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Serotonin does not increase the EPSP amplitude and IP_3 does not decrease the EPSP amplitude in *Orconectes* neuromuscular junction

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ABSTRACT

Serotonin (5-HT) is a neuromodulator known to facilitate neurotransmission in the neuromuscular junction (NMJ). It binds to presynaptic receptors, and activates G-proteins which then leads to the activation of second messenger molecule called IP_3. Our goal was to investigate whether IP_3 causes the release of internal Ca^{2+}, leading to facilitation. We investigated the effects of 5-HT and the role of IP_3 in crayfish (*Orconectes*) synaptic transmission. We hypothesized that 5-HT would increase the excitatory post-synaptic potential (EPSP) amplitude in crayfish (*Orconectes*) NMJ. Furthermore, we hypothesized that the inhibition of IP_3 receptors using 2-APB would block the effects of 5-HT and therefore prevent facilitation. In our experiment, 5-HT led to EPSP decreasing by 26%. The addition of 2-APB with 5-HT solution led to 61% higher EPSP amplitude compared to control (saline solution). However, our t-test ($p>.05$) showed that these differences were not significant. Thus, our results show that 5-HT and 2-APB do not affect the EPSP amplitude in the crayfish (*Orconectes*) NMJ.

INTRODUCTION

Serotonin (5-HT) is a neurotransmitter that enhances neurotransmission in the neuromuscular junction (NMJ). Delaney et al. (1991) observed that an increased concentration of 5-HT leads to an increased excitatory post-synaptic potential (EPSP) amplitude. They found that 5-HT leads to an increase in internal Ca^{2+} concentration. When serotonin binds to the receptors on the presynaptic cell, it activates G-proteins. This mechanism leads to the activation of IP_3, the second messenger molecule which typically releases Ca^{2+} from intracellular sites (Dixon and Atwood 1989). This usually occurs in the endoplasmic reticulum (Dixon and Atwood 1989). According to Dixon and Atwood (1989), increased Ca^{2+} levels lead to the enhancement of both evoked and spontaneous transmitter release. Furthermore, IP_3 activates C-kinase, an enzyme which is responsible for enhancement of neurotransmission over a longer period of time (~1h).

Dixon and Atwood (1989) tested the mechanism by which serotonin leads to facilitation in the crayfish NMJ. They added 5-HT and IP_3 into the opener muscle of *Procambarus clarkii,* and then added C-kinase inhibitor RA-233. The authors argued that IP_3 provokes short-term facilitation in NMJ, and that it also activates C-kinase which contributes to long-term facilitation. However, the short-term facilitation lasted longer when 5-HT was applied than when IP_3 was applied, leading to the conclusion that there might be another substance contributing to the short-term facilitation in crayfish NMJ.

Our objective was to better understand the role of IP_3 in synaptic transmission, and to confirm that 5-HT leads to facilitation in NMJ. Although Dixon and Atwood (1989) elaborated on the function of IP_3 in crayfish NMJ, we tested if inhibition of IP_3 will block the effects of 5-HT in crayfish NMJ. We assumed that blocking the IP_3 receptors would result in decrease in EPSP amplitude caused by adding of 5-HT. Therefore, we inhibited IP_3 with 2APB inhibitor, expecting a decrease in EPSP amplitude.

We hypothesized that the addition of 5-HT would lead to an increased EPSP amplitude in *Orconectes* NMJ. Our results showed no significant change in the EPSP amplitude when crayfish *(Orconectes)* was placed in the 5-HT solution. Furthermore, we hypothesized that the introduction of an IP_3 inhibitor (2-APB) would block the effects of 5-HT and lead to a decrease in EPSP amplitude. However, when we applied 2-APB to the 5-HT solution, we saw an increase in EPSP amplitude, though this increase was only marginally significant.

MATERIALS AND METHODS

Crayfish Preparation and Dissection

We anesthetized the crayfish (*Orconectes*) by submerging it in ice water prior to dissection. We used scissors to make one cut separating the abdomen from the crayfish, two cuts alongside the abdomen, and one cut across the posterior of the crayfish. These cuts were

completed to allow us to remove the ventral section of the crayfish. We removed excess muscle inside the crayfish, and left the deep extensor muscle cells along the dorsal section intact. The crayfish was then pinned onto a petri dish using Sylgard and four pins on four opposite sides of the crayfish tail. We collected data from six crayfish, using a new crayfish per day over a six-day trial period.

Solutions

We used a standard saline solution of 5.4 mM KCL, 196 mM NaCl, 2.6 MgCl, 10 mM HEPES and 6mM CaCl. The pH level of the saline solution was 7.4. We changed the saline solution to a fresh saline solution around every 40 minutes in an attempt to prevent deterioration of crayfish cells. For our serotonin (5-HT) solution, we mixed 250 µL of a 10 mM 5-HT solution into 250 mL of the saline solution in order to produce a final 5-HT solution of 10 µM. The dilution of 5-HT to saline solution was 1:1000. For our 5-HT+2-APB solution, we mixed 60 µL of a 100 mM 2-APB solution with 60 mL of the 5-HT solution we produced. The final concentration of 5-HT+2-APB solution we used was 100 µM. The dilution of 2-APB to 5-HT solution was 1:1000. For our 2-APB solution, we mixed 60 µL of 100 mM 2-APB solution with 60 mL of the crayfish saline solution to make a 100 µM 2-APB solution. The dilution of 2-APB to saline solution was 1:1000. We also separately prepared a DMSO solution to see if 2-APB would affect the EPSP amplitude by itself. We mixed 50 µL of DMSO solution with 50 mL of the saline solution.

Equipment

We used a World Precision Instruments PUL-1 microelectrode puller to create microelectrodes out of glass capillary tubes. The delay on the microelectrode puller was set at 3, and the heat was set at 7. We filled one microelectrode with a 3.0 M KCl solution using a syringe. This microelectrode was inserted into a crayfish muscle cell near the crayfish nerve cell that we were stimulating. We used sandpaper to blunt the sharp edge of a microelectrode in order to create a suction electrode that we used to suction, and stimulate, a crayfish nerve. This suction electrode was attached to a Grass Instrument Division SD9 Stimulator. For the setting on the SD9 Stimulator, we used voltage above threshold to generate an EPSP. A grounding electrode was attached to A-M Systems AC/DC Differential Amplifier 3000, and placed into solution. We placed the petri dish under a microscope, Leica Zoom 2000, which we used to view crayfish cells. To record the amplitude and test the resistance of the cells and microelectrode, we used LabChart attached to the A-M Systems AC/DC Differential Amplifier.

Testing and Methods

Initially, we inserted the microelectrode into solution to check for resistance (between 3 Ω to 10 Ω). We then inserted the microelectrode into the crayfish dorsal extensor muscle cells. We first measured the resting membrane potential of the crayfish muscle cells (between -40 mV to -120 mV). Then, we used the microelectrode to measure the EPSP amplitude of the crayfish cells when the crayfish nerve was stimulated using the suction electrode. We conducted these tests with the crayfish placed in saline, 10 µM 5-HT,10µM 5-HT+ 100 µM 2-APB, and 100 µM 2-APB. DMSO is present in 2-APB, so we also tested DMSO to make sure that it would have no effect on the EPSP amplitude of the crayfish (*Orconectes*) NMJ.

Data Analysis

We first conducted a t-test comparing the means of the 5-HT solution, 5-HT+2-APB solution, and 2-APB solution with the saline solution (control). Since we collected data over a period of six days using a different crayfish each day, we took the means of the EPSP amplitude for each separate day. This was done separately for each solution. We then calculated the mean of the separate means for each solution. This gave us the final mean which we used to conduct our t-tests. We then normalized or data to account for differences day to day using different crayfish. This was done using the formula $100 \times (EPSP - EPSP_{baseline})/EPSP_{baseline}$. For the $EPSP_{baseline}$, we took the mean of the last three EPSPs we received for the control data on that given day.

RESULTS

We wanted to test the role of 5-HT on the amplitude of EPSPs in the crayfish (*Orconectes*) neuromuscular junction (NMJ). More specifically, we wanted to confirm that 5-HT increases EPSP amplitude by causing intracellular calcium release. We also hypothesized that the increase in EPSP amplitude induced by 5-HT was completed through increasing IP_3.

We blocked the IP_3 receptors by adding 2-APB, an IP_3 inhibitor, to the extracellular solution. For the control condition, we measured the EPSP amplitude of the crayfish neuromuscular junction in standard saline solution. We then measured the EPSP for 3 more experimental conditions (5-HT, 5-HT+2-APB, and 2-APB).

We hypothesized that 5-HT interacts with IP_3 and causes a release of intracellular calcium which would result to an increase of EPSP amplitude. However, our

results did not align with our hypothesis. As seen in Figure 1, the mean EPSP amplitude decreased after adding 5-HT to the solution (from 19.9±1.32 mV to 15.9±0.96 mV). Nevertheless, after conducting a t-test, we came to the conclusion that the EPSP amplitude of crayfish NMJ in 5-HT solution is not significantly different from the EPSP amplitude of crayfish NMH in saline ($p>0.05$).

Additionally, since 2-APB inhibits the IP_3 receptors, we expected it to cancel out the effect of 5-HT to the neuromuscular junction. Therefore, we expected the EPSPs measured in the 5-HT + 2-APB solution to be of the same amplitude of those measured in control condition. However, 2-APB appeared to be reversing the effect of 5-HT; the EPSP amplitude was increased to higher levels compared to the control, reaching 26.7 mV (±0.88) (Fig.1). A t-test showed a marginally significant difference between 5-HT solution and 2-APB solution ($p=0.067$). Despite this apparent difference, an additional t-test suggests no difference between saline solution and 5-HT+2-APB solution ($p>0.05$), confirming our hypothesis that the EPSP amplitude in a 5-HT+2APB solution is not different from the EPSP amplitude in saline.

For the next experimental condition, we added 2-APB to a normal saline solution to block IP_3 and measured the EPSP amplitude. We hypothesized that the EPSP amplitude would not change compared to the control amplitude since IP_3 is activated only by serotonin. Therefore, blocking IP_3 would not affect the EPSP if 5-HT was not involved. We only saw a slight change in EPSP amplitude (from 19.9±1.32 mV to 21±1.21mV). A t-test confirmed that there is no significant difference between 2-APB solution and normal saline solution.

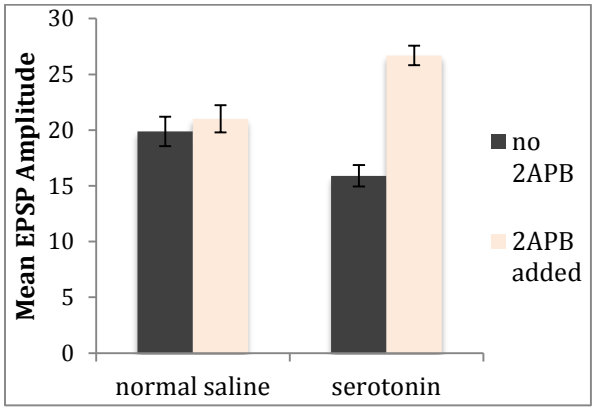

Figure.1: Mean EPSP amplitude of the control condition (saline solution) and each of the 3 experimental conditions (2APB solution, 5-HT solution, 5-HT + 2APB solution). Amplitude measured in mV. Error bars represent standard error.

Finally, as a separate trial, we added DMSO to normal saline to confirm that DMSO has no effect on EPSP amplitudes. A t-test suggested no significant difference between DMSO and control, confirming that DMSO does not affect EPSP amplitude.

Even though the statistical analysis suggested no significant difference between the conditions, we decided to explore our data further. Because EPSP amplitudes vary from one trial to the next, the results were normalized to the initial baseline EPSP amplitude. The initial baseline was the mean amplitude of the last 3 EPSPs we received for control in each trial. For each condition we calculated the percentage change of EPSP amplitude compared to control. We then took the means of the percentage changes. The mean percentage change of EPSP amplitude in serotonin solution is -25.99 (fig.2). We can see that adding serotonin caused the EPSP amplitude to drop by about 26%.However, no t-test was conducted, and therefore we cannot determine if they are significantly different or not. We believe that these results show the tendency of each solution to increase or decrease the EPSP amplitude. However, these results are insignificant. We see adding that 5-HT and 2-APB tended to cause the amplitude to increase by 61.4%.

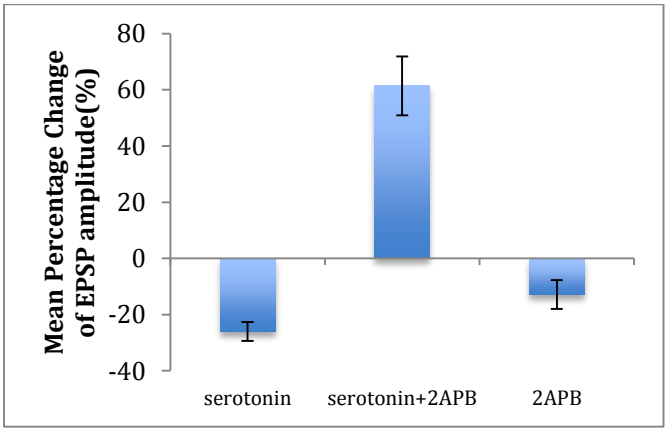

Figure.2 Percentage change of EPSP amplitude of each testing condition compared to control. Each bar represents the mean of all normalized values from the corresponding condition. Negative values represent a reduction in EPSP amplitude compared to control.

DISCUSSION

When we conducted a t-test comparing the experimental solutions against the saline solution (control), we found p-values of >.05. Our results show no significant evidence in support of our hypothesis that serotonin (5-HT) increases the EPSP amplitude of crayfish (*Orconectes*) neuromuscular junction (NMJ). Our hypothesis that 5-HT+2-APB would not significantly affect the EPSP amplitude of crayfish (*Orconectes*) NMJ was demonstrated by our results. However, we cannot solely attribute this lack of effect to the inhibition of IP_3. This is because the 5-HT solution also had no effect on the EPSP amplitude of crayfish (*Orconectes*). Dixon and Atwood

(1989) found that 5-HT affects IP_3, which then affects the EPSP amplitude. Thus, our results may be due to 5-HT not increasing the EPSP amplitude instead of IP_3 inhibition. Furthermore, 2-APB had no significant effect on the EPSP amplitude of the crayfish (*Orconectes*) neuromuscular junction, just as we hypothesized.

Our findings contradict previous research that found that 5-HT increases the release of neurotransmitters, thus increasing EPSP amplitude at the crayfish NMJ (Delaney et al. 1991). A possible contributing factor to the difference in results is that the species of crayfish used by Delaney et al. (1991) was different, as the researchers experimented on crayfish *(Procambus clarkii*) while our research was conducted on crayfish (*Orconectes*).

Prior research also contradicts our findings in another manner. Research conducted on rats indicates that 5-HT significantly decreased the amplitude of excitatory synaptic responses in rat deep dorsal horn neurons (Garraway and Hochman 2001). Our results showed no significant difference in EPSP. We believe that our results, which contradicts previous research conducted on rats and crayfish, are due to the existence of a separate part of the synaptic transmission mechanism that we have not tested.

We would like to conduct tests on the crayfish (*Orconectes*) in colder temperatures. Hamilton et al. (2007) found that the effect of 5-HT contractions and relaxations on the lobster dactyl opener muscle depended on temperature. The authors also found that another chemical, peptide DF_2 had a neuromodulatory effect three times as strong at temperatures of 7-9 degrees Celsius than at temperatures of 15-17 degrees Celsius (Hamilton et al. 2007). The crayfish (*Orconectes*) lives in colder temperatures relative to the crayfish (*Procambaryus clarki*). Our results may differ from previous experiments because the majority of our experiments were conducted with solutions at room temperature. This could make 5-HT ineffective at eliciting a change in EPSP amplitude at the crayfish (*Orconectes*) NMJ, if crayfish (*Orconectes*) superficial dorsal extensor cells react the way the lobster dactyl opener muscle cells reacted (Hamilton et al. 2007).

We would also like to conduct more experiments to test if another chemical opens calcium channels in the crayfish NMJ. Research conducted by Ullmer et al. (1996) on human pulmonary artery endothelial cells demonstrated that 5-HT leads to calcium release intracellularly, a release which the authors claim is independent of IP_3. The authors instead believe that the calcium release is connected to activation of ryanodine receptors (Ullmer et al. 1996). Therefore, we would like to conduct experiments testing the connection between 5-HT and ryanodine receptors using a ryanodine inhibitor. We believe that these experiments may elucidate why we received results that contradicts previous research.

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REFERENCES

Delaney, K., D.W. Tank, and R.S. Zucker. 1991. Presynaptic calcium and serotonin-mediated enhancement of transmitter release at crayfish neuromuscular junction. *The Journal of Neuroscience* 11(9): 2631-2643.

Dixon, D., and H.L. Atwood. 1989. Phosphatidylinositol system's role in serotonin-induced facilitation at the crayfish neuromuscular junction. *Journal of Neurophysiology* 62(1): 239-246.

Garraway, S.M., and S. Hochman. 2001. Serotonin increases the incidence of primary afferent-evoked long-term depression in rat deep dorsal horn neurons. *Journal of Neurophysiology* 85:5.

Ullmer, C., H.G. Bodekke, K. Schmuck, and H. Lubbert. 1996. 5-HT2B receptor-mediated calcium release from ryanodine-sensitive intracellular stores in human pulmonary artery endothelial cells. *British Journal of Pharmacology* 117:6.

Hamilton, J.L., C.R. Edwards, S.R. Holt, and M.K. Worden. 2007. Temperature dependent modulation of lobster neuromuscular properties by serotonin. *Journal of Experimental Biology* 210: 1025-1035.