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Article

Effects of copper sulfate exposure on the nervous system of the *Hirudo verbana* leech

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SUMMARY

Copper is a significant environmental contaminant resulting from electronic waste and pesticide use. It is also a micronutrient that is essential for life because it is an enzyme cofactor. However, excess exposure to this heavy metal can lead to adverse physiologic effects. Prior studies have demonstrated neurotoxic effects of copper exposure on arthropods, mollusks, amphibians, and mammals, via mechanisms ranging from oxidative stress to changes in neurotransmitter levels. We focused on toxic effects of copper exposure on the nervous system of the *Hirudo verbana* leech, hypothesizing that copper exposure leads to neurobehavioral changes associated with structural changes to and proteomic changes within nerve ganglia. After exposing leeches to graded concentrations of copper sulfate, we performed neurobehavioral testing to measure their ability to ingest liver and record their movements in a 3-D printed pool. We dissected out nerve ganglia and examined them with light microscopy following hematoxylin and eosin staining. We also extracted, identified, and quantified proteins from the nerve ganglia using high performance liquid chromatography-mass spectrometry. We found that copper exposure was associated with neurobehavioral changes including food avoidance, diminished dark-seeking behavior, and decreased motility. Furthermore, we found histopathologic changes and proteomic alterations within nerve ganglia of copper-exposed leeches. These findings contribute to our understanding of potential mechanisms for nervous system damage with copper exposure. The results are concerning as they indicate that copper accumulation in the environment has tangible neurological consequences for exposed organisms.

INTRODUCTION

Americans spent $71 billion on telephone and communication equipment in 2017 (1). When we buy a new electronic product, such as a phone, we tend to dispose of our prior one. This cycle of consumption has made electronic waste (e-waste) the world's fastest-growing solid-waste stream. In 2019, 53 million tons of e-waste were generated globally, a problem that disproportionately affects developing nations where e-waste is often shipped (1). Copper is the third most consumed metal in the world; three-quarters of its consumption goes towards electronics, such as electroplating and interconnects on silicon chips and in printed circuit boards (2).

Copper is also used as an effective fungicide and pesticide and is one of the most widely used algicides for the control of phytoplankton in lakes, reservoirs, and ponds (3). Being persistent pollutants, heavy metals like copper accumulate in the environment and consequently contaminate food chains (4). Therefore, between e-waste and agricultural use of copper, contamination of aquatic and terrestrial ecosystems with copper is an environmental pollutant of public health concern.

Copper exists in ionic, exchangeable, organic, precipitated, and residual forms in soil. Copper sulfate ($CuSO_4$), which was used in this study, is the most common naturally occurring compound of copper. In the United States, the copper content of soil is 2 to 100 parts per million (ppm), with an average value of about 30 ppm. Concentrations in drinking water can vary widely from ≤0.005 to 10 ppm (5).

Copper is a micronutrient essential for life, required as a cofactor for a number of enzymes including lysyl oxidase, cytochrome c oxidase, ferroxidase ceruloplasmin, and copper/zinc superoxide dismutase (6, 7). Since excessive exposure can lead to adverse effects, including kidney, liver, and heart failure in humans, it is necessary to detect and evaluate the impact of this pollutant on exposed organisms (7). Using physiological, behavioral, and biochemical studies to understand changes arising from sub-lethal exposure to copper is helpful in assessing potential toxicity.

Numerous studies have shown neurotoxic effects of copper exposure on arthropods, mollusks, and amphibians. Prior studies on carp showed that copper exposure decreased brain serotonin and dopamine neurotransmitter levels, which were associated with altered feeding behavior and locomotor control (8). Another study exposing tilapia fish to copper nanoparticles demonstrated that the oxidative stress enzymes glutathione, glutathione-s-transferase, and acetylcholinesterase were reduced in the brains of treated fish groups, which may affect their growth and development (9). In a study on zebrafish, copper exposure was associated with impaired behavioral function, muscle acetylcholinesterase activity, and oxidative stress in muscle samples (10).

In mammals, several multifactorial mechanisms of copper neurotoxicity have been proposed, suggesting its role in autism spectrum disorders and in neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Alzheimer's, Parkinson's,

Huntington's, and Wilson's diseases (11). Wilson's disease is an autosomal recessive disorder caused by mutations in ATPase7B, a hepatic enzyme responsible for transmembrane transport and excretion of copper into bile (12). The metabolic defect leads to accumulation of free copper in the liver, blood, brain, and kidneys. Patients present with tremors, involuntary muscle movements, clumsy gait, and speech difficulties. In Wilson's disease, impaired cellular homeostasis of copper may trigger neurodegeneration through various mechanisms, notably induction of oxidative stress, promotion of α-synuclein aggregation and fibril formation, and activation of microglial cells leading to inflammation and impaired production of metalloproteins (12).

We sought to examine damage to the nervous system of the *Hirudo verbana* leech with copper exposure. The leech nervous system consists of a ventral nerve cord which connects the cephalic ganglia (or 'head brain') to a prominent ganglion at the caudal end of the nerve cord (the 'tail brain') with 21 segmental ganglia in between (13). The leech is an ideal organism to study the implications of copper exposure because of the extremely simple structure of its nervous system. Additionally, even though the leech nervous system is much simpler than vertebrate nervous systems, principles of its function have been found to have counterparts even in humans (14).

Several studies have begun examining potential neurotoxic effects of copper on the *Hirudo verbana* leech. One study exposed leeches to a 0.2 ppm copper solution for 96 hours and then examined fibroblast cells and body wall structures using light and transmission electron microscopy, finding irregular and disordered muscle fibers, degenerated epithelial cells and fibroblasts, and short and scattered microvilli (15). Another study found changes in feeding activity as well as body posture and shape of leeches exposed to heavy metals like copper due to suppressed acetylcholinesterase activity (16). These studies suggest that exposure to copper leads to neurological, behavioral, and muscular changes. Further work examining potential neurobehavioral effects of copper toxicity in the leech is important to learn about ramifications of exposure to this environmental contaminant.

In this study, we used proteomics to identify and quantify proteins present in the ganglia as a novel approach to investigate the mechanisms by which copper damages the nervous system of the *Hirudo verbana* leech. Proteomics is the large-scale study of proteins, with the proteome being the entire set of proteins produced or modified by an organism or system. At least 238 proteins associated with *Hirudo verbana* have been identified (17). To determine proteins specifically associated with neural development, previous researchers severed interganglionic connections in leeches and found changes in the following: neuroinflammatory proteins, cytoskeleton proteins and modulatory components, neuronal sprouting and axon guidance proteins, and hemi-channels related to gap junction formation (18).

We hypothesized that copper exposure would damage the nervous system of the *Hirudo verbana* leech, leading to neurobehavioral changes, injury to the nerve ganglia as visible with light microscopy, and up- or down-regulation of proteins within the nerve ganglia. We included neurobehavioral testing to assess whether copper sulfate exposure would affect food ingestion, light avoidance, and motility, since feeding and ability to avoid predators are essential components to survival. Not only did we find impairment in each of these components of neurobehavioral testing with copper exposure, but we also observed damage to the nerve ganglia with histopathologic and proteomic analysis.

RESULTS

To study effects of copper exposure on the nervous system of the leech, we exposed leeches to graded concentrations of copper for 96 hours and then performed behavioral, microscopy, and proteomic studies. For neurobehavioral testing, we examined food avoidance, dark-seeking behavior, and motility. For food ingestion studies, we recorded the length of time the leeches attached to and ingested liver cubes. We performed light avoidance and motility testing by video-recording leech activity in a water-filled 3-D printed pool with open and opaque chambers. We dissected out and prepared nerve ganglia using hematoxylin and eosin staining to assess for damage visible with light microscopy. Lastly, we studied the proteins within nerve ganglia to determine changes with copper exposure.

We began with a mortality study to assess the maximal $CuSO_4$ concentration that the leeches could survive. One hundred percent of leeches survived with exposure to 0 (control group), 0.1, 0.2, 0.4, 2, and 5 ppm $CuSO_4$ exposure for 96 hours. However, at 10 ppm $CuSO_4$ and higher, no leeches survived (p = 0.001, one-way ANOVA test). In aggregate, we confirmed that the 0-5 and 10-500 ppm exposure groups differed significantly from each other ($p < 0.05$, post-hoc Tukey's test), but there was no difference within the 0-5 and 10-500 ppm groups. This indicated that $CuSO_4$ concentrations should not exceed 5 ppm for the rest of the experiments.

Copper-exposed leeches showed significant impairment in ingestion of liver such that with exposure to 0.5 ppm $CuSO_4$ or above, they did not attach to the liver cubes. Control leeches quickly moved in an average of 48 seconds to the liver, latched, and ingested for the remainder of the 30 minute observation period ($p = 1 \times 10^{-33}$, one-way ANOVA test, **Figure 1**). The 0, 0.2, and 0.5-5 ppm exposure groups differed significantly, with 96, 20, and 0 percent of feeding time respectively ($p < 0.05$, post-hoc Tukey's test). There was no significant difference within the 0.5, 2, and 5 ppm groups (p = 0.10, 0.08, 0.15).

Regarding motility, leeches exposed to 0.5 and 5.0 ppm $CuSO_4$ explored a much smaller proportion of their experimental habitat, a 3-D printed pool with open and opaque chambers (**Figure 2**). Copper-exposed leeches demonstrated a marked decrease in motility in center-of-mass track diagrams (**Figure 3**). We calculated a vitality index for each leech using a logarithmic scale, which quantified the total area of the pool explored. We created the vitality index to measure the total habitat footprint of the leeches, since some of the copper exposed leeches pivoted in circles but did not explore the chambers of

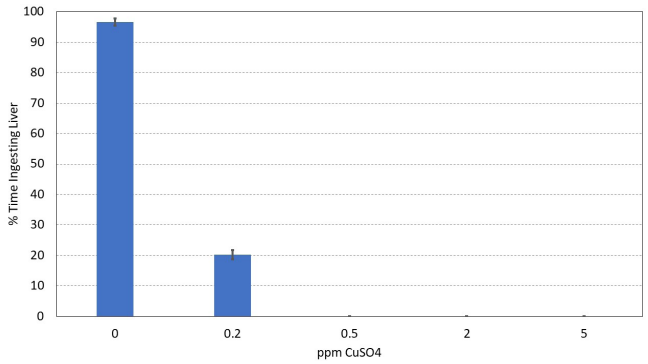

Figure 1: Liver ingestion study. As part of neurobehavioral testing, ingestion of liver was assessed. Five leeches per group (n = 25) were exposed to graded copper concentrations for 96 hours and then placed near 15 g liver cubes and observed for 30 minutes. With exposure to 0.2 ppm $CuSO_4$, liver ingestion was diminished, and at 0.5 and above ppm $CuSO_4$, leeches did not attach to liver cubes. The error bars represent the standard deviation. *p < 0.05.

the pool. The vitality index varied from a minimum of 16.0 X 10^0 for a 5 ppm leech to a maximum of 163 X 103 for a 0 ppm leech. Not only were the vitality indices for the copper-exposed leeches significantly decreased compared with controls, but the copper-exposed leeches also completely failed to seek out the opaque area of the pool (p = 1.26 X 10-6, one-way ANOVA test, **Figure 4**). The 0.5 and 5 ppm exposure groups had significantly lower vitality indices compared with controls (p < 0.05, post-hoc Tukey's test). There was no significant difference in vitality indices within the 0.5 and 5 ppm groups.

We performed histopathologic studies to examine visible effects of $CuSO_4$ exposure on components of the nervous system. Each neuron had a round cell body and a tail-like axon. Ganglia, or clusters of neurons, were clearly identified, containing central clumps of axons surrounded by groupings of neuron cell bodies (**Figures 5-6**). In control ganglia, the neuron cell bodies were uniformly round, with dense cytoplasm and distinct nuclei. Around the peripheries of the neuron cell bodies, there were definite borders of satellite cells, also known as glial cells which cover the surface of neuron cell bodies. The ganglia contained tightly packed whorls of

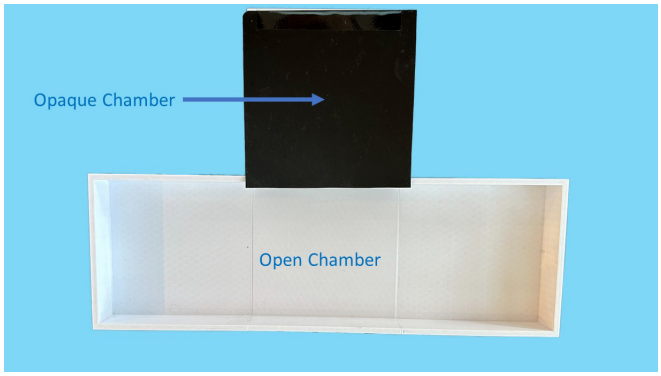

Figure 2: 3-D printed pool used for motility studies. The printed pool is 24 x 16 x 3 inches, filled with 1 inch of room temperature water, with open and opaque chambers for light sensitivity testing.

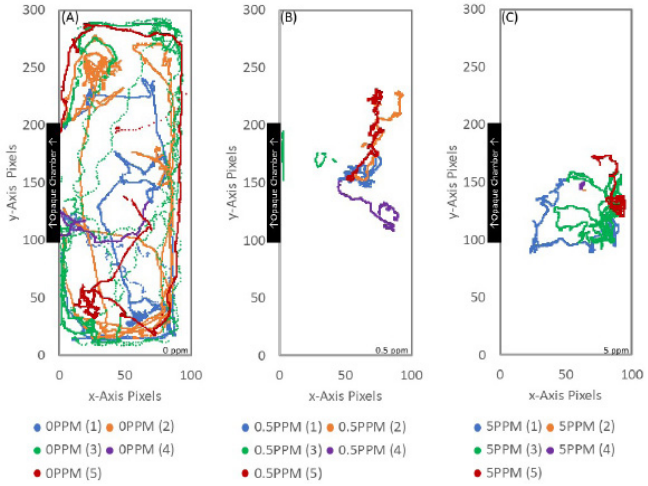

Figure 3: Center-of-mass track diagrams. To assess motility as part of neurobehavioral testing, 5 leeches per group were exposed to 0 (A), 0.5 (B), and 5 (C) ppm copper concentrations for 96 hours (n=15), and their movement in a 3-D printed pool filled with room temperature distilled water was tracked individually for 15 minutes. Leeches exposed to 0.5 and 5 ppm $CuSO_4$ explored a much smaller proportion of their experimental habitat compared with controls.

axons.

In the ganglia of leeches exposed to 0.5 ppm $CuSO_4$, there were diminished numbers of neuron cell bodies. The neuron cell bodies were no longer uniformly round; some were elongated and compressed. The neuron cell body cytoplasm varied in density, and nuclei were no longer visible. Some neuron cell bodies still had distinct satellite cell borders. The axons were loosely organized with space and vacuoles between.

In the ganglia of leeches exposed to 5 ppm $CuSO_4$, there were also markedly diminished numbers of neuron cell bodies. There was balloon degeneration of the neuron cell bodies with lacy breakdown of cytoplasm. The neuron cell bodies had indistinct borders, with absence of satellite cells. Furthermore, neuron cell body nuclei were absent. There were fibrillar changes, disruption, and disorganization of the axons.

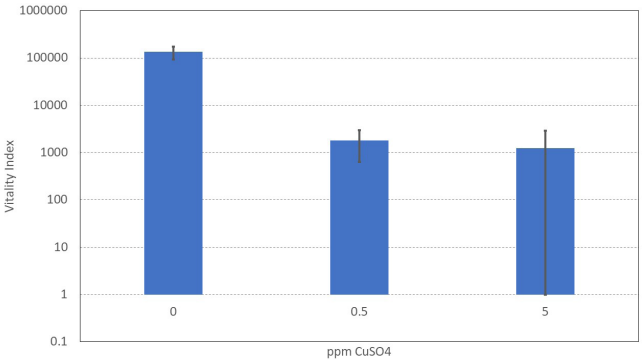

Figure 4: Vitality indices. To quantify and compare vitality, a vitality index was calculated for each leech: average velocity x maximum rectangular area explored. The above bar graph shows significant variation in mean and standard deviations of the vitality indices comparing 5 leeches exposed to 0, 0.5, and 5 ppm $CuSO_4$ (n=15). *p < 0.05.

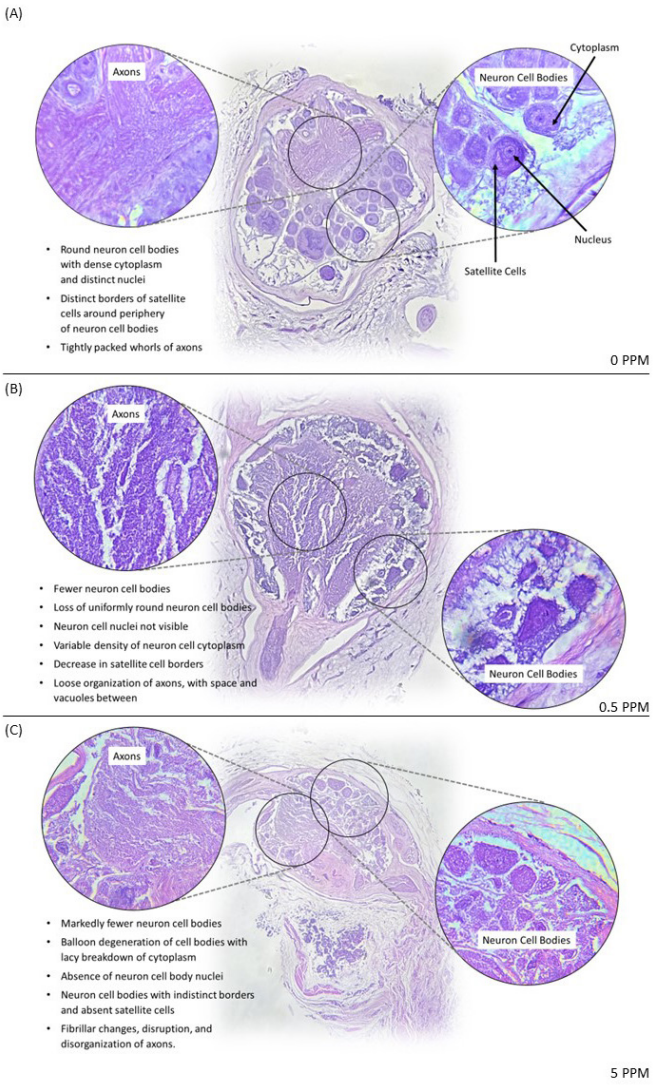

Figure 5: Histopathology testing: Effects of copper on ganglion structure. For histopathology testing, 5 leeches per group were exposed to 0 (A), 0.5 (B), and 5 (C) ppm copper concentrations for 96 hours. Nerve cords and ganglia were excised, stained, and examined using light microscopy. There was visible damage to the cellular structures of the ganglia with copper exposure.

Following 96 hours of $CuSO_4$ exposure from 0 to 0.5 ppm, we performed proteomic testing on ganglia to assess the effects of $CuSO_4$ exposure on the very protein makeup of the leech nervous system. Of the1220 total proteins identified, most of the proteins could not be quantified because either the mass spectrometry signal was too low or there was large interference signal from a co-eluting peptide that made it impossible to attribute the mass spectrometry signals to only one peptide. We quantified 28 proteins with certainty and found significant variation in ion intensity between experimental groups for 15 proteins (**Table 1**). Negative values indicate variation below the mean for ion intensity. Twelve proteins were significantly down- or up-regulated with $CuSO_4$ exposure (**Table 2**). Specifically, the RecQ family ATP-dependent helicase, patatin family protein, LysR family transcriptional regulator, tractin, and zona occludens

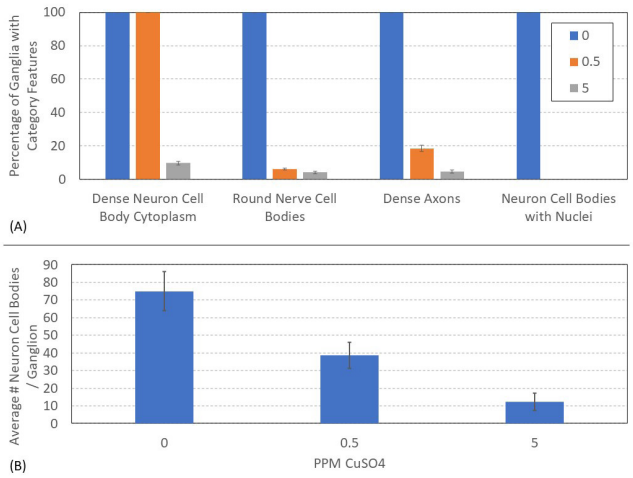

Figure 6: Histopathology testing. Histopathologic features from 4 ganglia from 5 leeches from each experimental group (n=60) were tallied: density of neuron cell body cytoplasm, roundness of neuron cell bodies, density of axons, and presence of nuclei in neuron cell bodies (A). Number of neuron cell bodies per ganglion were also counted (B). Mean ± standard deviation of the percentage of ganglia with the feature were recorded. Significant decreases in each of these observations was noted with copper exposure. *$p < 0.05$.

toxin were downregulated with copper exposure. The Bdellin B-3, Eglin C, and 2-methylcitrate dehydratase proteins were upregulated with copper exposure. These affected proteins are integral to nerve cell structure and function. Helicases unwind double-stranded DNA and RNA using energy from ATP hydrolysis. Patatin is a storage protein but also has enzymatic activity by catalyzing cleavage of fatty acids from membrane lipids. DNA transcriptional regulators orchestrate gene activity by regulating a cell's conversion of DNA to RNA (transcription). Tractin regulates axonal outgrowth, and zona occludens toxin increases membrane permeability by affecting the structure of intercellular tight junctions. Bdellin blocks trypsin, which is a serine protease that hydrolyzes proteins. Eglin C inhibits cathepsin G, an anti-inflammatory agent (19).

DISCUSSION

Treating leeches with graded concentrations of copper resulted in damage to the nervous system; copper exposure was associated with leech mortality and neurotoxicity as manifested by neurobehavioral, histopathologic, and proteomic changes in concentrations exceeding 5 ppm. Leeches exposed to copper showed impaired attachment to and ingestion of liver samples, indicating that they would be more likely to starve. As copper-exposed leeches were less motile, and did not explore their environment or seek out darkness, they would be more susceptible to predators.

Damage to the nerve ganglia was clear at the microscopic level, with multiple changes indicating destruction of the neuron cell bodies and axons. With copper exposure, neuron cell bodies lost their tight, uniformly round shape and showed loose balloon degeneration with the highest levels of copper exposure. The cytoplasm of control neuron cell bodies was dense,

PROTEIN	0 PPM	0.01 PPM	0.05 PPM	0.5 PPM	ANOVA
RecQ family ATP-dependent DNA helicase	1.59 +/- 0.26	-0.36 +/- 0.37	-0.42 +/- 0.37	-0.81 +/- 0.06	0.001
Uncharacterized protein	1.31 +/- 0.75	-0.35 +/- 0.16	-0.03 +/- 0.88	-0.93 +/- 0.17	0.001
Bdellin B-3	1.56 +/- 0.44	-0.56 +/- 0.36	-0.59 +/- 0.55	-0.42 +/- 0.18	0.001
Neurohemerythrin	1.44 +/- 0.75	-0.34 +/- 0.37	-0.46 +/- 0.69	-0.64 +/- 0.28	0.001
Intermediate filament gliarin	-0.86 +/- 0.41	0.27 +/- 0.71	-0.59 +/- 0.82	1.19 +/- 0.46	0.002
Patatin family protein	0.89 +/- 0.58	0.70 +/- 0.88	-0.57 +/- 0.59	-1.01 +/- 0.18	0.002
LuxR family transcriptional regulator	-0.35 +/- 0.28	-0.81 +/- 0.79	-0.16 +/- 0.21	1.31 +/- 0.95	0.003
Uncharacterized protein	-0.85 +/- 0.17	0.13 +/- 0.66	1.14 +/- 1.16	-0.43 +/- 0.48	0.011
Eglin C	0.96 +/- 1.18	0.40 +/- 0.52	-0.40 +/- 0.59	-0.96 +/- 0.27	0.012
LysR family transcriptional regulator	0.73 +/- 0.28	0.50 +/- 0.96	-0.05 +/- 1.05	-1.18 +/- 0.08	0.013
Flagellar hook-length control protein	-0.84 +/- 0.16	-0.57 +/- 0.92	0.71 +/- 0.67	0.70 +/- 0.99	0.022
Putative phosphoenolpyruvate synthase regulatory protein	1.09 +/- 1.10	0.11 +/- 0.14	-0.57 +/- 0.83	-0.64 +/- 0.70	0.029
Uncharacterized protein	-1.07 +/- 0.14	0.80 +/- 0.80	-0.04 +/- 1.34	0.32 +/- 0.10	0.032
Basal-body rod modification protein	1.01 +/- 1.30	-0.25 +/- 0.62	0.12 +/- 0.67	-0.88 +/- 0.05	0.036
Tractin	-0.92 +/- 0.09	0.41 +/- 1.26	-0.34 +/- 0.93	0.84 +/- 0.28	0.037
2-methylcitrate dehydratase	1.10 +/- 1.11	-0.29 +/- 0.62	-0.28 +/- 1.05	-0.53 +/- 0.28	0.065
Uncharacterized protein	-0.10 +/- 0.74	-0.78 +/- 0.01	0.99 +/- 1.31	-0.11 +/- 0.76	0.072
PTS-dependent dihydroxyacetone kinase operon transcriptional regulator DhaR	-0.71 +/- 0.24	-0.48 +/- 1.00	0.35 +/- 0.63	0.84 +/- 1.21	0.083
IS630 family transposase	0.71 +/- 1.01	0.48 +/- 0.82	-0.45 +/- 1.11	-0.74 +/- 0.31	0.099
Zona occludens toxin	-0.55 +/- 0.18	-0.21 +/- 0.56	-0.27 +/- 1.21	1.03 +/- 1.10	0.116
Uncharacterized protein	-0.50 +/- 0.45	-0.11 +/- 1.40	0.95 +/- 1.05	-0.35 +/- 0.17	0.173
Ribonucleoside-diphosphate reductase small chain	0.47 +/- 1.34	0.53 +/- 1.06	-0.17 +/- 0.64	-0.84 +/- 0.14	0.173
Uncharacterized protein 2	0.02 +/- 0.68	-0.41 +/- 0.16	0.89 +/- 0.64	-0.49 +/- 0.06	0.186
Intermediate filament macrolin	0.79 +/- 0.67	0.14 +/- 1.07	-0.69 +/- 0.64	-0.25 +/- 0.56	0.193
Regulator	0.17 +/- 0.57	0.58 +/- 1.63	0.04 +/- 0.64	-0.80 +/- 0.63	0.275
FAD-binding oxidoreductase	0.63 +/- 1.49	-0.58 +/- 0.46	-0.25 +/- 0.64	0.21 +/- 1.18	0.363
Putative potassium voltage-gated channel Shab subfamily 2	0.02 +/- 1.36	0.03 +/- 1.23	0.34 +/- 0.64	-0.40 +/- 0.17	0.808
Helix-turn-helix transcriptional regulator	-0.35 +/- 0.69	-0.04 +/- 0.96	0.22 +/- 0.64	0.16 +/- 0.26	0.879

Table 1: Effect of copper on normalized protein ion intensities. Following 96 hours of $CuSO_4$ exposure from 0 to 0.5 ppm, proteomic assays were performed on ganglia (n=8 total ganglia). Significant variation was found in in ion intensity between experimental groups for 15 proteins using one-way ANOVA testing setting p-value significance at 0.05. Green highlighted proteins varied significantly with different concentrations of copper. Ion abundances translate into peptides, allowing for protein identification.

but cytoplasm in the group with copper exposure became disorganized, containing spaces. The nuclei of the neuron cell bodies were no longer visible with copper exposure; this is concerning as the nuclei are the cellular "command centers." Numbers of neuron cell bodies also diminished with copper exposure. The string-like axons of the neurons became loosely organized with copper exposure, containing space and vacuoles between. All

PROTEIN	0 PPM			0.5 PPM			TTEST	Δ	Function
RecQ family ATP-dependent DNA helicase	0.73	+/-	0.28	-1.18	+/-	0.08	0.00042	DOWN	Serine-type endopeptidase inhibitor, inhibits trypsin, pepsin
Uncharacterized protein	1.59	+/-	0.26	-0.81	+/-	0.06	0.00020	DOWN	
Patatin family protein	1.31	+/-	0.75	-0.93	+/-	0.17	0.00780	DOWN	Lipyl acyl hydrolase activity
LysR family transcriptional regulator	0.96	+/-	1.18	-0.96	+/-	0.27	0.04377	DOWN	DNA transcriptional regulator
Tractin	1.56	+/-	0.44	-0.42	+/-	0.18	0.00114	DOWN	Muscle development, nerve formation
Zona occludens toxin	0.89	+/-	0.58	-1.01	+/-	0.18	0.00483	DOWN	Scaffolding protein, regulates assembly of cellular junctions
Uncharacterized protein	1.44	+/-	0.75	-0.64	+/-	0.28	0.00758	DOWN	
Regulator	1.09	+/-	1.10	-0.64	+/-	0.70	0.04536	DOWN	
Bdellin B-3	-0.86	+/-	0.41	1.19	+/-	0.46	0.00058	UP	Serine-type endopeptidase inhibitor, inhibits trypsin, pepsin
Eglin C	-0.35	+/-	0.28	1.31	+/-	0.95	0.03448	UP	Serine-type endopeptidase inhibitor, inhibits trypsin, pepsin
Uncharacterized protein	-0.92	+/-	0.09	0.84	+/-	0.28	0.00041	UP	
2-methylcitrate dehydratase	-1.07	+/-	0.14	0.32	+/-	0.10	0.00001	UP	Hydrolase, cleaves carbon-oxygen bonds

Table 2: Relative abundance of reporter ion intensities for proteins. The relative abundance of reporter ion intensities (number of standard deviations from the mean) was compared for the 0 and 0.5 ppm groups using Student's t-test, with 12 proteins being statistically significantly down- or up-regulated with $CuSO_4$ exposure.

of these observed changes with copper exposure were visible manifestations of copper-induced injury.

Regarding proteomic studies, extraction of proteins from nervous tissue and ganglia of *Hirudo verbana* leeches was possible. This has not previously been reported in the scientific literature as a means of evaluating effects of copper toxicity and proved to be an excellent way of assessing the effects of copper on the nervous system. Several proteins essential to nerve cell structure and functioning were significantly upregulated and downregulated with copper exposure, suggesting that the neuronal structure was wounded and the nervous system damaged. These alterations to multiple proteins that are essential to neural health contribute to our understanding of potential mechanisms for damage to the nervous system with copper exposure.

These findings ae concerning as they indicate that environmental copper accumulation from sources such as e-waste or pesticides may have tangible consequences for exposed animals and even humans. Moreover, excess copper exposure could have implications for multi-organ system dysfunction beyond the nervous system. The Environmental Protection Agency uses 1.3 ppm of copper in drinking water as an "action level," and our work noted neurological damage in the leech model at levels below this recommended regulatory level (20). Granted, given sheer differences in size and physiology comparing leeches and humans, the dramatic changes noted in the leeches in this study would not be anticipated to occur in humans at this level of exposure. However, it is disturbing that copper concentrations can exceed 17,000 ppm in contaminated soil, in which case harm to the human nervous system could be more likely (21). It is imperative that electronic waste is handled properly to avoid contamination of soil and water sources with heavy metals including copper. Additionally, copper-containing pesticides should be used sparingly since this is another common source of copper buildup in the environment (3).

Limitations to all aspects of the study included finite resources and time. It would have been most ideal to study a larger number of leeches and to repeat the experiments multiple times to be able to increase sample size. The study design was additionally limited by a relative paucity of information about the *Hirudo* proteome as it relates to the nervous system. Expanding this database will be essential to future proteomics studies involving this species. It is also possible that identification of proteins was limited by potential protein

aggregation in copper-treated groups, since copper can induce protein instability.

Regarding future studies, it would be interesting to repeat the experiments using a compound such as sodium chloride in place of $CuSO_4$ to verify that the observed changes were not due simply to changes in ionicity in the exposure water. The study could also be repeated using a separate copper salt to avoid any potential toxicology from excess sulfate exposure. These tests could be also repeated using another divalent cation to determine whether similar toxicity occurs at similar ppm values. Regarding the initial mortality studies, these could be repeated exposing the leeches to $CuSO_4$ concentrations between 5 and 10 ppm to pinpoint the concentration at which 50% of the leeches die. Nervous system damage was evident with exposure to $CuSO_4$ > 0.5 ppm. The exact concentration below 0.5 ppm at which detrimental effects begin to occur was not determined and could be important in potentially lowering action levels for contaminated soil and water. Future research should further quantify this dose-response effect. In our liver ingestion study, by 0.2 ppm $CuSO_4$, food avoidance was observed. Our subsequent studies limited exposure to 0.5 and 5 ppm $CuSO_4$, and this range should be expanded in future experimentation. Decreased motility, increased light avoidance, and histopathologic and proteomic changes were all observed with exposure at 0.5 ppm $CuSO_4$, but these changes may well occur at lower exposure levels. The threshold at which alterations begin to manifest should be better identified.

Additional future applications of this project include studying the effects of copper toxicity on other organ systems, such as the gastrointestinal or reproductive systems, and expanding exposure to additional heavy metals (e.g., lead, magnesium, aluminum, titanium, cadmium). Furthermore, it would be compelling to investigate sustainable ways of remediating copper contaminated soil and water by studying bioleaching and bio-immobilization approaches. For instance, patients with high levels of copper from Wilson's disease are treated using copper chelating agents (penicillamine, trientine, and dimercaprol), which lower blood and tissue copper levels (12). Perhaps a similar type of environmental chelating agent could be used to reduce copper levels in the environment. Lastly, exploring the public health and social justice aspects of the problem of heavy metal environmental contamination will be very relevant to pursue through correlating copper levels at different geographical locations with health outcomes.

MATERIALS AND METHODS

We obtained 86 *Hirudo verbana* leeches from Biopharm Leeches and maintained them at room temperature (65ºF) in distilled water.

To plan for further experimentation, we performed a leech mortality study to determine the maximal $CuSO_4$ (Carolina Biological Supply Company, 20%) concentration the leeches could survive. For 96 hours, we placed five leeches per group in room temperature distilled water containing the following concentrations of $CuSO_4$: 0, 0.1, 0.2, 0.4, 2, 5, 10, 50, 125, 250, and 500 ppm. We documented survival at the conclusion of the 96 hours.

Neurobehavioral Studies: Liver Ingestion, Light Avoidance, and Motility

For 96 hours, we placed 5 leeches per group in room temperature distilled water containing the following concentrations of $CuSO_4$: 0 ppm, 0.2 ppm, 0.5 ppm, 2 ppm, and 5 ppm. Then we placed each leech on a 10-inch white plate, three inches from a pre-weighed 15 g cube of liver. We observed the leeches for 30 minutes, and recorded the length of time the leeches attached to and ingested liver.

Using a 3-D printer, we created a 24 x 16 x 3 inch pool with open and opaque chambers, which was filled with one inch depth of room temperature water (**Figure 3**). For 96 hours, we placed five leeches per group in room temperature distilled water containing one of the following concentrations of $CuSO_4$: 0 ppm, 0.5 ppm, and 5 ppm. Following the exposure period, we separately placed each leech in the 3-D printed pool and recorded for 15 minutes. We recorded the patterns of movement and swimming, including whether the leech sought the opaque chamber ("hid"). We repeated these experiments three times per leech.

With MATLAB image processing techniques, we used the pixels associated with each leech to estimate the leech's center of mass. The contrast between the black leech and white pool created an environment where a constant threshold in intensity allowed for accurate binary images, with leech pixels equal to "1" and background "0". We used the center of mass between frames to compute the leech's velocity: center of mass distance covered/time. We measured and averaged the velocity over the number of frames the leech was visible. We calculated a vitality index for each leech: average velocity x maximum area covered by the leech.

Histopathology Studies

For 96 hours, we placed five leeches per group in room temperature distilled water containing one of the following concentrations of $CuSO_4$: 0 ppm, 0.5 ppm, and 5 ppm. Subsequently, we anesthetized the leeches with 8% ethanol. We dissected the nerve cords, including the ganglia, from the five leeches from each experimental group and prepared them for hematoxylin and eosin staining at Beth Israel Deaconess Medical Center, Boston. The tissues were placed in 10% neutral buffered formalin, passed through solutions of increasing alcohol concentration until 100% alcohol was reached to dehydrate, cleared with xylene, and embedded in paraffin wax. From these, we obtained three-micrometer thick sections, placed these on slides, deparaffinized with xylene solution, and rehydrated with decreasing alcohol concentration until 70% alcohol was reached. We stained the slides with hematoxylin, eosin, and nuclear (Harris hematoxylin) stains for 8 minutes, submerged them in water for 10 minutes, and counterstained with 1% alcoholic eosin for 6 minutes. To achieve dehydration, we performed the reverse process, by passing the samples through increasing alcohol concentrations and xylene. We covered the slides with slips and examined them with light microscopy (OMAX digital lab trinocular compound

LED microscope, 2500X magnification). Sixty ganglia were examined in total (4 ganglia from the 5 leeches from each of the 3 experimental groups). We used descriptive analysis for histopathology interpretation.

Proteomics Testing

For 96 hours, we placed two leeches per group in room temperature distilled water containing one of the following concentrations of $CuSO_4$: 0 ppm, 0.01 ppm, 0.05 ppm, and 0.5 ppm at the University of Massachusetts Center for Proteomics Research. Then, we anesthetized leeches with 8% ethanol and excised the nerve cord, including ganglia, from each leech. For each leech, we added 1 mL of protein extraction buffer (M-PER, Thermo Fisher) to two ganglia and the associated nerve cord in a 1.5 mL snap cap vial (22). We thoroughly homogenized the tissue, centrifuging the sample at 15,000 g for 10 minutes at 4°C. This produced two protein extracts from each leech. We performed a total protein assay on the supernatants, aliquoting the equivalent of 100 mg of total protein from the extract into a fresh 1.5 mL snap cap vial and keeping this on ice. We added a solution of phosphate buffered saline (PBS) to give a final volume of 100 mL. To reduce disulfide bonds, we added 4 µL of 500 mM dithiothreitol (DTT, Thermo Fisher), and incubated the samples at 80°C for 45 minutes. We left the samples to cool at room temperature for 10 minutes. Afterwards, we added 11.5 µL of 500 mM iodoacetamide (Thermo Fisher) in ammonium bicarbonate solution, and incubated the samples at room temperature in the dark for 1 hour to alkylate the reduced disulfides. We added pre-chilled, mass-spectrometry grade 100% acetone (460 µL, Fisher Chemical) and kept the samples at -20°C for 1 hour to precipitate the proteins. We centrifuged the samples for 10 minutes at 16,000 g and 4 °C. We dried and resuspended the protein pellets in 100 µL of 50 mM triethylammonium bicarbonate (TEAB). We added trypsin/lysine mixture (4 µL, Promega) to the protein sample, and incubated the samples at 37°C overnight. We stopped protein digestion in 10 minutes at -80°C. We labeled a total of 2 mg of each of the digested samples with a unique TMT16 reagent (Thermo Fisher). We combined, dried, and reconstituted the 16 TMT-labeled digests in 0.1% formic acid.

We performed all liquid chromatography-mass spectrometry (LC-MS) experiments using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher). We coupled the mass spectrometer to an EASY-nLC 1200 system (Thermo Fisher). The high-performance liquid chromatography (HPLC) mobile phases were 96.1:3.9 water/acetonitrile with 0.1% formic acid (A) and 20.0:80.0 water/acetonitrile with 0.1% formic acid (B). The flow rate was 300 nL/min, and the following gradient was used for each run: 0% B for 5 min, 0−5% B for 10 min, 5−30% B for 150 minutes, 30−90% B for 30 minutes, and 90% B for 10 minutes. We injected the equivalent of 750 ng of the combined TMT labeled digest onto a 75 µm × 150 mm EASY-Spray column (Thermo Fisher) for rapid separation liquid chromatography (RSLC). Electrospray ionization was performed at a voltage of 1.9 kV. We operated the mass spectrometer in the data-dependent mode. Survey scans were collected in a range of 400−1600 m/z in the orbitrap at a resolution of 120000 and an automatic gain control (AGC) target of 2×10^5 (or maximum injection time of 100 ms). Precursor ions were filtered by charge state (2−6 z), dynamic exclusion for 30 seconds at a 20 ppm mass width, and monoisotopic precursor selection. For charge states 2 and 3, the precursors were isolated in the ion trap with a 1.2 m/z isolation window and an AGC target of 2×10^4 with a maximum injection time of 50 ms. Collision-induced dissociation was performed on the isolated parent ions using 35% collision energy and 10 ms activation time. Electron transfer higher energy collision dissociation (EThCD) was used to dissociate precursors having charge states of 3−6 using a 1.2 m/z isolation window and an AGC target of 2×10^4 (or maximum injection time of 50 ms). We used the Proteome Discoverer (PD) Software Version 2.1 (Thermo Fisher) for peptide sequencing and protein identification. We used the SEQUEST search algorithm to analyze the data against fasta files from a *Hirudo verbana* database downloaded from Uniprot (16). We normalized the abundances of the reporter ions for each protein based upon the total reporter ion intensities for all mass spectrometry results recorded throughout the LC-MS run. For a given protein, we summed the normalized reporter ion intensities for all identified tryptic peptides. We downloaded the protein identification output from the PD search into a Microsoft Excel spreadsheet for further analysis. We determined the relative abundance of reporter ion intensities (number of standard deviations from the mean) for each experimental group and compared these relative abundances for the 0 and 0.5 ppm groups using a Student's *t*-test,

Statistical Analysis

Using Microsoft Excel, we performed ANOVA (one-way analysis of variance testing) calculations, setting *p*-value significance at 0.05 for analyses of leech mortality, liver ingestion, vitality indices, histopathologic changes, and reporter ion intensities among experimental groups. We performed post-hoc Tukey's testing setting *p*-value significance at 0.05 to indicate statistically significant difference between ANOVA-tested groups. We used Student's *t*-test for analysis of up- and down- regulation of proteins.

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REFERENCES

1. "Regional E-waste Monitor." United Nations Institute for Training and Research. www.ewastemonitor.info. Accessed 15 Jan 2020.
2. "Energy Efficiency." *Copper Alliance*. www.copperalliance.org/policy-focus/climate-environment/energy-efficiency. Accessed 19 Jan 2020.
3. Schwarzenbach, R. P., et al. "Global Water Pollution and Human Health." *Annual Review of Environment and Resources*, vol. 35, 2010, pp.109-136. DOI: 10.1146/annurev-environ-100809-125342.
4. Ali H, et al. "Environmental Chemistry and Ecotoxicology of Hazardous Heavy Metals: Environmental Persistence, Toxicity, and Bioaccumulation." *J. Chem.*, vol. 2019, 2018, pp. 1-14. DOI: 10.1155/2019/6730305.
5. Kumar V, et al. "Copper Bioavailability, Uptake, Toxicity, and Tolerance in Plants: a Comprehensive Review." *Chemosphere*, vol. 262, 2021, pp. 110-124. DOI: 10.1016/j.chemosphere.2020.127810.
6. Huffman D., et al. "Energetics of Copper Trafficking between the Atx1 Metallochaperone and the Intracellular Copper Transporter." *Biol. Chem.*, vol. 275, 2000, pp. 18611-18614. DOI: 10.1074/jbc.C000172200.
7. Desai V, et al. "Role of Copper in Human Neurological Disorders." *Am. J. Clin. Nutrition*, vol. 88, 2008, pp. 855S-858S. DOI: 10.1093/ajcn/88.3.855S.
8. De Boeck G, et al. "Brain Monoamine Levels and Energy Status in Common Carp after Exposure to Sublethal Levels of Copper." *Aquat. Toxicol.*, vol. 33, 1995, pp. 265-277. DOI: 10.1016/0166-445X(95)00022-V.
9. Al Ghais S., et al. "Effect of Copper Nanoparticles and Organometallic Compounds on Tilapia Fish." *J. Basic Appl. Zool.*, vol. 808, 2019, p. 808. DOI: 10.1186/s41936-019-0101-7.
10. Haveroth G., et al. "Copper Acutely Impairs Behavioral Function and Muscle Acetylcholinesterase Activity in Zebrafish (*Danio rerio*)." *Ecotoxicology and Environmental Safety*, vol. 122, 2015, pp. 440-447. DOI: 10.1016/j.ecoenv.2015.09.012
11. Bulcke F, et al. "Neurotoxicity of Metals." *Advances in Neurobiology*, edited by Aschner M. and Costa I, vol. 18, Springer, 2017, pp. 100-102.
12. Dusek P, et al. "The Neurotoxicity of Iron, Copper and Manganese in Parkinson's and Wilson's diseases." *J. Trace. Elem. Med. Biol*, vol. 31, 2015. pp. 193-203. DOI: 10.1016/j.jtemb.2014.05.007.
13. Garcia-Perez E., et al. "Spontaneous Electrical Activity and Behavior in the Leech *Hirudo medicinalis*." *Frontiers in Integrative Neuroscience*, vol. 1, 2007, pp. 1-9. DOI: 10.3389/neuro.07.008.2007.
14. "The Nervous System of the Leech is Similar to the Human Nervous System." *The Islam Papers*. www.islam-papers.com/2013/07/26/the-nervous-system-of-the-leech-is-similar-to-the-human-nervous-system. Accessed 3 Nov. 2022.
15. Kutlu H., et al. "Effect of Copper on the Body Wall Structures of the Medicinal Leech *Hirudo verbana*." *Fresenius Environmental Bulletin*, 2018, pp. 1187-88.
16. Petrauskiene L. "Water and Sediment Toxicity Assessment by Use of Behavioural Responses of Medicinal Leeches." *Environment International*, vol. 48, 2003, pp. 729-736 DOI: 10.1016/s0160-4120(02)00118-6.
17. "Uniprot blast." Uniprot. www.uniprot.org/uniprot/?query=hirudo+verbana&sort=score. Accessed 5 Jan 2020.
18. Meriaux C., et al. "Multiple Changes in Peptide and Lipid Expression Associated with Regeneration in the Nervous System of the Medicinal Leech." *PlosOne*, vol. 6, 2011, p. 18359. DOI: 10.1371/journal.pone.0018359.
19. "Enzyme, Zymogen, Coenzyme - Creative Enzymes." *Creative Enzymes*. www.creative-enzymes.com. Accessed 7 Feb. 2022.
20. "National Primary Drinking Water Regulations." *Environmental Protection Agency*. www.epa.gov/ground-water-and-dinking-water/national-primary-drinking-water-regulations. Accessed 15 Jan. 2022.
21. "Copper Public Health Statement." *Centers for Disease Control*. www.cdc.gov/TSP/PHS/PHS.aspx?phsid=204&toxid=37. Accessed 3 Jan. 2022.
22. Rodriguez-Quijada C, et al. "Protease Degradation of Protein Coronas and Its Impact on Cancer Cells and Drug Payload Release." *Appl. Materials and Interfaces*, vol. 11, 2019, pp. 14588-14596. DOI: 10.1021/acsami.9600928.