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Serotonin does not exclusively use the adenylate cyclase pathway to facilitate synaptic transmission at the crayfish neuromuscular junction

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ABSTRACT

The adenylate cyclase pathway is commonly used in neurons to send off second messengers to transmit signals within a cell. Certain neurotransmitters may bind to adenylate cyclase to activate the pathway. Studies have shown that serotonin utilizes the adenylate cyclase pathway and facilitates excitatory postsynaptic potentials (EPSPs) in the lateral giant interneuron (located in the central nervous system of the crayfish). However, serotonin has never been found to use the adenylate cyclase pathway at the neuromuscular junction of crayfish. To determine if serotonin facilitates EPSPs via the adenylate cyclase pathway in *Procambarius clarkii* crayfish, we compared EPSP amplitudes of serotonin alone to EPSP amplitudes of a control crayfish saline solution, and to serotonin combined with an adenylate cyclase inhibitor (SQ22,536). We found that serotonin does facilitate EPSPs, but the adenylate cyclase inhibitor (SQ22,536) does not block EPSP facilitation completely. Therefore, the adenylate cyclase pathway is not the only means by which serotonin facilitates EPSPs at the crayfish neuromuscular junction in the dorsal extensor muscle.

INTRODUCTION

Serotonin (5-hydroxytryptamine or 5-HT) is a neurotransmitter that has been shown to increase feelings of happiness and reduce anxiety (Charney et al. 1987). Serotonin alters presynaptic cell activity as a function of the level of neurotransmitter release, but the means by which this activity is altered are unknown (Hervas et al. 2001).

Serotonin plays a role in cell plasticity in many different species. In sea slugs, *Aplysia*, and rats, *Heterocephalus glaber*, serotonin has been shown to increase levels of EPSP facilitation (Schacher et al. 1990, Fuller et al. 2000). Therefore we understand serotonin's role in synaptic plasticity to be relatively consistent across various types of animals.

In the crayfish neuromuscular junction, previous research indicates that increased levels of serotonin facilitate excitatory postsynaptic potentials (EPSPs) at the dactyl abductor muscle (Dudel 1965).

Additionally in crayfish, serotonin has been shown to have a role as a regulator of aggressive behavior and anxiety (Dingman et al. 2009). Serotonin as a neuromodulator increases the release of glutamate, a neurotransmitter, by altering presynaptic cell activity (Alvarado-Alvarez et al. 2000).

Presynaptic cell activities can be altered by protein modification in the cell. Adenylate cyclase is a protein embedded in the cell membrane that turns adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA), which then changes the activity of target proteins within the presynaptic cell. PKA regulates neurotransmitter release by modifying the protein structures that are involved in the release of neurotransmitters (Leenders and Sheng 2005). This integral change allows the synapse to retain strength and for the cell to more easily participate in synaptic transmission.

The purpose of this study was to address the question of how serotonin facilitates synaptic transmission at the crayfish neuromuscular junction. No research has been done to indicate how this synaptic facilitation takes place at the crayfish neuromuscular junction. Araki et al. (2005) found that, in the crayfish lateral giant interneuron, serotonin uses the adenylate cyclase pathway to produce an increased release of the glutamate from the presynaptic neuron, and thus produce a greater EPSP. Once we understand how serotonin works at the crayfish neuromuscular junction, we can use this information to help us understand what happens in the synapses that are more medically pertinent, like those of humans.

Knowing how serotonin facilitates EPSPs is incredibly useful understanding the possible effects of selective serotonin reuptake inhibitors (SSRIs), one type of antidepressant. If serotonin creates greater activity in the muscles in crayfish, it is possible that antidepressant use in humans can cause problems with muscle activity after prolonged use of SSRIs. It is possible that antidepressants may lead to diseases such as Parkinson's disease, thus it is crucial to understand serotonin's

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mechanism of producing a greater release of glutamate from the presynaptic cell.

We hypothesized that serotonin uses the adenylate cyclase pathway to create this synaptic facilitation. We observed that serotonin facilitates EPSPs at the crayfish neuromuscular junction. However, the adenylate cyclase inhibitor (SQ22,536) does not block EPSP facilitation completely. Therefore, serotonin does not exclusively use the adenylate cyclase pathway to facilitate EPSPs.

MATERIALS AND METHODS

Crayfish Preparation.

We used a total of eight crayfish over eight days of testing. We anaesthetized each crayfish by keeping it in an ice-water bath. When the crayfish ceased to move, we revealed the muscle by first cutting the tail off the abdomen at the base of the tail. Then the head of the crayfish was placed back in the ice-water bath. To reveal the dorsal extensor muscles, we made incisions slightly above the ridges on the side of the crayfish tail, and cut toward the telson. We cut off this top portion of the tail that we separated from the bottom part of the tail. We used our thumbs to push away the excess muscle in the way of the dorsal extensor muscles. We pinned the crayfish specimen to a dissection bowl filled with crayfish saline until it covered the severed crayfish tail entirely. The saline solution consisted of 5.4 mM KCl, 200.7 mM NaCl, 12.3 mM $MgCl_26H_2O$, 5 mM sodium HEPES buffer, and 6.5 mM $CaCl_22H_2O$. We replaced the saline every 45 minutes to preserve the crayfish nerves and muscles. The crayfish in the dissection bowl was placed underneath a Leica Zoom 2000 microscope. Along with the microscope's light, we used a Fiber-Lite fiber optic illuminator to make the crayfish interior easier to see.

Intracellular Recordings.

We placed two reference electrodes in the solution to measure the extracellular solution compared to the intracellular voltage value. We used a Pul-1 microelectrode puller to make glass microelectrodes. We attached a recording microelectrode filled with 3 M KCl to a microelectrode holder (also filled with 3 M KCl) stationed on a Kanetec micromanipulator supported by a Craftsman Magnetic Base. We also placed a suction electrode on a Kanetec micromanipulator attached to the Craftsman Magnetic Base. We made the suction electrode by sanding down a microelectrode to a diameter of ~1/3 mm to give enough space to suck up a crayfish nerve in order to stimulate it.

We used the suction electrode to suck up the nerve with a suction syringe and pull the nerve up into the suction electrode. We also lowered the recording microelectrode into the saline solution, and set the voltage readings on LabChart to zero to see the voltage change easily once the microelectrode entered the muscle cell. We placed the recording microelectrode close to the targeted nerve so that when we stimulated the nerve it would affect the muscle cell that we entered. We used MacLab Bridge Amp and Powerlab 4/25 (AD Instruments) to measure the voltage of the crayfish cells. We read our voltage in the computer program LabChart on a MacBook Pro to make our measurements easier to read.

We stimulated each nerve with single pulse stimulation with a voltage of ~5-20 mV with duration on .1 millisecond and a frequency of .5 pps. Voltage varied due to different nerves responding differently to higher or lower voltage. Too high of a stimulation can cause twitching that would cause the recording electrode to come out of the muscle cell or the nerve to move out of the stimulating electrode due to twitching.

Experimental Conditions.

We tested four conditions. The first was our control solution, which was a crayfish saline solution composed of 5.4 mM KCl, 200.7 mM NaCl, 12.3 mM $MgCl_26H_2O$, 5mM sodium Hepes Buffer, and 6.6 mM $CaCl_22H_2O$. The second condition consisted of 10 µM serotonin, from a 10 mM serotonin stock solution. The third condition was a 10 µM serotonin solution with 100 µM SQ-22536, an adenylate cyclase inhibitor. The fourth condition consisted of testing 100 µM SQ-22536 by itself from a 100 mM stock solution. We tested this to ensure that 100 µM SQ22,536 did not affect EPSP amplitude by itself. In each condition, the solution sat for five minutes to allow the chemicals to work into the crayfish's system. We conducted a second condition of testing EPSPs in the 10 µM serotonin solution. For this condition, we waited fifteen minutes after applying the serotonin solution to the crayfish before we stimulated the nerve and observed EPSPs. We tested this condition that factors in time to make sure that serotonin still on the crayfish when we added in the 100 µM SQ22,536.

Every day we tested on a new crayfish, but multiple conditions were tested on that crayfish. We alternated the set of conditions every other day. On one day, we would test in the following order: control, 10 µM serotonin, 10 µM serotonin and 100 µM SQ22,536, and control. The next day would test the following, in order: control, 100 µM SQ22,536, and control. We alternated these different sets of conditions every other day of testing to obtain an equal amount of data for every condition.

Data Analysis.

We analyzed our data by comparing the average control solution EPSP amplitudes with the experimental conditions' EPSP amplitudes. We also compared EPSPs by calculating the percent change in EPSP amplitude from the control solution. We calculated percent change to show how much the conditions changed from the baseline EPSP. To calculate the percent change EPSP amplitude, we took the difference between the post-stimulus artifact resting membrane potential and the peak membrane potential of the recorded EPSP and divided that from the control solution EPSP amplitude: percent change in EPSP = [EPSPc —EPSPv]/[EPSPc], where c=control, and v=variable solution. We averaged EPSPs calculated over at least three trials for each solution; each trial contained 25 EPSP amplitudes. We used a two-tailed student t-test to determine significance.

RESULTS

We tested serotonin's use of the adenylate cyclase pathway to facilitate synaptic transmission. To study the effects of serotonin and the adenylate cyclase inhibitor (SQ22,536) on excitatory postsynaptic potentials (EPSPs), we used intracellular recording at the crayfish neuromuscular junction in the dorsal extensor muscle.

Serotonin Facilitation.

We observed that serotonin increased the percent change of EPSP amplitude compared to the standard saline solution. We tested two conditions of 10 µM serotonin. In the first condition, we waited five minutes after applying the serotonin solution to the crayfish before we stimulated the nerve and observed EPSPs. In the second condition, we waited fifteen minutes after applying the serotonin solution to the crayfish before we stimulated the nerve and observed EPSPs. Because there was a statistically insignificant difference on the %change of EPSP amplitude, we combined these two conditions to compare the effects of serotonin on EPSP facilitation.

We compared the EPSP amplitude in the standard saline solution to the EPSP amplitude in the 10 µM serotonin solution (Figure 1). A two-tailed student t-test showed evidence to reject the null hypothesis that there was a difference between the EPSP amplitude in the standard saline solution and the EPSP amplitude in the serotonin solution (p=0.005). A 10 µM serotonin concentration facilitates EPSPs in the crayfish neuromuscular junction at the dorsal extensor muscle.

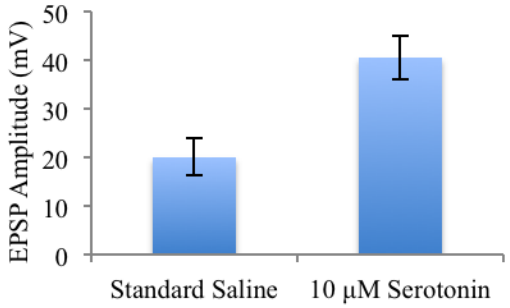

Figure 1. Mean excitatory postsynaptic potential amplitude in standard saline and 10 µM serotonin. Error bars represent standard error of the mean. In the standard saline, n=8 over eight trials with eight different crayfish. In the 10 µM serotonin, n=6 over six trials with six different crayfish. A two-tailed student t-test (p=0.005) showed that serotonin facilitated EPSP amplitude at the crayfish neuromuscular junction.

Inhibition of Adenylate Cyclase Reduced Serotonin Facilitation.

We wanted to know if serotonin exclusively uses the adenylate cyclase pathway to facilitate EPSPs at the crayfish neuromuscular junction. We compared %change in EPSP amplitude in the 10 µM serotonin solution and in the 10 µM serotonin & 100 µM SQ22,536 solution (Figure 2). A two-tailed student t-test (p=0.038) showed evidence to support that there was a difference in the percent change of EPSP in the 10 µM serotonin solution and the 10 µM serotonin solution & 100 µM SQ22,536 solution. Serotonin still produced an increased EPSP when SQ22,536 inhibited the adenylate cyclase pathway. However, this increased %change is significantly less than the EPSP amplitude %change when serotonin uses the adenylate cyclase pathway.

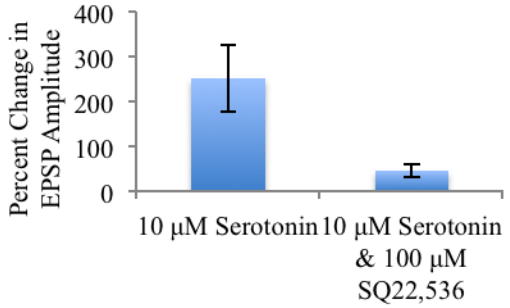

Figure 2. Mean %change in EPSP amplitude from a standard saline solution in 10 µM serotonin and 10 µM serotonin & 100 µM SQ22,536. Error bars represent standard error of the mean. In the 10 µM serotonin, n=6 over six trials with six different crayfish. In the 10 µM serotonin & 100 µM SQ22,536, n=3 over three trials with three different crayfish. A two-tailed student t-test (p=0.038) showed evidence to support that serotonin produces a greater %change in EPSP with the adenylate cyclase pathway than without the pathway.

Control Experiments.

SQ22,536 Had No Effect on EPSP Amplitude. We wanted to confirm that the %change in EPSP amplitude we observed in the 10 µM serotonin & 100 µM SQ22,536 solution was not due to SQ22,536. We compared the EPSP amplitude from the 100 µM SQ22,536 to the EPSP amplitude from the standard saline (Figure 3). A two-tailed student t-test (p=0.599) indicated that there was a statistically insignificant difference between the standard saline and the 100 µM SQ22,536. The adenylate cyclase pathway does not influence the standard EPSP amplitude.

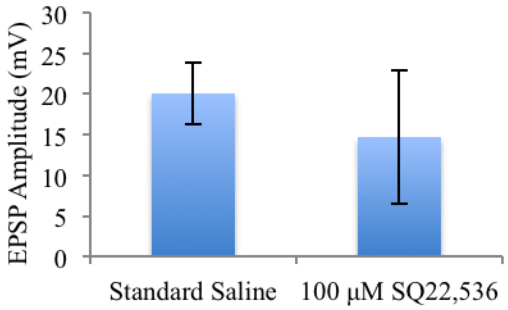

Figure 3. Mean excitatory postsynaptic potential amplitude in standard saline and 100 µM SQ22,536. Error bars represent standard error of the mean. In the standard saline, n=8 over eight trials with eight different crayfish. In the 100 µM SQ22,536, n=3 over three trials with three different crayfish. A two-tailed student t-test (p=0.599) showed that the 100 µM SQ22,536 has no effect on EPSP amplitude at the crayfish neuromuscular junction.

Time Did Not Affect EPSPs in Standard Saline. We wanted to account for time as a factor in EPSP amplitude production. We compared EPSP amplitude from the standard saline solution in each crayfish trial before and after we tested other solutions (Figure 4). This time period ranged from one to two hours. A two-tailed student t-test (p=0.238) showed that there was a statistically insignificant difference between the initial standard saline solution EPSP amplitude and the final standard saline solution EPSP amplitude. Mean EPSP amplitude did not differ, regardless of when the amplitudes were taken.

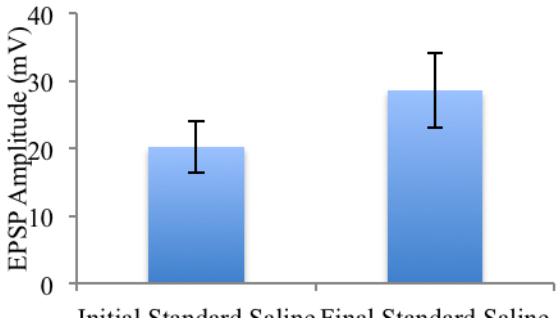

Figure 4. Mean excitatory postsynaptic potential amplitude in standard saline solutions before testing other conditions (Initial Standard Saline) and after testing other conditions (Final Standard Saline) for each trial with a new crayfish. Error bars represent standard error of the mean. In the initial standard saline, n=8 over eight trials with eight different crayfish. In the final standard saline, n=6 over six trials with six different crayfish. A two-tailed student t-test (p=0.238) showed the same EPSP amplitude was the same in both solutions at the crayfish neuromuscular junction.

DISCUSSION

Our results demonstrated that serotonin facilitated EPSPs, but the serotonin does not exclusively use the adenylate cyclase pathway to achieve this facilitation. The adenylate cyclase inhibitor SQ22,536 and serotonin combined produced higher EPSPs than the control solution, contrary to what we predicted. The SQ22,536 solution also generated insignificant statistics to say that SQ22,536 caused lower EPSPs than regular saline.

Serotonin Facilitation.

Dudel (1965) first demonstrated serotonin's facilitative effects in the crayfish neuromuscular junction. However, Dudel conducted his experiment in the dactyl abductor muscle of the leg. No previous research has shown the effects of serotonin in the neuromuscular junction at the dorsal extensor muscle found in the tail of the crayfish. When we compared the EPSP recording with the standard saline solution to the EPSPs recordings from the serotonin solution, they were significantly higher.

Serotonin created this facilitation because when serotonin binds to the presynaptic cell, it causes a change in the presynaptic cell, making it release heightened levels of glutamate. Glutamate is the neurotransmitter in crayfish equivalent to acetylcholine in humans—when released in the neuromuscular junction of the crayfish it signals the muscle to twitch (Dudel 1965). When more glutamate is available, more of them to bind to the postsynaptic cell, therefore creating a greater EPSP facilitation.

Inhibition o f Adenylate Cyclase Reduced Serotonin Facilitation.

The data show that there is a larger change in EPSP in serotonin alone than when we added SQ22,536 to the serotonin. This EPSP decreasing of facilitation from the serotonin saline condition supports our hypothesis and demonstrates that serotonin does use the adenylate cyclase pathway in the presynaptic cell to facilitate EPSPs. But because the SQ22,536 did not fully inhibit serotonin's effect on the EPSP amplitude, there must be another mechanism for how serotonin facilitates EPSPs.

We found these results in the dorsal extensor muscle. Maricq et al. (1991) found serotonin uses multiple second messenger systems to facilitate EPSPs at the chemical synapse. Their findings conclude that serotonin may activate many ion channels and proteins that release more neurotransmitters into the presynaptic cell, therefor increasing EPSP.

Somehow, serotonin allows more glutamate release at the neuromuscular junction. We wanted to determine the mechanism by which serotonin triggers the increased amount of glutamate release. Knowing that serotonin facilitates EPSPs is incredibly useful for understanding the possible effects of selective serotonin reuptake inhibitors (SSRIs), one form of antidepressants. If serotonin creates greater activity in the muscles in crayfish, it is possible that antidepressant use in humans can cause problems with muscle activity after prolonged use of SSRIs. It is possible that antidepressants may lead to diseases such as Parkinson's disease, thus it is crucial to understand serotonin's mechanism of producing a greater release of glutamate from the presynaptic cell.

We can also use the inhibition of the adenylate cyclase pathway to decrease the unwanted effect of serotonin at the neuromuscular junction. We can then discover new medicines that have the same effects as modern day antidepressants do while decreasing the unwanted side effects of using SSRIs (selective serotonin reuptake inhibitors) that target serotonin at the synaptic gap.

SQ22,536 Had No Effect on EPSP Amplitude.

EPSP amplitude when SQ22,536 combined with the crayfish saline, decreased in comparison to the control saline. SQ22,536 alone also produced lower EPSP amplitudes compared to the condition of SQ22,536 and serotonin together. We tested SQ22,536 alone to determine if the inhibition of adenylate cyclase produced any facilitation or depression in EPSP. In Dixon and Atwood (1989b) they found that when using an increase in cAMP to facilitate EPSP using the adenylate cyclase pathway, that it indeed increased EPSP amplitudes. We tested the inhibition of just adenylate cyclase and therefore the inhibition of cAMP on EPSP amplitude and expected, in comparison to the serotonin and the SQ22,536 together, they would produce the same results. This is because we expected the SQ22,536 to block the adenylate cyclase and thus prevent serotonin from facilitating EPSPs--producing no change in EPSP from the control solution.

We also expected SQ22,536 alone to have similar results to what we observed in our experimentation. If the percent change in EPSP amplitude of SQ22,536 alone is lower than serotonin and SQ22,536 combined, this suggests that SQ22,536 creates a depression in EPSPs. Even though it wasn't a huge change in EPSP this was found in Dixon and Atwood (1989a) that when an adenylate cyclase inhibitor was inserted into the neuromuscular junction and the EPSP depressed. When looking at the chemical synapse from a larger perspective, the inhibition of adenylate cyclase does depress EPSPs. The adenylate cyclase pathway thus is one way to affect EPSPs created in crayfish dorsal extensor muscles.

Time Did Not Effect EPSPs in Standard Saline.

We tested the standard saline solution before and after we were done testing all the conditions to ensure that the EPSP did not decrease. If the EPSP did decrease we might have had to assume that some tissue damage was present while stimulating the nerve in the neuromuscular junction. But because the EPSP did not decrease and actually increase (because of the residue of serotonin still present in the neuromuscular junction) we can assume that the neuromuscular junction was healthy enough to collect consistent data throughout the entire time that we stimulated the nerve.

Potential Experimental Errors.

There were several potential problems that may have affected the data we collected. When we inserted the 10 µM serotonin and 100 µM of SQ22,536 we did not insert the chemical directly into the chemical synapse. We first diluted the chemicals with around 100 mL of regular saline in order to fill the crayfish dish. Not having exact amounts chemical distribution amongst the crayfish might result in a different amount of chemicals actually making it to the specific neuromuscular junction that we were testing.

In our SQ22,536 testing, we may not have used enough of the chemical to totally inhibit EPSP. This may cause a larger EPSP and not accurate data in

regards to looking the adenylate cyclase not effecting EPSP.

For our final data analysis we compared the standard saline solution before and after each conditions and compared the EPSP amplitudes. As cell membrane becomes more adaptive to the microelectrode piercing its cell membrane, the resting membrane potential tends to rise. Also when we washed away the serotonin from the crayfish, the effects that the serotonin had on the crayfish may not have worn off. These may result in greater EPSP amplitudes.

Conclusion.

Our experiment was designed to find that serotonin used the adenylate cyclase pathway in the presynaptic cell. We found that serotonin does indeed use the adenylate cyclase pathway. However, serotonin uses more than just the adenylate pathway in the neuromuscular junction to increase EPSPs. Further research could be done to find out the other mechanisms where serotonin facilitates EPSPs in the synapse. Serotonin has been found to increase calcium influx in the presynaptic cell (Eliot et al. 1993) and therefore increases EPSPs. There could be research that could be done on the utilization of adenylate cyclase as a means of effecting EPSPs. And more research could be done to discover which other neuromodulators use the adenylate cyclase system to affect neuromuscular junction EPSPs.

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