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.

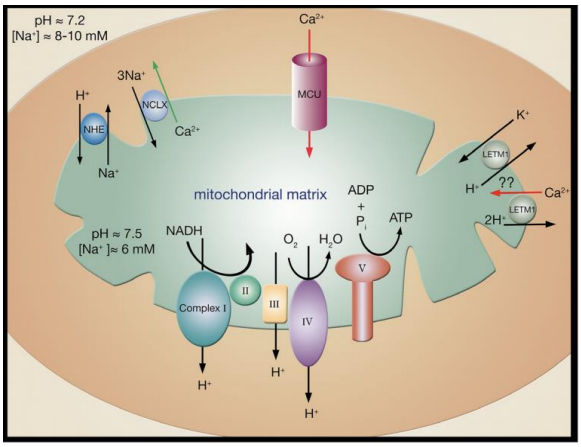

Figure 1. Mitochondrial Ion Transport Mechanisms (Finkel et al., 2015). Calcium enters through mitochondrial calcium uniporter (MCU). Calcium uptake enhances ATP production and regulates the internal calcium concentration. By blocking MCU with ruthenium red, the intracellular calcium concentration increases 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).

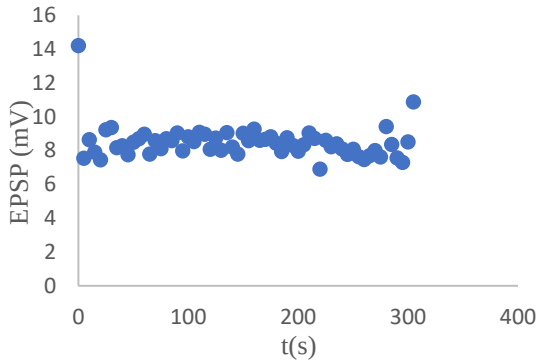

Figure 2. The baseline reading of EPSPs under low frequency stimulation. n=1.

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.

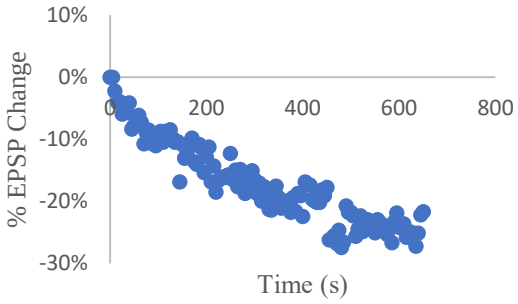

Figure 3. The effect of ruthenium red on EPSPs during low frequency stimulation. Ruthenium red was applied at t=-30. n=1.

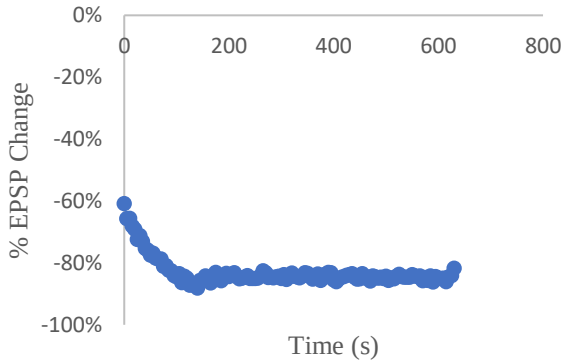

Figure 4. The maximum inhibitory effect of ruthenium red on EPSPs under low frequency stimulation. Ruthenium red was applied at t=-30. n=1.

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.

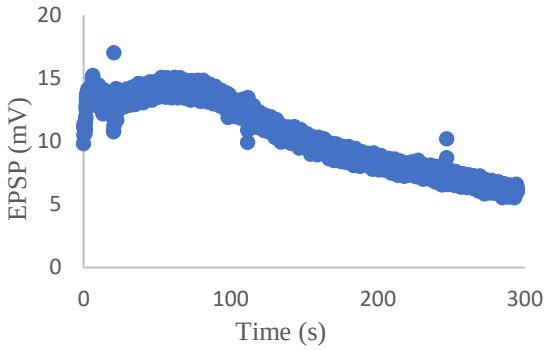

Figure 5. The baseline reading of EPSP during high frequency stimulation. No ruthenium red was added. n=1.

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.

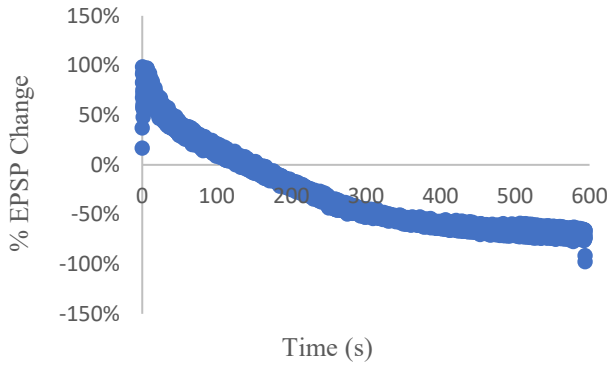

Figure 6. The inhibitory effect of ruthenium red on EPSPs under high frequency stimulation. Ruthenium red was applied at t=-30. n=1.

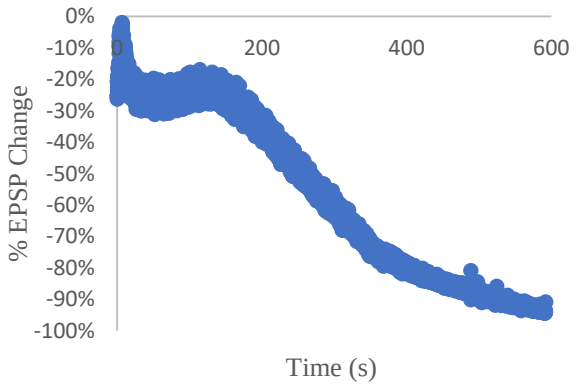

Figure 7. The inhibitory effect of ruthenium red on EPSPs under high frequency stimulation (continued). Ruthenium red was applied at t=-30. n=1.

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.

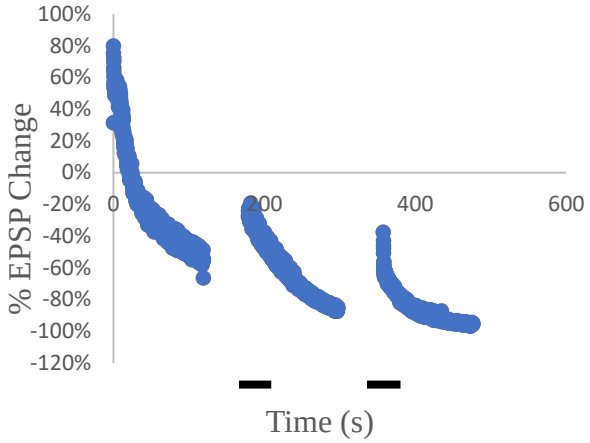

Figure 8. The inhibitory effect of ruthenium red during high frequency stimulation intervals. The black bars represent a 1-minute period of no stimulation. n=1.

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.

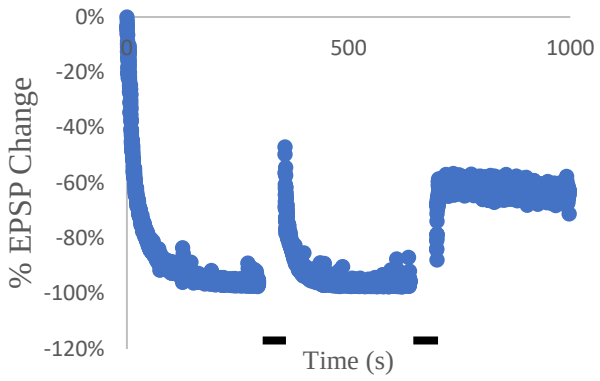

Figure 9. The removal of ruthenium red increases EPSP. The black bars indicate 1-minute breaks from stimulation. The first interval contains no chemicals. The second interval includes both 4-AP and ruthenium red. The last interval includes only 4-AP. n=1.

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.

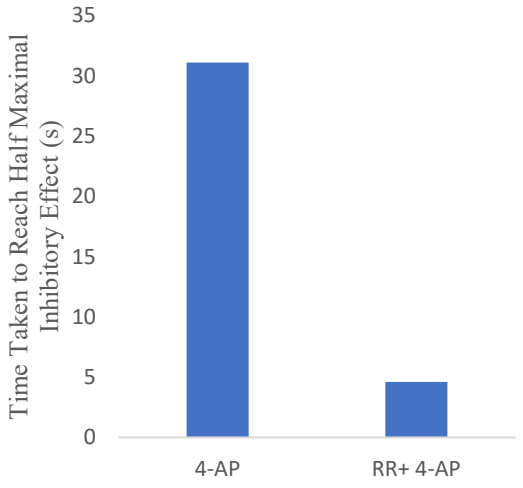

Figure 10. The time taken in seconds to reach half of the maximum inhibitory effect.

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.

REFERENCES

Bernath, Sandor & Vizi, E.S. (1986). Inhibitory effect of ionized free intracellular calcium enhanced by ruthenium red and *m*-chloro-carbonylcyanide phenyl hydrazon on the evoked response of acetylcholine. *Biochemical Pharmacology*, ***36*** (21): 3683-3687.

Finkel, T., Menazza, S., Holmstrom, K. M., Parks, R. J., Liu, J., Sun, J., Liu, J., Pan, X., & Murphy, E. (2015). The ins and outs of mitochondrial calcium. *Circulation Research*, ***116***: 1810-1819.

Kim, E., Owen, B., Holmes, W.R., & Grover, L.M. (2012). Decreased afferent excitability contributes to synaptic depression during high-frequency stimulation in hippocampal area CA1. *Journal of Neurophysiology*. ***108***(7): 1965-1976.

Lee, A., Hirabayashi, Y., Kwon, S. K., Lewis, T. L., Jr, & Polleux, F. (2018). Emerging roles of mitochondria in synaptic transmission and neurodegeneration. *Current Opinion in Physiology*, ***3***: 82–93.

Liao, Y., Dong, Y., & Cheng, J. (2017). The Function of the Mitochondrial Calcium Uniporter in Neurodegenerative Disorders. *International Journal of Molecular Sciences*, ***18***(2): 248.

Santo-Domingo, J., & Demaurex, N. (2010). Calcium uptake mechanisms of mitochondria. *Biochimica Et Biophysica Acta (BBA) - Bioenergetics*, ***1797***(6-7): 907–912.

Streit, J., Luscher, C., & Luscher, H.R. (1992). Depression of postsynaptic potentials by high-frequency stimulation in embryonic motoneurons grown in spinal cord slice cultures. *Journal of Neurophysiology*, ***68***(5): 1793, 1803.

Taipale, H. T., Kauppinen, R. A., & Komulainen, H. (1988). Ruthenium red prevents the voltage-gated increase in cytosolic free calcium in cortical synaptosomes from guinea-pig. *Biochemical Pharmacology, **38**: 1109-1113.*

Tong, J. J. (2007). Mitochondrial delivery is essential for synaptic potentiation. *The Biological Bulletin, **212***(2): 169-75.

Ying, W., Emerson, J., Clarke, M. J., & Sanadi, D. R. (1991). Inhibition of mitochondrial calcium ion transport by an oxo-bridged dinuclear ruthenium ammine complex. *Biochemistry, **30***: 4949-4952.