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The effect of silver nanoparticles on synaptic responses in the lateral giant escape circuit of the crayfish

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THE EFFECT OF SILVER NANOPARTICLES ON SYNAPTIC RESPONSES IN THE LATERAL GIANT ESCAPE CIRCUIT OF THE CRAYFISH

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ABSTRACT

The lateral giant neuron in the crayfish is a neuron that controls the reflexive escape response; the context and experience-dependent tuning of this response is critical for survival. Serotonin modulates synaptic responses in this neuron, and social experiences (stress) changes the modulatory role of serotonin. I investigated the mechanisms of induced synaptic response changes and serotonergic modulatory changes after silver nanoparticle exposure to ask if 1) contaminant induced stress changes serotonergic modulation in a manner consistent with social stress and 2) shed light on potential neurotoxic effects of widely used, but poorly understood nanomaterials. Our data show that stress induced by exposure to silver nanoparticles changes excitability of this neuron. Integrating these data concerning environmental stress with prior knowledge of behavioral and electrophysiological correlates of social stress we can ask if changes induced in synaptic responses and modulation induced by stressors of different kinds are generalizable.

CHAPTER 1

GENERAL INTRODUCTION

Marshall University in Huntington, WV, is located next to the Ohio River, which is an important transportation avenue as well as a source of drinking water for many in the Ohio Valley region. According to a toxic release inventory analysis conducted by the Environmental Protection Agency (EPA) Ohio River Valley Water Sanitation Commission, the Ohio River was ranked as the number one river in the country for receiving chemicals from 2001 to the time of the report (2013) (Ohio River Valley Water Sanitation Commission, 2013). With a number of industrial plants alongside the river and minimal governmental involvement with waste disposal regulations, the assumption can be made that many waste products are deposited into the Ohio River with no regulation and no detection methods after disposal, including waste from the fast-growing nanomaterial industry. Chemical discharge into the Ohio River is occurring at alarming rates and with the progressively greater use of nanoparticles, through applications ranging from clothing to wound dressings and technology, and no oversight for waste disposal, aquatic organisms in the Ohio River system are inevitably being impacted by these particles (Ray et al., 2009). Because the Ohio River is likely being polluted with nanoparticles, it is vital that their potential for harm is investigated as early as possible to control further nanoparticle disposal into this important drinking water source.

Despite the abundant use of nanoparticles, research investigating their potential for neurotoxic damage is still needed. Studies have shown that nanoparticles can have detrimental effects in numerous organisms including mitochondrial damage and genotoxicity in mammalian cells (Asharani et al., 2009). Though nanoparticle use is increasing, the neurological effects of nanoparticle exposure are still poorly understood. The abundant use, potential for human and/or

environmental harm, and lack of published health assessments on nanomaterials all present a need for investigation into the potential toxicity of the materials which prompted the EPA to classify nanomaterials as an "Emerging Contaminant" in 2014 (United States Environmental Protection Agency, 2014). Silver nanoparticles (AgNP) are very commonly used for their antibacterial properties and are applied to a variety of products ranging from medical tools to home goods and athletic clothing. However, with this increased use of AgNP, waste from nanoparticle use is greater than ever.

One very sensitive measurement of the health of an ecological system is the neurological health of the organisms living in the water. Because reflexes are a measure of an organism's health (Walker, 1990), a change in reflexive behaviors would be indicative of the pollutants manipulating the organisms' nervous systems. A change in an organism's nervous system will inevitably cause changes in the organism's behavior. These changes made as a result of exposure to pollutants can be informative when assessing the health of an environment. This study attempted to assess the neurotoxic effects of AgNP exposure on the crayfish reflex circuit, a behavioral response where modification would be bioindicative, and that is crucial for survival in the wild.

Using AgNP as an ecologically relevant environmental stressor, this study aimed to address the generalizability of the stress response in the crayfish escape reflex. This reflex circuit is commonly used to assess social stress paradigms and crayfish are often used as a bioindicator species for the health of the ecological system (Schilderman et al., 1999). Because crayfish are a bioindicator species, addressing the generalizability of their stress response could provide researchers with a model system for assessing stress on the nervous system incurred as a result of environmental stress. A lack of a generalized stress response to environmental stressors (like

AgNP exposure) could be indicative of an erratic and disorganized response further suggesting that the stressor may be unfamiliar to the crayfish.

Silver Nanoparticles in the Environment

Because the particle integrity and aggregation of AgNP is largely dependent upon the pH of the media - which is itself dictated by the presence of salts in the water - an ecologically relevant concentration is difficult to estimate (Gagne et al., 2013). Additionally, the electrostatic strength of the solution can affect AgNP aggregation with low ionic strength repelling aggregates and high ionic strength banishing the repulsion energy barrier (Bae et al., 2013). AgNP integrity has also been linked to the presence of ligands and organic materials present in the water as well as the capping agent utilized to prevent aggregation (El Badawy et al., 2010). Additionally, some nanoparticles, including AgNP, have been shown to reform after dissolution, resulting again in a high surface-area to volume ratio and further contributing to complications involving their detection in the environment (Jones et al., 2011). El Badawy *et al.* (2010) established that of the most commonly used capping agents, PVP-coated AgNP were most likely to maintain their zeta potential, or dispersion, in changing water chemistry, giving them the greatest potential for mobility. However, Doolette *et al*. (2013) established that when PVP-coated AgNP reached waste water treatment plants, not only would their surface coating likely be compromised, affecting their size, but also were transformed to ionic silver by sludge treatment. This transformation does not hinder the filtration system, but would likely have a negative impact on aquatic microbial communities, as already established (Mitrano et al., 2011). Because the environmental fate and agglomeration/dissolution tendencies are still relatively unknown,

determining the concentration of AgNP in a given water system is very difficult (Gottschalk et al., 2013).

Researchers with interest in the potential effects of nanoparticles in the environment have sampled their local watersheds and have found a wide range of concentrations. Mitrano, *et al.* (2011) found AgNPs in wastewater effluents at concentrations of 100 ng/L [0.0001 ppm] and a concentration of dissolved Ag^+ (a breakdown product of AgNP) of 60 ng/L [0.00006 ppm] in Black Hawk and Boulder, Colorado. Another group of researchers tested several Colorado waters and effluents upstream and downstream of several industrial plants and found a range of .3-1800 ng/L [0.003-18 ppm] of AgNP (Wen et al., 2002). This range is an indication that the presence of AgNP in water can vary tremendously, therefore making it difficult to pinpoint a specific ecologically relevant concentration. Whiteley *et al.* (2013) highlighted the challenges in AgNP risk assessment in an attempt to estimate the quantity of AgNP released per year. Given the varied surface reactions of AgNP to media components and consequent variable nanoparticle stability, ecologically relevant levels of AgNP are not available at this time, though with the growing interest in their anti-bacterial properties, AgNP have been proposed as an alternative water filtration system in developing countries (Mpenyana-Monyatsi et al., 2012). However, as this study showed, most of the AgNP applied to the filter materials had degraded into Ag^+ after 30 min. of filtration. Thus, the filter deposited Ag^+ into the drinking water at a concentration higher than that which is recommended by the World Health Organization at the cost of removing the bacteria. So, while the application of AgNP to filtration systems may not be in use now, many are seeking their feasibility in the near future, inevitably leading to more AgNP being introduced to water systems.

Asharani *et al.* (2008) studied oxidative stress in zebrafish after exposure to AgNP concentrations ranging 1-100 ppm. In order to maintain some constancy and comparability, other researchers have used this range of concentrations as a wide "blanket" concentration range until further ecological information is available (Struzynski et al., 2013). For the purposes of this study, we used concentrations 1ppm, 5 ppm, 10 ppm, and 50 ppm, which are standard in the AgNP research community. This wide range of concentrations should be applicable for inferring the potential toxicity of concentrations found in a variety of ecological systems as well as assessing the potential consequences of a large influx of AgNP, such as would be seen in an accidental spill. In additional support for the use of this concentration range, Mirzajani, *et al.* (2014) found that 1 ppm and 5 ppm were the minimal concentrations necessary to induce inhibition and death in *Bacillus thuringiensis,* a likely target organism; thus, for AgNP to be effective in their intended purpose, bacteria would need to be exposed to concentrations in the range of those used in this study.

Toxicity of Silver Nanoparticles

AgNP have been so widely utilized because of their anti-bacterial properties (Tian et al., 2007, Yoon et al., 2007). AgNP are applied to numerous medical products to prevent wound infection and, in turn, aid in proper healing. In addition to these medical uses, AgNP are also applied to a variety of consumer products boasting antibacterial properties, like cosmetics and clothing. With no regulation for application or waste disposal in place, the influx of AgNP into the waterways is greater than ever. Benn *et al.* (2010) showed that through average use of AgNP treated products, a consumer could release up to 470 μg Ag into the sewage system each day. This estimation includes only the release of Ag from consumer products (athletic T-shirt,

toothpaste, detergent, and shampoo), excluding the release from AgNP coated medical products. Though their antibacterial properties are the primary cause for their use, Bondarenko *et al.* (2013) wrote in a comprehensive review of AgNP toxicity that the inhibitory effects of AgNP on bacteria, the target organism, are less than that of the inhibitory effects on non-target organisms, like crustaceans and other aquatic species. So, for their application to be effective for the purposes advertised, AgNP would need to be applied to consumer products in concentrations that have shown to be harmful to non-target organisms. The use of these nanoparticles for consumer anti-microbial purposes at concentrations that are harmful to the aquatic food-web is cause for concern and raises the need to evaluate regulatory application and disposal procedures.

Considerable research has been dedicated to determining the causes of AgNP toxicity. From this research a number of studies have shown that AgNP break-down, resulting in ionic silver (Ag^+), is responsible for at least some of their toxic effects (Blinova et al., 2013, Newton et al., 2013). However, several other studies have shown that AgNP toxicity cannot be fully explained by liberated Ag^+, but the nanoparticle itself causes cellular damage independent of the Ag^+ content present (Carlson et al., 2008, Griffitt et al., 2008, Laban et al., 2010). In fact, it is likely that AgNP are toxic through a different mechanism than that of Ag^+. AgNP mode of toxicity is most commonly attributed to cell membrane damage (Shahverdi et al., 2007) where Ag^+ toxicity is likely a result of its interaction with protein thiol groups, rendering protein, including enzymes, inactive (Matsumura et al., 2003). Thus, enveloping the nanoparticles in coatings designed to prevent agglomeration and break-down, as is commonly done, may not mitigate their harmful effects. Ahamed *et al.* (2008) showed that polysaccharide-coated AgNP, when compared to non-coated AgNP, had more of an effect on the viability of mouse embryonic stem cells. This difference in effects suggests that the surface chemistry of the nanoparticle can

have different effects depending on the coating applied to the material. Lu *et al.* (2010) showed that citrate-coated AgNP were toxic after being exposed to sunlight while polyvinylpyrrolidone (PVP) -coated AgNP showed no cytotoxicity after three-week exposure to sunlight. From these findings, they suggest that PVP-coated AgNP should be used in day-to-day application. This suggestion, along with other promptings, has lead to PVP-coated AgNP being among the most popular AgNP for use in consumer products. However, other studies have shown that PVP-coated AgNP are also toxic and their common use should be reconsidered (Foldbjerg et al., 2009). In fact, Bondarenko *et al.* (2013) showed that overall, in existing literature, uncoated AgNP were less inhibitory to bacteria than PVP-coated AgNP. Additionally, PVP-coated AgNP aggregate more quickly, so they are more likely to fall into the sediment, therefore increasing the risk of ingestion by benthic species (Kwok et al., 2012). Due to the conflict in literature regarding the toxicity of coated vs. uncoated AgNP, as well as the abundant use of PVP-coated AgNP, this study will attempt to address the toxicity of PVP-coated AgNP.

Mechanisms of Toxicity

Though the modes of AgNP toxicity are still largely unknown, plenty of research has shown that AgNP are, indeed, toxic to many organisms. Heat shock protein 70 is a sensitive bioindicator of organisms undergoing stress (Ait-Aissa et al., 2000). Ahmed *et al.* (2010) identified that AgNP induce the up-regulation of heat shock protein 70, indicating a toxic effect of 50 and 100 ppm concentrations of AgNPs on *Drosophila melanogaster* larvae. Ahmed *et al.* (2010) discovered an increase in caspase-3 and caspase-9, proteases involved in the apoptosis pathway, showing that *Drosophila melanogaster* larvae began undergoing apoptosis after 24 hr. exposure to 50 and 100 ppm AgNP. Apoptosis in zebrafish embryos has also been identified

after exposure to 50 ppm AgNPs in the presence of common nanoparticle stabilizers (Asharani et al., 2008). Few studies have assessed the potential neurotransmitter disruption as a result of sub-lethal AgNP exposure. Of the existing available studies, Hadrup and Lam (2014) showed that oral dosage of 2.25 mg/kg body weight/day in rats induced changes in noradrenaline, dopamine, and serotonin concentrations in the brain. Zhaowei *et al.* (2009) found significant impairments to voltage-gated sodium channels in the rat hippocampal CA1 neurons after exposure to 5×10^{-5} g/ml [0.05 ppm]. These channels are involved in cellular processes such as action potential generation and propagation, so disruption of these channels could cause a disruption of cellular function.

Because of their characteristically high surface area to volume ratio (Christian et al., 2008) nanoparticles have the potential for surface-level reactions that could be detrimental to an organism, like their target organism bacteria. Silver is traditionally thought to be anti-bacterial because of its interference with –SH groups on enzymes, a crucial component for the integrity of protein structure (Jeon et al., 2003). AgNP are thought to be toxic to bacteria through their interaction with the bacterial membrane, creating perforations that increase leak and disrupt ionic balance, eventually resulting in cell death (Sondi and Salopek-Sondi, 2004). In support of this mode of toxicity for bacteria, Yoon *et al*. (2007) tested the resistance of *Eschericia coli* and *Bacillus subtilis* to AgNP exposure and showed that *E. coli* were more resistant to AgNP toxicity than *B. subtilis*. *E coli* have an outer membrane that is constructed mostly by tightly packed lipopolysaccharide molecules, creating a more resistant barrier to the nanoparticles. A number of proteins associated with cell wall management and oxidative stress mitigation have been identified and targeted in studies attempting to understand the causes for AgNP toxicity. Alanine dehydrogenase, for instance, is involved with peptidoglycan cell well synthesis and its

expression increased under AgNP exposure, suggesting that AgNP influence the peptidoglycan cell wall layer in bacteria (Mirzajani et al., 2014). This effect is thought to be a result of the attraction caused between the positively charged Ag and negatively charged bacterial membrane (Mpenyana-Monyatsi et al., 2012).

While the exact molecular mechanism behind nanoparticle toxicity to non-target organisms is still largely unknown, some findings have pointed to the possible causes for toxicity. Because AgNP are non-specific, it is likely that their route of toxicity to non-target organisms is similar to that of bacteria, thus their toxicity has been linked to significant damage to cell membrane integrity (Foldbjerg et al., 2009, Ahmed et al., 2010, Haberl et al., 2013). This compromise in the cell membrane would disrupt ionic balances crucial for cellular function and could cause the cell to be vulnerable to AgNP infiltration, leading to intracellular damage. Several studies have shown that nanoparticles also cause the generation of reactive oxygen species (ROS) which lead to oxidative stress (Hsin et al., 2008). In support of this method of toxicity, AgNP have been shown to cause up-regulation of proteins involved in neutralizing oxidative states (Mirzajani et al., 2014). This up-regulation, however, is accompanied with a down-regulation in proteins involved in cellular proliferation, suggesting that the cell is consumed with the need for survival and has compromised growth in order to do so. Because ROS are highly reactive, they can lead to significant cell damage via DNA damage, lipid peroxidation, and other signaling disruptions. Oxidative stress can cause a cascade of reactions including, at its mildest, transcription of antioxidant enzymes. With increase in ROS an increase in cellular response occurs including pro inflammatory responses, and at a maximum ROS level, compromised mitochondrial membrane and electron transport chain disruption, which can result in cell death (Manke et al., 2013). Oxidative stress was induced in *Drosophila melanogaster*

after exposure to 50 and 100 ppm AgNP for one and two day exposure periods (Ahmed et al., 2010). Genotoxic damage has been observed in fish heart and gill cell lines after exposure to 8, 16, 32, 64 ppm AgNP and is the presumed effect of ROS generation (Taju et al., 2014). Significant increases in ROS were found in THP-1 monocytes which correlated with DNA breakage and apoptosis and necrosis after exposure to 5 ppm AgNP for six hours. Hsin *et al.* (2008) showed that 5 and 50 ppm AgNP exposure caused intracellular ROS generation, leading to apoptosis in cultured mouse fibroblast and rat vascular smooth muscle cells. Concentrations 10 and 50 ppm AgNP induced oxidative stress leading to DNA damage in *C. Elegans* (Hunt et al., 2013); in A549 human alveolar epithelial cells, 50, 100, and 200 ppm AgNP exposure had toxic effects in the cells, likely via ROS-dependent toxicity in the cells and disruptioin of proliferation through cell-cycle phase arrest (Chairuangkitti et al., 2013). Oxidative stress has also been described in the crayfish hepatopancreas after exposure to 1, 5, 10, and 50 ppm AgNP (Struzynski et al., 2013). Kim *et al.* (2009) showed that the increase in oxidative stress after AgNP exposure is a direct effect of nanoparticle exposure independent of the toxicity induced by Ag^{+} released inside the cells.

A second proposed mechanism of toxicity is the cardiac dysfunction resulting in impeded blood flow. Asharani *et al*. (2008) quantified zebra fish embryo growth and development inhibition after AgNP exposure at concentrations 50 and 100 µg/ml. These researchers proposed that exposure to increasing concentration of AgNP impeded blood flow to the brain and spinal cord, resulting in abnormal growth. A potential cause for this cardiac irregularity is thought to be the interaction between AgNPs and cardiac muscle. AgNPs could directly or inadvertently block energy supplies to cardiac muscles (possibly through inducing oxidative stress), causing the heart muscles to weaken beyond ability to restore normal blood flow to developing tissues. Asharani *et*

al. (2008) found multiple morphological defects in zebrafish larvae after 50 and 100 ppm AgNP exposure. Another study attempted to assess the potential for organismal adaptation after pre-exposure to sub-lethal concentrations of AgNP. Results showed that pre-exposure of nematodes to lower concentrations of AgNP did not increase resistance to AgNP later, but rather increased stress by inducing deformations and/or immobility (Ellegaard-Jensen et al., 2012). AgNP also suppressed growth in *C. Elegans* larvae at concentrations 5, 10, 25, and 50 ppm (Hunt et al., 2013).

Cellular Uptake and Bioavailability of Silver Nanoparticles

Because AgNP and cellular structures are similar in size and because of the positively charged surface chemistry of AgNP, it is likely that the nanoparticles interact with cell membranes, creating pores and additionally compromising cell membranes. This interaction has been shown to lead to quick uptake of AgNP into the cell (Gaiser et al., 2009, Lu et al., 2010, Sirimuthu et al., 2010). In fact, this increase in permeability by pitting the cell membrane is thought by some to be the method through which AgNP are toxic to bacteria (He et al., 2012). Not only have AgNP shown their ability to penetrate cell membranes, but they have also shown biopersistance and biological barrier penetration. One study showed that Sprague-Dawley rats were able to clear ingested AgNP from most tissues, but were unable to clear the nanoparticles from the testes or brain, suggesting that while AgNP can cross biological barriers, exudating them back across biological barriers is not easily achieved (Lee et al., 2013). This biopersistence in the brain and testes indicates that while AgNP may be able to cross the blood-brain and blood-testes barriers, clearing the nanoparticles across those barriers presents further complications. Gagne *et al.* (2013) assessed AgNP bioavailability in freshwater mussels. They showed that

smaller nanoparticles (20nm) formed nano-sized aggregates, which were then ingested by mussels. They then showed that Ag^+ was liberated from AgNP by H_2O_2 interaction with the metallic AgNP surface in the tissues. This liberation of Ag^+ in tissues of the organism caused a series of toxic effects, including lipid peroxidation in the gills and digestive gland and a compromised immunocompetence. AgNP have been proposed as a targeted drug delivery system, carrying the drug to the target area across biological barriers, then releasing the active Ag^+ to induce toxicity in the target cells (Liu et al., 2010). However, given variable surface reactions and biopersistence of AgNP, it is important to consider the possible ramifications of this application.

Crayfish as a Model for Toxicity

Because aquatic animals will be healthy – and therefore will show healthy behaviors and physiological characteristics – if the animal lives in a well-suited environment, these characteristics and behaviors that change as a result of a changing environment can then be used to ascertain the health of that given home environment (Depledge and Galloway, 2005). Bioindicator species are species that readily present reflections of their environment. Ecological relevance and susceptibility to environmental stressors are two of the characteristics that define a bioindicator species (Kuklina et al., 2013). Crayfish are among the more commonly used bioindicator species because of their susceptibility to environmental stressors and their ecological relevance and widespread distribution. Additionally, crayfish are known to respond to environmental stressors with quick, adaptive changes that can be easily monitored (Bierbower and Cooper, 2009). Schilderman *et al*. (1999) conducted a study in the river Meuse located in western Europe using crayfish as a bioindicator for the environmental quality of the river.

Because crayfish are known to accumulate metallic pollutants in their tissues, these researchers measured the amount of heavy metal residue present in the crayfish hepatopancreas (a filtering organ similar to the mammalian liver) caught in the river Meuse against sediment and water samples from the same locations. From their findings showing reliable representations of inorganic and organic pollution between different geographical locations, they recommend that crayfish be used to assess water quality of river systems. Crayfish are also known to alter their behavior, specifically their escape responses, in dramatic ways as a result of their changing environment (Edwards et al., 1999, Schapker et al., 2002). Thus there is a precedent for use of crayfish in assessing environmental toxicity. This established precedent, in addition to the well characterized neurological system, makes the crayfish an ideal model for assessing the sub-lethal effects of AgNP on aquatic species.

Considerable research has been conducted to ascertain the effects of AgNP on a variety of species. Much of the research that has been done shows the adverse effects of AgNP, usually by means of describing the point of lethality in a given model system. However, little is known about the adaptive mechanisms that are launched in response to this toxicant. Describing sub-lethal effects of AgNP exposure can give us an idea of how the exposed organisms change in order to deal with the stressor. Using the base knowledge of adverse effects of AgNP exposure, we can now investigate more sensitive adaptive responses of crayfish, an aquatic species that is likely already being exposed to AgNP in nature. Crayfish are a keystone species, meaning any harmful effect on crayfish would cause residual harm to the ecological system as a whole. This investigation will not only add to the growing knowledge base of AgNP toxicity, but will also shed light on the adaptive responses of crayfish to environmental stressors.

Reflexes as a Measure of Physiological Stress

Reflexes have long been used as a measure of physiological health (Walker, 1990). Medical doctors have used the patellar reflex, for instance, to assess health for many years and a slow or absent reflexive response is understood as an indication of stress on the physiological health of the individual. Reflexes are an automated response to a given stimulatory input. Therefore, when a reflex is slow, or missing entirely, the assumption can be made something in the path between the sensory neurons and the efferent neurons is disturbed. In such a simple system, a disturbance is an indication of physiological stress because the quick reflexive response depends on available resources for fast synaptic communication; therein lies the justification for the use of a reflexive circuit as a measure of physiological health.

The crayfish lateral giant is a command neuron that responds to perceived attacks to the abdomen with a stereotyped escape reflex (Edwards et al., 1999). A command neuron is a single neuron that evokes the response of other cells, which results in a specific behavioral response. The command-nature of the lateral giant means that recording the activity of this single neuron can provide direct information about the subsequent behavioral activity of the animal. The lateral giant is a segmented neuron whose circuit consists of entirely electrical synapses (Zucker et al., 1971). This informational transmittance system allows for the greatest possible efficiency in cell communication. Stimulation of this neuron evokes a stereotypical, long lasting excitatory post-synaptic potential (EPSP) (Vu et al., 1997). The EPSP is characterized by depolarization of the cell relative to the strength of the stimulus. If the cell reaches threshold, an action potential is fired, generating a reflexive behavioral response. If the EPSP does not reach threshold, the cell repolarizes and does not fire an action potential, so the animal does not perform the tail-flip behavior. Because the LG escape reflex is a carefully tuned neural circuit, any change to the

EPSP is indicative of the organism remodeling the existing system; thus the study of this circuit can elucidate overall neurological effects of introduced stressors. Investigating synaptic changes between an afferent sensory neuron and the lateral giant as a result of AgNP exposure will shed light on potential neurotoxic effects of these materials. Further, researching the subtle changes that an organism must make in order to acclimate to the new surroundings will elucidate sub-lethal effects of AgNP.

The crayfish lateral giant-mediated escape circuit is made up entirely of electrical synapses, meaning that the communication from segment-to-segment is direct, fast, and directly proportional. A mechanosensory afferent neuron in the tail fan - which is innervated by the hairs on the tail fan - receives a signal and transmits that signal to the lateral giant neuron dendrites as well as interneurons through electrical synapses (Araki et al., 2005); the input from both the mechanosensory neurons and the interneurons produces the alpha and beta components of the lateral giant EPSP, respectively. If the stimulus is strong enough to prompt the lateral giant response to reach the point of threshold, the lateral giant then transmits the signal to the neuromuscular neurons, prompting the tail flip. The EPSP generated in the lateral giant after stimulation is a disynaptic response; the α component is characterized by fast depolarization, which is then followed by the slow, but greater amplitude depolarization making the β component of the EPSP. Measuring the response of this reflexive circuit allows for a direct measurement of the animal's behavior as well as the lack of behavior (measured in EPSPs) which is vital for the understanding of the circuit's modulation and disruption.

Serotonin Modulation

One key neurotransmitter that plays a large role in the functionality of the crayfish nervous system is serotonin (5-HT). 5-HT is a biogenic amine that binds to a metabotropic receptor in the cell membrane. 5-HT acts through both hormonal release and synaptic release in the crayfish nervous system (Musolf et al., 2009). 5-HT receptors vary in form and function, though their presence and function is conserved across species with the exception of 5-HT_3 subtype. 5-HT receptors (with the exception of 5-HT_3 mentioned previously) are G-protein coupled receptors that send chemical signals via a second messenger. That second messenger causes a cascade of effects in the cell, ultimately resulting in a net change in cellular properties. G_s protein activation causes an excitatory effect through the increase in cyclic AMP, protein kinase A, and adenyl cyclase. G_i activation results in an inhibitory effect by decreasing adenyl cyclase and cyclic AMP, as well as the opening of K^+ channels, hyperpolarizing the cell. G_o protein decreases the neurotransmitter release by shutting down Ca^{2+} channels. Each receptor has a different affinity for the amine, so the concentration of amine present in the synaptic cleft affects the receptors that will be activated, therefore changing the net effects of the neurotransmitter by means of second messenger cascade. This concentration-dependent activation affects the net effects of the amine on the cellular properties and allows for short and long-term plasticity. The ability for one neuromodulator to have opposing effects in a single neural circuit enables the animal to employ an efficient and appropriate response to a changing environment. With this available modulatory ability, it is not surprising that lateral giant excitability, and subsequent tail-flip escape behavior is modulated by 5-HT (Figure 1).

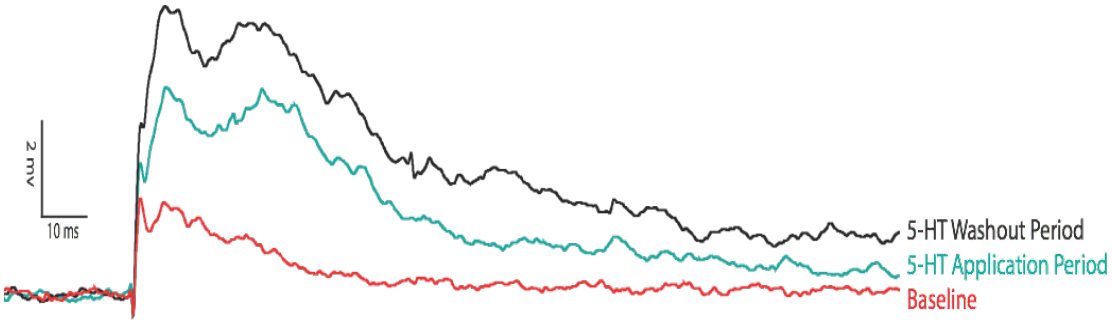

Figure 1 Sample Lateral Giant Recording Under Testing Conditions. Crayfish lateral giant (LG) recordings during 5-HT perfusion. Baseline recordings are taken while fresh crayfish saline is cycled through bath (black trace). 5-HT application typically causes LG to increase in excitability (as indicated by the increased voltage on the blue trace). After 5-HT is washed out by crayfish saline it is expected to decrease again, though the control animals in this experiment consistently maintained LG excitability after 5-HT washout, as indicated by the black trace.

The concentration of 5-HT at a synapse can have excitatory or inhibitory effects on a cell and this concentration-dependent response is also influenced by the rate of application (Teshiba et al., 2001) (Figure 2). This effect is also plastic as the social history of the animal has a meta-modulatory effect on the excitability of the lateral giant. The same concentration and rate of application regimen in socially subordinate animals causes inhibition of the lateral giant, while the opposite effect of 5-HT on the lateral giant excitability is seen in socially dominant animals (Yeh et al., 1996, Krasne et al., 1997, Panksepp and Huber, 2002, Cattaert et al., 2010, Issa et al., 2012). Studies have shown that this modulatory effect on membrane resistance - increasing or decreasing excitability of the cell in a concentration-dependent manner - can have dramatic effects on an organism's behavior. However, this effect is not static, but rather it can also decrease the excitability of the lateral giant when external influences warrant changes (Yeh et al., 1997). The proposed mechanism through which this modulation is achieved is a change in receptor density on the membrane (Yeh et al., 1996, Huber et al., 1997). These studies showed that the rate of change in lateral giant excitability after social interaction is slow, which likely

reflects the time it takes for a cell to adjust transcriptional factors and turn over the receptors present in the membrane (Yeh et al., 1997). Because 5-HT concentration manipulations cause more pronounced changes in the β component than the α component, it is thought that the modulatory action of 5-HT on this cell lies in the the β order of this response (Antonsen and Edwards, 2007).The vast knowledge of how 5-HT's modulatory role is influenced by stress is the basis for studying the serotonergic modulatory changes after AgNP exposure.

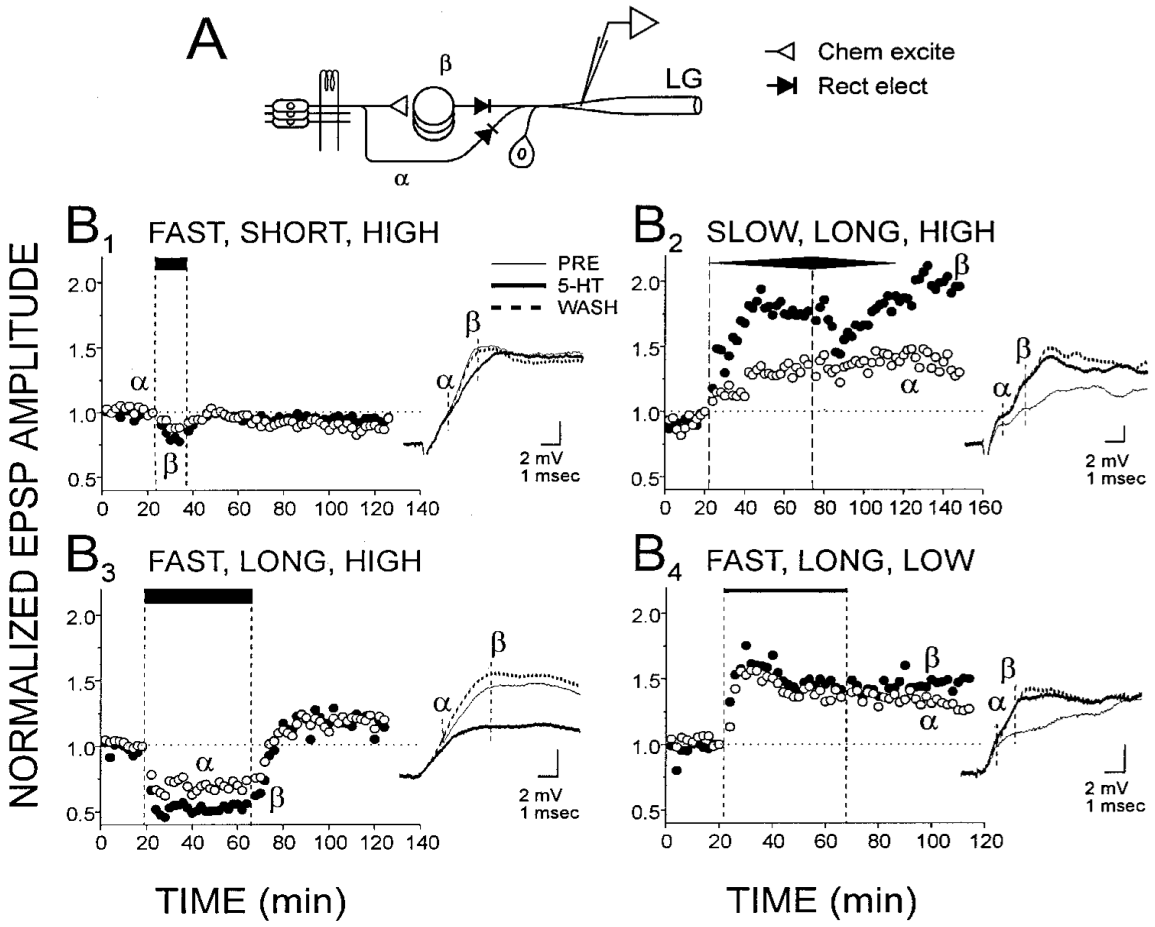

Figure 2 Rate/Dose Dependent Lateral Giant Response to 5-HT. Teshiba *et al.* (2001) showed rate/concentration dependence of lateral giant (LG) excitability to 5-HT modulation. A includes a schematic depicting the neural connections of the lateral giant neuron with mechanosensory neurons shown on the far left. Those innervate both the LG and interneurons. The synapse from mechanosensory neurons directly onto the lateral giant produces the alpha component of the LG EPSP while the synapse from interneurons to LG produces the beta component of LG EPSP. B_1 depicts the lateral giant excitatory post-synaptic potential (EPSP) over the course of fast 5-HT application (10 min) at a high (50 μM) concentration. LG was depressed for this course of application. B_2 shows LG's response to slow application (45 min) of high concentration (50 μM) 5-HT perfusion. In this time/concentration application, LG's response is excitatory. B_3 shows LG response to fast high concentration application held over a long period of time (45 min). In this time/concentration course, LG is depressed. B_4 shows fast application of low concentration 5-HT (0.01 μM) held over 45 min. In this time/concentration course LG response is excitatory. Figure used with permission from the Society of Neuroscience Central Office [57].

Stress Response in Crayfish

Stress and anxiety (in the absence of a stressor), have been shown in invertebrates and the mechanisms underlying the stress response in invertebrates have been a topic of study for many years. The perception of danger which underlies stress manifests itself in behavioral adaptations that are intended to deal with the stressor appropriately. In crayfish, an hypothalamic-pituitary-adrenal (HPA) homologue neuroendocrine system, called the X-organ/sinus gland, responds to a stressor by secreting hyperglycemic and steriodostatic hormones (Lorenzon et al., 2005). The X-organ neural cells project forward into the sinus gland, where they terminate (Hartenstein, 2006). Crustacean hyperglycemic hormone (cHH) is synthesized in the X-organ and secreted through the sinus gland located in the crayfish eyestalk. Amines such as serotonin and dopamine are known to play an important role in the secretion of cHH, which directly influences the circulating glucose levels (Lorenzon et al., 2005). Serotonin has been shown to increase the levels of circulating cHH (Lee et al., 2001), while dopamine was shown to inhibit the cHH secretion from the sinus gland (Sarojini et al., 1995). Fossat *et al.* (2014) showed that stressed crayfish who had undergone electrical impulses had behavioral adaptations indicative of anxiety as well as elevated 5-HT levels in the brain. Further, they demonstrated, through 5-HT injection, that the behavioral adaptations were controlled by the 5-HT levels and were homologous to other vertebrate anxiety paradigms. This finding provides support for the generalizability of stress research in invertebrates and suggests that the simpler, more conserved stress response of the crayfish can be a model system for the study of stress and anxiety.

Furthermore, increased levels of cHH have been seen after exposure to environmental stressors, such as increase in water temperature and changing salinity, but were not seen in eyestalkless crustaceans experiencing the same environmental stressor, providing further

evidence that the crayfish stress response is mediated by the X-organ/sinus gland secretion of cHH and that cHH levels change in response to environmental stress, further demonstrating the activity of this stress response to a variety of stressors. Exposure to the toxicant copper induced higher levels of blood glucose in shrimp, an effect that was shown to be caused by proportional secretion of cHH from the eyestalk organs (Fossat et al., 2014). The causative effects of an environmental toxicant on cHH and the subsequent rise in blood glucose levels provides further support for our methodology. It is presumed that, because AgNP are known to cause oxidative stress, which in turn negatively impacts physiological health, this potential neurotoxicant will cause synaptic changes in the reflex circuit. These changes are likely to be manifestations of an increase in 5-HT levels in the brain, which would lead to increases in circulating cHH, and higher blood glucose levels, resulting in an end effect of changes in the cell membrane.

Generalized Stress Response

Comparative biologists often attempt to find generalizable models for translational research. Because crayfish have a stereotyped social stress response that is well defined and relatively easy to study, more interest has been garnered for using this invertebrate and its reflexive responses as a model for stress response. This easily assessed stress response, coupled with the fact that crayfish are indicator species, makes this organism an attractive candidate for testing the stress that environmental contaminants are causing on aquatic organisms, and extrapolating what kind of stress that may cause for mammalian species, like humans.

A generalized stress response can be an advantage when an organism has a commonly occurring stressor in its natural environment. For instance, it would be advantageous for a population of animals to respond to the same stressor in a predictable and organized manner.

Crayfish social interactions are an example of the benefits of a generalized stress response. One can predict, based on an individual's social history, the response of that particular individual to an agonistic encounter as well as lingering effects that dictate how that individual would respond to further stimulation. The length of the fight and the frequency of future fights can also be predicted when the social history of the individual is known; therefore, the generalized social stress response in crayfish can result in shorter and fewer fights (Herberholz et al., 2001), likely preventing further physical harm to the crayfish involved. Without a generalized response to agonistic encounters, crayfish would likely fight longer, more frequently, and would see many more injuries and deaths as a result. In this case, a generalized stress response has likely evolved to maintain the population in an organized manner. We know that this organized lateral giant response to social stress utilizes the same machinery and neurotransmitter for dichotomous end behavioral effects.

Of course, while humans have a relatively generalized stress response (Selye, 1956), we do not respond to different types of stressors with identical responses, but rather with the same set of mechanisms. So, humans respond to social stressors with the same HPA axis and same hormones as they do physical stressors - the difference seen in behavioral response therein lies in specific biochemical pathways. Responses to stressors can be tailored to the specific stressor by the behavioral adaptions of the individual (Biondi and Picardi, 1999). While studying stress in humans can be enlightening, these contextual, individualistic responses to stress can be confusing and difficult to measure. However, studying stress in a simpler system, like the crayfish tail-flip reflex circuit, can begin to address the basic effects of a stressor. This neural circuit of escape behavior has already been shown to respond to stressful experiences, such as social dominance shifts, with synaptic changes in excitability and serotonergic role changes (Yeh

et al., 1997, Issa et al., 2012). Our understanding of this stereotyped adaptive behavior in crayfish makes it an excellent model for investigating the neurophysiological changes caused by environmental toxicants. We know the neural remodeling that occurs as a result of social stress, but social stress is a common, natural stressor for the crayfish, as their social status is determined by fighting (Huber et al., 1997) and encounters are predominately agonistic. We, however, do not know what remodeling must occur to accommodate an unnatural stressor, like an environmental contaminant. Once it is clear that the stressor does, indeed, disrupt the reflex circuit more complex questions can be built on this knowledge base.

Social stress is experienced by most animals in some form, whether it be caused by agonistic encounters, resource abandonment, or changes in the established social hierarchy. While the gross responses to stress are not always similar across species, the conservation of certain coping mechanisms, like the HPA axis, glucocorticoid secretion, and use of neuropeptides like 5-HT, is well described in vertebrates. As Summers (2002) discusses, while the behavioral outputs of the activity of these mechanisms will likely be different among different species, the commonality between taxa likely provided a framework from which species could develop an appropriate, adaptive behavioral response to the stressors they face. This specialized, but pseudo-generalized response is beneficial to employ when organisms face stressors that are common and context-specific for their given environment. For instance, crayfish respond to social stress in predictable ways, but the response is dependent on the social status of the animal. Thus, their stress response is systematic but also flexible enough to change with their changing social context. This flexible, yet mechanistic system allows both for the efficiency of having a generalized response system - so one does not need to develop and maintain multiple systems for every given stressor - as well as the appropriate response with the

necessary magnitude that the stressor requires. 5-HT is used by crayfish to modulate the lateral giant excitability after social encounters. However, while the same neurotransmitter is used in both situations, the subordinate and dominant animals' lateral giant neurons have markedly different responses to stimulation. This modulation circuit utilizes the same mechanisms to generate two opposite responses through the activation of specific receptor types.

Anxiety-like behavior has recently been shown in crayfish (Fossat et al., 2014) with similar behavioral endpoints as seen in rodents in the elevated plus maze after experiencing electric shock training. Previously it was questioned as to whether invertebrates were capable of exhibiting anxiety because it is a more complex emotion that is present when the stressor is not immediately distinguishable. The experimenters used electric shock to stress crayfish, then put them in a maze with light and dark arms. Crayfish typically explore their surroundings, but prefer to remain in shaded areas most of the time (Yamane and Takahata, 2002). The crayfish who had been exposed to electric shock avoided the light arms or hesitantly paused before entering them - a behavior that very similarly reflects risk-assessment behavior shown in rodents (Griebel et al., 1997). In both cases, the behavioral output, risk-assessment in rats or hesitation in crayfish, was achieved via 5-HT pathways. When Fossat *et al.* (2014) injected unstressed crayfish with 5-HT their time spent in light arms decreased further demonstrating the dependency of this behavioral response on 5-HT. Similarly, the risk-assessment behavior in rodents described by Griebel *et al.* (1997) was found to be sensitive to 5-HT as well; however an anxiolytic effect was seen with drugs that target 5-HT1A receptors, meaning that the anxious behavior decreased with activity of 5-HT1A, a receptor whose action is typically inhibitory (Griebel et al., 1997). So, while it would seem that similar biochemical pathways are at play in these two stress responses, the activity of the receptors' second messenger determines the

behavioral output of the animal. This distinction is particularly relevant when considering the behavioral output of the crayfish escape reflex. While the effects of 5-HT are well understood, prior exposure to stress (AgNP exposure) likely influences the 5-HT receptors that are present in the lateral giant membrane, which would determine the behavioral output as an effect of the second messenger cascade stemming from the type of 5-HT receptor present at the time of 5-HT perfusion.

However, a generalized stress response may not always be appropriate. Responding to environmental stressors, like an influx of AgNP, in the same manner as one responds to a social interaction could result in an inappropriate and potentially harmful stress response. Crayfish undergo social stress often because their social dominance hierarchy is determined through agonistic encounters. This social interaction is something that crayfish have, presumably, evolved to deal with appropriate modulation. However, we have not yet established the stress response launched in this system as a result of environmental stressors, like AgNP. Testing adaptive responses that are necessary for accommodating a sub-lethal stressor is a sensitive and useful tool for understanding the modulation that occurs due to AgNP exposure. Given the prior knowledge of crayfish reflex modulation after social stress, we can use our results to ask questions regarding the generalizability of the crayfish stress response.

Motivations

This project aimed to assess the potential neurotoxic effects of silver nanoparticles as well as ascertain the generalizability of the stress response in this neural system. Because stress is known to alter the excitability of the lateral giant (Yeh et al., 1997) and AgNP cause oxidative stress in crayfish (Struzynski et al., 2013) the hypothesis that drove this project was that AgNP

interfere with the excitability of the lateral giant and 5-HT-mediated neuron excitability. The findings from this project show a loss of fine-tuning control over the crayfish escape reflex circuit as a result of silver nanoparticle exposure. These results confirm the neurotoxicity of silver nanoparticles through disruption of a normally finely-tuned, efficient system and bring to light broader implications regarding the generalizability of stress.

CHAPTER 2

MATERIALS, METHODS AND RESULTS

Materials and Methods

Animals. *Procambarus clarkii* (crayfish) adults 4-6 cm in length were used to collect data. Crayfish were freely moving and kept physically, visually, and chemically isolated in 1 L tanks at least one week before and during treatment. Ambient room temperature was recorded each morning and evening and large temperature fluctuations were noted. Crayfish were exposed to AgNP via aquaria water for chronic (two weeks) or acute (overnight) treatment periods. Concentrations were 1 ppm, 5 ppm, 10 ppm, and 50 ppm. With the exception of 50 ppm chronic treatment, each concentration was tested in acute and chronic treatment periods. This wide treatment range was selected to maintain consistency and develop comparable data to previous work investigating toxicity of AgNP in aquatic organisms (Asharani et al., 2008). Additionally, 1 µg/ml [1 ppm] and 5 µg/ml [5 ppm] have shown to be the minimum concentrations necessary to induce toxicity in *Bacillus thuringiensis,* a potential target organism for the antibacterial effects of AgNP (Mirzajani et al., 2014). Crayfish were assigned at random to treatment groups and treatments were scheduled at random using a random number generator to avoid experimenter bias. 50 ppm chronic treatment showed lethality, which was not the aim of this project, so 50 ppm chronic treatments were terminated after two deaths.

A total of 147 crayfish were used for the purposes of this project. Of those, there were 75 complete data sets that could be used for analysis. The number of complete data sets used for analyses were the following: Control n=10, 1 ppm acute n=8, 1 ppm chronic n=10, 5 ppm acute n=9, 5 ppm chronic n=10, 10 ppm acute n=7, 10 ppm chronic n=10, 50 ppm acute n=9.

Silver Nanoparticles. 20 nm PVP-coated AgNP powder (99% purity) was ordered from NanoAmor (Houston, TX). AgNP were kept in dark container at all times. AgNP were dispersed into a 100 ppm stock solution of crayfish aquaria water. This solution was sonified to achieve maximum nanoparticle dispersion, then wrapped in aluminum foil to limit light exposure and kept at room temperature. The stock solution was diluted to desired concentrations before treatment. Any agglomeration visible in the container was dispersed again before diluting.

Serotonin Solution. 50 μM serotonin solution was made using crayfish saline and serotonin creatinine sulfate complex (Sigma-Aldrich). The solution was made no more than 24 hours before use and was kept refrigerated with limited light exposure at all times.

Experimental Preparations. Animals were cooled in ice water for 18-20 minutes to induce anesthesia. Cold submersion is an accepted form of inducing anesthesia in these animals. Anesthetization was confirmed by testing for a tail-flip reflex response to stimulation on the tail fan. Once the animal was no longer responsive to tail fan stimulation (a state confirming the anesthetization of the animal), the nerve cord leading to the brain was cut so that all input to the brain was terminated, thus the animal was no longer conscious. Antennae, appendages, and dorsal exoskeleton were removed. The ventral exoskeleton, head, and tail fan were kept intact, with the exception of cutting a small window in the rostral end of the telson exoskeleton in order to expose underlying terminal ganglion (A6) nerves more effectively. The nerve cord was exposed by removing surrounding tissues and musculature as cleanly as possible without damaging the nerve cord. Specifically, the uropod and telson musculature was removed as completely as possible. While nerves were left intact as much as possible, some animals' nerves feeding into flexor muscles were cut to prevent movement while the experiment was in progress; these nerves were always cut at the furthest point possible from the neuropil. A small piece of

Sylgard was placed underneath A6 to stabilize the ganglion for experiments. The partially dissected animal was then carefully moved to another dish containing about 18 ml of cool (19°C) crayfish saline and was pinned to Sylgard lining (Figures 3 and 4).

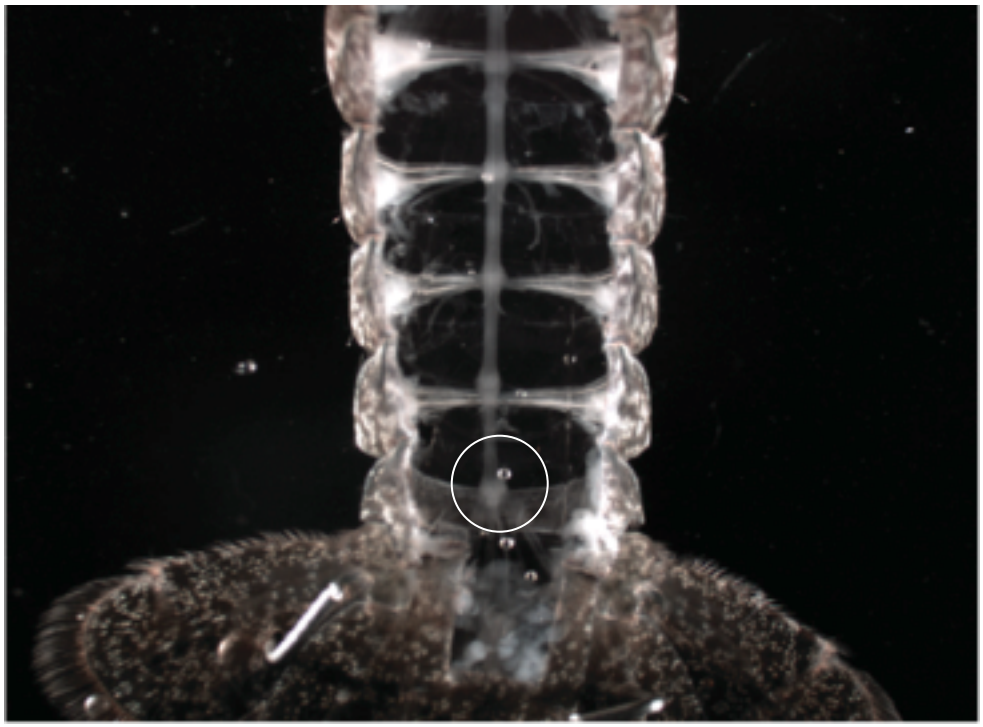

Figure 3 Experimental Preparation. Crayfish abdominal nerve cord with surrounding musculature removed. Terminal ganglion (A6) is indicated in white circle.

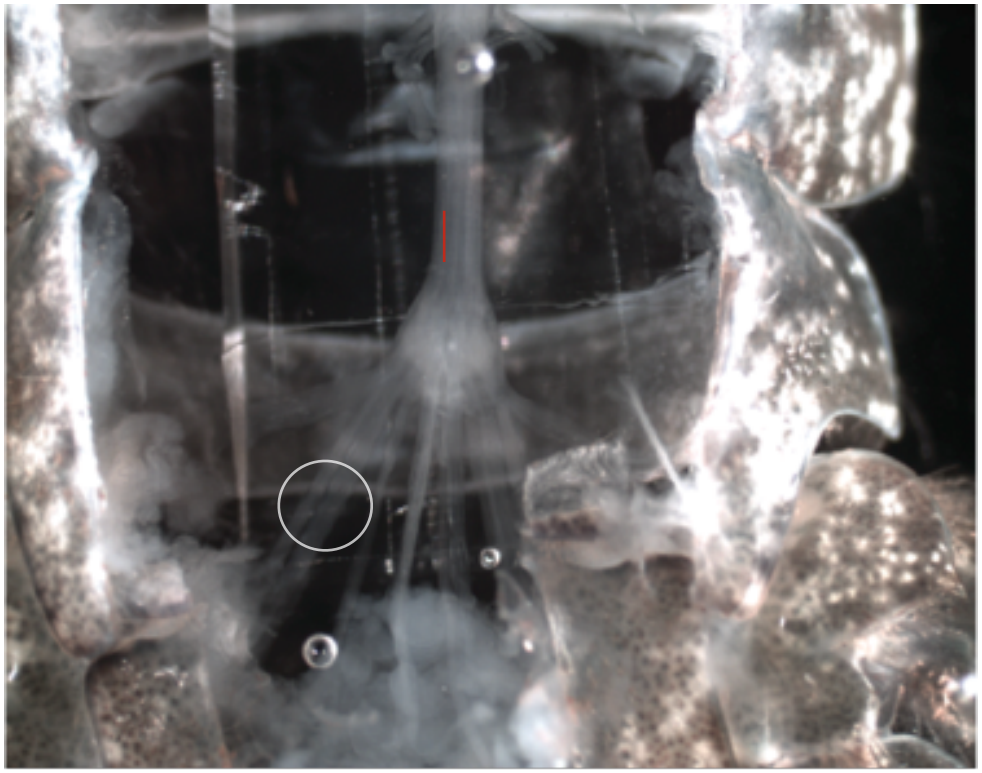

Figure 4 Experimental Preparation Magnified. Crayfish terminal ganglion (A6). Root nerves 2 and 3 indicated in white circle. Lateral giant (LG) integration center indicated in red.

Electrophysiology. Micropipettes were prepared using a micropipette puller (Flaming/Brown Micropipette Puller: Model P-97). Micropipettes were placed bottom-down into a tube containing 3 M KCl then stored in a humid container overnight to allow the tip to fill with 3M KCl without the influence of static electricity, which alters the resistance of electrodes. A syringe was used to fill micropipettes completely with 3M KCl. Recording electrodes 20-40 MΩ were used to record excitatory post-synaptic potentials (EPSP), resting membrane potential, membrane resistance, and cellular threshold before, during, and after washing the nerve cord with serotonin (described below). A silver/chloride interface electrode connected to the electrophysiological recording instruments was inserted into the micropipette and used to amplify current flow information from the cell to the computer software. An air-float table was

used to stabilize the prep and micromanipulators only when vibration was exceptionally noticeable and could potentially distort the electrophysiological recordings.

Recording electrodes were inserted into the integrating center of the LG A6. A stimulatory suction electrode was suctioned onto the 2nd and 3rd root of A6. Stimulus was delivered in variable amplitude, depending on the resistance of the cell; amplitudes applied ranged from 1.75-9V. Stimuli had a duration of 0.2 ms and began immediately upon the pressing of the spacebar. Cellular threshold was determined using stepwise stimulation. When a stimulus evoked an action potential, that stimulation level (amplitude) was considered threshold. Half of cellular threshold was used for recording EPSP. When a cell did not reach threshold before recording, half of the largest EPSP evoked was used. During recording, a perfusion pump cycled fresh crayfish saline (~18°C) into the bath and waste was removed at the same rate. Stimulus was applied via the suction electrode every three minutes for the entirety of the experiment. Baseline recording was at least 30 minutes (ten stimulus responses) or until the evoked EPSP was stable. Following baseline recording, 50 μM serotonin saline solution was bathed onto the cell at 1ml/min using a peristaltic pump. The bath serotonin concentration reached 50 μM after about 30 minutes. Evoked EPSP during serotonin exposure was recorded for 45 minutes (15 stimulus responses). The pump was switched back to crayfish saline for another 75 minutes (25 stimulus responses) (Figure 5).

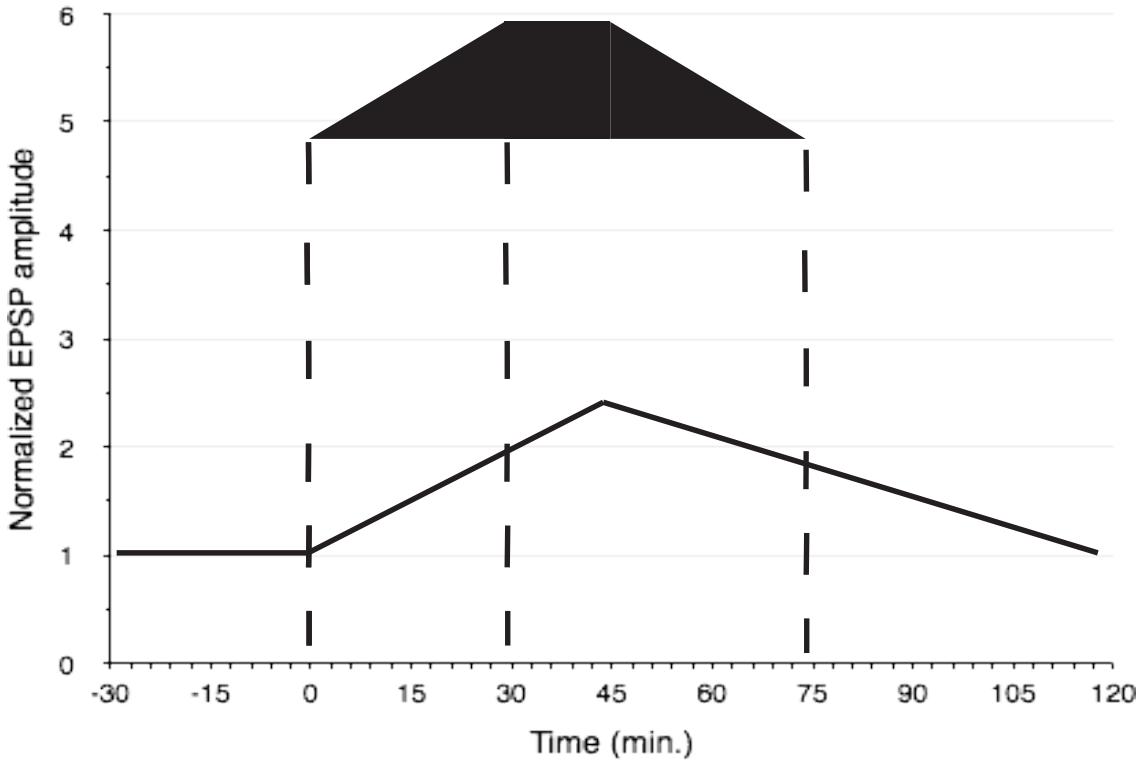

Figure 5 Experimental Timeline. Baseline recordings taken over first 30 min. of experiment. First dotted line indicates start of 5-HT drip. For 45 min. 50 µM 5-HT solution is pumped into the saline bath at a rate of 1ml/min. Peak 5-HT concentration in the bath is reached at 30 min. indicated by the second dotted line. Crayfish saline is pumped into saline bath at a rate of 1ml/min for the remainder of the experiment and is presumed to have washed out 5-HT completely after 30 min. indicated by the third dotted line. Black triangles at the top of the figure represent rising and falling 5-HT in the bath. Solid horizontal line depicts a hypothetical recording during the experimental trial.

Data Analysis. Only complete data sets were included in analysis. If the recording electrode or suction electrode had been manipulated in any way during the recording period, that data set was excluded from analysis. The electrophysiology software Clampfit 10.0 was used to collect EPSP data, including alpha (where possible) and beta components. These quantified data were normalized where each individual's data were normalized to its own baseline values. The data were then run through an unpaired t-test repeated measures analysis including an f-test using GraphPad Prism version 6 for Mac OS X. F-test is an accepted measure of variability differences between groups. A significant finding in an f-test suggests that the two groups tested have different variability.

Results

Increased excitability. Crayfish treated with AgNP showed a trend toward increased excitability during 5-HT perfusion and after 5-HT washout compared to control animals. Though not significant, this trend could suggest a change in the modulatory action of 5-HT on the LG. While 5-HT normally causes increased excitability in the LG, the AgNP treated crayfish show an exaggerated and longer lasting excitability under 5-HT perfusion compared to control animals (Figures 6 and 7). This excessive increase in LG excitability under 5-HT perfusion is uncharacteristic in this normally very finely-tuned, system. Over-excitability points to a loss of control over the circuit, which could be detrimental to the crayfish.

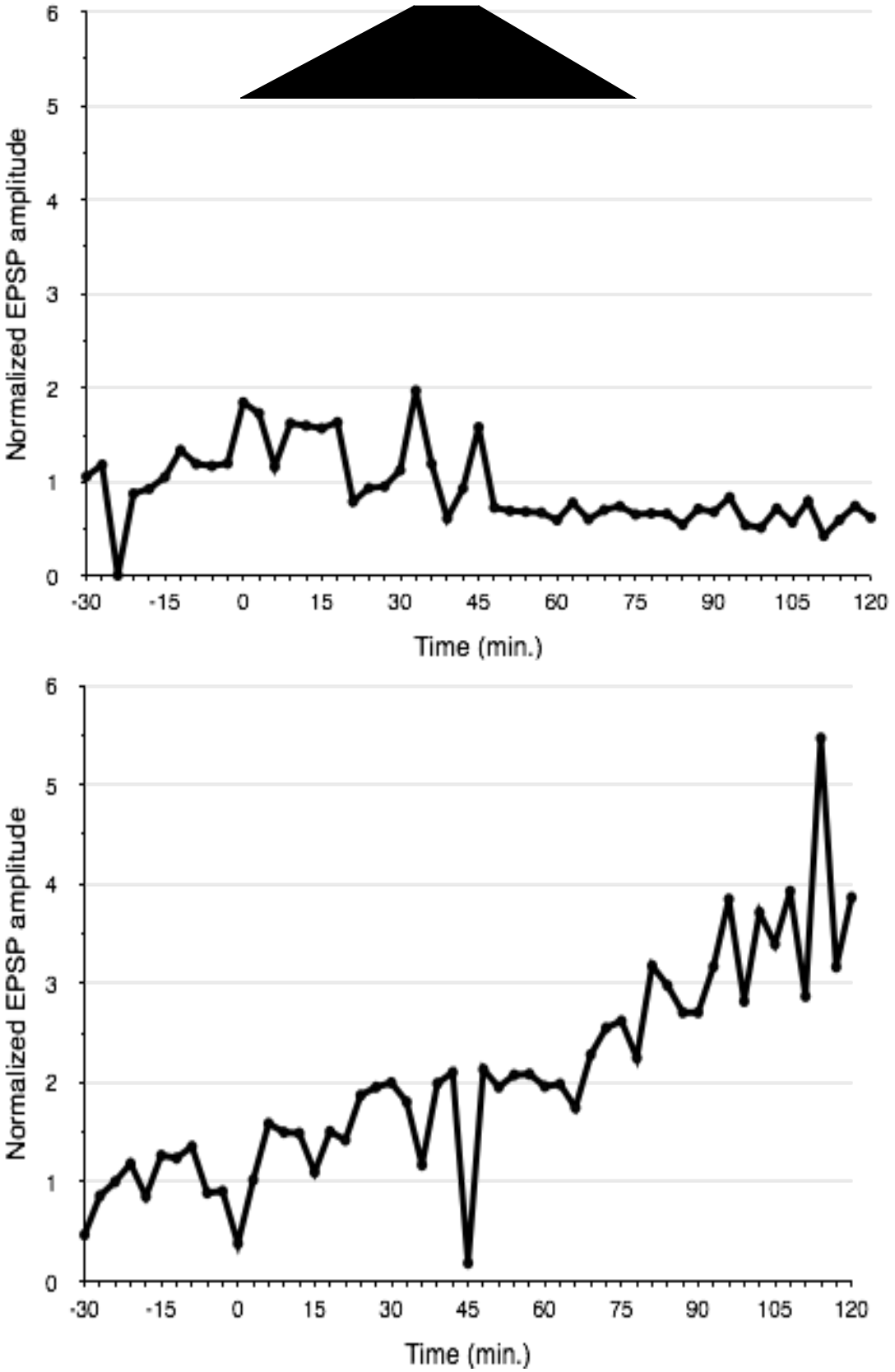

Figure 6 Representative Findings. Representative figures from control crayfish (A) and 5 ppm chronically treated crayfish (B). -30 – 0 min. baseline recording. Thickness of black bar at the top of the figures represents the concentration of 5-HT in the bath at the time of recording.

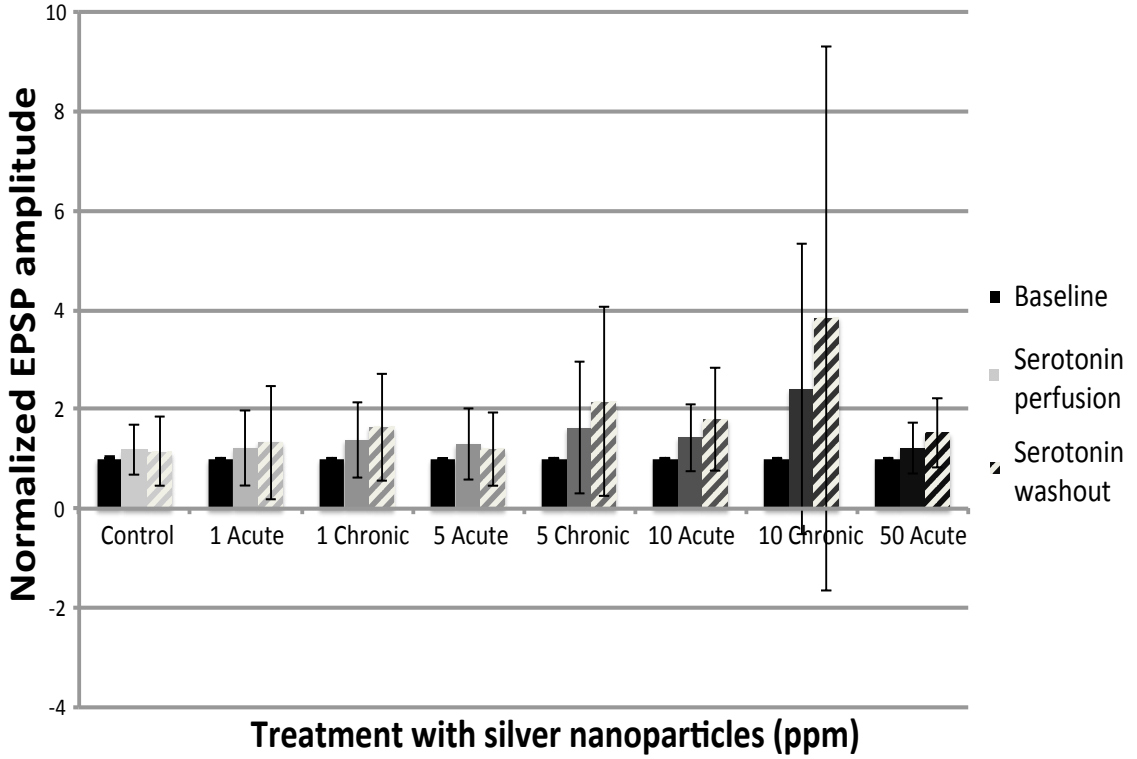

Figure 7 Results of AgNP Effects on 5-HT Modulation. Results depicting the normalized EPSP amplitude of the lateral giant in response to electric shock mimicking a natural stimulus. Treatment regimens are shown in parts per million. Solid bars show baseline recordings for each treatment group normalized to 1. Gray bars show lateral giant response to shock during 5-HT perfusion taken as a mean of the response during 5-HT application. Striped bars show lateral giant response to shock after the washout of 5-HT from the bath taken as a mean of the washout period.

Chronic Treatment vs. Acute Treatment. Chronically treated animals showed more exaggerated effects of AgNP on LG excitability during and after 5-HT perfusion than acutely treated animals. While this trend is not significant, it does allow for some inferences regarding the method by which AgNP can be neurotoxic. Because LG is known to be modulated by 5-HT and 5-HT's action on LG is metamodulated by the crayfish's context, it is reasonable to postulate that the crayfish's environment stimulates biochemical changes in the LG, specifically changes in the 5-HT receptor density, type, or second messenger cascade. Thus, the context in chronically treated animals has more time to influence the biochemical changes that are suspected to be made. Biochemical changes such as receptor density and type take time, therefore acutely treated

animals likely have not had the time needed to make the receptor turnovers that would make them more susceptible to 5-HT modulation. Additionally, AgNP have shown potential for bioaccumulation. This bioaccumulation is likely to have greater neurotoxic effects in chronically treated animals due to the greater accumulation and longer exposure period. This, compounded with the more time-consuming protein transcription that may take place as a result of exposure, could point to an increasing neurotoxic potential as crayfish are exposed to AgNP in their native habitat.

Variability. While there was a clear trend of increased excitability after AgNP, the results from this experiment were not statistically significant. Upon further examination, the lack of statistical significance appears to be caused by variability within treatment groups. While the trend suggests increased excitability of LG after AgNP exposure, the variability within treatment groups was very high. However, the individuals' responses over the entire recording was consistent. This result suggests that crayfish do not have a systematic response to AgNP exposure. While most crayfish responded to AgNP exposure by increasing LG excitability under 5-HT modulation, some crayfish did not. In fact, this increase in variability is significantly different from the variability seen in the control group and treatment groups *5 ppm chronic* ($p=0.003$) and *10 ppm chronic* ($p < 0.0001$), suggesting a disorganized and somewhat chaotic response to AgNP exposure (Fig. 8). Not only does this increase in variability point to neurotoxic effects of AgNP, it also speaks to the generalizability of stress. Crayfish do not have a characteristic response for this stressor as they do with social stress. A varied and uncharacteristic response suggests that in this modulated escape-reflex system, stress is not generalizable.

Additionally, the variability between chronically treated crayfish in 5ppm concentrations was significantly higher than those in acute treatment of the same concentration (p=0.0326). A similar finding was discovered in crayfish chronically treated in 10 ppm concentrations with greater variability than those in acute treatment of the same concentration (p=0.0001). An increase in variability after longer exposure to AgNP would suggest that the effects of AgNP are greater with longer exposure, meaning that the stress response of the crayfish is likely time-dependent.

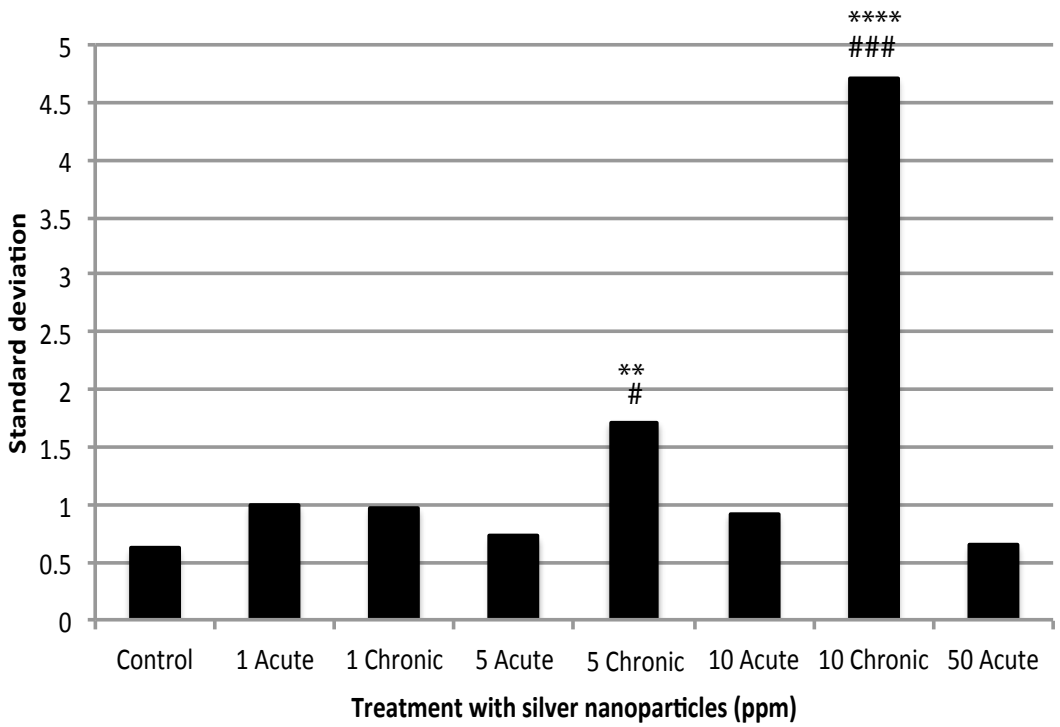

Figure 8 Results of AgNP Effects on Variability. Standard deviation of lateral giant response to shock during entire experimental protocol. (****p<0.0001,**p<0.005; ###p=0.0001,#p<0.05) Asterisk indicates significance against control, pound sign indicates significance against acute treatment of same concentration.

Decreased Muscle Mass. Anecdotal findings suggest that chronic exposure to AgNP causes a decrease in muscle mass. This change in muscle mass was not quantified for this

project, though the quantification of this decrease in muscle mass could be achieved in future studies. Representative examples of the differences in muscle mass are shown in Figure 9.

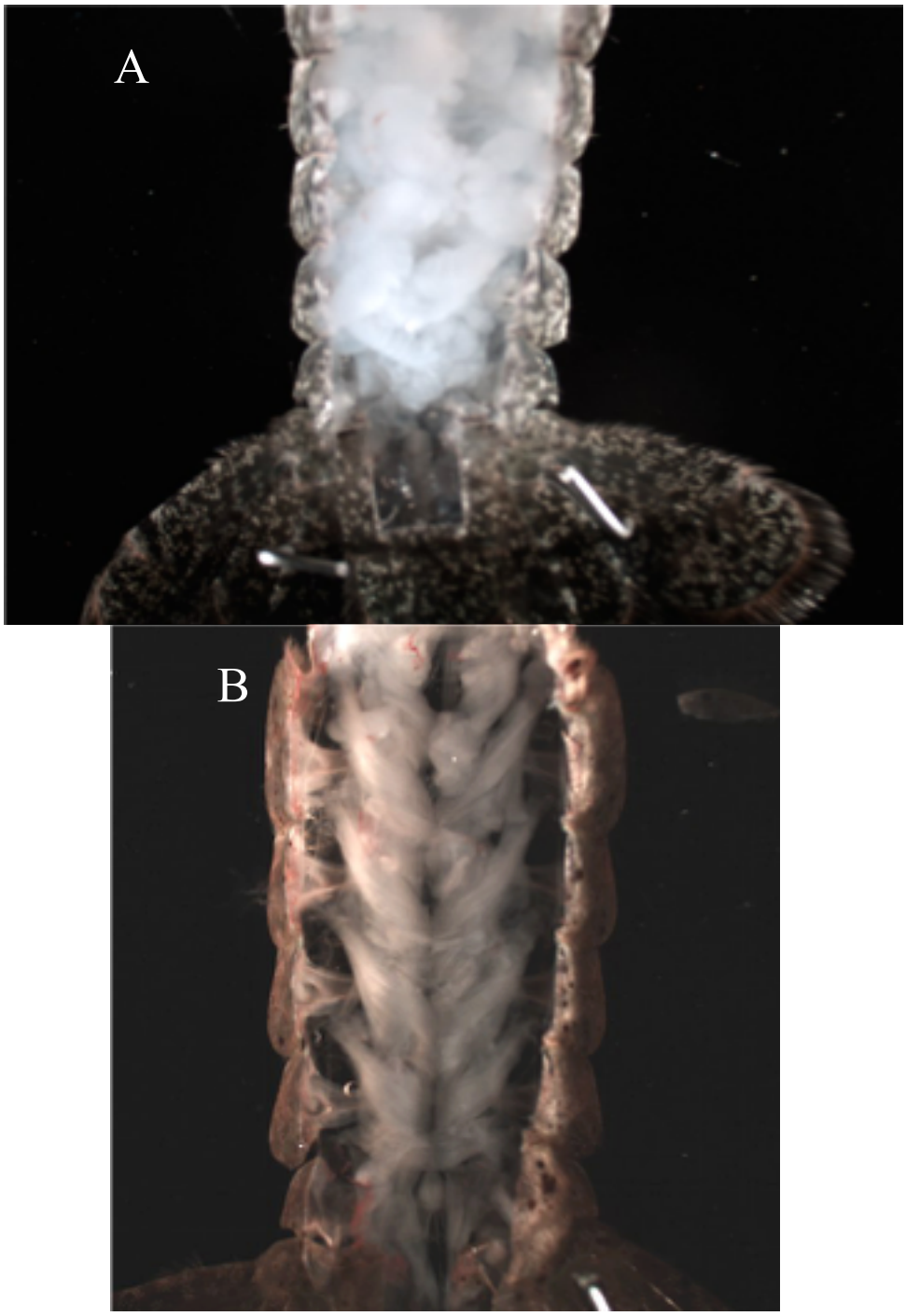

Figure 9 Decreased Muscle Mass. Representative example of control animal abdominal muscle tissue (A). Bottom figure representative example of decreased muscle mass observed in some chronically AgNP treated crayfish. Crayfish was treated with 1 ppm AgNP chronically (B).

Behavioral Changes. Anecdotal accounts of erratic behavior were noted in many chronically AgNP treated animals. Much of the erratic behavior was characterized by lethargy. Chronically treated crayfish stopped grappling for food and often stayed in the same position for multiple days (Figure 9 A). A few displayed exceptionally erratic behaviors such as that shown in Figure 9 (B).

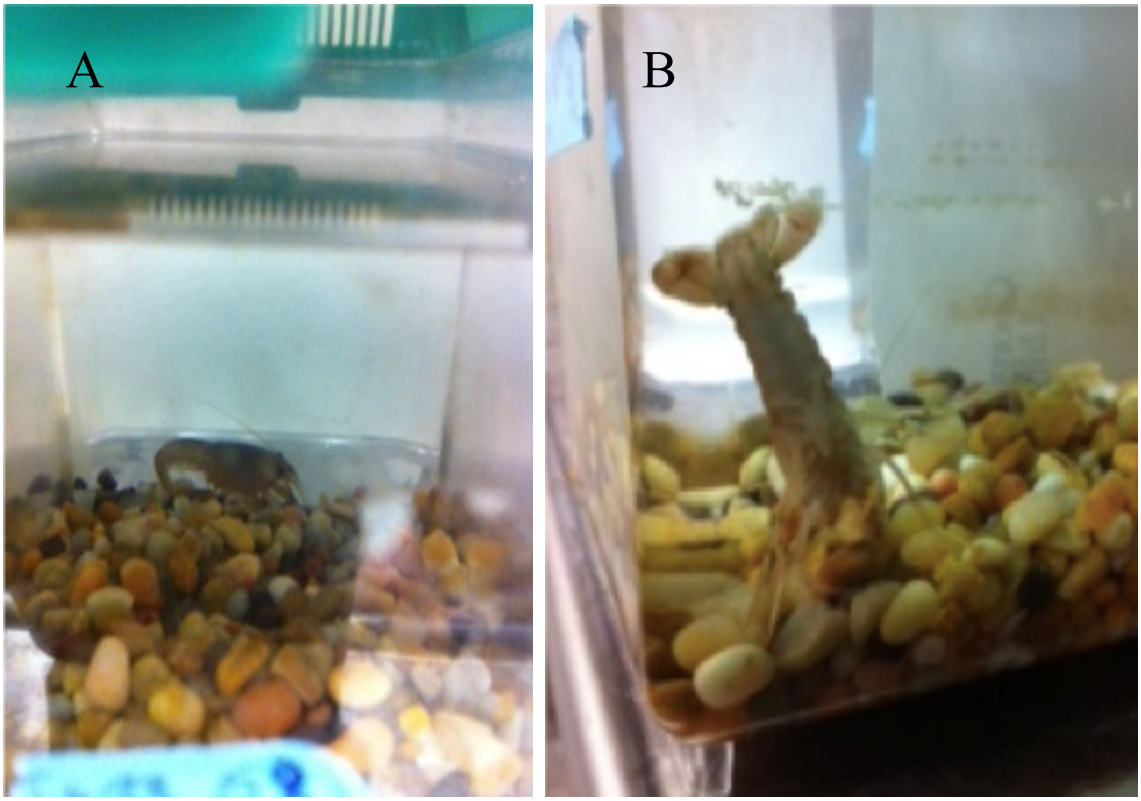

Figure 10 Behavioral Results. Image on left shows 10 ppm chronically treated crayfish displaying lethargic behavior (A). B shows 5 ppm chronically treated crayfish displaying erratic, unusual behavior.

Conclusions. Setting an appropriate gain for this tail-flip response is important for crayfish survival (Antonsen and Edwards, 2007). Over-excitability of the lateral giant neuron when that increased excitability is not necessary can cause the animal to tail-flip into the water column, which would make it more visible to predators, potentially resulting in detriment.

Comparatively, decreased excitability that is inappropriate for the animal's situation would likely result in the crayfish's death. As seen in this experiment, most animals treated with AgNP had an increased excitability compared to control animals exposed to the same stimulus level. This over-excitability indicates that the gain of the neural circuit was disrupted, which, in nature, could result in the animal putting itself in harm's way by propelling into the water column when the situation does not require it to do so.

The suspected change in receptor density that follows AgNP exposure is likely a manifestation of the loss of fine-tune control over the crayfish LG-mediated escape circuit. This system is normally carefully modulated by 5-HT which causes increases or decreases in LG excitability as needed and with the appropriate magnitude. After AgNP exposure most crayfish exhibited excessive excitability under 5-HT perfusion suggesting a change in 5-HT receptor density that goes beyond what is necessary. The loss of fine-tune control over this system, an escape circuit which is necessary for survival, is evidence for the neurotoxicity of AgNP. Additionally, the difference in variability between chronically and acutely treated crayfish provides further support for this difference being a manifestation of receptor change. Changing the receptors present in a membrane takes time and an acutely present stressor, while likely still acting as a stressor, does not allow the animal the time necessary to change the receptors necessary for the response seen in chronically treated animals.

CHAPTER 3

GENERAL DISCUSSION AND IMPLICATIONS

When crayfish encounter social stressors, the LG responds to 5-HT by either increasing or decreasing excitability, a well-characterized and predictable behavioral response. A crayfish's survival is dependent on setting the proper gain for the LG mediated escape reflex. The circuit should be set in a way that elicits a response of the appropriate magnitude for the crayfish's given circumstances. Thus, any change in the excitability of the LG neuron in this circuit can be indicative of the crayfish remodeling the gain of the escape reflex circuit. Crayfish have a well developed generalized response to social stressors and adjust the LG response appropriately; however, it would appear as though crayfish do not have a similarly organized, systematic response developed for dealing with AgNP exposure. Chronic AgNP treatment in high concentrations caused increased variability in the crayfish escape reflex. This increased variability within treatment groups is highly suggestive of a loss of control over what is normally a very organized, systematic behavioral response. Loss of control over such an important system which is vital for their survival is an alarming and unexpected response from such a resilient bioindicator species.

Time-Dependency of LG Response

Duration of AgNP treatment had a significant effect on the excitability of LG in 5 and 10 ppm treatment groups. The difference seen between chronically treated and acutely treated crayfish is likely a similar effect to that seen in crayfish after social pairings. Yeh *et al.* (1996) saw a change in LG excitability after prolonged pairing with other crayfish. Specifically, the difference seen between longer paired crayfish was an altered effect of modulation on the LG. The altered effect of 5-HT modulation on LG was thought to either be due to a change in

receptor density or a change in the gain of particular receptors' signaling pathway. This context-dependent plasticity of the modulatory action on LG allows for fine-tuning of the crayfish' behavior in changing social contexts.

Teshiba *et al.* (2001) showed timing-dependent 5-HT modulatory effects on the LG with fast and slow application of 5-HT. When high 5-HT concentrations are perfused quickly, the cell shows inhibitory response to stimulation; thus, the effects of a great influx of 5-HT quickly onto the cell membrane inhibits the cell's ability to depolarize. They propose that this inhibition is likely due to the signaling effects of one type of receptor (producing inhibitory effect in this case) interfering with the second modulatory receptor type's ability to generate a depolarizing effect. When 5-HT was added at a slower rate LG's response to stimulation was facilitory. This facilitation is likely because the type of modulatory receptors being activated first were facilitory, which allowed for their action on the cell to dominate the net effect that was observed. The activation of one modulatory pathway before another is likely dependent on the threshold of each, thus, the possibly faster, higher threshold pathways were activated with faster, higher concentrations of 5-HT, but the lower threshold facilitory pathway is activated with more gradual application of the same concentration.

Biological Applications of Time/Dose LG Response

Teshiba *et al.* (2001) proposed that there may be a biological correlate for the fast/slow application regimens often used in 5-HT modulation LG studies. They propose that 5-HT released by a serotonergic neuron that has contact with the dendritic segments of each ganglion could mimic the effects seen with the high concentration, fast application of 5-HT onto LG. The

slower application regimens may be a reflection of the 5-HT circulating in hemolymph, which would, presumably, reach the cell more slowly, therefore having a slower effect on the cell.

5-HT is known to influence the posture behavior in crayfish, with injection of 5-HT causing more aggressive posture. Because 5-$HT_{1\alpha Pro}$ receptors are clustered on the flexor neuromuscular regions, which play a role in posture, the activation of these receptors could cause the changes seen with 5-HT injection (Spitzer et al., 2007). The inhibitory effects seen in the LG may be a result of activation of 5-$HT_{1\alpha Pro}$ as activation of this receptor by meta-Chlorophenylpiperazine (mCPP) – (a 5-$HT_{1\alpha Pro}$ agonist) caused an inhibitory response, therefore the receptor may be activated by fast, synaptic application of 5-HT (Yeh et al., 1997). The facilitory response to 5-HT seen in crayfish may be due to the action of 5-$HT_{1\alpha Pro}$ or 5-$HT_{2\beta Pro}$ as α-Me5-HT, an agonist for these receptors, resulted in the facilitory effects of 5-HT_{α}. This facilitory effect could be a correlate of biological release of 5-HT from the pericardial organs into the blood. 5-HT receptor gene expression is thought to be linked to crayfish social status, though summed mRNA contents did not show significant differences between social groups. Therefore, the effects of social interactions on 5-HT-mediated responses are likely a result of a specific *pattern* of 5-HT receptor gene expression throughout the crayfish' nervous system (Edwards and Spitzer, 2006), not simply more or less 5-HT receptors. While these specific receptor subtypes may not be responsible for the LG tail-flip, similar models of a change in net effect after the activation of a specific receptor type could be at work. Localization of receptors and density of specific receptor types in confined regions could result in the dual 5-HT modulatory effects seen in LG.

Slow application of 5-HT has the most pronounced effects (a greater amplitude) on the β component of the LG EPSP (Antonsen and Edwards, 2003). This component-specific effect

implies that the modulatory effects of 5-HT seen in this experiment were likely through the mechanosensory interneurons that link to LG dendrites. The magnitude and direction of change seen in the EPSP after slow application of a high 5-HT concentration could mimic a physiological release of 5-HT from the pericardial organs with 5-HT slowly reaching the cell through the blood (Antonsen and Edwards, 2007). The net effects of 5-HT on the LG neural circuit are thought to be the result of activating second messenger cascades through binding to specific 5-HT receptor types (Araki et al., 2005). Specifically, the net effects seen in the crayfish 5-HT response are thought to be mediated by cyclic adenosine monophosphate (cAMP) which is produced by adenylyl cyclase. By blocking adenylyl cyclase, Araki *et al.* (2013) showed that the effects of 5-HT are cancelled when cAMP is not activated and they proposed that 5-HT's site of action likely lies in the sensory afferent neurons. Therefore, the effects of 5-HT perfusion after AgNP exposure seen in this study could be a manifestation of 5-HT receptor turnover in the sensory afferent neurons in the tail fan or could be due to modulatory action located on the central dendrites of LG (Bacque-Cazenave et al., 2013) or a combination of both biochemical changes. However, it is clear from the increased variability with longer exposure to higher concentrations of AgNP, crayfish are not employing the same biochemical mechanisms within the treatment groups, resulting in different net effects on the cell and ultimately different behavioral endpoints.

Generalized Stress Response

In the beginnings of psychological stress research and the development of what is now considered psychological dogma, researchers investigated the components of the human stress response. Many theories were developed regarding the generalizability of the stress response,

including Selye's famous general adaptation syndrome theory (Selye, 1956). This theory proposes that stress response evoked by different stress forms is the same, thus different types of stressors would elicit identical responses within an individual as well across several individuals. The general adaptation syndrome theory, however, has been challenged by many studies showing that the preparedness of the individual is more of a determinant of the stress response than the stressor itself (Biondi and Picardi, 1999). Indeed, inter-individual variation has been seen in groups with similar work loads and even similar perceptions of their work loads. However, this variation was observed when the individuals faced an unfamiliar, demanding stressor as opposed to stress they encounter as part of their daily experience. Additionally, the biochemical mechanisms employed to deal with a given stressor were still thought to be generalizable. It would appear as though, when facing a novel stressor, crayfish do not achieve the same behavioral modifications seen when they undergo a familiar stressor, like social stress. However, while the behavioral endpoint is markedly different, it is possible that the same biochemical machinery is at work, but is not being used in the organized, carefully tuned manner as it is normally employed. Similarly, it is unknown as to whether the same biochemical pathways are in use to produce the decreased muscle mass seen in some chronically treated crayfish. It is possible that the crayfish lost their ability to carefully set the gain of this system because the resources necessary to do so were allocated instead to the more urgent need for survival. This allocation of resources to survival could explain the lethargic and even erratic behavior because, in both instances, crayfish did not move from one location for long periods of time, sometimes days. What was counterintuitive then, was the increase in lateral giant excitability after chronic exposure to AgNP. Lethargic crayfish with symptoms of starvation would not be expected to be more excitable than crayfish with normal behavior. While the

increased excitability was not statistically significant, there was a clear trend indicating that crayfish who had been exposed to AgNP for chronic periods would be more likely to perform a tail flip under 5-HT perfusion. Given that crayfish's responses were different under 5-HT exposure, it is likely that the tail-flip differences seen between control and treatment groups is highly 5-HT dependent.

Decreased Biologically Available 5-HT

Because 5-HT is utilized as a hormone, neuromodulator, and neurotransmitter in crayfish, if AgNP induced stress caused the crayfish to exhaust their 5-HT resources, artificial introduction of high concentrations of 5-HT to cell through perfusion (like that used in this study) could be over-exciting the receptors present on the membrane resulting in a somewhat chaotic response. If AgNP caused 5-HT to be released onto the lateral giant in higher concentrations as a result of increased stress response, the receptors present on the membrane would be downregulated in order to prevent toxicity due to over excitation. Though, if 5-HT was less available because of resources being allocated elsewhere, then the receptors would have been upregulated due to lack of activity. Some evidence for this hypothesis was seen in preliminary (unpublished) data showing 5-HT present on the A6 ganglion after AgNP exposure. In this study, AgNP treated animals had less 5-HT fluorescence on the A6 ganglion than that seen in control animals. Additionally, chronically treated animals showed more consistency in location of 5-HT fluorescence where 5-HT fluorescence location in acutely treated animals was less consistent. 5-HT fluorescence that was observed in chronically treated animals was most consistently in the posterior region where the hindgut neurons (responsible for hindgut modulation) are located (Musolf et al., 2009). So the specific serotonergic activity while

undergoing AgNP is still relatively unknown, these data are suggestive that there is a change. If the cell had adjusted the number of receptors present on the membrane to accommodate the lower levels of 5-HT seen in these preliminary data, when the cell was exposed to high concentrations of 5-HT over a long period of time through perfusion, it may have been mimicking an over-excitation that would not be realistic if the animal had decreased levels of biologically available 5-HT. Thus, the cell would display non-adaptive characteristics like erratic responses of unpredictable magnitude. So, the toxicity of AgNP could lie in the decrease of biologically available 5-HT, not the change in receptors on the membrane.

Summers (2002) suggests that groups of crayfish employing the same biochemical mechanisms to generate different behavioral outputs is likely indicative of contextual adaptive evolution. For instance, agonistic encounters are common among crayfish, though if dominant and subordinate animals had a static response to social encounters plasticity of behaviors - and dominance heirarchies - would be lost. Having the ability to adapt to changing cultural contexts with different behavioral endpoints while using the same machinery can be very advantageous to an animal. Edwards and Spitzer (2006) further discuss the advantages - and necessity - of plastic behavioral outputs to variable contexts. Crayfish constantly receive input from their environment, which in part governs their cellular response to further input. With a changing environment influencing LG response to stimulation, one can see the benefit of modulating existing response systems to achieve variable behavioral responses. However, with the disruption of this generalized response, AgNP pose a threat to the adaptability to their changing environment. If the animals cannot match their environmental challenges quickly enough with appropriate responses, AgNP could be detrimental to the health of crayfish as well as their surrounding aquatic ecosystem.

Conclusions

The findings from this study indicate that, in the crayfish tail-flip reflex circuit, stress is not generalizable. When crayfish are faced with social stressors, the LG responds to 5-HT perfusion by either increasing or decreasing excitability, a well-characterized and predictable response. A socially subordinate crayfish will respond to 5-HT with decreased LG excitability while socially dominant animals respond to 5-HT perfusion with increased LG excitability. These adaptive responses to contextual changes are thought to be mediated by three types of 5-HT receptors. Two 5-HT receptors are responsible for the short-term change in LG excitability in response to a social context shift. A third type of receptor replaces these receptors and is responsible for the long-lasting LG response enhancement after 5-HT is no longer present (Yeh et al., 1996). The organized response of the crayfish escape circuit to social context is in stark contrast to the response seen in the current study to AgNP exposure. Crayfish have evolved a systematic response to social stress because agonistic encounters are the means by which they develop their social hierarchy. Therefore, social stress is a common, normal stressor that the crayfish would "expect" to encounter; thus it is logical that the crayfish would need an appropriate and quick response to this stressor to deal with it effectively and efficiently. However, AgNP in particular are a recent environmental contaminant that crayfish are certainly facing, yet it would appear as though they do not have a similar, systematic response developed for dealing with this type of stressor. Erratic and disorganized LG response to 5-HT is suggestive of a loss of efficient fine-tuned control of this escape circuit, which has been replaced with a chaotic and costly response. This increased variation and erratic magnitude of response shows that the escape circuit is disturbed in an unusual manner.

Crayfish are an important indicator species that are typically fairly resilient to changing environments - manifest in their ability to withstand harsh environments as well as environmental changes. The erratic response seen in this study should be an indication to us that AgNP which are being deposited into water systems are foreign to crayfish, and they do not have an appropriate, adaptive response for it, which is very harmful. Their response should raise alarm for us that we too may have unpredictable, erratic, and potentially dangerous responses to the same toxicant. While many stressors elicit a predictable response, mediated by the same mechanisms, it seems as though this type of stressor is an exception to the rule, and possibly the stress responses that have developed over many trials are not equipped to handle them. While the aim of this study was not to address AgNP regulation, the findings do suggest that the projection for increasing, unregulated use can be harmful to aquatic species like crayfish, and subsequently the surrounding aquatic ecosystem. The direction of AgNP use should be carefully considered and its application in consumer products evaluated.

REFERENCES

Ahamed M, Karns M, Goodson M, Rowe J, Hussain SM, Schlager JJ, Hong Y (2008) DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells. Toxicology and applied pharmacology 233:404-410.

Ahmed M, Posgai R, Gorey T, Mielsen M, Hussain S, Rowe J, Posgai R, Gorey T, Mielsen M, Hussain S, Rowe J (2010) Silver nanoparicles induced heat shock protein 70, oxidative stress and apoptosis in *Drosophila melanogaster*. *Tox Appl Pharmacol* 242.

Ait-Aissa S, Porcher J, Arrigo A, Lambre C (2000) Activation of the hsp70 promoter by environmental inorganic and organic chemicals: relationships with cytotoxicity and lipophilicity. Toxicology 145:147-157.

Antonsen B, Edwards D (2007) Mechanisms of serotonergic facilitation of a command neuron. *Journal of Neurophysiology* 98:3494-3504.

Antonsen BL, Edwards DH (2003) Differential dye coupling reveals lateral giant escape circuit in crayfish. The Journal of comparative neurology 466:1-13.

Araki M, Hasegawa T, Komatsuda S, Nagayama T (2013) Social status-depended modulation of LG-flip habituation in the crayfish. *The Journal of Experimental Biology* 216:681-686.

Araki M, Nagayama T, Sprayberry J (2005) Cyclic AMP mediates serotonin-induced synaptic enhancement of lateral giant interneuron of the crayfish. J Neurophysiol 94:2644-2652.

Asharani PV, Lian Wu Y, Gong Z, Valiyaveettil S (2008) Toxicity of silver nanoparticles in zebrafish models. Nanotechnology 19:255102.

Asharani PV, Mun GLK, Hande MP, Valiyaveettil S (2009) Cytotoxicity and genotoxicity of silver nanoparticles in human cells. *ACS Nano* 3.

Bacque-Cazenave J, Issa FA, Edwards DH, Cattaert D (2013) Spatial segregation of excitatory and inhibitory effects of 5-HT on crayfish motoneurons. J Neurophysiol 109:2793-2802.

Bae S, Hwang YS, Lee YJ, Lee SK (2013) Effects of water chemistry on aggregation and soil adsorption of silver nanoparticles. *Environmental Health and Toxicology* 28.

Benn T, Cavanagh B, Hristovski K, Posner JD, Westerhoff P (2010) The release of nanosilver from consumer products used in the home. Journal of environmental quality 39:1875-1882.

Bierbower SM, Cooper RL (2009) Measures of heart and ventilatory rates in freely moving crayfish. Journal of visualized experiments : JoVE.

Biondi M, Picardi A (1999) Psychological stress and neuroendocrine function in humans: the last two decades of research. Psychotherapy and psychosomatics 68:114-150.

Blinova I, Niskanen J, Kajankari P, Kanarbik L, Kakinen A, Tenhu H, Penttinen OP, Kahru A (2013) Toxicity of two types of silver nanoparticles to aquatic crustaceans Daphnia magna and Thamnocephalus platyurus. Environmental science and pollution research international 20:3456-3463.

Bondarenko O, Juganson K, Ivask A, Kasemets K, Mortimer M, Kahru A (2013) Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review. Archives of toxicology 87:1181-1200.

Carlson C, Hussain SM, Schrand AM, Braydich-Stolle LK, Hess KL, Jones RL, Schlager JJ (2008) Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species. The journal of physical chemistry B 112:13608-13619.

Cattaert D, Delbecque JP, Edwards DH, Issa FA (2010) Social interactions determine postural network sensitivity to 5-HT. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:5603-5616.

Chairuangkitti P, Lawanprasert S, Roytrakul S, Aueviriyavit S, Phummiratch D, Kulthong K, Chanvorachote P, Maniratanachote R (2013) Silver nanoparticles induce toxicity in A549 cells via ROS-dependent and ROS-independent pathways. Toxicology in vitro : an international journal published in association with BIBRA 27:330-338.

Christian P, Von der Kammer F, Baalousha M, Hofmann T (2008) Nanoparticles: Structure, properties, preparation, and behaviour in environmental media. *Ecotoxicology* 17.

Depledge M, Galloway T (2005) Healthy animals, healthy ecosystems. Front Ecol Environ 3:251-258.

Doolette CL, McLaughlin MJ, Kirby JK, Batstone DJ, Harris HH, Ge H, Cornelis G (2013) Transformation of PVP coated silver nanoparticles in a simulated wastewater treatment process and the effect on microbial communities. Chemistry Central journal 7:46.

Edwards D, Heitler WJ, Krasne FB (1999) Fifty years of a command neuron: the neurobiology of escape behavior in the crayfish. *Trends in Neuroscience* 22:153-161.

Edwards DH, Spitzer N (2006) 6. Social dominance and serotonin receptor genes in crayfish. Current topics in developmental biology 74:177-199.

El Badawy AM, Luxton TP, Silva RG, Scheckel KG, Suidan MT, Tolaymat TM (2010) Impact of environmental conditions (pH, ionic strength, and electrolyte type) on the surface charge and aggregation of silver nanoparticles suspensions. Environmental science & technology 44:1260-1266.

Ellegaard-Jensen L, Jensen KA, Johansen A (2012) Nano-silver induces dose-response effects on the nematode Caenorhabditis elegans. Ecotoxicology and environmental safety 80:216-223.

Foldbjerg R, Olesen P, Hougaard M, Dang DA, Hoffmann HJ, Autrup H (2009) PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. Toxicol Lett 190:156-162.

Fossat P, Bacque-Cazenave J, De Deurwaerdere P, Delbecque JP, Cattaert D (2014) Comparative behavior. Anxiety-like behavior in crayfish is controlled by serotonin. Science 344:1293-1297.

Gagne F, Auclair J, Fortier M, Bruneau A, Fournier M, Turcotte P, Pilote M, Gagnon C (2013) Bioavailability and immunotoxicity of silver nanoparticles to the freshwater mussel *Elliptio complanata*. *Journal of Toxicology and Environmental Health, Part A: Current Issues* 76:767-777.

Gaiser BK, Fernandes TF, Jepson M, Lead JR, Tyler CR, Stone V (2009) Assessing exposure, uptake and toxicity of silver and cerium dioxide nanoparticles from contaminated environments. Environmental health : a global access science source 8 Suppl 1:S2.

Gottschalk F, Sun T, Nowack B (2013) Environmental concentrations of engineered nanomaterials: review of modeling and analytical studies. Environmental pollution 181:287-300.

Griebel G, Rodgers RJ, Perrault G, Sanger DJ (1997) Risk assessment behaviour: evaluation of utility in the study of 5-HT-related drugs in the rat elevated plus-maze test. Pharmacology, biochemistry, and behavior 57:817-827.

Griffitt RJ, Luo J, Gao J, Bonzongo JC, Barber DS (2008) Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environmental toxicology and chemistry / SETAC 27:1972-1978.

Haberl N, Hirn S, Wenk A, Diendorf J, Epple M, Johnston BD, Krombach F, Kreyling WG, Schleh C (2013) Cytotoxic and proinflammatory effects of PVP-coated silver nanoparticles after intratracheal instillation in rats. Beilstein journal of nanotechnology 4:933-940.

Hadrup N, Lam HR (2014) Oral toxicity of silver ions, silver nanoparticles and colloidal silver--a review. Regulatory toxicology and pharmacology : RTP 68:1-7.

Hartenstein V (2006) The neuroendocrine system of invertebrates: a developmental and evolutionary perspective. The Journal of endocrinology 190:555-570.

He D, Dorantes-Aranda JJ, Waite TD (2012) Silver nanoparticle-algae interactions: oxidative dissolution, reactive oxygen species generation and synergistic toxic effects. Environmental science & technology 46:8731-8738.

Herberholz J, Issa FA, Edwards DH (2001) Patterns of neural circuit activation and behavior during dominance hierarchy formation in freely behaving crayfish. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:2759-2767.

Hsin YH, Chen CF, Huang S, Shih TS, Lai PS, Chueh PJ (2008) The apoptotic effect of nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells. *Toxicology Letters* 179:130-139.

Huber R, Orzeszyna M, Pokorny N, Kravitz EA (1997) Biogenic amines and aggression: experimental approaches in crustaceans. Brain, behavior and evolution 50 Suppl 1:60-68.

Hunt PR, Marquis BJ, Tyner KM, Conklin S, Olejnik N, Nelson BC, Sprando RL (2013) Nanosilver suppresses growth and induces oxidative damage to DNA in Caenorhabditis elegans. Journal of applied toxicology : JAT 33:1131-1142.

Issa F, Drummond J, Cattaert D, Edwards D (2012) Neural Circuit reconfiguration by social status. *The Journal of Neuroscience* 32:5638-5645.

Jeon HJ, Yi SC, Oh SG (2003) Preparation and antibacterial effects of Ag-SiO2 thin films by sol-gel method. Biomaterials 24:4921-4928.

Jones AM, Garg S, He D, Pham AN, Waite TD (2011) Superoxide-mediated formation and charging of silver nanoparticles. Environmental science & technology 45:1428-1434.

Kim S, Choi JE, Choi J, Chung KH, Park K, Yi J, Ryu DY (2009) Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells. Toxicology in vitro : an international journal published in association with BIBRA 23:1076-1084.

Krasne F, Shamsian A, Kulkarni R (1997) Altered excitability of the crayfish lateral giant escape reflex during agonistc encounters. *The Journal of Neuroscience* 17:709-716.

Kuklina I, Kouba A, Kozak P (2013) Real-time monitoring of water quality using fish and crayfish as bio-indicators: a review. Environmental monitoring and assessment 185:5043-5053.

Kwok KW, Auffan M, Badireddy AR, Nelson CM, Wiesner MR, Chilkoti A, Liu J, Marinakos SM, Hinton DE (2012) Uptake of silver nanoparticles and toxicity to early life stages of Japanese medaka (Oryzias latipes): effect of coating materials. Aquatic toxicology 120-121:59-66.

Laban G, Nies LF, Turco RF, Bickham JW, Sepulveda MS (2010) The effects of silver nanoparticles on fathead minnow (Pimephales promelas) embryos. Ecotoxicology 19:185-195.

Lee CY, Yang PF, Zou HS (2001) Serotonergic regulation of crustacean hyperglycemic hormone secretion in the crayfish, Procambarus clarkii. Physiological and biochemical zoology : PBZ 74:376-382.

Lee JH, Kim YS, Song KS, Ryu HR, Sung JH, Park JD, Park HM, Song NW, Shin BS, Marshak D, Ahn K, Lee JE, Yu IJ (2013) Biopersistence of silver nanoparticles in tissues from Sprague-Dawley rats. *Particle and Fibre Toxicology* 10.

Liu J, Sonshine DA, Shervani S, Hurt RH (2010) Controlled release of biologically active silver from nanosilver surfaces. ACS Nano 4:6903-6913.

Lorenzon S, Edomi P, Giulianini PG, Mettulio R, Ferrero EA (2005) Role of biogenic amines and cHH in the crustacean hyperglycemic stress response. J Exp Biol 208:3341-3347.

Lu W, Senapati D, Wang S, Tovmachenko O, Singh AK, Yu H, Ray PC (2010) Effect of Surface Coating on the Toxicity of Silver Nanomaterials on Human Skin Keratinocytes. Chemical physics letters 487.

Manke A, Wang L, Rojanasakul Y (2013) Mechanisms of nanoparticle-induced oxidative stress and toxicity. BioMed Research International 2013.

Matsumura Y, Yoshikata K, Kunisaki S, Tsuchido T (2003) Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. Applied and environmental microbiology 69:4278-4281.

Mirzajani F, Askari H, Hamzelou S, Schober Y, Rompp A, Ghassempour A, Spengler B (2014) Proteomics study of silver nanoparticles toxicity on Bacillus thuringiensis. Ecotoxicology and environmental safety 100:122-130.

Mitrano DM, Lesher EK, Bednar A, Monserud J, Higgins CP, Ranville JF (2011) Detecting nanoparticulate silver using single-particle inductively coupled plasma-mass spectrometry. *Environmental Toxicology and Chemistry* 31:115-121.

Mpenyana-Monyatsi L, Mthombeni NH, Onyango MS, Momba MN (2012) Cost-effective filter materials coated with silver nanoparticles for the removal of pathogenic bacteria in groundwater. International journal of environmental research and public health 9:244-271.

Musolf B, Spitzer N, Antonsen B, Edwards D (2009) Serotonergic modulation of crayfish hindgut. *Biol Bull* 50-64.

Newton KM, Puppala HL, Kitchens CL, Colvin VL, Klaine SJ (2013) Silver nanoparticle toxicity to the *Daphnia magna* is a funciton of dissolved silver concentration. Environmental Toxicology and Chemistry 32:2356-2364.

Ohio River Valley Water Sanitation Commission (2013) Ohio River Toxic Release Inventory analysis (TRI), Reporting year 2013. Cincinnati, Ohio: ORSANCO.

Panksepp JB, Huber R (2002) Chronic alterations in serotonin function: dynamic neurochemical properties in agonistic behavior of the crayfish, Orconectes rusticus. Journal of neurobiology 50:276-290.

Ray PC, Yu H, Fu PP (2009) Toxicity and environmental risks of nanomaterials: Challenges and future needs. *Journal of Environmental Health C Environ Carcino Ecotoxicol Rev* 27:1-35.

Sarojini R, Nagabhushanam R, Fingerman M (1995) Dopaminergic and enkephalinergic involvement in the regulation of blood glucose in the red swamp crayfish, Procambarus clarkii. General and comparative endocrinology 97:160-170.

Schapker H, Breithaupt T, Shuranova Z, Burmistrov Y, Cooper RL (2002) Heart and ventilatory measures in crayfish during environmental disturbances and social interactions. Comparative biochemistry and physiology Part A, Molecular & integrative physiology 131:397-407.

Schilderman PA, Moonen EJ, Maas LM, Welle I, Kleinjans JC (1999) Use of crayfish in biomonitoring studies of environmental pollution of the river Meuse. Ecotoxicology and environmental safety 44:241-252.

Selye H (1956) The Stress of Life. New York: McGraw-Hill.

Shahverdi AR, Fakhimi A, Shahverdi HR, Minaian S (2007) Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against Staphylococcus aureus and Escherichia coli. Nanomedicine 3:168-171.

Sirimuthu NM, Syme CD, Cooper JM (2010) Monitoring the uptake and redistribution of metal nanoparticles during cell culture using surface-enhanced Raman scattering spectroscopy. Analytical chemistry 82:7369-7373.

Sondi I, Salopek-Sondi B (2004) Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. Journal of colloid and interface science 275:177-182.

Spitzer N, Edwards DH, Baro DJ (2007) Conservation of structure, signaling and pharmacology between two serotonin receptor subtypes from decapod crustaceans, *Panulirus interruptus* and *Procambarus clarkii*. The Journal of Experimental Biology 211:92-105.

Struzynski W, Dabrowska-Bouta B, Grygorowicz T, Zieminka E, Stuzynska L (2013) Markers of oxidative stress in hepatopancreas of crayfish (*Orconectes limosus,* Raf) experimentally exposed to nanosilver. *Environmental Toxicology*.

Summers CH (2002) Social interaction over time, implications for stress responsiveness. Integrative and comparative biology 42:591-599.

Taju G, Abdul Majeed S, Nambi KS, Sahul Hameed AS (2014) In vitro assay for the toxicity of silver nanoparticles using heart and gill cell lines of Catla catla and gill cell line of Labeo rohita. Comparative biochemistry and physiology Toxicology & pharmacology : CBP 161:41-52.

Teshiba T, Shamsian A, Yashar B, Yeh SR, Edwards DH, Krasne FB (2001) Dual and opposing modulatory effects of serotonin on crayfish lateral giant escape command neurons. *The Journal of Neuroscience* 21:4523-4529.

Tian J, Wong KK, Ho CM, Lok CN, Yu WY, Che CM, Chiu JF, Tam PK (2007) Topical delivery of silver nanoparticles promotes wound healing. ChemMedChem 2:129-136.

Vu ET, Berkowitz A, Krasne FB (1997) Postexcitatory inhibition of the crayfish lateral giant neuron: a mechanism for sensory temporal filtering. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:8867-8879.

United States Environmental Protection Agency (2014) Nanotechnology & Nanomaterials Research.

Walker H (1990) Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd Edition. In: Chapter 72 (Walker, H. et al., eds) Boston: Buttorworths.

Wen L, Santschi P, Gill G, Tang D (2002) Silver concentrations in Colorado, USA, watersheds using improved moethodology. *Environmental Toxicology and Chemistry* 21.

Whiteley CM, Valle MD, Jones KC, Sweetmen AJ (2013) Challenges in assessing release, exposure and fate of silver nanoparticles within the UK environment. *Environmental Science -- Processes & Impacts* 15:2050-2058.

Yamane S, Takahata M (2002) Experimental modification of stepping course in spontaneously initiated locomotor behavior in the crayfish Procambarus clarkii Girard. Journal of comparative physiology A, Neuroethology, sensory, neural, and behavioral physiology 188:13-23.

Yeh SR, Fricke RA, Edwards DH (1996) The effect of social experience on serotonergic modulation of the escape circuit of crayfish. *Science* 271:366-369.

Yeh SR, Musolf BE, Edwards DH (1997) Neuronal adaptations to changes in the social dominance status of crayfish. *The Journal of Neuroscience* 17:697-708.

Yoon KY,

Hoon Byeon J, Park JH, Hwang J (2007) Susceptibility constants of Escherichia coli and Bacillus subtilis to silver and copper nanoparticles. The Science of the total environment 373:572-575.

Zhaowei L, Guogang R, Tao Z, Zhuo Y (2009) Action potential changes associated with the inhibitory effets on voltage-gated sodium current of hippocampal CA1 neurons by silver nanoparticles. *Toxicology* 264:179-184.

Zucker R, Kennedy D, Selverston A (1971) Neuronal circuit mediating escape responses in crayfish. *Science* 173:645-650.

APPENDIX

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Office of Research Integrity

February 19, 2014

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Huntington, WV 25755

Dear Ms. Adkins:

This letter is in response to the submitted thesis abstract entitled *"The Effect of Nanoparticles on Membrane Resistance in the Lateral Giant Neuron of the Crayfish."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) Chair has also deemed this not to be animal research requiring their approval. The information in this study is not considered human subject or animal research as set forth in the definitions contained in the federal regulations. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director