

## **No evidence of homocysteine or hydrogen peroxide influence at the crayfish neuromuscular junction.**

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### **ABSTRACT**

Due to the differences between crayfish and mammals, we researched how HCY and H<sub>2</sub>O<sub>2</sub> affect the crayfish neuromuscular junction. We hypothesized that H<sub>2</sub>O<sub>2</sub> would decrease synaptic activity at the crayfish NMJ, while HCY would enhance this inhibitory effect. We used electrophysiology to examine how the NMJ was influenced by HCY and H<sub>2</sub>O<sub>2</sub>. We concluded that there is no evidence of HCY or H<sub>2</sub>O<sub>2</sub> influence on synaptic activity at the crayfish NMJ.

### **INTRODUCTION**

Amyotrophic lateral sclerosis (ALS) is a progressive disease that affects motor neurons, the specialized nerve cells that control muscle movement (Wijesekera & Leigh, 2009). These nerve cells are found in the spinal cord and the brain. In ALS, motor neurons die (atrophy) over time, leading to muscle weakness, loss of muscle mass, and inability to control movement (Wijesekera & Leigh, 2009). Recently, several studies (Levin et al., 2010; Zoccolella et al., 2008) have shown that Homocysteine (HCY), a non-protein amino acid, has a strong relationship with ALS. Through an observational study of ALS patients, Levin (2010) concluded that HCY and methylmalonate are either directly involved in the neurodegenerative process or that both are surrogate markers for a neurodegenerative process. In addition to HCY, reactive oxygen species are also thought to contribute to the development of ALS by inhibiting synaptic transmission at the NMJ (Colton et al., 1989). Oxidative stress occurs when oxygen combines with other elements to create free radicals that harm the neurons in the body. To obtain more information about the function of HCY, Bukharaeva et al. (2015) investigated the effect of HCY on oxidative stress-induced impairment of transmitter release at the mouse diaphragm muscle. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), a mild oxidant, was added to the synapse which was pre-treated with HCY for two hours, and synaptic activity decreased. Through comparing the data of these different treatments, they concluded that HCY largely boosted the inhibitory effect of H<sub>2</sub>O<sub>2</sub>, via NMDA receptors in the mammalian neuromuscular junction (NMJ) (Bukharaeva et al., 2015).

To further understand the relationship between HCY, H<sub>2</sub>O<sub>2</sub>, and NMDA receptors, we performed a similar experiment at the crayfish NMJ. The crayfish NMJ is relatively underutilized as a medium for ALS research, as highlighted by the distinct lack of literature discussing the effects of HCY and H<sub>2</sub>O<sub>2</sub> at the crayfish NMJ. We used crayfish instead of rats because the NMJ is easily exposed and preserved. Since crayfish neuron cells are slightly different than mammals, with different pharmacological sensitivities of their Glutamate receptors (Shinozaki et al., 1992), we applied the same treatments to crayfish, and we observed whether the crayfish and the mammalian NMJ's reacted similarly to HCY and H<sub>2</sub>O<sub>2</sub>.

We observed how HCY and H<sub>2</sub>O<sub>2</sub> affected the crayfish NMJ. We hypothesized that HCY would amplify the neurodegenerative effect of H<sub>2</sub>O<sub>2</sub> in the crayfish NMJ similarly to how HCY and H<sub>2</sub>O<sub>2</sub> synergistically act in the mammalian NMJ. We found that HCY and H<sub>2</sub>O<sub>2</sub> had no consistent effect on the crayfish NMJ.

### **MATERIALS AND METHODS**

#### *Electrophysiology*

We measured synaptic activity through excitatory junction potentials (EJPs), which are the depolarizations of muscle cells as a response to a supra-maximal stimulus to the motor neuron. To measure the EJPs, we used two 1.2 mm glass capillary tubes. Using these capillary tubes, we made electrodes using an electrode puller (PUL 1, World Precisions Instrument). One electrode was slightly broken to create a suction electrode, and the other was filled with 3 M KCl to create a recording electrode. The recording electrode, with a resistance between 50 and 200 MΩ, pierced the extensor muscle of the crayfish, while the suction electrode sucked up a nearby nerve.

### Preparation

We prepared the crayfish tail according to the instructions in Wyttenbach et al. (1999). We put the crayfish on ice to anesthetize them before dissection. The tail was severed from the body of the crayfish and the ventral side of the tail was removed to expose the extensor muscles. We pierced these muscles with the recording electrode and sucked up a nerve with the suction electrode. We placed the prepared tail in a petri dish in Ringer's saline with HCY and  $H_2O_2$  treatments. All crayfish were pre-incubated for 150 minutes in either HCY or Ringer's saline, depending on the experimental treatment.

### Solutions

The different solutions we used include Ringer's saline (5.4 mM KCl, 196 mM NaCl, 2.6 mM  $MgCl_2 \cdot 6H_2O$ , 10mM Hepes Buffer, 13.5 mM  $CaCl_2 \cdot 2H_2O$ ), 330  $\mu$ M hydrogen peroxide ( $H_2O_2$ ), and 500  $\mu$ M homocysteine (HCY, 2-amino-4-sulfanybutanoic acid).

### Data Collection

We applied a paired pulse stimulus which stimulated the nerve twice in quick succession. The facilitation that occurred as a result of the paired pulse stimulus has been linked with presynaptic mechanisms (Colton et al., 1989). Using the electrodes, we measured the facilitation and the amplitudes of each respective EJP five times every five minutes. The delay between the two pulses was 100 ms. Resting membrane potentials varied from cell to cell, but all cells with a resting potential below -55 mV were rejected.

We conducted four different trials with four different crayfish. We collected two trials of the control group, with pre-incubation in Ringer's saline before the addition of  $H_2O_2$ , and two trials of the experimental group, with pre-incubation in HCY before the addition of  $H_2O_2$ .

### Data Analysis

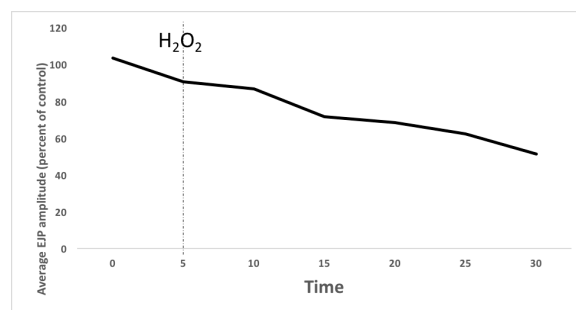
We used Excel for data analysis. We took the averages of the first 10 EJPs before applying the treatment for each trial, using that number as a standard for the rest of the EJPs in each trial to account for the variability between crayfish. Additionally, we calculated the percent change of the second pulse compared to the first pulse for each data set.

## RESULTS

To better understand the role of ALS at the NMJ, we conducted an experiment at the crayfish NMJ using

HCY and  $H_2O_2$ . We hypothesized that  $H_2O_2$  would inhibit the crayfish NMJ, and that pre-incubating the tail in HCY would amplify the inhibitory effect of the  $H_2O_2$ . Using intracellular recording techniques, we obtained EJPs every five minutes for 60-minute periods in four separate trials.

Our preliminary results indicated that  $H_2O_2$  depressed synaptic activity, as adding  $H_2O_2$  decreased average EJP amplitude by 40% over thirty minutes (Figure 1).

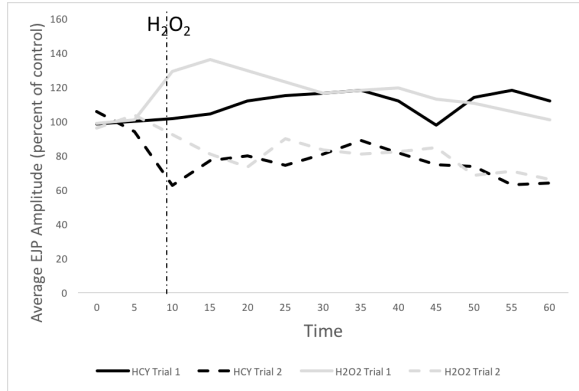


**Figure 1.** Figure 1 shows the average EJP amplitudes over the course of our preliminary experiments. We took the average of five measurements every five minutes for five minutes before adding  $H_2O_2$  and for 25 minutes after. All measurements taken before the addition of  $H_2O_2$  were considered control measurements. We averaged these control measurements to obtain a starting point, which we standardized to be 100 on the scale.

These preliminary experiments, however, were performed without the 150-minute pre-incubation period. With this pre-incubation period in the collection of our four trials, we noticed no consistent effect.

Regardless of whether the crayfish was incubated in HCY or Ringer's saline, synaptic activity remained relatively stable after adding  $H_2O_2$ . Under the control treatment, after adding  $H_2O_2$ , there was a 0.98% increase in EJP amplitudes (1.6 mV) in the first trial, and a 33.87% decrease (1.7 mV) in the second (Figure 2, Table 1). With pre-incubation in HCY, after adding  $H_2O_2$ , there was a 13.7% increase in EJP amplitudes (2.87 mV) in the first trial, and a 35.83% decrease (1.44 mV) in the second (Figure 2, Table 1). Because of the inconsistent effects of both  $H_2O_2$  and HCY, we have no evidence to support our hypothesis.  $H_2O_2$  did not significantly depress the average EJP amplitudes, and HCY did not amplify the depression. If anything, HCY actually mitigated the effects of the  $H_2O_2$ .

Additionally, we observed that at 10 minutes, after adding  $H_2O_2$ , all four trials fluctuate significantly (Figure 2). These fluctuations occur in no particular direction and may indicate some indirect effect of the physical act of adding  $H_2O_2$ .



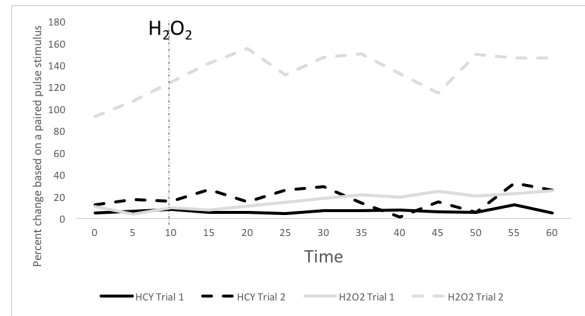
**Figure 2.** Figure 2 shows the average EJP amplitudes over the course of each of the four trials. We took the average of five measurements every five minutes for 10 minutes before adding H<sub>2</sub>O<sub>2</sub> and for 50 minutes after. All measurements taken before the addition of H<sub>2</sub>O<sub>2</sub> were considered control measurements. We averaged these control measurements to obtain a starting point in each trial, which we standardized to be 100 on the scale. The dark lines show average EJP amplitudes in the crayfish incubated in HCY, and the light lines indicate average EJP amplitudes in the crayfish incubated in Ringer's saline only.

Time	HCY Trial 1	HCY Trial 2	H2O2 Trial 1	H2O2 Trial 2
	20.95	4.24	15.63	4.82
		3.77	15.94	5.19
0	21.61	2.52	20.40	4.62
5	22.18	3.09	21.49	4.06
0	23.85	3.20		3.67
5	24.47	2.99		4.51
0	24.77	3.24	18.36	4.17
5	25.13	3.56	18.68	4.05
0	23.85	3.27	18.90	4.13
5	20.85	3.00	17.86	4.24
0	24.28	2.95	17.47	3.44
5	25.11	2.53	16.69	3.55
0	23.82	2.57	15.94	3.31

**Table 1.** Table 1 shows the average EJP amplitudes (mV) over the course of each of the four trials. We took the average of five measurements every five minutes for 10 minutes before adding H<sub>2</sub>O<sub>2</sub> and for 50 minutes after. All measurements taken before the addition of H<sub>2</sub>O<sub>2</sub> were considered control measurements. HCY trials indicate a pre-incubation in HCY. H<sub>2</sub>O<sub>2</sub> indicates pre-incubation in Ringer's. In both groups, H<sub>2</sub>O<sub>2</sub> was added at 10 minutes.

In an attempt to understand the mechanisms involved in the hypothesized HCY and H<sub>2</sub>O<sub>2</sub> induced synaptic depression, we observed paired pulse facilitation alongside EJP amplitude. Paired pulse facilitation is the short-term synaptic plasticity that results from a paired pulse stimulus. According to Colton et al., (1989), there is a link between paired pulse facilitation and presynaptic mechanisms. If paired pulse facilitation was depressed in a similar

manner to EJP amplitude, we could link the neurodegenerative effects to the presynaptic cell. Since we observed no such effects in the strength of the EJP amplitudes, we did not expect to find any consistent trends in paired pulse facilitation. We observed that the percent change based on a paired pulse stimulus did not significantly change after adding H<sub>2</sub>O<sub>2</sub> (Figure 4). If anything, the crayfish NMJ's ability to facilitate actually increased during each of the four trials (Figure 4).



**Figure 4.** Figure 4 shows the average percent change in EJP amplitudes based on a paired pulse stimulus over the course of the four trials. We took the average of five measurements every five minutes for 10 minutes before adding H<sub>2</sub>O<sub>2</sub> and for 50 minutes after. All measurements taken before the addition of H<sub>2</sub>O<sub>2</sub> were considered control measurements. The dark lines show average percent change in the crayfish incubated in HCY, and the light lines indicate average percent change in the crayfish incubated in Ringer's saline only.

## DISCUSSION

There is no evidence that HCY and H<sub>2</sub>O<sub>2</sub> have any consistent effects on synaptic activity at the crayfish NMJ. We hypothesized that HCY and H<sub>2</sub>O<sub>2</sub> would depress synaptic activity, however, the data does not support our hypothesis. We found no consistent trends in EJP amplitude or paired pulse facilitation (Figure 2, Figure 4). Previous research (Bukharaeva et al., 2015) used 500  $\mu$ M HCY and 332  $\mu$ M H<sub>2</sub>O<sub>2</sub>. Although solutions of these concentrations depressed synaptic activity at the mammalian NMJ, they did not have a consistent effect at the crayfish NMJ. Colton et al., (1989) used 1 mM H<sub>2</sub>O<sub>2</sub>, indicating that larger concentrations may be necessary. To more clearly observe the neurodegenerative influence of HCY and H<sub>2</sub>O<sub>2</sub> in crayfish, we suggest increasing the concentrations of both the HCY and H<sub>2</sub>O<sub>2</sub> applied to the NMJ.

Another possible explanation for the lack of trends in our data is the pre-incubation period. In our preliminary research, we did not incubate the crayfish for 150 minutes, and we observed a

larger decrease in EJP amplitudes (40%) than in any other experimental trials (Figure 1). It is possible that the neurodegenerative effects occurred during the 150-minute incubation period, and we collected data after synaptic activity was already depressed. We suggest either skipping the pre-incubation period or taking measurements before it, so as to monitor the effects this process has on the HCY and H<sub>2</sub>O<sub>2</sub> induced synaptic depression.

After increasing the concentrations of HCY and H<sub>2</sub>O<sub>2</sub> and controlling for the incubation period, we expect that HCY and H<sub>2</sub>O<sub>2</sub> will act similarly in the crayfish NMJ to how they did in the mammalian NMJ (Bukharaeva et al., 2015). Bukharaeva et al., (2015) indicated that HCY acted through NMDA receptors in the mammalian NMJ. If we can simulate the same neurodegenerative effects in the crayfish NMJ, it would be interesting to examine the impact of an NMDA blocker, such as AP-5. Bukharaeva et al., (2015) pre-incubated the mammalian NMJ in AP-5 and observed that the previously seen neurodegenerative effects of HCY and H<sub>2</sub>O<sub>2</sub> were blocked. To further this research, we suggest a post-application of AP-5 to determine its capacity to reverse HCY induced neurodegenerative effects.

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