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Transcriptional biomarkers and mechanisms of copper-induced olfactory injury in zebrafish.

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Abstract

Metals such as copper disrupt olfactory function in fish. Unfortunately, little is understood of the molecular consequences of copper olfactory impairment, thus hindering the development of relevant diagnostic tools of olfactory injury. To address this critical data gap, we analyzed gene expression within olfactory tissues of adult zebrafish exposed to CuCl_2 (6, 16, 40 ppb) for 24 hrs. Transcriptional markers of copper impairment within the entire olfactory system were identified and specific genes of interest (e.g. S100a, parvalbumin 8, olfactory marker protein, and calbindin 2-like protein) were confirmed with quantitative real-time PCR. In addition, we performed gene set analysis (GSA) using both *a-priori* and custom pathways of gene sets specifically targeting the olfactory signal transduction (OST) pathway. These analyses revealed down-regulated gene sets related to calcium channels and ion transport, g-proteins, and olfactory receptors. Collectively, these data demonstrate that copper causes a depression of transcription of key genes within the OST pathway and elsewhere within olfactory tissues, likely resulting in an olfactory system less responsive to odorants. Further, these data provide a mechanistic explanation in support of earlier studies of functional olfactory impairment in fish following copper exposure.

Introduction

A dysfunctional olfactory system can dramatically reduce the survival and fitness of many animals and is also associated with several human diseases. Consequently, a multidisciplinary interest in olfaction stemming from research in the fields of occupational health, medicine, conservation biology, and neurobiology has led to the identification of many conserved molecular pathways across species (1-5). In fish, the olfactory system is a sensitive target of metals and a number of pesticides (4,6,7). In the specific case of Pacific salmon, olfactory injury may contribute to population declines and the subsequent ecological, regulatory, political and economic ramifications (4,8). Fish models provide a simple and accessible vertebrate olfactory system to study *in vivo* olfactory function, behavioral effects and the fundamental molecular processes of olfaction (9-12). In this study, we utilized some of these advantages to further the molecular understanding of metal-induced olfactory injury in fish.

While a number of environmental contaminants have been shown to disrupt fish olfaction, copper (Cu) is a largely ubiquitous olfactory toxicant often associated with urban non-point source run-off (13,14). Furthermore, metals have been shown to move beyond the sensory cells to internal components of the olfactory system and brain (15). Using electro-olfactogram recordings from coho salmon (*Oncorhynchus kisutch*), exposures as low as 2

µg/L dissolved Cu will rapidly impair olfactory function (4,16). Depending on Cu concentration and exposure duration, olfactory function and damaged cells may recover (17-20). Under the appropriate exposure conditions, olfactory toxicants may induce apoptosis and cell death to an extent where cells in the olfactory system are permanently lost (7,19,20). In summary, the effects of toxicant exposure to the olfactory system can range from temporary olfactory impairment to permanent loss of olfactory function, all of which may be significant to fish health and survival. Furthermore, the regenerative properties and multiple olfactory cell types, critical for proper olfactory function, remain understudied targets of olfactory injury (21).

The zebrafish model offers powerful genomic and molecular tools to study vertebrate olfactory neurophysiology and anatomy (9,22-24). The multi-tissue olfactory system of fish extends from the olfactory rosettes, which are the sensory component in contact with the environment, to the olfactory bulb and neuronal networks within the brain (Supplemental Text, Supplemental Figure 1). The olfactory receptor neurons, located in the olfactory rosettes, express numerous types of olfactory receptors. Upon odorant binding, olfactory receptors translate this event into a relatively conserved pathway involving g-protein-coupled signaling cascade, membrane depolarization, and ensuing action potential (subsequently referred to as the olfactory signal transduction (OST) pathway) (11,25). Studies using mammalian epithelial cells of olfactory tissue have shown that exposure to copper and zinc prevent the closing of divalent ion channels leading to excessive neuronal excitability (26,27). Metals can also alter olfactory sensitivity, GTP-binding proteins, and block cyclic-nucleotide-gated channels (28-30), suggesting that there are multiple molecular targets in the olfactory system which may be impaired by metal exposure.

In the current study, we investigated the entire olfactory systems' transcriptional response to Cu and focused on the effects of Cu on the conserved processes of olfaction. This approach allowed us to test the hypothesis that Cu impairs the transcription of genes involved in olfaction, specifically those genes involved in OST. Furthermore, we sought to identify relevant transcriptional biomarkers and pathways of Cu-mediated olfactory impairment to further our understanding of olfactory impairment observed in wild fish, such as Pacific salmon. To achieve these goals, we analyzed olfactory gene expression in zebrafish exposed to environmentally relevant Cu concentrations known to be toxic to other zebrafish sensory cells (8,31). In addition, we exploited established and novel bioinformatic approaches to better define the transcriptional signature of Cu exposure to OST and to the entire olfactory system.

Materials and Methods

Animal care and maintenance

One year old adult AB zebrafish were a generous gift from the Marine and Freshwater Biomedical Sciences Center at Oregon State University. The animals were maintained in 10 gallon aquaria with re-circulating filtration at 4 animals/L. Fish were fed twice daily and water quality was recorded daily. The water source was Seattle municipal water passed through a treatment system containing 0.2 µm filtration, activated carbon, ionic and mixed bed filters. The resulting water pH was 5.5 and devoid of conductivity and chlorine. Water for fish was reconstituted freshwater using Instant Ocean® salts at 1000±100 µS, pH adjusted to 7.0 using Na₂HCO₃ and heated to 27°C in a holding reservoir. Tanks received a 20% (v/v) daily water change or more as necessary.

Copper exposures

Groups of 5 adult zebrafish were exposed for 24 hours in 3 independent replicates for each experimental concentration and control group. Nominal copper (Cu) concentrations for this study were Cu-L (6.3 ppb Cu or 0.1 μM Cu), Cu-M (16 ppb Cu or 0.25 μM Cu), and Cu-H (40 ppb Cu or 0.6 μM Cu). Cu samples were taken and analyzed to confirm these nominal concentrations. These methods and results are available in the Supplemental Text. A CuCl_2 stock (Alpha Aesar, Ward Hill, MA, USA) was prepared in distilled water. All Cu treatments were also spiked with 0.001% DMSO to equalize for carrier solvent effects related to ongoing laboratory comparisons of metals with other olfactory toxicants. Exposures were conducted in sealed glass jars maintained at 26°C using a water bath and were staggered by 1 hr to allow for tissue collection and so that no group was initiated (or terminated) the same time of day as the other replicates. After the exposure, fish health, pH, DO, and temperature readings were recorded. Because single gender studies were not possible for this study, each replicate pool had 2 males and 3 females (or vice versa) that were alternated so that across replicates ratios approximated 1:1 (males: females).

Olfactory tissue collection and total RNA isolation

Individual fish were euthanized by cervical dislocation, the head was secured in place under a dissecting microscope, and the brain cavity was opened to reveal the olfactory rosettes, telecephalon, and the underlying olfactory bulb (Supplemental Figure 1). The tissue was removed and tissues were immediately placed in Trizol (Invitrogen, Carlsbad, CA USA) and stored at -80°C for total RNA isolation until processing. The samples were thawed and homogenized using a hand-held tissue tearer followed by sonication. Total olfactory RNA was isolated using the Trizol method according to manufacturer's instructions with the exception that 100 μg glycogen was added to aid in the isopropanol precipitation step. Samples were then purified using the Qiagen RNeasy mini kit (Valencia, CA, USA) clean-up protocol followed by verifying RNA concentrations and purity on a ND100 nanodrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). RNA quality was then further analyzed using the Agilent 2100 Bioanalyzer (Santa Clara, CA) prior to processing for microarray hybridization. All olfactory RNA samples tested were devoid of contamination and RNA degradation as measured by the ratio of 28S to 18S RNA peaks.

Affymetrix microarray hybridizations

For each of the 12 samples, five μg of total RNA isolated from the olfactory tissues was aliquoted for processing and array hybridization. These samples included three arrays for the control group and three arrays for each of the three Cu treatments. The Affymetrix One Cycle Target Labeling and Control Reagents Kit were used according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Briefly, these methods included the following steps; 1) Synthesis of first- and second-strand cDNAs, 2) purification of double-stranded cDNA, 3) the synthesis of cRNA by in vitro transcription, 4) recovery and quantization of biotin-labeled cRNA followed by fragmentation, 5) hybridization to the Affymetrix GeneChip Zebrafish Genome Array and post-hybridization washings, and 6) detection of the hybridized cRNAs using streptavidin-coupled fluorescent dye. Hybridized arrays were scanned with an Affymetrix GeneChip® 3000 scanner. Image generation and feature extraction was performed using Affymetrix GCOS Software. CEL files were further processed in Bioconductor. The bioinformatic methods described in detail are found in the Supplemental Text due to space constraints and include the generation of -fold change and significance gene lists, Venn diagrams, K-means analysis, identification of over-enriched Gene Ontology Terms, and Gene Set Analysis.

SybrGreen quantitative real-time PCR confirmation of array

Primers for microarray validation were designed using the Affymetrix probe set sequence and Oligo Primer Analysis Software, v. 6.71 (Cascade, CO) (Supplemental Table 2). Genes selected from the bioinformatic analysis represented statistically significant differentially expressed genes, as well as potential molecular biomarkers. Each primer pair produced a single PCR product as evidenced by melt curve analysis and gel electrophoresis. Standards for quantification were created from gel-purified PCR products using QIAX II kit (Qiagen Inc.) and quantified before serial dilution from 10 to 0.01 picograms DNA. Total RNA was isolated as described previously and was treated with DNase (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from 2 µg total mRNA with an oligo (dT)18 primer using SuperScript II (Invitrogen) and 1 µl cDNA was used as templates for PCR amplifications. The PCR reaction mixture also contained SYBR Green master mix (Finnzymes, Espoo, Finland), 0.3 µM of each primer and water to reach the final reaction volume of 20 µL. PCR amplifications were performed using a Bio-Rad IQ5 thermocycler (Hercules, CA, USA) for 35 cycles with denaturation at 94°C for 10 s, annealing at optimum temperature for primers (45–58°C) for 20 s and extension at 72°C for 12 s. DNA amplification was quantified in picograms based on the C(T) value of standard curves. The quantified samples were normalized against β-actin quantities and ratios were calculated for treated samples compared with controls. Expression of β-actin did not differ among treatments based on either microarray analysis or reverse transcription-PCR and was therefore deemed an appropriate housekeeping gene for normalization in this study.

Results and Discussion

Identification of copper sensitive transcripts

Several bioinformatic tools were used to identify olfactory gene transcripts responsive to Cu. Initially, statistically significant ($p < 0.05$) differentially expressed transcripts, relative to controls, were segregated using 1.5-, 1.8-, 2.0 and 3.0-fold cut-off criteria. These cutoffs generated 341-455, 128-231, 75-141, and 19-52 differentially expressed genes, respectively (Supplemental Table 1). The 1.5 fold cut-off criteria provided a robust number of altered transcripts to compare gene signatures of the three Cu treatments using a Venn diagram (Figure 1A). For example, 119 transcripts that were altered 1.5 fold in every treatment are depicted where all treatment circles overlap. Further, 20 to 79 transcripts were shared between any two treatments and each concentration contained a large number of transcripts unique to each Cu concentration. To better visualize transcriptional trends, K-means analysis was employed to statistically cluster the altered transcripts by their responses with increasing Cu concentrations. We narrowed our search to a K-means with 12 clusters (± 1.8 fold, $p < 0.05$) (Figure 1A). The majority of these clusters (9/12) described genes with downward trends of expression associated with increasing Cu concentration, reflecting an overall depression of olfactory function by Cu (Figure 1B). We further identified clusters representing the trends across treatment providing many potential biomarkers of olfactory impairment, including those showing dose-dependent upregulation (Cluster 2), dose-dependent down regulation (Clusters 3 and 10), and persistent down regulation at all Cu treatments (Clusters 4 and 11, Figure 1C-D). Some of the most sensitive markers of Cu toxicity identified from K-means analysis included those showing an inverse relationship with Cu concentration, such as *parvalbumin 5*, *S100 calcium binding protein*, *parvalbumin 8* and *olfactory marker protein*.

Mechanisms of copper olfactory injury

To derive insight into the molecular pathways within the olfactory system targeted by copper, we inspected the microarray data for statistically significant ($p < 0.05$) over represented gene ontology terms (herein referred to as “Top GO terms”) within each of the

Cu treatments relative to controls (Supplemental Table 3). With exposure to low concentrations of copper (Cu-L, 6.3 ppb), calcium transport and channel related genes, among other GO categories were over-represented. At Cu-M (16 ppb), olfactory receptors, g-protein signaling and regenerative GO terms were over enriched in this treatment. These aforementioned Top GO terms also appeared at the highest dose of copper (Cu-H, 40 ppb) (Supplemental Table 3). In addition, some gene sets were significant at every Cu concentration tested, including those related to enzymatic transferase, peptidase, and phosphatase activities.

To further define potential mechanisms of copper-mediated olfactory injury, we applied Gene Set Analysis (GSA) to the entire microarray dataset. GSA allows the statistical evaluation and detection of coordinated changes in gene expression within similar gene sets and biochemical pathways, regardless of arbitrary cut-offs (32,33). Using GSA, we identified 169 gene sets from the treated groups which were significantly different ($p < 0.05$) from controls using the *a priori* gene sets from the Biological Process (BP, 543 gene sets) and Molecular Function (MF, 265 gene sets) databases (Supplemental Table 4). The coordinated expression of the genes within each gene set results in a net GSA score reflecting the relative magnitude and direction of the coordinated change relative to the control dataset. We consolidated these 169 significant gene sets into similar functional groupings (Figure 2). Among the most over represented functional grouping of gene sets included transport, di-valent ions and ion channels, stimulus response/detection, and g-proteins (Figure 2, black arrows).

Examination of the GSA results provided some intriguing insights regarding the nature of copper-induced olfactory impairment. Gene sets associated with di-valent ions, ion channels and g-proteins were overrepresented at the lowest dose of copper (Cu-L), whereas transport and stimulus response/detection gene sets appeared within the list of significant gene sets at higher concentrations (Supplemental Table 3). These data agree with the closely related abovementioned Top GO term analysis, and suggest an initial disruption of ion homeostasis within the olfactory tissue, possibly by preventing the closing of important ion channels, followed closely by inhibition of g-protein function (29,30). Several gene sets related to the regenerative properties of the olfactory system were present within the significant gene sets at Cu-L and Cu-H (Figure 2, dashed arrows). At the highest concentration, Cu perturbed a number of gene sets associated with metabolic and catabolic processes, potentially reflecting cellular toxicity. While GSA is performed on gene sets made up of several to hundreds of genes, the individual gene GSA scores and their relative ranking also provides valuable information. For example, closer examination of divalent ion and ion channel gene sets identified the presence of *parvalbumin 5* and *8*, *calbin2-like*, and *chloride intracellular channel 4 (CLIC4)* in multiple gene sets, and at every copper concentration. These genes often carried the most negative individual gene GSA scores, and therefore were likely dominant factors in driving many of these negatively shifted gene sets.

Olfactory Signal Transduction (OST) Pathway

Given the indication that Cu was targeting components of OST, we more closely examined how Cu was impacting this entire pathway. To achieve this goal, we created seven gene sets targeting the known conserved OST pathway using every gene available for this pathway on the array (Supplemental Table 5). The OST gene sets included a comprehensive overall pathway (OST-109), as well as more restrictive pathways based on the Affymetrix probe sequence with the greatest homology to the conserved OST pathway genes. We performed GSA using the microarray data and these custom gene sets within the BP database to generate GSA scores and significance for these pathways (Table 1). These data revealed that Cu exposure caused a significant coordinated depression in the expression of genes involved in the known conserved OST pathway located within the olfactory neurons, likely rendering

them insensitive to odorants. In addition, the OST-inhibitor pathway (OST-inh) targeting those genes involved in the negative inhibitory feedback on the transduction process was never statistically significant. This observation underscores the specific effects of Cu on olfaction and helps define a transcriptional signature for Cu in olfactory tissue.

To better illustrate these transcriptional changes we overlaid the average fold-change gene response on a schematic of the OST pathway with genes from the OST-39 gene set, the most restrictive pathway significant at all concentrations (Figure 4). This illustrates that Cu is targeting expression of olfactory receptors, g-proteins, and several ion channels thereby driving the significance of these OST pathway gene sets. This OST pathway provides a potentially valuable diagnostic tool to investigate how different olfactory toxicants and exposure conditions impact this conserved process in other species.

Mechanistically relevant genes of interest

Several of the most sensitive markers of copper olfactory toxicity identified in this study along with other representative genes of interest were confirmed using quantitative real time PCR (Figure 4). Many of these transcripts encoded proteins which could serve as potential markers of copper sensitive olfactory cell types. For example, the gene encoding *s100a* protein is a cellular marker for mature, likely competent, olfactory crypt cells involved in olfactory neuronal function found in multiple species (34,35). This transcript (originally annotated as *zgc:110464*), identified from the array study as having strong homology to *s100a* genes from mammals and fish, was found to have restricted expression in the olfactory tissue of zebrafish in ZFIN expression databases (35-37). There is a concentration-dependent decrease in the expression of this transcript by microarray and qPCR, suggesting a loss of olfactory crypt cells with exposure to Cu. Two additional genes encoding calcium binding proteins (*calbin2-like* and *parvalbumin 5*) with restricted expression in the olfactory epithelium were strongly down regulated in Cu treated animals (Figure 4A). In addition, the gene encoding olfactory marker protein, a marker of mature olfactory neurons (38,39) and olfactory receptors, such as OR 2.1 and 5.1, showed similar dose-dependent decreases with Cu exposure (Figure 2A). Olfactory marker protein is conserved in a variety of species, including salmon, and is a critical component in maintaining OST signaling through modulation of cAMP levels (40). Collectively, these genes may reflect a decline in the number of these cells within olfactory epithelium and provide a mechanistically relevant transcriptional profile to evaluate olfactory toxicants in fish sensory cells.

These data also revealed significant transcriptional changes in genes with known restricted expression in the olfactory bulb (Figure 4B). For example, expression of the calcium transport protein *parvalbumin 8* (*pval8*) decreased as Cu concentrations increased. The *pval8* protein is important in maintaining calcium ion balance in neuronal cells of the olfactory bulb and other sensory cells outside the olfactory system (41,42). A putative *ferritin heavy chain* mRNA was identified from the microarray and selected for PCR confirmation due to its altered regulation at the lower concentrations of Cu (Fig 4B). Some ferritin proteins have restricted expression in human and zebrafish olfactory bulb tissue, and a reduced presence of these proteins has been associated with the aging of the olfaction system (43,44). Accordingly, ferritin heavy chain has potential as a biomarker for olfactory bulb responses to metals at low concentrations. Together these transcriptional responses reflect impairment of ion homeostasis within the olfactory bulb.

Cu exposure also altered the expression of genes encoding neuronal proteins, including *gefilitin*, (Figure 4B), a neuronal intermediate filament protein which is important in neuronal regeneration in olfactory epithelial tissue (45,46). In addition, *muscle-specific beta-1 integrin binding protein* (*mibp*) encodes a neuronal protein important muscle cell differentiation (47). The expression of this gene has been associated with olfactory

ensheathing cells involved in olfactory receptor neuron regeneration (48). Therefore, the dose-dependent response of *mibp*, observed by microarray, may reflect an attempt at neuronal regeneration which will require further study. In addition to *gefilitin* and *mipb*, *neuropeptide FF-amide peptide precursor-like* mRNA was also upregulated with Cu exposure (Figure 4B). This protein belongs to a group of neuromodulatory peptides associated with gonadotropin releasing hormone activity (49), and was consistent with a dose-dependent increase in the mRNA expression of *gonadotropin releasing hormone 3* observed by microarray (Figure 1; Supplemental Table 1).

Olfactory impairment, injury, and toxicity are highly dependent on many factors including exposure concentration, length of exposure, life-stage, and exposure conditions. The microarray platform is one of many valuable tools necessary to elucidate unknown mechanisms of toxicity and establish relevant biomarkers of toxicity. In this study, we exploited the technology for biomarker discovery, hypothesis generation, and hypothesis testing. These data indicate that exposure to environmentally relevant copper concentrations impairs transcription of genes involved in olfactory signal transduction and other genes throughout the fish olfactory system. Collectively, the gene signatures, pathway analysis, and cellular markers identified in this study may contribute to future studies of toxicant-induced olfactory injury and provide biomarkers of olfactory injury in non-traditional and threatened species such as Pacific salmon.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Venn diagram and K means analysis of the transcriptional changes occurring with copper exposure. A.) Venn diagram: The total numbers of transcripts meeting the cut-off criteria for each copper treatment are contained within each labeled circle. The numbers where one or more treatment circles overlap show the number of transcripts which are shared between those treatments. There were 119 transcripts shared between all three treatments and between 20 to 79 genes shared between any two treatments. Interestingly, there was an increase in the number of transcripts unique to a treatment as the copper concentration. B.) K means analysis: the trends of these genes at each concentration were grouped statistically into 12 clusters (heat map coloring: red-increased transcription; green, decreased

transcription; color intensity indicates relative magnitude change). For example, clusters 2 and 8 show an upward trend with dose, while clusters 3, 4, 5, 6, 10 and 11 show definite downward trends. The remaining clusters 1, 7, 9 and 12 show various other trends. C.) Clusters-of-interest in greater detail showing gene trends plotted as log₂ fold change on y-axis vs. copper concentration on the x-axis. D.) Genes identified in these clusters are listed next to their individual fold change illustrated by color across treatment (left to right 0, 6, 16, 40 ppb). Fold change is shown as: Yellow-no change from control. Lime, green, dk green (>1.5, >2, >8 fold change respectively). Orange, red, maroon (>-1.5, >-2, >-8 fold change).

Figure 2.

Functional grouping of significant gene sets identified using Gene Set Analysis altered by copper exposure. Gene set analysis reveals impairment in signal transduction and ion balance in the olfactory tissue of zebrafish exposed to copper. Significant GSA ($p < 0.01$) scores (169 terms) from the molecular function (MF) and Biological Process (BP) Gene Ontology (GO) database were grouped together in similar functional groups and graphed according to the number of gene sets in each functional group. The cellular compartment (CC) GO database returned no significant gene sets. Overall, it is evident that there is a transcriptional signature indicative of impairment to signal transduction (black arrows). Also a number of gene sets involved in process likely related to the regeneration and development of olfactory tissue were revealed (dashed arrows). Other functional groupings include gene sets involved in metabolic and catabolic processes, three types of enzyme activity, and other cellular processes.

Figure 3.

Averaged Fold Change of OST-39 pathway genes linked by sequence homology to 15 of the 16 known conserved olfactory transduction pathway genes. Copper has a clear impact on olfaction causing a decrease in transcription of several key genes in olfactory signal transduction including olfactory receptors, g-proteins, ion channels. Each gene in the pathway (rectangles) within the olfactory signal transduction pathway is colored by the fold change (or average fold change from multiple genes in the gene set, e.g. OR) as obtained from the array data. Colors illustrating fold-change from control are: yellow $>+1.2$; orange $>+1.3$; red $>+1.5$; light green >-1.2 ; lime >-1.3 ; green >-1.5 . Large black arrows show the progression of olfactory transduction (OST root pathway). Orange arrows show positive feedback pathways. Lines with dark circles indicate negative feedback pathways. The only gene not linked to a zebrafish gene on the array was PKG (gray). Genes in the pathway include chloride channel, calcium activated (CLCA), calmodulin (CAM), calmodulin kinase II (CAM KII), phosphodiesterase (PDE), guanylate cyclase activator protein (GCAP), guanylate cyclase GC, cGMP, cAMP, adenylyl cyclase (AC), phopho kinase c-AMP

dependent (PKG), protein kinase, cAMP-dependent, alpha (PKA), phosducin (PDC), G protein alpha olfactory (Golf), olfactory receptor (OR), adrenergic receptor kinase (GRK), G protein-alpha inhibiting activity (Gipb).

Figure 4.

Quantitative real-time PCR confirmation of the array response. Selected genes identified in the array study were confirmed using quantitative PCR confirmation. The array response (mean, SD), shaded bars, for each treatment is matched to the real-time qPCR results, white bars, and the two methods compare favorably in both the direction and magnitude of the response. A) S100 protein (S100), calbindin 2, like (calb2l), olfactory receptor 2.1 (OR2.1), and olfactory marker protein (OMP) B) parvalbumin 8 (pvalb8), neuropeptide FF-amide peptide precursor-like (npffl), a putative ferritin heavy chain (ferritin), and the neuronal protein gefitin (gef). Dashed line across all data illustrates 2-fold change from controls.

Table 1

Gene Set Analysis (GSA) results showing the relative shift from controls (score) and significance of the seven custom pathways targeting olfactory signal transduction (OST) at each copper treatment. Significant pathways ($p < 0.05$) are in bold. Negative shifts in the treated datasets relative to controls are indicated in green. Positive shifts are in red.

Cu L Gene_set	Gene set name	Score	p-value	Gene set length
1792	Olfactory Transduction OST109 Pathway	-0.49	0.059	109
1791	Olfactory Transduction OST60 Pathway	-0.33	0.004	59
1790	Olfactory Transduction OST39 Pathway	-0.60	< 0.001	38
1789	Olfactory Transduction OST20 Pathway	-0.46	0.045	19
1788	Olfactory Transduction OST15 Pathway	-0.08	0.363	15
1794	Olfactory Transduction OSTroot Pathway	-0.64	0.045	17
1793	Olfactory Transduction OSTinhib Pathway	-0.06	0.439	31
Cu M				
1792	Olfactory Transduction OST109 Pathway	-0.54	< 0.001	109
1791	Olfactory Transduction OST60 Pathway	-0.41	< 0.001	59
1790	Olfactory Transduction OST39 Pathway	-0.67	< 0.001	38
1789	Olfactory Transduction OST20 Pathway	-0.17	0.148	19
1788	Olfactory Transduction OST15 Pathway	-0.06	0.328	15
1794	Olfactory Transduction OSTroot Pathway	-1.04	< 0.001	17
1793	Olfactory Transduction OSTinhib Pathway	-0.09	0.332	31
Cu H				
1792	Olfactory Transduction OST109 Pathway	-0.76	< 0.001	109
1791	Olfactory Transduction OST59 Pathway	-0.79	< 0.001	59
1790	Olfactory Transduction OST39 Pathway	-0.97	< 0.001	38
1789	Olfactory Transduction OST20 Pathway	-0.47	< 0.001	19
1788	Olfactory Transduction OST15 Pathway	0.47	0.11	15
1794	Olfactory Transduction OSTroot Pathway	-1.25	< 0.001	17
1793	Olfactory Transduction OSTinhib Pathway	-0.41	0.145	31