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Endogenous nitric oxide has inconclusive effect on long-term potentiation in crayfish neuromuscular junction.

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ABSTRACT

Nitric oxide has been shown to play a role in neurotransmitter release, and consequently, a role in long-term potentiation. However, its role in synaptic plasticity at the neuromuscular junction is debated. The effects of inhibiting nitric oxide production on long-term potentiation in the crayfish tail extensor muscle was studied. As previous research suggests that NO may play an important role in neurotransmitter release, we hypothesized that its presence is necessary for long-term potentiation at the neuromuscular junction. Crayfish tail extensor muscles were stimulated at a high frequency and excitatory postsynaptic potentials (EPSPs) were recorded in both a baseline saline solution and a solution containing the nitric oxide synthase inhibitor, L-NAME. Though our results showed the EPSP percent change of the post-tetanic stimulation LTP to be consistently less than that of the baseline recordings, the results were statistically insignificant. Further research into the role of NO on synaptic plasticity is prompted.

INTRODUCTION

While not the case for all chemical synapses, many demonstrate plasticity, the ability of synapses to strengthen or weaken over time in response to a change in activity (Lindgren, 2014). One type of synaptic plasticity is automodulation, where the activity in the presynaptic terminal will either increase or decrease the subsequent activity of the neuron (Graves et al., 2004).

An example of automodulation used by some synapses involves the production of nitric oxide (NO) at the neuromuscular junction (Graves et al., 2004). Neurotransmitter release into the synaptic cleft triggers nitric oxide synthesis in the postsynaptic cell and thus the release of NO into the synaptic cleft. The NO then binds to receptors on or near the presynaptic terminal and activates soluble guanylate cyclase (Hardingham et al., 2013). As a result, cGMP forms and activates protein kinase G (PKG) which, in some cases, increases the amount of neurotransmitter release (Arancio et al., 1996). Yet, NO has also been shown to play a role in synaptic depression, meaning that this pathway is able to decrease the exocytosis of neurotransmitters as well (Aonuma et al., 2000). Thus, in either scenario nitric oxide changes the presynaptic neuron's activity after initial neurotransmitter release and causes synaptic plasticity.

In addition to automodulation, another form of synaptic plasticity is long-term potentiation (LTP). LTP occurs when the synapse's connection is enhanced for a long period of time following a stimulus. It has been correlated with cognitive functioning such as memory formation and retention, and is therefore considered necessary for learning (Korshunova et al., 2014).

There is currently a lot of research being conducted about the role of NO in LTP induction, some with contradictory findings. Dachtler et al. (2011) found that neuronal nitric oxide synthase αNOS1 works in concordance with the glutamate receptor GluR1 to produce experience-dependent plasticity in the neocortex of adult animals. Similarly, and Balaban (2014) concluded that nitric oxide synthesis is necessary for long-term facilitation in terrestrial snails. Thus, there is currently strong evidence that nitric oxide synthesis is important to synaptic plasticity in vertebrates and invertebrates alike. Yet, Kober et al. (2010) conducted an experiment exploring the role of exogenous and endogenous nitric oxide in synaptic transmission at the crayfish neuromuscular junction and had mixed results. Their results suggest that exogenous nitric oxide increases synaptic transmission but nitric oxide synthase inhibition does not produce a statistically significant effect on long-term facilitation.

Therefore, our research planned to help resolve the debate about the role of nitric oxide in synaptic plasticity. We intended to build off the study of Kober et al. (2010) and identify the role of endogenous nitric oxide in LTP induction at the crayfish neuromuscular junction. To address this question we exposed crayfish muscles to standard crayfish saline and a 5 M solution of L-NAME, a nitric oxide synthase inhibitor. We then induced 10 seconds of 20 Hz stimulation to each muscle and compared the resulting EPSP amplitudes to baseline levels over time. We predicted that applying L-NAME would decrease the size of the EPSP, thus minimizing the

strength of long-term potentiation at the crayfish neuromuscular junction. Our results demonstrated that L-NAME generally mitigated long-term potentiation but this was not statistically significant.

MATERIALS AND METHODS

Crayfish Dissection

The experiments were conducted using the dorsal section of a crayfish that was anesthetized in a bucket of ice water which reduces activity of the nervous system making the dissection process more humane. The tail was cut at its base, and scissors were used to cut along the serrated edges down both sides of the tail near the ventral surface, which was then removed and discarded. The excess muscle was removed to isolate the extensor muscles and gain access to the nerves that innervate them. The dissected tail was pinned into a dissection dish and immersed in crayfish saline solution.

Electrode Fabrication

Glass microelectrodes were made from 1.2mm glass pipettes using a PUL-1 World Precision Instruments puller. One microelectrode was filled with 3 M KCl, rinsed in standard saline solution to wash off residual KCL, and placed in an electrode holder. We consistently tested the electrode's resistance during the experiment to ensure that it maintained a level of 5MΩ to 10 MΩ. If the resistance fell below this level, we replaced it with a new KCl filled microelectrode. A second microelectrode was serrated with sandpaper and used as a suction electrode for delivering a stimulus to the nerve.

Solution Preparation

N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), was obtained from Sigma Chemical Company. A stock solution of 5mM L-NAME was frozen in 300 microliters aliquots and stored in a freezer until use. We added 250 microliters from the aliquot to a 250ml volumetric flask of crayfish saline to achieve a final concentration of 5μM. For baseline measurements, a low calcium crayfish saline with concentration 5.4μM KCl, 200.7 μM NaCl, 12.3 μM $MgCl_26H_2O$, 5 μM Sodium Hepes Buffer and 6.5 μM $CaCl_22H_2O$ with a pH of 7.4 was used.

Electrophysiology

We located a nerve around the crayfish muscle and suctioned it into the suction electrode, then inserted the intracellular recording electrode into a muscle cell. The nerve was provided a 0.2 Hz stimulus using the SD9 Stimulator and the resulting EPSPs were recorded with an Intracellular Electrometer (Model 3200). A 20 Hz tetanic stimulus was applied to the cell for 10 seconds before returning the stimulus frequency to 0.2 Hz and the resulting EPSPs were recorded at 5 second intervals for approximately 5 minutes. This process was repeated after the crayfish tail was allowed to sit in 5 M L-NAME solution for approximately 5 minutes, which ensured that the nitric oxide synthase inhibitor had enough time to affect the neuromuscular junction. A different nerve and muscle section was used for each high frequency tetanic stimulation to account for muscle fatigue.

Data collection and analysis

The percent change in EPSP amplitude between the average pre-tetanus EPSP amplitude and post-tetanus EPSP amplitudes was calculated at 5 second intervals over the 5 minute period. Scatter plots were used to depict that change in EPSP amplitude over time. We used a two-tailed t-test with two-sample unequal variance to determine whether the difference in EPSP amplitude between the control and L-NAME groups at each 5 second interval had any statistical significance.

RESULTS

The objective of this experiment was to explore the effects of nitric oxide on LTP at the crayfish neuromuscular junction. We compared the effects of the NO-synthase inhibitor L-NAME on the EPSP amplitude after high-frequency stimulation to the effects of the same tetanus under control conditions. Once a consistent EPSP was established in a muscle cell via 0.2 Hz stimuli, we stimulated the same cell for 10 seconds at 20 Hz and then returned to 0.2 Hz for 5 minutes. The EPSP amplitudes before and after tetanic stimulation were recorded at 5second intervals over the time period. This process was repeated after exposing the crayfish to 5 M L-NAME solution for 5 minutes. A different nerve along the crayfish muscle was used to prevent overstimulation and harm to the nerve.

As illustrated in Figure 1, exposure to 5 M L-NAME solution inhibited long-term potentiation at the crayfish neuromuscular junction by reducing both the amplitude of EPSPs and the amount of time needed to return to normal EPSP levels. Immediately after tetanus, the percent change for the L-NAME group (29%) was on average lower than for the control (36%), yet after including the variance this difference is negligible. Additionally, both the L-NAME and control groups experienced a similar drop in percent change over the 5 minute interval of about 28% and 26%, respectively.

The main difference between the two groups is how quickly they returned to pre-tetanus EPSP

amplitudes. For the control, the average EPSP amplitude was larger than before the tetanus for the whole 5 minutes following tetanic stimulation. In contrast, the crayfish muscles exposed to L-NAME on average returned to baseline EPSP amplitude after 75 seconds post-tetanus. From 75 seconds on, the EPSP amplitudes did not dramatically decrease like the control group did, but rather stayed around pre-tetanus levels.

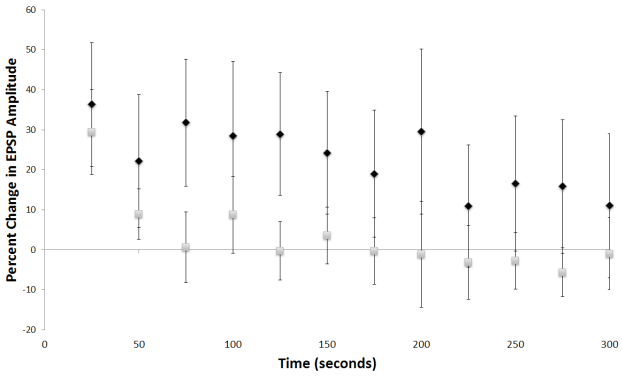

Figure 1. Percent change in EPSP amplitude after tetanic stimulation in control and L-NAME solutions. The average percent change in EPSP amplitudes after 20 Hz tetanic stimulation of 2 crayfish muscles exposed to either standard crayfish saline (black) or 5 M L-NAME solution (gray) is plotted over 5 minutes. The error bars indicate the S.E.M. across all trials of the same condition at that point in time. For the control and L-NAME groups, N=7 and N=6 , respectively. At all points $p>0.05$. Student's t-test (two-sample assuming unequal variance) was used to evaluate the significance of all electrophysiological data.

These results suggest that inhibiting the synthesis of nitric oxide reduces long-term potentiation at the crayfish neuromuscular junction and that NO does play a role in synaptic plasticity. Yet although our results showed that the average percent change in EPSP amplitude when L-NAME was applied was lower than the averages for the control trials, the differences are not great enough to be statistically significant.

DISCUSSION

Our research compared the effect of L-NAME on long-term potentiation in the crayfish neuromuscular junction to determine nitric oxide's role in automodulation. We found that L-NAME did decrease synaptic plasticity, in terms of long-term potentiation, but the difference in EPSP amplitudes post-tetanus between the L-NAME and control groups was not statistically significant. Thus, our results do not support our hypothesis that nitric oxide is necessary for long-term potentiation.

Despite having insignificant results, the general trend does suggest that nitric oxide may contribute to long-term potentiation at the crayfish neuromuscular junction, a hypothesis that has been supported in other studies. Aonuma et al. (2000) proposed that endogenous NO could have presynaptic modulatory effects on synaptic depression in crayfish since they found that applying an L-NAME bath partially blocked depression. Yet there still remains a larger debate about the role of NO in synaptic plasticity as other studies, like that conducted by Korshunova and Balaban (2014), argue that long-term potentiation requires nitric oxide.

Although our results were insignificant, they can still contribute to this debate since they suggested a correlation between nitric oxide and long-term potentiation at the crayfish neuromuscular junction through the application of an inhibitor. This trend can be explored in further research by revising the experimental procedure to account for more variables such as time or changing the duration of the 20 Hz stimulus. As time was not adequately controlled for, variability may have occurred due to inconsistencies in muscle fatigue. The number of times a single nerve and its innervated muscles were stimulated may have significantly contributed to variability in our results as well. Additionally, as some studies showed a relationship between nitric oxide and LTD (Aonuma 2000), testing different frequencies of tetanic stimulation may reveal differences in the type of automodulation present at the crayfish NMJ.

Although the role of exogenous nitric oxide has been shown to play a role in increasing long-term potentiation, results remain inconclusive about the relationship between nitric oxide inhibition and synaptic plasticity. Further research about NO inhibition may further illuminate how adding and inhibiting the molecule affects the crayfish neuromuscular junction. Observing their effects ont synaptic transmission and plasticity will allow us to fully consider under what conditions endogenous and exogenous NO influence the crayfish NMJ. Considering these elements in the future may help develop more insight into NO's modulatory effects on synaptic plasticity at the crayfish neuromuscular junction.

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