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Pharmacological Evidence for Cannabinoid Receptors in Glutamatergic Synapses at the Crayfish Neuromuscular Junction

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ABSTRACT

Endogenous cannabinoids and cannabinoid receptors are involved in retrograde messaging in neurons, a system believed to be restricted to the vertebrate nervous system. However, recent studies have begun to find evidence of both endogenous cannabinoids and their receptors in some invertebrates as well as at the neuromuscular junction of vertebrates. This experiment represents our attempts to link these two distinct areas of cannabinoid research by looking for evidence of cannabinoid receptors in the glutamatergic neuromuscular junction of crayfish (*Procambarus clarkii*). Using a combination of standard electrophysiological techniques and the chemical application of cannabinoid receptor agonists and cannabinoid receptor antagonists, we measured the excitatory post synaptic potentials (EPSPs) at this neuromuscular junction and compared the amplitudes of the EPSPs during the application of agonist, antagonist, and agonist with pre-applied antagonist. These data suggest that there are in fact cannabinoid receptors in the crayfish neuromuscular junction and that these receptors play a role in mediating synaptic inhibition. This research has the potential to redefine the evolutionary history of cannabinoids receptors and augment our current understanding of their role in the nervous system.

INTRODUCTION

Retrograde messenger systems provide a means for a neuron to control its pre-synaptic output. Recent research has shown that endogenous cannabinoids are key elements in a retrograde messenger system that is found in many vertebrates. This system includes several cannabinoid receptors (most commonly CB1) that modulate synaptic transmission. Much of the current research in the area of cannabinoids has been dedicated to vertebrates and, more commonly, the mammalian central nervous system (Levenes et al., 1998). While receiving some attention, relatively little work has been devoted to the study of endocannabinoids in the vertebrate peripheral nervous system or at any site in invertebrates.

Cannabinoids

Scientists have actively investigated cannabinoids for the past thirty years, though their research was limited to exogenous compounds such as delta-9-tetrahydrocannabiniol (THC), the active ingredient in marijuana (Montgomery and Madison, 2001). The presence of cannabinoids in neurological research was minimal until the 1990s, when the discovery and characterization of several endogenous compounds with cannabinimetric activity, or endocannabinoids, led to a heightened interest in their role in the nervous system (Freund et al., 2003). The identification of these endocannabinoids—anandamide, 2-arachidonoylglycerol (2-AG), virodhamine, and noladin ether—led to the discovery of their corresponding receptors, CB1 and CB2 (there is possibly a third receptor, CB3) (Freund et al., 2003).

Mechanisms of Cannabinoid Receptors

Cannabinoid receptors are known to operate in three broad categories of synapses: glutamatergic synapses where a depolarized-induced suppression of excitation (DSE) occurs (Kreitzer and Regehr, 2001), GABAergic synapses, where a depolarized-induced suppression of inhibition (DSI) occurs (Ohno-Shosaku et al., 2001), and cholinergic synapses in which a different form of automodulation occurs (Lindgren et al., unpublished). Various studies have investigated the biochemical processes—synthesis, transport, and inactivation—of these endogenous chemicals, and have established that cannabinoid receptors are located presynaptically (Ohno-Shosaku et al., 2001).

Recent research has found that these retrograde messengers are dependent on high concentrations of Ca^{2+} (Kreitzer and Regehr, 2001). Studies have shown that endocannabinoid release can be incited by a large influx of postsynaptic Ca^{2+} after a strong stimulation (Kreitzer and Regehr, 2001). Once released from the postsynaptic cell, these endocannabinoids diffuse to the presynaptic cell, activating CB receptors. Scientists have hypothesized that the cannabinoid receptors function via a G-protein mediated inhibition of Ca^{2+} channels, decreasing calcium influx following action potentials, which in turn reduces the amount of glutamate released (Montgomery and Madison, 2001).

Cannabinoid Lineage and Prevalence

Both CB1 and CB2 receptors exist throughout vertebrates (Elphick et al., 2003). Research of invertebrates, however, is still in its incipience. Anandamide, 2-AG, cannabinoid binding sites, and anandamide hydrolysis enzymes have been identified in the feeding response mechanism of the cnidarian *Hydra vulgaris*, one of the first organisms to develop a neural network (De Petrocellis et al., 1999). The nematode, *Caenorhabditis elegans*, however, lacks cannabiniod receptors (Elphick and Ergertová, 2001). Gene analysis of the insect *Drosophila melanogaster* failed to reveal the presence of either receptors or endocannabinoids (Elphick and Ergertová, 2001). In the locust, *Schistocerca gregaria,* radio-labeled cannabinoids failed to locate endocannabinoids or their receptors (Elphick and Ergertová, 2001).

Recent gene analysis suggests that the prototype of the CB1 and CB2 cannabinoid receptors originated in deuterostomian invertebrates (Elphick et al., 2003). This research, performed on the urochordate *Ciona intestinalis*, identified the gene CiCBR that codes for a protein similar to vertebrate cannabinoid receptors (Elphick et al., 2003). The CiCBR gene is phylogenetically distinct from vertebrate cannabinoid receptors, hinting at a common ancestor of the receptor (Elphick et al., 2003). This information has led some scientists to hypothesize that cannabinoid receptors, though they have developed in deuterostomian invertebrates (which include echinoderms, hemichordates, urochordates and cephalochordates as well as vertebrates) might be absent from all protostomian invertebrates (which include arthropods, nematodes, mollusks, annelids, platyhelminthes and several other phyla) (Elphick et al., 2003).

Cannabinoids at the Neuromuscular Junction

An early study of endogenous cannabinoids at the neuromuscular junction was performed in frogs, where anandamide was found to inhibit adenylate cyclase (Van der Kloot, 1994). This inhibition prevents the increase in quantal size normally seen after application of a hypertonic solution (Van der Kloot, 1994). Research on the neuromuscular junction in the mouse colon suggested that cannabinoid receptors are present in cholinergic neurons, nonadrenergic, and noncholinergic neurons (Storr et al., 2004). Studies of lizard cholinergic neuromuscular junctions have also suggested the presence of CB1 receptors in these organisms (Lindgren et al., unpublished).

From these various pieces of information we decided to perform an analysis of the role of cannabinoids in the peripheral nervous system as well as their role in invertebrates. The demonstration of cannabinoid signaling mechanisms in the crayfish neuromuscular junction would suggest that cannabinoid signaling systems are much more prevalent in the animal kingdom than previously thought. If evidence of these systems is not found in the crayfish neuromuscular junction, it would suggest that the evolution of the cannabinoid retrograde messenger system occurred in concordance with the theories based on current evidence in deuterostomian invertebrates.

The specific goal of our research is to detect cannabinoid receptors at the invertebrate neuromuscular junction. Although studies on some protostomian invertebrates preclude cannabinoid receptors, the ubiquity of such receptors in other glutamatergic synapses and the existence of cannabinoid receptors at the neuromuscular junction in higher level organisms suggest that some protostomian invertebrates might express cannabinoid receptors. Accordingly, we looked for evidence of cannabinoid receptors in the glutamatergic neuromuscular synapse of the crayfish, *Procambarus clarkii.* We hypothesized that exposure of the neuromuscular junction to a cannabinoid receptor agonist would result in a reduction in excitatory post-synaptic potentials (EPSPs) and that this effect would be prevented or notably reduced by a receptor antagonist. The application of a cannabinoid receptor agonist did in fact cause EPSP amplitude reduction, a reduction that was missing when the crayfish was pre-applied with a cannabinoid receptor antagonist. Because the changes observed agreed with the changes predicted, we concluded that cannabinoid receptors probably exist at the neuromuscular junction of this organism.

MATERIALS AND METHODS

Crayfish Preparation

We excised the ventral portion of the crayfish exoskeleton and removed all extraneous tissue to expose the extensor muscles. After pinning the crayfish tail in the observation dish, we submerged it in Ringer's solution (Table 1), which was replaced approximately every 20mins during the experiment to extend the longevity of the tissue.

Chemical	**Concentration (mM)**
NaCl	205.0
KCl	5.40
$CaCl_2$	135.0
$MgCl_2$	2.6
Tris(pH 7.4)	10.0

Table 1. Composition of standard crayfish Ringer's solution

Chemical application

Two chemicals were used to determine the existence of a CB1 receptor at the neuromuscular junction. The first, CB1 agonist *N*-(Cyclopropyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide or Arachidonylcyclopropylamide (ACPA), was added to the bath in a concentration of ~10 μM after a period of several minutes in which control readings were taken to determine if any potentially quick changes would occur independently of ACPA application (Lindgren et al., unpublished). We pipetted 10μL of stock ACPA (~ 1 to 1000 dilutions to achieve the ~10 μM end concentration) into the solution over the nerve and muscle involved in the recording. This allowed us to keep the electrode in the same cell for the duration of the recording session.

ACPA is highly insoluble in aqueous solutions and was first dissolved in Tocrisolve^{TM} 100 to facilitate dissolving into the Ringer's solution. ACPA acts on CB1 receptors in a manner similar to endocannabinoids, reducing the amount of glutamate released by the presynaptic cell through (it is believed) the indirect inhibition of calcium channels. Because glutamate evokes an excitatory response at the neuromuscular junction, this change can be measured by a reduction of the magnitude of EPSPs.

After a series of trials in which a decrease in the magnitude of EPSPs was recorded, a second set of trials were performed, using the aforementioned procedures. Control readings were taken to establish that the baseline EPSPs maintained a constant amplitude after which point the crayfish was bathed in Ringer's solution containing a CB1 receptor antagonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251). AM 251 acts by preventing the binding of endocannabinoids or cannabinoid receptor agonists and negating any effects they might incite through binding. AM 251 was dissolved in DMSO to facilitate its dissolving into the Ringer's solution. It was added in a concentration of 5μM (Lindgren et al., unpublished). After several minutes ACPA was added again to verify that the decrease previously noted in the magnitude of EPSPs was due to the effects of ACPA on a CB1 receptor and not the natural deterioration of the nerve or a side effect of Tocrisolve^{TM} 100. DMSO has been used in enough similar experiments that we felt it was not necessary to control against any effects it might have.

Electrophysiological Techniques

For the both sets of trials, a constant low-frequency stimulation (<0.5hz) was used over an extended period of time to allow any effects (short or long term) of the chemicals to be seen. The crayfish were bathed in standard crayfish Ringer's solution and were then placed beneath a dissecting microscope with an indifferent, ground electrode placed in the Ringer's solution. We attached a fine-tipped glass microelectrode, filled with 3M KCl, to a micro-manipulator, then lowered it into the Ringer's solution to measure resistance (all electrodes recorded from were in the range of 5MΩ and 20 MΩ). To account for junction potential we zeroed the voltage with respect to the Ringer's solution on the recording apparatus.

The crayfish were stimulated using two techniques, depending on the quality of the preparation. The first technique involved a suction electrode, a glass microelectrode with a larger diameter and smooth, fire polished tip. We sucked a nerve near the dorsal extensor muscle into the electrode and created a tight seal between the inside of the suction electrode and the nerve. We then sent an electrical current through the electrode to stimulate the nerve inside it. When there were no visible nerves to use with the suction electrode technique, a second technique was used. A two-pronged electrode was positioned on the extensor muscle in such a way that it straddled the gap between adjacent segments of the crayfish tail. This allowed a current to move across the gap, stimulating the nerves between the muscle segments. After we observed twitching of the muscle, we inserted a recording microelectrode into a dorsal extensor muscle cell to measure EPSPs as well as monitor the resting membrane potential, which ranged from ~ -40mV to -70mV. A MacLab data acquisition system provided us with a means to view and analyze our data. The primary data from each of the experiments was normalized to show the percent difference between the experimental and control EPSP amplitudes. All data has been averaged to provide more representative conclusions.

RESULTS

Electrophysiological recordings were used to determine the effects of a cannabinoid receptor agonist (ACPA) and a cannabinoid receptor antagonist (AM 251) on synaptic transmission in the crayfish glutamatergic neuromuscular junction. Nerves from the exposed crayfish fast extensor muscle were stimulated at a low frequency; the EPSPs resulting from this stimulation were measured for the duration of the stimulus before and after the applications of both chemicals. The data was summarized by averaging short time intervals and total exposure time periods. These averages were then normalized in each trial, and the resulting normalized data were averaged once more.

ACPA Agonist Trials

Trials in which ACPA was applied directly over the nerve and muscle produced consistently a reduction of varying degrees in EPSP amplitude (Figure 2): while

some trials showed no substantial drop in EPSP amplitude, others showed decreases of around 50% (Figure 1 and Figure 3). The timeframe for these drops took between 2 minutes and 10 minutes after ACPA application. Initial drops in EPSP amplitude were typically seen around 2 minutes after application of ACPA, though the differences here could have been the result of the variability in the application procedure (application rates are dependent on how long the ACPA and Tocrisolve™ 100 solution took to dissolve to the nerve). The rate of decrease (Figure 2) between t=1 and t=2 is fairly stable, after which time the drop becomes more precipitous. In Figure 3 a sharp drop can be seen approximately one minute after ACPA application. This was the quickest and strongest drop in EPSP amplitude observed during this research. Usually, after approximately ten minutes the EPSP amplitudes stabilized and showed no further substantial changes save those reflecting the natural decline of nerve impulse due to decay of the organism. In one trial the ACPA was rinsed from the crayfish specimen before being reapplied approximately twenty-five minutes later. The reduction recorded following reapplication was much stronger than that recorded during the first exposure to ACPA, implying that residual ACPA can augment the effects of the reapplied ACPA to provide a larger reduction in EPSP amplitude.

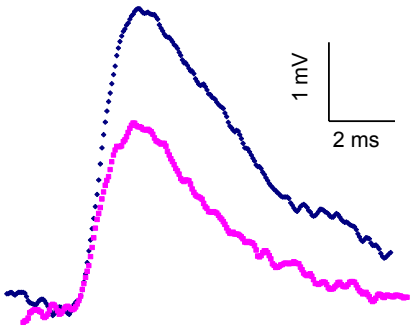

Figure 1. EPSP Traces. Both of these traces are examples of the shape of an EPSP. The upper trace is a baseline EPSP prior to ACPA application. The lower trace is an EPSP several minutes after the application of ACPA. Note the large reduction in EPSP amplitude caused by ACPA.

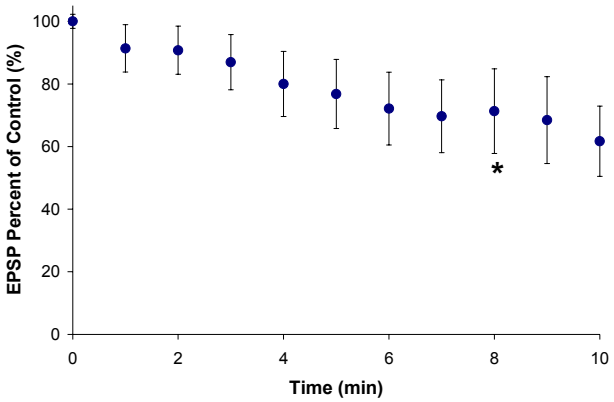

Figure 2. Effect of ACPA on EPSP amplitude. This graph shows the relative time scale and the average reduction of EPSP amplitude as seen after ACPA application (t=0). n=6 for t=1 through t=7. * note that there were data points missing for one trial (see Figure 2) n=5 for t=8 through t=10 which accounts for the small temporary increase in % of control. Data points represent an average for that time period and all data was also normalized in comparison with the control for that trial. t=0 corresponds to the percentage at which all other data points were normalized. t=0 was calculated from the average control EPSPs for each trial in this group.

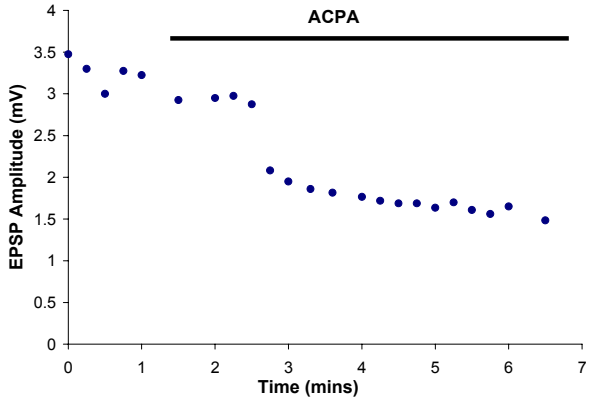

Figure 3. EPSP amplitude after ACPA application. In this trial ACPA was applied at ~1.5 min and the large reduction occurred ~1 minute after the application.

AM 251 with ACPA Antagonist Trials

Trials in which CB1 receptor antagonist AM 251 was applied to the crayfish supported the conclusions drawn from the results of the first trials, in which only ACPA was applied. Pre-application (application before ACPA) of AM 251 produced no marked change in EPSP amplitude (an effect that could indicate that there is an absence of endocannabinoids within the tissue). Some trials showed lower or higher EPSP amplitudes after application, but the average change was minimal. ACPA was applied several minutes (on average about eight) after AM 251. Data from these trials show that there was little or no reduction in EPSP amplitude following application (Figure 4).

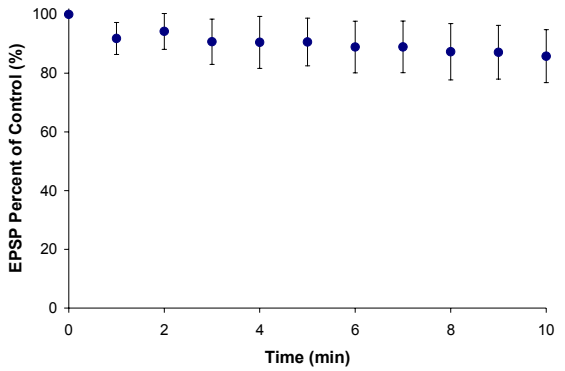

Figure 4. Effect of ACPA on EPSP amplitude with pre-applied AM 251. t=0 corresponds to the average EPSP % of control during AM 251application and prior to ACPA application. n=4 for all data points. Note the little to no reduction in EPSP amplitude of the ten minute interval.

This effect of AM 251 on the amplitudes of EPSPs demonstrates that the reduction noted in the ACPA-only trials can be prevented; that is, the reductions are due specifically to the effects of ACPA on cannabinoid receptors and not due to non-specific chemical effects of ACPA, natural nerve decomposition, lowering of junction potential, or a number of other environmental factors.

The general trends of ACPA and AM 251 application can be found in Figure 5. ACPA on average reduced EPSPs 40% from control. This evidence was produced fairly consistently over the trials, as can be seen from the relatively low standard error (~±6%). The AM 251 (Figure 5) shows a drop of about 10% from control; however these results gave a slightly larger standard error (~±12%) indicating greater variability in these readings. The ACPA application after AM251 pre-application shows a drop of about 20%, but again, with a fairly large standard error (~±15%). Despite the differences in the standard error these observations agree with our predictions.

Data Analysis

To determine whether these changes were due to variability in this experiment or to other extraneous variables, we statistically analyzed the average percent from control for the each trial under the different conditions. We used a two tailed t-test (two sample, assuming equal variances) with $\alpha<0.05$. The difference seen between the control values and the ACPA only trials were determined to be statistically significant (p=0.000136). The difference between the control and AM 251 was not statistically significant (p=0.471578), nor was that between the control and pre-applied AM 251 with ACPA (p=0.236949).

We also analyzed the relationships among the individual conditions, and found that the difference between ACPA only and AM 251 only was marginally significant (p=0.059174). However, the difference between ACPA and AM 251 with ACPA was not statistically significant (p=0.328827), nor was that found between AM 251 only and AM 251 with ACPA (p=0.583685). All of these tests provide substantial support for our initial hypothesis.

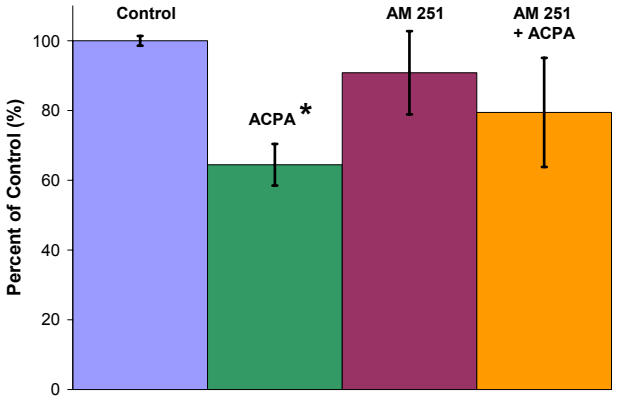

Figure 5. Percent reduction of EPSPs during ACPA application, AM251 application and ACPA application after pre-application of AM251 n=6 for ACPA. n=4 for both AM 251 and AM 251 + ACPA. * Statistically significant ($p<.05$) when compared to control and marginally significant when compared to AM 251($p\approx.05$)

DISCUSSION

EPSPs showed a statistically significant decrease in magnitude following application of the cannabinoid receptor agonist, ACPA. When the addition of ACPA was preceded by the application of AM 251, a cannabinoid receptor antagonist, the reduction in magnitude was not significant (if present at all). This supports our prediction that the application of ACPA would produce a statistically significant reduction in EPSP amplitude. Our data also support the hypothesis that a cannabinoid receptor antagonist would reverse this reduction, or at least inhibit it. The first set of data suggests the presence of CB1 receptors at the crayfish neuromuscular junction while the second set of data significantly lowers the possibility that non-specific or environmental factors caused the reduction.

The effects of ACPA and AM 251 in our experiment most likely occurred pre-synaptically, based on the evidence that in almost all previously studied systems the receptors are located pre-synaptically. ACPA and other receptor agonists act by simulating the ligands that typically bind to the receptors, or by enhancing the effect of the ligands (for example, by keeping them bound to the receptor for a longer duration) that are already

present at the site of the receptor. In this instance, the receptor receives endocannabinoids that reduce the amount of glutamate leaving the cell. Glutamate evokes an excitatory response in the post-synaptic potential that is lessened in magnitude when endocannabinoids or CB1 receptor agonists are present. Endocannabinoids are released after a calcium influx stimulates their production in the post-synaptic cell. Once released, they diffuse across to the pre-synaptic membrane where they bind to a receptor and incite a G-protein mediated suppression of calcium, possibly by blocking the voltage-gated calcium channels (Montgomery and Madison, 2001). The reduced EPSPs we recorded suggest that we are watching the effects of this system, implying the existence of CB1 receptors in the crayfish neuromuscular junction.

Cannabinoid antagonists, in turn, act by preventing cannabinoids from binding to the receptors, allowing glutamate to be released in normal (or, in cases where endocannabinoids commonly bind to CB1 receptors, heightened) amounts. The lowered reduction rate in trials in which AM 251 was applied further demonstrates that CB1 receptors probably exist at this synaps.

Future studies are needed to verify our results. Other chemicals, techniques, or recording preparations could be implemented to rule out any other possibilities for why the observed reduction could have occurred. Immunofluorescence imaging could be helpful in localizing these receptors specifically at the neuromuscular junction. Also experiments in which glutamate is applied in small quantities onto the synapse of the motor nerve could be used to confirm the location of the receptors. If, when ACPA is applied, no change is observed then it would mean the receptors are located pre-synaptically. Gene analysis could provide useful information into the origin of the receptor, while analysis of the structure of the protein could also determine the function and origin of these unique receptors. Our research could also lead to other invertebrate studies on the evolutionary origins of cannabinoid signaling.

This experiment provides the first evidence of CB1 receptors in protostomian invertebrates. Prior to this experiment, it was thought that cannabinoid receptors were limited to deuterostomian invertebrates, which are fairly distinct from the subphylum containing protostomian invertebrates in which the crayfish belongs. The idea that cannabinoids are not limited to deuterostomian invertebrates has wide evolutionary implications for the origin of cannabinoid receptors, endocannabinoids, and the retrograde messaging systems they mediate. Our experiment necessitates a reexamination of the origin of these mechanisms.

The discovery of cannabinoid receptors at yet another organism's neuromuscular junction is fascinating in itself as well. The ubiquitous functionality of endocannabinoids and knowledge that these systems are not limited to the central nervous system benefits the understanding of how nervous systems and neural networks operate.

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