*Pioneering Neuroscience*, 2017, **16**, 19-22

# 7-NI More Effectively Reduces the Inhibitory Effects of $H_2O_2$ on Synaptic Transmission in Crayfish Compared to L-NAME.

RACHEL BUCKNER, HEATHER SUH, and ANDREW TUCKER
Department of Biology, Grinnell College, Grinnell, Iowa

## ABSTRACT

Both oxidative stress and increased nitric oxide (NO) production have been linked to the progression of various neurological disorders. However, the specific pathogenesis of these disorders is unknown. To investigate the role of nitric oxide in cell death, we researched the effect of selective and non-selective nitric oxide synthase (NOS) inhibitors on synaptic transmission in crayfish during exposure to the oxygen derivative $H_2O_2$, which mimics oxidative stress. We hypothesized that n-Nitroarginine methyl ester (L-NAME), a nonselective NOS inhibitor, will be more effective than 7-Nitroindazole (7-NI), a selective nNOS inhibitor, at preventing the negative effects of $H_2O_2$ on the excitatory junction potential (EJP) in crayfish. We performed intracellular recording to measure the change in synaptic transmission before and after the addition of $H_2O_2$. Our results did not support our hypothesis, as 7-NI more effectively mitigated the inhibitory effects of $H_2O_2$ on synaptic transmission than L-NAME. We conclude that the ineffectiveness of L-NAME may be a result of the drug targeting NOS isoforms necessary for synaptic transmission.

## INTRODUCTION

The mode in which cell death is accelerated in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) is unknown (Schulz et al., 1997). Neurodegenerative diseases lead to loss or damage of neurons. Other neurodegenerative diseases include Alzheimer's, Parkinson's, and Huntington's, all of which result in neuronal cell death in different areas of the brain. Neurodegenerative diseases such as ALS lead to the eventual weakening of muscles and the inability to perform necessary motor functions. Existing research suggests that one cause of neuronal death is oxidative stress (Barnham et al., 2004; Jaro-Prado et al., 2003).

Increased nitric oxide (NO) production has been linked to the progression of various neurological disorders, and is indirectly caused by oxidative stress (Chabrier et al., 1999). Oxidative stress is the result of an overproduction of reactive oxygen species (ROS) that cause damage in neuronal cells (Barnham et al., 2004). Hydrogen peroxide ($H_2O_2$), a ROS, mimics oxidative stress in neurons and inhibits synaptic transmission (Bukharaeva et al., 2015). Normal cell activity produces $H_2O_2$; however, the derivative becomes toxic at too high a quantity (Colton, Fagni, & Gilbert, 1989). Meyer et al. (1996) found that in mammalian cells, $H_2O_2$ increases the concentration of intracellular calcium, but did not elucidate the mechanism for this calcium influx. This excess intracellular calcium activates calcium dependent enzymes that damage cell structures; one of these enzymes is nitric oxide synthase (NOS), which produces NO (Schulz et al., 1997).

As a neuromodulator, NO plays a role in regulating various bodily functions, including cognitive development, neuronal plasticity, and behavioral trends (Chabrier et al., 1999). In vertebrates, NOS presents itself in three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and induced NOS (iNOS). Of the three, nNOS and iNOS are more prominent in neurons while eNOS primarily operates to regulate vasodilation and blood pressure (Shesely et al., 1998). As a result, the NO produced by nNOS and iNOS is more likely to act as a neurotoxic agent when produced in high quantities (Chabrier et al., 1999). NO contributes to neuronal death through its reaction with the superoxide anion $O_2^{\cdot -}$ to form peroxynitrite: a highly reactive oxygen derivative. Since this bonding reaction occurs rapidly, peroxynitrite is formed faster than the body can safely neutralize $O_2^{\cdot -}$ and NO (Chabrier et al., 1999).

While research conducted by Schulz et al. (1997) discusses nitric oxide's role in NMDA neurotoxicity, little research has specifically examined the relationship between nitric oxide synthase and oxidative stress. Jara-Prado et al. (2003) investigated the effect of NOS inhibitors on synaptic transmission of vertebrates. They primarily focused on lipid peroxidation, one form of oxidative stress. Lipid peroxidation is the process in which phospholipids lose their function and integrity as a result of free radicals, leading to a calcium influx and cell death (Jara-Prado et al., 2003). Jara-Prado et al. (2003)

found that L-NAME was more effective than 7-NI in protecting against lipid peroxidation in vertebrates.

Our research expands on this study by examining the effect of L-NAME and 7-NI on synaptic transmission in invertebrates during $H_2O_2$-induced oxidative stress. We used intracellular recording to record the percent change of EJP amplitudes in invertebrates during paired-pulse facilitation. By mimicking mild oxidative stress with the addition of $H_2O_2$, we investigated whether endogenous NO and $H_2O_2$ have a synergistic effect. If selective or nonselective inhibition of NOS lowers the effect of $H_2O_2$ on synaptic transmission, that suggests that NO exacerbates the effect of $H_2O_2$. This research supports previous findings that NOS inhibitors aid in the treatment of ALS and other neurodegenerative diseases that cause oxidative stress. We hypothesized that L-NAME, the nonselective inhibitor, would be more effective than 7-NI, the selective nNOS inhibitor, at preventing the negative effects of $H_2O_2$ on EJP amplitudes in crayfish. We found that 7-NI is more effective at maintaining EJP strength than L-NAME when neurons experienced oxidative stress.

## MATERIALS AND METHODS

*Dissection*.

To test the effect of NO inhibition on excitatory junction potentials (EJPs), we used intracellular recording with voltage stimulation on the superficial extensor muscle of the crayfish. We conducted four trials: a baseline treatment, a treatment incubated in a nonselective nitric oxide synthase inhibitor (L-NAME), a treatment incubated in a selective nitric oxide synthase inhibitor (7-NI), and a treatment incubated in L-NAME without the addition of $H_2O_2$. We worked with a different dissected crayfish for each trial to measure the synaptic transmission on the superficial extensor muscle cells. We used an ice bath to anesthetize the crayfish. For dissection, we removed the tail of the crayfish with scissors, removing the ventral side of the tail and scooping out the muscles ventral to the gut (Wyttenbach et al., 1999).

*Solutions.*

We prepared three solutions: 100 mL of baseline Ringer's solution (5.5mM KCl, 196 mM NaCl, 2.6 mM $MgCl_26H_2O$, 10 mM Hepes Buffer, and 13.5 mM $CaCl_22H_2O$), 100 mL of Ringer's solution with a 300 µM concentration of L-NAME, and 75 mL of Ringer's solution with a 300 µM concentration of 7-NI. We prepared three of the same solutions, adding a 332 µM concentration of $H_2O_2$. The L-NAME only trial used 100 mL of Ringer's solution with a 300 µM concentration of L-NAME.

*Electrophysiology.*

To perform intracellular recording, we made a set of microelectrodes by pulling 1.2mm borosilicate glass capillary tubes in half with an electrode puller (PUL-1, World Precision Instruments), and filled the microelectrodes with 3M KCl (Wyttenbach et al., 1999). To stimulate synaptic transmission, we suctioned a nerve into a second electrode and impacted a neighboring muscle cell with a non-dulled glass microelectrode. We replaced the microelectrode if the resistance did not fall between 4-20MΩ. Using an SD9 stimulator**,** we delivered paired-pulse stimulation to the nerve by applying a supramaximal stimulus. Synaptic transmission was recorded using the PowerLab software by measuring EJP amplitude.

*Incubation*.

The baseline trial does not require incubation. For L-NAME and 7-NI, we added the inhibitor to the Ringer's solution and incubated for 30 minutes before exposure to $H_2O_2$. The $H_2O_2$ incubated for 10 minutes before we recorded EJPs. After recording synaptic transmission, we left the intracellular recording electrode in the muscle and added the $H_2O_2$ solution. Additionally, we conducted a separate trial testing the effect of L-NAME on synaptic transmission. We incubated for 30 minutes and recorded percent change before and after incubation.

*Data Collection.*

By using one muscle cell to measure the amplitude, we controlled for the variability of synaptic transmission across different muscle cells. After the incubation period, we recorded the synaptic transmission. For all recordings of synaptic transmission, we included the percent difference between $EJP_1$ and $EJP_2$ in paired-pulse facilitation before and after the addition of $H_2O_2$. We quantified the EJPs by measuring the amplitude and analyzing data on Excel.

## RESULTS

We investigated the impact of selective and nonselective nitric oxide synthase (NOS) inhibitors on synaptic transmission when neurons experience oxidative stress. Using intracellular recording, we found that the addition of $H_2O_2$ in the nonselective 7-NI treatment produced the smallest decrease in EJP amplitude, followed by the baseline treatment (i.e. no addition), then the selective L-NAME treatment. Specifically, the average percent change in EJP amplitudes from before to after the addition of $H_2O_2$ was -19% for the 7-NI treatment (Figure 1). For the baseline and L-NAME treatments, the percent changes were -42% and -87% respectively (Figure 1).

From the L-NAME only trial, we found that synaptic strength decreased by -33%.

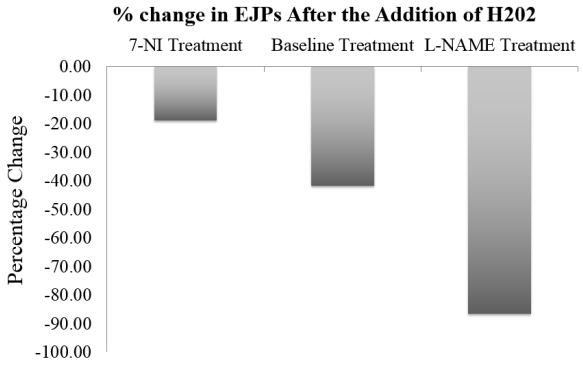


**Figure 1.** Percent difference in the average EJP amplitudes from before to after the addition of $H_2O_2$. The number of data points collected for each trial was 34 for the baseline treatment, 10 for the L-NAME treatment, and 27 for the 7-NI treatment. No errors bars are shown as we only conducted one trial for each treatment.

We recorded the paired pulse facilitation, or percent change in amplitude from $EJP_1$ to $EJP_2$ (($EJP_2$-$EJP_1$)/$EJP_1$) for the three treatments to calculate the percent difference in paired pulse facilitation from before to after exposure to $H_2O_2$. The results found that paired pulse facilitation increased by 101% after exposure to $H_2O_2$ for the baseline treatment, by -2% for the 7-NI treatment, and by -68% for the L-NAME treatment (Figure 2). The dramatic change in paired-pulse facilitation after the addition of $H_2O_2$ for the baseline treatment and the L-NAME treatment indicate the presence of a presynaptic change.

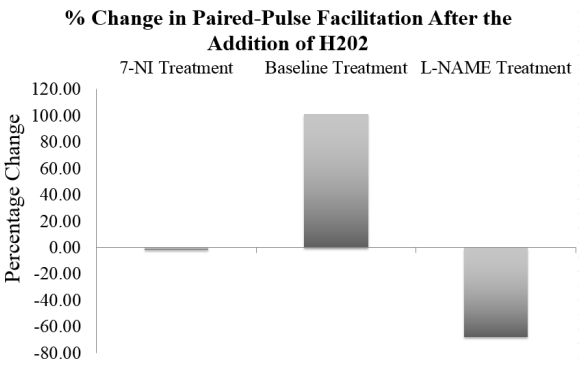


**Figure 2.** Percent change in the average paired-pulse facilitation from before to after the addition of $H_2O_2$, (average paired pulse facilitation calculated by using ($EJP_2$-$EJP_1$)/$EJP_1$). The number of data points collected for each trial was 34 for the baseline treatment, 10 for the L-NAME treatment, and 27 for the 7-NI treatment. No errors bars shown as we only conducted one trial for each treatment.

## DISCUSSION

Our results showed that following incubation in L-NAME, the strength of the EJP(s) before and after exposure to $H_2O_2$ decreased by over 80%, whereas following incubation in 7-NI, the EJP amplitude decreased by less than 20%. Our results did not support our hypothesis that L-NAME would be more effective than 7-NI in decreasing the EJP percent change with exposure to $H_2O_2$. We found that 7-NI was more effective at maintaining EJP strength than L-NAME when neurons were exposed to oxidative stress. This result may be due to the detrimental effects of blocking all types of NOS. NO is involved in the regulation of neuronal plasticity and is necessary for healthy neurological function (Chabrier et al., 1999). Exposing the crayfish superficial extensor muscle to the non-selective NOS inhibitor, L-NAME, for an extended period of time may have detrimental effects on the EJPs in the crayfish muscle. As L-NAME is a nonspecific NOS inhibitor, it also inhibits the synthesis of eNOS, which is crucial for proper vasodilation and circulatory function.

While various studies suggest that inhibiting NOS could delay or prevent the effects of various neurological disorders, these effects are not always positive nor permanent (Schulz et al., 1997; Chabrier et al., 1999). While partial inhibition of NOS activity has yielded positive results in delaying neurological degeneration, this delay lasts for less than a year (Chabrier et al., 1999). Furthermore, in some experiments that used nonspecific NOS blockers, symptoms not only failed to improve, but worsened as a result of the NOS inhibition (Chabrier et al., 1999). This effect may explain why L-NAME did not mitigate the effects of oxidative stress on synaptic transmission.

If some NO is necessary for neuronal function, exposing the crayfish muscle to a non-selective NOS inhibitor could damage neurons and decrease synaptic strength, even without exposure to $H_2O_2$ (Chabrier et al., 1999). Therefore, we incubated the crayfish muscle in L-NAME, and measured the synaptic strength over time without using $H_2O_2$ to simulate oxidative stress. We found that inhibiting all types of NOS decreases synaptic transmission by over 30%. These results suggest that using non-selective NOS inhibitors is not a beneficial treatment method for neurological degeneration, as the lack of all types of NO disrupts normal neural regulation and decreases synaptic strength. Also, these results offer an explanation for why we observed a significant decrease in synaptic strength in our trial with both L-Name and $H_2O_2$. Since L-NAME is itself detrimental to healthy synaptic function, this detriment would likely be exacerbated when paired with oxidative stress, such as exposure to $H_2O_2$. However, we recommend further replications of this study with alternative NOS inhibitors, to verify that the non-selective NOS inhibitor is what

causes the decrease in synaptic strength, and not a different quality specific of L-NAME.

Furthermore, Chabrier et al. (1999) shows evidence of increased NO production during neuronal degeneration due to excess stimulation of glutamate receptors. We recommend investigating the effect of adding excess nitric oxide with a nitrovasodilator on synaptic transmission. The vasodilator would accelerate the effects of excess NO on neurons by increasing circulation (Chabrier et al., 1999). This would help determine whether high levels of NO lead to cell death, which would improve our understanding of the pathogenesis of ALS and other neurodegenerative diseases.

Additionally, we recommend investigating the effects of exposing neurons to both NOS inhibitors and vasodilators. Inhibiting NOS drastically reduces the production of NO, which hinders normal neurological function. Specifically, the inhibition of eNOS, reduces the amount of NO that regulates vasodilation (Chabrier et al., 1999). Therefore, by pairing strong NOS inhibitors with a vasodilator, we could potentially decrease NO levels in neurons while maintaining proper circulation and blood pressure.

## ACKNOWLEDGEMENTS

We would like to thank Professor C. Lindgren for his patience and guidance throughout the entire experimental process. Thank you to the writing mentor, Tim Burnette, for helpful edits of the paper. We would also like to thank the lab assistant Ashley Wolterstorff and class mentor Julia Petrusan for their assistance and guidance in the lab.

## REFERENCES

Barnham, K. J., Masters, C. L., & Bush, A. I. (2004). Neurodegenerative diseases and oxidative stress. *Nature Reviews Drug Discovery*, *3*(3), 205-214.

Bukharaeva, E., Shakirzyanova, A., Khuzakhmetova, V., Sitdikova, G., & Giniatullin, R. (2015). Homocysteine aggravates ROS-induced depression of transmitter release from motor nerve terminals: potential mechanism of peripheral impairment in motor neuron diseases associated with hyperhomocysteinemia. *Front. Cell. Neurosci. 9*(391), 1-7.

Chabrier, P. E., Demerlé-Pallardy, C., Auguet, M. (1999). Nitric oxide synthases: targets for therapeutic strategies in neurological diseases. *Cellular and Molecular Life Sciences*, *55,* 1029-1035.

Colton, C. A., Fagni, L., Gilbert, D. (1989). The Action of Hydrogen Peroxide on Paired Pulse and Long-Term Potentiation in the Hippocampus. *Free Radical Biology & Medicine, 7,* 3-8.

Jara-Prado, A., Ortega-Vazquez, A., Ruano, L. M., Rios, C., & Santamaria, A. (2003). Homocysteine-induced brain lipid peroxidation: Effects of NMDA receptor blockade, antioxidant treatment, and nitric oxide synthase inhibition. *Neurotoxicity Research, 5*(4), 237-243.

Jiang, Q., Zhou, Z., Wang, L., Wang, L., Yue, F., Wang, J. & Song, L. (2013). A scallop nitric oxide synthase (NOS) with structure similar to neuronal NOS and its involvement in the immune defense. *PLoS ONE, 8(7)*: e69158.

Kim, H., Batista, L. A., Hoppes, J. L., Lee, K. J., & Mykles, D. L. (2004). A crustacean nitric oxide synthase expressed in nerve ganglia, Y-organ, gill, and gonad of the tropical land crab, *Gecarcinus lateralis*. *The Journal of Experimental Biology, 207,* 2845-2857.

Meyer, T. N., Gloy, J., Hug, M. J., Gregor, R., Schollmeyer, P., Pavenstadt, H. (1996). Hydrogen peroxide increases the intracellular calcium activity in rat mesangial cells in primary culture. *Kidney International, 49,* 388-395.

Schulz, J.B., Matthews, R.T., Klockgether, T., Dichgans, J., & Beal, M.F. (1997). The role of mitochondrial dysfunction and neuronal nitric oxide in animal models of neurodegenerative diseases. *Molecular and Cellular Biochemistry*, *174*, 193-197.

Shesely, E. G., Maeda, N., Kim, H.-S., Desai, K. M., Krege, J. H., Laubach, V. E., Sherman, P. A., Sessa, W. C. & Smithies, O. (1996). Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(23), 13176–13181.

Wyttenbach, R. A., Johnson, B. R., & Hoy, R. R. (1999). Crawdad: A CD-ROM lab manual for neurophysiology. Sunderland, MA: Sinauer Associates, Inc.