Research article

Re-partitioning of Cu and Zn isotopes by modified protein expression

Anette Büchl1, Chris J Hawkesworth1, K Vala Ragnarsdottir*1,3 and David R Brown*2

Address: 1Department of Earth Sciences, University of Bristol, Wills Memorial Building, Bristol, BS8 4EL, UK, 2Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, UK and 3School of Engineering and Natural Sciences, University of Iceland, Hjarðarhagi 6, 107 Reykjavík, Iceland

Email: Anette Büchl - c.j.hawkesworth@bristol.ac.uk; Chris J Hawkesworth - c.j.hawkesworth@bristol.ac.uk; K Vala Ragnarsdottir* - Vala.Ragnarsdottir@bristol.ac.uk; David R Brown* - bssdrb@bath.ac.uk

* Corresponding authors

Abstract

Cu and Zn have naturally occurring non radioactive isotopes, and their isotopic systematics in a biological context are poorly understood. In this study we used double focussing mass spectroscopy to determine the ratios for these isotopes for the first time in mouse brain. The Cu and Zn isotope ratios for four strains of wild-type mice showed no significant difference (δ65Cu - 0.12 to -0.78 permil; δ66Zn -0.23 to -0.48 permil). We also looked at how altering the expression of a single copper binding protein, the prion protein (PrP), alters the isotope ratios. Both knockout and overexpression of PrP had no significant effect on the ratio of Cu isotopes. Mice brains expressing mutant PrP lacking the known metal binding domain have δ65Cu isotope values of on average 0.57 permil higher than wild-type mouse brains. This implies that loss of the copper binding domain of PrP increases the level of 65Cu in the brain. δ66Zn isotope values of the transgenic mouse brains are enriched for 66Zn to the wild-type mouse brains. Here we show for the first time that the expression of a single protein can alter the partitioning of metal isotopes in mouse brains. The results imply that the expression of the prion protein can alter cellular Cu isotope content.

Background

Copper (Cu) and zinc (Zn) have essential roles in mammalian metabolism: copper in the formation of haemoglobin and red blood cells and Zn and Cu in several enzymes in a number of metabolic pathways. A number of neurodegenerative diseases are associated with abnormalities in the tissue distribution of these trace metals, such as Cu in prion disease [1,2], and Cu and Zn in Alzheimer’s disease [3]. Cu has the two isotopes 65Cu and 63Cu, Zn has the five isotopes 64Zn, 66Zn, 67Zn, 68Zn and 70Zn. Different isotopes of the same element have different masses, which leads to different behaviour, and this contribution is concerned with the extent these isotopes are fractionated by small changes in a complex biological system, the brain.

Precise analyses of the ratios of transition stable isotopes has only been possible since the development of multicollector inductively coupled plasma mass spectrometers and associated extraction techniques [4-7]. Variations in the isotopic composition are expressed by delta notation

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\delta^{66}_Zn = (66Zn/64Zn_{sample}/66Zn/64Zn_{standard} - 1) \times 1000, \text{ and}
\]

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δ$^{65}\text{Cu}$ = \left(\frac{^{65}\text{Cu}^{/}}{^{63}\text{Cu}^{/}}_{\text{sample}}/^{65}\text{Cu}^{/}/^{63}\text{Cu}^{/}_{\text{standard}} - 1\right) * 1000 \%

which is the deviation of a sample from an international standard in permil (1‰ = 0.1%). Variations in the isotopic composition of trace metals within organisms result from two effects. Biogeochronological processes in the environment lead to different isotopic compositions in, for example, soil, water, and plants. Isotope ratios may therefore be used to trace the origin, or source, of the element in question at the time it enters the body. Secondly, heavy stable isotope ratios fractionate during biochemical processes in organisms, and they are known to fractionate both during the uptake of trace metals into a cell, and as metals pass through membranes within the cell [8,9]. A study of Fe isotopes in human blood samples established that they were fractionated, and that the mean Fe isotope value is different in the blood of men and of women [10]. Such isotope fractionations reflect the fact that the isotope with a lower number of neutrons is kinetically more active and therefore used preferentially in biochemical processes.

This study focuses on the extent that small changes in a complex system affect trace metal Cu and Zn isotope ratios. If they do change such isotopes may be used as a new medical tool to investigate the pathways and partitioning of trace metals in human beings. Cu and Zn concentrations are routinely measured to determine their distribution in the brain, and the involvement of these elements in, for example, protein or enzyme function has been studied in detail [11,12]. Both metals are distributed throughout the brain with zinc concentrations being approximately twice that of copper (Cu being approximately 5 parts per million). Both metals are associated with large number of proteins and are important co-factors in the activity of many enzymes. Currently, no suitable method exists to examine pathways by which naturally occurring isotopes of metals are transported and partitioned within the animal body or whether they could be used to provide markers of abnormal transport and partitioning of trace metals in disease. Thus the question is the extent to which the isotope ratios of a metal are influenced by the expression of one particular protein in a biological system. Mice brains were analysed due to the availability of a suitable range of transgenically manipulated mice, allowing examination of the effects of alterations to a single proteins on the isotope ratios in a complex system like the brain.

The protein we chose to study was the prion protein, as it is a Cu-binding glycoprotein that can bind up to four Cu atoms, it is concentrated at synapses and may protect them from oxidative stress [13]. The metal ions (usually Cu) bind to a specific domain in the protein called the octameric repeat region. Deletion of this region from the protein abolishes metal binding to the protein [14]. Together with Cu the protein forms a complex that possesses anti-oxidant activity, and that may have important implications for synaptic homeostasis [13]. Prion protein misfolding is associated with the development of animal or human prion diseases (Scrapie, Bovine spongiform encephaloathy, Creutzfeldt-Jacob disease). Other trace metals such as Zn and Mn can substitute for Cu at the binding site [14]. However, while the affinity for Cu is high, that for Zn is very low. It is now well established that the prion protein (PrP) has an influence on cellular copper metabolism. As an example exposure of cells to high Cu concentrations increase PrP expression [15] and caused PrP to internalise [16], delivering copper into the cell [17]. Because PrP has a low zinc affinity it would be out competed by other zinc binding proteins and is unlikely to play any role in zinc metabolism.

This study presents for the first time accurate measurements of the ratio copper and zinc isotopes as they occur naturally in the brain. By studying these ratios in the brains of transgenic mice we sought to establish whether altered expression of a single protein can alter these isotopic ratios. We have shown that altered expression of the prion protein, in the normal cellular, non-aggregating isform, can selectively modify Cu isotope ratios. This implies that Cu isotopes are sensitive to the presence of different Cu-binding sites in the brain.

**Methods**

**Mouse brains**

The brains of different mouse strains were collected from adult (4 month old mice) or newborn mice. Mice studied included four lines of wild-type mice which included: MF1, 129Sv, FvB and C57BL/6 (Harlan). Also studied was a transgenic line included as a control for transgenic effects known as Harry [18] and three lines of mice in which the prion protein had been modified. The prion protein transgenic mice included prion protein knockout mice (PrP<sup>ko</sup>) [19], mice overexpressing the protein (Tg20) [20] and mice which express a version of the prion protein lacking the octameric repeat region on a prion protein knockout background (C4) [21]. The comparable control mouse for the prion transgenic mice was the 129Sv mouse.

**Isotope analyses**

A method to separate Cu, Fe and Zn from silicate samples using a strongly basic anion resin has been described in detail by Marechal et al. (1999) [6]. This protocol, using AG MP-1 resin (Bio-Rad, CA, USA) was adopted here to accommodate smaller sample sizes and to decrease the size of the environmental blank contribution. It follows the approach of Archer and Vance [7] who provide more details of the analytical technique. The samples were digested using concentrated HNO<sub>3</sub> + H<sub>2</sub>O<sub>2</sub> and concentrated HCl in a second step. Following sample digestion
samples were loaded onto the column in 1 ml 7 M HCl + 
H₂O₂. The majority of matrix elements were removed by 
addition of a further 2 ml of 7 M HCl + H₂O₂, before col-
lecting Cu in 8 ml 7 M HCl + H₂O₂; Fe was eluted by pass-
ing 4.5 ml 2 M HCl + H₂O₂ before finally collecting Zn in 
4 ml 0.5 M HNO₃ [7].

All analyses were performed on a ThermoFinnigan Nep-
tune double focusing mass spectrometer at the University 
of Bristol (see [7] for a detailed description). Purified ana-
lyte fractions were introduced into the mass spectrometer 
in 2% HNO₃ by means of a CETAC (Omaha, NE, USA) 
Aridas desolating spray chamber fitted with a CPI 
(Amsterdam, Neth.) PFA nebuliser and spray chamber to 
give enhanced sensitivity. Instrumental mass fractiona-
tion was corrected for using external normalisation tech-
niques described by Marechal et al. [6], with careful 
attention being paid to matrix matching of samples and 
standards (cf. Archer and Vance [7]). We used the Cu 
standard from NIST and the Zn standard from Lyons JMC,
and ⁶⁰Ni was monitored to correct for the isobaric inter-
ference of ⁶⁴Ni on ⁶⁴Zn.

Statistical analysis of the data was carried out using "Analy-
sis of Variance" (ANOVA) or with a two tailed Student's 
t-test.

Results and discussion

Cu and Zn isotopes were analysed by MC-ICP-MS and the 
results are presented in Table 1. The internal reproducibil-
ity for measurement of Cu and Zn isotopes was 0.03‰ (2 
sigma). The external reproducibility for Cu and Zn by 
sample bracketing was 0.08‰ (2 sigma) and for Zn with 
a double spike 0.04‰ (2 sigma). All the observed shifts 
in isotopic ratios lie on the mass fractionation line (Fig. 
1), and thus they all obey the mass fractionation law.

In order to eliminate age or diet effects, only mice held 
under controlled conditions were chosen. The mice were 
4 months old and they had all been fed with the same 
mouse pellets. Cu and Zn isotopes were analysed in 'nor-
mal' wild type mouse brains from 4 different strains with 
no genetic modifications (n = 19), in prion protein over-
expressed mouse brains (TG20, for details see [20]) (n = 
6), in brains in which the prion protein had been deleted 
(PrP⁰/⁰, for details see [19]) (n = 4), in brains in which the 
copper-binding region of the prion protein had been 
knocked out (C4, for details see [21]) (n = 4) and in other 
transgenic mice from the line Harry which carry a luci-
ferase transgene driven by promoter/enhancer elements 
from the Igf2/H19 locus (for details see [18]) (n = 4). Four 
new born mice brains in which the prion protein had 
been deleted were also analysed to assess the influence of 
age on the Cu and Zn isotopic composition. In all cases 
whole brains were dissolved in concentrated nitric and 
hydrochloric acid for analysis.

The δ⁶⁵Cu values in the wild type mice brains of four dif-
ferent strains (MF1, 129, C57 BL/6 and FvB) varied 
between -0.12 and -0.78 permil, and δ⁶⁶Zn between -0.23 
and -0.48 permil (the results are plotted on a diagram of 
δ⁶⁵Cu against δ⁶⁶Zn in Figure 2). The range of Cu and Zn 
isotopic ratios in the wild-type mice brains is not under-
stood, but there was no significant difference between the 
isothe ratios of the different wild type mice strains. In 
more detail the two sample t-test, indicates that for most 
of the samples the wild-type mice brains show no signifi-
cant differences to each other (e.g. 129 – C57: δ⁶⁵Cu: t = 
0.09, df = 7, p = 0.92, δ⁶⁶Zn: t = 1.47, df = 7, p = 0.18; 
δ⁶⁶Zn: t = 0.25, df = 7, p = 0.80). This implies that the 
different genetic backgrounds had no significant influence 
on the isotopic ratios. These results present the first assess-
ment of Cu and Zn isotope ratios in the brain.

There has been one other study on the fractionation of Cu 
and Zn isotopes in biological tissue. Cu uptake in vitro 
into the apoprotein of azurin expressed in Escherichia coli 
fractionates the Cu isotopes, and the azurin (δ⁶⁵Cu -1.64) 
was enriched in the lighter isotope relative to the source 
material (δ⁶⁵Cu -0.11) [9]. In an in vivo experiment, 
where the intact Cu protein azurin was synthesised 
directly inside cells of the bacterium Pseudomonas aeru-
ginosa, the Cu isotope composition of the azurin (δ⁶⁵Cu - 
1.09) was also enriched in the lighter isotope [9]. It 
appears that the Cu isotopic ratios of the mouse brains lie 
within the range of other biological samples.

In order to determine if any form of genetic manipulation 
could alter isotope ratios a transgenic line was chosen at 
random and the isotope ratios in the brain compared to 
that for the brains of the matching mouse strain from 
which they were derived (C57BL/6). The δ⁶⁵Cu and δ⁶⁶Zn 
values of the wild type mice brains overlap with those 
from transgenic mouse brains from the line Harry in 
which the genetic changes have no connection with Cu or 
Zn (Fig. 2). These results show that transgenic manipula-
tion on its own did not change the Cu or Zn isotopic com-
position significantly.

We then studied a series of different transgenic mice in 
which transgenic manipulation of a single protein is 
known to alter the copper content of the brain. The prion 
protein in these mice was either either overexpressed 
(Tg20) deleted (PrP⁰/⁰) or modified to lack the main 
metal binding domain (C4). All three groups plot in dif-
f erent fields to the wild type and line Harry mice brains on 
the plot of Cu versus Zn isotopes (Fig. 2). Specifically the 
prion protein overexpressed (Tg20) deleted (PrP⁰/⁰) or 
modified to lack the main metal binding domain (C4) 

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Table 1: Cu and Zn isotopic compositions of the mouse brains

| Sample          | \(\delta^{65}\text{Cu}/^{63}\text{Cu}\) | \(\delta^{66}\text{Zn}/^{64}\text{Zn}\) | \(\delta^{67}\text{Zn}/^{64}\text{Zn}\) |
|-----------------|-----------------------------------------|-----------------------------------------|-----------------------------------------|
| MF1             | -0.59                                   | -0.27                                   | -0.47                                   |
|                 | -0.35                                   | -0.30                                   | -0.46                                   |
|                 | -0.33                                   | -0.35                                   | -0.54                                   |
|                 | -0.49                                   | -0.29                                   | -0.47                                   |
|                 | -0.43                                   | -0.23                                   | -0.30                                   |
|                 | -0.47                                   | -0.29                                   | -0.44                                   |
| I29             | -0.29                                   | -0.25                                   | -0.47                                   |
|                 | -0.23                                   | -0.42                                   | -0.69                                   |
|                 | -0.78                                   | -0.37                                   | -0.56                                   |
|                 | -0.77                                   | -0.32                                   | -0.52                                   |
| Fvb             | -0.12                                   | -0.37                                   | -0.53                                   |
|                 | -0.16                                   | -0.39                                   | -0.52                                   |
|                 | -0.38                                   | -0.32                                   | -0.44                                   |
|                 | -0.3                                     | -0.36                                   | -0.51                                   |
| C57             | -0.35                                   | -0.31                                   | -0.46                                   |
|                 | -0.46                                   | -0.41                                   | -0.57                                   |
|                 | -0.7                                     | -0.43                                   | -0.59                                   |
|                 | -0.73                                   | -0.48                                   | -0.65                                   |
|                 | -0.42                                   | -0.40                                   | -0.60                                   |
| PrP-KO (adult)  | -0.56                                   | -0.20                                   | -0.26                                   |
|                 | -0.16                                   | -0.19                                   | -0.23                                   |
|                 | -0.14                                   | -0.11                                   | -0.13                                   |
|                 | -0.16                                   | -0.02                                   | -0.04                                   |
| PrP-KO (newborn)| -0.33                                   | -0.23                                   | -0.30                                   |
|                 | -0.17                                   | -0.19                                   | -0.18                                   |
|                 | -0.1                                     | 0.00                                    | 0.07                                    |
|                 | -0.38                                   | -0.10                                   | -0.15                                   |
| C4              | -0.02                                   | -0.15                                   | -0.14                                   |
|                 | 0.31                                    | -0.22                                   | -0.29                                   |
|                 | 0.04                                    | -0.21                                   | -0.26                                   |
|                 | -0.09                                   | -0.03                                   | -0.06                                   |
| TG20            | 0.18                                    | 0.08                                    | 0.12                                    |
|                 | -0.36                                   | -0.04                                   | -0.05                                   |
|                 | 0.26                                    | -0.09                                   | -0.13                                   |
|                 | -0.44                                   | -0.12                                   | -0.17                                   |
|                 | -0.37                                   | -0.25                                   | -0.34                                   |
|                 | -0.58                                   | -0.21                                   | -0.28                                   |
| Line Harry      | -0.14                                   | -0.26                                   | -0.32                                   |
|                 | -0.17                                   | -0.36                                   | -0.46                                   |
|                 | -0.21                                   | -0.25                                   | -0.29                                   |
|                 | -0.46                                   | -0.36                                   | -0.5                                    |

The Cu and Zn isotope ratios of mice brains, expressed in the delta notation (as discussed in the text. The different samples are: MF1, I29, FvB and C57 BL/6 – wild-type mouse brains; PrP-KO (adult) prion protein deleted mouse brains, adult; PrP-KO (newborn) prion protein deleted mouse brains, newborn; C4 – Cu binding domain of the prion protein deleted; TG20 – prion protein overexpressed; Line Harry – transgenic mouse brains with the genetic changes have no connection with Cu or Zn.
brains all have more positive Zn isotope ratios than the wild type and line Harry mice brains ($\delta^{66}\text{Zn}$: $R^2 = 0.70$, $p < 0.001$; $\delta^{67}\text{Zn}$: $R^2 = 0.76$, $p < 0.001$). In contrast, only the C4 samples, those without the Cu binding domain of the prion protein, have consistently more positive Cu isotope ratios (Fig. 2), as do two samples from TG20. The average $\delta^{65}\text{Cu}$ value of the C4 brains is 0.57 permil enriched in the heavy Cu isotope ($^{65}\text{Cu}$) compared to the wild type mice brains, and this difference was highly significant (ANOVA, $R^2 = 0.45$, $p = 0.01$). Analysis was also carried out for these results with the Student’s t-test. The analyses confirm the inferences from the figures, namely, that $\delta^{65}\text{Cu}$: $t = -3.339$, $df = 6$, $p = 0.02$; $\delta^{66}\text{Zn}$: $t = -3.269$, $df = 5$, $p = 0.02$; $\delta^{67}\text{Zn}$: $t = -5.004$, $df = 5$, $p = 0.01$) and that $\delta^{65}\text{Cu}$/PrP$^0/0$ show no significant differences for the Cu isotope ratios ($\delta^{65}\text{Cu}$: $t = -1.454$, $df = 6$, $p = 0.19$), but are different for the Zn isotope ratios ($\delta^{66}\text{Zn}$: $t = -3.792$, $df = 6$, $p = 0.01$; $\delta^{67}\text{Zn}$: $t = -5.746$, $df = 6$, $p = 0.001$). Finally, we tested whether changes in isotopes could be something that occurs with increased age. We examined the metal isotope ratios in new born mice and compared them to those from the adults in this study. The one-day old mice brains with the prion protein deleted ($\delta^{65}\text{Cu}$ ranges between -0.10 and -0.38, and $\delta^{66}\text{Zn}$ between 0.00 and -0.23 permil) fell within the field of the older mice brains in which the prion protein had been deleted ($\delta^{65}\text{Cu}$ ranges between -0.14 and -0.56 permil, and $\delta^{66}\text{Zn}$ between -0.02 and -0.20 permil, Table 1). Comparison between PrP$^0/0$-adult and PrP$^0/0$-young using the Student’s t-test confirm that these two groups show no significant differences from each other ($\delta^{65}\text{Cu}$: $t = -0.082$, $df = 6$, $p = 0.98$; $\delta^{66}\text{Zn}$: $t = 0.00$, $df = 6$, $p = 1.00$; $\delta^{67}\text{Zn}$: $t = -0.272$, $df = 6$, $p = 0.80$). This strongly suggests that changes with age did not result in analytically significant differences in Cu and Zn isotopic ratios, at least in the first 4 months. Neither overexpression nor the lack of expression of PrP alters the partition of the Cu isotopes. However, expression of a form of PrP that cannot bind Cu results in increased levels of the heavier Cu isotope. The implication is that the presence of PrP inhibits mechanisms that would otherwise regulate the Cu content to maintain the normal Cu isotope ratio. When PrP can bind Cu these alternative mechanisms are not required. When PrP is not
expressed the alternative mechanisms are activated and able to compensate for the loss of expression. At present, if these alternative balancing mechanisms exist, they remain unknown. Tg20 mice express 10 time the normal level of PrP with little measurable variation in the level of the protein times the normal levels of PrP [20]. However, previous studies have shown the amount of Cu bond per molecule of PrP in Tg20 mice was greatly reduced when compared to wild-type [22]. This implies that the impact of increased expression of PrP on Cu isotopes is limited by the availability of Cu to bind to it. However, it is important to note the huge variation in the values for the TG20 mice. This variation is greater than between different wild-type mouse lines and suggests that overexpression of PrP causes disturbance to maintenance of isotope ratios at an individual level. This would imply that expression of PrP is not the prime mechanism by which a cell maintains the ratio balance.

A very interesting observation is that wild-type mice show little variation in the ratios of the three Zn isotopes (Fig. 1). This again implies tight regulation of Zn isotope content of the brain. However, any form of manipulation of PrP resulted in a significant change to the ratios. It is possible that either loss or overexpression of a copper binding protein could have a similar effect on the way a cell processes Zn. In the case of deleting PrP it is possible that compensatory mechanisms, increasing Cu uptake or utilisation could be non selective and also alter Zn uptake. In the case of overexpression of PrP, PrP itself could alter internalisation of Zn either by directly binding Zn as has been suggested [14] or indirectly increasing transport of Zn. In the latter case other non selective transport proteins such as divalent metal transporter (DMT-1) could show an increase transport of Zn due to decreased availability of Cu (due to it being bound to PrP). Again, the mice overexpressing PrP showed high variability in Zn isotopic ratios in the brain. This variation was similar to that seen for Cu ratios. Although there is no information concerning the mechanism, it does suggest that there is some form of co-regulation of this process maintaining isotope ratios for different metals. At the level of individuals the greater variation in values implies an increased complexity of the alternative routes for metal entry into cells.

In terms of the relationship of Zn isotopes ratios to PrP, the simplest explanation of the results is that they are unrelated to the metal binding capacity of PrP. While changes in Cu isotopes can clearly be related to the loss of metal binding to the protein, the change in Zn isotopes is only consistent with a genetic modification of the mice involving the PrP gene. This might suggest that Zn isotope ratios are more sensitive to the modification of cellular metabolism that Cu isotope ratios. This means that the study of Cu isotope ratios is more likely to uncover mechanisms that could be utilised to understand how a protein alters copper metabolism than the study of Zn isotope ratios. This possibly relates to the different nature of the two metals and the greater role of Cu in reactions that can cause damage to cells through oxidative stress.

**Conclusion**

This study establishes for the first time that a minor change to a biological system can alter the distribution of metal isotopes in ways that could not be predicted. Until this study it has not been possible to establish the wild-type ratios of Cu and Zn isotopes. The observed differences in the Cu and Zn isotope composition of mouse brains with different protein expression profiles supports the hypothesis that trace metal isotopes can be used to examine processes leading to brain damage and disease and pathways by which metals are transported through the animal body. As the altered ratios were not observed in the Harry transgenic mouse line it is clear that these changes are specific for relevant metal binding proteins. These observations may open up a new field of medical research using geochemical tools. Other applications may include other neurodegenerative diseases that are related to trace metals like Alzheimer's disease [3,23,24] or Parkinson's disease [25]. However, further studies of the basic mechanisms involved are essential before such information could have any meaning in a diagnostic sense. Nevertheless, these findings highlight that an essential unexplored aspect of metalochemistry has a significant biological relevance. How different metal isotopes are utilised biologically remains unknown but our data provides evidence that they are used differently. If cells in the brain selectively regulate the isotopes that enter the brain, then differential utilisation of these isotopes by the brain might lead to important consequences for important cellular processes. The consequences of altering this differential utilisation might have a significant impact on health issues.

**Authors’ contributions**

AB carried out the experimental procedures and assisted with manuscript preparation. DRB provided mouse brains and background for biological aspects of the project and contributed to experimental planning and manuscript preparation. CJH and KVR were responsible for supervision, project planning and preparation of the manuscript.

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