Effects of Extra-Organismal Caffeine

How Extra-Organismal Caffeine Affects Crayfish Neuromuscular Synapses

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The effect of extra-organismal caffeine exposure upon crayfish neuromuscular activity was explored as a novel method to characterize the physiology of synaptic contacts in the crayfish superficial flexor muscle (SFM) system. Crayfish were housed in 0.5 L of aerated tap water or 3 mM caffeine for one and five days. Muscle junction potentials (JPs) were measured at 1 Hz and 10 Hz frequencies of nerve stimulation. At both frequencies and at both lengths of exposure, external application of caffeine either unexpectedly appeared to enhance or failed to affect synaptic transmission, contradicting the well-documented depressive effect of caffeine upon direct application to the dissected SFM system. This finding merits further investigation to confirm the effect of external application of caffeine. This means of drug exposure has potential future applications to studies of nerve regeneration in the SFM system.

Introduction

Past work on the superficial flexor muscle (SFM) system of the crayfish *Procambarus clarkii* has looked at effects of exposing the dissected SFM system to various drugs in an effort to better characterize the physiological properties of these synaptic contacts (1-5). This project seeks to expand the possibilities and implications of drug research in the crayfish model system by exposing living crayfish to caffeine via more realistic pathways, namely extra-organismal exposure of a living crayfish to caffeine. Exploring the usefulness of this extra-organismal mode of exposure stands to expand the usefulness of the crayfish as a model organism for understanding the physiology of synaptic contacts.

It was anticipated that depression of synaptic transmission in the SFM system would be observed upon external application of caffeine. This hypothesis was based upon prior studies that have exposed the dissected SFM system to caffeine and have observed a depression in synaptic transmission (1-3). Any detectable behavioral changes were therefore expected to support the hypothesis of decreased overall activity, meaning that the crayfish was expected to spend more time hiding in pots in the tank, show less aggressive behavior to other crayfish, and perform the tail flip escape response less frequently and strongly.

Initial studies of synaptic effects of exposure of the SFM system to caffeine hypothesized enhancement of synaptic transmission following caffeine exposure, based upon studies that established that 5 mM to 10 mM caffeine exposure, far above toxic levels in humans, enhance synaptic transmission (6). On a molecular level, caffeine at mM concentrations enhances synaptic transmission by stimulating Ca^{2+} release, inhibiting phosphodiesterase, and blocking $GABA_A$ receptors (6). The mechanism of caffeine's effect in crayfish is still unknown, other than that caffeine must act to decrease internal calcium concentrations, thereby inhibiting vesicle release into the synapse (2).

There is a great precedent in scientific literature for studying behavioral and synaptic effects of non-human ingestion of drugs, such as those among rats and mice (7-9). Precedent for external application of drugs to arthropods is found in NASA's work on comparing shapes of webs spun by drugged spiders and non-drugged spiders (10). Additional work on extra-organismal drug exposure has been undertaken to study the effects of chronic exposure of crayfish to herbicides, though this study focused on the survival, growth, and other life-cycle consequences of drug exposure, rather than the neurobiological effects (11). This project thus explores a promising new direction for research with the crayfish model organism.

Materials and Methods

Experimental Design

Southern red crayfish *Procambarus clarkii*, ordered from Carolina Biological Supply Company, were used in this experiment. Prior to use, the crayfish were housed together in a large aerated freshwater aquarium. Crayfish were divided into three groups: control, caffeine one day, and caffeine five days. Each crayfish was kept in its own medium square (946 mL size) plastic Ziploc container containing 0.5 L of water and a small terra-cotta pot for hiding. Containers were aerated and covered. Crayfish in the control group were kept in room-temperature tap water for one day. Crayfish in caffeine groups were kept in room-temperature tap water with 3 mM caffeine, a level previously determined to depress SFM synaptic activity when applied to the dissected SFM system (5). For all groups, data on neuromuscular activity were collected.

Data Collection Methods

Crayfish were sacrificed, and the tail was dissected to expose the SFM neuromuscular system. Enzymes released by cutting abdominal deep muscle cells were rinsed away using cold Ringer's solution and then the tail was bathed in fresh cold Ringer's solution. Ringer's solution contains 210 mM NaCl, 14 mM $CaCl_2$, 5.4 mM KCl, 2.6 mM $MgCl_2$, and 2.4 mM $NaHCO_3$, the ion concentrations found in crayfish plasma that maintain normal nerve and muscle activity in the SFM system (12).

Data collection followed the conventional methods used to research the crayfish SFM system (1-5). Two suction electrodes were used, one to stimulate the SFM nerve and the other to record nerve activity. The nerve was stimulated using a Grass SD9 Stimulator, and all nerve signals were displayed on a Tektronix storage oscilloscope after passing through a Grass P15 Preamplifier. The nerve was stimulated at two frequencies, 1 Hz and 10 Hz, displaying facilitation at 10 Hz. One microelectrode, filled with 3 M KCl, was used to record resting potentials and junction potentials in muscle cells. All data were collected from the second-to-last abdominal segment during the evening hours. Some data were collected in morning hours, but were not included in analysis in order to avoid introducing another variable into the experiment.

Data Analysis

Junction potential (JP) sizes were averaged, and the standard deviation (*s*) was then calculated using $s = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n-1}}$ where x_i represents an individual JP, x represents the average JP size, and n represents the number of JPs in the sample. Standard error of the mean (*SE*) was calculated using $SE = \frac{s}{\sqrt{n}}$. Values for standard error of the mean were converted into error bars on a column graph of average JP sizes.

Results and Discussion

In the control group, two crayfish were used to obtain measurements from 5 SFM muscle cells, and one crayfish was used for each of the caffeine exposure groups. Data were obtained from 9 cells for the group with one day caffeine exposure and from 14 cells for the group with five days of caffeine exposure. It should be noted that, due to the extremely small sample size, none of the results obtained in this experiment were statistically significant. Additionally, to strengthen the methodology, there should ideally have been two separate control groups: a group of crayfish kept for one day and a group of crayfish kept for five days, both groups in tap water in the plastic Ziploc container.

That said, the experiment yielded interesting and unexpected results (Fig. 1). Both caffeine groups had a larger average JP size than that of the control group, at stimulation frequencies of 1 Hz and 10 Hz. As expected, JPs obtained from 10 Hz stimulation displayed facilitation.

Data on average JP size are also displayed as a column graph in Fig. 2, along with error bars representing standard error of the mean for each data series. Extra-organismal exposure of the crayfish to 3mM caffeine both for one day and for five days induced an increase in average JP size. However, the data on JP sizes fall within the range of the error bars, so it is possible that the external application of caffeine had no effect on neuromuscular synaptic transmission in the crayfish. This data is not statistically significant as only one crayfish was measured at each of the time points for caffeine exposure, and only two crayfish were measured for the control group. This experiment gives only a preliminary indication that extra-organismal caffeine exposure may enhance or may not change synaptic transmission in the SFM system.

While not statistically significant, these results are interesting and unexpected, and merit further investigation. If these results are accurate, it is unclear by what mechanism extra-organismal caffeine exposure would enhance or fail to affect synaptic transmission, whereas direct application of caffeine to the dissected SFM system has a well-documented depressive effect (1-3,5). It is further unclear why the mechanism of caffeine's action upon the SFM system would depend upon whether caffeine exposure occurred via external application or via direct application to the dissected system.

Behavioral data could not be collected in this experiment for two reasons. First, and most importantly, the size of the plastic container was too small to allow a great deal of crayfish movement, and thus any behavioral differences between the control and the caffeine groups were difficult to discern. Additionally, a number of the stated measures for crayfish behavior were based upon crayfish interactions, and these interactions were impossible to observe as only one crayfish was placed in each container. Future researchers wishing to observe behavioral effects of caffeine exposure should use a larger tank.

An additional result observed over the course of the study was crayfish mortality at various time points in both the control and caffeine groups. One crayfish in the control group died at five days – effectively eliminating the control five days group – and crayfish in the caffeine groups died at two, three, and seven days. These results suggest that, independent of caffeine exposure, a space-constrained environment is not conducive to crayfish survival. Future experiments considering drug exposure at the organismal level should use a tank that is substantially larger than the approximately 1 L sized container used in this experiment. The plastic material of the container may have factored into the observed crayfish deaths; future experiments should consider using standard glass tanks. An additional confounding factor might be that the crayfish did not have enough food to survive, as crayfish might have been hungry even before being placed into the plastic container and might have starved in the plastic container. However, based upon prior lab experience, the likelihood of starvation seems very small, as this is a highly atypical cause of crayfish death. To limit mortality, future experiments should also limit the length of caffeine exposure, as much higher mortality rates were observed in the caffeine group versus the control group.

For a number of reasons, this experiment would be best pursued as a multi-term project. First, there will inevitably be various troubleshooting issues that the researcher will need to address, including – but certainly not limited to – dealing with equipment problems and determining the appropriate method and concentration of caffeine exposure. Second, there is a very steep learning curve for the crayfish dissection and proper equipment usage, both of which take up to one or two months to gain sufficient experience to obtain good data consistently. Third, there is no guarantee that every crayfish that is dissected will provide good data. Any given crayfish may be molting, stressed, or may be demonstrating synaptic repression for other reasons, such as seasonal variation (13). A significant representative sample of muscle cells would constitute at least 7-10 cells from each crayfish, and statistically, the minimum total number of crayfish is three crayfish. Therefore, assuming approximately a 30% average success rate in obtaining sufficient JP data from a crayfish, the researcher would need to dissect 10 crayfish for each of the desired groups in this experiment – control one day, control five days, caffeine one day, and caffeine five days. This would total 40 crayfish, a number for which there was insufficient time in the

Experimental Group	# Muscle Cells	1 Hz		10 Hz	
		JP Size (mV)	Std Error	JP Size (mV)	Std Error
Control 1 day	5	2.2	0.374	4.2	0.663
Caffeine 1 day	9	2.7	0.289	5.3	0.441
Caffeine 5 days	14	2.6	0.272	5.4	0.429

Image courtesy of Tara Kedia

Figure 1: Average muscle junction potential (JP) size at 1 Hz and 10 Hz frequency of stimulation of the crayfish SFM nerve. Two crayfish were in the 1 day Control group, and one crayfish was in each of the caffeine groups. Crayfish were kept in 0.5 L of 3mM caffeine in an aerated 1-L plastic Ziploc container for either 1 day or 5 days. Stimulated JPs were measured from the second-to-last SFM segment. Standard error of the mean was calculated for all data.

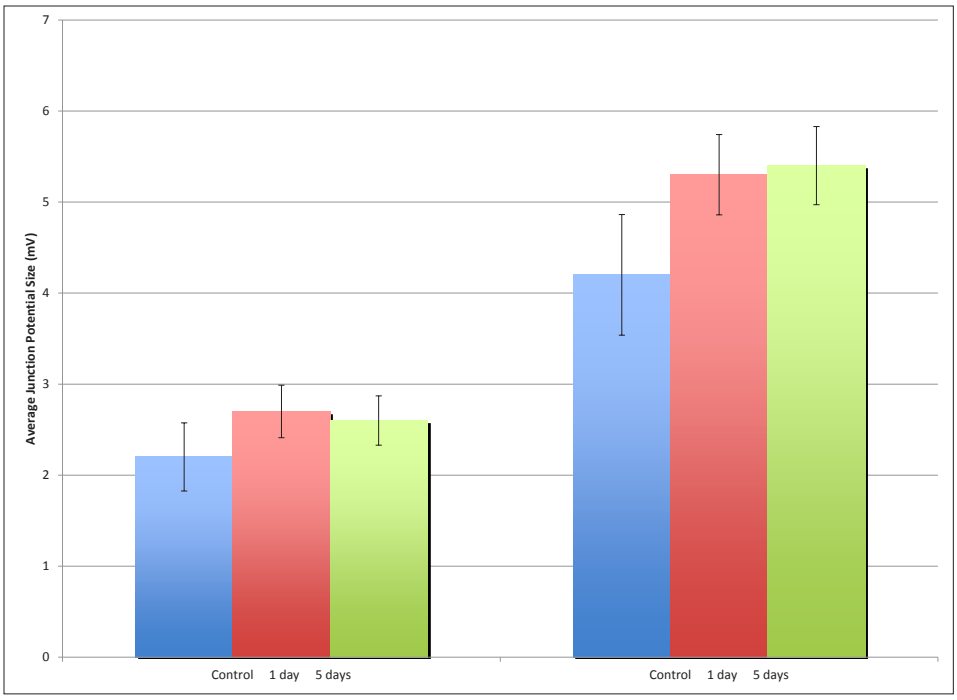

Image courtesy of Tara Kedia

Figure 2: Effects of extra-organismal 3mM caffeine exposure on size of stimulated junction potentials (JPs) in crayfish SFM muscle cells. The vertical axis displays the average size of JPs. Data on JP size was collected at two different frequencies of stimulation: 1 Hz and 10 Hz. Error bars represent the standard error of the mean. The number of cells recorded is as follows: Control 1 day: 5; Caffeine 1 day: 9; Caffeine 5 days: 14.

one term that I pursued this experiment. The primary limiting factor in this research project was the lack of time to complete enough dissections to obtain sufficient data.

Looking ahead, this external application of caffeine promises to have useful applications in many areas of future research on the crayfish SFM system. Past work on the crayfish SFM system has studied neuronal regeneration and transplantation and has attempted to characterize the physiology of these new synaptic contacts (14-18). This project proposes a novel method for characterizing the physiology of SFM synapses, and, once developed, this method would better simulate conditions that organisms are exposed to during recovery from real – rather than simply surgical – nerve damage. This method of extra-organismal exposure to caffeine would allow exploration of the effects of chronic drug exposure on nerve regeneration.

In summary, the premise for this project was that exposure of the SFM system to drugs should occur via a more realistic mode of transmission. This project explored the effects of crayfish exposure to 3 mM caffeine dissolved in tank water, but other concentrations and/or modes of exposure might prove more effective. In human cases of nerve damage, nerve regeneration occurs while the organism is exposed to various environmental factors, such as stress and ingested drugs, including caffeine and ethanol. In order to most effectively use data from the crayfish SFM system to model human nerve regeneration, it is proposed that the nerve-damaged crayfish be exposed to similar environmental factors throughout the process of nerve regeneration, rather than after the nerve has already regenerated. However, before such studies can be undertaken, we must establish the physiologically relevant concentration of caffeine, as well as the best method of chronic administration.

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Acknowledgements

Thanks to Dr. Samuel Vélez for his advice and guidance over the course of the experiment. This research was conducted for Biology 95 using departmental funds.