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The effects of nitric oxide on long-term potentiation at the crayfish neuromuscular junction

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ABSTRACT

Nitric oxide (NO) has been discovered to function as a novel inter- and intracellular messenger—including as a neurotransmitter (Miller, 1993). In this study, we observed the effects of NO on long-term potentiation (LTP) in the crayfish neuromuscular junction (NMJ) by increasing the amount of free NO in the crayfish Ringer solution through the addition of the NO donor DEANO. Previous studies such as Gainey et al. (2003), Baxter et al. (2005), and Boehning et al. (2003) indicate that NO may cause LTP in the crayfish NMJ. Though we hypothesized that exposing the crayfish NMJ to NO would initiate LTP, our results do not indicate that this is the case. Our results do allude to NO, increasing post-tetanic potentiation (PTP). However, none of our results' means were significant at the 0.05 level (Student's T-test), therefore we cannot conclude that the addition of NO through DEANO affects potentiation in the crayfish NMJ.

INTRODUCTION

Nitric oxide (NO) was once considered only a toxic environmental contaminant; recently, however, it has been discovered to function as a novel inter- and intracellular messenger — including as a neurotransmitter (Miller, 1993). NO's many functions include making light production possible in fireflies (Trimmer et al., 2001) and modulating genital erection in mammals (Kim et al., 2001). Recently, because of its vasodilatory effects (i.e. increasing blood flow to muscles), NO precursors have been utilized by bodybuilders hoping to increase muscle mass.

The purpose of this study is to observe in the crayfish NMJ the effects of NO on LTP, a type of synaptic plasticity characterized by a modification of the synaptic terminal which causes more neurotransmitter to be released after subsequent stimulation. LTP was chosen as the emphasis for our study because it is one of the lesser studied forms of synaptic plasticity in neuromuscular junctions.

Previous research conducted on NO and neurotransmission alludes to the potential for NO to increase LTP. The basis of this research has been done using the hippocampus and cerebral cortex of various vertebrates, including rats (Bon et al., 2003 and Baranano et al., 2001). These studies have shown that NO is released from the postsynaptic cell in response to a high-intensity stimulus, causing the activation of NMDA receptors and an accumulation of calcium. The calcium buildup activates a calmodulin protein that binds to NO synthase, which then synthesizes NO from a precursor L-arginine. NO causes synaptic plasticity by diffusing back to the presynaptic cell and activating guanylyl cyclase, leading to a buildup of cGMP. Specifically in causing LTP, cGMP appears to cause a small cluster of proteins, puncta, to form near the synaptic terminal (Wang et al., 2005), thus changing the terminal's structure and altering neurotransmitter release.

Moreover, Gainey et al.'s (2003) study of NO's role in muscle potentiation in clam gills, a preparation similar to the crayfish NMJ, showed that NO can increase potentiation in invertebrate cells different from the rat hippocampuses.

Past research on NO and neurotransmission in the crayfish NMJ has also been conducted. Baxter et al. (1985) demonstrated LTP in the crayfish neuromuscular junction. Another study of NO and synaptic plasticity at the crayfish NMJ showed that L-NAME, a NO-synthase inhibitor, failed to prevent long-term depression (LTD), suggesting that NO exposure may be more important for LTP than LTD (Anderson et al., 2000). Boehning et al. (2003) found that although NO can cause either LTP or LTD, it often plays only one role at a given synapse. Finally, Chon et al.'s (2002) study demonstrated that the NO donor SNP causes an increase in the size of EPSPs. These findings suggest that some degree of LTP may have occurred in the experiment.

To study the effects of NO on LTP, we created a substantial amount of free NO through the addition of the NO donor, DEANO, into the crayfish Ringer solution. We hypothesized that exposing the crayfish NMJ to 10 mM DEANO would initiate LTP. LTP was measured by examining the changes in the average EPSP response to a low-frequency stimulus. Our experiment, however, did not support this hypothesis. Although we found no evidence of LTP, our experiment did show that NO possibly increased post-tetanic potentiation (PTP), a

shorter-term form of synaptic facilitation that lasts a few minutes (Fisher et al., 1997). This evidence, however, was not statistically significant, therefore we did not find any conclusive evidence that the addition of NO through 10 mM DEANO has any significant effect on potentiation in the crayfish neuromuscular junction.

MATERIALS AND METHODS

Organism Preparation and Dissection

At the beginning of each trial, a crayfish was placed in an ice bath for twenty minutes or until it stopped moving. Next the tail was removed from the body, and the viscera were dissected to expose the superficial extensor muscle fibers on the dorsal shell. The tail was then pinned down in a Sylgard-coated chamber and fully immersed in Ringer Solution. Four experimental trials and four control trials were conducted, with each trial using a different crayfish.

Experimental Solutions

The crayfish were bathed in standard crayfish Ringer solution comprised of 205 mM NaCl, 5.4 mM KCL, 13.5 mM CaCl2, 2.6 mM MgCl2, and 10.0 mM Tris (pH7.4). We increased the amount of free NO in our experimental conditions through the addition of the NO donor, DEANO, into the Ringer solution. The DEANO was diluted into the Ringer solution so that it reached 10.0 μM. The DEANO solution was composed of a mixture of sodium hydroxide (to keep the DEANO stable) and DEANO; it was kept on ice and added to the Ringer solution only immediately before use. 5 mL of the Ringer solution in both the control and experimental conditions was replaced every ten minutes to increase the longevity of the preparation.

EPSP Induction and Recording

Intracellular recording in the superficial extensor muscle was used to measure EPSPs created by electrical stimulation of the motor nerve. Microelectrodes were created using a microelectrode puller (World Precision Instruments' PUL-1). Recording electrodes were filled with 3 M KCL and attached to a micromanipulator and voltage recording equipment. At first suction electrodes were used to stimulate the nerve, however, because we were unable to consistently induce EPSPs with this technique, after the first two control experiments we began using a two-pronged wire electrode. The suction electrode stimulated the nerve by suctioning it into the tip of the electrode and then delivering electrical pulses to the nerve through a Grass DS9 stimulator. The two-pronged wire electrode only differed from the suction electrode in that it stimulated the nerve through a two-pronged wire which straddled the junction between two tail segments.

Experimental Procedure

After locating a healthy muscle cell we recorded five baseline EPSPs at a rate of one per minute, which were delivered using the lowest suprathreshold voltage on the stimulator. The amplitude of each EPSP was defined as the difference between the minimum membrane potential following the stimulus and resting membrane potential. At the beginning of the sixth minute, a burst of five threshold pulses was delivered at 50 Hz, a stimulus protocol that has been found to allow LTP to occur in the presence of exogenous NO without causing significant post-tetanic potentiation (PTP) or LTP in and of itself (Bon et al., 2003). Simultaneously, during the experimental trials, DEANO solution was added. During control trials, however, no DEANO was added. EPSPs then were recorded, at a rate of one per minute, for the duration of the 30 minute time.

RESULTS

Data from this experiment consisted of 8 series of EPSP amplitudes recorded from the crayfish dorsal extensor muscles, four experimental trials with DEANO and four control trials with only Ringer solution. Pulses were delivered at a rate of 1 per minute for 30 minutes during each trial. The addition of DEANO to the experimental trials coincided with a brief (0.1s) high-intensity (50Hz) stimulus given after the fifth reading (i.e. 5 minutes into the trial.) This stimulus was also given at the same point during the control trials.

The EPSP amplitudes from each trial were converted to a relative scale by dividing them by the corresponding trial's EPSPs baseline, which was calculated by finding the mean of the first 5 EPSPs of the trial. These scaled values were then averaged within each experimental condition to give a picture of the typical change in EPSP amplitude in each condition.

Overall, the data proved to be highly variable in both control and experimental conditions. Raw, unscaled EPSP amplitudes ranged from 1.5mV to more than 25mV. Both conditions included trials where EPSP amplitudes were mostly increasing and trials where EPSP amplitudes were mostly decreasing. Average control and experimental EPSP amplitude proportions are roughly equal, with overlapping standard error bars, for almost every recorded time (Figure 1). Between 8 and 10 minutes, however, average experimental EPSP amplitude proportions were much higher than average control EPSP amplitude proportions (Figure 2).

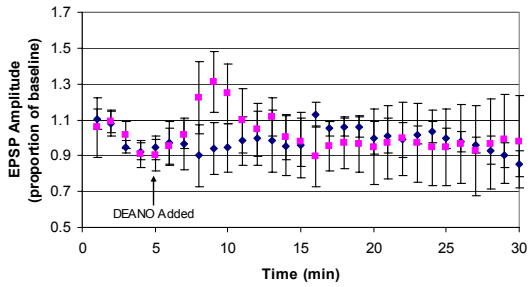

Figure 1. Changes in EPSP amplitudes over time. Light squares correspond to the average EPSP proportion for experimental trials, dark diamonds correspond to the average EPSP proportion for control trials (n=4 for each case.) Error bars show the standard error for each set of values. DEANO solution added to experimental trials after the 5th measurement.

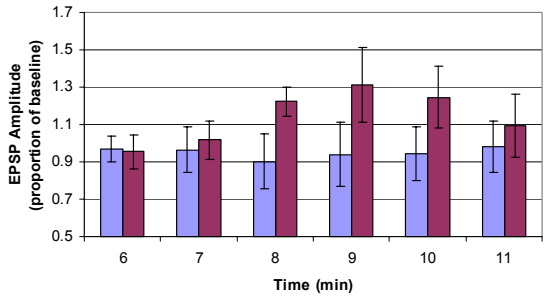

Figure 2. DEA/NO-dependent synaptic facilitation after administration of a brief stimulus. Data are identical to figure 1, but zoomed in to highlight possible PTP. Light bars represent the average relative EPSP in control trials, dark bars represent the average relative EPSP in experimental trials. Error bars show SE.

Student's t-tests were performed for the set of four EPSPS that make up each average data point. None of the differences observed between the control and experimental trials were significant at the *p=0.05* level. In fact, most had p-values greater than 0.5. The facilitation witnessed shortly after the stimulus burst was not significant either, with the lowest p-value being 0.17.

DISCUSSION

Our results do not support our hypothesis that the NO donor DEANO leads to LTP in the crayfish NMJ. This is because the p-values pertaining to LTP obtained from our t-test were greater than 0.05. In fact, these p-values were all above 0.10. However, our results may suggest that DEANO increases PTP. Data recorded between eight and ten minutes after the first reading (three minutes after the addition of DEANO) indicates that the average amplitude difference between control and experimental EPSP proportions was about 0.3. Yet, this data was not significant at the 0.05 level after conduction of t-tests (the average p-value between 8 and ten minutes was .35). Overall, none of our data was significant, therefore we are left to conclude that, based on this experiment, the addition of NO through DEANO does not effect potentiation in the crayfish neuromuscular junction.

There are four possible explanations for our experiments' lack of significance. For one, we only completed four control and four experimental trials when we planned to conduct at least seven trials of each. More experiments could have yielded significant data. Second, we also encountered numerous times where the recording electrode dislodged from the muscle cell.

Third, after the first three control experiments, we changed stimulation devices from using a suction electrode to using a two-pronged wire electrode. The change was made because we had great difficulty stimulating EPSPs with the suction electrode, while the two-pronged wire electrode could consistently stimulate EPSPs. The two-pronged electrode, however, was less precise than the suction electrode because, while the suction electrode continued to stimulate the same nerve bundle, if the preparation moved as a result of muscle twitches, the electrical pulse sent by the two-pronged electrode would change position relative to the nerves. Also, the two-pronged electrode affected a larger section of the preparation and thus often stimulated more than one nerve at a time, creating multiple EPSPs and making the EPSP threshold voltage difficult to determine. This change may have affected our data's reliability because the trials conducted with the suction electrode were more accurate than data used with the two-pronged electrode and because the data from the two-pronged stimulator may have been unreliable in and of itself.

Finally, we did not account for the possible removal of DEANO during solution changes. Although, DEANO has a half life of approximately two minutes (Invitrogen, 2005), DEANO may have been removed during the first solution change at ten minutes, since the DEANO was applied at the beginning of the sixth minute. This may have prevented LTP from occurring.

Although one might think the use of other NO donors might have yielded more accurate data, this does not seem to be the case. For example, Abi-Gerges et al. (2002) found that multiple NO donors give indistinguishable results. Moreover, none of the studies we referenced consider differences in donors to be an issue. DEANO is a widely used and easily quantifiable donor.

We feel that it may be possible to obtain significant data in this experiment by the use of more accurate stimulating instruments. The use of precisely crafted suction electrodes may be one way future researchers could find significant data in our experiment. Further investigation with better equipment should be conducted on this relatively new topic in order to better understand the role of NO in neurotransmission.

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