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Evidence from Paired-Pulse Experiments Suggest that Endocannabinoids Do Not Inhibit Neurotransmitter Release by Reducing Calcium Influx

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ABSTRACT

Endocannabinoids are involved in retrograde messaging in neurons. Recent studies have found evidence of endocannabinoids and CB1 receptors at the neuromuscular junction of vertebrates as well as in some invertebrates. We attempted to explain how endocannabinoids affect neurons by examining the influence of CB1 receptors on paired-pulse facilitation at the neuromuscular junction, *Procambarus clarkii*. We measured excitatory postsynaptic potentials (EPSP) at the neuromuscular junction using standard electrophysiological techniques with twin-pulse stimulation and applied the endocannabinoid receptor agonist, ACPA and antagonist AM 251. We then compared the ratio of twin-pulse amplitudes of the EPSPs during the application of agonist and antagonist to our baseline to determine whether there had been any change to inracellular Ca^{2+} in the presynaptic cell. We hypothesized that the ratios of $EPSP_2$ to $EPSP_1$ would vary between the conditions with ACPA, AM 251 and the baseline. This would have indicated that Ca^{2+} concentrations were different after the application of an agonist. Although our data suggests that ACPA does have an effect on inhibiting the release of neurotransmitters in the crayfish neuromuscular junction, our evidence shows that ACPA does not mediate synaptic inhibition of neurotransmitters through inhibition of calcium ion channels. This research has the potential to redefine the current studies of endocannabinoids receptors in invertebrate and augment our understanding of their role in the nervous system.

INTRODUCTION

Retrograde messenger systems provide a means for a neuron to control its pre-synaptic output. Endocannabinoids are the messengers in these retrograde systems. Endocannabinoids are released from the postsynaptic cell, diffuse across the membrane to the presynaptic cell, where the endocannabinoids bind to specific G-protein-coupled receptors and inhibit neurotransmitter release for tens of seconds or longer (Kreitzer, 2002). The release of a retrograde messenger causes presynaptic inhibition. This leads to depolarization-induced suppression of inhibition (DSI) or excitation (DSE) in central neurons (Guo and Ikeda, 2003). Guo and Ikeda propose that the sequence of events underlying DSI/DSE in mammals starts with the depolarization of a postsynaptic neuron that stimulates the synthesis of endocannabinoids via Ca^{2+} influx through voltage-gated Ca^{2+} channels. Then, the newly synthesized endocannabinoid escapes the postsynaptic neuron and diffuses across the synaptic cleft to receptors. Next, the activation of G-protein coupled receptors results in inhibition of voltage-gated Ca^{2+} channels and subsequently the release of glutamate (Guo and Ikeda, 2003). These systems are believed to be present in mammalian vertebrates (primarily in the central nervous system otherwise known as the CNS) (Kreitzer, 2002). Other studies have examined the pathways through which endocannabinoids inhibit neurotransmitter release in non-mammalian vertebrates. McAllister et al (1999), experimented on the G protein-coupled inwardly rectifying potassium channels in frogs, as a mechanism of endocannabinoid effects. It was demonstrated that endocannabinoid receptors are present in vertebrates, mammalian and non-mammalian alike. Several previous studies have led us to believe that endocannabinoid receptors are present in invertebrates (Green, 2005). Furthermore, evidence suggests that endocannabinoid receptors exist in the peripheral nervous system (PNS) of invertebrates (Green, 2005). However, it remains undetermined whether endocannabinoids affect neurotransmitter release through calcium pathways, or another method. Although recent studies have identified the endocannabinoid receptors such as CB1, CB2 (and possibly CB3) and their presence in vertebrates as well as invertebrates, but even as new research develops, the pathways through which retrograde messengers function in invertebrates are not known (Freund et al., 2003). It is currently assumed that endocannabinoids inhibit neurotransmitter release via calcium channels in invertebrates, however this claim has yet to be supported. Building on this existing research, we indirectly examined the calcium influx in the presynaptic cell with and without the presence of an endocannabinoid agonist in *Procambarus clarkii*.

We hypothesized that endocannabinoid agonists would reduce the amount of neurotransmitters released by

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inhibiting calcium channels in the presynaptic cell of these crayfish. By applying the endocannabinoid agonist Arachidonylcyclopropylamide (ACPA), the neuromuscular junctions will imitate a cell exposed to endocannabinoids. Using standard electrophysiological techniques, we recorded $EPSP_1$ and $EPSP_2$ after paired-pulse-facilitation. Paired-pulse facilitation represents the presynaptic effect of residual Ca^{2+} (Jiang and Abrams, 1998). If the calcium channel is inhibited by the ACPA, then there will be less calcium in the presynaptic cell. The first EPSP would be lower and the second EPSP would be higher than normal due to a higher Ca^{2+} concentration gradient outside of the cell. We hypothesized that the calcium channels would be blocked the ACPA, and the ratio between the second and first EPSP would be greater than one.

However the evidence did not support the hypothesis. We found that endocannabinoid agonist ACPA and antagonist AM 251 did not significantly change the ratio between $EPSP_2$ and $EPSP_1$. Therefore the drugs did not alter the calcium influx in the presynaptic cell as predicted. Calcium is not the pathway through which endocannabinoids inhibit glutamate release in crayfish.

MATERIALS AND METHODS

Crayfish Preparation

After being iced for 15 minutes or until unresponsive, the tail of the *Procambarus clarkii,* which was supplied to the laboratory by Carolina Biologicals (North Carolina, USA), was separated from the thorax and the ventral part of the crayfish exoskeleton and all extraneous tissue were removed for the viewing of the extensor muscles. The crayfish was then placed in a bowl half filled with silicone elastomer, pinned down, and submerged in the crayfish saline. This solution was replaced approximately every 20 minutes to stretch the longevity of the tissue.

Chemical	**Concentration (mM)**
NaCl	196.0
KCl	5.4
$CaCl_2 2H_2O$	6.0
$MgCl_2 6H_2O$	10.1
Sodium Hepes Buffer	10
pH	7.4

Table 1. Low calcium crayfish solution

Chemical Application

The first chemical used in this experiment to mimic the actions of an endocannibinoid was the CB1 agonist ACPA (N-(Cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraenamide Arachidonylcyclopropylamide). The solution was made by adding a 100µl aliquot to a 100ml graduated cylinder filled with the crayfish saline. The aliquot was rinsed several times with the saline from the graduated cylinder to guarantee the use of all the ACPA. This solution was then inverted several times to mix.

When this chemical was applied, 90ml of the crayfish saline was removed from the bowl and 100ml of the ACPA solution was then added. Measurements were recorded immediately after application and every minute after for 10 minutes following the initial recording.

The second chemical used in this experiment was the CB1 receptor agonist AM 251 (N-(Piperidin-1-yl)-5-(4iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide). This drug effectively binds to endocannabinoid receptors to negate any effects that agonists like ACPA might cause. This solution was made a similar manner as that of the ACPA; first, a 50 µl aliquot of the drug was added to a 50ml graduated cylinder filled with the crayfish saline. The aliquot was then rinsed several times with the saline in the graduated cylinder to ensure all the solution was gone from the aliquot. Because each vial only made 50ml of solution, the process was done an additional time (100ml), and then inverted several times to mix.

Application of this drug was not only similar to ACPA, but followed it. The specimen was washed with the crayfish saline several times, upon being filled with the saline again. Once this was done, 90ml of the saline was removed from the bowl and 100ml of the new solution were then applied. Each minute after the application, for the next ten minutes, measurements were recorded and written down.

Electrophysiological Techniques

The crayfish were placed in the low calcium concentration saline underneath the microscope. Also found in the saline was a ground electrode. A fine-tipped microelectrode, filled with 3M KCl, was then attached to a micromanipulator and placed in the crayfish saline to measure the resistance (5MΩ -20MΩ). The voltage was zeroed to account for the junction potential.

The crayfish were stimulated using a technique involving a suction electrode, and the previously mentioned microelectrode. A nerve near the dorsal extensor muscles was sucked into the suction electrode until a tight seal was made. Paired-pulse stimulation of 10 volts was then used to stimulate the nerve located in the suction electrode. Additional settings of the stimulator

were a frequency of 0.2Hz, an inter-pulse delay of 25ms, and duration of 0.5ms. The microelectrode was then inserted into a dorsal extensor muscle cell to measure the resting membrane potential. Once this was found, the stimulator was turned on and the microelectrode was again used to measure the EPSPs of the muscle cell. The MacLab data acquisition system gave us the ability to view and analyze our data.

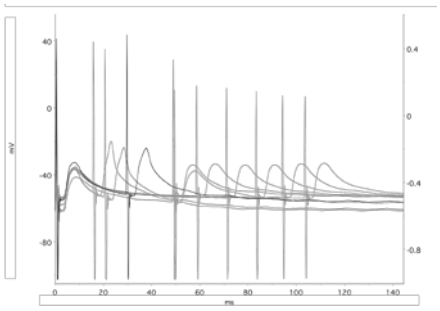

Figure 1. Preliminary experiments were conducted to distinguish the optimal delay required for this experiment. These experiments tested delay ranging from 15-100ms whereupon discovering 25ms to be ideal. The y-axis represents millivolts and the x-axis represents milliseconds.

RESULTS

In first part of our experiment we confirmed the effects of ACPA on the crayfish's neuromuscular junction. If ACPA was working correctly, it should lower the amplitude of $EPSP_1$ by inhibiting the release of neurotransmitters from the presynaptic cell.

Our results indicate that ACPA did lower the first EPSP of the paired-pulse facilitation. Typically the ratio of ACPA $EPSP_1$ and the baseline $EPSP_1$ was 0.7.

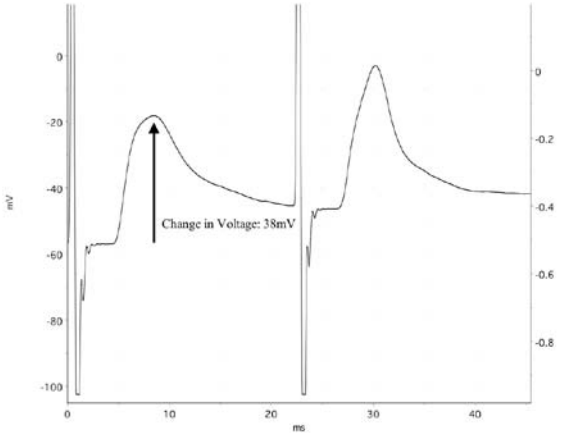

Figure 2. Baseline. This figure shows the $EPSP_1$ and $EPSP_2$ of a crayfish's neuromuscular junction with no added drugs. The arrow indicates the amplitude of the first EPSP. The y-axis represents millivolts and the x-axis represents milliseconds.

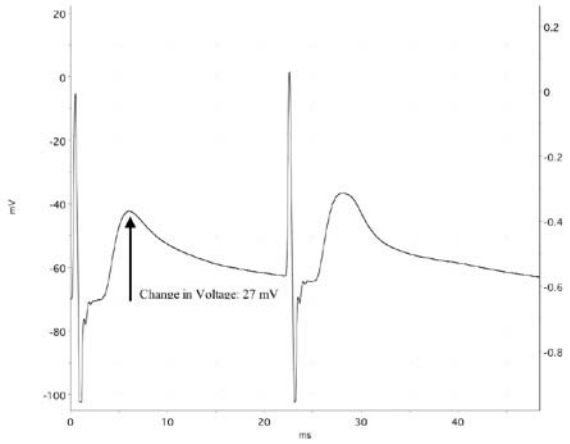

Figure 3. This figure shows the $EPSP_1$ and $EPSP_2$ of a crayfish's neuromuscular junction after the application of ACPA. The arrow indicates the amplitude of the first EPSP. The y-axis represents millivolts and the x-axis represents milliseconds.

After establishing that ACPA inhibits the release of neurotransmitters, we then tested the cells looking for an increase in facilitation. This required paired-pulse stimulation. The ratio between the second and first EPSP represents the Ca^{2+} concentration in the presynaptic cell. The ratio was then calculated using this formula:

$$\frac{EPSP_2}{EPSP_1}$$

Several of these measurements were taken before the application of the ACPA. The average ratio of the two EPSP measurements was 1.0352.

The ACPA results were measured following the measurement of the baseline results. The ratio between the first EPSP and the second EPSP was calculated to be 1.059. Using this ratio, we conducted a standard t-test, and determined that there was no significant difference between the baseline and the ACPA tests. The p-value was greater than 0.556, thus we did not reject the null hypothesis. The ratios between the first and second EPSP were not significantly different from the ratios of the baseline conditions.

After applying ACPA, the specimen was washed and AM 251 was added to the new Ringer's solution. The results from AM 251 come from the EPSPs measured in the crayfish after the ACPA was washed out and AM 251 was added. The ratio between $EPSP_1$ and EPSP2 was found to be 09939. A t-test was conducted on the ratio of the AM 251, comparing the ratio between the first and second EPSP of the baseline with the ratio of the ACPA experiment. The p-value was 0.700, indicating that we should not reject the null hypothesis. Once again, the drug had no significant affect on the ratio between the first and second EPSP.

Even comparing the ratios between ACPA and AM 251 conditions, the p-value was greater than 0.5. With a p-value of 0.758, there is no significant difference between ACPA and AM 251 ratios as seen in figures 2 and 3. Figure 3 visually highlights the similarities between the spread of data.

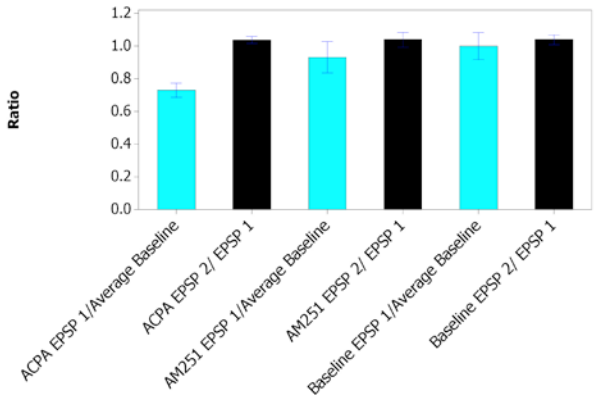

Figure 4: The blue bars represent the 1st EPSP of each treatment compared to the 1st baseline EPSP. The black bars are the same as figure 1. They are the average of EPSP2 to EPSP1 ratio under different treatments. Each of the standard error bars represents one standard error from the mean. It should be noted that there were 46, 72, and 11 recording for the Baseline, ACPA, and AM251. After doing a t-test, we found that the ratios between first EPSP of baseline and ACPA were significantly different. ($p = 0.005$). Also the ratios between baseline and AM251 were not significantly different ($p = 0.593$).

In comparing the ratios of $EPSP_2$ to $EPSP_1$ under baseline conditions, application of ACPA and application of AM 251, there is no significant difference between these ratios. The evidence does not support our hypothesis that ACPA inhibits Ca^{2+} channels.

DISCUSSION

The present study demonstrates that the agonist ACPA and antagonist AM 251 do not significantly change the ratio between the first and the second EPSP during pair-pulse stimulation. This implies that calcium channels are not the pathway through which endocannabinoids regulate neurotransmitter release. This finding contradicts our hypothesis that calcium channels were responsible for the inhibition of neurotransmitter with the presence of endocannabinoids.

In previous research, ACPA and AM 251 altered the amplitudes of the EPSP (Green, 2005). However, this study did not examine the ratio of $EPSP_2$ to $EPSP_1$ after pair-pulse stimulation. Our results invalidate the assumption that endocannabinoids inhibit neurotransmitter release through the inhibition of Ca^{2+} channels. If Ca^{2+} channels are not responsible, then endocannabinoids function through another mechanism in the presynaptic cell. It is possible that the SNARE complex or another pathway may be responsible for the inhibition of neurotransmitter release.

Future studies are essential for verification of our results. Other experiments could be performed as well in attempt to rule out old knowledge and/or discover new knowledge. Other chemicals could be used to rule out any possibilities for why these results occurred. For instance, glutamate applied in small quantities onto the synapse of the motor nerve could be used to confirm the location of the receptors (Green, 2005). Otherwise, different agonists and antagonists could be used. With different endocannabinoids come different responses, so a different endocannabinoid could be used. The endocannabinoid anandamide, for instance, is known to exert its physiological effects through endocannabinoid receptors, but unlike ACPA and most endocannabinoids, it inhibits T-currents independently from the activation of CB1/CB2 receptors, G-proteins, phospholipases and protein kinase pathways (Chemin, 2001). Using a different endocannabinoid or agonist such as this could illuminate otherwise unknown endocannabinoid pathways.

The difference between the use of CB1 receptors and CB2 receptors should be noted in the future. Although both are endocannabinoid receptors, in a study by Begg et at. (2001), the selective CB1 receptor ligand inhibited an outward current while the CB2 receptor did not. In the future, the difference between these two receptors could be further examined.

On another note, with proper equipment (and sufficient time allowance) immunofluorescnce imaging could be helpful in identifying the exact location of these CB receptors. Information into the origin of the CB receptor could potentially be provided by a gene analysis. Determining the origin of these receptors could prove useful too. Our research experiment necessitates further studies on invertebrates and their nervous system, more specifically, retrograde signaling such as that of endocannabinoids.

This newfound information, though not damaging to the discovery of endocannibinoids in the neuromuscular junction of invertebrates, provides further information regarding the nervous system of these specimens and invertebrates alike.

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