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

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

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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.

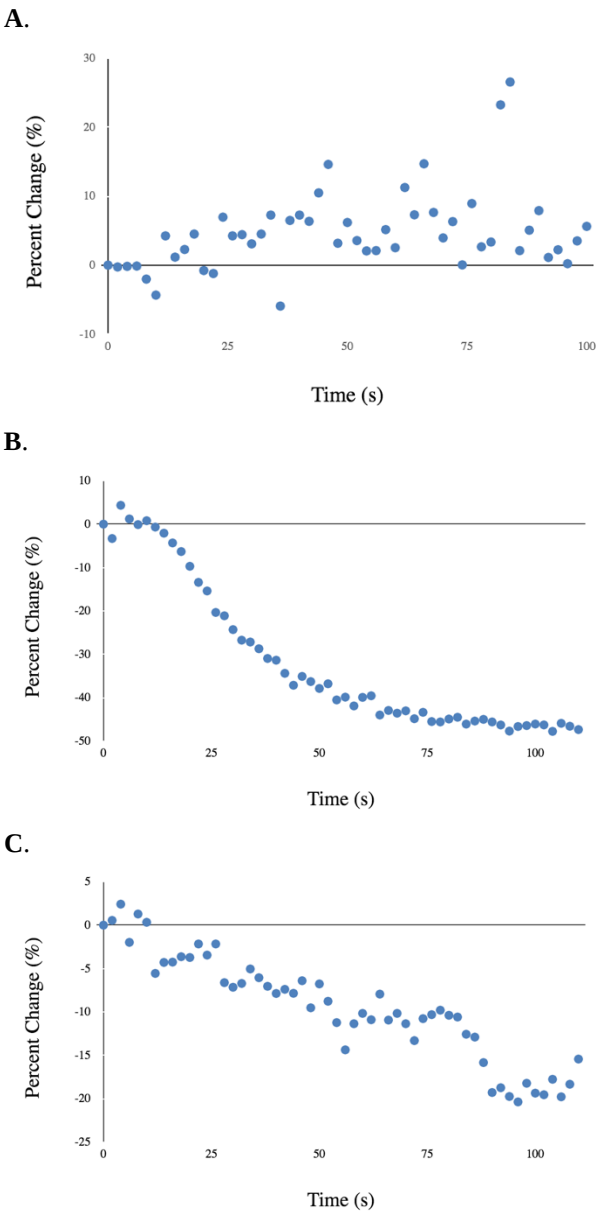


Figure 1. Percent change in EPSP amplitude across time for the first 100 seconds after 10 mM glutamate was added to a crayfish preparation that had previously been in a normal crayfish ringer's solution. A, B, and C each represent data from a separate trial.

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

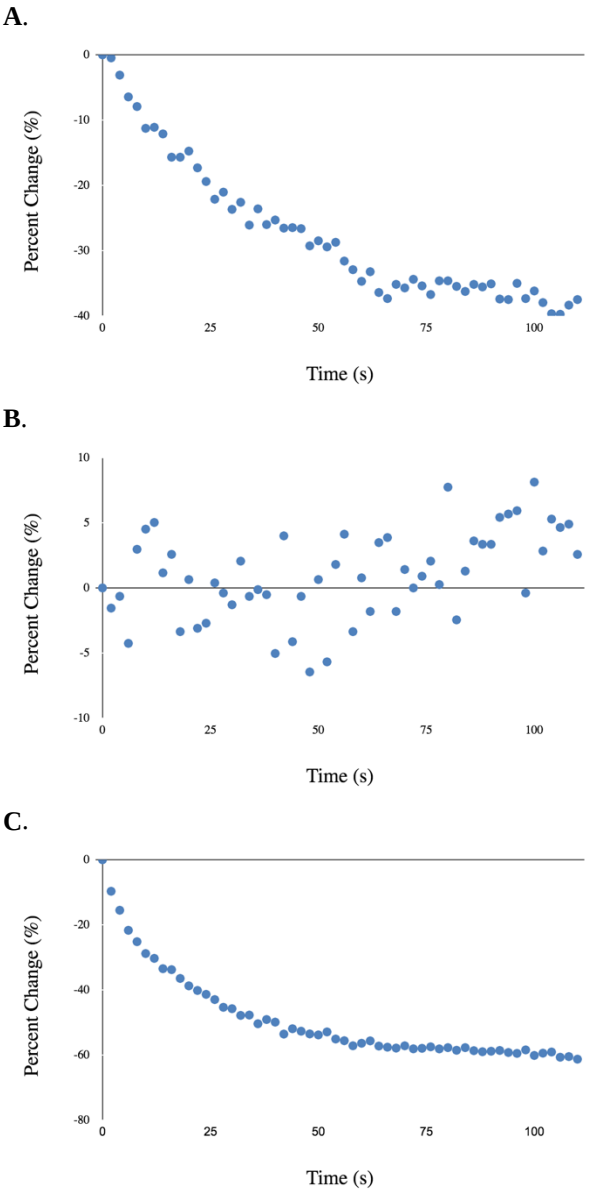


Figure 2. Percent change in EPSP amplitude across time for the first 100 seconds after 10 mM glutamate was added to a crayfish preparation that had previously been in a 50 µM riluzole solution. A, B, and C each represent data from a separate trial. One trial was excluded due to a large, sudden jump in 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

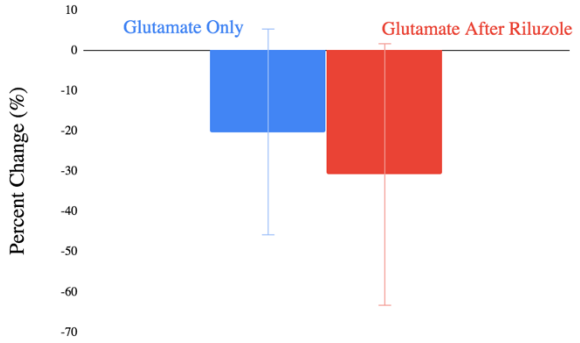


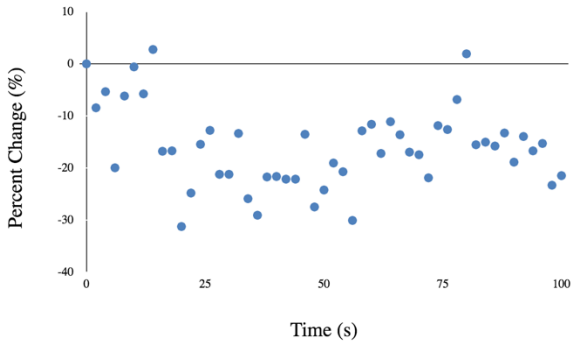
Figure 3. Average percentage change in EPSP amplitude for 10mM glutamate only and 10mM glutamate and 50µM riluzole between 90 and 110 seconds after the addition of each solution. N=3 for both conditions. The percent change in EPSP amplitude between the value immediately after 10mM glutamate was added 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.



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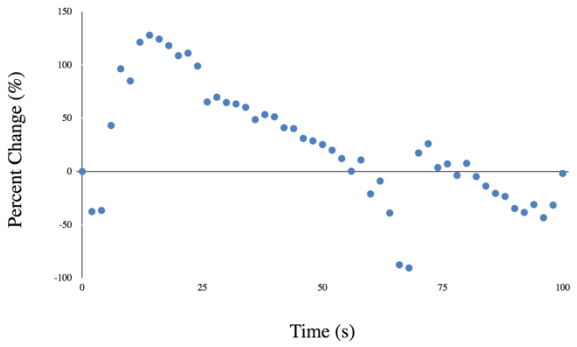

Figure 4. Percent change in EPSP amplitude across time for the first 100 seconds after a crayfish preparation that had previously been in a 50 µM riluzole solution was switched to **A)** a solution with 20 mM glutamate and no riluzole and **B)** a solution with 20 mM glutamate and 50 µM riluzole. In **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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