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# EPSP amplitudes at crayfish neuromuscular junctions are sensitive to methods of increasing glutamate concentrations in the synaptic cleft.

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## ABSTRACT

Glutamate is the primary excitatory neurotransmitter in crayfish, and theoretically, increasing glutamate concentrations in the synaptic cleft should have similar excitatory results regardless of the method causing the increase. To test this hypothesis, we conducted three different experimental trials using crayfish neuromuscular junctions: increasing glutamate by high frequency stimulation, submersion in 2mM glutamate bath, and submersion in a glutamate uptake inhibitor, 1-aminocyclobutane-trans-1, 3-dicarboxylic acid. We found that high frequency stimulation caused depression rather than excitation, addition of glutamate to the bath caused no change in EPSP amplitudes, and inhibition of glutamate uptake caused greater depolarization in the postsynaptic cell. The results carry significant implications regarding the sensitivity of glutamatergic synapses, and demonstrate that for healthy excitation, glutamate must be both present in the cleft and able to diffuse away from the cleft. The only trial resulting in an increase in EPSP amplitude was the uptake inhibitor trial, possibly because this was the only condition that met the sensitive needs of the glutamatergic synapse for excitation.

## INTRODUCTION

Glutamate is important to the development of excitatory post-synaptic potentials (EPSPs) across species. In humans, for example, high glutamate levels can cause the over-excitation of neurons characteristic of epilepsy, and changes in glutamate receptors contribute to cortical dysplasia, a common cause of epilepsy (Najm et al., 2000). However, the human nervous system is too complex to study glutamate interactions at the synaptic level. Because of its relative simplicity, the crayfish neuromuscular junction is a good model to study the role of glutamate. Kawagoe, Onodera and Takeuchi (1981) found that glutamate is released in crayfish neuromuscular junctions, and seemed to be the primary excitatory neurotransmitter in these synapses. They showed that iontophoretic application of glutamate to the synaptic area caused a depolarization, while acethylcholine (ACh), another possible excitatory neurotransmitter, did not. Iontophoretic application means that an electric charge pushes a specific amount of an ion, in this case glutamate, from a micropipette aimed at the synaptic cleft. Thus, it seems that glutamate levels also contribute to EPSPs in crayfish. Theoretically, if levels of glutamate in the synaptic cleft are increased, excitatory post-synaptic potentials will increase because more glutamate will be present in the cleft to activate more post-synaptic receptors. Thus, we hypothesized that any manipulation used to increase glutamate levels should have a similar effect on EPSP amplitudes, and this effect should be due to higher glutamate concentrations, not the method of increase. Our study will explore the excitatory capabilities of glutamate at crayfish neuromuscular junctions, using three different methods to increase glutamate levels.

Previous research has shown there are a number of ways to increase glutamate levels at synapses. First, Kawagoe, Ondera and Takeuchi (1981) reported that frequent stimulation of the presynaptic nerve causes greater amounts of

glutamate to be released by the neurons into the synaptic cleft. In this situation there are two variables which may influence the EPSP: the frequency of activation of the synapse and the glutamate level. To determine if results from this trial are due to glutamate levels, independent of the frequency of stimulation, we will have a first trial with high frequency electronic stimulation, and a second trial in which glutamate levels in the synaptic cleft are increased by the addition of glutamate to the bathing solution of the crayfish tissue. This technique of glutamate application varies from most previous research, in which glutamate was added via a pipette directly to the synaptic cleft, so our results may show different effects (Kawagoe, Onodera, Takeuchi, 1981; Dudel, Franke, Hatt, 1990) Comparing these trials will show the effects of electric stimulation versus submersion as methods of increasing glutamate. Third, we will inhibit glutamate uptake with 1-aminocyclobutane-trans-1, 3 dicarboxylic acid, a chemical shown to prevent glutamate uptake in mammals and birds (Fletcher et al, 1991; Lewin et al, 1996). This will cause glutamate to remain in the cleft longer, in greater quantities, by reducing its escape from the cleft via uptake into surrounding cells.

Our results failed to support the hypothesis; only the uptake inhibitor increased EPSP amplitude. This suggests the method of increasing glutamate does influence EPSP amplitude at the crayfish neuromuscular junction. This information should be helpful in future research dealing with glutamate in invertebrates.

## MATERIALS AND METHODS

*Preparation*

We made glass microelectrodes from micropipettes, using the PUL-1 microelectrode puller by World Precision Instruments. Each microelectrode was filled with a 3M KCL solution, and was plugged into an adapter filled with the same solution. The microelectrodes were tested in the saline solution, outside of a cell, to verify that their resistance fell between 5MΩ and 10MΩ. We mounted the microelectrodes on a micromanipulator to more accurately control their movement.

We received a chilled crayfish tail at the beginning of each trial and submerged the tail in a standard crayfish saline solution for 20 seconds prior to dissection. We first cut through the dorsal portion of the tail shell along the longitudinal indentations. We removed the dorsal side of the shell from the ventral by cutting the muscle tissues connecting the two, and then discarded the ventral portion. We used pins to fasten the dorsal region to the bottom of a petri dish lined with Sylgard.

We placed a stimulating electrode on one of the crayfish motor nerves and positioned the microelectrode into a cell in a medial muscle to the anterior of the nerve. An indifferent electrode was also placed in the crayfish saline to complete the electrical circuit. We used the Scope v3.3 computer program to measure and display resting potentials of the muscle fibers. After finding a resting potential, we administered electric stimuli to the nerve to elicit EPSPs. The stimulus shocks were delivered at 52V for .2mS, at 1.5Hz. We repeated this procedure to obtain five control EPSPs on each crayfish.

*Data Anaylsis*

After obtaining 15 control and 15 test EPSP measurements for each experimental condition, we examined the EPSPs and calculated the overall means for controls and tests of each experimental procedure by combining the data from all three crayfish (Fig. 1). We ran t-tests to compare the control and trial data. In addition, by subtracting the means of the control measurements from the means of the tests, we also obtained the mean differences in EPSP amplitude for each crayfish. We averaged these differences to obtain a mean difference for each experimental condition (Fig. 2). To determine if EPSP time course changed with amplitude, we normalized the control data to match the amplitude of the test data for one EPSP sample from a glutamate inhibited crayfish (Fig. 3).

## RESULTS

*High Frequency Stimulation*

Our first experimental procedure was to increase the frequency of the electrical stimuli to 20Hz to increase the amount of glutamate released into the synaptic cleft. We stimulated the presynaptic nerve cells for 20 seconds at 54V while observing the EPSPs on the computer as they were recorded. We then changed the frequency back to 2Hz immediately following the high frequency stimulation and adjusted the voltage until an EPSP was observed. This usually
occurred within 20 seconds. We repeated this procedure to obtain three test measurements. When we attempted to obtain further measurements and had to change the microelectrode, the crayfish muscle cells in the preparation died. To prevent this from happening in future trials, we added 10mL of fresh saline via pasteur pipettes and removed 10mL of used saline at three minute intervals. We repeated this experiment on two other crayfish, obtaining five control measurements for both, and eight and four test measurements respectively. The EPSP results do not support our hypothesis that high frequency stimulation increases glutamate levels in the cleft, thus increasing the size of the EPSPs due to glutamate's excitatory nature (control EPSP mean: 19.6 mV, test EPSP mean: 14.1 mV). We are unable to determine if glutamate levels increased due to a lack of precise instruments, and had been assuming that an increase in glutamate would reflect itself through the EPSP amplitude. However, we found a significant average decrease in EPSP size ($t=2.857$, $p=0.013$) following the stimulation, which is termed depression (Fig. 1). The difference between the control and test measurements was approximately a 5.4mV decrease following activation (Fig. 2).

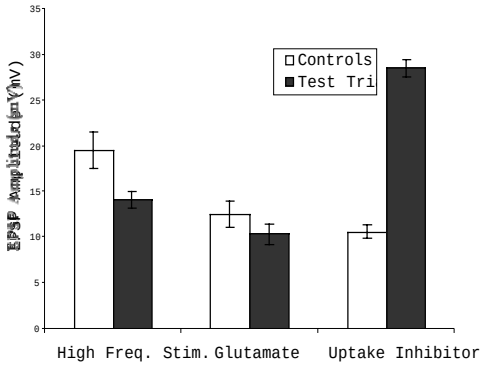


**Figure 1.** Mean control and test trial results for each experimental condition. The EPSP sizes are averages of three crayfish, except the glutamate test trial which was averaged from two crayfish. Each average was taken from five readings of EPSPs. Notice the shock trial shows a decrease in EPSP size; we propose this decrease resulted from depression. There was no change in the glutamate trial, presumably caused by desensitization. The uptake inhibitor trial showed a significant increase following the addition of the inhibitor to the saline bath. * $p<.05$ ** $p<.0001$

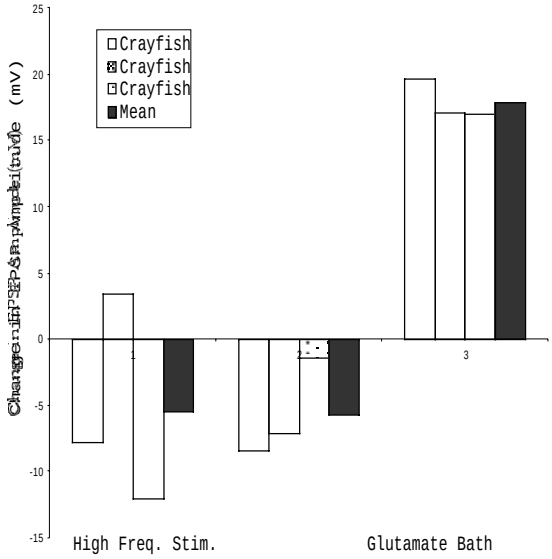


**Figure 2**. EPSP Sizes for crayfish 1 through 3 are the differences in EPSP size for the test measurements minus the control measurements. The averages show the average difference among the three crayfish for each trial, so the fourth bar is an average of the first three. This shows the uptake inhibitor is more consistent in the EPSP results among crayfish. It also shows the Shock Trial and Glutamate Trial had similar overall changes between control and test trials.

*Glutamate Addition*

For the second experimental condition, the addition of glutamate into the saline bath, we altered the bath to a 2mM glutamate concentration, assuming this increase would also produce an increase in EPSP size. Theoretically, the glutamate would have entered the cleft through diffusion and could mimic the actions of glutamate released from the pre-synaptic cell. We prepared 300mL of 2mM glutamate solution for each of three crayfish. We used pasteur pipettes to remove most of the standard crayfish saline from the petri dish, and then refilled the dish with the new solution. We repeated this procedure a second time to ensure enough standard saline had been removed to obtain the goal concentration of 2mM glutamate. Following the change in solution we moved the recording electrode back into a cell and stimulated at 2Hz, at 52V for .2mS to elicit EPSPs. The second preparation of cells died, but for crayfish 1 and 3 we obtained 5 controls and 10 and 5 test measurements respectively. The results from these crayfish preparations also failed to support our hypothesis (see Fig. 1). For this trial, the control measurements averaged 5.6mV higher than the test measurements, and there was no significant change between control and test (control EPSP mean: 12.6 mV, test EPSP mean: 10.4 mV) ($t=1.72$, $p=0.108$).

*Glutamate Uptake Inhibitor*

Our final procedure utilized 1-aminocyclobutane-trans-1, 3 dicarboxylic acid, a glutamate uptake inhibitor, and this chemical did produce results in support of the hypothesis. We prepared a 10mM inhibitor solution from 100ml inhibitor stock and 100 mL of saline, and removed the standard saline from the petri dish using pasteur pipettes. This solution was replaced with the 10 mM inhibitor solution. Our supply of the inhibitor was limited, so we could not flush the dish twice as in the glutamate trial. We waited for three minutes before taking EPSP measurements, and used 2Hz, 52V stimuli delivered for .2mS to stimulate the motor nerves. Throughout the trial we replaced two pipettes of the used solution with new solution every two minutes to ensure that the cells would remain alive and the inhibitor would remain active. When we began measuring EPSPs, we observed a marked increase in their size (mean EPSP control: 10.6 mV, test EPSP control: 28.6 mV). In fact, the average test results were 17.9mV greater than the average control measurements for this trial, which was a highly significant increase ($t=-23.86$, $p<.0001$). The duration of the EPSPs also increased (Fig. 3); the test EPSPs lasted an average of 7.7ms longer than the controls. We obtained this number by measuring the duration of the EPSPs on the normalized graph, using the place where the curves flattened out as the stopping points of the EPSPs.

## DISCUSSION

Only the glutamate uptake inhibition trial supported the original hypothesis that increased glutamate in the cleft causes larger EPSPs. However, a main part of the hypothesis was that each of the three trials would have the same effect on EPSP size, because theoretically, each of the conditions would cause similar increases of glutamate in the cleft. Since two of the three trials did not show results consistent with the hypothesis, we have evidence that the three methods of increasing glutamate actually had different effects on glutamate levels and excitation.

First we will examine the first experimental condition with increased frequency of electronic stimulation. Kawagoe, Onodera and Takeuchi (1981) reported that high frequency nerve stimulation increased levels of glutamate release in the crayfish neuromuscular junction. In 1983 they conducted a study with nine minutes of stimulation at 20Hz and found that glutamate levels inside the junction were significantly increased. However, the effects of this stimulation on their crayfish EPSP sizes were not reported. Our study, with 20Hz stimuli for 20 seconds, elicited a statistically significant depression in EPSP size, but we are unsure of the level of glutamate release. Since we had no method to measure glutamate levels in synaptic clefts, we assumed that glutamate levels could be inferred by the size of EPSPs, as glutamate is a primary

excitatory neurotransmitter in crayfish. But there seems to be a flaw in this planning, because long trains of high frequency stimulation cause depression, whether or not glutamate levels are increased. We think the reason our stimulation caused depression was the length of the high frequency stimulation period. Our 20 seconds was significantly shorter than the nine minutes of the previous experiment, so the shocking may not have even led to an increased release of glutamate. Also, a study by Alford, Zompa and Dubuc (1995) elicited potentiation in lamprey with 50 shocks in one second. Potentiation is caused by a short train of high frequency stimulation, and our 20 seconds was too long to lead to this growth in EPSP size. Therefore, the combination of the length of time of high frequency stimulation and our inability to measure glutamate release explains why our results differ from the hypothesized results and from previous research on high frequency shocking at the crayfish neuromuscular junction.

Our trial with submersion of the crayfish in 2mM glutamate solution also failed to support the hypothesis. The second preparation of cells died

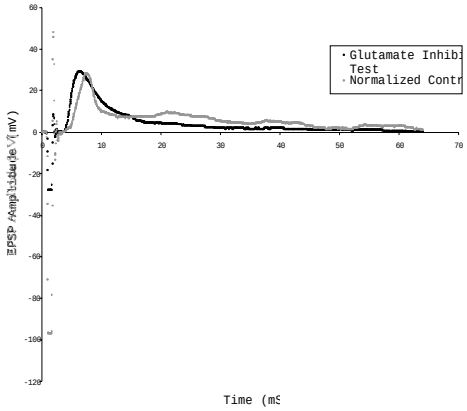


**Figure 3.** The Glutamate Inhibitor Test shows the amplitude of a sample test EPSP from crayfish 2 in the inhibitor trial. The normalized control shows a control EPSP normalized so its highest point is equal to the test trial's highest point. This allows us to see what the duration of the control EPSP would be had its amplitude been as large as the test. We see that the duration of the test EPSP was longer than the control, approximately 7.7 ms longer. This is logical due to the increased presence of glutamate at the synaptic cleft resulting from the inhibitor.

after the glutamate was added to the bath, and the other two crayfish showed no change after the addition of glutamate. We suspect the glutamate caused desensitization of glutamate post-synaptic receptors, and thus did not allow enhanced EPSPs to develop. Desensitization means the receptors temporarily stop responding to glutamate. This reasoning follows a study by Dudel, Franke and Hatt (1990). They used a micropipette to insert 1mM to 10mM of glutamate, the range of concentrations effective in activating receptors, into a glutamatergic synapse every 0.2ms to maintain high glutamate concentrations and make up for uptake that naturally reduces glutamate levels in the cleft. They noted that maximum activation of the receptors occurred 0.4ms after the addition of glutamate, at which point desensitization occurred for a period of at least 2ms. Because our 2mM glutamate concentration fell within a range that would have activated receptors, we believe that the submersion of the cells in it would have caused a desensitization in an average of four out of five receptors at any given time. We derived this from the ratio of 0.4ms required for activation, to the 2ms duration of desensitization (Dudel et al., 1990). This desensitization would inhibit the muscle's ability to achieve enhanced depolarization. However, because at least some of the receptors remained open at all times, the cell was still able to achieve standard EPSPs. The problem was unique to this trial because in other trials glutamate left the synapse by diffusing. However, diffusion is not possible in an already concentrated bathing solution.

The final trial, the addition of the glutamate uptake inhibitor 1-aminocyclobutane-trans-1, 3-dicarboxylic acid, produced significant results in the direction proposed by our hypothesis. Normally, after the release of glutamate into the synaptic cleft, its actions are quickly terminated by uptake into the pre-synaptic cell through protein transporters (Fletcher et al, 1991). Our chemical is a restricted analogue of L-glutamate that has been found to

restrict glutamate uptake; however, most previous research has been conducted on glutamatergic synapses in mammals and birds (Fletcher et al, 1991; Lewin et al, 1996). Restricting glutamate uptake means exit mechanisms are limited to diffusion, so glutamate will remain in the cleft longer, and theoretically will activate more post-synaptic receptors for a longer period of time. Our results show a major increase in EPSP amplitude following the addition of the chemical, suggesting it is also a highly effective glutamate uptake inhibitor in crayfish. We also noted that the duration of EPSPs in this trial increased an averaged of 7.7ms during test trials, which is consistent with the increased availability of glutamate caused by the inhibitor (Fig. 3).

Based on our findings, we have learned that specific conditions must exist at the neuromuscular junction to cause enhanced EPSPs. First, glutamate must be able to diffuse from the cleft in order to prevent desensitization of the receptors. However, prevention of uptake does not inhibit the development of EPSPs. This occurs because glutamate is still capable of diffusion, but levels of glutamate are increased enough to activate more receptors and increase EPSP amplitudes. It seems that the ability to diffuse is more important than uptake for limiting glutamate levels and preventing desensitization. Additionally, we learned that high frequency stimulation, though theoretically increasing glutamate concentrations, causes depression in the postsynaptic cell. Further research can explore the exact effects of high frequency stimulation on glutamate levels in the synapse, in addition to the time and frequency necessary for these effective levels to develop. It would also be interesting to study how actual levels of glutamate affect EPSP amplitudes, as we were unable to actually measure the levels of glutamate, and instead assumed that frequent stimulation would increase these levels based on a previous experiment (Kawagoe et al., 1980).

In conclusion, we determined that the uptake inhibitor 1-aminocyclobutane-trans-1, 3 dicarboxylic acid is effective as a glutamate uptake inhibitor in crayfish, as well as in birds and mammals. As previous research has shown, we noted that the submersion method of glutamate application led to constant desensitization in most of the post-synaptic receptors, thus preventing enhanced EPSPs from developing. Overall, we noted that the method used to increase glutamate concentration affects EPSP development, as the glutamatergic synapse is quite sensitive to glutamate levels and the frequency of electrical stimulation.

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