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AP5 and CNQX decrease post-tetanic potentiation at the crayfish neuromuscular junction.

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ABSTRACT

This study looks at the roles of the glutamate receptors alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) in post-tetanic potentiation (PTP) at the neuromuscular junction of *Orconectes*. In previous studies, researchers used ketamine to antagonize the NMDA receptors in crayfish; however, ketamine also affects other glutamate receptors including AMPA. Research on the individual roles of the AMPA and NMDA receptors in crayfish may lead to a better understanding of their influence in humans. We introduced the AMPA receptor antagonist CNQX and the NMDA receptor antagonist AP5 into crayfish saline solution to block the receptors and observe their individual roles in synaptic transmission and plasticity. Using electrophysiological techniques, we measured changes in excitatory postsynaptic potentials (EPSPs) and PTP resulting from CNQX and AP5. We found that while CNQX and AP5 did not affect EPSP amplitude, both drugs significantly decreased PTP.

INTRODUCTION

In this research, we aimed to better understand synaptic transmission and synaptic plasticity at the neuromuscular junction of *Orconectes*. To do this, we studied the glutamate receptors alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA), which have been implicated in memory and learning processes as well as in many neurodegenerative diseases (Traynelis et al. 2010). These receptors are important in synaptic transmission, as glutamate is the most common excitatory neurotransmitter in both humans and crayfish (Zhou and Danbolt 1996; Parnas et al. 1994). Previous research found that the NMDA receptor antagonist ketamine (Sleigh et al. 2014), which also affects the AMPA receptor, increases paired-pulse facilitation at the crayfish neuromuscular junction (Hoang et al. 2014). More recently, ketamine has been applied to the treatment of pain and mood disorders in humans (Witkin et al. 2007). By exploring the individual roles of the glutamate receptors AMPA and NMDA in crayfish using specific antagonists, we may be able to obtain a better understanding of their particular influence in synaptic transmission and synaptic plasticity.

Over-activated AMPA receptors are involved in chronic neurodegenerative diseases such as Parkinson's disease and Huntington's disease (Jayakar and Dikshit 2004). Jayakar and Dikshit (2004) also reported that regulating AMPA receptors may lead to new methods in reducing or preventing these excitotoxicity-induced neurodegenerative diseases; therefore, it is important to develop a better knowledge and understanding of this receptor and its antagonists. The location of the AMPA receptor in crayfish has not been investigated thoroughly; however, it is found in the postsynaptic cell in vertebrates and in the invertebrate *Aplysia* (Whitlock et al. 2006; Quan et al. 2005). Therefore, we believe that the AMPA receptor is also located in the postsynaptic cell at the crayfish neuromuscular junction and thus functions to promote facilitation.

When the NMDA receptor does not function properly, it plays an important role in clinical depression (Pittenger et al. 2007). Pittenger et al. (2007) also describe how drugs targeting the NMDA receptors produced antidepressant effects. Advances in the knowledge of the NMDA receptor in crayfish may allow for the development of a new and better treatment for clinical depression. In their study on NMDA glutamate receptors in crayfish, Feinstein et al. (1998) identified NMDA receptors on the presynaptic membrane.

Because glutamate functions as an excitatory neurotransmitter, we hypothesized that the specific AMPA receptor antagonist CNQX would decrease EPSP amplitude and decrease post-tetanic potentiation (PTP) at the crayfish neuromuscular junction. We found that CNQX had no effect on EPSP amplitude, but trends in our data indicate that it may decrease amplitude. Our findings also showed that CNQX significantly decreased PTP as we had hypothesized. Parnas et. al. (1996) found that NMDA, an agonist of the NMDA receptor, decreased neurotransmitter release at the crayfish neuromuscular junction. We therefore hypothesized that blocking the NMDA receptor with the specific NMDA receptor antagonist AP5 would increase neurotransmitter release, which would in turn increase EPSP amplitude and promote facilitation. While AP5 had no effect on EPSP

amplitude, it significantly decreased PTP – contrary to our hypothesis.

MATERIALS AND METHODS

Crayfish Dissection

We first anesthetized the *Orconectes* by placing it in ice water. The tail was cut from the thorax and an incision was made on the ventral sides of the ridge of the tail. We then removed the ventral surface and the main muscle mass of the tail in order to expose the deep extensor muscle system. We used seven crayfish throughout this study and no crayfish was exposed to both CNQX and AP5.

Microelectrode Preparation

The microelectrodes used in this study were made with borosilicate glass capillary tubes (Kwik-Fil; World Precision Instruments Inc.), pulled with a PUL-1 microelectrode puller (World Precision Instruments Inc.) and filled with 3M KCl using a fine syringe. A first electrode was sanded down to create an opening large enough for a nerve. In a second microelectrode, we removed bubbles and checked for a resistance between 10 and 20 MΩ in order to avoid a low current that could skew data by preventing a flow of charge through the electrode.

Stimulation and Intracellular Recordings

The first suction electrode, which was hooked to an SD9 stimulator (GRASS Medical Instruments), was placed into a micromanipulator and used to stimulate nerves found on the sides of the crayfish abdomen. Stimulation frequency was recorded with LabChart (version 8.0.5) on a MacBook Pro which was connected to the stimulator through an AD Instruments PowerLab 26T. The second micro-electrode was placed into a microelectrode holder which was also put into a micro-manipulator. This electrode was inserted into the deep phasic extensor muscle in order to record EPSPs. The micro-manipulator was connected to LabChart through an AM Systems, Inc. intracellular electrometer and PowerLab. We used a Leica Zoom 2000 stereo microscope to view our dissections.

Solutions

The control saline solution used in this study consisted of 5.4 mM KCl, 196 mM NaCl, 2.6 mM $MgCl_2$, 10 mM HEPES buffer, and 6 mM $CaCl_2$ with a pH of 7.4. To obtain a solution with a 100 µM concentration of CNQX (Parnas et al. 1996), we combined 250 µl of a stock solution of 100 mM CNQX in dimethyl sulfoxide (DMSO), a compound needed to dissolve CNQX in saline, with 250 ml saline solution. To obtain a solution with a 10 µM concentration of AP5 (Parnas et al. 1996), we combined 250 µl of a 10 mM AP5 stock solution with 250 ml saline solution. We submerged the crayfish in each solution for 5 minutes before taking measurements, and every solution was changed in 30 minute intervals. To make our DMSO control solution, we combined 50 µl DMSO and 50 ml saline solution. The conditions for our experiment were the saline control, CNQX solution, CNQX wash, AP5 solution, AP5 wash, and DMSO control.

Data Collection

We first stimulated the nerve in single mode three times to record EPSP amplitude before potentiation. We then stimulated the nerve for 10 seconds at a frequency of 50Hz, duration of 0.035ms, and voltage just above threshold to evoke EPSPs. Immediately after potentiating, we stimulated it again three times in single mode. We defined a trial as each time we collected three amplitude data points, potentiated, and collected three more data points. After PTP data were recorded in AP5 and CNQX solutions, we washed the crayfish with the control saline solution and EPSP amplitudes were again recorded before and after PTP. The wash was done to insure the drugs' effects were reversible and did not induce any long term effects.

We performed a DMSO control by measuring EPSPs before and after PTP in a control saline solution with a 50mM concentration of DMSO. This control was done to eliminate DMSO as a variable that would affect EPSP amplitude.

Data Analysis

We calculated the mean and standard error of EPSP amplitude measured in the control, CNQX, and AP5 solutions. We then performed two two-tailed t-tests comparing the mean EPSP amplitude in the control with that in the CNQX solution and the mean EPSP amplitude in the control with that in the AP5 solution.

We took the percent change of the average EPSP amplitudes measured before and after PTP for each trial. Each average contained three EPSP amplitudes. We then calculated the mean and standard error of every percent change for each of our conditions. Two-tailed t-tests were performed comparing these last calculated means, i.e. our t-tests compared the means of the mean percent changes across our different conditions.

RESULTS

We investigated the effects of the specific AMPA antagonist CNQX and specific NMDA antagonist AP5 on EPSP amplitude and PTP. To measure synaptic transmission, we collected EPSP amplitude data by

stimulating nerves in control, CNQX, and AP5 solutions and measuring the resulting EPSP amplitudes. We then measured the percent change in EPSP amplitude resulting from PTP in control, CNQX, CNQX wash, AP5, AP5 wash, and DMSO solutions.

CNQX has no effect on EPSP amplitude

Figure 1 shows that the control solution had a mean EPSP amplitude of 12.21mV (SE=1.07) and the CNQX solution had a mean EPSP amplitude of 10.19mV (SE=0.82). From our t-test, we found that there was no significant difference between the mean EPSP amplitude of the control and the mean EPSP amplitude of the CNQX solution ($p > 0.05$); therefore CNQX had no effect on EPSP amplitude. However, we did observe a trend indicating that CNQX may decrease EPSP amplitude. If we used a larger sample size, we might have observed a significant difference between the two conditions.

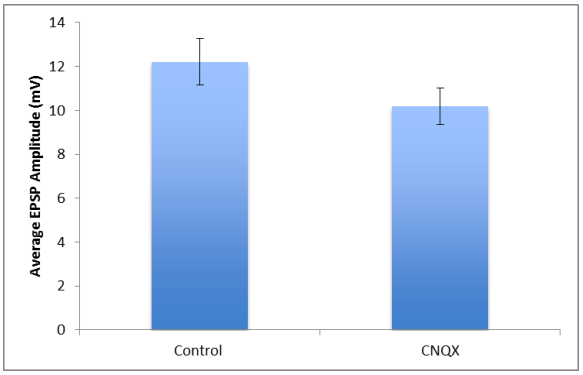

Figure 1. CNQX decreases average EPSP amplitude (mV). EPSP amplitude of the CNQX solution was not significantly different than that of the control ($p > 0.05$). Error bars represent standard error of the mean. N=57 for the control and n=45 for CNQX.

AP5 has no effect on EPSP amplitude

Figure 2 shows that the control saline solution had a mean EPSP amplitude of 12.21 mV (SE=1.07) and the AP5 solution had a mean EPSP amplitude of 13.38 mV (SE=0.37). However, our t-test comparing the mean EPSP amplitude of the control to the mean EPSP amplitude of the AP5 solution showed there was not a significant increase in EPSP amplitude ($p > 0.05$). Our small sample size may have affected our results making them insignificant.

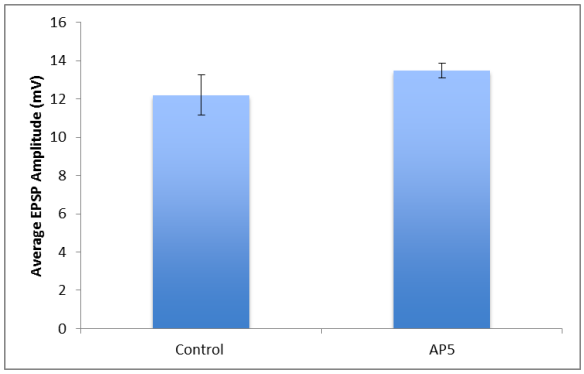

Figure 2. AP5 increases EPSP amplitude (mV). EPSP amplitude of the AP5 solution was not significantly different than that of the control ($p > 0.05$). Error bars represent standard error of the mean. N=57 for the control and n=27 for AP5.

CNQX decreases PTP

From Figure 3, it can be seen that CNQX decreased mean percent change in EPSP amplitude with the control having a mean of 53.12% (SE=17.06) and the CNQX solution having a mean of 9.60% (SE=4.32). The t-test comparing the mean percent change in EPSP amplitude of the control to the mean percent change in EPSP amplitude in the AP5 solution showed that there was a significant difference between the two conditions ($p < 0.05$).

The saline wash performed after inducing PTP in the CNQX solution showed that removing CNQX caused the decrease in PTP to reverse. The mean percent change in EPSP amplitude after the CNQX wash (mean=96.32%, SE=15.04) was significantly greater than that of the control ($p < 0.05$) and significantly greater than that of the CNQX solution ($p < 0.05$). Because these findings support our hypothesis, we believe that the AMPA receptor functions to promote facilitation under normal circumstances.

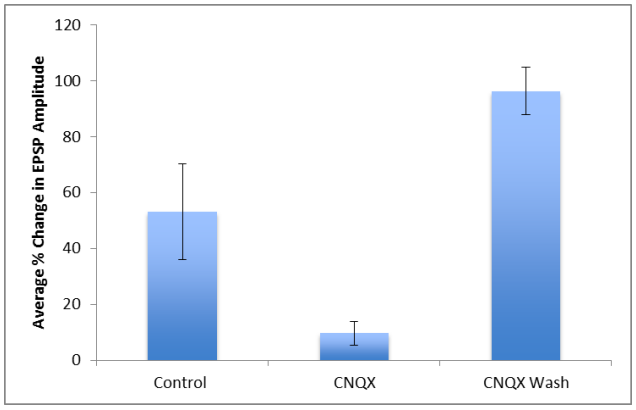

Figure 3. CNQX decreased PTP. PTP after the saline wash was greater than PTP measured in the control. Average % change in EPSP amplitude in the CNQX solution was significantly different from that in the control ($p < 0.05$). Average % change in EPSP amplitude after the saline wash was significantly different from that of the control ($p < 0.05$) and that of the CNQX solution. Error bars represent standard error of the mean. N=12 for the control, n=10 for the CNQX solution, and n=3 for the CNQX wash.

DMSO decreases PTP

In figure 4 it can be seen that compared to the control (mean=53.12%, SE=17.06), DMSO (mean=13.29%, SE=6.71) decreased the mean percent change in EPSP amplitude. From our t-test comparing the mean EPSP amplitude of the control to the mean EPSP amplitude of the DMSO solution, we found that this decrease was statistically significant ($p < 0.05$). We may have observed a decrease in PTP due to DMSO because we were only able to collect data using one crayfish, whereas three crayfish were used for the control condition. This finding, however, does suggest that we cannot be sure if the effects of CNQX that we observed were due to the drug or to DMSO.

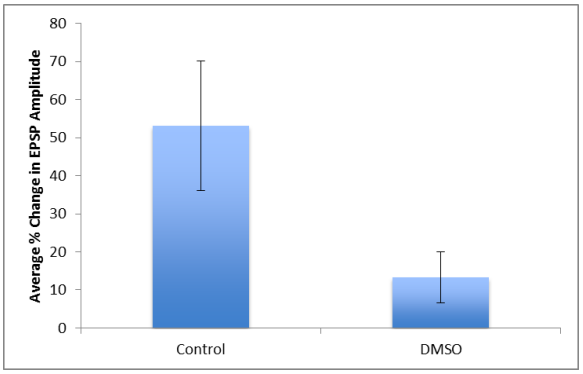

Figure 4. DMSO decreased PTP. Average % change in EPSP amplitude in the DMSO solution was significantly different from that in the control ($p<0.05$). Error bars represent standard error of the mean. N=12 for the control and n=5 for the DMSO solution.

AP5 decreases PTP

Figure 5 shows that AP5 (mean percent change=4.49%, SE=7.09) caused a decrease in PTP compared to the control (mean percent change=53.12%, SE=17.06). Our t-test comparing the mean percent change in EPSP amplitude of the control to the mean percent change in EPSP amplitude in the AP5 solution indicated that this decrease was significant ($p < 0.05$).

The AP5 wash condition, which had a mean percent change of -36.31% (SE=15.04), further decreased PTP compared to AP5. The mean percent change in EPSP amplitude after the AP5 wash was significantly less than that of the control ($p < 0.05$) and significantly less than that of the AP5 solution ($p < 0.05$). We may have observed a decrease in PTP following the wash because our AP5 concentration was too high leading to cell death or because NMDA mechanisms are more complex than we previously believed.

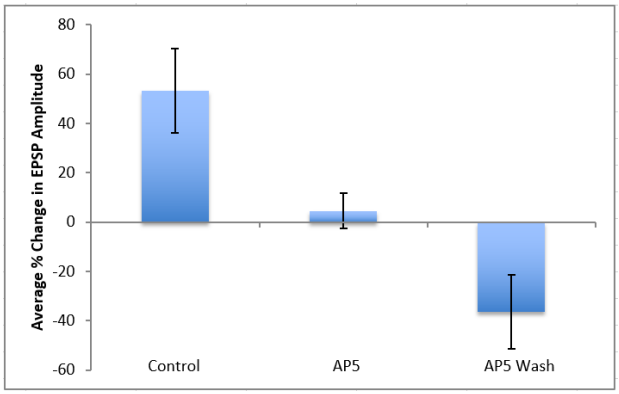

Figure 5. AP5 decreased PTP. PTP decreased further and depression occurred following a saline wash. Average % change in EPSP amplitude in the AP5 solution was significantly different from that in the control ($p < 0.05$). Average % change in EPSP amplitude after the saline wash was significantly different from that that of the control ($p < 0.05$) and that of the AP5 solution ($p < 0.05$). Error bars represent standard error of the mean. N=12 for the control, n=14 for the AP5 solution, and n=3 for the AP5 wash.

DISCUSSION

Our findings are inconsistent with those of Hoang et. al. (2014). They found that ketamine, an NMDA antagonist which also affects the AMPA receptor, results in a reduction in EPSP amplitude and an increase in paired-pulse facilitation. We hypothesized that AP5 would have a similar effect: reducing EPSP amplitude and decreasing PTP. However, our data contradicted our hypotheses and their research. We found that both the NMDA antagonist AP5 and the AMPA antagonist CNQX had no effect on EPSP amplitude and decreased PTP.

The inhibition of AMPA receptors caused a decrease in PTP

CNQX caused no change in EPSP amplitude compared to the control. However, we observed a trend in the means of the two conditions indicating that CNQX may decrease EPSP amplitude with a larger sample size. CNQX significantly decreased PTP, which supported our hypothesis. This backs our assumption that AMPA receptors are postsynaptic at the crayfish neuromuscular junction, as antagonizing postsynaptic receptors associated with excitatory neurotransmitters should decrease facilitation.

PTP after the wash was significantly greater than that of the control. That PTP increased after CNQX was removed indicates that CNQX did not induce any long term effects. We believe that our CNQX wash was significantly greater than our control because we only collected data using one crayfish after the wash, whereas we used four crayfish for our control. EPSP amplitudes can differ greatly among crayfish, therefore a single crayfish is not an accurate representation of the entire population.

We performed a DMSO control in order to ensure that any effects of CNQX were not due to DMSO. However, our data indicated that there was a significant difference between the control and the DMSO solution. Therefore, we cannot be certain that the effects we observed were a direct result of CNQX. It is also possible that the change we observed as a result of DMSO may be due to our small sample size, as we also only used one crayfish in collecting our DMSO control.

The inhibition of NMDA receptors caused a decrease in PTP

We believe there are several possibilities for why AP5 decreased PTP. The first is that our AP5 concentration may have been too high, as there is little research on blocking the NMDA receptor with AP5 in crayfish. If our concentrates were too high, it is possible that cell death occurred. This is backed up by our AP5 wash, which showed that after AP5 was removed, facilitation ceased altogether and depression occurred. The other possibility is that mechanisms of the NMDA receptor are more complex than we previously believed. Kano et. al (2008) described how in

possible that blocking NMDA increases neurotransmitter release, but because of other mechanisms acts to decrease instead of increase PTP.

Limitations

One of the largest limitations of this study was a restricted time frame to conduct experiments. This also greatly reduced the amount samples that we were able to use. Because we had a limited sample size, physiological differences in individual crayfish had a larger effect on our data. We based our concentrations off those used by Parnas et al. (1996), as this was the only study that used AP5 and CNQX at the crayfish neuromuscular junction. It is possible therefore that our concentrations were either too low to induce an effect or so high (in the case of AP5) that they caused cell death.

Further Research

Further research is necessary to validate and improve upon our findings. A replication of our experiment, allowing a longer time frame and using a larger sample size of crayfish, would be helpful in eliminating errors in our data. To expand upon the results that we obtained, future research could also investigate the causes of disparities between our hypothesis and results by looking at different potential mechanisms of NMDA and AMPA-induced facilitation. For example, previous research suggests that AMPA receptors require the presence of certain auxiliary proteins to function (Zheng et. al 2006). Walker et. al (2006) found that stargazin-like proteins were necessary in *Drosophila* and *C. elegans* for receptors to contribute to long-term potentiation. Our results could have been affected by the absence of these auxiliary proteins in crayfish or by their role in the AMPA receptor mechanism, which we did not account for in our hypotheses. Furthermore, although we know NMDA receptors are present presynaptically, it is possible that they are also located on the postsynaptic cell. Answers to these questions would contribute to scientific understanding of the glutamate receptors in crayfish and synaptic transmission and plasticity at the neuromuscular junction.

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