

Effects of Caffeine on the Crayfish Neuromuscular Junction

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ABSTRACT

The effect of caffeine on the nervous system was studied by exposing the crayfish neuromuscular junction to caffeine and stimulating nerves within proximity of glutamatergic synapses to elicit excitatory post-synaptic potentials (EPSPs), a measure of synaptic transmission. Previous work has shown that caffeine increases the probability of neurotransmitter release and increases the amount of available neurotransmitters in the pre-synaptic cell. Using a preparation of the fast extensor muscles in the tail of adult crayfish, we compared the amplitudes of the EPSPs elicited in 0.75, 1.00, and 1.25 mg of caffeine per liter of Ringer's solution to the EPSPs elicited in normal Ringer's solution. Observed trends and previous works suggest that caffeine increases EPSP amplitude and that this increase is time-dependent, concentration-dependent, and reversible; however, we were unable to show statistically significant conclusions from our data. Nevertheless, the trends we were able to see do support previous research concerning the effects of caffeine in other organisms.

INTRODUCTION

Millions of Americans start their days with a caffeinated drink of some kind. Caffeine is widely known to be a central nervous system (CNS) stimulant, and is commonly used in medicine to treat, among other things, migraines and asthma. Also known as 1,3,7-Trimethylxanthine, caffeine ($C_8H_{10}N_4O_2$), is a bitter, white alkaloid commercially derived from tea and coffee.

Previous work suggests that caffeine increases synaptic transmission. Wilson (1973) studied the effect of a 1.0 mM caffeine solution on end plate potentials when applied to the rat diaphragm. By comparing the effects of caffeine to that of increased Ca^{2+} concentration, Wilson concluded that caffeine acts on internal calcium stores in the presynaptic cell and also increases the mobilization rate of neurotransmitter.

It has since been shown that caffeine activates the endoplasmic reticulum Ca^{2+} release channel by increasing the frequency and the duration of open events (Meissner et al. 1989). Specifically, caffeine is an agonist of the ryanodine-sensitive endoplasmic reticulum Ca^{2+} release channel, enhancing Ca^{2+} -induced Ca^{2+} release in rat osteoclasts (Shankar et al. 1995) and rat ventricular myocytes (O'Neill et al. 1990).

More recently, Jacobson (1998) reviewed six of his previous studies on human reactions to ingesting caffeine. He suggests that caffeine stimulates certain neurotransmitters, both inhibitory and excitatory. This manifests itself in affected muscle groups by either strengthening or weakening the action.

However, the preparations used for these studies are not directly applicable to the human central nervous system at the cellular level. We therefore used the crayfish neuromuscular junction (NMJ) in order to bridge

this gap, as it is an ideal preparation for understanding the human CNS. The glutamatergic synapses of the fast extensor muscles correlate directly to those of the human CNS, which are also predominately glutamatergic synapses. The nerves are easily accessible by way of a relatively simple dissection, and the specimens are relatively inexpensive.

This study examined synaptic transmission at the cellular level with the use of several concentrations of caffeine, ranging from .75 to 1.25 mg/L. Based on the findings of Wilson and Jacobson, we anticipated that the effect of caffeine would be excitatory in the crayfish NMJ, that the amplitudes of the EPSPs would be relatively higher at higher concentrations of caffeine, that the effect grows as time progresses, and that the effect would be reversible. Although we did observe these trends, our data did not yield statistically significant results due to high variability of EPSP amplitudes which we attributed to differences in intracellular conditions among the multiple cells from which we took readings.

MATERIALS AND METHODS

Overview

Our investigation into the effects of caffeine on the crayfish neuromuscular junction (NMJ) was focused specifically on changes in EPSP amplitude in the crayfish tail. Our test subjects were crayfish (*Procambarus clarkii*) provided by

the Grinnell Biology department.

Basic Preparation

We used a dissection of adult crayfish that isolates superficial fast extensor muscle fibers. The details of this dissection are discussed in “Crawdad Lab Manual for Neurophysiology” (Wytenbach et al. 2002). Depending on our successes at eliciting EPSPs on any given day, we used either high or low Ca^{2+} Ringer’s. The composition of Ringer’s solution is as follows:

Component	Concentration
KCl	5.4 mM
NaCl	205 mM
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	9.35 mM OR 2.6 mM
NaHCO_3	2.3 mM
Dextrose	2.0 mM
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	6.75 mM OR 13.5 mM

Table 1. Composition of Ringer’s Solution. When two values are reported, the first applies to Low Ca^{2+} Ringer’s and the second applies to High Ca^{2+} Ringer’s.

We cycled the saline solution inside the dish every 15 minutes by removing approximately one fourth of the total solution with a syringe and then pouring in fresh solution.

Stimulation and Recording

We stimulated local nerves with a suction electrode that is connected to a stimulator. The suction electrodes were made from recording electrodes (described below) with the tips ground off so that the electrode could be affixed to the surface of a nerve by suction without piercing it or completely enveloping it. Stimulations ranged from .13V to 60V for different preparations, but we set the stimulator for a duration of 1 millisecond and a frequency of 2 Hz for all experiments.

We used standard intracellular recording techniques to measure membrane potential in the innervated muscle fiber, as described by DiPolo and Latorre (1971). We made recording electrodes by pulling heated glass capillary tubes to a point with diameter less than 1 micron. We tested recording electrodes during use for resistance and rejected any electrodes with resistance below 4 M Ω . The data was recorded using Scope.

Data Collection

In each of our experiments we started by taking EPSP recordings from a normal preparation (no caffeine added). This established a baseline for comparison that would account for the variability of each specific crayfish for each day’s experiments. Through the course of the study, we discovered it best to take measurements from a variety of different muscle cells for each individual set of

recordings. In these sets, 5 slides were taken of each cell, where all of the cells were innervated by a common nerve. If we were unable to elicit an EPSP using low Ca^{2+} Ringer’s, we changed out the solution to high Ca^{2+} Ringer’s, allowing time for the natural diffusion of Ca^{2+} into the organism.

We then prepared a caffeine solution using Ringer’s solution, with Ca^{2+} matching that of the bathing solution. We accomplished this by diluting stock solution (1 mg/mL) to specific concentrations using a micropipette. Throughout our experiments we used concentrations of 0.75, 1.00, and 1.25 mg/L caffeine diluted with Ringer’s solution to test whether changes in caffeine concentration have an effect on how the crayfish responds.

After adding the caffeine to the preparation we began to take recordings immediately for approximately ten to twenty minutes. We also recorded time to the accuracy of 30 second intervals in order to observe the change in effect over time.

If possible, we also tested for reversibility of the effects on the treated crayfish. We washed the crayfish preparation with Ringer’s three times (one minute per wash), then submerged the washed preparation in caffeine-free Ringer’s solution of the same Ca^{2+} concentration as was used for recordings previously and took readings in the manner described above.

Control

We did a control experiment to account for the effects of repeated stimulation over time on the crayfish tail. In this experiment we used no caffeine and instead recorded the EPSPs of a normal preparation for 100 minutes.

Data Analysis

EPSP amplitude was determined by using the tools in scope to find the difference between the peak of the EPSP and the resting potential. The values for EPSP amplitude generally ranged between 2 and 30 mV.

We used linear regressions to fit time- and concentration-dependency data. The use of t-tests on reversibility data was originally planned, but proved to be unnecessary as the data was inconsistent.

RESULTS

Crayfish Roles for Data Reporting

We attempted tests on many crayfish, with

varying levels of success. Table 2 shows the laboratory conditions and successful tests from each of the crayfish. In reporting our results, we used data from as many crayfish as applicable, so in the case of reversibility, for example, only data from three subjects is reported because only three reversibility tests were conducted successfully.

Name	[Ca ²⁺]	[Caffeine]	Reversibility	Cells
Jorge	Low	1.00 mg/L	No	Single
Rasputin	Low	1.00 mg/L	Yes	Multiple
Rufus	High	1.00 mg/L	Yes	Multiple
Rufus 2	High	1.25 mg/L	No	Multiple
Dabrat	High	0.75 mg/L	No	Multiple
Lotus	High	n/a	No	Multiple
Chester	High	1.25 mg/L	Yes	Multiple

Table 2. Legend to Crayfish Roles. Rufus 2 refers to the second application of caffeine to Rufus after completing a full cycle of being caffeinated and then washed for a reversibility test.

In the case of Jorge, we took recordings from only one cell throughout the duration of the experiment to create a time-dependence model. This removed enough experimental error to allow the discovery of a correlation between time and the change in EPSP amplitude.

Lotus did not receive a caffeine treatment as Lotus was used as a baseline for the time-dependence experiment in an effort to establish a baseline for comparison.

The data from Jorge is not included in some figures in the interest of consistency, as recordings were taken from a single cell for Jorge whereas in the other five successful caffeine trial recordings were taken from multiple cells.

Variation and Number of Samples

We found a great deal of variation among cells and crayfish with respect to EPSP amplitudes, which has a direct impact on the change in EPSP amplitudes that we were attempting to see after caffeination. To address this, standard error bars are included on figures when applicable. The number of samples, or n-values, are reported in Table 3.

Name	Normal	Caffeinated	Wash
Dabrat	7	5	n/a
Rufus	4	10	3(*)
Rasputin	2(**)	8	3
Rufus 2	3(*)	8	n/a
Chester	5	21	5

Table 3. Sample sizes (n-values) for reported averages. (*) The wash data from Rufus (first trial) and the normal data from Rufus 2 (second trial) are the same. (**) The two readings taken for Rasputin under normal conditions have a remarkably small standard error of .005, and thus the standard error bars for these data in Figures 2 and 5 appear to be missing.

Excitatory Effect and Concentration

In preliminary tests, we observed a dramatic increase in EPSP amplitude at the caffeine concentration of 1 mg/L. However, this effect did not prove to be consistently repeatable. As shown in Figure 1, there was a great amount of variation in average EPSP amplitudes among different crayfish and also a great amount of variation within cells of individual crayfish. Figure 2 displays the percent increase in EPSP amplitude after adding caffeine to the preparation. In two of five cases, the average EPSP amplitude actually decreased after adding caffeine. Averaging all cases, the overall effect is that caffeine increases EPSP amplitude, but a t-test does not find these results to be statistically significant.

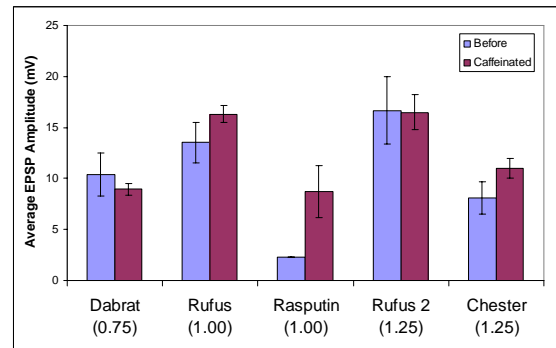


Figure 1. EPSP amplitudes before and after administering various concentrations of caffeine. The values beneath the crayfish names correspond to the caffeine concentrations in mg/L that were applied. Rufus 2 corresponds to the second application of caffeine to Rufus after he underwent one full cycle of caffeination and washing. Error bars represent standard error using the n-values reported in Table 3.

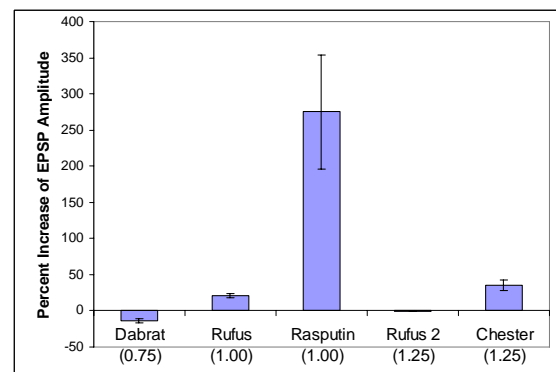


Figure 2. Percent change of EPSP amplitudes after adding caffeine. Numerical values beneath the crayfish names correspond to the concentration of caffeine applied in mg/L. The EPSP amplitude actually decreased in the cases of Dabrat and the second application of caffeine to Rufus. Rasputin shows an alarming increase in EPSP amplitude, but also with the largest proportional error. The error bars represent the proportion of standard error from the largest standard error (by percent) among the averages used to calculate each increase.

From this data, we cannot draw any definite conclusions concerning the effect of caffeine on EPSP amplitude due to the overwhelming variability in our recordings. However, the time-dependence data shown below suggests that caffeine does increase EPSPs. The erratic range of EPSP increase percentages, as displayed in Figure 2, disallows us from drawing conclusions concerning the relative effects of using various caffeine concentrations. To create a regression relating caffeine concentration and EPSP amplitude increase requires much more precise data than has been collected during this study.

Time Dependence

We began our investigation into the time course of change caused by caffeine by establishing a baseline. For the crayfish Lotus we did not apply any caffeine and observed the EPSP amplitudes in multiple cells over time. The results from this are seen in Figure 3.

The apparent finding from this data is that the EPSP amplitudes diminish over time. Administering caffeine to a crayfish caused an effect that goes against this trend.

By taking recordings from a single cell within the crayfish Jorge over time, we observed that EPSPs climbed at a steady rate immediately after administering caffeine for a number of minutes. The time course of the change is shown in Figure 4.

Since the EPSPs of a dissected crayfish would naturally decrease over time (Figure 3), we attribute the effect seen in Figure 4 to the presence of caffeine. Because caffeine appears to be responsible for a slow increase in EPSP amplitude over time, it can be suggested that caffeine does, in fact, cause an overall increase in EPSP amplitude.

Reversibility

The data does not permit a decisive conclusion about reversibility. In one instance, the average EPSP amplitude almost returned to normal after washing, but in another instance it did not, and in a third the EPSPs actually increased dramatically. It is worth noting that the most precise data (the data with the least amount of variability among cells) came from the crayfish showing reversibility, but there is not enough data to draw a secure conclusion from this observation.

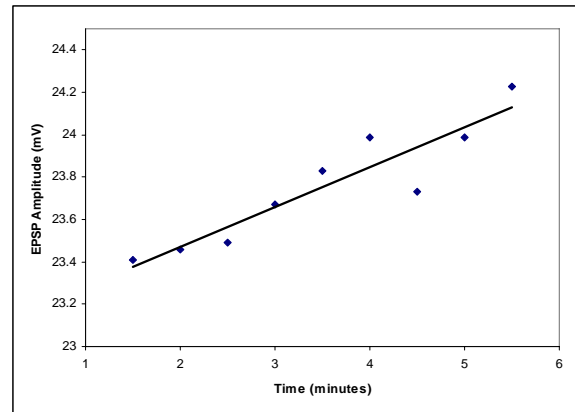


Figure 4. Increasing EPSPs immediately following caffeine administration. This data, taken from Jorge, appears to display that caffeine takes time to fully affect its target. The R2 value for this test is .86, suggesting that there is indeed a strong correlation.

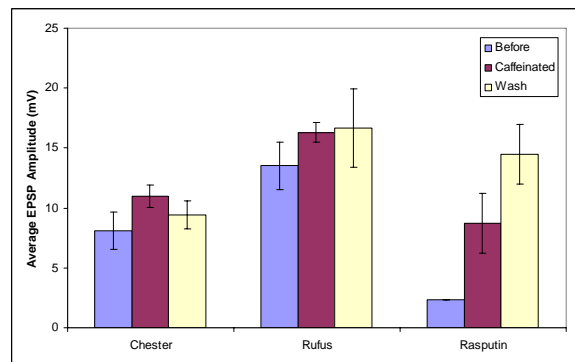


Figure 3. Reversibility of Caffeine on three test subjects. All three test subjects in these reversibility tests showed an increase in EPSP amplitude after administering caffeine, but they all show dramatically different results after washing. The error bars represent standard error using the n-values reported in Table 3.

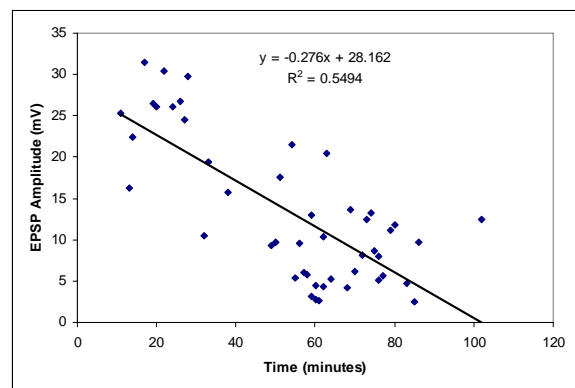


Figure 3. EPSPs of a repeatedly-stimulated crayfish over time. As time progressed, EPSP amplitude generally decreased. Each data point represents the average of five recordings from one cell within one minute. We concluded this experiment after 100 minutes because the crayfish no longer responded to stimulation.

DISCUSSION

We were unable to conclusively answer our questions regarding the effect of caffeine on the crayfish neuromuscular junction based on the collected data, as from cell to cell the resting potentials and EPSP amplitudes varied greatly. However, we did observe trends that are in agreement with our hypothesis.

The excitatory effect that was generally observed is consistent with the work done by Jacobson (1998) and Wilson (1973). The amplitudes of the EPSP signals usually increased after administering caffeine, which suggests that the increase in performance seen by Jacobson may be attributed to enhanced neurotransmission between nerves and muscle fibers. This finding is also consistent with the findings of Wilson, who suggested that caffeine would cause more neurotransmitters to release, thus resulting in a larger EPSP.

Although it was not a part of this study, we also noticed that the addition of caffeine also generally resulted in less synaptic delay. This is consistent with Wilson's finding that caffeine increases the rate of mobilization of neurotransmitters.

For future research, it may prove worthwhile to focus on taking recordings from one cell throughout the duration of the experiment. The alternative would be to collect much more data using the same method (recording from multiple cells) under more controlled conditions in hopes that more statistically significant trends may be identified and verified.

Although our data did not yield statistically significant results, we maintain our hypothesis that caffeine increases the amplitude of EPSPs and that this effect is dependent on time and concentration and is reversible. Future tests may prove or disprove this theory, but are necessary before we can move forward in understanding the effects of caffeine in more concrete and analytical terms.

ACKNOWLEDGEMENTS

We thank Professor Clark Lindgren, Sue Kolbe, and lab assistants Laura Dobbs and Priya Malik. We also would like to acknowledge Rudy, Jorge, Rasputin, Stimpy, Thor, Rufus, Dabrat, Lotus, Spike, and Chester for their significant contributions to this project.

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