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Nitric Oxide Plays a Significant Role as a Retrograde Messenger that Causes Long-Term Facilitation at the Crayfish Neuromuscular Junction

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ABSTRACT

Nitric oxide (NO) is a compound that acts as a retrograde messenger in a variety of organisms. It also plays a role in the strengthening of synapses and facilitation of synaptic plasticity. In our study, we asked whether NO plays a similar role in the crayfish neuromuscular junction. Our results indicate that NO does indeed act as a retrograde messenger that is produced in the postsynaptic muscle fiber and affects the presynaptic cell, increasing synaptic plasticity and causing long term facilitation. When NO production was chelated in the postsynaptic cell, EPSP amplitude decreased considerably, and when NO production was catalyzed, EPSP amplitude increased, supporting our hypotheses.

INTRODUCTION

Neurons communicate with one another through synapses. The release of neurotransmitters into the synaptic gap transmits signals from the presynaptic cell to the postsynaptic cell (Lindgren, 2010). If a presynaptic cell is stimulated consistently over time there will be an increase in neurotransmitter release and therefore an increase in EPSP amplitude. This process is called long-term facilitation (LTF) (Sherman & Atwood, 1971). LTF has been found to occur in the crayfish neuromuscular junction (NMJ) (Dixon & Atwood, 1989). For the most part, synaptic action moves from the presynaptic cell to the postsynaptic cell however there are compounds that move the opposite way called retrograde messengers (Taqatqeh et at., 2009). Nitric oxide (NO) is a chemical compound created by the enzyme nitric oxide synthase (NOS). NOS is found in both the presynaptic cell as well as the postsynaptic cell so the directionality of nitric oxide as a messenger is debated. Many theorize however that NO is a retrograde messenger that can induce, or at least exacerbate, LTF (Kristie et al., 2010). However, the effect of NO as a retrograde messenger in crayfish has yet to be investigated.

LTF facilitates a strengthening of synapses, which is a crucial part of synaptic plasticity. Increases in synaptic performance have been shown to be a possible mechanism of memory (Teyler & DiScenna, 1987). Our research on the possible role of nitric oxide (NO) as a retrograde messenger and neuromodulator that facilitates synaptic plasticity is relevant in a larger scientific context as it relates to previous research done on long lasting effects of synaptic plasticity in the lateral amygdala. This testing was done in rats subjected to pavlovian fear conditioning. This article theorizes that synaptic plasticity created by the retrograde messaging of NO serves as the connection between these post and pre-synaptic areas (Ota et al, 2010). This research suggest that synaptic plasticity in the lateral amygdala, following auditory fear conditioning promotes synaptic plasticity in presynaptic sites by way of retrograde signaling driven by NO (Ota et al, 2010). Another study suggests a connection between the NO-producing enzyme (NOS) and mental disorders such as schizophrenia and bipolar disorder. The research argues that further understanding the mechanism of NOS isoforms could advance understanding and production of drugs associated with treatments of these disorders (Silberberg, et al, 2010). Our research has the potential to further cement the role of NO as a retrograde messenger and facilitator of synaptic plasticity, which would have larger implications for the fields of neuroscience as well as psychology.

In order to supplement research on the directionality and function of NO as a messenger we created an experiment that tested both. We used the NO scavenger, Carboxy PTIO, which decreases the presence of NO and theoretically would decrease EPSP amplitude and therefore LTF. We also chose to incorporate DEANO, an NO activator to see if we could reverse the effects of Carboxy PTIO. Experimentally, we asked whether NO plays a role as a retrograde messenger in the crayfish NMJ, and whether NO being released in the postsynaptic cell can induce LTF. Our hypotheses were that NO does serve as a retrograde messenger, and that it's presence in the crayfish NMJ would cause LTF to occur. Our data supported our hypotheses.

MATERIALS AND METHODS

Tissue Preparation

The crayfish, *Procambarus clarkii*, were supplied by Carolina Biological Supply Company (North Carolina, USA). The crayfish were placed in an ice bath before dissection. The tail was removed for our experiment and the thorax was returned to the ice bath. We removed the ventral surface of the tail by cutting at a 45° angle with our scissors. Then, using our finger we removed the remaining muscle on the dorsal half of the abdomen. We continued to remove this muscle until the white superficial extensor muscle was visible and accessible. Then we pinned the remaining part of the crayfish on either side in a 100 mL bowl with silicone elastomer on the bottom. We added saline solution to keep the muscle functioning.

Saline Solution

High Calcium Saline The saline solution was made up of: 5.4 mM of KCl, 195 mM of NaCl, 2.6 mM $MgCl_2.6H_2O$, 10 mM of Sodium Hepes Buffer, and 13.5 mM $CaCl_22H_2O$. The saline solution had a pH of 7.4 before use and was made one day before our laboratory usage.

Low Calcium Saline The saline solution was made up of: 5.4 mM or KCl, 196 mM of NaCl, 10.1 mM $MgCl_2.6H_2O$, 10 mM of Sodium Hepes Buffer, and 6.0 mM of $CaCl_22H_2O$. pH was 7.4. This was made one day before laboratory usage.

Making Electrode

We heated a thin glass tube that was 11 cm in length and 1.2 mm in diameter. We heated this singular glass tube at a cooler temperature than normal (3 on the scale) so that it would split into two tubes half the length with tips small, but large enough to inject liquids through. We filled our control electrodes with 3 M KCl and our experimental electrodes with Carboxy PTIO and then dipped the tips of both types of electrodes in saline solution. The electrode's resistance varied, but was near 10 mega ohms.

Chemicals Used

Carboxy PTIO Carboxy PTIO is a nitric oxide scavenger. In our experiment the final solution of Carboxy PTIO was 400 μM in 3M KCl. We were given a stock solution of 40mM Carboxy PTIO and diluted this in KCl. This was done by mixing a graduated cylinder filled with 10ml of 3M KCl and 40 mM of Carboxy PTIO. After this was mixed we filled our experimental electrodes with this solution.

DEANO DEANO is a nitric oxide donor. In our experiment the final solution of DEANO was 100 μM in crayfish saline. We were given a stock solution of 100mM DEANO and diluted it with the crayfish saline. This was done by pipetting 200 μl of DEANO into a graduated cylinder we filled with 200 ml of crayfish saline. After mixing this together, our 100 μM of DEANO was ready to replace the normal saline preserving the crayfish.

Injection Device

In order to inject different solutions into the cell we used a tank of compressed N_2 to provide the air pressure. This tank was attached to an instrument that applied precise pulses of pressure to the back of the microelectrode. For most of our experiments we applied either 1 or 5 second-long pulses at a pressure of 30 psi, but when our air tank was low we had it set at either 1 or 5 second at 20 psi.

Measurement of resting potential in saline

We placed our reference electrode into the edge of the bowl filled with saline solution. Then we attached our electrode to the micromanipulator and moved the electrode down until it touched the surface of the saline solution. We used the program Scope on our computer to measure the voltage. We zeroed the voltage reading to set the voltage of the saline solution as a baseline to measure intracellular voltage. We did this every time a fresh solution was added. Then, we used the handles on the micromanipulator to lower the electrode toward the crayfish muscle cell. We had one person looking through the microscope to watch when the tip entered the muscle and one person watching the Scope program to see when the voltage became more negative. Once the electrode was safely in the cell we recorded the negative voltage across the membrane. In one control, we measured the membrane potential in high-calcium saline, which made the muscle twitch. In the second control we measured the membrane potential in low-calcium saline solution because the muscle twitching would inhibit our ability to inject solutions into the muscle and maintain stability.

Locating a Nerve

We placed one of two silver wire electrodes into the edge of the bowl filled with saline solution. Then we looked into the microscope to locate a nerve on the crayfish. Throughout the experiment we found nerves on both sides of the crayfish both closer to the muscle and closer to the outer edges. Once we

located a nerve we used the micromanipulator to move the suction electrode directly over the nerve. Then we used the syringe to suck enough saline to reach the second silver wire inside the suction electrode. In this process the nerve was also sucked into the electrode. We then applied a voltage to stimulate the nerve in the electrode.

Measurement of EPSP amplitude with injection of KCl

Once we successfully stimulated the nerve to produce an EPSP in a muscle cell we were measuring, we began injecting KCl into said muscle. We manipulated our injecting apparatus so that it would pulse 30 psi for one second when activated. We measured the EPSP amplitude after our specific pattern of this injection. After taking these measurements we manipulated our apparatus to inject KCl into the muscle at 30 psi for 5 seconds. We measured these amplitudes following our set injection pattern. After finishing these injections we determined that the EPSP amplitude was not affected by injection of a neutral solution and therefore justified injecting our experimental solution of Carboxy PTIO.

Measurement of EPSP amplitude with injection of Carboxy PTIO

Once we successfully stimulated the nerve to produce an EPSP in a muscle cell we were measuring, we began injecting Carboxy PTIO. We manipulated our injecting apparatus so that it would pulse 30 psi for one second when activated. We measured the EPSP amplitude after our injection pattern. After taking these measurements we manipulated our apparatus to inject Carboxy PTIO into the muscle at 30 psi for 5 seconds. We measured these amplitudes after applying our injection pattern. After finishing these injections we measured the EPSP amplitude to compare to the control injections done with KCl alone.

Measurement of EPSP amplitude with introduction of DEANO

Once we had injected Carboxy PTIO and recorded the change in EPSP amplitude, we drained the 130 ml of crayfish saline and replaced it with 130 ml of DEANO. The nerve would need to still be in the suction electrode and the electrode would still need to be in the same muscle. The next 10 minutes we measured the EPSP amplitude every minute following the addition of DEANO.

RESULTS

We sought to determine whether nitric oxide (NO) exists in the crayfish NMJ, where it is produced, whether or not it serves as a messenger and, if so, if it is retrograde or not. If NO was shown to be a messenger in the crayfish NMJ, we also wanted to know if it's release had any effects on EPSP strength in the postsynaptic muscle fiber. In testing our hypothesis, we electrically stimulated presynaptic nerves and then measured the amplitudes of corresponding EPSPs in the postsynaptic muscle fibers. Chemicals were later introduced to the crayfish NMJ that changed NO concentrations to see if it is produced in the postsynaptic cell, if it is a retrograde messenger, and to see what, if any effects it has on EPSP amplitude. Our testing determined that NO is a retrograde messenger produced in the postsynaptic cell, and that its release increases EPSP amplitude, and, by extenstion, synaptic strength and induces LTF.

Control testing was first conducted to provide a baseline from which to compare the effects of NO on the crayfish NMJ. Change in EPSP amplitude over time was measured in the NMJ in saline solutions with low (Fig. 1a) and high Ca^{2+} (Fig. 1b) concentrations while stimulating the nerve at a high frequency. These resluts would provide a baseline with which to compare our experimental testing. EPSP amplitude decreased linearly in the low calcium saline, but did not in the high calcium saline. However, we believe that the data suggested a downward trend, as explained further in the discussion section. We thus conclude that EPSP amplitude decreased over time in both tests, likely due to cell death and decreases in the cleanliness of the extracellular saline solution.

Change in EPSP amplitude over time was then measured while KCl was being injected into the postsynaptic muscle cell in an extracellular solution of low calcium saline (Fig. 1c). This testing also yielded results that suggested a decrease in EPSP amplitude as time passed. The slope of the regression line drawn for these data was more negative than that which was drawn for our high Ca^{2+} saline data, but was not significantly different at a 95% confidence level. These data suggest that the injection of KCl into the postsynaptic cell did not have a significant effect on EPSP amplitude when compared to EPSP amplitude in normal conditions.

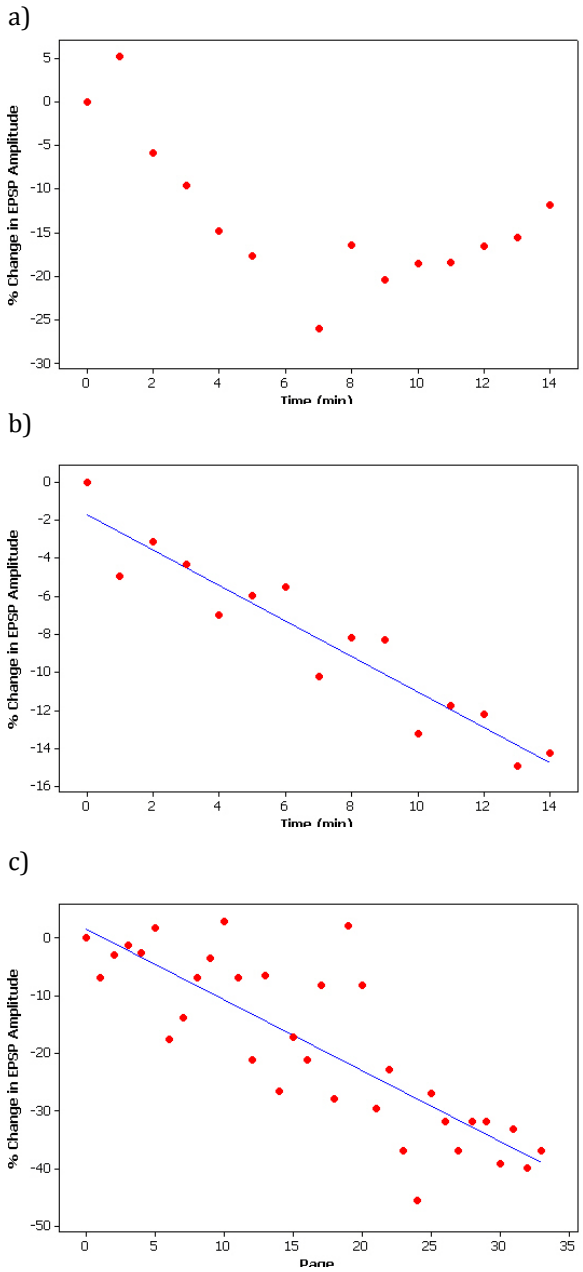


Figure 1. Control testing on the crayfish NMJ to provide a baseline trend for how EPSP amplitude would changein the muscle fiber when frequently stimulated under normal conditions. **a)** Percent change in EPSP amplitude was measured over time in the crayfish NMJ when the muscle was submurged in a high calcium saline solution. A regression line was not drawn for this data because they did not suggest a linear trend. **b)** The same test was repeated, but in low calcium saline. **c)** Percent change in EPSP amplitude was measured over time when the crayfish was frequently stimulated and KCl was being injected into the postsynaptic cell.

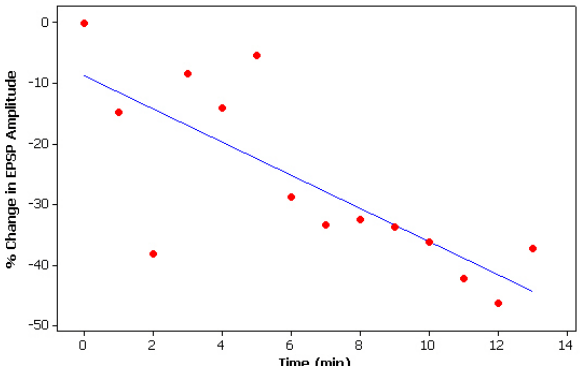
To test whether NO is produced in crayfish postsynaptic muscle fiber cells, and to determine whether is has an effect on EPSP amplitude in the NMJ as a retrograde messenger, we first tested the change in EPSP amplitude over time while Carboxy-PTIO, a NO scavenger, was injected into the postsynaptic cell along with KCl (which has to be in the electrode in order to measure an EPSP) while the NMJ was in an extracellular solution of low calcium saline (Fig. 2a). A roughly linear decrease in EPSP amplitude over time was observed. A t-test was conducted comparing these results with those from the KCl-only injection and concluded that the decrease in EPSP amplitude observed when carboxy-PTIO was injected was significantly larger. This conclusion supports our hypothesis that Carboxy-PTIO does decrease synaptic strength. We believe this is true because it inhibits NO accumilation in the postsynaptic cell, preventing it from messaging the presynaptic cell, increasing EPSP amplidue, and causing LTF.

To support these conclusions, we attempted to reverse the effects of Carboxy PTIO by applying exogenous NO. We replicated our injection of Carboxy PTIO into the muscle cell, but then added the chemical DEANO. DEANO is an NO donor and therefore should simulate the effects of NO production even while the endogenous NO is chelated by Carboxy PTIO (Fig. 2b). Under these conditions, EPSP amplitude decreased at a smaller rate before it levelled out after roughly 11 minutes. These results show that the presence of extracellular DEANO minimized the reduction in EPSP amplitude produced by Carboxy PTIO injection. These results also further suport our hypothesis that NO is a retrograde messenger that increases EPSP amplitude in the crayfish NMJ and facilitates LTF.

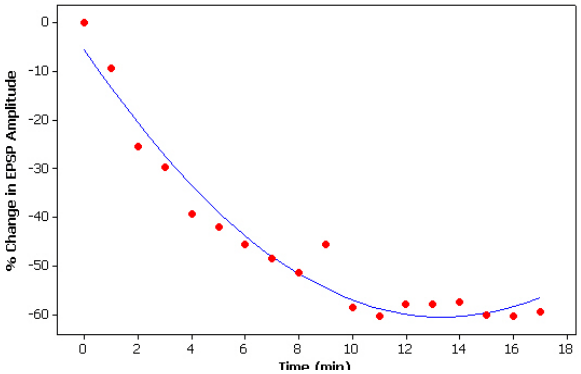
For our final set of data collection, we continued testing on the same organism, but stopped the injection of Carboxy-PTIO into the postsynaptic muscle fiber to test the effect of DEANO on EPSP amplitude when the presence of NO and it's production is no longer blocked in the postsynaptic cell (Fig. 2c). EPSP amplitude immediately began increasing, and continued to increase EPSP amplitude over time in a linear fashion. These results further demonstrate that DEANO, simulating the presence of NO, increases EPSP amplitude. This evidence also supports all of our hypotheses, as explained in the previous paragraph.

Blocking the production and presence of NO in the postsynaptic cell caused EPSP amplitude to significantly decrease over time. Simulating this production by introducting DEANO caused an increase in EPSP amplitude. This suggests that NO increases EPSP amplitude and could induce LTF.

a)



b)



c)

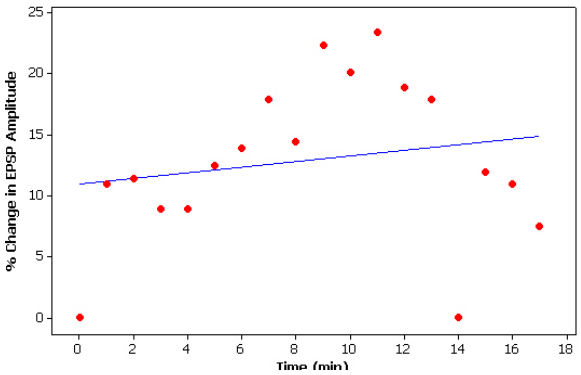


Figure 2. Experimental testing. Chemicals that manipulated NO concentrations were introduced, and change in EPSP amplitude was recorded to see if the changes in NO concentrations had an effect on EPSP amplitude. **a)** Percent change in EPSP amplitude over timewas recorded as KCl and carboxy PTIO were injected into the postsynaptic cell. **b)** Change in EPSP amplitude was recorded in the same crayfish, while KCl and carboxy PTIO were injected into the postsynaptic muscle fiber and DEANO was present in the synaptic gap. A quadratic regression line was drawn in correlation with the trends observed in the data. **c)** Change in EPSP amplitude was recorded in the same NMJ as part b, but Carboxy PTIO and KCl were no longer being injected into the postsynaptic cell while DEANO was still present in the synaptic gap.

Furthermore, our testing that showed that the introduction of an NO inhibitor in the postsynaptic cell results in a decrease in EPSP amplitude suggests that NO is produced in the postsynaptic cell and targets receptors in the presynaptic cell that would facilitate LTF. Thus,we believe that the results of all of our testing supported our initial hypotheses that NO serves as a retrograde messenger and that it's presence in the crayfish NMJ would cause LTF to occur.

DISCUSSION

The result of our experiments support our initial hypothesis that nitric oxide functions as a retrograde messenger in the crayfish neuromuscular junction and that its release causes an increase in EPSP amplitude, which strengthens synapses and produces long term facilitation. The results of our control testing (in high calcium and low calcium), which provided us with a baseline from which to compare the effects of NO on the crayfish NMJ, showed a decrease in EPSP amplitude over the hour and fifty minute testing period. When these results were put into a scatter plot, they showed a clear downward trend, although in the case of low calcium not a linear one. We have attributed the apparent decrease in EPSP amplitude over time to a variety of factors, including, inattentiveness to the amount of time between saline changes, tissue degradation and cell death. The first factor, not changing the saline solution often enough is a highly plausible explanation, as we were still becoming acquainted with the procedural aspects of this experiment and often neglected to change the saline at the recommended fifteen-minute intervals. It is also possible that infrequent changing would allow the saline temperature to increase causing an increase in the natural rate of the cell death and tissue degradation of the crayfish tissue. Accelerated tissue degradation and cell death over time could also explain why our graph (figure 1a.) Of the decrease in EPSP amplitude in the low calcium saline was not linear.

When the results of our testing of the effect of the KCl were subjected to statistical analysis they confirmed that the injection of KCl had a statistically significant impact on EPSP amplitude. However when the results of this testing were put into a scatter plot the regression line for the KCl injection had a lower slope than the regression line for the graph of the results of control testing. One possible explanation for this difference is, when KCl is injected into the cell, the concentration of both K and

Cl ions in the cell increase, increasing the concentration gradient of both ions. Thus, there would be more of a concentration force pushing both ions out of the cell. However, in crayfish cells, potassium is more permeable to the cell membrane than chloride. The increased concentration gradient of potassium would cause it to leave the cell, resulting in the resting membrane potential of the postsynaptic cell becoming more polar as KCl is injected. This change in resting membrane potential could also potentially affect EPSP amplitude. It is also important to note that we only conducted one test for the control and one test for the KCl injection meaning that natural could be variability a reason for the difference in EPSP amplitude between the control and KCl testing.

In the next phase of our testing we injected Carboxy PTIO, an NO scavenger, into the muscle cell, in hopes of determining whether or not NO served as a messenger in the crayfish NMJ. We hypothesized that if the EPSP amplitude decreased with the injection of Carboxy PTIO, decreasing the amount of NO in the NMJ it would support our hypothesis that NO was in fact a messenger. When the results of this testing were subjected to statistical analysis we confirmed that the introduction of Carboxy PTIO did cause a significant decrease in EPSP amplitude. To further confirm the role of NO as a messenger we added switched our low calcium saline solution for a DEANO saline solution. When the DEANO was first introduced we continued to inject Carboxy PTIO and the results showed a leveling out of the decrease in EPSP amplitude that was occurring prior to the introduction of DEANO (figure 2b). When we stopped injecting Carboxy PTIO the EPSP amplitude began to rise. The upward trend of EPSP amplitudes after the Carboxy PTIO injection was stopped and DEANO was introduced was further confirmed when the results of this test where entered into a scatter plot, displaying a clear positive slope. Although some discrepancies that could be accounted for by natural variability and error, we still firmly believe that our results support our hypothesis. For further research we would replicate the same experiment to see if we could generate similar or more significant results. We could also try introducing different NO antagonists or agonists to see if they had the same effects on EPSP amplitude.

Our research relates well to previous research done on the role of nitric oxide as a messenger and facilitator of synaptic plasticity while investigating various neurological abnormalities in humans. In particular the implication of abnormal NO signaling as a possible contributing factor in the development of degenerative neurological conditions such as Alzheimer's disease, multiple sclerosis and Parkinson's disease (Steinert, 2010). Continued research into the role of nitric oxide as a messenger, in varieties of systems but particularly in the human brain could be crucial to gaining a better understanding of the causes, progression and possible treatment options for degenerative and debilitating Neurological pathologies.

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