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The effect of endocannabinoids at the crayfish neuromuscular junction

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ABSTRACT

Endocannabinoids are a widely studied family of molecules that act as neurotransmitters and influence synaptic plasticity. Endocannabinoids have been extensively studied in the frog and lizard neuromuscular junction. However, this study assessed the effects of arachidonylcyclopropylamide (ACPA), an agonist to the CB1 cannabinoid receptor, in the crayfish neuromuscular juction. We found that the addition of ACPA decreased the excitatory post synaptic response. We cannot conclusively determine if an endocannabinoid analogue utilizes the CB1 receptor pathway in the crayfish neuromuscular junction.

INTRODUCTION

Cannabinoids are a group of molecules that function as neurotransmitters and include Δ^9-tetrahydro-cannabinol, or THC, the active ingredient in marijuana. These molecules have motor as well as psychoactive effects that occur upon their interaction with receptors on nerve cell membranes. There are two subtypes of cannabinoid receptor, CB_1 and CB_2, and activation of either receptor leads to activation of a G-protein, then decreases in levels of protein phosphorylation and intracellular cyclic AMP (cAMP). The endogenous cannabinoids, or endocannabinoids, which activate these receptors are the fatty acids 2-arachidonylglycerol (2-AG) and anandamide (Sánchez-Pastor et al. 2007).

At the frog neuromuscular junction, anandamide has been shown to activate CB_1 receptors and block the enzyme adenylate cyclase, which converts ATP into cAMP (Van der Kloot 1994). Administration of cannabinoids at the frog neuromuscular junction also decreases MEPP frequency through the activation of presynaptic CB_1 receptors. These receptors interact with $G_{i/o}$ proteins and activation seems to lead to the blockage of N-type Ca^{2+} channels (Sánchez-Pastor et al 2007).

Also in the frog neuromuscular junction, two CB_1 receptor agonists, WIN and ACEA, have opposite effects on quantal acetylcholine (ACh) release. Introduction of WIN and the CB_1 antagonist AM 251 nullified WIN's depression of ACh release. Introduction of ACEA yielded an increase in ACh quantal release, and this increase was not negated when AM 251 was introduced. Silveira et al. (2010) discovered that ACEA targets the vanilloid receptor (TRPV2) and is blocked by capsazepine, the TRPV1 antagonist.

Cannabinoids have also been studied in the lizard neuromuscular junction. Newman et al. (2007) examined the presence and location of CB_1 receptors via fluorescence microscopy. They also questioned whether M_3 muscarinic ACh receptors inhibit neurotransmitter release via endocannabinoids. By utilizing multiple combinations of agonists and antagonists, the researchers also determined whether Ca^{2+} influx was actively being inhibited in the nitric oxide-dependent system. The researchers discovered that *Anolis carolinensis* do in fact have CB_1 receptors that are localized on the presynaptic nerve terminal. When muscarine and AM 281, a CB_1 antagonist, were applied they determined that the depression of EPP was due to cannabinoid receptors. They also discovered that endocannabinoids cause a decrease in transient Ca^{2+} that accounts the later depression of synaptic transmission. In conclusion, Newman et al. (2007) discovered that through the M_3 muscarinic receptor mediated system, muscle cells synthesize endocannabinoids (2-AG) to bind to CB_1 receptors on the nerve cell.

Our study hoped to fill the void bridging endocannabinoids in the crayfish NMJ, which have shown to play a part in synaptic plasticity. Ultimately we hope to draw comparisons with other previously studied model organisms. By drawing these comparisons, we can further test if the specific mechanisms published in the frog and lizard also are expressed in the crayfish. The goal(s) of this study are/were to test whether the crayfish NMJ is sensitive to activation of CB1 receptors, and to determine whether the response seen, due to cannabinoid addition, affects the CB1 receptor pathway.

We hypothesized that, similar to the lizard and frog NMJ, the crayfish would show analogous effects when the CB1 agonist, ACPA, was introduced – a decrease in excitatory post synaptic potential (EPSP). We also hypothesized that any effect seen due to the addition to endocannabinoids would be caused by augmentation of the CB1 receptor.

MATERIALS AND METHODS

Experimental preparation and solutions

Crayfish were first immersed in crushed ice for approximately 10 min. Next, we removed the tail and isolated the tail extensor muscle. This muscle and the attached exoskeleton were pinned down in a Sylgard®-coated dish. We covered the preparation in approximately 40 ml of fresh physiological saline solution (5.4 mM KCl, 196 mM NaCl, 13.5 mM $CaCl_2 \bullet H_2O$, 2.6 mM $MgCl_2 \bullet H_2O$, 10 mM HEPES).

Arachidonylcyclopropylamide (ACPA) was obtained in Tocrisolve®, a soy oil and water emulsion. It was diluted in physiological saline before being added to the crayfish preparation at an approximate final concentration of 10 µM. ACPA was purchased from Tocris Cookson (Ellisville, MO, USA).

Electrophysiology

End-plate potentials were evoked by stimulating the motor nerve axon at 1-10 V for 5 ms, at 0.2 Hz. We used a glass micropipette filled with 3 M KCl and with resistance between 5 and 15 MΩ to measure EPPs and recorded data using a MacLab data acquisition system (AD Instruments, Colorado Springs, CO, USA).

For our first experiment, we recorded from a number of separate, randomly chosen muscle cells both before and after treatment with ACPA. The average EPP amplitudes in each condition were compared using a student's *t*-test (two-sample assuming equal variance).

For our second experiment, we recorded from several separate, randomly chosen muscle cells before the addition of ACPA. After drug treatment, EPPs were measured in one cell every 30 sec for 2 min and then every 1 min for 18 min. Mean EPP amplitudes before and after treatment were compared as above.

For our third experiment, we recorded from one muscle cell both before and after ACPA treatment. EPPs were averaged over 8 stimuli for recording. We recorded two averages before treatment, and several averages at a number of time intervals after treatment. In Figure 2, each bar after ACPA addition is the mean of four recordings. Mean values for consecutive pairs of averages were compared using a student's *t*-test (two-sample assuming equal variance).

RESULTS

In order to determine whether cannabinoids have an effect at the crayfish neuromuscular junction, we performed electrophysiology of the tail extensor muscle. We recorded EPSPs while stimulating the motor nerve at low frequency, both with and without the presence of the CB_1 receptor agonist ACPA.

We first compared the average value of five EPSPs recorded from randomly selected muscle cells before and after ACPA treatment (Figure 1). This recording did not show a significant change in EPSPs due to the addition of ACPA.

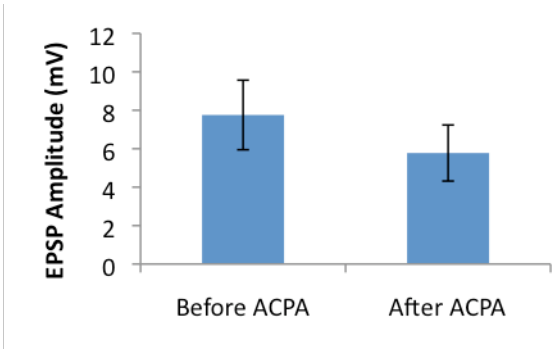

Figure 1. Mean EPSP amplitude. Each bar represents the mean of six EPSPs, and all data was recorded from one crayfish muscle preparation.

Second, we performed a time course experiment in which we recorded from one cell both before and after ACPA treatment (Figure 2). We found a significant difference between the mean EPSP before ACPA addition (35.60 mV) and the mean EPSP after addition (15.66 mV) ($p = 0.024$). ACPA decreased the EPSP.

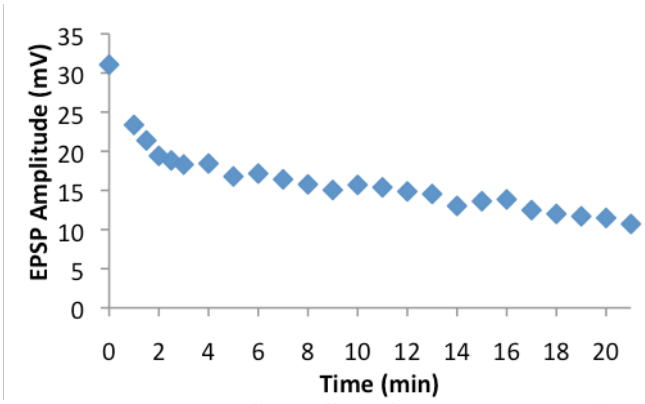

Figure 2. Time course of the effect of ACPA on the crayfish neuromuscular junction. ACPA was added at 1 min. Each point represents one recorded EPSP (n=1). After ACPA addition, EPSPs were recorded every 30 s for 2 min and then every 1 min for 18 min.

Third, we performed an experiment in which we recorded EPSPs averaged over eight nerve stimuli, also in one cell (Figure 3). We found a significant difference between the mean EPSPs recorded before treatment with

ACPA and those recorded from 0 to 2.5 minutes, 3.5 to 6 minutes, and 7 to 10 minutes after treatment (p values = 0.002, 0.0003, and 2×10^{-5}, respectively). In this experiment, as well, ACPA decreased the EPSP.

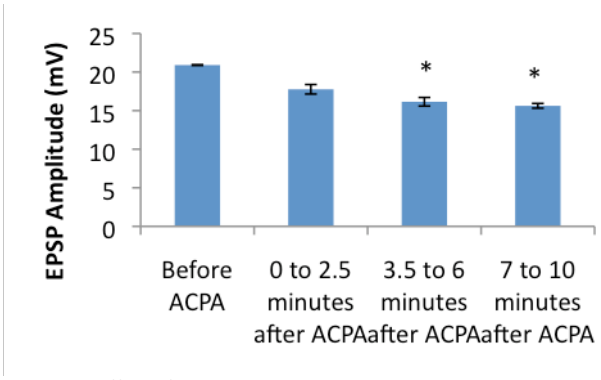

Figure 3. Effect of ACPA on average EPSP amplitude (± SEM). Each bar after ACPA addition represents the mean of four average recordings, while the bar before ACPA represents the mean of two. * indicates $p < 0.05$ compared to EPSP before ACPA. (n=1)

DISCUSSION

Experiment 1 did not provide any statistically significant results (Figure 1). This lack of significance is primarily since there is a large variation in EPSP amplitude across different muscle cells. To counteract this difficulty, it might help to survey cells that were similar distances from the nerve that was being stimulated. We could have also used a larger sample size, which might have allowed us to find a statistically significant result. Additionally, we did not allow any time to pass for our first measurement after we added ACPA, which may have affected the results of our experiment. Due to these limitations, the results of this experiment neither validate nor disprove our hypothesis.

In the second trial, the addition of ACPA showed a statistically significant effect of decreasing the EPSP 43.9% (Figure 2). With the exception of a few points, the time course showed a consistent decrease as each successive measurement was taken. This was the first indication that the addition of a CB_1 receptor agonist would decrease the EPSP in the crayfish NMJ. This result supports our hypothesis.

In our third experiment, we found a significant effect of ACPA (Figure 3). The agonist led to EPSP depression which increased over time. Although the methodology of this experiment was somewhat different from the previous one, they are similar enough that their results reinforce each other's validity. Therefore, this experiment supports the conclusions we drew from our second experiment. Overall, we find that the CB_1 receptor agonist ACPA leads to EPSP depression which increases over time. We hypothesize that this consistent enhancement in EPSP depression may be caused by continuing activation of some receptor which responds to ACPA. This may be the CB_1 receptor or an analogue of the CB_1 receptor.

One of the major limitations in our study was that all three trials employed different methodologies. This inherent lack of continuity calls into question the replication and significance of our results. The opportunity to replicate each of our experiments would have greatly increased the confidence with which our results may be interpreted. However, because our second two experiments are methodologically similar, they can be viewed as confirming each other's results in the absence of exact replication.

Another major limitation is that we were not able to probe the question of whether an endocannabinoid analogue affects a CB_1 receptor in the crayfish NMJ, utilizing both an agonist and an antagonist. If blocking any present CB_1 receptors with an antagonist eliminated the EPSP depression in response to agonist treatment, we would be able to conclude that depression is due to a CB_1 receptor. Although we confirmed our hypothesis that adding a CB_1 agonist does depress EPSP amplitude, we cannot definitively determine whether the effect of endocannabinoids influences the CB1 receptor specifically in the crayfish NMJ.

Future studies teasing apart the relationships of endocannabinoids in the crayfish NMJ would help to extend the results of this study. Further replication of our experiments is needed to strengthen our conclusions. Moreover, it would be helpful to determine the involvement of the CB_1 receptor using an antagonist. Additionally, further studies could use fluorescence microscopy to spatially locate CB_1 receptors on the synapse, for example using fluorescently tagged CB_1 antibodies, or add various combinations of agonists and antagonists to the NMJ. In conclusion, we determined that addition of an endocannabinoid analogue, ACPA, decreased the EPSP at the crayfish NMJ.

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