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Impact of Aging on Copper Isotopic Composition in the Murine Brain

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9 **Key words:** aging, copper isotopes, biomarker, Alzheimer's disease, metallomics, MC-ICP-MS

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11

12 **Abstract**

13 Aging is the main risk factor for Alzheimer's disease (AD). AD is linked to alterations in metal
14 homeostasis and changes in stable metal isotopic composition can occur, possibly allowing the latter
15 to serve as relevant biomarkers for potential AD diagnosis. Copper stable isotopes are used to
16 investigate changes in Cu homeostasis associated with various diseases. Prior work has shown that
17 in AD mouse models, the accumulation of ⁶³Cu in the brain is associated with the disease's
18 progression. However, our understanding of how the normal aging process influences the brain's
19 isotopic composition of copper remains limited. In order to determine the utility and predictive power
20 of Cu isotopes in AD diagnostics; we aim - in this study - to develop a baseline trajectory of Cu
21 isotopic composition in the normally aging mouse brain. We determined the copper concentration
22 and isotopic composition in brains of 30 healthy mice (WT) ranging in age from 6 to 12 months, and
23 further incorporate prior data obtained for 3-month-old healthy mice; this range approximately
24 equates to 20-50 years in human equivalency. A significant ⁶⁵Cu enrichment has been observed in the
25 12-month-old mice compared to the youngest group, concomitant with an increase in Cu

26 concentration with age. Meanwhile, literature data for brains of AD mice display an enrichment in
27 ⁶³Cu isotope compared to WT. It is acutely important that this baseline enrichment in ⁶⁵Cu is fully
28 constrained and normalized against if any coherent diagnostic observations regarding ⁶³Cu
29 enrichment as a biomarker for AD are to be developed.

30

31

32

33 1. Introduction

34 The process of aging provokes wide-ranging effects on the brain, involving various cellular and
35 molecular changes. Mitochondrial dysfunctions in brain cells lead to a decline in energy metabolism
36 efficiency. DNA repair mechanisms become compromised resulting in increased genomic instability.
37 Furthermore, there is an accumulation of soluble (and sometimes neurotoxic) protein precursors [1],
38 misfolded and aggregated proteins, dysfunctional organelles and elevated levels of oxidative stress
39 [2]. Numerous studies have established a direct association between oxidative stress, aging and
40 cognitive decline in humans [3-7]. The progressive increase in oxidative stress during normal aging
41 is attributed to the imbalance between the production of reactive oxygen species and the ability of
42 cellular antioxidant defense systems to counteract them.

43 Additionally, neuro-inflammation is a hallmark of the aging brain. Senescent glial cells,
44 and the infiltration of immune cells into the brain contribute to a chronic state of neuro-inflammation
45 (see fig1). This process is characterized by elevated levels of pro-inflammatory cytokines and a
46 decline in anti-inflammatory compounds [8, 9]. Chronic neuro-inflammation not only promotes
47 neurodegeneration but also accelerates the overall aging process of the brain [10].

48 Aging is recognized as the primary risk factor for neurodegenerative diseases, including
49 Alzheimer's disease (AD) [11, 12]. Alzheimer's disease is a global health concern, currently affecting
50 50 millions of individuals worldwide, with an estimated increase to nearly 113 million by 2050 [12,
51 13]. It is the fifth leading cause of death in high income countries [14, 108].

52 At the cellular level, AD is characterized by two major patho-physiological features: the
53 presence of extracellular amyloid plaques (A β -plaques), formed by the agglomeration of the A β 42
54 peptides, and the formation of intracellular neurofibrillary tangles (NFTs), resulting from abnormal
55 hyperphosphorylation of the tau protein [12, 15-17]. The latter is mainly due to clinical diagnosis *via*
56 positron emission tomography (PET) imaging, however it is noted that this technique is predicated
57 on significant deposition of A β -plaques to breach the fidelity threshold of current imaging
58 instrumentation, which are generally unable to detect the soluble A β fraction [48-50]. Current *in vivo*
59 detection methods are invasive and onerous, involving imaging techniques such as positron-emission
60 tomography (PET) to visualize amyloid- β deposits [18], or measuring the levels of amyloid- β or tau
61 proteins in cerebro-spinal fluids [19, 20].

62 During the initial phase of the disease, A β -plaques begin to accumulate in the extracellular
63 space, yet clinical symptoms, including loss of memory, difficulties in performing executive functions
64 and solving problems, cognitive decline, apraxia may not manifest for up to 20 years [14, 21]. This
65 asymptomatic/ preclinical stage of the disease holds great significance for the development of early
66 diagnosis techniques and the optimization of AD treatments.

67 In addition to other neurodegenerative diseases such as Wilson disease, Menkes disease,
68 Amyotrophic Lateral Sclerosis (ALS) [22 – 24, 109, 110], AD has also been associated with a change
69 in the metal homeostasis [25 – 27]. Previous works by Moynier et al., (2019, 2020) have produced
70 evidences that the changes in the metal isotopic composition, of elements like copper serve as relevant
71 biomarkers for the future diagnosis of AD [28, 42], and there is a growing body of literature that more
72 generally suggests viability of stable metal isotopes in disease biomarker development [112].

73 Copper plays a crucial role in various biological processes in the brain, functioning as a
74 cofactor and structural component of several enzymes involved in essential functions. It participates
75 in redox metabolism, energy metabolism (cytochrome c oxidase), antioxidative defense (Zn,Cu-
76 superoxide dismutases), iron metabolism (ceruloplasmin), neurotransmitter synthesis (dopamine- β -

77 monooxygenase), regulation of inflammation, and neuropeptide synthesis (peptidylglycine- α -
78 amidating enzyme) (see fig1) [29- 33].

79 The concentration of Cu in the human brain is typically found to be between 3 and 5 $\mu\text{g/g}$
80 wet weight [34, 35]. Similarly, a mouse brain contains on average 7 $\mu\text{g/g}$ for a female and 4 $\mu\text{g/g}$ for
81 a male [43]. Copper has two stable isotopes: ^{63}Cu and ^{65}Cu with different relative abundances in
82 nature of 69% and 31%, respectively. Isotopes undergo fractionation due to changes in bonding
83 environments within the body [27, 37 - 44]. Isotope fractionation is a general term referring to the
84 process of creating isotopic variability of a particular element among coexisting species or reservoirs
85 hosting this element [40, 41]. Heavy isotopes tend to accumulate in environments characterized by
86 stiffest bonds [37, 38, 45]. For instance, ^{65}Cu binds preferentially to histidine, glutamate and aspartate
87 [38, 112]. As a result of the fractionation, the isotopic composition of copper differs in organs: ^{65}Cu
88 accumulates in the brain, liver and kidney whilst ^{63}Cu is enriched in red blood cells (RBC) and
89 plasma. Changes in the isotopic composition of copper in body fluids and in different organs have
90 been used as proxies to identify cancers [46 – 49], liver diseases [50 – 54] and neurogenerative
91 diseases [24, 28, 38, 40, 42, 55, 56].

92 In AD mice models expressing the amyloid-beta precursor protein (APP), the accumulation
93 of ^{63}Cu in the brain has been shown to be related to the progression of the disease [42]. Besides,
94 nuclear magnetic resonance (NMR) studies have established that Cu (II) ions interact directly with
95 A β forming a Cu (II)-A β complex *in vitro* [32, 57 – 62]. Copper might increase the number of β -sheet
96 and α -helix structures in amyloid peptides, which could be a cause of β -amyloid aggregation [63].
97 Copper and Zinc might also be, in healthy individuals, linked with the degradation of soluble A β [64].
98 Amyloid- β sequestering copper, the change in bonding environment due to the emergence of protein
99 aggregates, amyloid plaques (A β) and neurofibrillary tangles (NFT) is a key to understand this shift
100 in copper isotopic composition in AD brains.

101 However, prior to any of the above mechanistic pathways for Cu isotope fractionation being
102 identified, it is requisite to first bolster our current understanding regarding the impact of the normal

103 aging process on the isotopic composition of copper in the brain remains limited, such that if present,
104 this effect can be accounted for in the development of any future biomarker based on stable Cu
105 isotopes. The objective of this study is to establish a baseline for the changes in Cu isotopic
106 composition in the brains of normally aging mice, as a proxy for that in humans. We determined the
107 copper concentration and isotopic composition in a total of 30 mice (15 males and 15 females) aged
108 6, 9 and 12-months. Our findings reveal that there is no significant difference in the Cu isotopic
109 composition of male and female mice (i.e. no sex-dependence). Therefore, we combined the data for
110 all mice in our analysis. We observed that ^{65}Cu is enriched in the 12-months old mice compared to
111 the younger age groups, which show no distinguishable difference. Additionally, the overall copper
112 concentration in the brain tends to increase with age

113 2. **Materials and Methods**

114 2.1. **Mice.** *At Hopital Bichat.*

115 The 30 WT mice used in this study were collected from VTS INSERM 1148, Hopital Bichat, Paris.
116 All the 30 mice had the same genotype: C57BL/6JRj. The mice were housed in 6 different cages, 5
117 same-sex mice per cage. The dry food given to the mice has a $\delta^{65}\text{Cu} = 0.55 \pm 0.01$ (2SD) [43].

118 2.2. **Sample collection.** *At Hopital Bichat.*

119 Mice_aged 6, 9 and 12-months were euthanized by receiving a lethal dose of ketamine and xylazine,
120 followed by exsanguination. Throughout their life cycle, mice were all given the same diet -
121 previously analyzed in Cu isotopic composition- and kept in similar conditions. The brains were
122 collected directly after death using instruments in stainless steel. The brains were then conserved in
123 a -20 degrees Celsius freezer.

124 The biological standard used was tuna fish ERM-CE464 (TF ERM-CE464).

125 2.3. **Digestion and chemical purification** *At Institut de physique du Globe de Paris (IPGP)*

126 The dissolution, chemical purification and mass-spectrometry analyses are a close modification of
127 the protocols established previously [28, 42].

128 **Dissolution.** In polyfluoralkyl (PFA) beakers, whole brain samples and biological standard (TF ERM-
129 CE464), all at masses around 100mg, were dissolved in a few droplets of H₂O₂ and concentrated
130 distilled HNO₃ for a day and a half. Once the dissolution was complete, the beakers were placed
131 opened on a hot-plate at 120°C overnight.

132 **Copper extraction.** The Cu purification is conducted using anion- exchange chromatography
133 following a protocol adapted from [65]. The samples are redissolved in 1mL of distilled 7M HCl then
134 loaded on 1.6 mL of AG-MP1 resin. Matrix elements are taken away by passing 8 mL of 7M HCl
135 through the resin. The copper is collected in 16 mL of 7M HCl. This procedure is replicated after
136 evaporation of the acid to ensure pure Cu fractions. The resin is washed following a day-long protocol
137 alternating three series of rinses with MQ water and HNO₃ 0.5N in between the two replicates. The
138 copper was extracted from the standard following the same procedure as for the samples. The yield
139 of Cu is >99%.

140 **2.4. Mass Spectrometry At Institut de physique du Globe de Paris (IPGP)**

141 Copper isotope ratios were determined with multi-collection inductively-coupled-plasma
142 mass-spectrometers (MC-ICP-MS) at the Institut de Physique du Globe de Paris France. Part of the
143 samples were analyzed using a Nu-Instrument Sapphire and the other part using a Thermo-Fisher
144 Neptune. Depending on instrument performance and availability on the day of analyses; no analytical
145 differences are observed between instrument platforms (figS1; Table1B). The samples were analyzed
146 between 1 and 5 times each depending on the amount of copper in each sample (Table1B).

147 The sample-standard bracketing (SSB) method was applied and the Cu concentration in the
148 samples and standards where match within 10%. This method consists in measuring a standard before
149 and after each sample and using the average of the two standards to normalize the sample ratio
150 [40,44]. During analytical session, the standard utilized was IPGP-Cu, previously determined to have
151 a $\delta^{65}\text{Cu}$ of $+0.271 \pm 0.006 \text{ ‰}$ (2SE) relative to the NIST SRM 976 [28]. All data herein have been
152 converted to $\delta^{65}\text{Cu}$ relative to NIST SRM 976 by adding 0.271.

153 **2.5. Statistical analysis**

154 Statistical analysis were performed using GraphPad Prism.

155

156 3. Results

157

158 3.1. Effect of age on the isotopic composition of Cu in the brain

159

160 We incorporated the data from a previous study conducted by Moynier et al., [43] on 3-months
161 old mice that were bred together with the mice studied here. All data are reported in Table 1A and
162 1B and displayed in fig2. The isotopic composition of the biological standard (TF ERM-CE464) was
163 analyzed every 12 samples on both the Nu-Instrument Sapphire and Thermo-Fischer Neptune
164 instruments. The value obtained (0.09 ± 0.02 , 2SD, N=4) was consistent within error with the value
165 reported by [66, 116], (0.11 ± 0.14 , 2SD) (Table 2). The Cu isotope compositions of all materials are
166 expressed as the ratio ^{65}Cu to ^{63}Cu relative to that of the internationally recognized standards SRM-
167 976, in per mil (per thousand) notation, or $\delta^{65}\text{Cu}$, and have been calculated using the formula:

168

$$169 \quad \delta^{65}\text{Cu} = \left(\frac{\left(\frac{^{65}\text{Cu}}{^{63}\text{Cu}} \right)_{\text{sample}}}{\left(\frac{^{65}\text{Cu}}{^{63}\text{Cu}} \right)_{\text{NIST SRM 976}}} - 1 \right) \times 1000$$

170

171 Two brain samples were analyzed on both instruments to ensure the consistency of the results.
172 The results obtained were similar within error: brain-1 gave a $\delta^{65}\text{Cu} = 0.86 \pm 0.06$) on the Neptune
173 and, 0.90 ± 0.05) on the Sapphire. Brain-2 has a $\delta^{65}\text{Cu}$ of 0.70 ± 0.08 on the Neptune, and of 0.75 ± 0.06)
174 on the Sapphire.

175 The values of $\delta^{65}\text{Cu}$ for mice aged 3 to 12 months were analyzed with One-way ANOVA test
176 ($p\text{-value} < 0.0001$; R squared= 0.4934). Our data were analyzed with Mann-Whitney test for each age
177 categories as the data are not normally distributed and the number of data is close to 30. Mann-

178 Whitney test: (age 9 vs age 6; p-value = 0,0051); (age 6 vs 12; p-value = <0.0001); (age 3 vs age 6;
179 p-value = 0.7915, ns); (age 12 vs age 9; p-value = 0,0008) (see fig2A).

180 The results (see fig2A, B, C) reveal a notable evolution in the isotopic composition of copper
181 as a function of age. Mice aged between 3 and 6 months exhibit a relatively consistent composition
182 of copper isotopes, with a $\delta^{65}\text{Cu}$ value of 0.85 ± 0.23 (2SD). However, from the age 9 months onwards
183 (mean: $\delta^{65}\text{Cu} = 0.98\pm 0.22$ (2SD)) there is a noticeable shift towards heavier copper content and
184 dispersion of $\delta^{65}\text{Cu}$ values, particularly evident in the 12 months old mice (mean: $\delta^{65}\text{Cu} = 1.32\pm 0.61$
185 (2SD)) (fig2A and 2B). The simple linear regression (fig2C) plotted using the values displayed in
186 fig2B has a positive slope and shows a linear increase in the values of $\delta^{65}\text{Cu}$ depending on time. An
187 un-linear curve showing was plotted as well in fig2C and is also displaying a heavier copper isotopic
188 composition as a function of age. Taken as a whole, these data strongly suggest that the isotopic
189 composition of copper undergoes significant changes with age. Specifically, the copper composition
190 becomes progressively heavier with advancing age. Consequently, the aging process is associated
191 with a shift in the isotopic composition of copper, particularly in the brain, which demonstrates an
192 age-dependent increase in the proportion of ^{65}Cu compared to ^{63}Cu .

193

194 **3.2. Effect of age on copper concentration**

195

196 Several studies in the literature have reported an age-related increase in copper levels in
197 various organs. In healthy individuals, it has been observed that copper tends to increase with age in
198 human serum [67, 68]. However, in cortical tissue affected by Alzheimer's disease (AD), the level of
199 copper is actually decreased [69]. This decrease is believed to be associated with the binding of copper
200 to senile plaques [70]. Other studies have shown that copper tends to decrease in salivary sediment
201 and hair samples [71]. In rats aged 15 to 49 weeks, Cu level increase in the serum, kidney, liver and
202 five distinct parts of the brain (cortex, corpus striatum, hippocampus, midbrain+medulla and

203 cerebellum) [72]. These findings further support the notion of age-dependent changes in copper
204 amounts across different tissues and species.

205 Copper concentrations aren't the main focus in the study. They are determined as a matter of
206 comparison with other studies that determine the concentrations the same way. Our objective was to
207 investigate whether there were any variations in copper concentration within our samples. To
208 accomplish this, we quantified the copper concentration in each brain sample (n=30). For each
209 sample, we performed a factor 100 dilution (10 μ L of sample in 1mL of 0.5N HNO₃) and measured
210 the signal in volts obtained on the MC-ICP-MS. Given that the concentration and signal were
211 precisely known for the standard (NIST SRM 976), we could compute the concentration of copper in
212 each of the samples. The weight collected for each sample was 100mg \pm 30. The data are in Table 3
213 and displayed in fig3. The concentrations are given in ppm, equivalent to a concentration given in
214 μ g/g. The means were calculated for each age category: mice aged 6 months (mean: C_{copper} = 3.73
215 ppm, 2sd = 2.50, n=10), mice aged 9 months (mean: C_{copper} = 4.39 ppm, 2sd = 5.58, n=10), mice aged
216 12 months (C_{copper} = 6.02 ppm, 2sd = 4.56, n=10) (see fig3).

217 We performed a Mann-Whitney test: age 12 vs 9 (p-value = 0,2475, ns) and age 12 vs age 6
218 (p-value = 0,0089, **). Ordinary one-way ANOVA and 2way ANOVA were not conclusive.
219 However, we observe a qualitative increasing trend as a function of age. The data obtained for mice
220 aged 9 months are very disperse compared to 6 and 12 months-old mice (see fig3).

221

222

223 **4. Discussion**

224 **4.1. Effect of sex on copper isotopic compositions and concentrations**

225

226 Previous studies have demonstrated differences in the isotopic composition of Cu and Fe in
227 function of sex notably in the serum and red blood cells [28, 73, 74]. In archeological human bones,
228 Jouen et al., have shown that the isotopic signature is dependent on sex for Cu and Fe [74].

229 Furthermore, several studies have indicated sex-related differences in the development of
230 neurodegenerative diseases. For instance, men have a higher disposition for Parkinson's disease [75-
231 77] while women constitute two-thirds of AD patients [78]. Moreover, the progression of the disease
232 progression appears to be accelerated in men [79]. Although these sex-dependent differences are
233 known, their underlying causes remain elusive and require further investigation. To better constrain
234 the sex effect on brain isotopic composition and concentration, we conducted a comparison between
235 the results obtained for males and females, irrespective of age. However, no significant difference
236 was observed between the two groups. Furthermore, we further examined the data by plotting the
237 results for males and females separately in each age category. Even after considering age as a factor,
238 no notable or statistically significant differences between the sexes were found (see fig4; Table4;
239 Table 5).

240 We conducted a Mann-Whitney test to compare the $\delta^{65}\text{Cu}$ values between males and females,
241 which revealed no statistical difference (p-value = 0.1276). The mean $\delta^{65}\text{Cu}$ for males was $0,98\pm 0.48$
242 (2SD), while for females it was 1.13 ± 0.67 (2SD) (see fig4A). Additionally, males and females could
243 not be statistically differentiated based on copper concentrations (see fig4B).

244 Although there was no significant difference in $\delta^{65}\text{Cu}$ between males and females, it is worth
245 noting that the data for females exhibited a slightly broader distribution compared to males.
246 Furthermore, there is a tendency in the evolution of $\delta^{65}\text{Cu}$ values between males and females as a
247 function of time (fig4D), however more samples from future work might drive this further towards
248 statistical significance. The difference in copper isotopic composition between males and females
249 tends to increase as a function of time. As reported in the study by Moynier et al., (2019), at age 12,
250 there was a discernible difference in $\delta^{65}\text{Cu}$ values between males and females. However, in our study,
251 it did not reach statistical significance (see fig4C)

252

253 **4.2. $\delta^{65}\text{Cu}$ decreases after 12 months.**

254 To provide a better understanding of the evolution of $\delta^{65}\text{Cu}$ with age, we combined our data
255 with literature [43, 107] (fig5). Moynier et al., (2022) measured 4 brains from healthy mice aged 3
256 months, Morel et al., (2022) measured 29 brains from mice aged 6, 16 and 24 months. All mice have
257 the same genotype. The data show an increase in $\delta^{65}\text{Cu}$ values before 12 months and a decrease after
258 12 months. Combining the data from Morel et al., (2022) with our data, we could observe that both
259 data from mice aged 6 months are consistent. Performing Mann-Whitney test for the 6 and 9 months,
260 p-value = 0.0004, ***, which is even lower when the data are combined with Morel et al., (2022)
261 compared to our data alone (p-value = 0.0051). Even if the measurement method and copper
262 extraction vary slightly between the studies, the results are consistent.

263

264 **4.3. 12 months old WT and AD mice have different brain copper isotopic compositions**

265

266 Combining our data for 12 months old healthy mice with those from Moynier et al. (2019)
267 [28], we obtained a mean $\delta^{65}\text{Cu} = 1.32 \pm 0.61$ (2SD) for healthy 12 months-old mice (WT), in our
268 study. For 12 months-old AD mice (AD), the mean was $\delta^{65}\text{Cu} = 0.50 \pm 0.29$ (2SD). We also took into
269 account the $\delta^{65}\text{Cu}$ values for healthy mice brains reported in Moynier et al., (2019). The data are
270 statistically differentiable (Mann-Whitney, p-value < 0.01, **). The results (see fig6A; Table 6) show
271 a decrease in the isotopic composition between WT and AD at 12 months. However, we could only
272 have five samples for AD mice ages 12 months. Despite the limited data, this difference is considered
273 robust, as a statistical difference of well over 0.5 per mil is over an order of magnitude larger than
274 typical analytical precision within this and other studies.

275 This suggests that AD brains are isotopically lighter than healthy ones in terms of copper
276 isotopes (see fig6B). This is also what is found in literature in the brains of AD and WT humans [42].
277 This can be explained by the change in bonding environment caused by the appearance of amyloid
278 plaques and neurofibrillary tangles, as copper directly interacts with $\text{A}\beta$ [42,57,58, 70]. The new
279 environment created by the presence of amyloid plaques in the brain might encourage the binding of

280 lighter isotopes and thus generating a shift in the natural isotopic fractionation of copper. Copper
281 being a redox active metal [25] and Cu (II) ions binding preferentially to A β plaques this also could
282 be a source of explanation for this shift [42,57,58, 70]. Conversely, the presence of Cu (0) has been
283 detected in AD human brains [80].

284 Normal aging and AD have different signatures in terms of copper concentration as well.
285 In the literature, it is reported that AD is accompanied by a deficiency in copper in the brain [69,81,
286 82]. Interestingly, the amount of Cu in aged human brains has been shown to have a negative
287 correlation with the advance of A β plaques [83]. Normally aging individuals tend to have copper
288 accumulating in the brain as a function of age. Thus, AD and healthy individuals appear to display
289 different copper signatures regarding copper isotopic composition and copper concentration.

290

291 **4.4. Copper related oxidative stress might be a key to understand normal aging in the brain**

292

293 Oxidative stress is thought to be one of the main causes of aging in mammals [3-6]. In the
294 brain, the levels of antioxidants are low: it makes it susceptible to oxidative stress induced by the
295 redox-active nature of Cu [84]. Superoxide dismutases (SODs) are a category of ubiquitous enzymes
296 representing one of the main antioxidant defense systems against reactive oxygen species (ROS).
297 SODs catalyze the dismutation reaction of O₂^{•-} to H₂O₂ which is then reduced to H₂O by the action
298 of catalase [85]. The reaction requires alternate reduction and reoxidation of a redox active transition
299 metal (Cu or Mn). In mammals, there are three isoforms that are all bound to catalytic metals:
300 intracellular SOD1 or CuZn-SOD, mitochondrial SOD2 or Mn-SOD and extracellular SOD3 [86].
301 SOD1 is intracellular [87, 88].

302 Warner et al., (1994), have shown, through genetic manipulations increasing CuZn-SOD
303 activity, a resistance to most forms of oxidative stress [89]. CuZn-SOD deficiency increases the
304 vulnerability to oxidative stress and thus potentially normal aging. Copper is bound to several
305 proteins, frequently enzymes, including metallothionein. It has been shown that SOD1 was enriched

306 in ^{65}Cu relative to the metallothionein (MT) in human cortex [90]. Thus, probably due to specific
307 catalytic micro-environments, SOD1 seems to be a source of Cu fractionation.

308 The distributions of Cu isotopes between MT and SOD1 in AD patients is also different
309 compared to healthy patients [90]. CuZn-SOD activity is negatively correlated with age [91] which
310 might partly explain the increase of ROS in cells and tissues. However, while the activity of the
311 enzyme seems to decrease with age, its concentration appears to follow the opposite tendency. It was
312 shown that in the cerebro-spinal fluid (CSF), the concentration of SOD1 tends to increase with age
313 [111]. As we mentioned, SOD1 binding preferentially ^{65}Cu , which might explain the increase in
314 $^{65}\text{Cu}/^{63}\text{Cu}$ ratio in CSF compared to the decrease in the $^{65}\text{Cu}/^{63}\text{Cu}$ ratio observed in the brain [90]
315 (fig2). The variations observed in this study and in other studies (fig6) [107] for both $\delta^{65}\text{Cu}$ values
316 and Cu concentrations (ppm) as a function of age might be related to SOD1 concentration and activity
317 during aging and -more generally - to enzymes binding copper in the brain. Further investigations
318 regarding this aspect would be interesting to understand precisely the mechanisms involved, notably
319 including the parallel-operating influence of MT and the influence this may have on Cu isotope
320 composition.

321

322 **4.5. Effect of glial cells and neuroinflammation on copper contents in the brain**

323

324 Astrocytes are a specific type of glial cells in the central nervous system (CNS) that are
325 reported to have - amongst others - a role in the maintenance of extracellular ion homeostasis [92,
326 93]. They are key regulators of Cu and Fe in the brain [94 – 96]. Astrocytes express copper
327 transporter 1 (Ctr1) [97] as well as MT [98] and glutathione (GSH) [99]. Ctr1 is the main copper
328 transporter in eukaryotic cells and is responsible for copper entrance in the cell [100]. When the brain
329 ages, astrocytes encounter senescence and their functions become dysregulated [101]. This is thought
330 to have an impact on the accumulation of debris and especially $\text{A}\beta$ plaques [102, 103]. Furthermore,
331 copper has been shown to activate the secretion of inflammatory products such as IL- 6, NO and

332 TNF- α by interacting with microglia [104]. Microglia are as well as astrocytes a type of glial cell and
333 the main type of immune cell in the brain [105]. Inflammation in the brain promotes the appearance
334 and development of neurodegenerative diseases [10,106]. Ashraf et al., (2019), demonstrated that
335 glial senescence may induce differential regional content of metals (Cu, Zn, Fe) [106]. In chronic and
336 acute inflammatory conditions, copper is reported to be at higher concentrations in the brain. In the
337 same conditions, labile copper was found to be also at higher concentrations in microglia. It has been
338 shown in copper-deficient rats that a lack of copper is linked with a sensitivity to acute inflammatory
339 agents [113]. Copper seems to be implied in the regulation of inflammation in the brain. Moreover,
340 the copper complex copper*bis*(thiosemicarbazones) (Cu^{II} (at-sm)) was shown to be a potential
341 therapeutic candidate for strokes and acute brain injuries [114]. This same copper complex has also
342 been shown to have protective effects in animal models of neurodegenerative diseases such as ALS
343 and AD [114, 115]. Cu^{II} (at-sm) was reported to have an anti-inflammatory action by potentially
344 restoring the brain copper homeostasis [114]. We propose that the senescence of glial cells, as well
345 as neuro-inflammation and its peripheral processes during ageing, could be key contributing factors
346 to understanding the accumulation of copper, and potentially other metals, in the ageing brain, and
347 therefore hold relevance in understanding the evolution of Cu isotope compositions in the brain as a
348 function of age as well as potential therapeutic candidates.

349

350 5. Conclusions

351 This study has provided evidence that the isotopic composition of copper in the brain
352 undergoes changes with normal ageing, manifesting an increase in the $^{65}\text{Cu}/^{63}\text{Cu}$ ratio with age. These
353 results suggest that the copper isotopic signature becomes progressively heavier as individual ages,
354 accompanied by a tendency towards Cu accumulation in the aging brain. Notably, we observed a
355 distinct shift in $^{65}\text{Cu}/^{63}\text{Cu}$ ratio between healthy and AD model mice at 12 months. While the ultimate
356 utility of Cu isotopes in AD diagnostics remains to be clear, the present work provides an invaluable
357 baseline trajectory of Cu isotope compositions in the brain with age. This baseline progression must

358 be well-understood so that it can be deconvolved from other alterations to Cu isotope compositions,
359 namely the observed light Cu isotope excursion in the AD brain; this is paramount to mechanistically
360 and statistically isolating a potential biomarker signal induced by the disease. These findings greatly
361 contribute to the development of a baseline Cu isotope signature associated with normal aging,
362 enhancing our ability to understand Cu isotope changes induced by neurodegenerative diseases such
363 as Alzheimer's.

364

365 **6. Acknowledgments**

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370 ACAV+.

371 **7. Disclosure statement**

372 The authors have no actual or potential conflicts of interest.

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374 **8. Data availability**

375 The data underlying this article are available in the article and in its online supplementary material.

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378 **Figure captions**

379 **Fig1.** The roles of copper in a healthy brain. Made with BioRender.

380

381 **Fig 2.** A. $\delta^{65}\text{Cu}$ in healthy mouse brain in function of age. Boxes extend from the 25th and 75th
382 percentile, the line inside the box represents the median, the whiskers show the minimum and

383 maximum of the values. p-value < 0.001; **, p-value < 0,0001; ****. B. $\delta^{65}\text{Cu}$ in healthy mouse
384 brain in function of age. Each point corresponds to the mean for one mouse, n corresponds to the
385 number of brains measured per age category. C. Simple linear regression for the data displayed in
386 figure 2B (black full line) with 95% confidence intervals (dotted line), $R^2= 0.3946$ and quadratic
387 second order polynome (red full line), $R^2= 0.5126$. GraphPad Prism.

388 **Fig 3.** Concentration of copper (ppm) in function of age. Boxes extend from the 25th and 75th
389 percentile, the line inside the box represents the median, the whiskers show the minimum and
390 maximum of the values. p-value < 0.02, *. GraphPad Prism.

391 **Fig 4.** A, B, C. $\delta^{65}\text{Cu}$ in function of sex (A), copper concentration in function of sex (B), $\delta^{65}\text{Cu}$ in
392 function of sex and age (C). (D) is the evolution of the p-values for unpaired-t tests between males
393 and females as a function of age, $R^2 = 0.9988$. Boxes extend from the 25th and 75th percentile, the line
394 inside the box represents the median, the whiskers show the minimum and maximum of the values.

395 **Fig5.** $\delta^{65}\text{Cu}$ in healthy mouse brain in function of age. Data from (Moynier et al., 2022) and (Morel
396 et al., 2022) were incorporated to the data from this study. Boxes extend from the 25th and 75th
397 percentile, the line inside the box represents the median, the whiskers show the minimum and
398 maximum of the values. p-value < 0.001, ***. GraphPad Prism.

399 **Fig 6.** A, B. Isotopic composition of copper in the brain of healthy and AD mice. p-value < 0.001, **
400 (A), copper isotopic composition of a healthy (green) and Alzheimer brain (red) (B). Boxes extend
401 from the 25th and 75th percentile, the line inside the box represents the median, the whiskers show the
402 minimum and maximum of the values. GraphPad Prism, Bio Render.

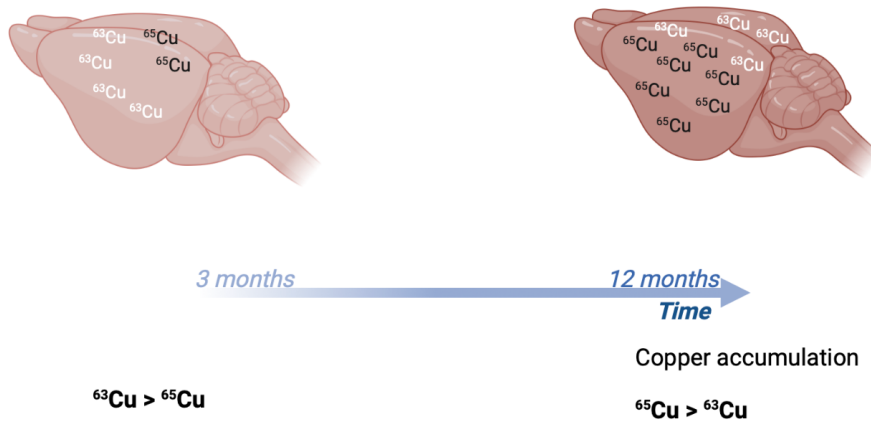
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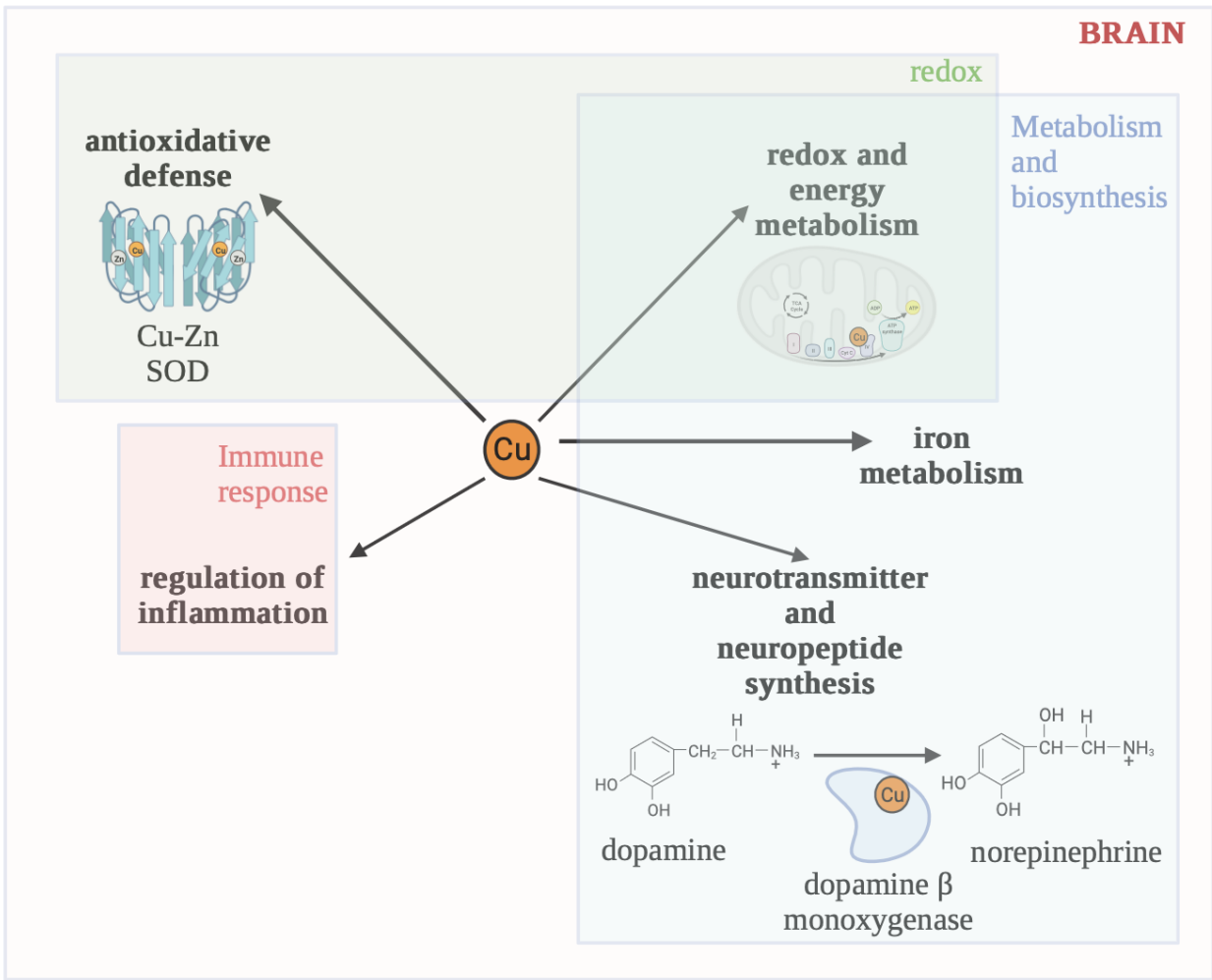
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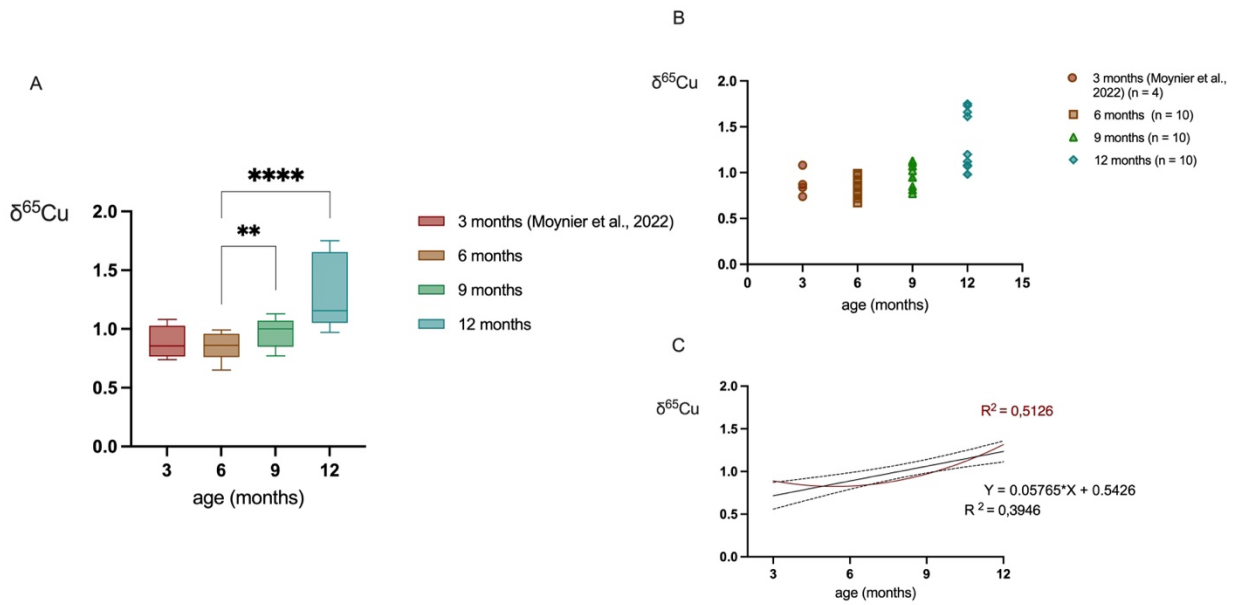
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Fig1. The roles of copper in a healthy brain. Made with BioRender.



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448 Fig 2. A. $\delta^{65}\text{Cu}$ in healthy mouse brain in function of age. Boxes extend from the 25th and 75th

449 percentile, the line inside the box represents the median, the whiskers show the minimum and

450 maximum of the values. p-value < 0.001; **, p-value < 0,0001; ****. B. $\delta^{65}\text{Cu}$ in healthy mouse

451 brain in function of age. Each point corresponds to the mean for one mouse, n corresponds to the

452 number of brains measured per age category. C. Simple linear regression for the data displayed in

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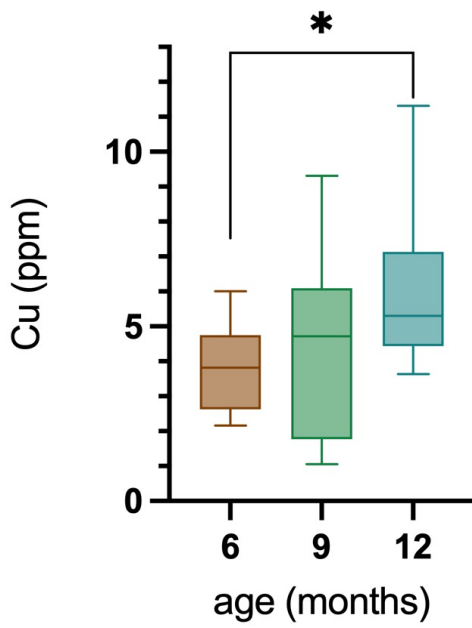
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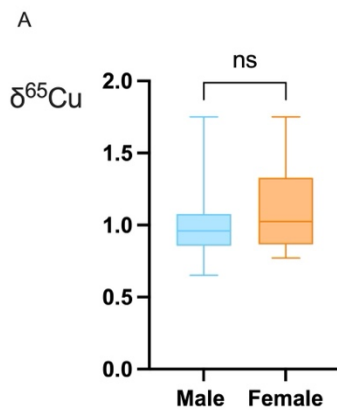
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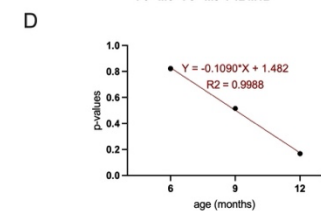
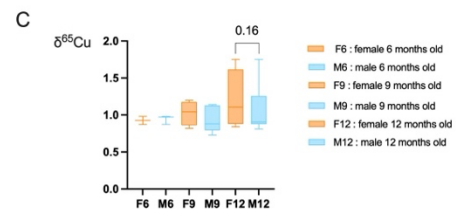
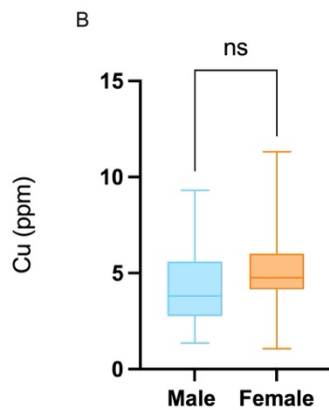
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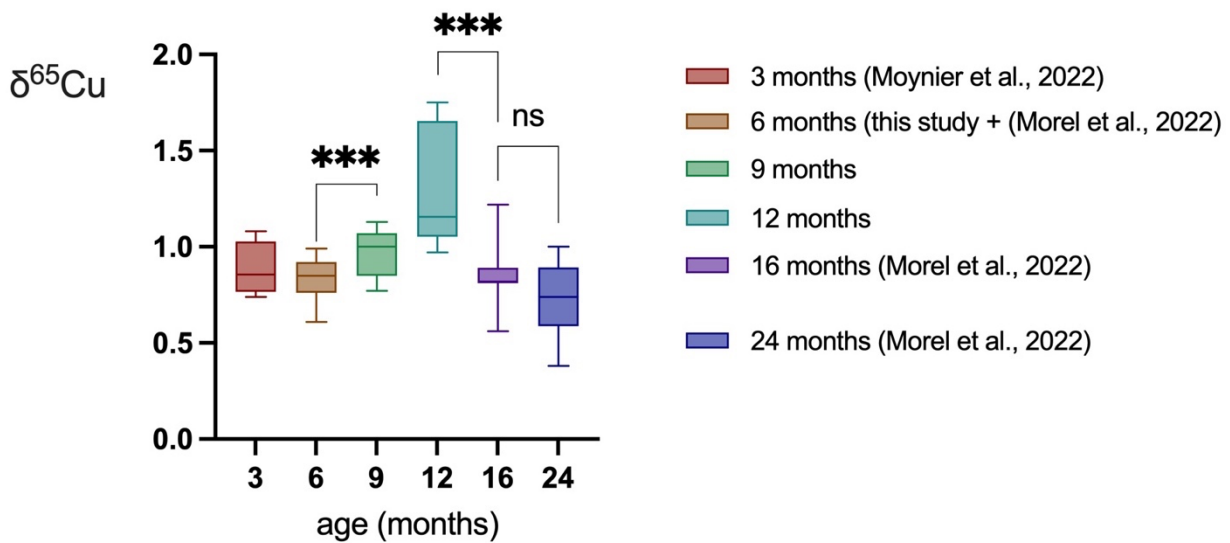
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477 Fig 4. A, B, C, D. $\delta^{65}\text{Cu}$ in healthy mouse brain in function of sex (A), copper concentration in
478 healthy mouse brain in function of sex (B), $\delta^{65}\text{Cu}$ in healthy mouse brain in function of sex and age,
479 p-value = 0.16, non-significative (C). (D) is the evolution of the p-values for unpaired-t tests between
480 males and females as a function of age, $R^2 = 0.9988$. Boxes extend from the 25th and 75th percentile,
481 the line inside the box represents the median, the whiskers show the minimum and maximum of the
482 values. ns: not significative.

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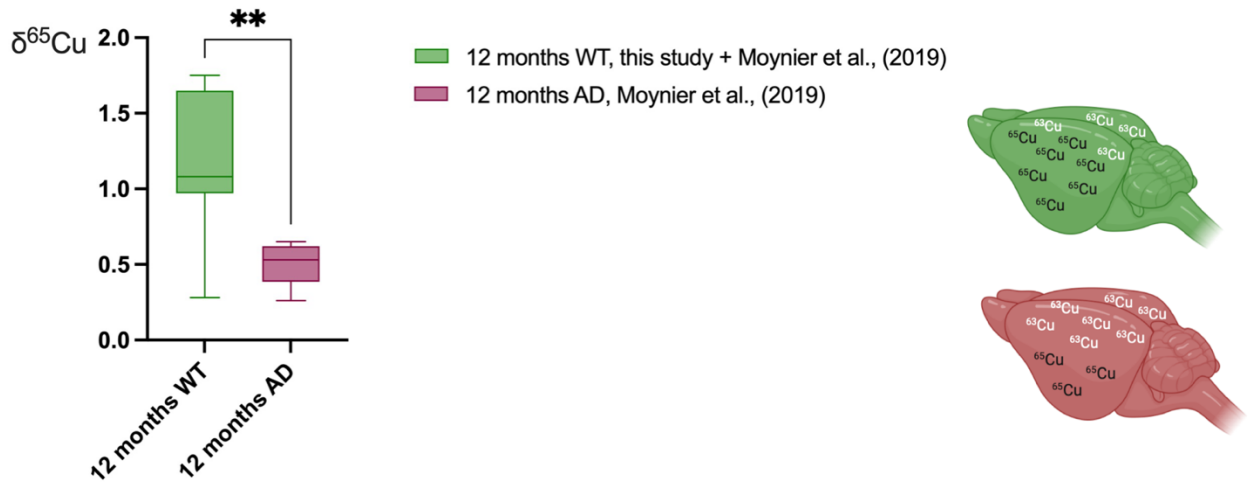
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488 Fig5. $\delta^{65}\text{Cu}$ in healthy mouse brain in function of age. Data from (Moynier et al., 2022) and (Morel
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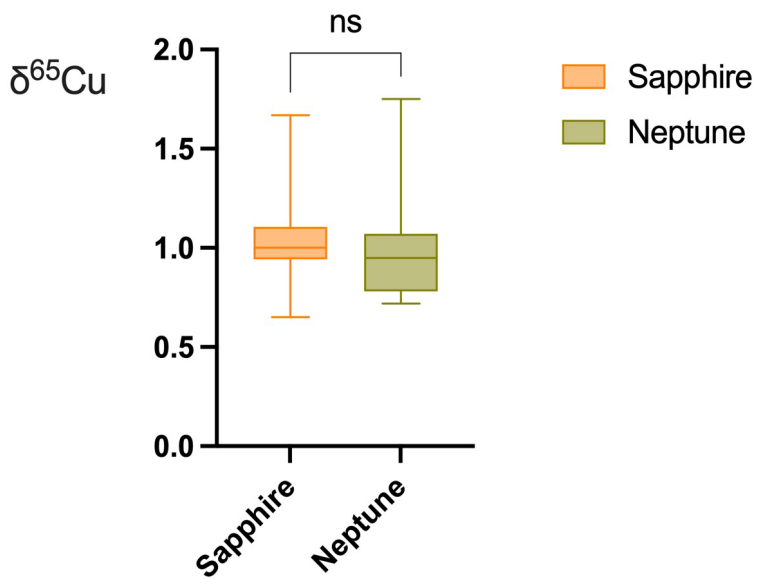
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516 **FigS1.** Copper isotopic composition of healthy mice brains depending on the MC-ICP-MS used for
517 the measure. ns = not significant. GraphPad Prism.

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