Biometals (2012) 25:337–350
DOI 10.1007/s10534-011-9504-8

Transition metal abnormalities in progressive dementias

Hiroyasu Akatsu · Akira Hori · Takayuki Yamamoto · Mari Yoshida · Maya Mimuro · Yoshio Hashizume · Ikuo Tooyama · Eric M. Yezdimer

Received: 18 November 2010 / Accepted: 21 October 2011 / Published online: 12 November 2011

Abstract Abnormal distributions of transition metals inside the brain are potential diagnostic markers for several central nervous system diseases, including Alzheimer's disease (AD), Parkinson's disease, dementia with Lewy bodies (DLB), bipolar disorders and depression. To further explore this possibility, the total concentrations of iron, zinc, copper, manganese, aluminum, chromium and cadmium were measured in post-mortem hippocampus and amygdala tissues taken from AD, DLB and Control patients. A statistically significant near fifty percent reduction in the total copper levels of AD patients was observed in both the hippocampus and amygdala. The statistical power of the hippocampus and amygdala copper analysis was found to be 86 and 74% respectively. No statistically significant deviations in the total metal concentrations were found for zinc, manganese, chromium or aluminum. Iron was found to be increased by 38% in AD amygdala tissues, but was unchanged in AD hippocampus tissues. Accounting for differences in tissue water content, as a function of both tissue type and disease state, revealed more consistencies with previous literature. To aid in the design of future experiments, the effect sizes for all tissue types and metals studied are also presented.

Keywords Copper · Iron · Alzheimer's disease · Lewy body · Amygdala · Hippocampus

E. M. Yezdimer—formerly at Industrial Summit Technology

H. Akatsu · A. Hori · T. Yamamoto · Y. Hashizume
Choju Medical Institute, Fukushimura Hospital, Toyohashi, Japan

M. Yoshida · M. Mimuro
Department of Neuropathology, Institute for Medical Science of Aging, Aichi Medical University, Nagakute, Japan

I. Tooyama
Research Promotion Organization for Intractable Neurological Disease, Molecular Neuroscience Research Center, Shiga Medical University, Otsu, Japan

E. M. Yezdimer (✉)
State College, PA, USA
e-mail: eyezdimer@yahoo.com

E. M. Yezdimer
Industrial Summit Technology, Parlin, NJ, USA

Introduction

Abnormal distributions of transition metals inside the body are potential diagnostic markers for several central nervous system diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), dementia with Lewy bodies (DLB), bipolar disorders and depression. A growing amount of evidence in recent years has implicated iron, zinc and copper as playing vital roles in the neuropathology of several progressive dementias. The reduction of the bioavailability of copper and zinc reduces the activity of amyloid

degrading enzymes (Strozyk et al. 2007). Amyloid plaques are known to sequester copper, zinc and iron (Lovell et al. 1998; Smith et al. 1997); potentially leading to further decreases in their bioavailability and the creation of a feedback mechanism favoring runaway amyloid accumulation. The amyloid precursor protein (APP) has also been shown to reduce Cu^{+2} to Cu^{+} ions (Multhaup et al. 1996), suggesting APP may be involved in copper endocytosis. The transmembrane protein β-secretase (BACE1), that catalyses the rate-limiting step in the amyloidogenic processing of APP, contains a cytoplasmic Cu^{+} binding domain and is believed to form a complex with the copper chaperone protein for superoxide dismutase 1 (Dingwall 2007). The aggregation of α-synuclein into Lewy bodies, one of the hallmark features of both PD and DLB, is also copper dependent, arising from strong affinities of α-synuclein to copper. The potential formation of a neurotoxic copper-dopamine complex that is recognized by dopamine transporters may also provide a possible pathway for the selective death of dopamine active cells (Paris et al. 2001).

Iron has been theorized to play a major role in the pathology of PD by facilitating the death of dopamine producing cells via localized excesses of iron inside the substantia nigra (Dexter et al. 1991). The proposed mechanism of cellular damage is through the destruction of lipids and other molecules, following the release of free radicals generated by the reaction of H_2O_2 with increased amounts of iron ions. Interestingly evidence of an anticipated link between PD and hereditary haemochromatosis, a disease that leads to increased iron inside the basal ganglia and other brain tissues (Berg et al. 2000), has not been forthcoming (Aamodt et al. 2007). While effective treatments targeting this mechanism have not yet emerged, the documented iron increase still may have a diagnostic utility. Clinical symptoms of PD are often not observed until dopamine levels become severely depleted, so measurements of iron distributions within the brain may be capable of predicting the onset of PD. Using multiple field MRI techniques, iron loaded ferritin molecules can be detected and correlate well with post-mortem brain iron concentrations (Bartzokis et al. 2007).

Similar diagnostic schemes may be possible for early detection of other neurological diseases like DLB or AD, provided a characteristic metal abnormality is present. Previous studies directly measuring metal concentrations inside human brain tissues are however often contradictory. For example hippocampus levels of zinc in AD patients have been reported as unchanged (Panayi et al. 2002; Rulon et al. 2000), significantly increased (Deibel et al. 1996) and significantly decreased (Corrigan et al. 1993; Ward and Mason 1987). Additional research is required to firmly establish the expected metal distributions and concentrations in both healthy and diseased populations. To these ends, we report the concentrations of several key transition metals from post-mortem human brain tissues taken from AD and DLB patients.

Materials and methods

The long-term goal of this research is to determine if the distributions of transition metals inside the brain are a characteristic biochemical feature of progressive dementias. The availability of tissues and other resources were significant limiting factors on the scope of this research. To accomplish the most wide ranging impact possible given our current resources, we have chosen to focus on three short term goals. The first goal was to obtain preliminary effect sizes for metal abnormalities across different tissue types and diseases states. These initial estimates would be highly instrumental in designing future, more comprehensive studies. The second goal was to conduct a more complete study of the metal distributions inside the memory and emotional areas of the brain, namely the hippocampus and amygdala. The third goal was to investigate more deeply the apparent discrepancies in previous literature.

Tissue samples

Post-mortem tissue samples were taken from the Choju Medical Institute's brain bank repository located at Fukushimura Hospital. Sample tissues were selected from patients confirmed at autopsy to be either AD, DLB or a non-demented Control. The procedures detailing the pathology classification have been described elsewhere (Akatsu et al. 2002). The pathological examination and classification was conducted using either the abnormal hemisphere (as evidenced by CT scan) or the left hemisphere if no differences between the left or right hemispheres were apparent. The hippocampus and amygdala samples

used in this study were taken from the hemisphere opposite of that used for the pathological examination. The samples used in this study were not exposed to formalin or any other fixation agent. After extraction, each tissue sample was placed inside a sealed flip-top 1.5 ml polyproplene test tube and frozen at −20°C for long-term storage.

Patients from both the AD and DLB groups were clinically diagnosed with dementia. Patients in the Control group were determined to be neurologically healthy following clinical exams. The average age of onset for the AD and DLB group was 76.6 ± 8.4 years and 74.8 ± 11 years respectively. The average disease duration was 11 ± 5.2 years and 8.4 ± 6.3 years for the AD and DLB groups. Care was taken during patient selection to avoid cases of mixed dementia. Gallyas-Braak staining of the temporal lobe revealed that the selected AD patients had a high density of neurofibrillary tangles (ranging between 30 and 175 counts/1.1 mm^2). All selected AD subjects were classified has either Braak stage 4 ($N = 2$), Braak stage 5 ($N = 4$) or Braak stage 6 ($N = 12$). The Gallyas-Braak staining of the Control and DLP groups however showed virtually no neurofibrillay tangles in the temporal lobe (<3 counts/1.1 mm^2), indicating that any AD pathology present in these groups had not progressed beyond the Braak 3 stage. The presence of Lewy bodies was confirmed in the DLB group by ubiquitin and α-synuclein immunostaining. Our DLB subject pool was also subclassified as described earlier (Perry et al. 1997) and contained occurrences of the common limbic type ($N = 6$), the common neocortical type ($N = 4$) and the pure limbic type ($N = 1$).

Sample preparation

Each wet (or "fresh") sample was removed from its original storage flip-top plastic test tube, weighed and placed into a pre-cleaned polypropylene drying tube. A rinse aliquot of 150 µl of ultrapure water was added to each of the original storage containers. The rinse water was then vortexed to help mix it with any remaining residue and was then added to the corresponding sample. The content of each drying tube was then centrifuged briefly, frozen at −20°C and stored for a few days prior to drying. The samples were dried under a vacuum over the course of 3 days or until a consistent weight had been reached. Following the completion of the drying treatment all samples had reached a consistent weight. The final dry weight of each sample was recorded and the samples were then returned to the freezer pending digestion.

The samples were divided into a series of several digestion sets for manageability. Each sample was allowed to reach room temperature before beginning the digestion procedure. The dried sample pellets generally did not adhere tightly to the tube walls and were transferred into a pre-cleaned Teflon digestion vessel. In order to assure complete removal of any remaining residue in the drying tube, 0.5 ml of hydrogen peroxide (Fluka brand TraceSelect Ultra grade 30%) was added to each drying tube and was allowed to sit for approximately 1 hour. Each of the drying tubes was then vortexed and its liquid added to the corresponding digestion vessel. This process was repeated with an additional 0.5 ml of H_2O_2, excluding the initial hour wait time. The process was then repeated a third time using 1.05 ml of nitric acid (Fisher brand Optima grade nitric acid). Alongside each digestion set, a reference sample of bovine liver (NIST SRM 1577) was also digested using the same method and was used as an external reference standard. Blank vessels consisting of an empty digestion vessel with only the digestion reagents were also included in each test set.

The sealed samples were digested using a Milestone Ethos Plus microwave system. A two step digestion process was used. The first step used a microwave power setting of 400 W and a temperature set point of 140°C for 10 min. The second step used a power setting of 600 W with a temperature set point of 190°C for 25 min. The microwave frequency was 2450 MHz. The vessels were then allowed to cool to room temperature. Following this process all the samples were completely decomposed into a colorless aqueous solution.

After digestion, the samples were transferred (using ultrapure water rinsing) into pre-cleaned polyethylene Nalgene bottles. The digestates were brought up to a total mass of approximately 20 g and the exact mass of each digestate was determined. A solution of internal standards Be, Sc, and In was then added into the digestates. The relativity high concentrations of some of the target metals necessitated the preparation of multiple dilutions which were analyzed in separate analytical runs. The dilution levels varied from 10× to 40× because of variations in the sample masses. Dilutions were prepared gravimetrically, using 2%

HNO_3 as a dilutent, in order to calculate exact mass dilution factors.

ICP-MS measurements

The samples were analyzed using a VG Axiom high-resolution ICP-MS located at the University of Missouri Research Reactor. The instrument was calibrated separately before each of the analytical runs using a five-point linearity standard series (0, 5, 10, 20, and 50 ppb) that had been prepared by gravimetrically diluting a commercial high purity multi-element stock solution. The internal standards of Be, Sc and In were also included in each linearity standard. Each sample was tested for the following isotopes: ^{27}Al, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{63}Cu, ^{65}Cu, ^{66}Zn, and ^{111}Cd.

The original ICP-MS units of measurement were μg of metal/gram of dried tissue. These units however are somewhat divorced from their original biological context. For reporting and discussion purposes the measurements have been converted into units of moles/fresh tissue volume. For simplicity the conversions also assumed a uniform brain tissue density of 1 g/ml. Density effects due to differences in temperature and salinity were ignored.

Background contamination

The brain tissue samples were collected over a period of several years. No special precautions to limit trace metal contamination were employed during the sample collection and storage. While all samples were treated using the same systematic procedure (regardless of their future group classification), we felt it was still imperative to conduct an investigation regarding several possible sources of trace metal contamination. Our investigation focused on two potential sources of metal impurities: the autopsy tools and the tissue storage containers. Metal impurities introduced from either personnel or airborne particles were not evaluated.

To estimate the degree of metal contamination resulting from direct tissue contact with the autopsy tools, we placed a series of metal surgical tools into a 50 ml bath of ultrapure water for 15 min. Ultrapure water operates as a mild leaching agent and served as an analog to the biological fluids encountered by the tools during an autopsy. The quantity of 50 ml was selected because it gave a sufficient volume to completely immerse the functioning part of the tool (i.e. the handles were not immersed) and it was a crude approximation for the amount of pooling fluid that a tool could have been exposed to during the autopsy. The 1.5 ml flip-top plastic test tubes used for long-term storage of the tissues were also tested by filling them with 1.0 ml of ultrapure water for a few days. Aliquots of the tool bath and storage tube incubation water were then acidified by the addition of concentrated nitric acid (Fisher brand Optima grade, 150 μl) and analyzed by ICP-MS using a procedure analogous to the tissue measurements. The sample limit of detection (LOD) was calculated for each element by using stock ultrapure water and multiplying the instrument LOD by the total sample dilution. The instrument LOD for each element was taken as three times the standard deviation of the concentration measured over 10 runs of a zero point standard.

Statistical analysis

To investigate the statistical significance of our data set we used the analysis of variance (ANOVA) method. The data from the hippocampus and amygdala samples were divided into 3 groups that corresponded to the AD, DLB and Control classifications described earlier. The null hypothesis for the experiment was defined as the following: "No difference exists in the average metal concentrations of the patient populations." The α level to reject the null hypothesis was selected as 0.05 and the alternative hypothesis was defined as "a difference exists between the average metal concentrations of the patient populations." If the null hypothesis was rejected, a posteriori comparison of the dementia mean and control mean was performed using Tukey's honestly significant difference procedure. Effect sizes and power estimations were conducted using the tables and recommendations from Cohen (1988).

The appropriate employment of ANOVA however does require that several fundamental assumptions are upheld. These assumptions are the distributions are normal, the variances between groups are the same and the number of samples/group is equal. Small departures from these assumptions usually have a negligible effect, particularly when the study is well balanced. Limited tissue availability and resources however forced us to produce study design that was

only semi balanced. For these reasons we believed a more exhaustive investigation of the nature of each population distribution was required. Investigation of the skewness and kurtosis of each data set was conducted in order to estimate the degree of non-normalcy present. Rather than attempt to determine the validity of an ANOVA treatment to our dataset, we instead chose to use the more robust, but less information rich, Mann–Whitney form of Wilcoxon statistic as an additional check. The Mann–Whitney test makes no assumptions regarding the normalcy of the distributions and is not affected by outliers. It is also a more natural method to treat populations with data points near or under the LOD. The conclusions of both tests are reported.

Results

The external liver reference standard was found to have excellent agreement with the standardized literature value (Table 1). The analysis of possible metal contamination from the autopsy tools revealed that the expected level of contamination was generally much lower than the corresponding tissue concentrations (Table 2). Zinc was the largest contaminate by far for every tool examined, including iron contaminants from stainless steel instruments. The majority of tissue samples used in this study ranged between 0.1 and 0.3 g. Crude estimates of the magnitude of contamination expected can be obtained by considering the hypothetical effect of spiking a tissue sample with an aliquot extracted from one of the ultrapure water leaching solutions. Example calculations revealed that if a 0.1 g tissue sample, taken from an average Control subject's hippocampus, was spiked with an 1.0 ml aliquot of the leaching solution (exposed to the polypropylene storage container) it would only register a very small percentage increase for most metals. This hypothetical spiking would produce a 0.7, 0.2, 0.01, 0.02 and 0.4% increase in the measured concentrations of Cr, Mn, Fe, Cu and Zn respectively. Aluminum contamination was a more serious concern with the above example potentially producing a 17% increase. These estimations are in line with a previously published study where the use of either stainless steel, Lexan plastic, titanium or Teflon-coated stainless steel dissection tools did not have a detectable

Table 1 NIST SRM 157 bovine liver external reference

Element	Al	Cr	Mn	Fe	Cu	Cu	Zn	Cd
Measurement isotope	^{27}Al	^{52}Cr	^{55}Mn	^{56}Fe	^{63}Cu	^{65}Cu	^{66}Zn	^{111}Cd
This study[a]	1.16 ± 0.2	0.08 ± 0.07	9.5 ± 0.4	253 ± 7	189 ± 5	191 ± 5	137 ± 5	0.31 ± 0.02
Consensus[b]	1.2 ± 0.5	0.11 ± 0.05	10.2 ± 0.7	263 ± 22	190 ± 10	190 ± 10	131 ± 8	0.28 ± 0.02

Given in units of ppm defined as (μg of element)/(g of dried tissue weight)

[a] These results were averaged over all digestion sets measured

[b] Roelandts and Gladney 1998

Table 2 Autopsy tool surface containments as measured from ultrapure water exposure

Element	Al	Cr	Mn	Fe	Cu	Cu	Zn	Cd
Measurement isotope	^{27}Al	^{52}Cr	^{55}Mn	^{56}Fe	^{63}Cu	^{65}Cu	^{66}Zn	^{111}Cd
Metal tray	<LOD	3.2	12.9	74.6	4.4	4.8	266.6	<LOD
Trimming blade no. 260 type (L)	<30.0	0.2	2.2	47.5	0.9	1.0	65.1	<LOD
FEATHER disposable scalpel stainless steel no. 10	<26.7	0.2	1.3	7.1	1.2	1.2	100.9	<LOD
FEATHER stainless brain autopsy blade #325	<31.9	3.5	0.8	3.5	0.6	0.7	68.4	<LOD
Igarashi stainless tweezers	41.8	0.2	1.6	7.3	1.5	1.7	83.6	<LOD
Stainless steel spatula	<40.0	<0.3	2.0	8.3	3.3	3.6	154.5	<LOD
Storage container	<49.3	<0.2	1.8	7.8	0.9	<1.0	111.4	<LOD
Sample LOD	24.5	0.1	0.2	0.4	0.4	0.4	1.0	0.1

Given in units of nM

effect on the measured metal tissue concentrations in marine bluefish (Heit and Klusek 1982).

An upper limit to the contamination expected following exposure to the storage containers is available by examining the effect of strong acid leaching on plastic storage containers (Moody and Lindstrom 1977). Adapting the findings of Moody and Lindstrom (1977) allows us to calculate that if a linear polyethylene storage container (with a 10 cm^2 surface area) was incubated for 7 days with a 1:1 mixture of pure nitric acid and ultrapure water, and that solution was then used to spike our Control hippocampus samples only a 0.8, 1 and 4% contamination in the total measurement of Fe, Cu and Zn concentrations would be expected, respectively. Hypothetical measurements of aluminum and chromium would be very seriously compromised in this strong acid leaching example, with a contamination level expected to exceed the natural trace-level abundance of these metals in brain tissue. The brain tissue concentration of other more biologically active metals, such as copper, zinc and iron, however were not at trace levels, making their analysis more robust in the face of trace level contaminates.

The average brain metal concentrations are presented in Tables 3, 4 and 5. The distributions generally appeared normal, where normal was defined as a skewness (γ_1) between -1 and 1 and a kurtosis (γ_2) of less than 4. Although the following exceptions to our definition of normalcy were found. The chromium distributions were strongly skewed and very tail heavy with γ_2 values ranging up to 7. The manganese distribution within the AD amygdala samples yielded $\gamma_1 = 3.0$ and $\gamma_2 = 8.4$. Cadmium populations within both the Control hippocampus and Control amygdala groups showed $\gamma_1 = 1.97$ and $\gamma_1 = 1.69$, respectively. Iron distributions were mildly skewed, with γ_1 values ranging up to 1.6. Aluminum distributions inside DLB hippocampus and AD hippocampus tissues displayed a mild skewing that was similar in magnitude to that of iron. Copper distributions inside the Control amygdala group also showed a slight skewness of 1.1.

The absolute differences between the ^{63}Cu and ^{65}Cu channel measurements averaged between 1.0–2.0%. Individual differences did range as high as 9.3%. No statistically significant correlation between the channel differences was found. Usually the ^{65}Cu channel reported the higher of the two measurements, although higher values from the ^{63}Cu channel were not uncommon and occurred in about 25% of the samples. Analysis of tissue copper concentrations were conducted using the ^{63}Cu, ^{65}Cu and a composite ($^{63}Cu + {}^{65}Cu$)/2 channel. Conclusions about the statistical significance of copper distributions were unaffected by the choice of channel. The results for

Table 3 Brain metal concentrations in dementia with Lewy bodies

	Hippocampus	Amygdala
N	11	11
Age	83.7 ± 8.5	83.1 ± 8.8
Male/Female	6/5	6/5
Dry/Fresh	0.192 ± 0.013	0.183 ± 0.011
Iron	733 ± 131	771 ± 154
Zinc	285 ± 38	257 ± 26
Copper	43.3 ± 12.5*	44.0 ± 8.1
Manganese	11.7 ± 6.6	11.9 ± 7.6
Aluminum	5.6 ± 6.1	6.9 ± 5.9
Chromium	0.9 ± 2.5	1.1 ± 2.0
Cadmium	0.8 ± 0.3	0.8 ± 0.3

All metal concentrations are given as μM of wet tissue. The errors denoted as ± are the standard deviation of the distribution. Statistically significant deviations from the corresponding control group were determined by a Tukey analysis (* $P < 0.05$).

Table 4 Brain metal concentrations in Alzheimer's disease

	Hippocampus	Amygdala
N	15	18
Age	87.5 ± 7.6	87.5 ± 7.6
Male/Female	5/9	6/11
Dry/Fresh	$0.162 \pm 0.014^{**++}$	$0.163 \pm 0.016^{**++}$
Iron	697 ± 323	$955 \pm 454^{+}$
Zinc	293 ± 45	273 ± 54
Copper	$29.5 \pm 8.9^{**++}$	$27.8 \pm 9.1^{**++}$
Manganese	7.4 ± 2.2	8.8 ± 5.2
Aluminum	4.4 ± 3.8	6.4 ± 5.5
Chromium	0.2 ± 0.2	$0.7 \pm 1.1^{+}$
Cadmium	1.0 ± 0.5	$1.0 \pm 0.4^{+}$

All metal concentrations are given as μM of wet tissue. The errors denoted as ± are the standard deviation of the distribution. Statistically significant deviations from the corresponding control group were determined by a Tukey analysis (* $P < 0.05$ and ** $P < 0.01$). Statistically significant deviations from the corresponding control group were determined by a Mann–Whitney test ($^{+}$ $P < 0.05$ and $^{++}$ $P < 0.01$)

Table 5 Brain metal concentrations in non-dementia controls

	Hippocampus	Amygdala
N	12	16
Age	84.0 ± 6.7	85.9 ± 7.3
Male/Female	6/6	6/10
Dry/Fresh	0.194 ± 0.008	0.181 ± 0.014
Iron	669 ± 152	694 ± 190
Zinc	297 ± 60	264 ± 28
Copper	57.7 ± 16.7	53.2 ± 25.1
Manganese	8.7 ± 4.8	7.8 ± 4.9
Aluminum	2.8 ± 2.0	6.0 ± 6.5
Chromium	0.3 ± 0.3	0.4 ± 0.9
Cadmium	0.7 ± 0.5	0.7 ± 0.4

All metal concentrations are given as μM of wet tissue. The errors denoted as ± are the standard deviation of the distribution

^{63}Cu are usually considered to be superior to those of ^{65}Cu because of the higher natural abundance of the ^{63}Cu isotope. For this reason the copper concentrations reported in this paper were solely taken from the ^{63}Cu channel, unless otherwise specified.

The average copper concentrations inside the hippocampus and amygdala varied sufficiently enough to reject the ANOVA null hypothesis. The average copper concentrations in the AD hippocampus and AD amygdala tissues were 51 and 52% of the Control average, respectively. Copper abnormalities within the DLB hippocampus and amygdala were also decreased 25 and 17%. The experimental ICP-MS procedure reports only on the total amount of metal in a sample, as any organic material is acid-microwave digested prior to analysis. Any metal sequestered inside an amyloid or Lewy body deposit is therefore released during this procedure. This implies that, particularity in the case of AD, even steeper reductions in the bioavailability of copper may be present. No statistically significant correlation of copper with age, total brain mass, tissue storage time or death-to-autopsy time was found. Figure 1 shows the distribution of copper concentrations for both the amygdala and hippocampus as a function of subject age.

Iron concentrations rejected the ANOVA null hypothesis within the amygdala ($P < 0.04$), but not within the hippocampus ($P < 0.8$). Figure 2 shows the distributions of iron as a function of patient age. No statistically significant correlation of iron with age, total brain mass, tissue storage time or death-to-autopsy time was found. Pairwise evaluation between the DLB/AD and Control populations using Tukey's honestly significant difference method failed to meet the $P < 0.05$ significance criteria. The Mann–Whitney test however found the iron concentration inside the amygdala of AD patients was significantly elevated ($P < 0.05$) by 38%. Hippocampus and amygdala tissues from the DLB group also showed 10% increases in iron, but were not statistically significant using either of the evaluation metrics. Our amygdala dataset also contained a notable number of iron outliers with 1 DLB patient, 6 AD patients and 2 Control patients having iron concentrations greater than 1.0 mM (i.e., near twice the standard deviation of

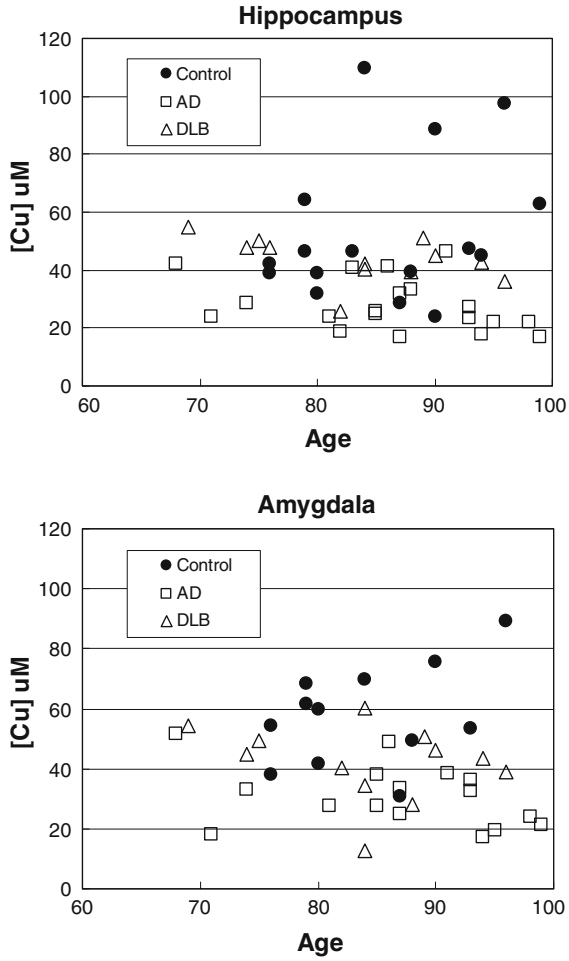

Fig. 1 Age dependence of copper. The *top figure* shows the copper dependence for the hippocampus dataset and the *bottom figure* shows the copper dependence for the amygdala dataset. *Closed filled circles* denote the Control, *open squares* denote AD, and *open triangles* denote DLB

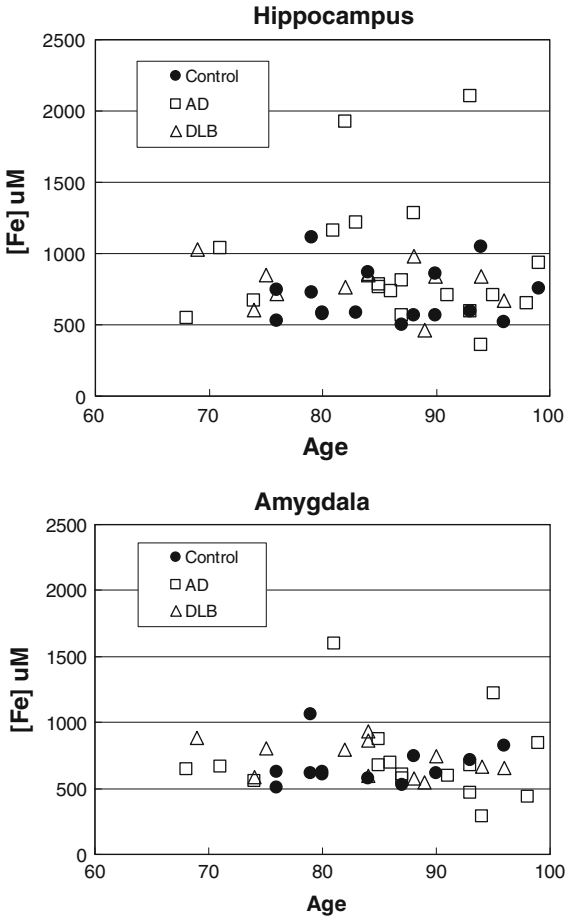

Fig. 2 Age dependence of iron. The *top figure* shows the iron dependence for the hippocampus dataset and the *bottom figure* shows the iron dependence for the amygdala dataset. *Closed filled circles* denote the Control, *open squares* denote AD, and *open triangles* denote DLB

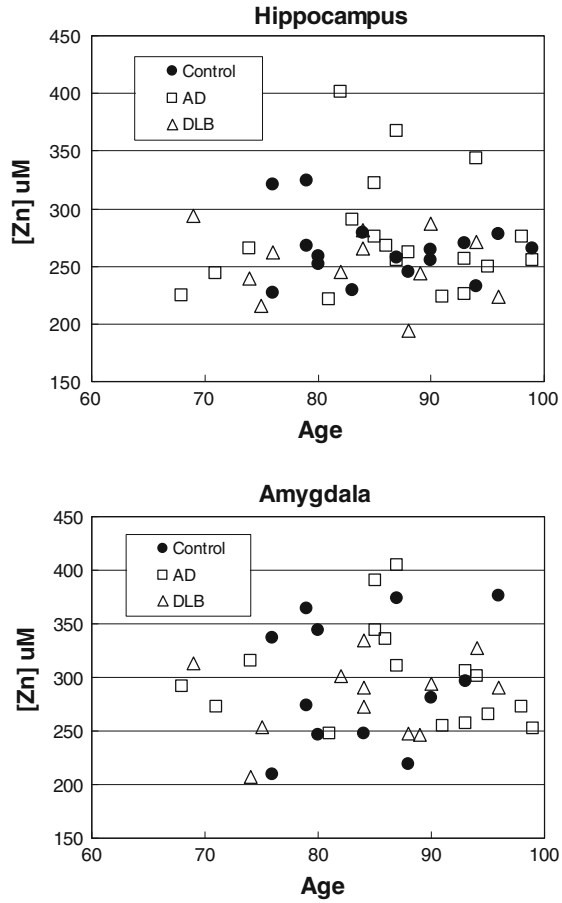

Fig. 3 Age dependence of zinc. The *top figure* shows the zinc dependence for the hippocampus dataset and the *bottom figure* shows the zinc dependence for the amygdala dataset. *Closed filled circles* denote the Control, *open squares* denote AD, and *open triangles* denote DLB

iron in the Control amygdala group). Of the nine iron amygdala outliers, three were female with the highest iron value of 2.1 mM being from a 93 year old female patient.

Zinc populations failed to reject the ANVOA null hypothesis in both tissue types. Figure 3 gives the distributions of zinc as a function of age. No statistically significant correlation of zinc with age, total brain mass, tissue storage time or death-to-autopsy time was found. In addition no significant changes in the manganese or aluminum data sets were found. Manganese levels in DLB tissues are increased on average, but with only $P < 0.24$ and $P < 0.19$ for the hippocampus and amygdala respectively. The examination of aluminum was hampered because the amount of aluminum in the tissue samples was often close to or under the LOD and our impurity analysis indicated that significant aluminum contaminates may have been present. Cadmium failed to reject the ANOVA null hypothesis for both tissues types, but did meet the significance criteria for the AD amygdala samples using Mann–Whitney test. The average cadmium concentration in the hippocampus is identical and the reported 0.7 to 1.0 μM increase in AD patients may become more firmly established with a larger sample population.

There also were notable correlations between the metal concentrations of the amygdala and hippocampus. Figure 4 shows these correlations for copper, iron, and zinc. The strongest correlation was found with copper (yielding Pearson correlation coefficients

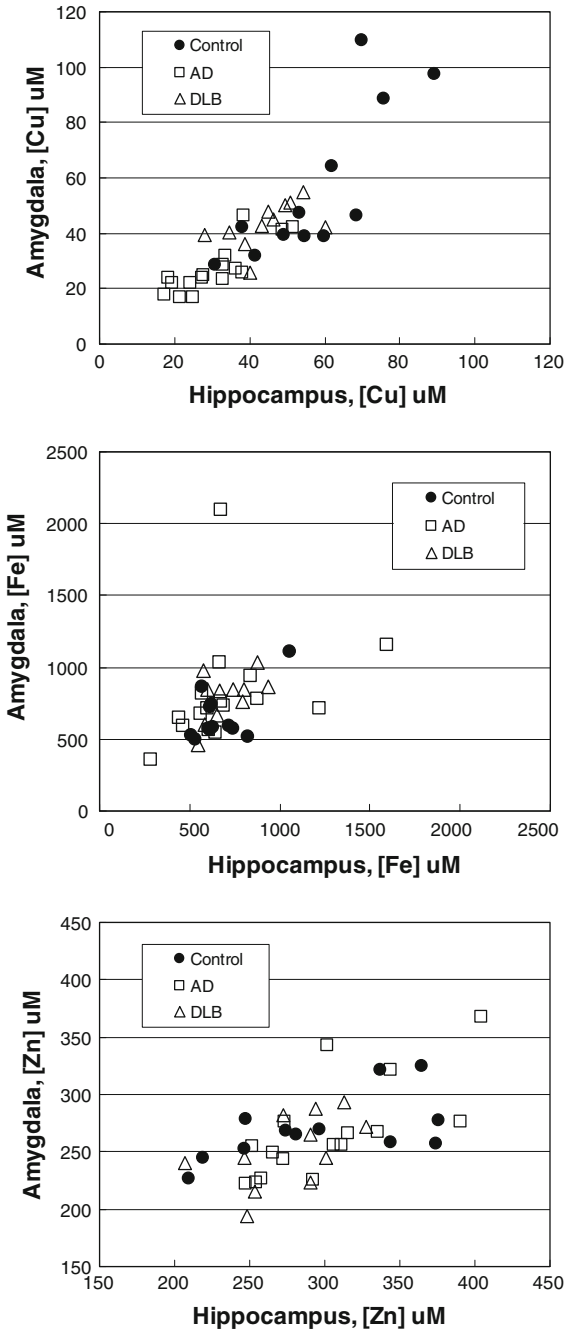

Fig. 4 Correlation between hippocampus and amygdala tissues. *Closed filled circles* denote the Control, *open squares* denote AD, and *open triangles* denote DLB. Subfigure **a** gives copper the correlation, **b** gives the iron correlation, and **c** gives the zinc correlation

of 0.57, 0.71, and 0.81 for the DLB, AD, and Control groups respectively). Neurodegeneration was also generally higher in AD tissues compared to those from DLB patients. The average brain mass of the AD, DLB and Control groups was found to be 1038 ± 143 g, 1143 ± 17 g and 1128 ± 116 g respectively. The dry/wet tissue mass ratios indicated that the AD tissues had a statistically significantly higher water content than the DLB and Control tissues. A post ad-hoc analysis was also conducted where all the common neocortical DLB samples were removed from the dataset, but was not found to significantly alter the aforementioned findings.

Table 6 Statistical effect sizes

	Hippocampus	Amygdala
Iron	0.08	0.26
Zinc	0.08	0.12
Copper	0.57	0.45
Manganese	0.28	0.20
Aluminum	0.23	0.04
Chromium	0.16	0.15
Cadmium	0.22	0.25

The effect size is defined as $f = \sqrt{\eta^2/(1-\eta^2)}$, where $\eta^2 = \sigma^2_{\text{means}}/\sigma^2_{\text{total}}$, σ^2_{means} is the variance between the means and σ^2_{total} is the total variance of the combined super-population which is comprised of all data points from all groups

Table 6 contains the ANOVA effect sizes for each metal and disease type. The effect sizes for copper were large and yielded a power value (at $\alpha = 0.05$) of 0.86 and 0.74 for copper inside the hippocampus and amygdala. The effect sizes for zinc were vastly smaller suggesting that hundreds of samples/group would be required to reach an 80% power value. A similar result was found for iron in the hippocampus. An improved situation was present for iron in amygdala tissues, requiring around 45–50 samples/group to achieve an 80% power value. This power analysis leads us to recommend that future studies of this type strive to reach a sample group size of approximately fifty subjects.

Discussion

Literature

Several inconsistencies are prevalent in the literature, most notably regarding the percent change in transition metal concentrations during central nervous system disorders. One initial area of contention is

whether a study reports concentrations using either dried or wet tissue weights. Consider the hypothetical example of a diseased and control tissue both measured to contain 10 μg/g (dry) of a metal. Converting between dry and wet tissue units requires the use of the dry/wet tissue ratio, gram(s) of dry tissue/gram(s) of wet tissue. Assuming the dried tissue ratio to be 0.194 for the control tissue and 0.162 for the diseased tissue (as was found in this study), the conversion would give 10 μg/g (dry) × 0.194 g (dry)/g (wet) = 0.194 μg/g (wet) and 10 μg/g (dry) × 0.162 g (dry)/g (wet) = 0.162 μg/g (wet). The percent reduction of metals between the diseased and healthy tissue is then calculated as a $1 - 0.162/0.194 = 16.5\%$. This conclusion is quite different from the original unchanged measurement using only the dried tissue weight.

This study found that the dry/wet ratio of tissues varied significantly according to their disease classification. To assist in our literature comparison we adjusted studies reporting in units of μg/g (dry) to units of μg/g (wet), using the ratios from this study when they were not otherwise unavailable. After this correction the agreement between zinc, copper and iron in the AD hippocampus measurements of Ward and Mason (1987) and Corrigan et al. (1993) became quite good (Table 7). Agreement is less pronounced with work of Deibel et al. (1996) where no change in zinc concentration was found and iron was reported to increase, rather than decrease. The work of Panayi et al. (2002) and Rulon et al. (2000) also reported that zinc in the hippocampus was unchanged in averaged AD populations and is at odds with a study from Andrási et al. (1995) that reported zinc is reduced by 59%.

In this work we found a statistically significant 49% decrease in copper, a 3% insignificant decrease in zinc and an 4% insignificant increase in iron within the hippocampus in AD patients. These results were more in-line with the results of Deibel et al. (1996), Panayi et al. (2002) and Rulon et al. (2000). Our measurements also showed a statistically significant 48% decrease in copper, a borderline significant 38% increase in iron and an insignificant 3% increase in zinc within the amygdala of AD patients. These findings were consistent with the concentrations reported by Deibel et al. (1996) and Rulon et al. (2000).

It is also instructive to examine the absolute value of the metal concentrations between studies. Figure 5 compares the absolute value of the copper, zinc and iron measurements for hippocampus Control samples. The results for copper and iron are generally consistent, with the reported value for iron from Ward and Mason (1987) being notably high. It is important to highlight that our data seems to report elevated levels of zinc inside the Control hippocampus as compared to other literature. Similar elevated levels of zinc were also seen inside the Control amygdala tissues. One possible explanation could be the presence of an unknown zinc impurity source. Potential zinc impurities were the largest of all the metals examined in this study and even through our initial analysis indicated their contribution was small, prudence may demand additional investigations relating to zinc impurities. Another source of literature discrepancies could simply be an insufficient number of samples. Effect sizes >0.3 are required to obtain reasonable power levels (>0.60) with 20 samples/group. The effect sizes were more commonly between 0.15–0.3, implying that in many cases the group size should range between 30–120 in order to reach repeatable conclusions. The dry/wet tissue mass ratios may also differ significantly based on the exact autopsy sampling positions. Individual variations in the dry/wet ratios can affect the conclusions of a study profoundly by influencing the population variance. Each individual measurement should be converted to a μg/g (wet) value before undertaking any statistical analysis of the data. This is because the rejection of the null hypothesis for experimental groups close to the significance criteria can depend on whether a measure of μg/g (dry) or μg/g (wet) was employed.

Another possible explanation of the literature discrepancies could be biochemical differences regarding the exact tissue position being sampled at autopsy. Thin slice laser ablation ICP-MS studies of the human hippocampus revealed that the distributions of copper, zinc, and iron were generally not uniform (Becker et al. 2005). Distinct localized patterns were present, with each metal having a unique distribution on the millimeter scale. It is quite possible that the studies of Ward and Mason (1987) and Corrigan et al. (1993) sampled the hippocampus from a similar position, while the studies of Deibel et al. (1996), Panayi et al. (2002) and Rulon et al. (2000) sampled from a slightly alternate location.

The simplified conclusion is that when considering fresh tissues from the hippocampus of AD patients:

Table 7 Literature summary for zinc, copper and iron inside the hippocampus and amygdala during AD

Study	Metal	Method	Control (μg/g)	AD[b]	Δ% (dry)	Δ% (Wet)[c]
Hippocampus						
Corrigan et al. 1993	Zn[a]	ICP-MS	12.76 (wet)	8.13		−36
	Cu		5.11 (wet)	4.46		−13
	Fe[a]		49.80 (wet)	39.85		−20
Deibel et al. 1996	Zn[a]	INAA	72.0 (dry)	85.1	18	1
	Cu[a]		16.8 (dry)	12.6	−25	−37
	Fe[a]		216 (dry)	288	33	11
Ward and Mason 1987[d]	Zn[a]	NAA	67.62 (dry)	52.75	−22	−35
	Cu		13.72 (dry)	14.02	2	−15
	Fe[a]		456.4 (dry)	417.6	−9	−24
Panayi et al. 2002	Zn	ICP-MS	14.65 (wet)	14.55		0
Andrási et al. 1995[e]	Zn[a]	ICP-AES	86 (dry)	46	−47	−59
	Cu		19 (dry)	20	5	−19
	Fe[a]	INAA	230 (dry)	310	35	4
Rulon et al. 2000	Zn	INAA	15.2 (wet)	16.3		7
Amygdala						
Deibel et al. 1996	Zn[a]	INAA	75.9 (dry)	89.9	18	7
	Cu[a]		19.8 (dry)	13.0	−34	−41
	Fe		243 (dry)	322	33	19
Rulon et al. 2000	Zn	INAA	15.7 (wet)	16.6		6

[a] Denotes the average was reported as statistically significant

[b] Same units as the control column

[c] If not directly reported, the Δ% (wet) was calculated using an average dry/wet ratio of 0.194 for the control condition and 0.162 for AD condition (concurrent to this study). The results of Andrási et al. (1995) are an exception because although the metal concentrations were reported as μg/g (dry) the average dry/wet ratios were also provided. Those ratios had values of 0.1831 for the control and 0.1413 for the AD groups. The quantity Δ% (wet) was determined using those ratios

[d] Eastern Canada population. A generalized non-control-disease dry/fresh ratio conversion of factor of 0.125 was reported for AD

[e] The tissue source was described as from Ammon's horn

copper levels appear to be always reduced, zinc may be unchanged or significantly reduced and a clear consensus regarding iron has not be reached. Similar conclusions within the amygdala of AD patients were also found, but with a stronger weighting toward increased iron concentrations.

Pathology

Our predominant finding was that copper distributions were severely affected in AD, typically resulting in an approximate 50% decline. Similar 39 to 45% decreases in copper concentrations of the temporal, frontal, parietal and occipital lobes of AD patients has also been reported (Plantin et al. 1987). The same study of AD subjects found no statistically significant changes in the concentrations of Cr, Fe, Zn and Cd in any of the areas studied, with the exception of a statistically significant 16–18% decrease in the manganese concentrations of the frontal and parietal lobes. Increasing the bioavailability of intracellular copper has been shown to restore cognitive function in rodents by inhibiting the accumulation of β-amyloid trimers and phosphorylated tau (Crouch et al. 2009). An associative link between the deregulation of serum copper and declines in cognitive function during AD has been found (Squitti et al. 2009). The concentration of serum copper has been found to distinguish (at $P < 0.001$) between AD (average = 17.2 ± 5.9 μM) and both vascular dementia patients (average = 12.9 ± 2.3 μM) and healthy controls (average = 12.6 ± 2.5 μM) (Squitti et al. 2005). Based on this

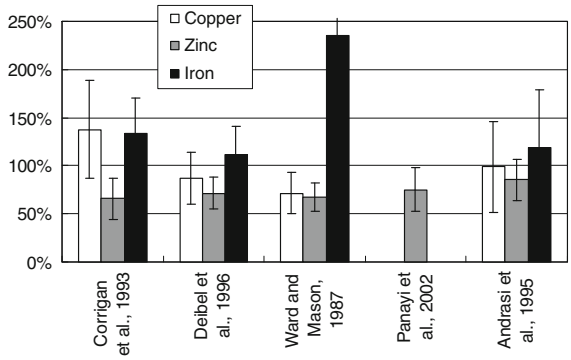

Fig. 5 Agreement between Controls for copper, zinc and iron inside the human hippocampus. The *y-axis* shows literature Control concentrations divided by the Control concentration found in this study as expressed by percentage. The error *bars* are displayed using a single standard deviation of the propagated error

data the concentration ratio of copper in the brain to copper in the serum is 4.3:1 in neurologically normal patients and only 1.7:1 in late-stage AD patients. This data leads us to hypothesize that the copper trafficking system of the blood–brain barrier may be dysfunctional during AD pathology. Further study is necessary to determine if the delocalization of neurological copper is a disease specific marker of AD pathology or is a symptomatic consequence of neurodegeneration. Increases in the zinc levels of serum in AD cases have also been reported (Rulon et al. 2000). While the observation of zinc decreases in AD limbic tissues is more sporadic than for copper, it is not inconceivable that an analogous biochemistry may exist between the two metals. The larger overall concentration of neurological zinc compared to that of copper may also serve to obscure pathologically relevant changes in zinc homeostasis.

Copper serum levels in 20–30 year old control patients have been reported as 13 ± 4 μM (Mustak et al. 2008) and are in excellent agreement with the control serum data found from much older patients (average = 71.1 years) (Squitti et al. 2005). This consistency illustrates that serum copper levels are usually stable over an entire lifetime. Recent articles and editorials (Brewer 2010b; Brewer 2010a) however have made the case that prolonged and elevated copper intake from contaminated drinking water may be playing a significant role in AD pathology. Our findings directly refute this conclusion. While increased copper intake and/or copper overload can indeed be highly toxic and lead to pronounced neurological symptoms, AD brains appear to be uniquely devoid of copper atoms.

Our secondary finding was a large 38% increase in iron levels inside AD amygdala tissues, but not within the hippocampuses of AD patients. The true pathological relevance of this finding is however masked by the presence of several elevated iron outliers across the entire sample population. Indirect but highly correlative multiple field MRI (fDRI) estimations of iron levels in a large population set ($N = 165$, age 19–82) have suggested that a statistically significant sex difference in ferritin iron levels is present within the brain (Bartzokis et al. 2007). Subdivision of the population into healthy older males (age > 55 years) with either the hemochromatosis H63D or transferrin TfC2 alleles (IRON+) showed a statistically significant 14% increase in fDRI intensity in the caudate nucleus ($P = 0.034$) as compared to the IRON− population (Bartzokis et al. 2010). The same study however found no statistically significant change in fDRI intensity between the male IRON+ and IRON− subpopulations in the globus pallidus, putamen, thalamus, frontal lobe white matter, genu of the corpus callosum or hippocampus. In the same study no similar variation between the IRON+ and IRON− genotypes in similarly aged, healthy females were found. Our healthy male test population was too limited ($N = 6$) to detect small changes in iron concentrations due to the presence of the IRON+ and IRON− genotypes. The average iron concentration for our male Control patients was 683 ± 185 μM and 740 ± 218 μM within the hippocampus and amydgala respectively. One of six male Control patients however was an iron outlier, having iron concentrations of 1058 and 1111 μM within his hippocampus and amydgala tissues. A similar iron spike in the amydgala of one of our Control female patients cautions against assigning the causality of this spike to any specific genotype and a detailed genotyping of our patient population was unfortunately beyond the scope of the current work.

The metals levels in DLB patients were found to be statistically unchanged with the one exception of copper inside the hippocampus. It is unclear however whether the decreases in DLB copper levels found in this study represents a possible characteristic feature of DLB or is simply an artifact of a co-present early or mid phase (Braak stage ≤ 3) AD pathology. The

single DLB subject that was subclassified as pure limbic type had only slightly above average copper concentrations, 45 and 48 μM inside his hippocampus and amygdala respectively. Further research is required to confirm the pathological origins of observed copper decreases in DLB patients, however if the decrease is associated with the presence of an AD-type pathology it would suggest that the reduction of limbic copper may be an early marker of AD pathology.

Levels of iron, copper, manganese and zinc in the substantia nigra were previously found to be unaltered in patients with incidental Lewy body disease (Dexter et al. 1994). These findings are somewhat inconsistent with the conclusions of other pathological and cellular research. Copper overloads, as it occurs in Wilson's disease (Kitzberger et al. 2005), leads to extrapyramidal symptoms in a significant number of cases. Concordant bicompartmental dopaminergic deficits in neurologic Wilson disease have also been observed (Barthel et al. 2003), suggesting that the pathologies of Wilson disease and PD are related. There is also a strong correlation ($r = 0.95$; $P < 0.01$) of serum copper and serum peroxides (Squitti et al. 2006), highlighting the ability of excess copper to induce oxidative stress in a manner similar to that of iron. Direct challenges to SH-SY5Y cells with copper yielded an increase in α-synuclein (Grunberg-Etkovitz et al. 2009), raising the possibility that Lewy body formation could be a neuroprotective response to elevated heavy metal concentrations. Despite this evidence pointing to a metal overload mechanism in DLB, an increase in the total copper concentration inside the limbic system during DLB was not observed in this data set. Iron was increased by an average of $\sim 10\%$ in DLB patients, although the change was not statistically significant. Substantia nigra Lewy bodies in PD are known to stain iron positive while cortical Lewy bodies in cases of variant AD stain iron negative, suggesting the two types of Lewy bodies may be of different structures (Castellani et al. 2000). Our conclusion is that while Lewy bodies may be capable of sequestering metal atoms, limbic Lewy bodies as they occur in DLB may be primarily involved in sequestering some other chemical species or may only sequester metals in localized regions that are too small to affect the overall tissue concentration.

Acknowledgments The authors would like to sincerely thank both the patients and their families for their generous donations, without which this research would not be possible. The authors would like to acknowledge the researchers at Elemental Analysis Inc. (Kentucky, USA) for their invaluable assistance with the inductively coupled mass spectrometry experiments. The authors would also like to acknowledge financial support from the New Energy and Industrial Technology Development Organization (Tokyo, Japan).

References

Aamodt AH, Stovner LJ, Thorstensen K, Lydersen S, White LR, Aasly JO (2007) Prevalence of haemochromatosis gene mutations in Parkinson's disease. J Neurol Neurosurg Psychiatry 78:315–317

Akatsu H, Takahashi M, Matsukawa N et al (2002) Subtype analysis of neuropathologically diagnosed patients in a Japanese geriatric hospital. J Neurol Sci 196:60–63

Andrási E, Farkas É, Scheibler H, Réffy A, Bezúr L (1995) Al, Zn, Cu, Mn and Fe levels in brain in Alheimer's disease. Arch Gerontol Geriatr 21:89–97

Barthel H, Hermann W, Kluge R, Hesse S, Collingridge DR, Wagner A, Sabri O (2003) Concordant pre- and postsynaptic deficits of dopaminergic neurotransmission in neurologic Wilson disease. Am J Neuroradiol 24:234–238

Bartzokis G, Tishler TA, Lu PH, Villablanca P, Altshuler LL, Carter M, Huang D, Edwards N, Mintz J (2007) Brain ferritin iron may influence age- and gender-related risks of neurodegeneration. Neurobiol Aging 28:414–423

Bartzokis G, Lu PH, Tishler TA et al (2010) Prevalent iron metabolism gene variants associated with increased brain ferritin iron in healthy older men. J Alzheimers Dis 20:333–341

Becker JS, Zorly MV, Pickhardt C, Palomero-Gallagher N, Zilles K (2005) Imaging of copper, zinc, and other elements in thin section of human brain samples (hippocampus) by laser ablation inductively coupled plasma mass spectrometry. Anal Chem 77:3208–3216

Berg D, Hoggenmuller U, Hofmann E, Fischer R, Kraus M, Scheurlen M, Becker G (2000) The basal ganglia in haemochromatosis. Neuroradiology 42:9–13

Brewer GJ (2010a) Copper toxicity in the general population. Clin Neurophysiol 121:459–460

Brewer GJ (2010b) Risks of copper and iron toxicity during aging in humans. Chem Res Toxicol 23:319–326

Castellani RJ, Siedlak SL, Perry G, Smith MA (2000) Sequestration of iron by Lewy bodies in Parkinson's disease. Acta Neuropathol 100:111–114

Cohen J (1988) Statistical power analysis for the behavioral sciences. Psychology Press, New York

Corrigan FM, Reynolds GP, Ward NI (1993) Hippocampal tin, aluminum and zinc in Alzheimer's disease. Biometals 6:149–154

Crouch PJ, Huang LW, Adlard PA et al (2009) Increasing Cu bioavailability inhibits Abeta oligomers and tau phosphorylation. PNAS 106:381–386

Deibel MA, Ehmann WD, Markesbery WR (1996) Copper, iron, and zinc imbalances in severely degenerated brain regions

in Alzheimer's disease: possible relation to oxidative stress. J Neurol Sci 143:137–142

Dexter DT, Carayon A, Javoy-Agid F, Agid Y, Wells FR, Daniel SE, Lees AJ, Jenner P, Marsden CD (1991) Alterations in the levels of iron, ferritin and other trace metals in Parkinson's disease and other neurodegenerative diseases affecting the basal ganglia. Brain 114:1953–1975

Dexter DT, Sain J, Rose S et al (1994) Indices of oxidative stress and mitochondrial function in individuals with incidental Lewy body disease. Ann Neurol 35:38–44

Dingwall C (2007) A copper-binding site in the cytoplasmic domain of BACE1 identifies a possible link to metal homoeostasis and oxidative stress in Alzheimer's disease. Biochem Soc Trans 35:571–573

Grunberg-Etkovitz N, Lev N, Ickowicz D, Avital A, Offen D, Malik Z (2009) Accelerated proteasomal activity inducted by Pb(+2), Ga(+3), or Cu(+2) exposure does not induce degradation of alpha-synuclein. J Environ Pathol Toxicol Oncol 28:5–24

Heit M, Klusek CS (1982) The effects of dissecting tools on the trace element concentrations of fish and mussel tissues. Sci Total Environ 24:129–134

Kitzberger R, Madl C, Ferenci P (2005) Wilson disease. Metab Brain Dis 20:295–302

Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR (1998) Copper, iron and zinc in Alzheimer's disease senile plaques. J Neurol Sci 158:47–52

Moody JR, Lindstrom RM (1977) Selection and cleaning of plastic containers for storage of trace element samples. Anal Chem 49:2264–2267

Multhaup G, Schlicksupp A, Hesse L, Beher D, Ruppert T, Masters CL, Beyreuther K (1996) The amyloid precursor protein of Alzheimer's disease in the reduction of copper(II) to copper(I). Science 271:1406–1409

Mustak MS, Rao TSS, Shanmugavelu P, Sundar NMS, Menon RB, Rao RV, Rao KSJ (2008) Assessment of serum macro and trace element homeostasis and the complexity of inter-element relations in bipolar mood disorders. Clinica Chimica Acta 394:47–53

Panayi AE, Spyrou NM, Iversen BS, White MA, Part P (2002) Determination of cadmium and zinc in Alzheimer's brain tissue using inductively coupled plasma mass spectrometry. J Neurol Sci 195:1–10

Paris I, Dagnino-Subiabre A, Marcelain K, Bennett LB, Caviedes P, Caviedes R, Olea-Azar C, Segura-Aguilar J (2001) Copper neurotoxicity is dependent on dopamine-mediated copper uptake and one-electron reduction of aminochrome in rat substantia nigra neuronal cell line. J Neurochem 77:519–529

Perry R, McKeith I, Perry E (1997) Lewy body dementia-clinical, pathological and neurochemical interconnections. J Neural Transm 51:95–109

Plantin L-O, Lying-Tunell U, Kristensson K (1987) Trace elements in the human central nervous system studied with neuron activation analysis. Biol Trace Elem Res 13:69–75

Roelandts I, Gladney ES (1998) Consensus values for NIST biological and environmental standard reference materials. Fresenius J Anal Chem 360:327–338

Rulon LL, Robertson JD, Lovell MA, Deibel MA, Ehmann WD, Markesbery WR (2000) Serum zinc levels and Alzheimer's disease. Biol Trace Elem Res 75:79–85

Smith MA, Harris PL, Sayre LM, Perry G (1997) Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. PNAS 94:9866–9868

Squitti R, Pasqualetti P, Forno GD et al (2005) Excess of serum copper not related to ceruloplasmin in Alzheimer disease. Neurology 64:1040–1046

Squitti R, Barbati G, Rossi L et al (2006) Excess of nonceruloplasmin serum copper in AD correlates with MMSE, CSF beta-amyloid, and h-tau. Neurology 67:76–82

Squitti R, Bressi F, Pasqualetti P et al (2009) Longitudinal prognostic value of serum "free" copper in patients with Alzheimer disease. Neurology 72:50–55

Strozyk D, Launer LJ, Adlard PA et al (2007) Zinc and copper module Alzheimer ABeta levels in human cerebrospinal fluid. Neurobiol Aging 30:1069–1077

Ward NI, Mason JA (1987) Neutron activation analysis techniques for identifying elemental status in Alzheimer's disease. J Radioanal Nucl Chem 113:515–526