution into the micropipettes, creating micropipettes with a resistance between 4MΩ and 25MΩ.

Experiment and Recording

To carry out our measurements, we sucked the nerve of the extensor muscles into a suction electrode and inserted a microelectrode into a muscle cell near the nerve. We stimulated the nerve every two seconds using a GRASS SD9 Stimulator and recorded the resulting EPSPs from the muscle cell using an amplifier.

In all trials, we first measured EPSP amplitudes in the normal crayfish Ringer's solution. We then switched the solution using the SLURP. In the glutamate conditions, we switched from Ringer's solution to the solution comprised of only Ringer's and either 10mM or 20 mM glutamate, performing three trials for each concentration. We continued to record EPSPs continuously until 15 minutes after the switch. We did not use riluzole in these trials in order to examine the effect of glutamate itself on the muscle cells. In the riluzole conditions, we first switched from the Ringer's solution to a solution containing Ringer's solution and 50µM riluzole. We continued recording for 10 minutes so that the riluzole had time to affect the nerve and muscle cells. Then we exchanged the solution again, switching to the solution comprised of Ringer's solution and either 10mM or 20 mM glutamate for four trials and one trial respectively. Finally, in one trial, we applied 50µM riluzole and Ringer's solution to the crayfish for 10 minutes, and then applied 20mM glutamate and 50µM riluzole with Ringer's solution simultaneously in order to test the direct effect of riluzole on glutamate.

We continued to stimulate the nerve and record the EPSP amplitudes once every two seconds for at least 15 minutes after the final switches.

Data analysis

Across trials different crayfish preparations had widely varying EPSP amplitudes in Ringer's solution. In order to compare results across these preparations we calculated percent change in EPSP amplitude, which is a more standardized measure. The formula for percent change is:

$$(EPSP_{measured} - EPSP_{initial})/EPSP_{initial} \times 100$$

In this formula, $EPSP_{initial}$ is the first amplitude measured after glutamate was added and $EPSP_{measured}$ is the amplitude at a given time after the addition of glutamate.

RESULTS

To investigate whether riluzole reduces the excitotoxicity induced by excessive glutamate at the crayfish neuromuscular junction, we conducted experiments on a crayfish preparation in baseline and experimental conditions. In the baseline condition, we submerged the crayfish in 10mM glutamate and 20mM glutamate for three trials of each concentration. By adding only glutamate in these trials, we intended to induce excitotoxicity in the crayfish preparation and determine a baseline of what EPSP amplitudes were like when excitotoxicity was induced. In the experimental condition, we first submerged the crayfish in a solution with 50µM riluzole and Ringer's solution

and then changed the solution to one containing either 10mM glutamate or 20mM glutamate, conducting four trials and one trial respectively with each concentration. These trials were meant to test whether exposure to riluzole protected the neuromuscular cells from excitotoxicity. If we found that the amplitudes of the EPSPs in this condition decreased less than they did in the condition with glutamate only, then we would conclude that riluzole reduces excitotoxicity in crayfish neuromuscular cells. In one additional trial, we applied 50µM riluzole and Ringer's solution to the crayfish for 10 minutes, and then applied 20mM glutamate and 50µM riluzole with Ringer's solution simultaneously. We performed this trial in order to test whether the effect of riluzole on excitotoxicity would be different if the preparation were still being directly exposed to riluzole than if it had only been exposed to riluzole previously. However, since we only performed one of these trials, we did not get enough data to make any conclusions about that question.

During the three trials of 10mM glutamate we conducted, we saw a clear change in EPSP amplitude by 100 seconds as shown in Figure 1, though we did not see the same changes in every trial. As shown in Figure 2 we also saw clear changes in EPSP amplitude in the 100 seconds after adding glutamate in the three trials where the preparation had been treated with 50µM riluzole prior to the switch to 10 mM glutamate, although again, the responses over that time were varied. We interpreted these changes to mean that the glutamate took effect during this time. Because of this we chose to analyze the percent change in EPSP amplitude obtained between 90 seconds and 110 seconds for both the conditions. To do this, we averaged the percent change at all points in the 90-110 second time interval for each trial, then found the mean of those averages across the three trials in each condition.

As shown in Figure 3, 10mM glutamate changed the EPSP amplitude by an average of -20.3%. However, when 50µM riluzole was added to the solution, the EPSP amplitude changed by -30.9%.
Results from a two-tailed independent groups t-test of the difference between the average percent change in EPSP amplitude for 10mM glutamate only and the average percent change in EPSP amplitude for 10mM glutamate after riluzole showed that there is no statistically significant difference between the two conditions (t=0.4432, p= 0.6805, df=4). The 95% confidence interval of the difference indicated that the actual percent change in EPSP for 10mM glutamate after riluzole is between 55.71 percentage points lower and 76.869 percentage points higher than the actual percent change in EPSP for 10 mM glutamate only.

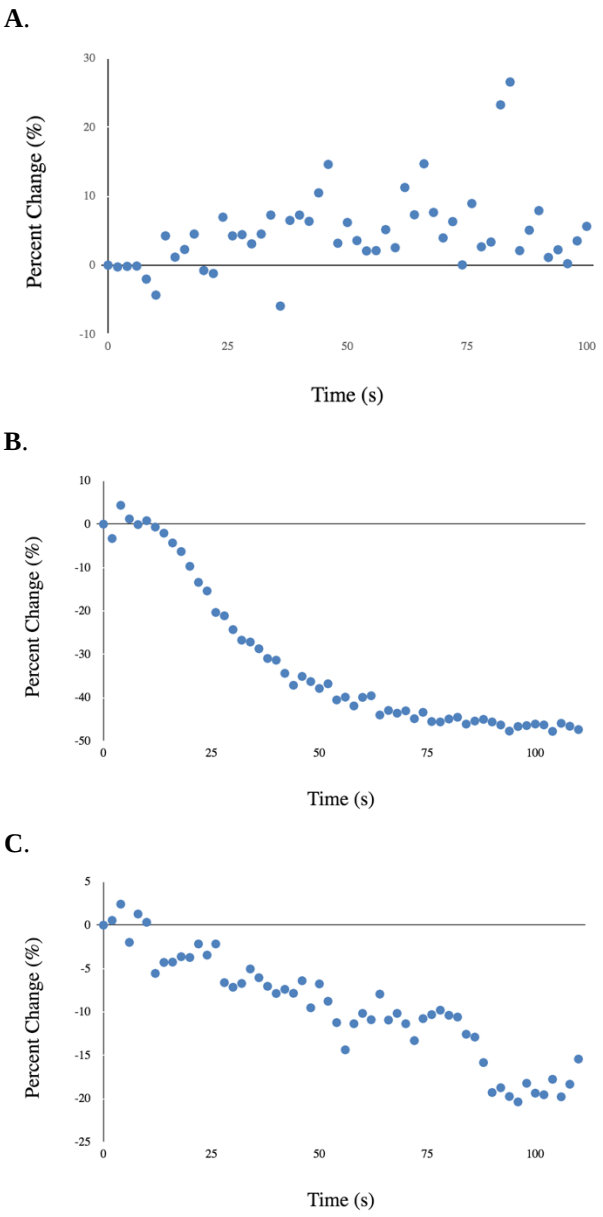


Figure 1. Percent change in EPSP amplitude across time for the first 100 seconds after 10 mM glutamate was added to a crayfish preparation that had previously been in a normal crayfish ringer's solution. A, B, and C each represent data from a separate trial.

This means that we did not find any evidence in these trials that riluzole protected the cells from excitotoxicity, especially since the difference we did find, though it was not significant, was in the opposite direction than we would expect if riluzole were protecting the cells-- the EPSPs decreased more in the preparations treated with riluzole than in the preparations that were not.

We suspected that the reason we did not see a protective effect of riluzole was that the 10 mM concentration of glutamate was not enough to induce

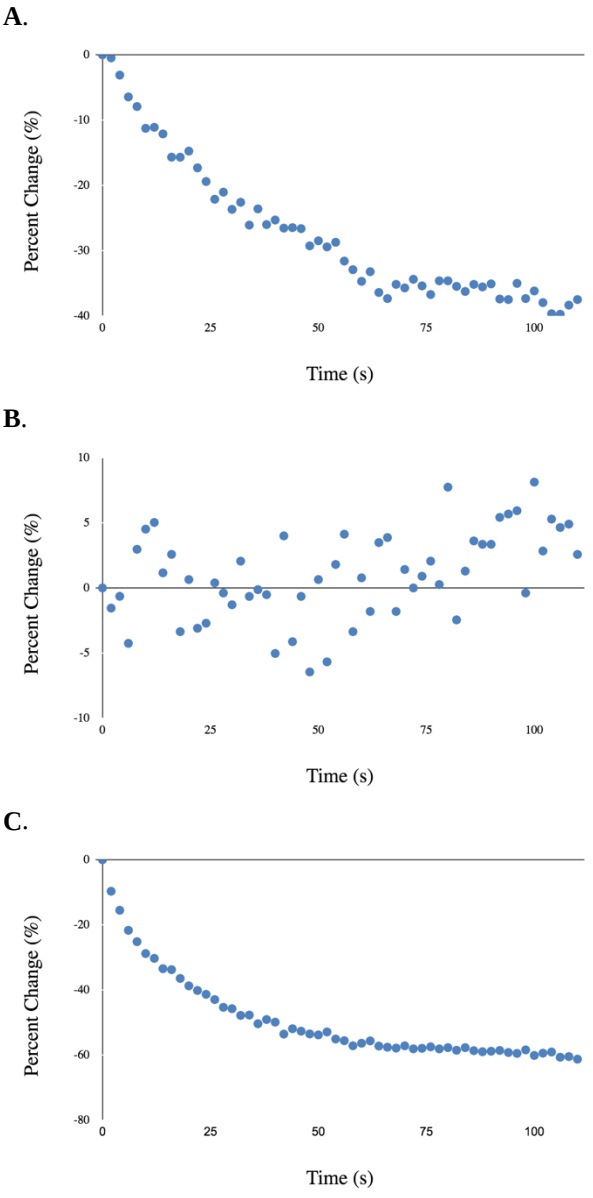


Figure 2. Percent change in EPSP amplitude across time for the first 100 seconds after 10 mM glutamate was added to a crayfish preparation that had previously been in a 50 µM riluzole solution. A, B, and C each represent data from a separate trial. One trial was excluded due to a large, sudden jump in EPSP during the 0-100 second period which indicated that the motor axons were not being consistently activated.

excitotoxicity, since the reduction we saw in EPSP amplitude for trials with 10 mM glutamate and no riluzole was not dramatic. The mean of percent change between 90 and 110 seconds in these trials indicates that the EPSPs retained 79.7% of their original amplitude on average, which is not drastically smaller. Because of this, we conducted three more glutamate only trials using a higher concentration of glutamate, 20mM rather than 10 mM. In all three trials with 20mM glutamate, we lost the EPSP immediately after the

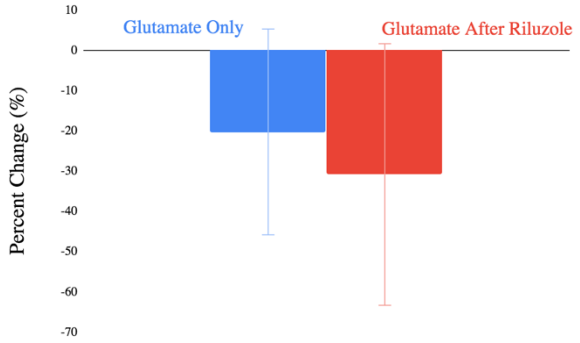


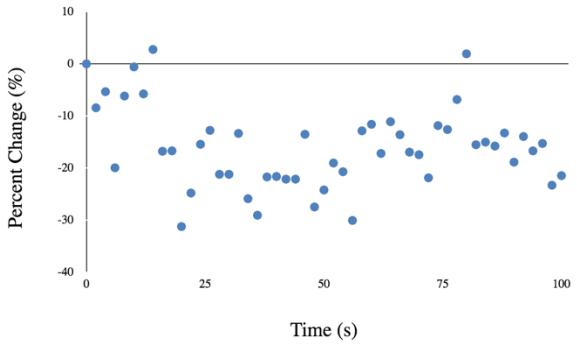
Figure 3. Average percentage change in EPSP amplitude for 10mM glutamate only and 10mM glutamate and 50µM riluzole between 90 and 110 seconds after the addition of each solution. N=3 for both conditions. The percent change in EPSP amplitude between the value immediately after 10mM glutamate was added and the value at each individual point was averaged for all points during the 90-110 seconds time interval for each of the three trials in both conditions. Error bars indicate one standard deviation from the mean. One trial was excluded from the glutamate after riluzole condition because of large, sudden jumps in EPSP during the 90-110 second time interval.

addition of glutamate. Because we lost the EPSP and were unable to recover it, our results from these trials are inconclusive. However, because this loss of EPSP occurred across all of our trials of 20 mM glutamate and in none of the trials in any other experimental conditions, we believe that this effect was not due to experimental error but rather the effect of the glutamate itself. Because of this we believe that we successfully induced excitotoxicity with 20mM glutamate very quickly after its application.

To test whether riluzole would protect the neuromuscular cells from excitotoxicity with this 20mM concentration of glutamate, we conducted two more trials. One trial was conducted with a 20 mM glutamate solution after the crayfish was exposed to 50µM riluzole for 10 minutes. The other was conducted with a solution containing both 20mM glutamate and 50µM riluzole after the crayfish was exposed to a solution containing only 50µM riluzole for 10 minutes. We did not lose the EPSP during either of these trials, and consistently observed a strong EPSP amplitude for the duration of the trials. As shown in Figure 4, the EPSPs stayed within at least 50% of the initial amplitude for every measurement in the first 100 seconds of these trials. In the first trial the electrode briefly came out of the preparation after the 102 second mark, making the data from 102 to 110 seconds unusable, but between 90 and 102 seconds after the glutamate solution was added there was an average decrease in amplitude of only 17.99 percentage points. In the second trial there was an average decrease of 4.64 percentage points in the 90-110 second time interval. This is very different from what we found in the 20 mM glutamate only trials, where the loss of

EPSP can be interpreted as a 100% average decrease in EPSP amplitude. This indicates that in these trials riluzole did have a protective effect.

A.



B.

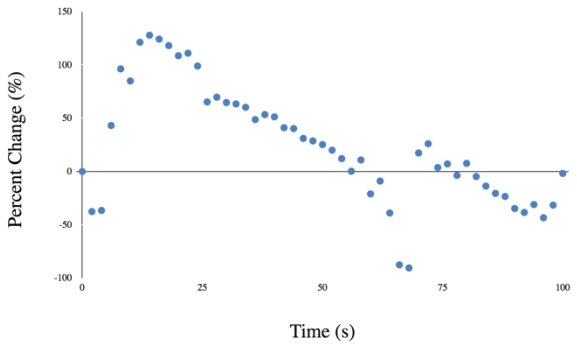


Figure 4. Percent change in EPSP amplitude across time for the first 100 seconds after a crayfish preparation that had previously been in a 50 µM riluzole solution was switched to **A)** a solution with 20 mM glutamate and no riluzole and **B)** a solution with 20 mM glutamate and 50 µM riluzole. In **B**, the two points at 66 and 68 seconds which are close to -100% change are indicative of the electrode popping out of the cell briefly. The electrode was tapped back into the cell after those two points.

DISCUSSION

We hypothesized that riluzole would reduce the excitotoxicity induced by excessive glutamate in crayfish muscle cells. However, our hypothesis was not supported by the main set of trials we did with 50µM riluzole and 10mM glutamate. An independent sample t-test showed that the difference between the average percentage change of EPSP amplitude for 10mM glutamate only and 10mM glutamate and riluzole was insignificant. There are two possible explanations for this result. The first is that riluzole does not reduce excitotoxicity induced by excessive glutamate in crayfish. However, we believe that a second explanation, that 10 mM glutamate was not sufficient to induce excitotoxicity, is more likely. There are two main reasons for this conclusion.

First, when we did additional experiments where we used 20mM glutamate instead of 10mM glutamate, the EPSPs disappeared completely a short time after initial exposure to glutamate. As shown in Figure 3, this was not the case for 10 mM glutamate; 10mM glutamate changed the EPSP amplitude by an average of -20.3% after between 90 and 110 seconds of exposure to glutamate, leaving EPSPs which still maintained about 79.7% of the initial EPSP amplitude. In contrast, by 90 seconds after exposure to 20 mM glutamate the EPSPs had disappeared completely. This disparity in EPSP response indicates completely different processes occurring between the two concentrations. The process which is the most likely explanation for this difference is excitotoxicity, which would have occurred in the 20mM glutamate condition but not the 10mM glutamate condition.

Second, when we conducted trials with both 20mM glutamate and riluzole, EPSPs remained stable and did not disappear like they did when the preparation was treated with only 20mM glutamate. In fact, the EPSPs in these trials decreased less on average than the EPSPs in trials with 10mM glutamate and riluzole. This reinforces the idea that the disappearance of the EPSPs after exposure to 20mM glutamate was caused by excitotoxicity, because riluzole, which is known to protect cells from excitotoxicity in other contexts, seems to have prevented that disappearance. This also provides preliminary evidence that the protective effects of riluzole against excitotoxicity do in fact hold true for the crayfish neuromuscular junctions.

Furthermore, there were a number of limitations to our research. As our experiment monitored the progression of the change in EPSP amplitude continuously in different solutions, we could not take the average when the experiment was running. Thus, when the microelectrode moved out from the muscle cell, we obtained a different reading, which made it hard to analyze the actual effect of riluzole and glutamate on the muscle cells.

We also had difficulty recording the exact moment the glutamate was added, since the solution switching process took varying amounts of time across trials. Therefore, the specific definition of the time marked as the end of the glutamate addition process varied; glutamate may have been in contact with the crayfish longer for some trials than others before the time marked as the beginning, making the data less consistent.

Current research suggests that there are a few mechanisms by which riluzole reduces excitotoxicity. Firstly, riluzole reduces excitotoxicity by inactivating Kv1.4 potassium channels (Xu, Enyeart, & Enyeart, 2001). Kv1.4 potassium channels are distributed in the axons and nerve terminals of the brain and are hypothesized to release glutamate (Xu, Enyeart, &

Enyeart, 2001). The inactivation of Kv1.4 potassium channels by riluzole is likely one mechanism by which riluzole inhibits glutamate release. The other major mechanism shown to be responsible for riluzole's anti-excitotoxic effect is astrocytic reuptake in the brain and spinal cord, a process in which astrocytes, the CNS's glial cells, absorb extracellular glutamate (Pittenger *et al.*, 2008).

While these mechanisms focus heavily on presynaptic effects of riluzole and effects in the CNS, our research focused on the postsynaptic effect of riluzole in the PNS, which is an area that has not been explored as fully. In future research, we hope to further explore the mechanism by which riluzole acts on postsynaptic neurons to reduce excitotoxicity induced by excessive glutamate. One potential next step is to isolate crayfish glial cells to test their viability as a mechanism for riluzole's effect by measuring their response to glutamate with and without riluzole. Future research should also involve performing more trials using 20 mM glutamate in order to get a large enough sample to find statistical significance and to confirm that our findings were not due to experimental error. Additionally, future trials should test various glutamate concentrations between 10 and 20 mM to more specifically determine the threshold for inducing excitotoxicity.

ACKNOWLEDGEMENTS

We thank Dr. Clark Lindgren, Emily Kozik, and Ashley Wolterstorff for their time, help, lab space, and constant positivity. We thank our Bio 150-02 classmates for good music and dedicated, fun lab time.

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