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The Dependence of Optimal Post-Tetanic Potentiation in Crayfish Muscle Cells on Nitric Oxide Concentration.

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ABSTRACT

We conducted this research to determine how synaptic activity, namely post-tetanic potentiation (PTP) amplitude and duration, in crayfish muscle cells is affected by differing levels of nitric oxide (NO). We hypothesized that, relative to control conditions, medium (100 µm sodium nitroprusside) levels of NO would have no effect on PTP, high (200 µm sodium nitroprusside) and low (50 µm sodium nitroprusside) levels of NO would negatively impact PTP, and an absence of NO would result in a lack of PTP. Through the use of two drugs, L-NAME and sodium nitroprusside, we exposed a dissected crayfish to various levels of NO before using intracellular recording to measure PTP several times for each NO concentration. Compared to control levels, we found that all NO concentrations below or equal to that provided by 100 µm nitroprusside resulted in a decrease of PTP while high NO levels resulted in an increase in PTP thus disproving our hypothesis.

INTRODUCTION

Motor neuron diseases, namely Amyotrophic Lateral Sclerosis (ALS), are currently an important area of study due to their causes and cures remaining unknown (Zarei et al., 2015). There are several hypotheses concerning ALS causation that are currently being investigated. One is the "Dying Back Hypothesis" which postulates that the dysfunction of nerve terminals precedes the neurodegeneration of motor neurons in ALS (Moloney et al., 2014; Pollari et al., 2014). Unfortunately, the exact mechanisms behind nerve terminal dysfunction in motor neurons (which is necessary knowledge for supporting or rejecting the "Dying Back Hypothesis") has also yet to be definitively determined due to the complexity and interrelated functioning of synaptic transmission.

There is evidence (Bukharaeva, et al., 2015) that increased levels of the amino acid homocysteine witnessed in ALS conditions, through its hyperactivation of NMDA (N-methyl-D-aspartate) receptors, significantly augments the inhibition of transmitter release caused by oxidative stress while simultaneously contributing to oxidative damage on presynaptic terminals of motor neurons. Bukharaeva et al. (2015) also implicate the increased nitric oxide (NO) release associated with the aforementioned hyperactivation of NMDA receptors as a possible cause of or contributor to the synaptic damage enacted by homocysteine.

Despite research documenting NO as a possible contributor to motor neuron diseases, its beneficial, cytoprotective, and modulatory effects on numerous cell processes such as synaptic transmission are also well documented (Wong et al., 2015). These conflicting effects of NO are most likely concentration dependent though more research is required to fully understand NO's varying impact on motor neurons. We studied NO's concentration dependent effects on synaptic transmission through the use of two drugs: (1) L-NAME, which inhibits the production of NO; and (2) sodium nitroprusside, which decays consistently into NO. The proper concentrations of these drugs to achieve various levels of NO were established previously by Wang et al. (2003). Unlike other studies that witnessed NO's effect on synaptic mechanisms using the mammalian neuromuscular junction (NMJ), the primary neurotransmitter of which is acetylcholine (Bukharaeva, et al., 2015), our experiment used the NMJ of a crayfish, the primary neurotransmitter of which is glutamate (Kawagoe et al., 1981). The crayfish NMJ is a more appropriate model for human motor neuron synapses due to its similar usage of glutamate as the primary neurotransmitter (Zhang et al 2011; Kawagoe et al., 1981). By determining the effects of various concentrations of NO on synaptic activity in the crayfish NMJ, our experiment will aid the effective development of ALS medical treatments involving nitric oxide. Such medical treatments are presently being considered though more research into NO's ambiguous effects needs to be conducted first (Bukharaeva et al., 2015). We conducted this experiment to determine the effects of various concentrations of NO (control, none, low, medium, and high) on synaptic activity, specifically post-tetanic potentiation (PTP) amplitude and duration.

We predicted that the plasticity of a synapse is dependent on the concentration of nitric oxide present, whether it is exogenous or endogenous. While this

concept had not been explored in crayfish, work had been done in the amygdala of mice that shows the dependence of long-term potentiation (LTP) on nitric oxide signaling (Lange et al., 2011). LTP and PTP differ in several ways but we believed that they will be similarly dependent on nitric oxide. This corresponding dependence allowed us to adapt the findings of Lange et al. (2011) onto a time frame similar to that of PTP. We sought to know the effect of various levels of nitric oxide on synaptic plasticity in the crayfish NMJ. We hypothesized that medium (100 µM sodium nitroprusside) nitric oxide levels will create PTP similar to control levels, that low (50 µM sodium nitroprusside) and high (200 µM sodium nitroprusside) levels of nitric oxide would negatively affect PTP, and that no nitric oxide would result in a lack of PTP. Using intracellular recording to measure PTP amplitude and duration in crayfish muscle cells exposed to various levels of NO, we concluded that all NO concentrations below or equal to that provided by 100 µm nitroprusside resulted in a decrease of PTP while high NO levels resulted in an increase in PTP. These findings led us to reject our hypothesis.

MATERIALS AND METHODS

Organisms

After anesthetizing the crayfish in ice water, we removed the tail and exposed the superficial extensor muscles of its tail by cutting the sides laterally and carefully removing the ventral mass of flexor muscles and other tissue (Wyttenbach, Johnson, & Hoy, 1999).

Solutions

We placed the tail in Ringer's solution (pH 7.4; 5.4 mM KCl, 196 mM NaCl, 2.6 mM MgCl2 * 6H2O, 10 mM Hepes buffer, 13.5 mM CaCl2 * 2H2O). To achieve different levels of nitric oxide we first inhibited the means of natural production of nitric oxide, nitric oxide synthase, through the use of L-NAME (300 µM). After L-NAME application, we reintroduced nitric oxide back into the saline solution by using sodium nitroprusside which decays continuously into nitric oxide (Grossi & Dangelo, 2005). We allowed a 15 minute incubation period after the application of L-NAME and sodium nitroprusside. By applying different concentrations of sodium nitroprusside, different levels of nitric oxide were produced for different trials. Table 1 shows how we achieved the different levels of nitric oxide using L-NAME and sodium nitroprusside.

Nitric Oxide	Use of	Concentration of
Control	0 µM	0 µM
None	300 µM	0 µM
Low	300 µM	50 µM
Medium	300 µM	100 µM
High	300 µM	200 µM

Table 1. Use of L-NAME and Sodium Nitroprusside to obtain different concentrations of Nitric Oxide

Stimulation

We delivered an electrical stimulus to the nerve innervating the crayfish muscle via a selected nerve. Then we recorded the change in membrane potential resulting from this stimulus using a recording electrode that has been inserted into the superficial extensor muscle of the crayfish tail on the same side and segment of the nerve. Stimulation began at a rate of 0.2 Hz, increased to 20 Hz for 5 seconds, and then returned to 0.2 Hz.

Recording

The amplitudes of these excitatory junction potentials (EJPs) were recorded before and after tetanic stimulation. We recorded five EJPs prior to tetanic stimulation and then continued to record EJPs every 15 seconds for two minutes after tetanic stimulation.

We measured the resting membrane of the extensor muscles with intracellular electrodes that were made using capillary tubes (1.2 mm) and an electrode puller. The recoding electrodes were filled with 3M KCl using a syringe with a microfil needle and then inserted into a muscle cell. The stimulating electrodes were shaved down so that they were wide enough suck up a nerve.

Data Analysis

Five EJP amplitudes were recorded prior to tetanic stimulation for each muscle. These were averaged to find the normal EJP amplitude of that neuromuscular junction. After tetanic stimulation, percent change was calculated by dividing the EJP amplitudes recorded by the normal EJP amplitude and then multiplying the quotient by 100. Finally, percent change at each time interval was averaged for all trials. We conducted T tests (two-tail equal variance) for each of our trials relative to the control to determine the statistical significance of each trial.

RESULTS

Using intracellular electrodes, we recorded the amplitudes of excitatory junction potentials in crayfish muscles at different levels of nitric oxide before and after tetanic

stimulation. We inhibited natural nitric oxide through the use of L-NAME and then reintroduced it through different concentrations of sodium nitroprusside, a compound that decays consistently into NO. Finding percent change relative to the mean EJP amplitude prior to tetanic stimulation allowed us to see post tetanic potentiation (PTP).

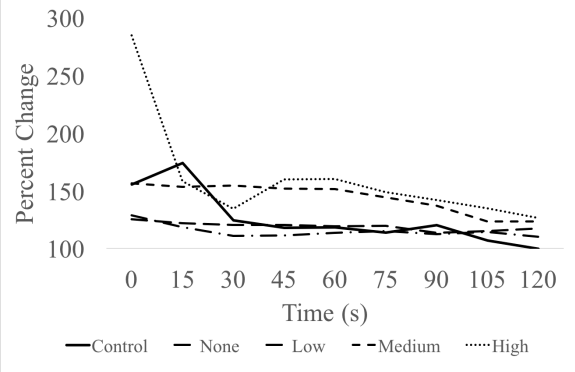


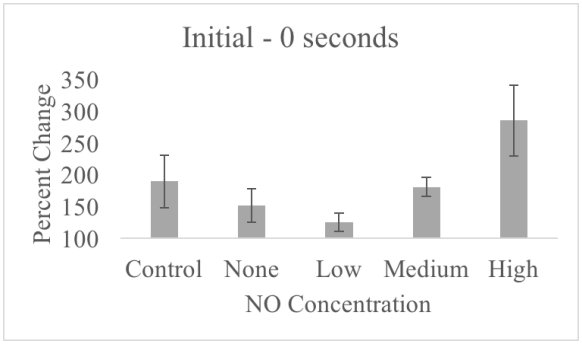
Figure 1. Percent change of EJP amplitudes at increasing NO concentrations following tetanic stimulation. Percent change in EJP amplitude is shown following tetanic stimulation. Time 0 represents the moment when tetanic stimulation was stopped and we began to record EJP amplitudes. Compared to control levels (n=5), we found that all NO concentrations below or equal to that provided by 100 µm nitroprusside (none (n=4), low (n=3), medium (n=4)) resulted in a decrease of PTP while high NO levels (n=4) resulted in an increase in PTP.

We observed PTP at all levels of NO. Compared to control trials, at every NO concentration below or equal to that provided by 100 µm nitroprusside, PTP decreased, while at high NO levels PTP increased (Figure 1). Due to the variance in PTP duration, we looked at three different time intervals (0s, 60s, 120s) to quantify our results (Figure 2)

Across all time increments we witnessed a trend relating to NO concentration; as NO concentration increased, PTP also increased. Directly following tetanic stimulation (0s), we observed that average EJP amplitudes were highest at high NO concentrations (285% ± 55%), and that percent gradually decreased as NO concentration decreased so that at a low concentration of NO, percent change was only 125% ± 14% greater than normal levels. This trend continued throughout all time intervals that were examined.

Compared to control trials, the use of L-NAME seemed to prolong the duration of PTP. While the control trials had returned to normal levels by 120 seconds, all other trials were still elevated (Figure 2). Unfortunately, as we did not record past 120 seconds we are unaware how long PTP lasted in these trials.

A



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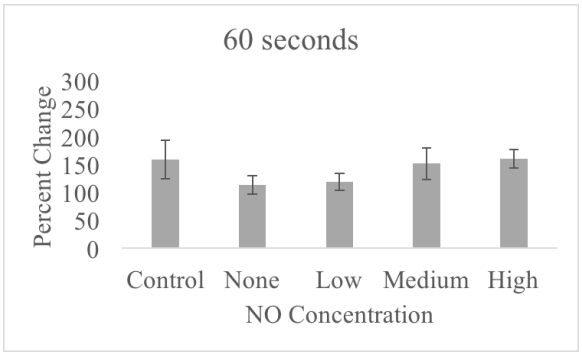

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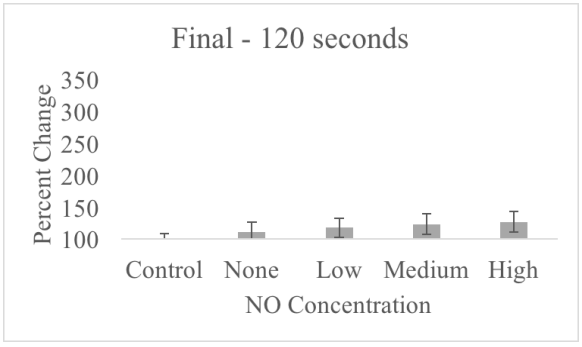

D

Control (n=5) Compared to	P value at 0 seconds	P value at 60 seconds	P value at 120 seconds
None (n=4)	0.49	0.32	0.41
Low (n=3)	0.29	0.44	0.33
Medium (n=4)	0.86	0.88	0.17
High (n=4)	0.20	0.91	0.08

Figure 2. Percent change of EJP amplitudes at increasing NO concentrations following tetanic stimulation. Percent change in EJP amplitude is shown at selected times. Time 0 represents the moment when tetanic stimulation was stopped and we began to record EJP amplitudes. Error bar show standard error of the mean. (A) Initial percent change is shown. (B) Percent change 60 seconds after tetanic potentiation is shown (C) Final percent change (120 seconds) is shown. (D) P values calculated using a two-tail equal variance t-test are shown comparing each trial's significance relative to control.

DISCUSSION

We found that all NO concentrations below or equal to that provided by 100 µm nitroprusside resulted in a decrease of PTP while high (200 µm nitroprusside) NO levels resulted in an increase in PTP. Because these results did not match our predicted effects of NO concentrations on PTP, we rejected our hypothesis.

Previous research implicating increased NO levels as a possible contributor to the degeneration of synaptic functionality witnessed in ALS conditions (Bukharaeva, et al., 2015) originally led us to predict that NO, though beneficial at normal levels (Wong et al., 2015), would decrease synaptic plasticity at high levels. However, our findings suggest that high levels of NO, at least as high as created by 200 µM sodium nitroprusside, actually increased PTP relative to control levels. Additional testing is needed to understand if high amounts of NO are only damaging in inflammatory conditions such as those studied by Bukharaeva, et al. (2015) or if NO can be ruled out as a possible contributor to synaptic damage in motor neuron diseases.

Additional testing is needed to clear up some of the ambiguities of our data. One difficulty we faced is not knowing whether we have added enough sodium nitroprusside to obtain a "normal" level of nitric oxide in the saline solution. We tried to counteract this limitation by basing our use of sodium nitroprusside on the work of other researchers with nitric oxide donors. Wang et al. (2003) found that 100µM of the nitric oxide donor DEA/NO completely reversed the effects of L-NAME. Since DEA/NO decays at a similar rate to sodium nitroprusside, we used 100 µM as a reference point as we try to simulate a normal level of nitric oxide in the saline solution surrounding the crayfish tail. However, no research had explored the use of L-NAME and sodium nitroprusside in crayfish muscle cells prior to our experiment. Because we found that medium (100 µM of sodium nitroprusside) levels of NO resulted in a decrease of PTP relative to that of control levels of NO, we believe that 150µM of sodium nitroprusside may be a more accurate representation of normal NO levels in crayfish. This hypothesis requires further testing to confirm.

Another difficulty we had in interpreting our results was not knowing if NO affected synaptic mechanisms through the creation of peroxynitrite, or through presynaptic neurotransmitter release inhibition. Future experimentation differentiating the dualistic damage enacted by NO could be useful. Additionally, we recognize that the body's natural means of production for nitric oxide, nitric oxide synthase, may produce other reactive nitrogen species (RNS) besides NO, which could have unknown ramifications on cellular processes (Alderton et al., 2001). Since we inhibited NOS, and reintroduced only NO, these reactive nitrogen species were not present in our experiment which may have altered the synaptic activity we recorded. This lack of RNS may also be responsible for the increased duration of PTP we witnessed as control trials returned to normal EJP amplitudes during the 120 second recording period, while trials with L-NAME were still experiencing PTP when we stopped recording. Repeating this experiment while inhibiting NO directly, rather than through NOS inhibition, could clarify the separate effects of both NO and other RNS species.

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