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The Effects of the Endocannabinoid Agonists, AM356 and ACPA, on the Crayfish Neuromuscular Junction.

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ABSTRACT

Endocannabinoids are retrograde transmitters, which inhibit the release of neurotransmitters. We hypothesized that endocannabinoids would operate similarly in crayfish neuromuscular junctions as they do at other synapses, lowering the amplitude of the excitatory post-synaptic potentials (EPSPs). We tested this by exposing the superficial extensor muscles in the crayfish tail to two synthetic CB_1 agonists, arachidonylcyclopropylamide (ACPA) and methanandamide (AM356). We found that ACPA decreased the EPSPs as expected. AM356, however, did not; in fact when exposed to AM356 the EPSP amplitudes increased.

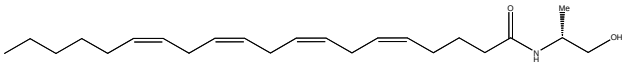
INTRODUCTION

Endocannabinoids acting as retrograde signaling molecules, is an emerging field of study. The endocannabinoid system consists of enzymes and proteins necessary for the synthesis, transport, and degradation of 2-AG or anandamide (Johnston *et al.*, 2008). In many brain regions, endocannabinoids allow postsynaptic cells to modulate their synaptic inputs. Endocannabinoids have been shown to alter various ion channels and synaptic strength (Brenowtiz & Regehr, 2003).

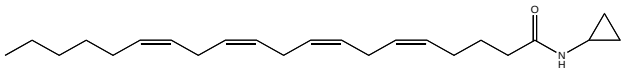
CB_1 and CB_2 receptors have been found in several vertebrates. CB_1 receptors are present mainly at synapses while CB_2 receptors are found mainly in the immune system (Brenowtiz & Regehr, 2003). CB_1 and CB_2 receptors are activated by endocannabinoids. A recent study at Grinnell College was performed to test the endocannabinoid system at the crayfish neuromuscular junction (Johnston *et al.*, 2008). CB_1 receptors have been confirmed to exist in crayfish (Garcia *et al.*, 2007). Johnston *et al.* (2008) found that the exposure of superficial extensor muscles in the crayfish tail to the synthetic CB_1 agonist, arachidonylcyclopropylamide (ACPA) decreased the amplitudes of the EPSPs.

We built upon the findings of Johnston *et al.* (2008). The question we addressed was: do endocannabinoids work the same way at the crayfish neuromuscular junction as they do at other synapses? We tested this question by testing two different synthetic CB_1 agonists. We used two experimental groups, each of which had their own control. The two experimental solutions contained ACPA and methanandamide (AM356), both of which were dissolved in Tocrisolve™ 100. The control solutions contained the solvent Tocrisolve™ 100.

We hypothesized that just as Johnston *et al.* (2008) found, ACPA would decrease EPSPs. We also hypothesized that AM356 as another synthetic CB_1 agonist would have a similar effect. These results would further confirm the presence of endocannabinoids at the crayfish neuromuscular junction. The results of our research confirmed that ACPA does in fact decrease EPSP amplitudes at crayfish neuromuscular junctions; however, AM356 had the opposite result. The AM356 results contradict the physiological evidence shown by the ACPA findings that the endocannabinoid signaling system works the same in crayfish as other organisms.



AM356



ACPA

MATERIALS AND METHODS

Dissection

After a crayfish (*Procambarus clarkii* from Carolina Biological Supply Company, Burlington, North Carolina) was iced for 15 minutes, we detached the tail and disposed of the body. The tail was cut along both sides anterior to posterior as ventral as possible. The tissue was removed to give proper access to the superficial extensor muscles of the crayfish tail. The prep was pinned onto a dish and covered with Ringer's solution consisting of 5.4 mM KCl, 196 mM NaCl, 2.6 mM $MgCl_2 \cdot 6H_2O$, 10 mM Sodium Hepes Buffer and 13.5mM $CaCl_2 \cdot 2H_2O$. The solution

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was brought to a pH of 7.4 directly before the beginning of lab.

Microelectrodes and Measurement

Microelectrodes were made from 1.2 mm diameter capillary glass tubing using a PUL-1 electrode puller. The electrodes were filled with a 3M KCl solution. The resistance of the electrodes ranged from 4 MΩ to more than 10 MΩ. The electrode was hooked up to a computer so that the voltage difference between the microelectrode and the reference electrode could be measured and recorded using the program Scope. A suction electrode was used to suck up a nerve on the crayfish tail. The suction electrode was connected to a stimulator, which was used to stimulate the nerve, generating an action potential in the nerve and creating EPSPs.

Solutions

The EPSPs were measured in three different solutions. The control was a 1:1000 dilution of Tocrisolve™ 100, a solvent in which the drugs were dissolved, with the aforementioned Ringer's solution (All drugs from Tocris Bioscience, Ellisville, Missouri). The two solutions for the trials with drugs were 1:1000 dilutions of ACPA and AM356, both of which were dissolved in Tocrisolve™ 100. The final molarity of the ACPA solution was 14.55 µM and the final molarity of the AM356 solution was 13.83 µM. The muscles were immersed in the solutions for 5-10 minutes before any measurements were made. A fresh crayfish was used for each drug.

RESULTS

The purpose of our experiment was to determine the effects of AM356 and ACPA on crayfish neuromuscular junctions. We prepared a crayfish and used microelectrodes and a stimulator to measure EPSPs. We measured control EPSPs (Tocrisolve™ 100). We then exposed the crayfish to AM356 and ACPA and measured the EPSPs of each, using a fresh prep for each drug.

We found the EPSP increased with exposure to AM356 and decreased with exposure to ACPA. We determined that there was a statistically significant change in the EPSP amplitudes between each control and its experimental using a student two-sample t-test with p-values <0.001.

Using synthetic CB_1 receptor agonists, we expected the EPSP amplitudes to decrease. This is because the agonists would activate the CB_1 receptors leading to decreased release of neurotransmitters. This was based on the research done by Johnston *et al.* (2008). We found this to be true for ACPA, but not for AM356 which increased the EPSP amplitude.

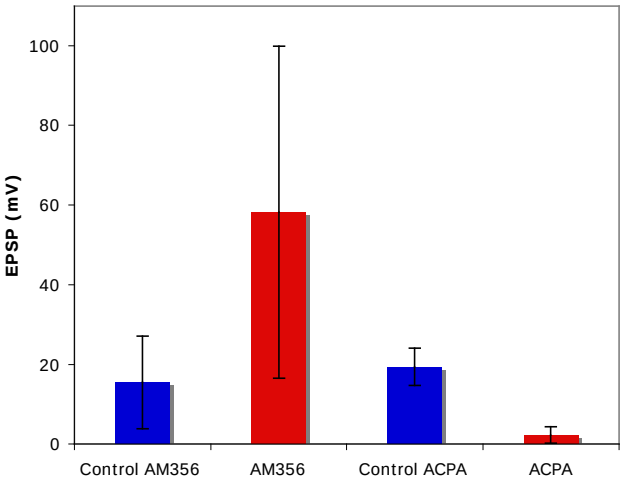

Figure 1. Graph shows average control, AM356, and ACPA EPSP amplitude values. The AM356 control n=6, AM356 n=15, ACPA Control n=12, ACPA n=9. The error bars indicate the standard error.

DISCUSSION

We hypothesized that both ACPA and AM356 would make the amplitudes of the EPSPs decrease. We found that the ACPA did indeed lower the EPSP amplitudes and that AM356 did not lower the EPSP amplitudes but in fact increased the EPSP amplitudes. The findings for the ACPA support our hypothesis, reconfirming the results found by Johnson *et al.* (2008). However, the findings for AM356 do not support our hypothesis, suggesting that the endocannabinoid signaling system does not operate the same in crayfish as in other organisms. This also suggests a difference between the way that the endocannabinoid system works in invertebrates and vertebrates. Matsuyama *et al.* (2008) found that AM356 decreases EPSP amplitudes when working with guinea pigs. Our work on crayfish does not support their finings, but that could be because of the differences between vertebrates and invertebrates.

After recording increased EPSP amplitudes with the addition of AM356, we returned the crayfish to the control solution in order to verify that the initial control amplitudes were not lower by mistake. Once in the control solution the EPSP amplitudes returned to the control level.

Our contradiction of the results of Matsuyama *et al.* (2008) suggests that future work should retest the part of the experiment with AM356. Furthermore, another logical step leading from our research is to try the addition of both ACPA and AM356 at once, which we were unable to do because we ran out of ACPA.

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