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Serotonin and fluoxetine cause paired pulse depression in typically facilitating synapses in crayfish neuromuscular junction.

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ABSTRACT

Serotonin, a neurotransmitter in humans, affects synaptic plasticity when used as a modulator with and without the presence of fluoxetine, a serotonin reuptake inhibitor. Most previous research has shown that serotonin causes synaptic facilitation when subjected to long-term stimulation, and experiments on fluoxetine have had mixed results, with some showing facilitation and others showing depression. Our experiment tested the effect of serotonin and serotonin with fluoxetine. We recorded EPSPs after paired pulse stimulation in the crayfish neuromuscular junction while exposed to these chemicals to learn if the chemicals cause paired pulse stimulation or depression in crayfish, which use serotonin as a modulator. Our results showed that serotonin as well as serotonin with fluoxetine caused paired pulse depression in postsynaptic potentials when the control condition yielded paired pulse facilitation. However, the percent change in EPSP amplitude was greater in just serotonin than in serotonin plus fluoxetine. These results indicate that when used as a modulator in the crayfish neuromuscular junction, as opposed to a neurotransmitter, serotonin does not facilitate enhanced synaptic plasticity. Fluoxetine, however, decrease the paired pulse depression caused by serotonin.

INTRODUCTION

Neurons communicate with other nerve and muscle cells by releasing neurotransmitters into the synaptic gap, where they can bind to receptors on the postsynaptic cell and cause a postsynaptic potential. This potential could either be excitatory or inhibitory depending on the neurotransmitters and their function. Postsynaptic potential strength can be altered based on prior stimuli. This process is called synaptic plasticity and it is measured by change Excitatory Postsynaptic Potential (EPSP) amplitude. If a chemical facilitates synaptic transmission, then a second stimulus to the presynaptic cell will cause a larger EPSP than the stimulus that came before it.

In crayfish serotonin is a chemical that acts as a modulator, meaning that it can change the amount of primary neurotransmitter released by a presynaptic neuron. The primary neurotransmitter in the crayfish neuromuscular junction is glutamate. When glutamate is released from the presynaptic cell it binds to receptors on the postsynaptic cell, which signal EPSPs. Exposing crayfish to serotonin can increase the amount of glutamate released, which would affect the size of EPSPs and, under paired pulse stimulation, the plasticity. A study done by Johnstone et al. (2008) provided a possible explanation for why serotonin could cause facilitation in crayfish. Using high frequency stimulation (5 Hz over 10 minutes) the scientists found that after being treated with serotonin, presynaptic cells were more likely to release vesicles from the reserve pool to the readily releasable pool. Because this study used stimulation that depleted the readily releasable pool of vesicles, the effect was a delayed depression of EPSP and not facilitation of EPSP. However, this effect of serotonin could logically cause facilitation if applied to a muscle undergoing short-term stimulation, since more vesicles filled with glutamate are available without exhausting the previous supply. The experiment by Sparks and Cooper in 2004 tested the extensor muscle of a crayfish leg, and found that when the crayfish muscle tissue was treated with serotonin and stimulated using short-term paired-pulse stimulation, facilitation occurred. The scientists also concluded that the facilitation was a result of more glutamate (a primary neurotransmitter in crayfish) being released into the synaptic gap, and when the reserve pool was exhausted there was not as great of an increase in glutamate released.

In humans, serotonin acts as a primary neurotransmitter. Drugs called Selective Serotonin Reuptake Inhibitors (SSRIs) work in humans to regulate levels of serotonin by preventing its reuptake into a presynaptic cell. Fluoxetine is an SSRI, so the effect of adding it to an extracellular solution also containing serotonin should be that there is more serotonin the synaptic gap to bind with presynaptic receptors. Therefore, fluoxetine should enhance paired pulse facilitation caused by serotonin. Knowing how these chemicals affect neuromuscular plasticity can possibly provide the insight into the mechanisms serotonin uses to affect EPSPs. We chose to test serotonin and fluoxetine

together in order to shed light on how fluoxetine effects nerve to cell communication in an animal which does not use serotonin as its primary neurotransmitter.

Previous research on fluoxetine has been done on animals. Graves, et al. (2002) used tetanic stimulation a crayfish tail exposed to fluoxetine showed increased EPSPs compared to a control tail only exposed to saline. Other research has been done using both tetanic and paired-pulse stimulation on other animals such as rats which show that fluoxetine does indeed cause facilitation but not as much as other drugs like amitriptyline that have similar functions to fluoxetine (Zarei et al. 2014). In contrast, Zu and Luk (2010) found indicated that fluoxetine causes a decrease in EPSP amplitude. The researchers tested tetanic stimulation in Lymnaea tissue, and found that fluoxetine not only caused a decrease in EPSP amplitude, but also physically degenerated the neurons.

Our research was aimed at determining exactly how exactly serotonin as well as serotonin with fluoxetine affect the plasticity on the crayfish muscular junction. We will test using paired pulse stimulus to monitor the change in plasticity as transmissions are propagated from the presynaptic cell to the postsynaptic cell. We predicted that both of these drugs would cause paired pulse facilitation. But, because fluoxetine will increase the amount of serotonin available to bond with receptors, we predicted that fluoxetine would cause higher paired pulse facilitation. Our results show that serotonin only caused paired pulse facilitation in the muscle that showed paired pulse depression in the control condition. The muscle that yielded paired pulse facilitation in the control condition showed paired pulse depression when exposed to serotonin. Serotonin with fluoxetine caused also caused paired pulse depression, but the depression was less than that caused by serotonin alone.

MATERIALS AND METHODS

Subject Preparation

We prepared our crayfish by submerging it in ice water, fully anesthetizing it. Once the crayfish was no longer moving, the tail was removed at the base using scissors. Using scissors, both ends of the tail were cut near the ventral side all the way to the last segment. The ventral surface was pulled away and discarded. The bulk of the muscle tissue was pushed away using a fingertip, and was also discarded. The fully dissected tail was then fastened to a dissection bowl using four pins.

Conditions Preparation

Our control trials measured EPSP amplitude while the crayfish tail was submerged in low calcium saline. The saline consisted of 5.4 mM KCL, 200.7 mM NaCl, 12.3 mM $MgCl_2H_20$, 5 mM Sodium Hepes Buffer, and 6.5 mM $CaCl_22H_20$, and was provided for us. Our serotonin condition was a 10µM solution of serotonin. We prepared this by measuring 100mL of low calcium saline into a flask, adding 100µL of serotonin using a micropipette, and inverting to mix. Our serotonin and fluoxetine condition used a 10µM solution of serotonin and 10µM of fluoxetine. We prepared this solution by measuring 100mL of low calcium saline into a flask, and adding 100µL of serotonin, then 100µL of fluoxetine, and inverting to combine.

Procedure

To test the effect of the chemicals on paired pulse facilitation, we first placed the tail in low-calcium crayfish saline and used a suction electrode to stimulate a nerve bundle, while having recording electrode inserted into a muscle. After measuring the control condition, we removed all the saline from the dish using a syringe. We inserted the solution (either 10µM serotonin or 10µM serotonin with 10µM fluoxetine into the crayfish dish and continued recording from the same muscle cell and stimulating the same nerve as in the control trial. Occasionally during this process of changing solution, our nerve wasn't completely grasped in the suction electrode. When this occurred, we used our syringe to pull the same nerve back into our electrode again. We let the serotonin solution sit in the dish for five minutes before collecting data. Following this process we removed the chemical, replaced it with low-calcium saline, and collected data on a second control condition that we called the follow-up control. We repeated the procedure for another postsynaptic muscle cell.

Control for Time

The purpose of the follow-up control was to allow us to conclude that the change in EPSP amplitude was due to the chemical and not tissue death. It served as a way to control for time, since it took varying amounts of time to find an EPSP in each crayfish.

Data Collection

To collect data on change in EPSP amplitude we used a process of presynaptic paired pulse stimulation and postsynaptic recording. To accomplish this we used a suction/stimulation microelectrode attached to a Grass SD 9 Stimulator to stimulate a presynaptic nerve at .5 Hz. Each stimulus lasted .5 ms and a delay of 20 ms between the paired pulses. The voltage was adjusted for each muscle in order to find EPSPs. We made the suction/stimulation electrode by pulling a glass electrode and filing down the tip using sandpaper until the opening

was wide enough to hold a nerve. We recorded the EPSP amplitude through a glass microelectrode filled with 3 M KCl solution and inserted into a postsynaptic muscle cell. This recording electrode sent the EPSPs through Bridge Amp and Power Lab to be recorded on a laptop with LabChart software. Each set of two pulses created two EPSPs, which were saved on one LabChart page. Each condition within each muscle recorded for about 20 seconds, and we tested two muscles for both serotonin and serotonin with fluoxetine. We saved the LabChart Pages themselves, as well as recording resting membrane potential, amplitude of first EPSP, and amplitude of second EPSP.

Data Analysis

Our experiment dealt with measuring plasticity, meaning the difference between EPSP amplitudes after paired pulse stimulation. In order to objectively measure plasticity, we took our preliminary data on first and second EPSP amplitude and calculated percent change in EPSP. This calculation looks like:

$[(EPSP_2-EPSP_1)/EPSP_1] * 100$

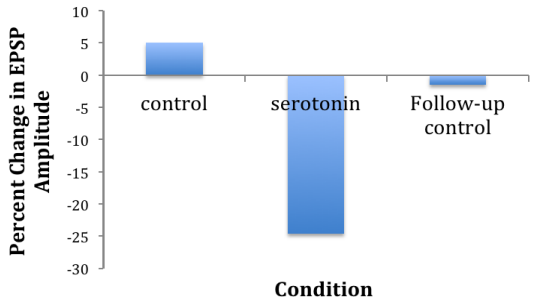
After calculating percent change in EPSP, we analyzed the mean percent change in EPSP over 10 pages for each muscle exposed to the conditions. We also calculated percent error for each condition.

RESULTS

Our measurements of percent change in EPSP amplitude using paired pulse stimulation showed the effect of serotonin on crayfish tail muscle cells. We tested two muscle cells in our crayfish, each with separate control conditions. Our first muscle showed paired pulse facilitation under control conditions, and then paired pulse depression when exposed to serotonin (Figure 1A). The follow-up control showed paired pulse depression while the control showed paired pulse facilitation.

Our second muscle cell treated with serotonin showed paired pulse depression in the control condition and paired pulse facilitation when exposed to serotonin (Figure 1B). In both muscles serotonin caused a reversal of the control condition; if the synaptic transmission originally caused paired pulse facilitation than serotonin caused paired pulse depression, and if synaptic transmission started with paired pulse depression then serotonin caused paired pulse facilitation.

A.



B.

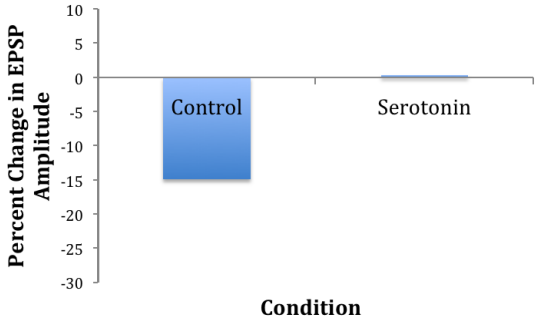


Figure 1. The mean percent change in EPSP amplitude of the second pulse compared to the first pulse in each pair, for control and serotonin conditions. In Figure A the muscle started with paired pulse facilitation in the control condition, with a 5.01% increase in EPSP amplitude. When serotonin was added, it caused paired pulse depression, with a 24.5% decrease in EPSP amplitude. The follow-up control showed paired pulse depression (-1.46). n=1. Figure B shows that the second muscle started with paired pulse depression in the control condition, with a 14.8 % decrease in EPSP amplitude (n=1). When serotonin was added, it caused paired pulse facilitation, with a .33% increase in EPSP amplitude (n=1).

In order to test our second hypothesis, whether fluoxetine causes further paired pulse facilitation when added to serotonin, we tested the change in EPSP amplitude using paired pulse stimulation in a control condition, serotonin and fluoxetine, and a follow-up control in two crayfish tail muscle cells from a different crayfish. In both muscles treated with serotonin with fluoxetine the control condition showed paired pulse facilitation and the follow-up control showed paired pulse depression, so we averaged the data for both muscles. The serotonin and fluoxetine condition yielded paired pulse depression, and had a range of 15.6.

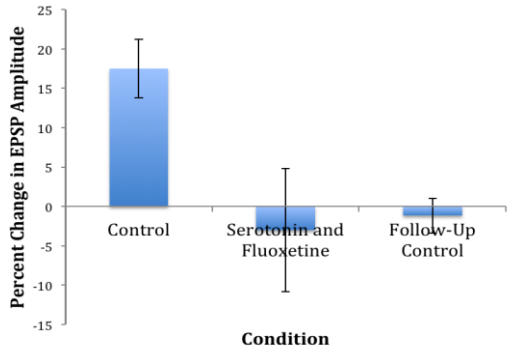


Figure 2. . The mean percent change in EPSP amplitude of the second pulse compared to the first pulse in each pair, for control, serotonin with fluoxetine, and follow-up control conditions. The control condition yielded paired pulse facilitation, with an increase in EPSP amplitude of 17.5%. Serotonin and fluoxetine showed paired pulse depression, with a decrease in EPSP amplitude of 2.9%. The follow up control yielded paired pulse depression, with a decrease in EPSP amplitude of 1.17%. The error bars represent the range between the highest and lowest values for each condition (7.5, 15.6, and 4.4 respectively). (n=2).

DISCUSSION

Our results did not fully support our hypothesis. Our first hypothesis, that serotonin would cause paired pulse facilitation, was only true when the control condition yielded paired pulse depression. When the control condition yielded paired pulse facilitation, the addition of serotonin caused depression. Experiments done by Johnstone et al. (2008) showed when crayfish muscles were subjected to long-term stimulation yielded facilitation under paired pulse stimulation since serotonin increases the probability that vesicles filled with neurotransmitter will be released from the reserve pool to the readily releasable pool. We assumed that this facilitation would hold for short-term facilitation, but our data shows this may not be the case. Serotonin shows different effects as a modulator in the crayfish neuromuscular junction under short-term stimulation, although the exact reason for this is unclear. Beaumount and Zucker (2000) performed an experiment in crayfish that suggests that activating serotonin receptors affects the CAMP system, which in turn modulates synaptic plasticity. A similar experiment by Enyeart (1981) found that when cyclic AMP levels increased in crayfish, the levels of transmitter released after a stimulus also increased. Since serotonin can cause increased levels of neurotransmitter release, this study suggests another link between the effects of serotonin and the cAMP system. A follow-up to our experiment could test several pathways serotonin might uses to affect plasticity. Experimenters could use drugs to inhibit certain steps in cyclic AMP and cyclic GMP pathways, and see if either of these is involved in serotonin's short-term effects.

Another aspect of our data that warrants explanation is our follow-up control data. All of our trials showed that the follow-up data was different than the control data. This was because each time we removed the solution with serotonin or serotonin and fluoxetine and poured in fresh saline, our recording electrode would come out of the muscle and we would need to reinsert it. Each time that happen, the electrode likely hit a different muscle cell, which would explain the different EPSP amplitudes.

Our second hypothesis, that serotonin and fluoxetine would show facilitation, was also not entirely correct, although the addition of fluoxetine did cause a smaller paired pulse depression than serotonin alone. The from this test showed that the control condition yielded paired pulse facilitation, and fluoxetine with serotonin caused paired pulse depression, though it was less than the depression from the serotonin only trial. A possible reason for this is that while fluoxetine inhibits the reuptake of serotonin, experiments by Luk and Xu (2010) showed that fluoxetine could damage or degenerate the physical nerve tissue in a neuron. The lowered paired pulse depression from fluoxetine and serotonin could be caused by the serotonin degenerating the serotonin receptors, meaning that the depression caused by the serotonin would not occur as strongly. A follow-up experiment could test the effect of fluoxetine on other receptors for neurotransmitters whose effects are well known, so the effect of fluoxetine would be clearer.

Our research yielded results that, although they do not support our hypothesis, leave many opportunities for future experimentation. We found that serotonin causes paired pulse facilitation in the crayfish neuromuscular junction only in cases when the control condition showed paired pulse depression. When the control condition showed paired pulse facilitation, then the addition of serotonin caused paired pulse depression. Fluoxetine with serotonin caused paired pulse depression, although it was less severe than the serotonin alone. Future research in the effects of these chemicals is needed to understand exactly what systems serotonin uses to effect plasticity as a modulator, and whether fluoxetine can have damaging effects on nerves and receptors.

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REFERENCES

Beaumount, V., R.S. Zucker. 2000. Enhancement of synaptic transmission by cyclic AMP modulation of presynaptic Ih channels. *Nature Neuroscience.* 3:133-141

Enyeart, J. 1981. Cyclic AMP, 5-HT, and the modulation of transmitter release at the crayfish neuromuscular junction. *Journal of Neurobiology*. 12:505-513

Graves, A., K. McCarthy, E. Mudoh. 2002. Increased EPSP amplitude at the crayfish neuromuscular junction for acute but not chronic exposure to fluoxetine. *Pioneering Neuroscience* 3:49-52.

Johnstone, A.F.M., S.S. Kellie, and R.L. Cooper. 2008. Presynaptic depression in phasic motor nerve terminals and influence of 5-HT on vesicle dynamics. *Open Neuroscience Journal* 2:16-23.

Sparks, G.M., R.L. Cooper. 2004. 5-HT offsets homeostasis of synaptic transmission during short-term facilitation. *Journal of Applied Physiology* 96:1681-1690.

Xu, F., C. Luk. 2010. Antidepressant fluoxetine suppresses neuronal growth from both vertebrate and invertebrate neurons and perturbs synapse formation between Lymnaea neurons. *European Journal of Neuroscience* 31:994-1005.

Zarei, G., P. Reisi, H. Alaei, and S.H. Javanmard. 2014. Effects of amitriptyline and fluoxetine on synaptic plasticity in the dentate gyrus of hippocampal formation in rats. *Advanced Biomedical Research* 3:199.