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Endocannabinoids Inhibit EPSPs at the Crayfish Neuromuscular Junction

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ABSTRACT

Previous research has shown that cannabinoid (CB) receptors are present at the pre-synaptic nerve terminals in crayfish neuromuscular junctions and that endocannabinoids alter the release of neurotransmitters in humans and other animals. Our research was directed towards finding physiological evidence of endocannabinoids at the crayfish neuromuscular junction. We hypothesized that by applying an exogenous cannabinoid to the crayfish neuromuscular junction, the excitatory post-synaptic potentials (EPSPs) would decrease. To test this, we first measured a control group of EPSP amplitudes with a crayfish exposed to normal saline solution; we then emptied the dissection dish and refilled it with a diluted solution of arachidonylcyclopropylamide (ACPA), a synthetic cannabinoid. By measuring the subsequent EPSP amplitudes and comparing the results to that of the control group, we discovered that endocannabinoids do indeed have a function at the crayfish neuromuscular junction.

INTRODUCTION

The endocannabinoid system is a new and evolving area of neuroscience. The first major breakthrough occurred in 1990 with the cloning of the cannabinoid$_1$, or CB_1, receptor. Since then, research has yielded many interesting findings about the endocannabinoid signaling system. The endocannabinoid system is made up of enzymes and proteins that are responsible for the synthesis, transport and degradation of either 2-Arachidonylyl Glycerol (2-AG) or anandamide. The cannabinoid receptors are the second aspect of the endocannabinoid system, with the CB_1 and CB_2 receptors currently cloned and characterized. CB_1 receptors mostly exist in the brain and spinal cord in the central nervous system, while CB_2 receptors are found throughout immune cells, including white blood cells. The downstream signaling molecules are the last components of the endocannabinoid system. The ultimate result of these three components is the inhibition of neurotransmitter release during synaptic transmission (Basavarajappa 2007).

While CB_1 receptors have been found in vertebrates (mammals, birds, amphibians and fish) and CB_2 receptors are also known to exist in vertebrates, the study of cannabinoid receptors is still fairly recent. Through the study of invertebrate ancestry of endocannabinoid signaling, it has been found that the ancestor of vertebrate CB_1 and CB_2 receptor are present in a deuterostomian invertebrate (Elphick, et al. 2003). More recently, Grinnell College Biology 150 students performed an experiment using anti-body selective immunofluorescence staining and microscopy to find that CB_1 receptors exist at the crayfish neuromuscular junction (Sahel, Garcia, Truong, 2007). A study similar to the one that we have conducted showed that in isolated buccal and parieto-visceral ganglia of *Aplysia californica,* tetrahydracannibol (THC) repressed nerve cell excitability the same way it does in mammals, showing the presence of endocannabinoids in that invertebrate organism (Salzet and Stefano 2002).

Building on this previous research, we ask the question: are endocannabinoids present at the crayfish neuromuscular junction? To test this, we set up two groups: a control group and an experimental group. The experimental group would include trials in which we applied the synthetic form of the endocannabinoid arachidonylcyclopropylamide (ACPA).

Our hypothesis was that the addition of ACPA would decrease excitatory post-synaptic potentials (EPSPs), thus providing us with physiological evidence for the presence of endocannabinoids at the crayfish neuromuscular junction. The results of our research suggest that endocannabinoids are present in crayfish neuromuscular junctions, thus providing physiological evidence that the endocannabinoid signaling system works the same way in crayfish as it does in humans.

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MATERIALS AND METHODS

Preparation

The crayfish we used throughout our trials were of the species *Orconectes rusticus.* To prepare our specimens, we first cut the tail from the body of a crayfish that had been submerged in ice for at least 15 minutes. Then, the sides of the shell on either side of the tail were cut and split open with scissors to gain access to the tissues on the inside of the tail. The tissues were cleared out to expose the extensor muscles that lie on the dorsal inner surface of the tail of the crayfish. The crayfish tail was set in a large dissection dish (11cm in diameter and 5cm in width) that was then filled with 100mL of saline solution to preserve the muscle cells. The saline mimics the environment that the cells are used to, prolonging the life of the cells. The composition of the saline was as follows: 5.4mM KCl; 196mM NaCl; 2.6mM MgCl; 13.5mM CaCl; 10mM Hepes Buffer.

Measuring the Voltage inside the Muscle Cell and Measuring EPSPs

To measure the voltage inside the muscle cell, we used microelectrodes approximately 2 inches in length, pulled from 4in. capillary tubes. We used the PUL-1 electrode puller by World Precision Instruments to divide a full-length capillary tube into two separate pieces, both with a very fine tip. The electrodes were then filled with 3M KCl solution and dipped in a beaker full of Ringer's solution to rinse the KCl off of the exterior of the electrode. We then attached the electrode to an apparatus connected to an input amplifier made by AD Instruments PowerLab 4/25. The input amplifier was connected to the computer, which displayed the voltage over time. After attaching the electrode to the holding apparatus, we used a Leica Zoom 2000 microscope to observe the insertion of the electrode into the muscle cells. After we successfully inserted an electrode into a muscle cell, we applied a stimulus to a nerve to provoke an action potential in the nerve, which then released neurotransmitter onto the post-synaptic muscle cell, producing an EPSP. The stimulus was applied using a two-prong electrical stimulator connected to a Grass SD9 Stimulator (Grass Medical Instruments) where we were able to control voltage application, duration, frequency, and delay of the stimulus pulse.

Testing Potentials with ACPA

First, we measured EPSPs when the crayfish was exposed to regular saline solution and we used these measurements as our controls. Then, we switched out the saline solution with a solution containing ACPA. The stock solution of ACPA that we used had an initial concentration of 14.55mM. For our experimental trials, we diluted a portion of the stock solution with saline to reach a final concentration of 1μM ACPA. We first measured control EPSP amplitudes with the crayfish exposed to normal ringers solution. Afterwards, we emptied the dissection dish and we refilled it with the diluted solution of ACPA. We measured the subsequent EPSP amplitude(s) and we performed this procedure 4 times.

RESULTS

The purpose of our experiment was to find evidence of endocannabinoid function at the crayfish neuromuscular junction. We first measured control EPSP amplitude values when the crayfish was exposed to normal crayfish ringers solution. We then exposed the crayfish to a solution containing 1μM ACPA and recorded its EPSP amplitudes. After doing this 4 times, we noticed that the size of the EPSP amplitude when the crayfish was exposed to ACPA was indeed smaller than the EPSP amplitudes when the crayfish was exposed to normal saline.

While we thought that our data was sufficient enough to make a viable conclusion, we had to perform a statistical test in order to better analyze our results. After performing a paired, two-tailed t-test assuming equal variances, we discovered that our t-value was 0.015, indicating that our data is statistically significant.

Trial #	Control (Saline Exposed)	ACPA Exposed
1	26 mV	21 mV
2	22 mV	16 mV
3	15 mv	10 mV
4	22 mV	21 mV

Figure 1. Table showing EPSP amplitude before and after exposure to ACPA.

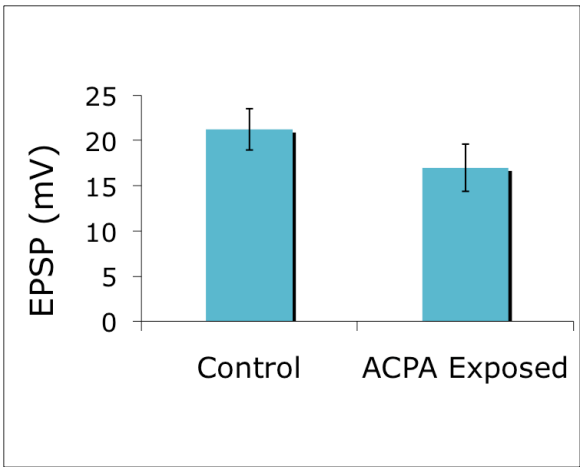

Figure 2. Graph shows average control and ACPA EPSP amplitude values.

DISCUSSION

Building on research performed by students in a previous Biology 150 class, our experiment provides physiological evidence of the existence of CB_1 receptors at the pre-synaptic terminal of the crayfish neuromuscular junction (Sahel, Garcia, Truong, 2007).

Initially, our data collection was inhibited due to the low-calcium saline solution that we were using. At the beginning of our experiment, we used a saline solution with a low calcium concentration because we kept producing action potentials and not EPSPs. We hoped that the low calcium solution would solve this problem, as a lesser amount of calcium would probably inhibit a higher number of action potentials. However, the use of said solution proved difficult, as we were unable to record EPSPs large enough to measure. After using different nerve stimulators, we came to the conclusion that the low-calcium saline was the problem and we proceeded with the regular saline solution. For purposes of ease we decided to use the two-prong electrical stimulator as opposed to the suction-electrode stimulator. As figure 1 shows, after fixing our initial problem, we were much more successful in obtaining data.

Our purpose was to find out if endocannabinoids work the same way in crayfish as they do in humans, and our results show that they do. This means that we can be confident any further research we do regarding endocannabinoids in crayfish can also be applied to humans. This allows us to perform a greater variety of experiments regarding how our endocannabinoid system works, and even how the entire crayfish nervous system works.

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