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## Endogenous Concentrations of Biologically Relevant Metals in Rat Brain and Cochlea Determined by Inductively Coupled Plasma Mass Spectrometry

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### Abstract

Manganese (Mn), iron (Fe), copper (Cu), and zinc (Zn) are essential nutrients which aid in the proper functioning of cells, but high concentrations of these metals can be toxic to various organs. Little is known about the endogenous concentrations of these metals in the cochlea, the auditory portion of the inner ear which is extremely small and difficult to access. To fill this gap, a trace quantitative digestion and inductively coupled plasma mass spectrometry method was developed to determine the concentrations of these metals in the stria vascularis, organ of Corti, and spiral ganglion, three critically important parts of the cochlea ( ~ 1.5 mg); these values were compared to those in specific brain regions ( ~ 20 mg) of rats. Rats were sacrificed and the cochlea and brain regions were carefully isolated, digested, and analyzed to determine baseline concentrations of Mn, Fe, Cu, and Zn. In the cochlea, Mn, Fe, Cu, and Zn concentrations ranged from 3.2–6, 73–300, non-detect, and 13–200 µg/g respectively. In the brain, Mn, Fe, Cu, and Zn concentrations ranged from 1.3–2.72, 21–120, 5.0–10.6, and 33–47 µg/g respectively. Significant differences (p

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Conflict of Interest

The authors state that they have no conflicts of interest that affect the objectivity of this publication.

< 0.05) were observed between the tissue types within the cochlea, and between the cochlea and brain. This validated method provides the first quantitative assessment of these metals in the three key subdivisions of the cochlea compared to the levels in the brain; Mn, Fe, and Zn levels were considerably higher in the cochlea than brain.

### Keywords

ICP-MS; cochlea; trace analysis; auditory pathway; iron; manganese; zinc; copper

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### Introduction

Essential transition metals, including iron (Fe), zinc (Zn), copper (Cu), cobalt (Co) and manganese (Mn), play a critical role in normal functioning of all cells because they act as required cofactors for a variety of enzymes in addition to their participation in maintaining basic mitochondrial behavior and integrity (Kuo et al., 2001, Nose et al., 2006, Roth et al., 2013). They catalyze a diverse array of redox reactions that are critical for cellular respiration, chemical detoxification, metabolism, and neurotransmitter synthesis (Erikson et al., 2002, Sharp, 2003, Jenkitkasemwong et al., 2012). These metals are required nutrients, but when exposure greatly exceeds homeostatic nutritional requirements, they can become neurotoxicants (Aschner, 1997, Erikson et al., 2002, Reaney et al., 2006, Mizuno and Kawahara, 2013, Liu et al., 2014). Overexposure to other heavy metals, such as lead (Pb), strontium, rubidium, and cadmium (Cd), which have no known biological function, can also stimulate cell toxicity, presumably by interacting and interfering with metal binding components of the cell (Bartley and Reber, 1961, Fieve and Meltzer, 1974, Alexander and Meltzer, 1975, Murata et al., 1993). Heavy metal neurotoxicity normally progresses as a continuum of symptoms and consequences, which depend on the properties of the metal, the dose, the duration of exposure and genetic factors.

All organs in the body can potentially be adversely affected by heavy metals, yet considerable specificity exists with regard to their interaction with distinct cellular sites within any given tissue. In terms of metal toxicity, one organ which has received relatively minor attention is the cochlea even though studies have clearly identified a number of heavy metals associated with hearing impairment, including Cd (Ozcaglar et al., 2001, Agirdir et al., 2002, Choi et al., 2012), Pb (Choi et al., 2012), arsenic (Jacobsen, 1998); Co (Ikeda et al., 2010, Pelclova et al., 2012, Apostoli et al., 2013) and Mn (Ding et al., 2011b, Choi et al., 2012). Several of these metals have been shown to accumulate in the auditory portion of the inner ear, and based on their known toxicological properties, have the potential to selectively influence cell viability within the individual cellular compartments within the cochlea, thus potentially affecting hearing. Heavy metal exposure studies from our laboratory (Ding et al., 2011b, Ding et al., 2014, Liu et al., 2014) using cochlear organotypic cultures have demonstrated preferential toxicity to specific cellular components within the cochlea. Toxicity is most commonly associated with the production of reactive oxygen species which leads to apoptosis; though other cell death pathways such as autophagy and necrosis may be involved (Oberto et al., 1996, Zou et al., 2001, Hirata, 2002, Zhang et al., 2011, Unuma et al., 2013, Zhang et al., 2013, Wang et al., 2014). The rate limiting steps controlling metal

toxicity are not fully understood but likely involve uptake of the metals into the different cells within the cochlea. Previous studies from our lab have identified different isoforms of the major transporter for many of these metals; divalent metal transporter I (DMT1), Zip8, Zip14 and transferrin receptor, in 3 day old and mature cochlea (Ding et al., 2014). These four proteins were selectively expressed in different cell populations within the cochlea and their cellular and subcellular distribution changed with development. Thus, the cochlea, like other organs differentially expresses specific proteins required for the uptake of several potentially toxic metals.

Although treatment of rats with heavy metals can lead to their accumulation in the cochlea, the normal levels of these metals within the different regions of the cochlea that contain the neural, sensory cells and supporting cells is largely unknown. Since abnormal levels of essential metals can potentially have deleterious effects on the cochlea, it is important to determine the normal concentration and distribution of these metals within the various regions in order to better understand their normal and potential impact on function. Accordingly, in this paper, the levels of four essential metals, Mn, Fe, Cu, and Zn, were assessed in the modiolus (M) where the spiral ganglion neurons (SGN) reside, the basilar membrane (BM) containing the sensory hair cells, and the stria vascularis (SV) which contains supports cells responsible for the import and export of key ions and molecules into the cochlea. To put the data from the cochlea into perspective, we also measured the concentration of Mn, Fe, Cu and Zn in two regions of the brain within the auditory pathway, the inferior colliculus (IC) and cochlear nucleus (CN), as well as the hippocampus (HP), striatum (S) and globus pallidus (GP) in the central nervous system (CNS).

## Experimental procedures

### Animals

Female SASCO<sup>®</sup> Sprague Dawley Rats ranging in age from 3–4 months were used for these experiments. Experiments were performed according to the rules and regulations of the Institutional Animal Care and Use Committee of the State University of New York at Buffalo and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Tissue Harvesting

Rats were anesthetized with ketamine (40 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.), then decapitated with a small guillotine. The bulla was quickly removed under a dissection microscope and placed in phosphate-buffered saline. The bony walls of the cochlea were opened. The organ of Corti containing the hair cells, the spiral ligament including the SV, and the soft tissue in the cochlear M including the SGN, were carefully dissected out. Brains were removed from the skull and sliced by using 1 mm intervals coronal rat brain slicer matrices (Plastics One Inc.) on ice. Regions of interest including the S, IC, GP, CN, and HP were identified using Paxinos & Watson rat brain atlas, and separated using a biopsy punch (Glowinski and Iversen, 1966). Each component of the cochlea and brain was placed in a separate metal-free polypropylene centrifuge tube. Individual tubes contained tissues from four pooled animals.

### Solid Sample Digestion

Rat tissue and animal food samples were digested using trace metal-grade nitric acid (Aristar Ultra) and hydrogen peroxide (J. T. Baker) following a method adapted from USEPA 3050B (1996) for use at the micro scale. The samples were air dried at room temperature under filter paper and weighed to the nearest tenth of a microgram using a microbalance (Mettler Toledo). Polytetrafluoroethylene coated tweezers and metal-free polypropylene centrifuge tubes and caps were used to prevent metal contamination. Samples were refluxed with concentrated trace metal nitric acid for 2–3 h in metal-free 1.5 ml polypropylene centrifuge tubes in a hot block. Samples were refluxed for another 1–3 h after the addition of hydrogen peroxide. Method reagent blanks were prepared using the same procedure, and contained only the chemical reagents. An internal standard of  $^{103}\text{Rh}$  (Sigma Aldrich) was added (50  $\mu\text{l}$  of 1 ng/ $\mu\text{l}$  in 2% nitric acid) to the samples prior to injection. The internal standard was used for volume and instrument drift correction. Samples were diluted to 5 ml with Nanopure® water, and then filtered with 0.45- $\mu\text{m}$  polypropylene membrane syringe filters (VWR International).

### Water Samples

Samples of rat drinking water were obtained and adjusted to 2% nitric acid concentration using trace metal-grade nitric acid. Concentrated acid was used to reduce dilution effects. The internal standard was added prior to injection (50  $\mu\text{l}$  of 1 ng/ $\mu\text{l}$   $^{103}\text{Rh}$  in 2% nitric acid) to account for volume differences and instrument drift.

### Elemental Analysis

Samples were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) on an X-Series 2 instrument (Thermo Scientific) using a 1 ml sample loop. Manganese, Fe, Cu, and Zn, were quantified using isotope standards for  $^{55}\text{Mn}$ ,  $^{57}\text{Fe}$ ,  $^{65}\text{Cu}$ , and  $^{64}\text{Zn}$  respectively. All samples were normalized to the  $^{103}\text{Rh}$  signal. Iron was analyzed using collision cell technology to reduce polyatomic interferences. Operating conditions of the ICP-MS are presented in Table 1. Quantification was performed with external calibration curves (n 3) in 2% nitric acid at concentrations from 0.1 to 250 ng/ml. A standard metal mix (82026–108, BDH Aristar® Plus, VWR International) was used to quantitate all metals.

### Statistical Analysis

Outlying data points were removed using a Q-test at the 95% confidence level. Statistical analysis was performed using Origin Pro 9.0. One-way analysis of variance (ANOVA) was performed using a Tukey post-hoc test with significance at  $p < 0.05$ .

### Analytical Figures of Merit

Method limits of detection (mLOD) and quantification (mLOQ) were determined for each element and tissue type including homogenized brain (HB) as summarized in Table 2. Like tissue digests were pooled and spiked at 4 levels (0, 2.5, 10 and 50 ng/ml); each level was injected 7 times to produce a calibration curve. Correction for the endogenous concentrations was performed using multipoint standard addition. The mLOD and mLOQ were determined by dividing 3 or 10 times the standard deviation of the matrix blank by

the slope of the calibration curve respectively. Reported values represent recovery-corrected concentrations present prior to sample preparation. The HB limits were applied to the CN and HP as no tissue specific limits were obtained. Extraction recoveries in homogenized brain were evaluated at three spiking levels: 10, 20 and 100  $\mu\text{g/g}$  with  $n = 3$  samples per level. As analyte free matrix could not be obtained, spiking values were in addition to the endogenous concentrations. Recoveries were corrected by background subtracting the endogenous levels. Values are reported as averages and standard errors for the recovery levels used. Mn and Cu values are reported for all three recovery levels, Fe values are reported at the 20 and 100  $\mu\text{g/g}$  levels, and Zn recoveries are reported only for the 100  $\mu\text{g/g}$  level. Recovery values are  $108 \pm 1\%$ ,  $110 \pm 10\%$ ,  $112 \pm 5\%$ , and  $120 \pm 20\%$  for Mn, Fe, Zn, and Cu respectively.

## Results

### Analysis of Metals in Food and Drinking Water

The concentrations of Mn, Fe, Zn and Cu present in rat drinking water and standard laboratory rodent chow are shown in Table 3. As expected, all four metals were present in rat chow and drinking water at varying concentrations.

### Manganese in the Cochlea and CNS

Mn concentrations were determined for three regions of the cochlea, the M, BM, and SV, and five regions of the brain. As illustrated in Fig. 1, Mn was detected in all of the tissues examined, though the highest concentrations were found in the cochlear samples. In the cochlea, tissue concentrations ranged from about 3.0 to 5.8  $\mu\text{g/g}$  tissue dry weight with highest levels expressed in the BM, followed by the M and then the SV. Statistical analysis revealed that levels of Mn within these areas of the cochlea were significantly different from each other. The data further indicates that Mn concentrations in brain were statistically lower than the cochlea, ranging from approximately 1.2 to 2.8  $\mu\text{g/g}$  tissue dry weight, with the highest levels present in the IC.

### Iron in Cochlea and CNS

All regions of the cochlea and brain contained detectable levels of Fe as shown in Fig. 2. In the cochlea, tissue concentrations of Fe ranged from about 70 to 300  $\mu\text{g/g}$  tissue dry weight with the highest levels expressed in the SV, followed by the BM and then the M. However, only the SV and M were statistically different from one another. In brain, Fe concentrations ranged from approximately 20 to 120  $\mu\text{g/g}$  tissue dry weight with highest levels expressed in the IC and lowest in the CN, yet statistical comparison indicated that none of the brain areas were significantly different from each other.

### Zinc in Cochlea and CNS

Zn concentrations measured in the cochlea and brain are presented in Fig. 3. Within the cochlea, highest levels were observed in the SV which contained approximately 210  $\mu\text{g/g}$  of tissue. The SV was statistically different from both the M and BM which contained essentially identical concentrations of Zn at around 140  $\mu\text{g/g}$  of tissue. Like Mn, all of the cochlear sections contain statistically higher levels of Zn than any of the brain areas

examined. The levels of Zn within brain were similar ranging between 30 and 45  $\mu\text{g/g}$  of tissue.

### Copper in the Cochlea and CNS

Cu was detected in all of the brain specimens examined and values are displayed as  $\mu\text{g/g}$  tissue dry weight in Fig. 4. Within the brain, the highest levels of Cu were observed in the IC which contained approximately 11  $\mu\text{g/g}$  of tissue followed by the S which contained about 9  $\mu\text{g/g}$  of tissue. Levels of Cu in the other brain areas ranged between 5 and 6  $\mu\text{g/g}$  of tissue. Statistical analysis of the brain tissue revealed that with the exception of GP, when compared to CN and HP, all other areas were statistically different from each other. As opposed to the trend for the other three metals examined, the levels of Cu present in all three compartments of the cochlear tissues of the rat were below detectable limits.

### Discussion

The cochlea has been reported to accumulate a variety of essential and nonessential metals including Mn, Pb, Co and Cd. Accumulation is regulated, at least in part, by their affinity for the different transport systems which are ultimately responsible for uptake across the blood-cochlea barrier and subsequent transport into the cells within the cochlea. The unique and selective properties of the different metal transporters discreetly controls endogenous levels of the essential metals within the cochlea, thereby, influencing normal cellular processes as well as potentially provoking neurotoxic responses leading to hearing impairment in conditions of excess metal exposures. The overall net accumulation of each metal is, thus, contingent on the discerning and opposing capacities of the import and export transport systems within each part of the cochlea. Since unequal compartmentalization of metals in the cochlea can lead to distinctive hearing deficits, it is important to evaluate endogenous levels of these metals in normal animals in order to understand or explain the origins of metal-induced damage to different anatomical structures in the cochlea.

In this paper, endogenous levels of four essential transition metals, Fe, Mn, Cu and Zn, were examined. All four metals share, at least in part, common transport proteins for cellular uptake including, DMT1, ZIP8, and ZIP 14 (Garrick et al., 2006, Jenkitkasemwong et al., 2012), though a number of other proteins are responsible for the uptake of Cu including Ctr1 (Kuo et al., 2001) which is important in the ototoxic actions of the anti-cancer drug cisplatin (Sharp, 2003, Ding et al., 2011a). Export proteins include ferroportin for Zn, Fe and Mn, and ATP7A and B for Cu (Gupta, 2014, Kaler, 2014). SLC30A10 is a selective exporter for Mn and Zn, and mutations in this gene lead to Mn toxicity characterized by dystonic movements similar to those observed in Parkinson's disease (Quadri et al., 2012, Stamelou et al., 2012). It should be noted that the measured intracellular levels of these metals are also significantly influenced by binding proteins within the cell involved in their sequestration.

Prior studies in our laboratory have reported the selective expression of several metal transporters in different cell populations of the cochlea (Ding et al., 2014) including DMT1 and the two ZIP proteins, 8 and 14. In addition, our studies demonstrated that the Cu transporter, Ctr1 and the Cu export pumps, ATP7A and ATP7B, are selectively localized within the organ of Corti, which contains the hair cells, the vascular epithelium of the SV,



and the SGN (Ding et al., 2011a). In general, the pattern of existing transporters represents redundant and overlapping systems for maintaining homeostatic levels of essential metals.

### Cochlear Compartments

The metal concentrations in the cochlea were measured in three functional subdivisions, the M, BM, and SV. The M, located within the central core of the cochlea, is mainly populated by SGN myelinated by Schwann cells. The BM is the sensory hair cells which transduce sound vibrations into neural activity. The sensory cells rest on the BM and are surrounded by a diverse array of supporting cells. The SV and spiral ligament form the lateral wall of scala media. The marginal, intermediate and basal cells of the SV, which contains numerous small blood vessels, play an important role in fluid and ion transport into scala media and production of endolymph which contains a high concentration of potassium and low concentration of sodium. Quantitative analysis of metal content in the individual cochlear components was particularly challenging as tissue sample sizes were extremely small (1.5 mg dry weight). Nevertheless, the micro-scale extraction procedure and ICP-MS method developed, validated, and described in this paper enabled us to accurately assess endogenous levels of all four metals with the exception of Cu. In general, our results show a consistent trend for the levels of these metals within individual tissue compartments with  $Fe \approx Zn \gg Mn$ . Cu concentrations were below detectable levels in all specimens of the cochlea examined, yet the levels of Cu in food and water were well within our measurement range. The reasons for this are not known, and are puzzling given that cuprous ions are transported by DMT1, which was previously identified in the cochlea (Ding et al., 2014). Cu is also a component of ceruloplasmin and hephaestin which are required for the export and oxidation of Fe from cells and uptake across the endothelium of the blood-cochlea barrier. Despite our inability to detect Cu in the samples, the apparent low abundance of Cu in the cochlea must be sufficient to account for the accumulation of Fe in the inner ear (McCarthy and Kosman, 2014). Our findings are also surprising, given that the Cu transporter, Ctr1, and Cu export pumps ATP7A and ATP7B, have previously been identified in the organ of Corti, epithelium of the SV, and SGN (Ding et al., 2011a). Thus, all the machinery is present within the cochlea for Cu utilization despite our inability to detect them with our methods. With regard to the relative concentrations of the other three essential metals within the individual components of the cochlea, there was no clear evidence of selective localization of Fe, Zn or Mn. Although the differences in the concentrations were in some cases statistically significant, the differences were relatively small.

### Brain Regions

One of the most significant findings in this study was the observation that, with the exception of Cu, the levels of Fe, Mn, and Zn were considerably higher in the cochlea compared with any of the brain regions examined, specifically the IC, CN, GP, S, and HP. The IC and CN were selected as they are part of the auditory pathway in the brainstem and midbrain (Rose et al., 1966, Young et al., 1992). Additionally, the IC is one of the more metabolically active parts of the brain (Sokoloff, 1981) in which Mn is known to accumulate in rats (Takeda et al., 1999), and due to its high metabolic rate, the IC is an important region to monitor for metal concentration changes. The GP and S are components of the basal ganglia, and were chosen due to their involvement in motor dysfunction related to metal

toxicity. The GP, a Mn rich region of the brain, readily accumulates additional Mn which leads to neuron death (Bird et al., 1967, Larsen et al., 1979, Larsen et al., 1981, Aschner, 1997, Roth et al., 2013).

The trend for the overall distribution of the metal levels in all the brain samples measured in this study follows the order Fe > Zn >> Cu > Mn. Previously reported values of these metals in rat brain (Table 4) follow a similar trend and our measurements fall within the concentration range previously reported (Donaldson et al., 1973, Fjerdingsstad et al., 1974a, 1977, Seth et al., 1977, Chan et al., 1983, Han et al., 1999, Reaney et al., 2006).

In general, the levels of Zn within the brain tissues studied do not follow the same pattern as Mn and Fe, suggesting that Zn may be transported via different transporters than Mn and Fe. It has been suggested, however, that Zn is transported by DMT1 though it has a considerably lower affinity for the transporter than either Fe or Mn (Garrick et al., 2006). Interestingly, the relative pattern for Cu measured within our brain tissue sections are similar to those of Fe suggesting that similar transport mechanisms may be involved in their accumulation. This is supported by the observation that Fe deficiency, which causes an increase in DMT1, results in an increase in both Zn and Mn concentrations in brain (Erikson et al., 2004). Additionally, Fe is known to be transported by Crt1, which may account for the similarities in uptake patterns (Nose et al., 2006). However, the non-detectable Cu concentrations in the ear tissues suggest that the distribution of transporters present in the brain tissues is distinct from that in the cochlea.

## Conclusions

A sensitive analytical method for the trace quantification of metals in tissue samples, less than 20 mg was successfully developed and validated. Endogenous concentrations of Fe, Zn, Mn and Cu were measured in three separate compartments of the cochlea for the first time and compared to those in several parts of the central auditory system plus other sections of the brain. Importantly, our results indicate that Fe, Mn, and Zn levels in the cochlea are greater than those detected in the other regions of rat brain specimens examined. Surprisingly, Cu levels within the cochlea were below the detection limit (28.0 – 101 µg/g) and therefore far below those in the brain.

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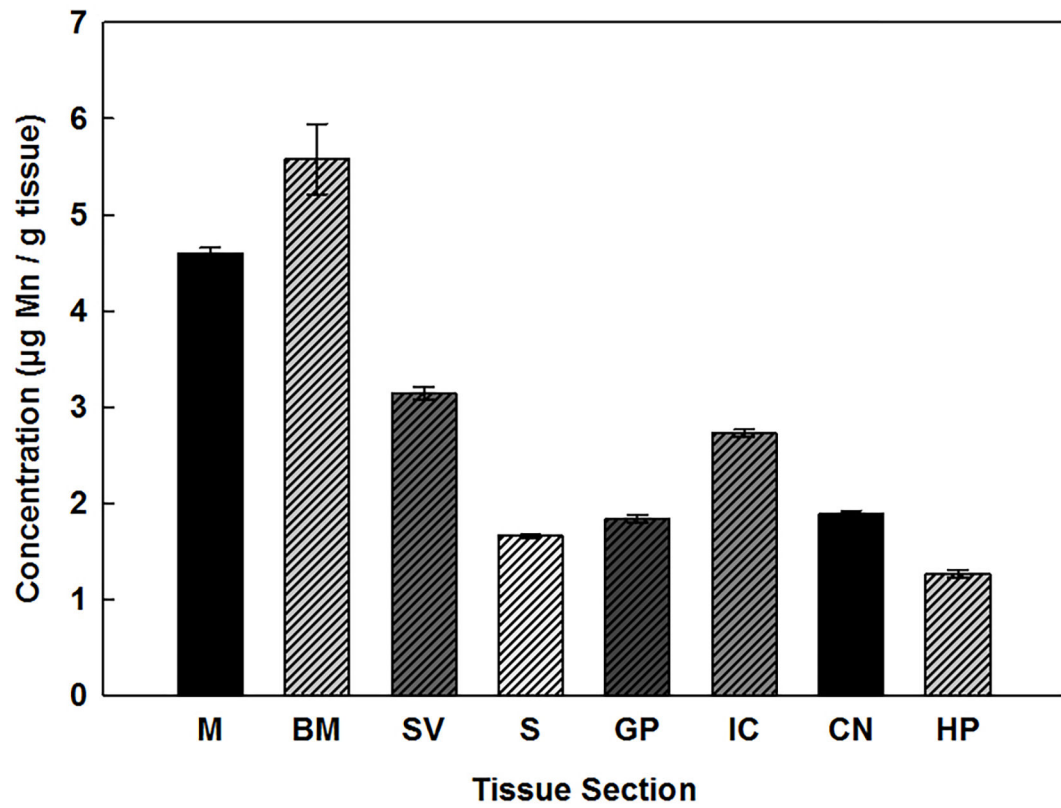
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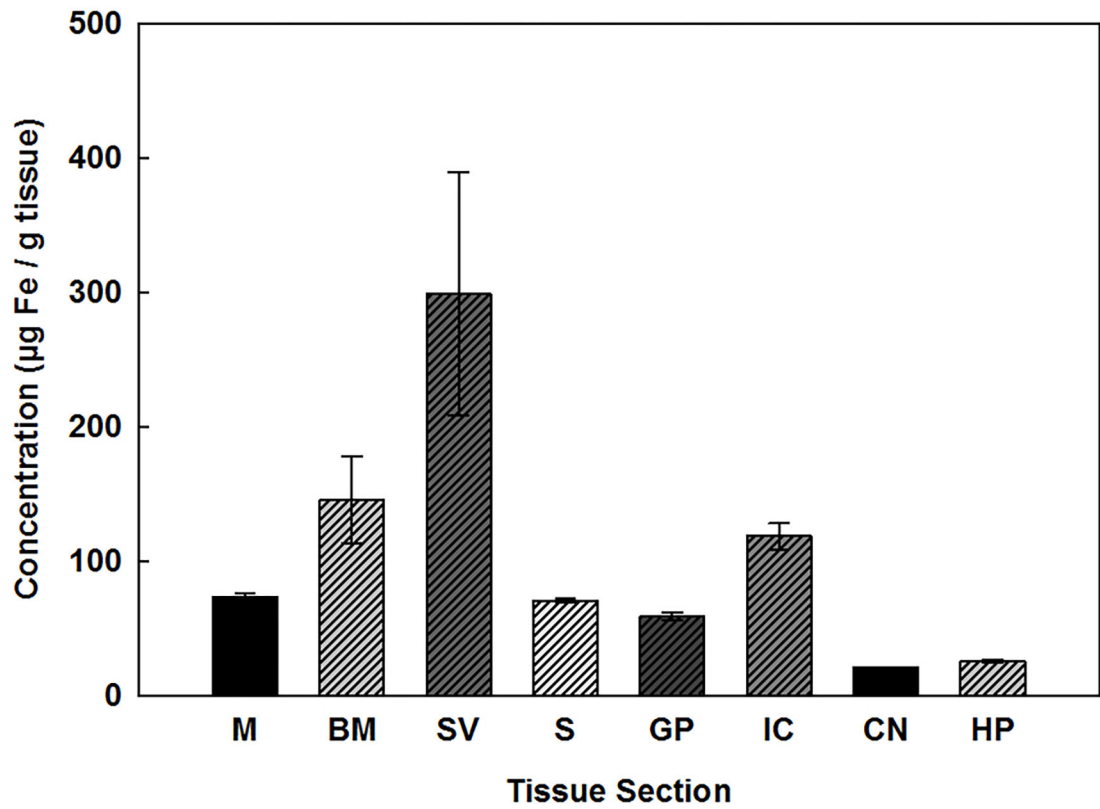
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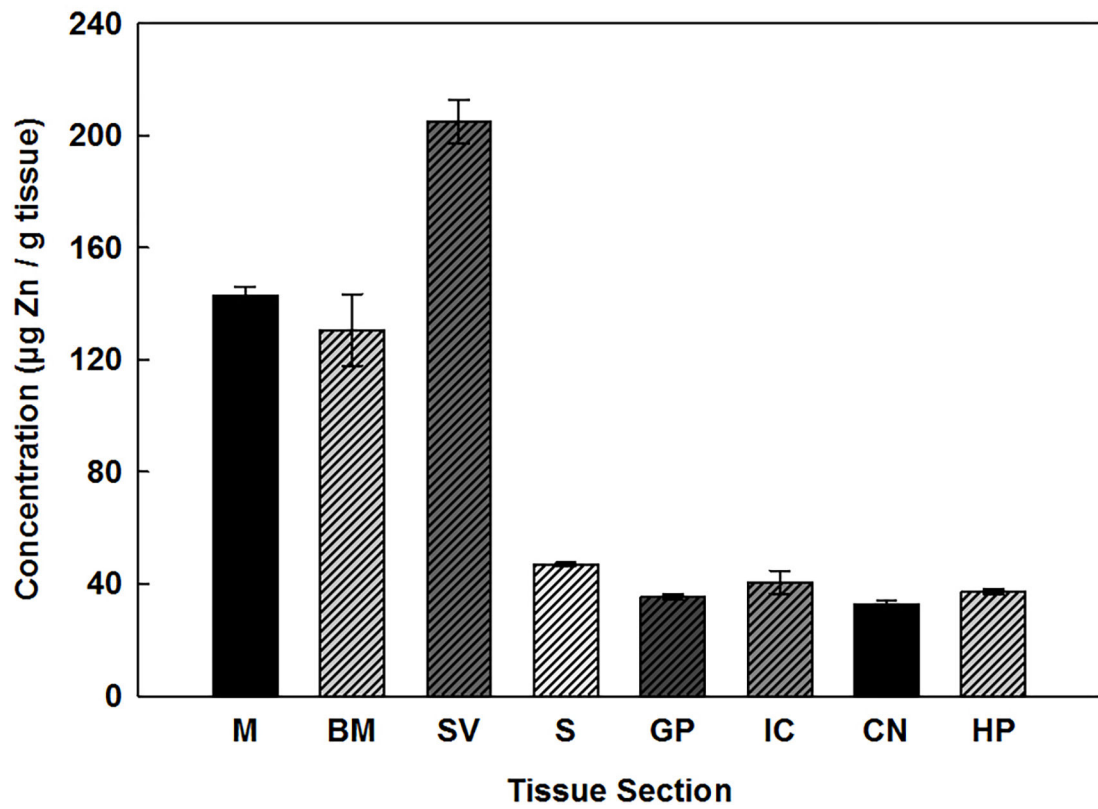


**Fig. 1.** Mn concentrations ( $\mu\text{g/g}$ ) present in the cochlea: M, BM, SV, and brain: S, GP, IC, CN, and HP of rats. Error bars represent standard error ( $n = 9$ ). g of tissue are expressed as dry weight.



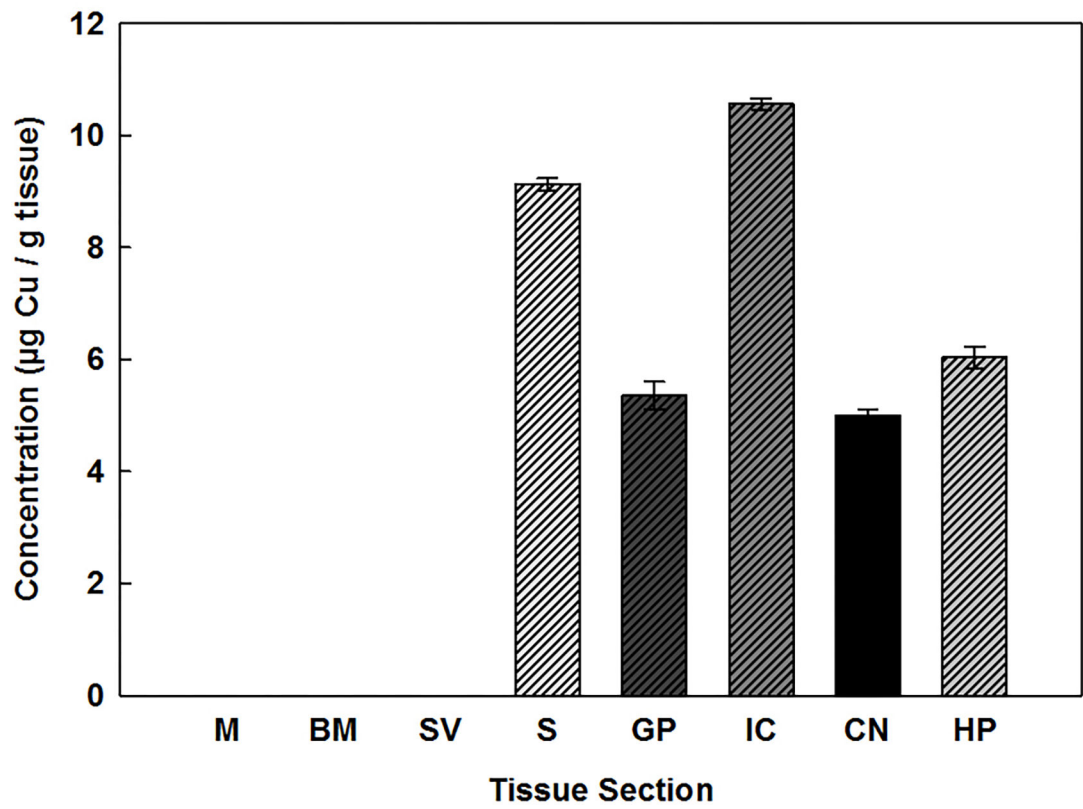
**Fig. 2.**

Fe concentrations ( $\mu\text{g/g}$ ) present in the cochlea: M, BM, SV, and brain: S, GP, IC, CN, and HP of rats. Error bars represent standard error ( $n = 4$ ). g of tissue are expressed as dry weight. The cochlear nucleus is represented by a pooled sample, and therefore has no calculated error.



**Fig. 3.** Cu concentrations ( $\mu\text{g/g}$ ) present in the cochlea: M, BM, SV, and brain: S, GP, IC, CN, and HP of rats. Error bars represent standard error ( $n = 6$ ).  $\mu\text{g}$  of tissue are expressed as dry weight. The absence of a data bar indicates that values are less than the limits of detection.





**Fig. 4.** Zn concentrations ( $\mu\text{g/g}$ ) present in the cochlea: M, BM, SV, and brain: S, GP, IC, CN, and HP of rats. Error bars represent standard error ( $n = 9$ ). Grams of tissue are expressed as dry weight.

**Table 1.**

## Operating Conditions of the Inductively Coupled Plasma Mass Spectrometer

Parameter	Setting
Forward power	1403.92 W
Cooling gas flow (argon)	13.02 L/min
Auxiliary gas flow (argon)	0.70 L/min
Nebulizer gas flow (argon)	0.98 L/min
Pole bias [(Mn, Cu, Zn) / Fe]	-5.80 V / -17.00 V
Hexapole bias [(Mn, Cu, Zn) / Fe]	-4.00 V / -20.00 V
Scanning mode/s	Survey and peak jumping
Collision cell gas (H <sub>2</sub> /He) (Fe analysis only)	2.00

**Table 2.**

Method Limits of Detection and Quantification for Mn, Fe, Cu, and Zn

		Limit ( $\mu\text{g/g}$ )						
		BM	M	SV	S	GP	IC	HB
Mn	mLOD	11.5	59.6	13.6	49.4	65.5	67.2	26.7
	mLOQ	38.3	199	45.3	165	218	224	89.0
Fe	mLOD	$1.15 \times 10^3$	$7.51 \times 10^3$	$9.07 \times 10^2$	$5.41 \times 10^3$	$2.11 \times 10^3$	$2.95 \times 10^3$	$1.93 \times 10^3$
	mLOQ	$3.83 \times 10^3$	$2.50 \times 10^4$	$3.02 \times 10^3$	$1.80 \times 10^4$	$7.02 \times 10^3$	$9.69 \times 10^3$	$6.43 \times 10^3$
Cu	mLOD	28.0	101	32.0	292	195	684	298
	mLOQ	93.4	337	107	974	649	$2.28 \times 10^3$	992
Zn	mLOD	$1.06 \times 10^3$	$4.77 \times 10^3$	$1.34 \times 10^3$	$2.17 \times 10^3$	$1.99 \times 10^3$	$3.10 \times 10^3$	$1.51 \times 10^3$
	mLOQ	$3.52 \times 10^3$	$1.59 \times 10^4$	$4.46 \times 10^3$	$7.24 \times 10^3$	$6.63 \times 10^3$	$1.03 \times 10^4$	$5.03 \times 10^3$

The HB limits were applied to the CN and HP tissues.

**Table 3.**

Concentrations of Mn, Fe, Cu and Zn in food

	<b>Food (<math>\mu\text{g/g}</math>)</b>	<b>Water (ng/ml)</b>
Mn	91 $\pm$ 3	0.226 $\pm$ 0.003
Fe	200 $\pm$ 40	135 $\pm$ 3
Cu	18 $\pm$ 4	18.90 $\pm$ 0.05
Zn	47 $\pm$ 2	9.84 $\pm$ 0.04

Mean ( $\pm$ SEM) concentrations of Mn, Fe, Cu and Zn in food (n=9) and water (n=6). Methods are described in Experimental Procedures.

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**Table 4.**

Range of Metal Concentrations in Rat Brain Compared to Previous Literature

Element	Previous Studies $\mu\text{g/g}$		Present Study $\mu\text{g/g}$	
	Min.	Max.	Min.	Max.
Mn	0.887	- 5.878	1.31	- 2.72
Fe	71	- 149	21	- 120
Cu	8.61	- 54.85	5.0	- 10.6
Zn	31.9	- 400	33	- 47

Concentrations are expressed in  $\mu\text{g/g}$  tissue dry weight. Values from (Donaldson et al., 1973, Fjeringstad et al., 1974b, Fjeringstad et al., 1977, Seth et al., 1977, Chan et al., 1983, Han et al., 1999, Reaney et al., 2006)

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