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Investigating the Effects of Homocysteine as an Agonist on Invertebrate Glutamatergic Synapses

Elizabeth Grau
University of Kentucky, elizabeth.grau@uky.edu

Alexandra E. Stanback
University of Kentucky

Alec Bradley
University of Kentucky, alec.bradley@uky.edu

Danielle Cantrell
University of Kentucky, daniellecantrell@uky.edu

Samantha Eversole
University of Kentucky, samantha.eversole@uky.edu

See next page for additional authors

Authors
Elizabeth Grau, Alexandra E. Stanback, Alec Bradley, Danielle Cantrell, Samantha Eversole, Carolyn Grachen, Kaylee Hall, Danielle Hawthorne, Claire Kinmon, Paula Ortiz Guerrero, Bhavik Patel, Kaitlyn Samuels, Chinni Suryadevara, Gia Valdes, Samuel Wycoff, and Robin L. Cooper

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Department of Biology, University of Kentucky, Lexington, KY, USA

Hyperhomocysteinemia (HHcy) in mammals can produce neurological deficits, such as memory loss. The cause of the neurological issues is assumed to be due to homocysteine (HCY) binding to glutamatergic receptors in the central nervous system (CNS). High levels of HCY in the CNS are also associated with Amyotrophic Lateral Sclerosis (ALS) and Parkinson's disease. Thus, understanding the detailed mechanisms of HCY in model preparations could be useful in developing potential treatments to neurodegenerative diseases with overlapping symptoms to HHcy. The aim of this study is to investigate the efficacy of HCY as an agonist at glutamatergic synapses in invertebrates. The glutamatergic synapses of the larval *Drosophila melanogaster (D.* melanogaster) and *Procambarus clarkii* (*P. clarkii)* neuromuscular junctions (NMJs) were utilized to examine the effects of applying HCY. Measurements of evoked synaptic transmission in both preparations revealed that 100 mM of HCY did not have any consistent effect. The expectation was that the acute action of HCY would have activated the glutamate receptors and then desensitized them so evoked transmission would be blocked. The pharmacological receptor profile of these NMJ receptors are of a quisqualate subtype and not a kainate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or N-methyl-D-aspartate receptor (NMDA) subtype. Consequently, HCY may not have any action on quisqualate glutamate receptor subtypes. The findings of this experiments could provide clinical implications regarding relevant pharmacological treatments in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Parkinson's disease.

Abbreviations: ACURE— authentic course-based undergraduate research experience; AMPA— α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; EPSP— excitatory postsynaptic potential; HCY— homocysteine; HHcy— Hyperhomocysteinemia; NMDA— N-methyl-D-aspartate receptor; NMJ— neuromuscular junction

Keywords — ACh, glutamate, Homocysteine, *Drosophila melanogaster*, invertebrate, pharmacology, *Procambarus clarkii*, receptor

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Introduction

Homocysteine (HCY) is an amino acid intermediate produced in the central nervous system (CNS) in the metabolism of methionine to cysteine. The actions of HCY in mammals and the associated pathological state of high plasma HCY levels, known as hyperhomocysteinemia (HHcy), in part, revolve around the actions of HCY on glutamatergic receptors (Lipton et al., 1997; Poddar and Paul, 2013; Abushik et al., 2014; Bukharaeva et al., 2015). Glutamate is an excitatory neurotransmitter that acts at glutamatergic receptors, particularly N-methyl-D-aspartic acid (NMDA) receptors. HCY is

produced in the CNS and can act as an excitatory transmitter activating both NMDA and non-NMDA glutamate receptors in mammalian models. In this study we examined the potential action of HCY on the non-NMDA glutamate receptors. In particular, we tested the action of HCY on the quisqualate receptor subtype which is of a non-NMDA subtype Even at the acetylcholine (Ach) transmitting neuromuscular junctions (NMJs) of mammals, it has been established that there are NMDA receptor subunits which are affected by HCY (Bukharaeva et al., 2015).

Glutamate receptors are defined as NMDA (N-methyl-D-aspartic acid) and non-NMDA subtypes (i.e., AMPA, kainate, δ and quisqualate) based on their pharmacological sensitivity (Lee et al., 2009). The importance of the receptor subtypes is in understanding the action of the cells expressing them. NMDA receptors are ionotropic and allow an influx of Ca^{2+} ions when open, which can lead to a number of cellular responses and even cell death (Verdoorn et al., 1991). Kainate receptors in mammals are of a metabotropic subtype and work through second messenger cascades. They are even known to be present on presynaptic nerve terminals to reduce evoked transmitter release via autoreceptors on the presynaptic terminals (Park et al., 2006). NMDA and kainate subtypes produce a lower percentage of desensitization than the quisqualate receptor subtype (Thio et al., 1991). The compounds kainate, NMDA and AMPA do not activate the glutamate ligand gated ion channels at the larval *Drosophila melanogaster (D. melanogaster)* NMJ but quisqualate does (Bhatt and Cooper, 2005; Dudel et al., 1992) (

The action of HCY at the mammalian NMJs can be blocked by application of the glutamate competitive antagonist AP-5 (Bukharaeva et al., 2015). Additionally, there is a strong correlation with HHcy in mammals with high plasma and cerebrospinal fluid levels of HCY to the motor neuronal disease, amyotrophic lateral sclerosis (ALS) (Bukharaeva et al., 2015; Veeranki and Tyagi, 2013). Over-activation of glutamatergic drive on motor neurons has been shown to facilitate the disease state of ALS through subsequent degradation of the motor neurons (Vucic and Kiernan, 2009). Riluzole, a glutamatergic antagonist within the central nervous system (CNS), works as an effective treatment in ALS patients through reduction of glutamatergic activation on motor neurons (Vucic and Kiernan, 2009; Miller et al., 2012; Doble, 1996). As highlighted by Yohay et al., (2014), Riluzole also doubles as an effective treatment in rodent models of CNS injury as well as neurodegenerative states such as Parkinson's disease. Thus, HCY has the potential to maybe act similarly to Riluzole for potential treatments.

HCY has also been shown to restore sensitization of glutamatergic receptors in rodent neurons, which may account for the larger ionic currents of the glutamate receptors (Bolton et al., 2013). It has been demonstrated that an enzyme deficiency in rodents of methylenetetrahydrofolate reductase leads to an increase in neuronal damage by HCY and results in an increase in γ-aminobutyric acid (GABA) within the CNS which is assumed to compensate for the higher HCY levels (Jadavji et al., 2015). Also, HCY has an antagonistic action on GABA receptors (Tyagi et al., 2005). The actions on GABA receptors could, in part, account for the effect of HCY and the resulting pathologies in mammals exhibiting HHcy (Tyagi et al., 2005). GABA is an inhibitory receptor subtype, conducting actions via G-proteins to limit Ca^{2+} within the cell during synaptic transmission (Olsen and DeLorey, 1999). Antagonistic actions of HCY on GABA receptors could result in prevention of inhibition of glutamatergic receptor action. Therefore, the result of such an interaction could be the production a summative excitatory response that would contribute to neuronal death and subsequent blood brain barrier degradation experienced in the presence of excess HCY.

How the precise mechanisms of action of HCY on various neurotransmitter receptors and synaptic transmission may be altered are still

being investigated. It is established that HCY can act on both NMDA and non-NMDA receptors in rodent models (Yuzaki and Connor, 1999). The glutamatergic NMJs of larval *D. melanogaster* and *Procambarus clarkii* (*P. clarkii)* are of a non-NMDA subtype. Given that HCY is known to be synthesized in *D. melanogaster* (Caggese et al., 1997), it is likely it is present in other arthropods such as the crayfish *P. clarkii*. The investigations to date are primarily focused on mammalian CNS models; however, some aspects of precise quantal measures of synaptic transmission are more challenging in mammalian models compared to those at invertebrate NMJs. This difference is due to the ability to record at synaptic sites in invertebrate models. Furthermore, the potential effects of HCY on synaptic facilitation and synaptic depression have not been thoroughly investigated in mammalian models.

However, such information could provide clinical implications regarding diseases that have been shown to exhibit relationships with HCY pathology, such as Parkinson's disease and ALS. Thus, the purpose of this experiment is to gain insight into the mechanisms of action of HCY at the NMJs of model organisms. These experiments were designed to test the prediction that application of HCY to model systems would produce an initial overstimulation of glutamatergic receptors followed by quick desensitization.

P. clarkii NMJs have served as a classic model in glutamatergic synaptic facilitation and depression (Richet, 1879; Katz, 1949; Sherman and Atwood, 1971; see recent reviews Cooper and Cooper, 2009; Titlow and Cooper, 2018). *D. melanogaster* NMJs have also been utilized for the same purpose due to their utility as a genetic model for mutational defects in synaptic transmission afflicting humans (Jan and Jan 1976a,b; Usherwood, 1977; DiAntonio, 2006; see recent review Titlow and Cooper, 2018). In addition, the *P. clarkii* NMJs have both glutamatergic and GABA-ergic innervation profiles, (Bazemore et al., 1956; Van Harreveld and Mendelson, 1959; Dudel and Kuffler, 1961a; Florey, 1961) which are used for assays of pharmacological agents (Van Harreveld 1959,1980; Shinozaki and Ishida, 1981a,b,c; Shinozaki et al., 1982; Bhatt and Cooper, 2015; Lee et al., 2009). Alterations in both quantal amplitudes and area of quantal shapes are indicative of action on fundamental synaptic properties by neurotransmitters. These are readily measured at *P. clarkii* and larval *D. melanogaster* NMJs (Cooper et al., 1995).

Both preparations produced graded evoked responses which are related to the synaptic efficacy of multiple quantal fusion events and spontaneous single quantal events. These are readily obtained with intracellular and extracellular recordings on identifiable muscle fibers (Dudel and Kuffler, 1961b; Pawlu et al., 2004). Thus, these two model synaptic preparations were utilized in this study to further assess the effects of HYC on synaptic transmission. Any changes in the amplitude of evoked excitatory postsynaptic potentials (EPSPs) were investigated for HYC effects on glutamate receptors at these two model NMJs. To investigate any potential presynaptic contributions by HYC and the influence of Ca^{2+} on facilitation and depression, varied concentrations of Ca^{2+} in bathing saline were used with exposure to HYC. This study was designed and executed with the intention of progressing understanding of the mechanistic actions of HCY on glutamatergic non-NMDA receptors. Such information could be utilized to provide improved treatment of HHcy pathologies and symptoms, including difficulty forming new memories, therefore providing a deeper understanding of neuropathologies such as Alzheimer's disease and Parkinson's disease.

Material and Methods

Animals

Both *P. clarkii* and *D. melanogaster* glutamatergic were utilized. These synapses are commonly used to assay pharmacological agents.

The graded EPSPs of the *P. clarkii* opener NMJ are easier to measure reliably when the responses are facilitated to obtain an amplitude of the synaptic responses above the noise level. Thus, ten stimuli delivered at 40 Hz produces a reasonable EPSP response for the 10th response to measures (Figure 1A). The changes over time were measured by change in response of the 10th EPSP during exposure to HCY (100 µM). A representative preparation in which the EPSPs were monitored over time is illustrated (Figure 1B).

Experiments were performed using *P. clarkii*, measuring six– eight cm in body length (obtained from Atchafalaya Biological Supply Co., Raceland, LA, USA). Six *P. clarkia* were individually stored in an aquatic facility at room temperature (20-21°C) and were fed commercial fish food pellets (Aquadine) for at least two weeks prior to experimentation.

The general maintenance in culturing *Drosophila* as well as staging of instars and the developmental cycle are described in Campos-Ortega and Hartenstein (1985). All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium as described in Cooper and Cooper (2004). Wild type Canton S (CS) flies were used for analyses via the semi-intact method. This method involves cutting along the dorsal midline to expose the body wall muscles and the nervous system (Stewart et al., 1994). This *Drosophila* strain has been isogenic in the lab for several years and was originally obtained from Bloomington Fly Stock. To obtain 3rd instar staged larvae, the larvae were held at 25 °C in a 12 h light/dark incubator before being selected.

Electrophysiological recordings of NMJs in P. clarkii

In brief, the excitatory neuron is isolated from the inhibitor neuron and stimulated in the meropodite segment. The most distal fibers of the opener were always used for recording synaptic responses (Figure 2A). The stimulation paradigm consisted of providing a train (series of electrical pulses) of 20 pulses at 40 Hz with ten seconds between trains. An average of 20 trains in each of the conditions was used for measures of EPSPs. Preparations were not stimulated during control periods to obtain spontaneous quantal events.

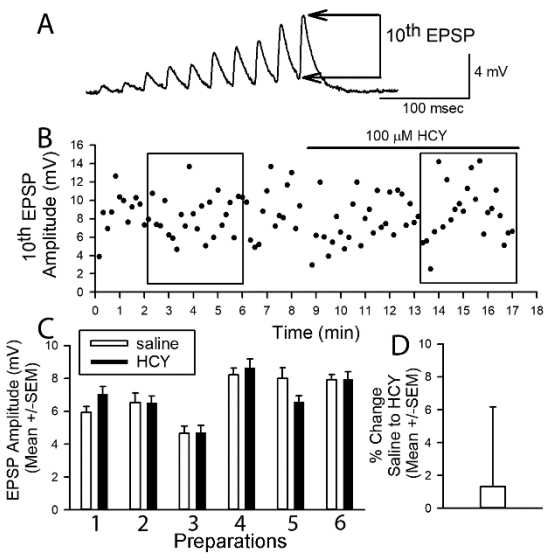

Figure 1: The effects of homocysteine (HCY) on the glutamatergic synaptic responses at the *P. clarkii* neuromuscular junction. (A) The amplitude of tenth EPSP produced by 40Hz stimulation is used as an index of synaptic transmission. (B) A representative response from a preparation in which the tenth EPSP is monitored before and during the exposure to HCY (100 µM). The box outlines indicate the time period used to obtain a mean amplitude of the EPSPs to be used to calculate percent differences in amplitude due to exposure to HCY. (C) The mean amplitude of the EPSPs before and during exposure to HCY for each of the six preparations. (D) The mean percent difference in the six preparations obtained before and during exposure to HCY. No significant effect in EPSP amplitude was noted due to exposure to HCY.

Dissected preparations were maintained in saline; a modified van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 $CaCl_2 \cdot 2H_2O$, 2.45 $MgCl_2 \cdot 6H_2O$, and 5 HEPES adjusted to pH 7.4). All the experiments were performed at room temperature. Intracellular recordings were made with a sharp glass electrode filled with 3.0 M KCl, and both recorded and analyzed via Scope and LabChart software (AD Instruments). The

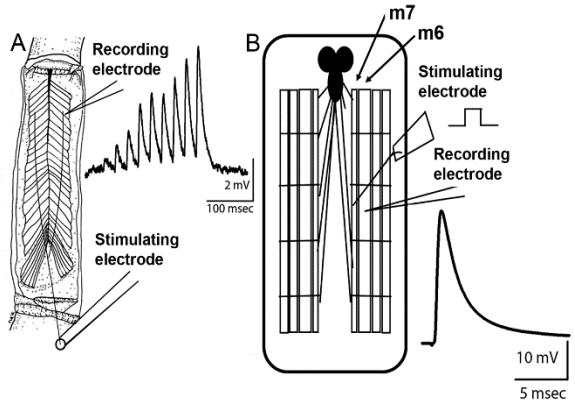

Figure 2: The *P. clarkii* and larval *D. melanogaster* preparations. (A) The most distal muscle fibers of the *P. clarkii* opener muscle in the first walking leg provides a common target among preparations. The opener muscle does show regional differences in amplitudes of EPSPs. The excitatory motor neuron is selectively stimulated in a more proximal segment of the leg while monitoring the EPSPs produced. (B) The body wall muscle named m6 of the third or fourth segments in the dissected early third instar larvae are used to measure the evoked EPSP. Body wall muscle m6 and m7 are shown in each segment. The segmental nerve to the segment of interest is stimulated with a suction electrode which contains the segmental nerve.

amplitudes of the EPSPs spontaneous quantal events (mEPSPs) elicited by the motor nerve were recorded. Intracellular responses were recorded with a 1 x LU head stage and an Axoclamp 2A amplifier (Molecular Devices, Sunnyvale, CA, USA). HCY was made fresh in the physiological saline the day of experimentation. All chemicals were obtained from SIGMA-Aldrich (St. Louis, MO, USA).

Electrophysiological recordings of NMJs in larval Drosophila

The synaptic responses at the larval *D. melanogaster* NMJs were recorded by standard procedures (Lee et al., 2009) with stimulation at 0.5 Hz (Figure 2B). The modified HL3 saline was used for physiological measures (Stewart et al., 1994) at a pH of 7.1 (de Castro et al., 2014). Saline solution (in mM): 1.0 $CaCl_2 \cdot 2H_2O$, 70 NaCl, 20 $MgCl_2$, 5 KCl, 10$NaHCO_3$, 5 trehalose, 115 sucrose, 25 5N,N-bis(2-hydoxyethyl)-2-aminoethanesulfonic acid (BES)) was utilized. HCY (100 µM) was made fresh in the physiological saline the day of experimentation. All the experiments were performed at room temperatures (20–21◦C). EPSPs were measured by intracellular recordings with a sharp glass electrode (3 M KCl) and AxoClamp-2 B amplifier (Molecular Devices, Sunnyvale, CA, USA). Stimulations were made with a Grass S88 dual stimulator (Natus Neurology Incorporated, Middleton, WI, USA). Preparations were used immediately after dissection. Electrical signals were recorded online to a computer via a PowerLab/4s interface (AD Instruments, Colorado Springs, CO, USA). Seven larval *D. melanogaster* preparations were dissected as previously described (Li et al., 2001) for early third instars. Each *D. melanogaster* preparation was used to measure EPSPs in the same muscle fiber in both saline and in the presence of HCY, therefore giving a baseline control for that individual preparation. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All measures were made in muscle six of segments three or four.

Statistical analysis

All data are expressed as an average value along with the standard error of the mean (i.e., $\pm$ SEM). The rank sum pairwise test or a sign test was used to compare the differences in responses before and after exchanging a solution with saline containing the compounds. This analysis was performed with Sigma Stat software. P of $\leq$ 0.05 is considered to be statistically significant.

Results

To examine for an effect due to exposure to HCY, the mean amplitude of the tenth EPSP from an average of at least 20 responses was compared during saline exposure and during HCY exposure (Figure 1C). The percent difference in each of the six preparations before and during exposure to HCY revealed no

significant trend (P > 0.05, sign test) to an effect on the EPSP amplitude at the *P. clarkii* NMJ (Figure 2D). The mean percent difference was 1.33 (4.8+/- SEM).

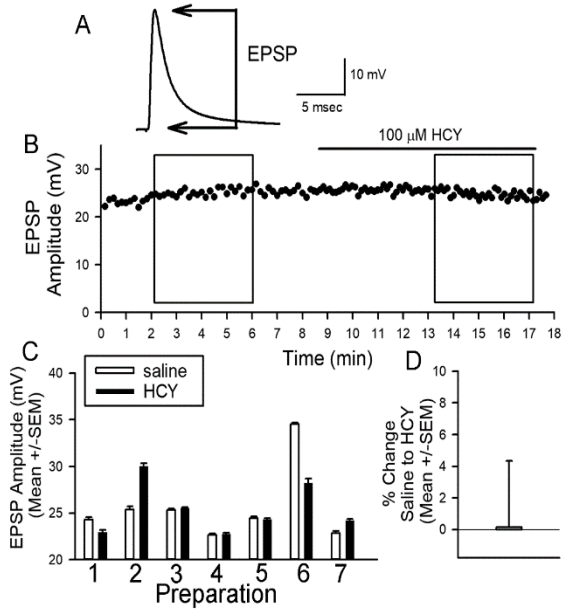

Figure 3: The effects of homocysteine (HCY) on the glutamatergic synaptic responses at the *larval D. melanogaster* neuromuscular junction. (A) The amplitude of the EPSP used as an index of synaptic transmission. (B) A representative response from a preparation in which the EPSP is monitored before and during the exposure to HCY (100 μM). The box outlines indicate the time period used to obtain a mean amplitude of the EPSPs to be used to calculate a % differences in amplitudes due to exposure to HCY. (C) The mean amplitude of the EPSPs before after during exposure to HCY for each of the seven preparations. (D) The mean percent difference in the seven preparations with before and during exposure of HCY. No significant effect is noted due to exposure of HCY.

The graded evoked response at the larval *D. melanogaster* NMJ on muscle 6 produced a large enough EPSP that facilitation of the synaptic response was not necessary as for the *P. clarkii* opener NMJs (Figure 3A). The amplitude of the EPSPs over time for a representative preparation illustrated that no large changes occurred upon exposure to HCY (100 μM) (Figure 3B). The blocked regions in the trace indicated the region of time used to calculate the mean EPSP amplitude used for quantification in determining a percent change due to HCY exposure. The mean amplitudes for each of the seven preparations before and during exposure to HCY is shown (Figure 3C). The percent difference in the seven preparations before and during exposure to HCY revealed no significant trend (P > 0.05, sign test) in an effect on the EPSP amplitude at the larval *D. melanogaster* NMJ (Figure 3D). The mean percent difference was 0.13 (4.2+/- SEM).

Discussion

In this study, we demonstrated that HCY at 100 μM did not have any effect on synaptic transmission at the NMJs of the *P. clarkii* and larval *D. melanogaster*. This differed from our hypothesis that application of HCY to glutamatergic synapses in *D. melanogaster* and *P. clarkii* would be inhibited by a high HCY concentration via competition for the release of glutamate at the NMJ. Despite these glutamatergic synaptic preparations having non-NMDA receptor subtypes postsynaptically, and that it was established that HCY could influence non-NMDA receptors in mammalian CNS preparations (Yuzaki and Connor, 1999), no detectable differences in the amplitude of the evoked EPSPs could be ascertained. It is likely the extracellular domain of the quisqualate subtype glutamate receptors of the *P. clarkii* and larval *D. melanogaster* NMJs is significantly divergent such that they are unreceptive to HCY (Lee et al., 2009). It is known that the GluN2A subunit of the NMDA receptors is key to HCY having an effect (Sibarov et al., 2016). Potentially, the extracellular domains of the different receptor make up could be compared with bioinformatics to estimate the 3-dimensional configuration required for HCY sensitivity. It is known that exposure to the *P. clarkii* or larval *D. melanogaster* NMJs to 100 μM of glutamate that depolarization will occur followed by desensitization and a reduce amplitude in the evoked EPSP (Lee et al., 2009; Titlow and Cooper, 2018). So, if HCY was to act as an

agonist we would have expect some alteration in response at a similar concentration.

The lack of an acute or longer term effect by HCY at such high concentration is most likely explained that the glutamate receptors on both preparations is not sensitive to HCY. If one was interested in knowing the precise structural elements required for HCY to have an action, various glutamate receptors subunits, with sequence alterations, could be expressed in a cell line or frog oocytes and examined for responses to HCY.

In mammals, HCY acts on the CNS to alter various functions such as sympathetic activity, and may even be related to inducing cardiovascular pathologies (Zhong et al., 2017). The physiological profiling of the glutamatergic receptors within the CNS or the equivalent ventral nerve cord of larval *D. melanogaster* and *P. clarkii* has not been performed. Perhaps there may be actions of HCY on the central glutamatergic synapses in these invertebrate preparations as for mammals. Further studies will need to be conducted to determine if these invertebrate models have similar central responses to HCY as mammals.

Since *D. melanogaster* are known synthesize HCY, (Caggese et al., 1997) it is probable other insects and crustaceans synthesize it as well due to physiological and biochemical similarities between species of arthropods. The crustaceans are similar to insects in many ways related to neural function. At this point, investigations have yet to be conducted to determine if HCY is present in the CNS of crustaceans. However, one of the proposed long-range treatments by health food supplement aids for HHcy in humans is to have a diet high in marine crustaceans (Tou et al., 2007; Naushad et al., 2017) as it has been shown that a diet of Arctic krill for rodents reduces HCY levels in the blood (Bjørndal et al., 2015). We could not find any scientific studies using humans to directly show that a diet of marine crustaceans was beneficial for human treatment of HHcy pathologies. The mechanisms in the rodents for reducing HCY is most likely due to the n-3 polyunsaturated fatty acids in the muscle and vitamins B12, B6, and folate in krill (Bjørndal et al., 2015; Krajcovicová-Kudlácková and Blazícek, 2002).

Future studies would be of interest to determine any potential central effects of HCY in invertebrates, particularly *D. melanogaster*, as genetic models of degradation of HCY could potentially be utilized to look at long term consequences on neural development with raised HCY as it relates to consequences in mammals. As a comparative investigation, it would be of interest to know if crustaceans also produce HCY in the CNS. This could be determined through analysis of HCY levels in varied areas of interest within the CNS, such as the hippocampus and blood-brain barrier both at baseline neural stimulation and in differing states of stimulation. Also of interest would be to determine if an altered diet, such as with vitamin B complexes, would lower HCY in the CNS of *D. melanogaster*, thus furthering implicating a relationship in diet, HCY levels, and possible resultant neuroprotective or neurodegenerative responses. The understandings that would be provided by these further elaborations of this experiment could translate to development of treatments of aforementioned diseases such as Parkinson's disease and ALS at a much later point.

The results of this experiment lead to several possible implications regarding the clinical relevance of using *P. clarkii* and *D. melanogaster* as models for HHcy and diseases with similar pathologies. However, utility of these preps remains useful for establishing a basic understanding of HCY action at glutamatergic receptors, but subsequent experiments attempting to draw conclusions regarding clinical application would require a model with a subtype of glutamatergic receptors that is more similar to those found in mammals.

Endnotes

Many of the authors were students in a neurophysiology lab based class who addressed authentic scientific based questions in regard to the topic of examining pharmacological agents known in other models to block stretch activated ion channels. This course project is part of a trend in teaching science to undergraduates (Linn et al., 2015). Course-based undergraduate research experiences (CUREs) are an approach being adopted by science educators in high schools and colleges (Bakshi et al., 2016). Our hope is to present a new acronym for authentic course-based undergraduate research experience (ACURE).

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Corresponding Author

Dr. Robin L. Cooper
Dept. of Biology, 675 Rose Street.
University of Kentucky, Lexington, KY 40506
Phone: 859-559-7600; Fax: 859-257-1717
Email: RLCOOP1@uky.edu

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