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Nitric oxide synthase inhibitor L-NAME decreases long-term depression at the crayfish neuromuscular junction

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ABSTRACT

Nitric oxide (NO) is a gaseous neuromodulator in the central nervous system that acts as a retrograde messenger and has been found to both facilitate and depress synaptic transmission in various synapses. However, the role of NO remains unclear in the crayfish neuromuscular junction (NMJ). We investigated NO's effect on synaptic plasticity, the strengthening and weakening of synapses over time, in the crayfish NMJ using the nitric oxide synthase inhibitor L-NAME. First, we hypothesized that applying L-NAME exogenously would decrease percent change in excitatory postsynaptic potentials (EPSPs), and thus restrain long-term facilitation (LTF) or long-term depression (LTD) at the crayfish NMJ. Second, we hypothesized that increasing concentrations of L-NAME would amplify the decrease in percent change in EPSP and further restrain synaptic plasticity. We compared the percent change in EPSPs between the control (0 mM L-NAME), 0.075 mM L-NAME, and 0.15 mM L-NAME conditions. Our results showed a large percent change in EPSP and thus strong LTD in the control condition that was significantly different from the small percent change in EPSP and thus weak LTD in 0.15 mM L-NAME. However, we found no significant difference in percent change in EPSP between the 0.15mM and 0.075 L-NAME conditions, and therefore deduced that inhibition of nitric oxide synthase (NOS) restrains LTD at the crayfish NMJ but could not confirm that increased concentrations of L-NAME amplify resulting changes in plasticity.

INTRODUCTION

The majority of messengers that play a role in synaptic transmission act as anterograde messenger, which are released from the presynaptic cell to act on the postsynaptic cell. Inversely, nitric oxide (NO), a neuromodulator in the nervous system (Lindgren and Laird 1994; Aonuma et al. 2000; Thomas and Robitaille 2001), acts as a retrograde messenger in synapses, traveling from the postsynaptic cell to the presynaptic cell (Arancio et al. 1996). Specifically, after nitric oxide synthase (NOS) enzymes produce NO in the postsynaptic cell, NO diffuses out of the postsynaptic cell, across the synapse, and into the presynaptic cell. In the presynaptic cell of the hippocampus, NO activates guanylate cyclase, thereby stimulating the production of cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG) (Arancio et al. 1996). The production of these proteins increases the release of neurotransmitters, ultimately increasing excitatory postsynaptic potentials (EPSPs) in the postsynaptic cell. Additionally, Arancio et al. (1996) found a correlation between tetanic stimulation and NO synthesis. They proposed that tetanic stimulation may cause an influx of calcium ion (Ca^{2+}) in the postsynaptic cell, which activates NOS to produce NO. This suggests that tetanic stimulation increases the number of neurotransmitters released from the presynaptic cell and increase EPSPs in the postsynaptic cell.

Wojtowicz and Atwood (1988) classified long-term facilitation (LTF) as a phenomenon in which the amplitude of EPSPs progressively increases during the stimulation of a nerve at a frequency above 10 Hz. Although NO has been shown to play an integral role in long-term potentiation (LTP) in the hippocampus, little research has been done to study the effect of NO on LTF in the crayfish neuromuscular junction (NMJ), assuming that LTF is the specific form of plasticity that NO facilitates in that particular synapse. However, NO has been found to inhibit the presynaptic release of neurotransmitters in the frog neuromuscular junction (NMJ) (Lindgren and Laird 1994), and to induce long-term depression (LTD) in the crayfish NMJ (Aonuma et al. 2000). This contradiction stresses the different roles neuromodulators have in different organism's synapses, such as NO's role as an inhibitory or excitatory factor in the NMJ of different organisms. Thus, we are unable to generalize the role of NO on synaptic plasticity for all synapses.

When added to the extracellular concentration of a crayfish muscle cell, hydrochloride chemical L-NAME acts as an inhibitor and directly blocks the functions of the protein NO synthase and thus prevents NO production (Knowles et al. 1994). Our research is an extension of experiments conducted by Hochstein (2008), in which L-NAME was used to decrease NO levels in the synapse in

order to characterize NO's function in the crayfish NMJ. Hochstein (2008) measured EPSP amplitudes before and after a high frequency stimulation (50 Hz for 10 seconds) for both the control (0 mM L-NAME) and the L-NAME extracellular solution (0.3 mM L-NAME). Though Hochstein (2008) observed a decrease in facilitation when NO was inhibited by L-NAME, it did not provide statistical evidence to determine the significance of the results. In addition, unlike Hochstein (2008), which used only one concentration of L-NAME to compare to a control condition without L-NAME, we analyzed two different concentrations of L-NAME (0.075 mM and 0.15 mM) to observe how the effect L-NAME has on synaptic plasticity changes with the change in the chemical's concentration.

Our objective was to better understand the role of NO on synaptic plasticity in the crayfish NMJ by inhibiting NOS and to confirm the findings of Hochstein (2008). We also hoped to characterize NO's role in plasticity at crayfish synapses in comparison to other model organisms' synapses from previous research.

We first hypothesized that applying the NOS inhibitor L-NAME to the extracellular solution would decrease percent change in EPSP, and thus restrain LTF or LTD at the crayfish NMJ. Our findings support our first hypothesis in that synaptic plasticity observed in the control condition, LTD, was restrained by the application of L-NAME to the extracellular solution. We concluded this by observing a much greater amplitude of percent change in EPSP in the control condition than in the L-NAME concentration (0.075 mM). Secondly, we hypothesized that increasing the concentration of L-NAME would amplify the resulting change in plasticity. However, our findings did not support our second hypothesis because the difference in percent change in EPSP between the two L-NAME concentrations (0.075 mM and 0.15 mM) was not significant.

MATERIALS AND METHODS

Crayfish tail preparation

Each crayfish (*Procambarus clarkii*) was anesthetized by submergence in an ice-filled container for approximately 20-30 minutes. Once the crayfish was immobilized, we separated the tail from the body by extending the tail and cutting it at its base and discarded the body back into the ice container. In order to expose the dorsal extensor muscle for experimentation, we used scissors to cut along the crayfish's ventral surface until the posterior end. We grasped the swimmerets to pulled the muscle mass from the tail and pushed the remaining muscle mass out to expose the extensor muscles. We pinned the crayfish tail ventral side up into a gel bottom dish and covered it with the respective volume of standard crayfish saline needed for the condition tested. After 45 minutes, we replaced the saline with fresh saline containing corresponding concentrations of L-NAME. Data were collected from a total of 5 crayfish, with two crayfish used for two conditions and the remaining three used for only one condition.

Solutions

For our extracellular solutions, we obtained a 5 mM stock solution of L-NAME and a low calcium standard saline solution containing 5.4 mM KCl, 200.7 mM NaCl, 12.3 mM $MgCl_2{*}6H_2O$, 5 mM sodium hepes buffer, and 6.5 mM $CaCl_2{*}\ 2H_2O$. For the first solution, we added 30 mL of the standard saline solution and used this as our control (no L-NAME). For our second extracellular solution, we combined 450 µL of the L-NAME solution with 29.55 mL of the saline solution to create an experimental solution of 0.075 mM L-NAME concentration. For our final extracellular solution, we combined 900 µL of the L-NAME solution with 29.1 mL of the saline solution to create a final experimental solution of 0.15 mM L-NAME concentration.

Microelectrode preparation and instrument placement

We made microelectrodes from Borosilicate glass capillary tubes, 1.2 millimeters in diameter, using a PUL-1 microelectrode puller by World Precision Instruments with the delay set at 2 and the heat set at 7.1. We filled the microelectrode and the microelectrode holder with 3.0 M KCl^- and later dipped the tip of the microelectrode into standard saline to remove any exterior KCl^-. We created the suction electrode by blunting the microelectrode tip with sandpaper so it was large enough to suck the crayfish nerve bundle in place. We then put the recording electrode in the electrode holder and the suction electrode into the electrode holder connected to the syringe. We placed the dish containing a crayfish tail and the respective saline solution underneath a Leica Zoom 2000 microscope, moving both micromanipulators and the dish so that the crayfish tail could be seen under the microscope. We placed the reference electrodes into the saline-filled basin. Using the micromanipulators, we positioned the recording electrode to penetrate muscle cells and the suction electrode to suck the crayfish nerve bundle using a syringe.

Electrophysiology and data collection

For each trial, we placed the microelectrode in saline to check for adequate resistance (between 4 Ω and 13 Ω) and used Analog Digital Instruments MacLab Bridge Amp to zero the junction potential. The microelectrode was inserted into the crayfish muscle

tissue to record an adequate membrane potential (between -40 mV and -100 mV). At the end of our data collection period, we only analyzed the data for which the resting membrane potential throughout the data collection was between -40 mV and -100 mV. After we successfully sucked a nerve into the suction electrode with the syringe, we stimulated the nerve using a Grass Instruments SD9 Stimulator. We ran a repeated frequency of 0.5 Hz for a period of 1 minute and used Analog Digital Instruments PowerLab 4/25 to measure the resulting excitatory postsynaptic potential (EPSP) amplitudes. Voltage and duration were varied in order to obtain EPSPs without making the muscles twitch. After collecting our baseline EPSP measurements, we applied a high frequency stimulation of 50 Hz for 10 seconds, then reverted to a frequency of 0.5 Hz and recorded EPSP amplitudes for a duration of 8 minutes. The 0.5 Hz frequency allowed for 30 EPSP measurements per minute.

Data analysis

In order to account for the potential differences between individual crayfish cells, we normalized all measured EPSP amplitudes relative to the baseline EPSP amplitude by calculating percent change in EPSP from baseline EPSP. Baseline EPSP was calculated by averaging the 30 samples of EPSP taken continuously during the 1 minute period before tetanic stimulation. With this, we calculated the percent change in EPSP amplitude from the baseline for each EPSP sample measured every 2 seconds after tetanic stimulation (for a period of 8 minutes) with the equation: Percent change = [(EPSP - $EPSP_{baseline}$) / $EPSP_{baseline}$] x 100 % (Badhwar et al. 2006).

We also accounted for the effect of crayfish muscle tissue death on percent change in EPSP by keeping the experimental time constant across all conditions and crayfish. Thus, the rate of tissue death was approximately the same across all conditions. Therefore, by using a control condition and looking at the difference in percent change in EPSP over time, we compared the effect of L-NAME without constituting crayfish muscle tissue death.

RESULTS

We tested the role of nitric oxide (NO) on synaptic plasticity in the crayfish neuromuscular junction (NMJ) by inhibiting nitric oxide synthase (NOS) via addition of L-NAME to the extracellular solution of the crayfish muscle cell. Using intracellular recording, we measured EPSP amplitudes for three conditions (control with 0mM L-NAME, 0.075 mM L-NAME, and 0.15 mM L-NAME), both before and after applying a tetanic stimulation of 50 Hz for 10 seconds. EPSPs were measured for 1 minute before tetanus and for 8 minutes after tetanus.

L-NAME decreases percent change in EPSP

We observed an initial difference in percent change in EPSP between the control condition and the 0.075 mM L-NAME condition. A strong LTD occurred after tetanic stimulation in the control condition (Fig. 1). In the first minute, the change in EPSP was -29.6%, and in the eighth minute, the change went down to -60.1%. In the 0.075 mM L-NAME condition, there was an increase in percent change in EPSP from the first to second minute (-18.9% to -7.88%). Subsequently, the percent change leveled out, exhibiting a weak LTD (Fig. 1). From the second to eighth minute, the change in EPSP decreased by 3.32%. As seen in the error bars in Figure 1, one trial in the 0.075 mM L-NAME did not show LTD during the second and third minute.

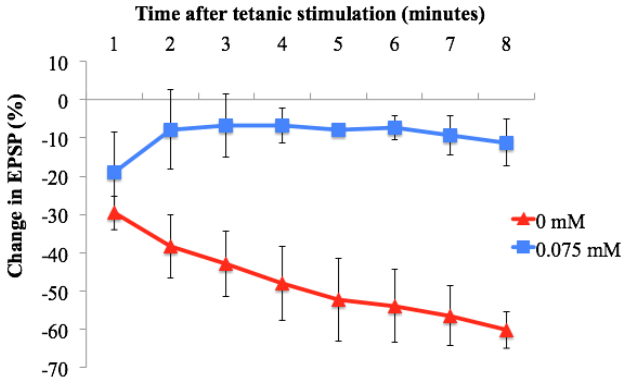

Figure 1. Percent change in EPSP amplitude after tetanic stimulation for the control condition (0 mM) and the low L-NAME condition (0.075 mM). A total of 30 EPSP measurements were recorded per minute (for each condition) for 8 minutes following tetanus. Each data point shown in the figure represents the mean of the 30 measurements taken over each one minute period. The effect of stimulation stabilized after 3 minutes in both conditions. Error bars represent range. The control condition resulted in a strong LTD, while low concentration resulted in a weak LTD. n = 2 for both conditions.

The effect of the tetanic stimulation stabilized after approximately 3 minutes (Fig. 1). We therefore factored out time and conducted Student's t-test to observe difference in percent change in EPSP between the control and 0.075 mM conditions from the third to eighth minute. The mean percent change in EPSP in the control condition (M = -52.2%) was significantly lower than the 0.075 mM condition (M = -8.31%, $p < 0.0001$, Fig. 2). Thus, we concluded that the 0.075 mM concentration of L-NAME restrained synaptic plasticity in the crayfish NMJ.

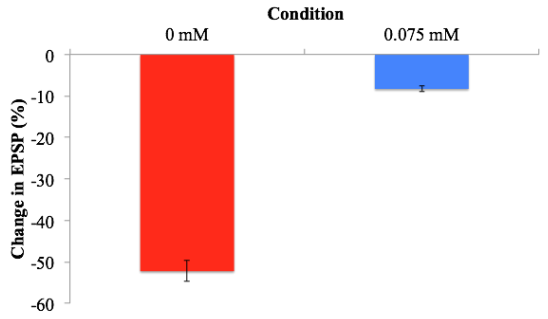

Figure 2. Mean percent change in EPSP from the 3rd minute to 8th minute for the control condition (0 mM) and low L-NAME condition (0.075 mM). The amplitude of percent change in EPSP for the control condition was significantly larger than the 0.075 mM L-NAME condition ($t = -16.86$, $p < 0.0001$); synaptic plasticity was restrained in the 0.075 mM L-NAME condition. Error bars represent standard error of the mean. n = 12 for both conditions.

Increase in L-NAME concentration does not affect percent change in EPSP

After we observed a significant difference between the 0.075 mM and control, we compared two different L-NAME concentrations in order to see if increasing the L-NAME concentration amplifies its effect on percent change in EPSP. Both 0.075 mM and 0.15 mM L-NAME conditions resulted in weak LTD (Fig. 3). Percent change in EPSP in the 0.15 mM L-NAME condition increased to positive values from the second (0.21%) to fourth minute (0.88%), followed by a gradual decline (-15.5% in the eighth minute).

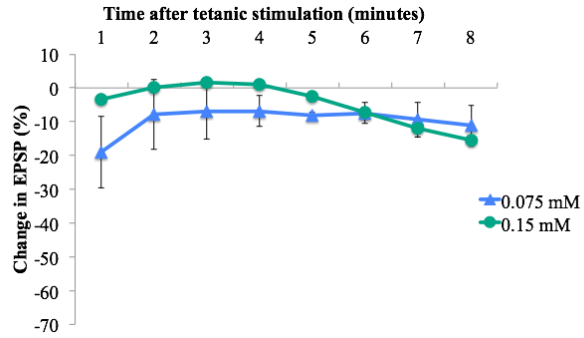

Figure 3. Percent change in EPSP amplitude after tetanic stimulation between low (0.075 mM) and high (0.15 mM) L-NAME conditions. A total of 30 EPSP measurements were recorded per minute (per condition) for 8 minutes following tetanus, and each data point shown in the figure represents the mean of the 30 measurements taken over each one minute period. Both conditions resulted in a weak or no LTD. The effect of stimulation stabilized after 3 minutes in both conditions. n = 2 for 0.075 mM and n = 1 for 0.15 mM. Error bars represent range (i.e. the highest and lowest measurements taken per minute).

Similar to our first analysis, the effect of stimulation stabilized after 3 minutes, and thus we factored out time and conducted Student's t-test to observe difference in percent change in EPSP between the 0.075 mM and 0.15 mM L-NAME conditions from the third to eighth minute.

We found no significant difference in percent change in EPSP between the 0.075 mM (M = -8.31%) and 0.15 mM L-NAME conditions (M = -5.78, $p > 0.05$, Fig. 4). We concluded that an increase in the concentration of L-NAME from 0.075 mM to 0.15 mM did not amplify the change in synaptic plasticity from the control condition.

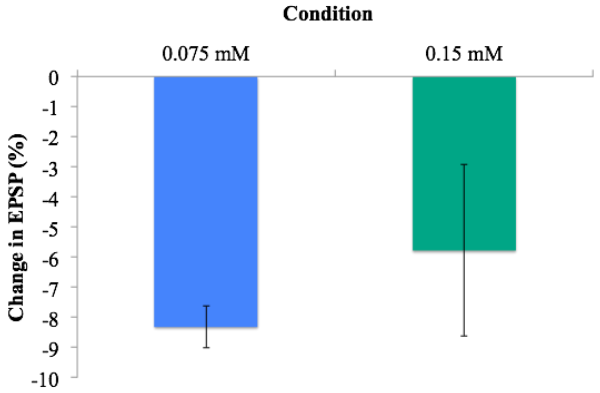

Figure 4. Mean percent change in EPSP from the 3rd minute to 8th minute for the low L-NAME (0.075 mM) and high L-NAME (0.15 mM) conditions. There was no significant difference in percent change EPSP between these two conditions ($t = -0.86$, $p > 0.05$). Error bars represent standard error of the mean. n = 12 for 0.075 mM condition and n = 6 for 0.15 mM condition.

DISCUSSION

In order to identify NO's effect on synaptic plasticity in the crayfish neuromuscular junction (NMJ), we applied NOS inhibitor L-NAME to the extracellular solution of crayfish muscle cells. We found that the 0.075 mM L-NAME experimental solution demonstrated a significant difference in percent change in EPSP in comparison to the control condition (0mM L-NAME). In addition, the control condition with no application of L-NAME resulted in strong LTD, and we observed relatively weak LTD for both concentrations (0.075 mM and 0.15 mM) of L-NAME. These results support our first hypothesis that application of L-NAME will decrease percent change in EPSP and thus restrain synaptic plasticity at the crayfish NMJ. In the case of our research, the specific form of synaptic plasticity that was restrained was LTD.

Although percent change in EPSP in the 0.075 mM L-NAME condition was significantly different from the control condition, percent change in EPSP between the 0.075 mM and 0.15 mM concentrations of L-NAME

was not significantly different. From this lack of significance, we are unable to support our second hypothesis that a higher concentration of L-NAME will further decrease its restraining effect on LTD. However, we are also unable to reject our hypothesis because both of our tested concentrations of L-NAME lie on the upper bound of the L-NAME dose response curve, which means that even our relatively low concentration of L-NAME (0.075 mM) is at the high end of the L-NAME concentration scale, and therefore had already achieved the maximum effect of L-NAME on synaptic plasticity. This may explain the lack of significant difference between the 0.075 mM to 0.15 mM conditions. This possibility is supported by the findings of Müller (1996), who found the inhibition of NOS to sharply increase between 0 mM and 0.1 mM L-NAME concentrations, at which point (0.1 mM) the NOS inhibition reached its peak and leveled out.

Although we intended to expand upon the findings of Hotchstein (2008) by experimenting two concentrations of L-NAME (0.075 mM and 0.15 mM), we did not find difference in percent change in EPSP between them. Nevertheless, our results corresponded with Hotchstein (2008) in that L-NAME decreases synaptic plasticity. Additionally, we provided statistical significance absent in his results. However, Hotchstein (2008) observed a decrease in synaptic plasticity through a decrease in LTF, while we observed a decrease in LTD.

Research conducted by Albensi et al. (2007) found that in the hippocampal synapse, LTD occurs as a result of prolonged low frequency stimulation and LTP occurs as a result of high frequency stimulation, contradicting our findings. However, additional research suggests that the specific form of synaptic plasticity induced is dependent upon extracellular calcium concentrations in addition to the frequency of nerve stimulation. In particular, the calcium concentration needed to induce LTD is significantly lower than the calcium concentration needed to induce LTP (Artola and Singer 1993; Hansel et al. 1997). This research supports the legitimacy of our findings that LTD was the specific form of plasticity that ensued following tetanic stimulation. In short, it suggests that despite the fact that we used high frequency stimulation, which typically results in LTP/LTF, our low calcium saline solution did not have a high enough concentration of calcium to induce LTF. However, the saline solution *did* have a high enough concentration of calcium to induce LTD, which consequently ensued.

Although Hochstein (2008) found LTF in the crayfish NMJ following high frequency stimulation, the research lacks crucial information regarding the chemical solutions used to create the calcium control solution of standard saline as well as the concentrations of those chemical solutions. If the saline solution were a normal or high calcium solution, then it would be plausible that the concentration of calcium was high enough to produce LTP following a high frequency stimulation.

Additional research supports our results that inhibiting NO decreased LTD. Aonuma et al. (2000) suggested that in vertebrates, NO induces an increase in cGMP levels, which in turn decreases intracellular Ca^{2+} levels. Zucker and Regehr (2002) proposed that intracellular Ca^{2+} acts to increase neurotransmitters. From these two studies, we can predict that NO induces a decrease in neurotransmitter release. Thus, inhibiting NO with L-NAME increased neurotransmitter release, which in result decreased the amplitude of LTD (Fig. 1).

Future research is needed to accommodate for the insignificant difference between L-NAME concentrations by testing a lower range of L-NAME concentrations on the crayfish NMJ. We will be able to have a better understanding of the effect that L-NAME has on synaptic plasticity in the crayfish NMJ by having tested a more comprehensive range of the concentrations represented on the dose response curve.

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