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Serotonin increases EPSP amplitude by the activation of IP_3-gated calcium channels and ryanodine receptors in *Procambarus clarkii*

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ABSTRACT

Serotonin (5-HT) is a neurohormone found to increase the excitatory presynaptic potential (EPSP) amplitudes recorded at the neuromuscular junction of crayfish. Serotonin is believed to cause this EPSP increase by indirectly activating the inositol triphosphate (IP_3)-gated calcium channel on the endoplasmic reticulum (ER) in a neuron by producing IP_3. Serotonin is also known to indirectly activate ryanodine receptors through a complex, not yet understood secondary messenger system as well. Our hypotheses were that serotonin would increase overall EPSP amplitude, that the two inhibitors separately would significantly lower EPSP amplitudes, and that both inhibitors added at once would produce an additive effect that seemingly negates the effect of serotonin on EPSP amplitudes. From our experiments, we found that serotonin affects both IP_3-gated calcium channel and the ryanodine receptors, by eliciting internal calcium release through both pathways. Through electrophysiology, we saw that the addition of serotonin does, indeed, increase the EPSP at the neuromuscular junction of a crayfish deep extensor muscle cell. We also found that by blocking the aforementioned receptors, the effects of serotonin would be negated, resulting in an EPSP even lower than the EPSP observed in our first control, a basic crayfish saline solution. Addition of 100 µM 2-Aminoethoxydiphenyl borate, 2-APB, blocked the IP_3 receptors and decreased the observed EPSP; addition of 10 µM Dantrolene blocked the ryanodine receptors and also decreased the observed EPSP. In conjunction, the two chemicals resulted in a miniscule EPSP.

INTRODUCTION

Although serotonin (5-HT) is commonly known for its role as a neurotransmitter in the central nervous system, our experiment looked at serotonin as a neurohormone. We examined how serotonin affected the release of the internal calcium stores found in the endoplasmic reticulum (ER) of the nerve adjacent to the deep extensor muscle cells of the species of crayfish: *Procambarus clarkii.* Two receptor proteins found on the ER that are specifically affected by serotonin are the inositol triphosphate (IP_3)-gated calcium channels and the ryanodine receptors (Dropic et al. 2005). Serotonin is claimed to produce IP_3, which leads to the activation of the IP_3-gated calcium channel ( ), and is also believed to play a role in the activation of the ryanodine receptor (Ullmer et al. 1996). The release of the calcium ions from these stores triggers the eventual exocytosis of synaptic vesicles containing neurotransmitters by activating the various SNARE proteins that induce the process. Neurotransmitters released from the presynaptic terminal into the synaptic gap cross the gap and bind to their specific postsynaptic receptors eliciting a change in voltage across the membrane of the postsynaptic cell. The change in voltage triggers the opening of postsynaptic cation channels, which causes an influx of positively charged ions that produce an excitatory postsynaptic potential (EPSP) (Etzkorn et al. 2006). The magnitude of the resultant EPSPs shows the relative quantity of neurotransmitters released from the presynaptic cell (i.e. a larger EPSP indicates more neurotransmitters were released into the synaptic gap).

Our experiment sought to observe the effect of serotonin on the EPSP amplitudes measured at the neuromuscular junction in the deep extensor muscles of the crayfish. Prior research shows that IP_3, a secondary messenger molecule, participates in serotonin-mediated intracellular calcium ion release (Delaney et al. 1991). This finding was tested by adding IP_3 directly to the solution and measuring the resultant EPSP amplitudes through electrophysiology (Dixon and Atwood 1989). Dixon and Atwood observed that IP_3 raised the overall EPSP amplitude. Our methods differed from Delaney et al. because we indirectly facilitated IP_3 production through increased levels of serotonin. Serotonin has also been claimed to indirectly activate ryanodine receptors by locking them in an open-state leading to the depletion of the internal calcium stores found inside the ER. Again, these results were tested by recording the resultant EPSP amplitudes through electrophysiology (Ben-Or et al. 2010).

Past research has only observed the relationship between IP_3 and serotonin without examining the effect ryanodine has on the aforementioned relationship (Dropic et al. 2005). By inhibiting both the IP_3-gated calcium channels and ryanodine receptors through the addition of

$(C_{14}H_{10}N_4O_5)$, we saw whether serotonin caused an increase in calcium ion release from the ER stores through one or both of the receptors in question. We examined both variables in conjunction with one another to determine which internal calcium release pathway was affected more by serotonin. With this knowledge, doctors can utilize the most efficient pathway to combat neurodegenerative diseases in which serotonin deficiency is a contributing factor, such as Huntington's Disease (Reynolds et al. 1999). Our research on the most efficient pathway for internal calcium release can lay the foundations for further studies on possible medication for neurodegenerative diseases affected by serotonin deficiency. By bypassing the direct effects of serotonin and directly activating the channel proteins, the release of neurotransmitters at the neuromuscular junction can occur. Release of needed neurotransmittes could minimize the effects of the disease.

We hypothesized that serotonin facilitates internal calcium release through both the IP_3-gated calcium channel and ryanodine receptor pathways. We believed that the addition of serotonin would produce the largest EPSP amplitudes. We observed that serotonin does in fact increase EPSP amplitudes, which confirms the first part of our hypothesis. We also predicted that inhibiting either of the previously mentioned receptors would result in significantly lower EPSP amplitudes. We saw that applying both inhibitors separately yielded lower EPSP amplitudes, confirming the second part of our hypothesis. We predicted that inhibiting both receptors would result in the lowest EPSP amplitudes. If the effects of serotonin on internal calcium release were only present in these two systems, we predicted to observe no serotonin-induced EPSP. As hypothesized, inhibiting both receptors produced our smallest EPSP amplitudes, all of which were almost negligible.

MATERIALS AND METHODS

Dissection and Preparation of Crayfish

The crayfish we dissected belonged to the species *Procambarus clarkii* purchased from Carolina Biological Supply Co. We began our dissection of the tail by cutting the carapace along the ventral ridges of the shell. We then removed and discarded the superficial muscle to expose the deep extensor muscle. After muscle removal, both the nerves connected to the deep extensor muscles and the muscles themselves were accessible for observation. This procedure can be found on Wyttenbach and Hoy's "Crawdad: A Lab Manual for Neurophysiology" under the subheading "Muscle Resting Potential." The crayfish was anesthetized under ice for thirty minutes before dissection to ensure humane experimentation. The dissected crayfish tail was submerged in a bath of basic crayfish saline solution with a pH of 7.4 that consisted of 5.4 mM of KCl, 200.7mM of NaCl, 12.3 mM of $MgCl_2$*6H20, 5 mM of Sodium Hepes Buffer, and 6.5 mM of $CaCl_2$*$2H_20$. Submersion in this saline solution preserved cell integrity and prevented cell fatigue as we conducted our experiments. We also changed the saline every 45 minutes in order to preserve cell health. We examined our prepared crayfish specimen through a Leica *ZOOM* 2000 microscope.

Mixing and Testing Chemical Solutions for Experimental Conditions

We conducted trials with three controls and three experimental conditions in our experiment. Our first control was the crayfish tail immersed in a solution consisting of a basic crayfish saline only. Our second control, the Serotonin-only trial, was 10 µM serotonin which was prepared by adding 10µL of 10 mM stock serotonin solution to 100 mL of basic crayfish saline. The third control, the DMSO-only trial, was 10µL of DMSO added to 100mL of basic crayfish saline. DMSO was the solvent our inhibitors were dissolved in. Our first experimental condition, the Serotonin + 2-APB trial, was 100 µM 2-APB and 10 µM serotonin which we prepared by adding 10 µL of 100 mM stock 2-APB solution and 10 µL of 10 mM stock serotonin solution to 100 mL of basic crayfish saline. The second condition, the Serotonin + Dantrolene trial, was 10 µM Dantrolene and 10 µM serotonin, which we prepared by adding 10 µL of 10 mM stock Dantrolene solution and 10 µL of 10 mM stock serotonin solution to 100 mL of basic crayfish saline. Our final condition, the Serotonin + 2-APB + Dantrolene trial, consisted of 100 µM 2-APB, 10 µM Dantrolene, and 10 µM serotonin which we prepared by adding 10 µL of each stock solution to 100 mL of basic crayfish saline. We had two separate protocols for testing the three controls and conditions in order to avoid contamination. The first protocol tested basic crayfish saline, the DMSO-only trial, the Serotonin-only trial, the Serotonin + 2-APB trial, and the Serotonin + 2-APB + Dantrolene trial in that order. Our second protocol tested the same controls and conditions yet our Serotonin + 2-APB trial was switched with our Serotonin + Dantrolene trial. We divided the two protocols separately to ensure that the difference in EPSP under each condition would not be affected by the previous chemical. Successive tests did not change

chemicals, they simply added chemicals to the existing mix. Were we to test Dantrolene and 2-APB in succession, our experiment would risk cross contamination between the 2-APB and Dantrolene trials.

Making Microelectrodes for Intracellular Recording

We pulled two types of glass microelectrodes using the PUL-1 electrode-pulling machine that was provided in our lab and manufactured by World Precision Instruments. The microelectrode that was to be used for recording was filled with 3.0M of KCl and was tested for an optimal resistance of 5-10Ω using the A-M Systems Inc. Intracellular Electrometer. The junction potential from the recording microelectrode was zeroed in the basic crayfish saline solution using the program LabChart. The microelectrode that was to be used for suction was sanded down to effectively capture the nerve that would receive the stimulation. Both microelectrodes were secured in micromanipulators to ensure stability when piercing the muscle cell or capturing the nerve.

Recording and Analysing Data

To begin recording data, we captured a nerve using the suction microelectrode. We then pierced a deep extensor muscle adjacent to the suctioned nerve with a recording microelectrode by using a micromanipulator. A resting membrane potential was found using the program LabChart. The captured nerve then received a 3.5 Volt stimulation with a 0.55 Hz frequency and a 0.06 ms duration from a Grass SD9 Stimulator. The resultant EPSP amplitudes were recorded using LabChart. We took between forty and fifty EPSP recordings for every experimental condition. We allowed each condition a ten to fifteen minute diffusion period before recording data to allow the chemicals to diffuse into the cells. Using a module of LabChart, DataPad, we recorded the amplitude of the EPSP and transferred the data into Microsoft Excel for statistical analysis and comparison. In order to analyze our data, we used ANOVA tests to compare the results. Each ANOVA test compares multiple conditions observed in a single day. The ANOVA test indicated that the conditions were significantly different. We used the calculated p-value to determine whether we would reject the null hypothesis. The null hypothesis indicates discrepancies between values observed in the two conditions can be attributed to arbitrary factors and not a relationship between the two conditions. We took the mean EPSP amplitudes for each experimental condition, and then averaged the mean EPSP amplitudes for each day, removing outliers. We then calculated the percent change between each experimental condition by using the equation:

$$\left[\left(EPSP_{CONDITION} - EPSP_{CONTROL}\right) / EPSP_{CONTROL}\right] * 100 = \%\ change$$

For data that indicated a change over time as opposed to consistent results, we compared quartile averages instead of an overall average for the trial. We used this method in order to account for discrepancies between the EPSP values recorded at the start and end of the trial. We used the standard error of the mean in our graphs to indicate the deviation from the mean and take into account any variance between data sets.

RESULTS

Serotonin Increases EPSP Amplitudes

We observed a 157.75% increase between the EPSPs recorded in the basic crayfish saline control and the Serotonin-only control, as is presented in Figure 1. The resultant p-value from a t-test comparing the two controls was 0.036, indicating we could reject the null hypothesis and confirm that the addition of serotonin has an effect on the EPSP amplitudes at the crayfish neuromuscular junction and caused a significant increase.

DMSO does not Increase EPSP Amplitudes Significantly

Calculating the percent change between the basic crayfish saline control and the DMSO-only trial, we found a 25.55% increase (see Figure 1). The t-test comparing the basic crayfish saline control and the DMSO-only control indicates that we should accept the null hypothesis because the p-value was ≥0.05, indicating DMSO does not increase EPSP amplitudes significantly.

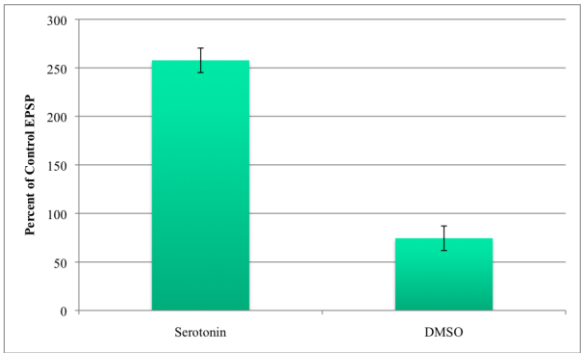

Figure 1. Percent change in EPSP amplitude comparing Serotonin-only and DMSO-only trials to our basic crayfish saline control. Adding serotonin to basic crayfish saline resulted in an EPSP 257.75% of the EPSP amplitudes recorded from the control trial (N=8). Adding DMSO to basic crayfish saline resulted in an EPSP 74.45% of the EPSPs recorded from the control trial (N=2). The p-value calculated comparing the control and the serotonin trials was 0.036. The p-value calculated comparing the control and DMSO was 0.44. Error bars represent the standard error of the mean (SEM). The SEM for the Saline-only trial versus the Serotonin-only trial is ± 12.6535. The SEM for the Saline-only trial versus the DMSO-only trial is ±12.6543.

2-APB/Dantrolene Decrease EPSP Amplitudes Produced by the Effects of Serotonin

The percent change between the Serotonin-only trial and the Serotonin + 2-APB trial was a 65.42% decrease in EPSP amplitude. The percent change observed between the Serotonin-only trial and the Serotonin + Dantrolene trial was similar to the Serotonin + 2-APB trial, a 61.67% decrease. We observed a 99.18% decrease between the Serotonin-only trial and the Serotonin + 2-APB + Dantrolene trials. These data are illustrated in Figure 2. The p-values we observed were all smaller than 0.001, allowing us to easily reject the null hypothesis for all experimental conditions.

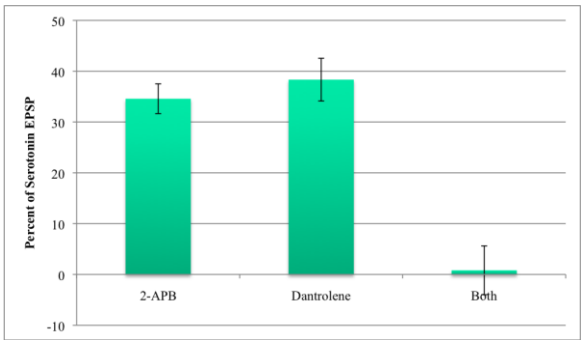

Figure 2. Percent change in EPSP comparing inhibitory conditions to our Serotonin-only trial. Our Serotonin + 2-APB trial (N=3) resulted in an EPSP 34.58% of the EPSP recorded from the Serotonin-only trial. Our Serotonin + Dantrolene trial (N=2) resulted in in an EPSP 38.33% of the EPSPs recorded from the Serotonin-only trial. Our Serotonin + 2-APB + Dantrolene trial (N=2) resulted in an EPSP 0.82% of the EPSPs recorded from the Serotonin-only trial. We took forty to fifty samples for each condition. The p-value calculated comparing the Serotonin-only and the Serotonin + 2-APB trials was ≤ 0.05. The p-value calculated comparing the Serotonin-only and the Serotonin + Dantrolene trials was ≤ 0.05. The p-value calculated comparing the Serotonin-only and the Serotonin + 2-APB +Dantrolene trials was ≤ 0.05. Error bars represent standard error of the mean. The SEM for the Serotonin-only versus the Serotonin + 2-APB trial is ±2.93. The SEM for the Serotonin-only versus the Serotonin + Dantrolene trial is ±4.20. The SEM for the Serotonin-only versus the Serotonin + 2-APB + Dantrolene trial is ±4.80.

Dantrolene Decreases EPSP Amplitude as Stimulus is Applied over Time

The data recorded from the Serotonin + Dantrolene trial showed a downward trend over time; with repeated stimulus, the observed EPSP amplitudes gradually decreased in amplitude as seen in Figure 3. The first EPSP amplitude recorded after the addition of Dantrolene was greater than the EPSP amplitude recorded during the serotonin trial, 67.93 mV and 66.67 mV respectively. We observed that after a few stimuli, the resultant EPSP amplitudes began to decrease dramatically. Although the steepness of the slope over time began to decrease, the overall downward trend in the recorded magnitude of the EPSP amplitudes continued.

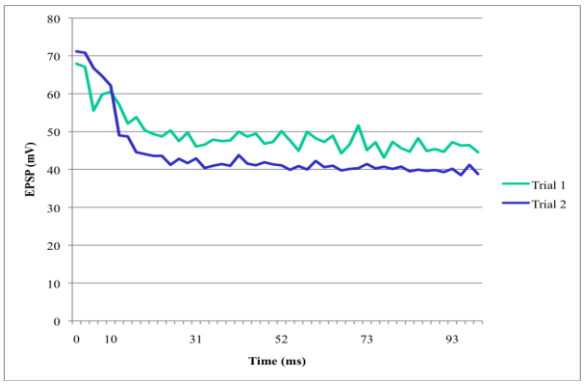

Figure 3. Observed trend in EPSP amplitudes of the Serotonin + Dantrolene trials over time. The observed downward trend of EPSP amplitudes after repeated stimulus is shown. Trial one indicates the first consecutive stimulations of the nerve and their resultant EPSPs. Trial two indicates the consecutive stimulations of the nerve after we allowed a five-minute replenishing period and their resulting EPSPs. Trial one and two were recorded for ten minutes each and were performed on the same crayfish specimen, stimulating the same nerve.

DISCUSSION

We observed that the addition of serotonin resulted in higher EPSP amplitudes. Our findings suggest serotonin increases EPSP amplitudes through some sort of intercellular system, which we hypothesized to be the internal calcium release system found on the ER. To examine this hypothesis, we added either 2-APB, an IP_3-gated calcium channel antagonist, or Dantrolene, a ryanodine receptor antagonist, which significantly lowered the EPSP amplitudes initially raised by serotonin's reported effect on internal calcium release (MacMillan et al. 2005). This decrease in recorded EPSP amplitudes due to the addition of either of our inhibitors supported our hypothesis that serotonin affects the internal calcium release system through both the IP_3-gated calcium channel and the ryanodine receptors. This finding coincides with the work done with Dantrolene and 2-APB by Zucchi et al. (1997) and Etzkorn et al. (2006) respectively. This was further supported when we examined both inhibitors in our final experimental condition. The addition of both Dantrolene and 2-APB to our serotonin and basic crayfish saline solution resulted in almost negligible EPSP amplitudes. These data suggest serotonin facilitates internal calcium release primarily through the IP_3-gated calcium channels and ryanodine receptors, and not some other major channel protein.

From our third control, the DMSO-only trial, our resultant EPSPs showed an increase in EPSP amplitude from the basic crayfish saline control. We carried out this control to examine whether DMSO, the solvent our

inhibitors were dissolved in, had an effect on resultant EPSP amplitudes. Our p-value was ≥0.05 for this control indicating we should accept the null hypothesis. Tsvyetlynska, N. A. et al. (2005) stated DMSO can affect EPSP amplitudes in large quantities, yet only slightly. In our experiment, we used only 10µL of DMSO added to 100mL of basic crayfish saline, which was lower than the concentration used in Tsvyetlynska, N. A. et al. We concluded that DMSO did increase EPSP amplitudes slightly, but not significantly, for the EPSP amplitudes we recorded for the Serotonin + 2-APB, Serotonin + Dantrolene, and Serotonin + 2-APB + Dantrolene trials. This could also explain why we still produced almost negligible EPSP amplitudes when we inhibited both internal calcium release pathways.

The data we recorded from our Serotonin + Dantrolene trial raised questions concerning the possibility of a correlation between the IP_3-gated calcium channel and [Ca^{2+}]-induced[Ca^{2+}] release (CICR). When recording EPSPs for this experimental condition, the EPSPs we recorded at the beginning of the stimulation decreased with every subsequent stimulation. This lead us to believe that internal calcium release could also depend upon a positive feedback loop concerning cytosolic Ca^{2+} ions, a hypothesis which coincides with Fabiato's findings (1985). Because of this CICR we saw that after every stimulation the nerve received, the EPSP amplitudes decreased. Not stimulating the nerve for a recovery period of five minutes effectively reset the next observed EPSP to a value proximal to its starting amplitude. When we began stimulating the nerve again, the trend repeated itself and the observed EPSPs decreased over time.

A possible explanation for this could be that the initial stimulation of the nerve opens presynaptic calcium ion channels, which allow external calcium to enter the cytosol. External calcium ions bind to the [Ca^{2+}] activation sites on the ER, leading to the release of internal calcium. From there, due to CICR, a positive feedback loop begins, causing further calcium ion release from the ER stores due to the cytosolic calcium binding to the [Ca^{2+}] activation sites, a process which leads to the eventual depletion of the calcium ion stores found in the ER (Zucchi et al. 1997). Our data suggest that EPSP amplitude decreases because the calcium ion stores deplete, leaving only an insignificant amount of residual calcium to activate the SNARE proteins necessary to trigger the exocytosis of the presynaptic vesicles. The decreased rate of exocytosis ultimately means fewer neurotransmitters are released into the synaptic gap, leading to a decrease in EPSP amplitude. Not stimulating the nerve for a period of time raised the next observed EPSP amplitude because the lack of stimulation allowed for the internal calcium stores to replenish themselves. What we could not determine is why inhibiting the ryanodine receptor allowed for this positive feedback loop to take place. Our Serotonin + 2-APB trial resulted in EPSPs that were overall lower, and did not show such a change over time. Through further experimentation, we could possibly determine whether this CICR positive feedback loop has ties to the IP_3-gated calcium channel or the inhibition of the ryanodine receptor. We could place the specimen in a no-calcium solution, and run the Serotonin + Dantrolene trial once more. If our resultant EPSP amplitudes show consistent results and no change over time, then we can assume that the positive feedback loop does occur and is tied to the IP_3-gated calcium channel. Though, due to time constraint, we were not able to run this experiment.

Although the previously stated finding was interesting and served to answer a portion of our main question, our final experimental condition lead us to believe that serotonin affects internal calcium release primarily through the ryanodine receptors and the IP_3-gated calcium channels. When we added both inhibitors, 2-APB and Dantrolene, to the serotonin and basic crayfish saline solution, our EPSP recordings were nearly non-existent, never exceeding an amplitude change of 0.5 mV. This illustrates that serotonin increased internal calcium release through these two channel proteins and not some other pathway. Zucchi et al. (1997) and Etzkorn et al. (2006) saw decreased EPSPs amplitudes in their experiments with Dantrolene and 2-APB, so it follows that adding both inhibitors would result in even lower EPSP amplitudes. Dropic et al. (2005) also recorded similar EPSP amplitudes when examining serotonin's effects on IP_3-gated calcium channels and ryanodine receptors. Our basic crayfish saline had a low concentration of [Ca^{2+}], meaning only a small amount of external calcium ions would flow into our presynaptic cell. Because of the inhibition of both internal calcium release pathways and the low amount of external calcium ion concentration, our almost negligible EPSP amplitudes support our hypothesis that serotonin works on internal calcium release primarily through both IP_3-gated calcium channels and ryanodine receptors. When both receptors are inhibited, serotonin cannot cause internal calcium release, which explains the ≤0.5 mV EPSP amplitudes we recorded from our final experimental condition.

Our experiments concluded that serotonin does in fact facilitate internal calcium release through both IP_3-gated calcium channels and ryanodine receptors. Furthermore, we found that serotonin worked primarily through these receptors because of the negligible EPSP amplitudes we recorded when we inhibited both receptors. Moreover, the possible correlation between the IP_3-gated calcium channels and the CICR positive feedback loop could serve as a basis for future experimentation. By

researching serotonin's effect on the neuromuscular junction, medical professionals can assess the most efficient pathway to combat neurodegenerative diseases, which are commonly associated with a serotonin deficiency. This deficiency would lead to a decreased rate of neurotransmitter release from the presynaptic gap, a possible cause of such neurodegenerative diseases such as Alzheimer's or Huntington's disease. As serotonin facilitates internal calcium release by indirectly activating these two receptors, perhaps patients with a serotonin deficiency could receive medication that skips the intermediary steps of serotonin and directly activate these internal calcium release receptors.

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