Pioneering Neuroscience, 2014, **14**, 31-36.

Octopamine affects neurotransmitter release in crayfish neuromuscular junction through residual calcium

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ABSTRACT

Octopamine and serotonin produce opposite responses in both crayfish behavior and in the crayfish neuromuscular junction. With this correlation in mind, we sought to discover whether octopamine and serotonin shared a pathway responsible for facilitating neurotransmitter release. We explored a specific aspect of this pathway, namely the effect of residual calcium on EPSP amplitudes in the crayfish superficial extensor neuromuscular junction. Calcium plays an important role in signal transmission, and there are several ways in which it can do so. Residual calcium facilitates transmission by prompting the release of more synaptic vesicles through exocytosis into the synaptic gap. Research has shown that serotonin does not use residual calcium to facilitate synaptic transmission in crayfish muscle cells, so we hypothesized that octopamine would not either. In order to test this, we stimulated the second ganglionic nerve with a paired set of two electrical pulses in quick succession, and measured the amplitudes of the EPSPs they produced, both with and without octopamine. We then measured the percent change from the first to the second EPSP to quantify the effect of residual calcium on signal transmission, as excess calcium ions remain in the presynaptic cell between pulses. We found that, contrary to our hypothesis, octopamine does affect the amount of residual calcium in the cell. In fact, there is a significant decrease in facilitation by residual calcium in the presence of octopamine.

INTRODUCTION

Calcium ions are an essential component in synaptic transmission. In the presynaptic cell, they bind to SNARE proteins and synaptotagmin, which are proteins that regulate synaptic vesicles containing neurotransmitters. Once these proteins have been activated, they facilitate the fusion of the vesicles to the membrane of the presynaptic cell. On completion of exocytosis, the neurotransmitters are released into the synaptic gap. Many of the neurotransmitters reach the receptors of the postsynaptic muscle cell, and an excitatory postsynaptic potential (EPSP) is created as a result. Thus, the calcium ions allow for signal transmission, with the amount of neurotransmitter released dependent on the amount of calcium triggering exocytosis (Pearson, 2011).

The concentration of calcium ions that trigger exocytosis can increase in the presynaptic cell in a variety of ways. One such path is by an influx of calcium ions through voltage-gated ion channels during depolarization. This influx may be modified by changing the composition of the ion channels to let in either more or fewer calcium ions. Another way is by the IP_3 pathway (Delaney et al., 1991), where internal vesicles of calcium ions are released from the endoplasmic reticulum, which has collected them during an earlier period of influx.

Yet a third way in which calcium concentrations increase is through residual calcium left in the cell from a previous signal that had not yet bound to a SNARE protein, diffused out of the cell, or been collected by the endoplasmic reticulum (Delaney et al., 1991). This final method occurs when signals propagate in quick succession. Once the second signal occurs, the residual calcium from the first facilitates the propagation of the next signal by release of extra neurotransmitters, and thus results in a greater EPSP in the postsynaptic cell.

Octopamine is a neurohormone released in the blood stream that binds to the presynaptic cell. Fischer and Florey (1983) found that octopamine causes an increase in the amplitude of excitatory postsynaptic potentials (EPSPs) in the crayfish neuromuscular junction, indicating an increase in neurotransmitter release. There are many possible ways in which octopamine could increase neurotransmitter release, but we tested for one way specifically: namely, residual calcium. Previous research indicates that octopamine has the opposite effect from serotonin in crayfish neuromuscular junction (Glanzman and Kranse, 1983). Octopamine and serotonin have also been found to give rise to opposing changes in crayfish behaviors such as posturing (Brummer et al., 2000), spontaneous and reflex leg activity (Gill and Skorupski, 1996), and escape reaction (Glanzman & Kranse, 1983). This

effect further indicates a correlation between the two. Drawing on Delaney et al. (1991), which found that serotonin does not use residual calcium to enhance neurotransmission in crayfish, as well as on the suggested correlation between serotonin and octopamine, we hypothesized that octopamine, like serotonin, does not use residual calcium to facilitate an increased release of neurotransmitters into the crayfish neuromuscular junction.

Facilitation by residual calcium can be detected by administering two stimuli and then comparing the percent change in EPSP amplitude. A significant difference between percent change of EPSP amplitude between the experiments with and without octopamine would therefore indicate that octopamine causes facilitation of synaptic transmission by residual calcium.

MATERIALS AND METHODS

Dissection and preparation of Crayfish.

As a model for synaptic transmission, the neuromuscular junction of nerve and muscle cells in the superficial extensor muscle were used. Before dissecting to reach this tissue, a live crayfish was placed in an ice bath for 30 minutes in order to anesthetize it. The tail was cut off using laboratory scissors, and the flat ventral half of the tail was cut off along the outer edge of the curved side. The flat ventral part was then pulled by hand and cut off completely exposing. We scraped the flexor muscles out of the carapace with a thumb nail, exposing the superficial extensor muscle underneath.

Solutions and trials.

The low-calcium crayfish ringer saline solution was composed of 5.4 mM KCl, 200.7 mM NaCl , 15.55 mM $MgCl_26H_2O$, 5 mM Sodium Hepes Buffer, 3.25 mM $CaCl_22H_2O$, with a pH of 7.4. To make the octopamine solution, we diluted 250 µl octopamine into 250 ml of the low-calcium solution for a final concentration of 10 µM. We experimented with a total of four trials with varying conditions: low-calcium saline solution, octopamine solution, low-calcium saline solution then octopamine solution, and octopamine solution then low-calcium saline solution. The last two trials were conducted to control for the cell death associated with time after dissection or a potentially irreversible effect of octopamine. When switching from octopamine solution to low-calcium saline solution, we rinsed the crayfish four times with the low-calcium saline solution. When switching from low-calcium saline solution to octopamine solution, we waited 5 minutes before taking measurements to allow the octopamine to diffuse throughout cells of the tissue.

Electrode Fabrication.

We made glass pipette electrodes with a World Precision Instruments PUL-1 electrode puller. Suction electrodes were modified by sanding down the tip to a .3 mm diameter at the opening. Recording electrodes were filled with 3M KCl then dipped in a beaker of saline solution (composition: 5.4 mM kCl, 19.6 mM NaCl, 2.6 mM $MgCl_2 \cdot 6H_2O$, 10 mM Sodium Hepes Buffer, 13.5 mM $CaCl_2 \cdot 2H_2O$) to rinse away any residual 3M KCl from the exterior. Electrodes were replaced whenever the measured resistance was less than 5 MΩ.

Excitatory postsynaptic potential (EPSP) measurement.

With a suction electrode, we captured a second ganglionic nerve, which was stimulated using a Grass SD9 stimulator. We then inserted an electrode filled with 3M KCl into an adjacent cell and put reference electrodes into the solution for PowerLab to register the signal. We delivered two quick suprathreshold stimuli to the nerve, at least .12 volts each, and measured the resulting EPSPs in the postsynaptic cell. We stimulated at least 10 times per cell.

Twin Pulse Facilitation and Data Analysis

We delivered two quick suprathreshold stimuli to the second ganglionic nerve with a 20-40 ms delay between the two pulses. We calculated the ratio of the two EPSPs as a percent of the first EPSP:

$$\% \text{ change} = ((EPSP_2 - EPSP_1) / EPSP_1) * 100$$

This percent change directly relates to the amount of facilitation caused by residual calcium. We waited 30 seconds between each paired-pulse stimulus to allow the cell time to clear out calcium and minimize the effects of residual calcium from prior twin-stimuli.

Two-tailed student t-tests run through Microsoft Excel were used for statistical analysis. A two-tailed t-test allowed for us to compare two sets of data, and determine whether their means were significantly different from each other. From a t-test, we obtain a p-value, which indicates the probability that the results were obtained by chance. A p-value less than .05 allows us to reject the null hypothesis (i.e. the two tested data sets are not significantly different), and thus interpret the means to be significantly different and draw further conclusions.

RESULTS

In order to measure residual calcium facilitation in the presence of octopamine, a twin pulse stimulus was applied to the crayfish neuromuscular junction and EPSPs were measured. As seen in figure 1, we first looked at the amplitudes of the EPSP from the first stimulus. To measure amplitude we subtracted the resting membrane potential of the postsynaptic cell from the peak of the measured EPSP. The two conditions were averaged and compared as seen in Figure 1. For the baseline modified low calcium crayfish solutions, the average amplitude was 0.4 mV (SEM=±1mV). The experimental condition with octopamine in modified low calcium saline solution had a much greater average EPSP amplitude 14mV (±1mV). This 13.6 mV difference between the baseline and experimental conditions is statistically significant with a t-test p-value of 4*10^-9. Since this p-value is less than .05, we can confidently say that octopamine increases EPSP amplitude.

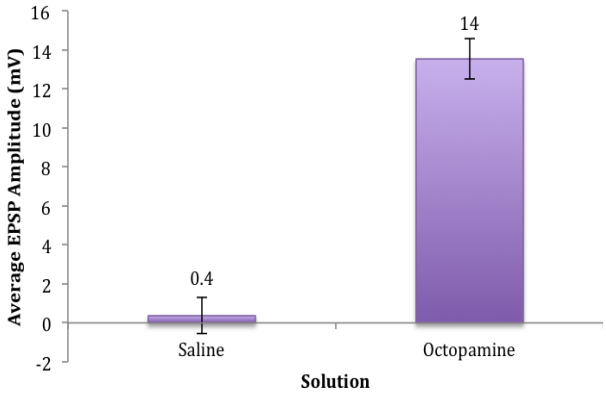

Figure 1. *Average EPSP amplitude recorded in crayfish muscle cells in Baseline saline solution and octopamine solution*. In both cases, amplitude was calculated by subtracting the voltage at the peak of the first EPSP of the twin pulse from the resting membrane potential before the stimulation. The amplitudes were averaged for each condition. The average EPSP amplitude in the crayfish muscle cell in the low-calcium crayfish saline solution is graphed with error bars representing Standard Error (±1mV). N=105. The average percent change of EPSP in the crayfish muscle cell submerged in the 10 µM octopamine solution is graphed with error bars representing S.E. (±1mV). N=78.

In order to determine the relative amount of facilitation of neurotransmitter release by residual calcium, we looked at the percent change of EPSP amplitude from a twin pulse stimulation. The percent change of EPSP amplitudes from the second to the first pulse was calculated by subtracting the resting membrane potential from the voltage recorded at the peak of the EPSP of the twin pulse. The average of all the EPSPs were calculated for each condition. As seen in Figure 1, the baseline condition in the modified 3.25mM Ca2+ crayfish saline solution showed a 58% (±5%) increase in EPSP from first to second pulse. In the experimental condition with 10 µM octopamine solution in modified 3.25mM Ca2+ crayfish saline solution, the EPSPs were increased by 19% (±1%). Thus, there is a 39% difference between the baseline and octopamine conditions. Running a Student's t-test, we obtained a p-value of $3*10^{-11}$. This p-value, which is far less than .05, allowed us to reject the hypothesis that octopamine does not affect facilitation through residual calcium. Therefore, our data suggests that octopamine significantly decreases facilitation by residual calcium.

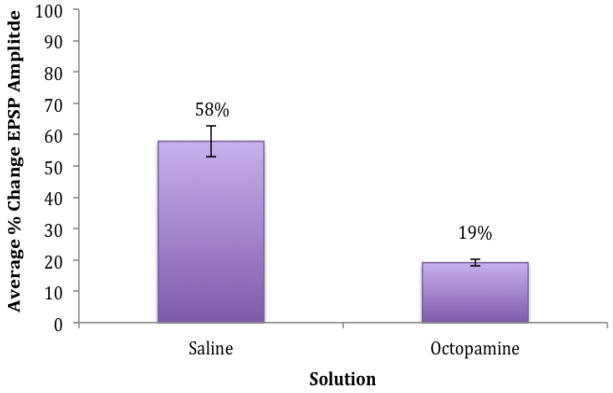

Figure 2. *Average percent change of EPSP of crayfish muscle cells in Baseline saline solution and octopamine solution.* In both cases, percent change was calculated by taking the difference between the second and the first EPSPs from the twin pulse stimulation divided by the first EPSP, and then percent changes were averaged for each condition. The average percent change of EPSP in the crayfish muscle cell in the low-calcium crayfish saline solution is graphed with error bars representing Standard Error (±5%). N=64. The average percent change of EPSP in the crayfish muscle cell submerged in the 10 µM octopamine solution is graphed with error bars representing S.E. (±1%). N=66.

To account for time, we conducted one experiment with the crayfish submerged in the low-calcium crayfish saline solution for the first half of measurements and the octopamine solution for the second half. Another experiment was done in reverse. There was a significant difference found in the post-octopamine-flushed saline trial and the rest of the baseline saline trials. A t-test reveals a p-value of $6*10^{-14}$ indicating that these results did not happen by chance. Therefore, these data points have been taken out of the average baseline condition EPSP percent change in our results.

DISCUSSION

Our results showed that octopamine does significantly increase neurotransmitter release while significantly reducing facilitation through residual calcium. While this finding is contrary to our hypothesis, which stated that octopamine would have no effect on facilitation through residual calcium, it does raise interesting alternatives which might further the current understanding of octopamine's synaptic role, and its relationship with serotonin.

We found that facilitation due to residual calcium decreased when octopamine was introduced, as evidenced by lower second EPSP amplitudes produced by twin-pulse stimulation, while neurotransmission overall was greatly facilitated, when compared to the control. This finding suggests octopamine is responsible for the decrease in residual calcium ions in the cytoplasm. There are two possible explanations for these results.

Decreased facilitation in the presence of octopamine could be a direct result of a greater release of neurotransmitters. Theoretically, neurotransmission by residual calcium is relative to the size of the EPSP. If the same number of synaptic vesicles is released by residual calcium in both a large EPSP and a small EPSP, then the portion of the EPSP transmitted by residual calcium is relatively smaller for the large EPSP and relatively larger for the smaller EPSP. Therefore, it is more difficult for facilitation by residual calcium to occur after a large EPSP than a small one. This trend would explain why we observed, on average, low EPSP amplitudes with high facilitation for the saline trials and high EPSP amplitudes with low facilitation for the octopamine trials. However, because facilitation decreased by such a large percentage, we believe that other factors may be at play.

As an alternative, the decrease in residual calcium concentrations in the presence of octopamine could be attributed to an octopamine-facilitated reuptake of calcium ions into the endoplasmic reticulum. Similarly, Delaney et al. (1991) suggests that serotonin may signal second messenger IP_3 to release calcium from the endoplasmic reticulum, we believe that octopamine may trigger an opposing second messenger pathway responsible for storing residual calcium in the ER. This idea is further supported by the suggested inverse relationship between serotonin and octopamine in crayfish on a behavioral level (Glansman & Krasne 1983; Gill, M.D., & P. Skorupski. 1996; Brummer et al., 2000; Momohara, Y., A. Kanai, and T. Nagayama. 2013). Should it be proven, this theory would provide support to further link the two neurohormones on a molecular level.

In humans, dopamine is considered to be the equivalent of octopamine, and similarly, it has been linked to opposing changes in behavior from serotonin. While dopamine and octopamine, and for that matter, humans and crayfish are not fully comparable, because crayfish are used so widely as a model for synaptic behavior, research showing similarities or other correlations between the octopamine and serotonin pathways could provide a preliminary synaptic-level support for the dopamine-serotonin connection which could open the doors to further experiments. In humans, the dopamine-serotonin relationship is a critical one. Dopamine and serotonin are considered to function on opposite ends of a behavioral spectrum. Serotonin has been linked to compulsive behaviors seen in people with OCD and anxiety disorders, while dopamine has been shown to cause impulsive behaviors such as those exhibited by gambling addicts and people diagnosed with ADD/ADHD, and additionally is used to treat patients with neurodegenerative diseases such as Parkinson's (Dongju, S. & C.J. Patrick. 2008; Fineberg et al., 2010; Seibell, P.J. & E. Hollander. 2014). An attempt to link dopamine and serotonin synaptically would potentially shed light on the origins of a wide range of behaviors, as well as give insight into possible treatments for behavioral disorders and devastating neurodegenerative diseases influenced by a lack or excess of either neurotransmitter. Our findings only scratch the surface of the molecular and synaptic mechanisms involved in the balance of these neurohormones. Further research on specific pathways that are potentially influenced by octopamine, dopamine or serotonin would surely yield informative results and lay the foundation for continued research on the relations of neurohormones and their impact on systems as a whole.

ACKNOWLEDGEMENTS

We thank Clark Lindgren, our professor, Sue Kolbe, our lab assistant, Jason Parks, our lab technician, and Allie Byrne, our mentor, for helping us with our research. We thank Grinnell College for funding our research. We also thank our crayfishes, Bananaconda, Cray Cray the Crayfish, Patricia, Rick, and Walt, as well as the other, unnamed crayfishes that gave their tails and lives for science.

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