Elevated hippocampal copper in cases of type 2 diabetes

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Summary

Background Type-2 diabetes (T2D) is characterized by chronic hyperglycaemia and glucose-evoked organ damage, and displays systemic copper overload, elevated risk of impaired cognitive function, and epidemiological links to sporadic Alzheimer's disease (sAD). Contrastingly, sAD exhibits impaired cerebral-glucose uptake, elevation of cerebral glucose but not blood glucose levels, and widespread cerebral-copper deficiency. We hypothesized that sAD-like brain-metal perturbations would occur in T2D.

Methods We measured nine essential elements in an observational case–control study of T2D without dementia (6 cases and 6 controls) in four brain regions and compared the results with those from our study of brain metals in sAD (9 cases and 9 controls), which employed equivalent analytical methodology. We evaluated intergroup differences by supervised and unsupervised multivariate-statistical approaches to contrast between T2D cases and controls, and to compare them with cerebral-metal patterns in sAD.

Findings Unexpectedly, we found that hippocampal-copper levels in T2D were markedly elevated compared with controls (P = 0.005 and 0.007 by Welch's t-test in two technical-replicate experiments), to levels similar to those in cases of untreated Wilson's disease (WD), wherein elevated cerebral copper causes neurodegeneration. By contrast, hippocampal-copper levels in sAD were markedly deficient. Multivariate analysis identified marked differences in patterns of essential metals between hippocampal datasets from cases of T2D and of sAD.

Interpretation Elevated hippocampal copper could contribute to the pathogenesis of cerebral neurodegeneration and cognitive impairment in T2D, similar to known impacts of elevated brain copper in WD. Therapeutic approaches with copper-lowering agents similar to those currently employed in pharmacotherapy of WD, may also be applicable in patients with T2D and impaired cognitive function. Further studies will be required to replicate and extend these findings and to investigate their potential therapeutic implications.

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Keywords: Type 2 diabetes; Hippocampal copper; Neurodegeneration; Sporadic Alzheimer's disease; Wilson's disease; Copper homeostasis; Mass spectrometry

Introduction

Diabetes mellitus is a metabolic disease characterised by chronic hyperglycaemia, which can cause glucose-evoked tissue damage that impacts numerous organs including the brain, heart, kidneys, and arteries, through mechanisms involving both microvascular and macrovascular processes.[1] Due to its ever-increasing worldwide prevalence, rising in the adult population from 4.7 to 8.5% since 1980, it is now considered to be a global health epidemic.[2,3] For T2D in particular, which accounts for ≥90% of all cases of diabetes, this increase is largely attributable to modifiable risk factors such as diet, obesity, and lack of exercise.[1,3] T2D has a complex and incompletely understood molecular pathogenesis that comprises islet beta-cell damage with islet amyloid formation,[4] relative insulin deficiency, and insulin

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Research in Context

Evidence before this study

We searched Pubmed for articles published up until September 17, 2021, without language restrictions, using the keywords "Diabetes mellitus", "Type 2 diabetes", "Brain metal deposition", "Brain metal levels", "Cognitive impairment", "Cerebral atrophy", "Neurodegeneration", "Metal dyshomeostasis". Extensive plasma- and serum-metal dyshomeostasis in T2D are evident within the existing literature; however, only a few studies were identified that reported brain-metal dysregulation in T2D. Moreover, these studies only investigated brain-iron deposition via clinical imaging in type 2 diabetes and reported mixed results. In recent years, cognitive impairment and hippocampal atrophy have slowly become acknowledged as significant features of T2D, yet the mechanisms by which they occur are largely unknown. Although such mechanisms may not be directly attributable to brain-metal dysregulation, reports of brain-metal levels in sAD, for which T2D is a major risk factor, and other neurodegenerative diseases potentially point towards similar brain-metallomic patterns in T2D.

Added value of this study

We present for the first time, to our knowledge, multiregional-metal levels measured in T2D *post-mortem* brain tissue. Whereas previous measurements of brain metals in diabetic patients have utilised quantitative susceptibility-weighted MRI imaging, here we used inductively coupled plasma mass spectrometry, which offers high sensitivity and the ability to determine multiple metal concentrations simultaneously, to probe specificity. Using this technique, we found strong evidence for increased hippocampal copper in T2D cases when compared to non-diabetic controls. Moreover, the metal pattern identified in T2D contrasted strongly with our previous sAD metallomic dataset, which identified widespread brain-copper deficiency therein. Contrary to our initial hypothesis, these data support the potential existence of different neurodegenerative processes in T2D and in sAD.

Implications of all the available evidence

The findings described in this study shed light on the previously unreported regulation of metals in T2D brain. Furthermore, the observation of increased copper in T2D hippocampal tissue may reflect a mechanism that could lead to hippocampal atrophy and cognitive impairment in diabetes. A similar pattern of copper toxicity occurs in WD, an inherited copper disorder wherein increased copper causes neurodegeneration. These findings may be crucial to the field of diabetes as copper-chelation therapy, which is used successfully to treat WD, may also be beneficial to the treatment of T2D.

resistance.[5] Current pharmacological approaches to treating T2D have largely focused on agents that increase insulin secretion or lower blood glucose by other means; however, such strategies often cause adverse effects and have failed as significant disease-modifying treatments.[6] These observations highlight the need to identify novel therapeutic targets to guide treatment of T2D.

Essential metals are vital for normal physiological functioning of biological systems. They are often present in metalloprotein/metalloenzyme complexes and mediate a variety of processes, including mitochondrial function, transcriptional regulation, and cell metabolism.[7] However, the presence of essential metals at concentrations outside their physiological ranges can lead to severe cellular dysfunction.[8] Defective brain-metal metabolism occurs in various neurodegenerative diseases including sAD and Parkinson's disease,[9,10] but there is no current consensus concerning the role of defective metal homeostasis in the pathogenesis of either disease.

Most metallomics studies in diabetes have focused on urinary- or plasma-metal concentrations. In a fully residential balance study of patients with T2D but without complications that provides high-quality evidence, plasma levels of copper, iron, zinc, calcium, magnesium, and manganese did not differ significantly between cases and controls, whereas by contrast, urinary excretion of copper, iron, zinc, manganese, and calcium was elevated in patients.[11] This RCT describes a hyperglycaemia-driven pathogenic abnormality of copper homeostasis in T2D that is reversible by selective chelation.[11] Conversely, in patients with established diabetes-associated complications, those with retinopathy, hypertension, or microvascular disease had higher plasma copper concentrations compared with both diabetic subjects without complications and with control subjects, and these levels worsened with severity.[12] Similar findings have also been reported in patients with diabetic nephropathy.[13] However, there are no available reports of brain-tissue-copper levels in T2D, and whether brain-copper dyshomeostasis contributes to the pathogenesis of cognitive impairment in T2D is unknown. In the afore-mentioned balance study, T2D patients demonstrated increased copper balance compared with controls, consistent with the presence of a systemic copper-overload state.[11] The authors concluded that, in conjunction with therapies aimed at decreasing hyperglycaemia, treatment with a compound that can extract divalent copper from the body might be a potential way of suppressing the heightened risk of cardiovascular complications in diabetes.

Longitudinal epidemiological studies have implicated chronic hyperglycaemia and microvascular disease in the pathogenesis of T2D-related cognitive

dysfunction, but the causal pathway that underlies the statistical associations between T2D and dementia is unknown.[14] There is robust evidence for increased rates of lacunar infarction and cerebral atrophy in T2D[15,16] where the hippocampus is severely affected,[17] even in the early stages of disease.[18] Hippocampal atrophy is also a prominent aspect of neurodegeneration in sAD,[19] where hippocampal-copper levels are severely lowered,[10] to values similar to brain-copper levels in Menkes' disease, a genetically-mediated disorder wherein low copper causes neurodegeneration.[20] A recent meta-analysis of 56 studies investigating copper biomarkers in AD (mainly sAD) has been reported (where pooled totals were 182 cases of AD and 166 non-demented controls, HC) respectively in human brain, and serum/plasma (pooled totals of 2929 AD and 3547 HC). This meta-analysis provides robust evidence for substantively decreased copper in AD brain tissue, increased copper and non-bound caeruloplasmin (non-Cp) copper in serum/plasma, and unchanged circulating Cp. Moreover, copper excess in serum/plasma was associated with a three- to four-fold increase in AD risk.[21]

Our team is applying multi-omic methodologies to probe molecular linkages between different age-related neurodegenerative diseases, including sAD,[22–24] Huntington's disease,[25–28] Parkinson's disease,[29–32] and vascular dementia,[33] and to uncover their linkages with T2D.[22,23,34] Our underlying hypothesis is that the multi-omic approach will show that these neurodegenerative diseases have major metabolic mechanisms in common that can be therapeutically targeted by similar approaches.[11,35–37] The current study was designed to characterize aspects of the metabolic linkage between processes that may cause dementia in T2D and sAD.

Based on the reported epidemiological linkages between T2D and sAD, our research hypothesis here was that brain-copper levels in T2D would be markedly lowered, to levels similar to those reported in sAD, with the hippocampus being a severely affected region. We measured the concentrations of eight essential metals (sodium, magnesium, potassium, calcium, manganese, iron, copper, and zinc) and the metalloid, selenium, using inductively-coupled-plasma mass spectrometry (ICP-MS) in short-*post-mortem*-delay (PMD) human-brain tissue from cases diagnosed with T2D (n = 6) and non-diabetic controls (n = 6) that were selected by inspection by National Disease Research Interchange (NDRI; Philadelphia, PA) pathologists to be similar in sex, age, and PMD, to compare and contrast values from four brain regions: frontal cortex (FC), temporal cortex (TC), hippocampus (HP), and anatomically related meninges (MN). Unexpectedly, hippocampal-copper levels were strikingly elevated in T2D compared with controls, to levels consistent with those reported in the brain in cases of WD (a genetically transmitted disorder that causes neurodegeneration due to copper toxicity) whereas, contrastingly, hippocampal-copper levels in sAD were markedly deficient.

Elevated hippocampal copper could contribute to the pathogenesis of neurodegeneration in T2D. Agents similar to those used to lower brain copper in overload states such as WD represent a new experimental therapeutic approach for the treatment of impaired cognitive function and dementia in T2D.

Methods

Ethics

All experiments were performed in accordance with relevant UK and international guidelines and regulations as stated below. The case–control studies of *post-mortem* human brain tissue were approved by the University of Manchester Research Ethics Committee (Ref: 2019-5675-10520). Informed consent for collection of tissues for the T2D/control study was provided by the NDRI, which hosts the Human Tissues and Organs for Research Resource Program funded by the National Institutes of Health (https://ndriresource.org/).

Consent for collection of tissue for the sAD/control study was as previously stated.[10]

Acquisition and sampling of human brain tissue

Tissue from T2D cases and controls was obtained through the NDRI programme. Donor ages were restricted to 60–80 y, to be consistent with our comparator case–control sAD data set,[10] and were similar for sex, age, body-mass index (BMI), and PMD. Cases and controls were contemporaneous and acquired specifically for this study. For clarification, no statistical packages/models were used for selection of the cases and controls in this study.

Tissue samples were acquired at *post-mortem* examination from four brain regions of cases diagnosed *ante-mortem* with T2D (n = 6) or controls (n = 6), by partner organizations of the NDRI in the USA.

Tissues from four brain regions (FC, TC, HP, MN) from T2D cases and controls were obtained specifically for this study by partner organizations of the NDRI; the samples were then transferred to the University of Auckland where they were dissected by pathologists and stored at –80 °C until all cases and controls had been recruited, after which they were transferred to the University of Manchester and stored at –80 °C until analysis.

Case/control selection was performed by pathologists working in NDRI-partner organizations with the *a priori*-agreed objective of producing a cohort not significantly different for sex, age, and PMD. In the event, there was a significant difference in age between T2D cases and controls in the final study group of uncertain significance.[29] Brains from T2D cases were collected and tissue samples corresponding to regions designated as

"hippocampus", "temporal cortex", and "frontal cortex" were dissected by pathologists working at NDRI centres in the USA. For purposes of this study, hippocampus comprises Brodmann area (BA) 35; temporal cortex BA 20/21 and 38; and frontal cortex BA 8/10 and 44/46. Exact Brodmann areas were not necessarily the same between replicates within an individual case, or between different cases, and no differentiation between white and grey matter was made. The sample size was restricted by the marked scarcity of available human T2D-brain tissue with short PMD.

Wet-weight aliquots of 50 ± 5 mg were dissected using a ceramic scalpel to avoid metal contamination and dried to constant weight in a centrifugal concentrator (Savant Speedvac™; Thermo-Fisher, Waltham, MA). Dry weights were determined by weighing with a Discovery semi-micro analytical balance (DV215CD; Ohaus, Northamptonshire, UK). Dry-weight measurements are preferred for the determination of tissue metal levels (e.g., of tissue-copper for the diagnosis of WD in clinical laboratories) and are commonly also employed for brain-metal measurements,[10] and *post-mortem* metal levels thus determined are found to be stable, robust, and replicable.[29]

Diagnosis and severity

T2D cases were diagnosed by clinical history whereas corresponding controls had no *ante-mortem* evidence of diabetes. Neither cases nor controls had historical or *post-mortem* evidence of dementia or other brain disease. The presence of cognitive impairment was not recorded in the NDRI metadata, nor was it excluded by formal mental-state examination. For the sAD/control study, diagnosis, and severity of sAD were determined by a consultant neuropathologist as described.[10] Group characteristics for both T2D/control and sAD/control cohorts are as shown in Table 1 and individual NDRI patient characteristics, including age and *post-mortem* delay in Table 2. All case and control brains entered into this study were obtained from individuals whose *post-mortem* examination for approved indications were not related to their inclusion in this study, and as a consequence, are considered to be representative of the individual populations under study (that is, T2D, sAD, and controls).

Tissue digestion

Prior to digestion, all samples were briefly centrifuged at 2400×*g* (Heraeus Pico 17 Centrifuge; Thermo Fisher Scientific, MA, US) to ensure that tissue aliquots sat at the bottom of the tubes. Concentrated nitric acid (A509 Trace Metal Grade; Fisher, Loughborough, UK) and 5% Agilent Internal Standard mixture (5183-4681; Agilent Technologies, Cheadle, UK) were combined to make the tissue digestion mixture. Calibration standards were prepared to the appropriate dilutions (Table S2) using Environmental calibration standard mixture (Agilent 5189-4688) and 2% (v/v) nitric acid digestion solution. For these dry-weight analyses, 200 µL of digestion solution was added to each sample including two empty 2-mL microcentrifuge tubes as digestion blanks. Tube lids were punctured with a septum remover to prevent pressure build-up before being transferred into a Dri-Block DB3 heater (Techne, Staffordshire, UK) at room temperature. Temperature was set to 60 °C for 30 min and then increased to 100 °C for the remaining 3.5 h. Thereafter, 100 µL of each sample or blank was added to 5 mL of LC/MS grade water in 15-mL Falcon tubes (Greiner) and samples retained at room temperature pending ICP-MS analysis.

Mass spectrometry

Metal concentrations were determined using an Agilent 7700x ICP-MS spectrometer equipped with a MicroMist nebulizer (Glass Expansion, Melbourne, Australia), a Scott double-post spray chamber and nickel sample and skimmer cones. Samples were introduced into the spray chamber using an Agilent integrated autosampler (I-AS). Before each analysis, the peristaltic-pump sample tubing was replaced to limit abnormal sample delivery to the nebulizer. ICP-MS system optimisation and performance reports were generated on Agilent MassHunter Workstation software (G7201A, A.01.01) prior to each analysis to ensure consistent system performance.

To remove spectral interferences, two collision-cell gas modes were employed. All elements were analysed in helium mode (5.0 mL min^{-1} helium), except for selenium which was analysed in high-energy helium

NDRI

Variable	Control	T2D
Number	6	6
Age (y)	76 (69–79)	70 (66–75)*
BMI (height in m/weight in kg^2)	20.7 (16.3–25.1)	30.5 (20.8–40.1)
Post-mortem delay (h)	11 (5.6–14.2)	9 (4.2–17.4)
Male sex, n (%)	3 (50)	3 (50)

Auckland

Variable	Control	sAD
Number	8	9
Age (y)	71 (63–78)	70 (60–80)
Post-mortem delay (h)	10 (5.5–13)	7 (4–12)*
Male sex, n (%)	4 (50)	5 (56)

Age, BMI, and PMD are mean (±95% CI); *$P < 0.05$, T2D compared with controls; all other differences are non-significant. To determine the possible effect of this difference in PMD between sAD cases and controls, we fitted a one-way ANOVA as follows: (hippocampal copper) $^{contin} \sim$ {(sAD/control) + PMD). There is evidence that the sAD/control variable evoked a significant effect on hippocampal copper in this model ($P = 0.045$) whereas PMD did not ($P = 0.86$). This finding is similar to that for the T2D case.

Table 1: **Cohort sample-group characteristics.**

NDRI No.	Diagnosis	Age at death	Cause of death	PMD (h)	Sex
ND05378	Non-diabetic	78	Complications of CVA	6.7	F
ND05475	Non-diabetic	76	Stomach cancer with unknown metastases	5.8	M
ND05764	Non-diabetic	76	AAA rupture	14.2	F
ND06063	Non-diabetic	79	Cardiac arrest	8.7	M
ND06116	Non-diabetic	69	Tonsil cancer	5.6	M
ND08354	Non-diabetic	77	Respiratory failure	12.5	F
ND05498	T2D	69	Colonic adenocarcinoma	8.5	M
ND05499	T2D	70	Myocardial infarction	17.4	F
ND06157	T2D	70	Endometrial cancer	11.5	F
ND07412	T2D	66	Pulmonary embolism	12.9	M
ND07636	T2D	70	ESRD	4.2	M
ND08151	T2D	75	Myocardial infarction	9.9	F

Causes of death were the primary causes listed on the death certificate. Abbreviations: AAA, Abdominal aortic aneurysm; CVA, Cerebrovascular accident; ESRD, End-stage renal disease; PMD, *Post-mortem* delay; T2D, type-2 diabetes.

Table 2: **Individual patient characteristics of T2D cases and controls.**

mode (HEHe; 10 mL min^{-1} helium) following Agilent's recommendation to reduce interference by polyatomic ion formation. Germanium and indium internal standards were analysed in both modes. Integration times for relevant trace metals were 3 s for selenium; 0.01 s for iron; 0.03 s for manganese, copper, and zinc; and 0.1 s for sodium, magnesium, potassium, and calcium. A multi-element method using serial dilutions of environmental calibration standards (Table S2; Agilent 5183-4688) was implemented for each analytical batch. 50 µg/L and 5 µg/L internal standard calibration standard solutions were used as periodic quality controls 1 and 2, respectively. Consistent with our established method for tissue-copper measurement,[10] we employed ^{63}Cu as the reference isotope in these studies.

Statistics

All ICP-MS datasets were first exported to individual Microsoft Excel (2010) worksheets where they were corrected for sample weight and dilution and then converted to units of mmol/kg or µmol/kg as appropriate. Means (±95% CI) were calculated and the significance of case–control differences determined by unpaired Welch's *t*-tests to allow for unequal variances and sample sizes, following the confirmation of normal distribution using the Shapiro–Wilk test for normality. Between-group variances were not assessed as there is little difference in statistical power between the Welch's *t*-test (which assumes unequal variance) and the Student's *t*-test (which assumes equal variance). As a result, the Welch's *t*-test is generally the preferred test regardless of variation, provided that data are normally distributed.[38] Correction for multiple comparisons was not performed because the study examined functionally distinct brain regions. Statistical calculations were performed using GraphPad Prism v8.1.1 (GraphPad; La Jolla, CA) unless stated otherwise. *P*-values <0.05 have been considered significant.

To identify cluster separation for the nine essential elements measured here between T2D and our previous sAD metal dataset,[10] multivariate principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were applied using the R-platform MetaboAnalyst.[39] To assess the suitability of each dataset for PCA, the Kaiser-Meyer-Olkin measure for sampling accuracy and Bartlett's test of adequacy were first performed using SPSS v23 (IBM; Armonk, NY). Before multivariate analysis, all metal datasets were mean-centred and divided by the standard deviation of each variable. Metals with variable importance in projection (VIP) scores based on the PLS-DA model of >1.5 were considered to contribute to group separation.

Sensitivity analysis

Sensitivity analysis may help to ensure that interpretation of clinical studies is appropriate, effective, and robust. Here, we performed sensitivity analysis using hippocampal copper levels in the T2D cases and controls according to recommended approaches.[40,41] This sensitivity analysis focussed on hippocampal copper levels since our initial analysis excluded robust case–control differences in any of the other metals and selenium. Data for each of the replicate runs were analysed by ICP-MS and then compared. Box plots were fitted for detection of putative outliers in the copper data (S-PLUS v8.2). Although the T2D cases were shown to be marginally younger than corresponding controls, which might introduce bias from this source, the sensitivity analyses performed here could reduce this risk.

STROBE statement

We performed a completed "Strengthening the Reporting of Observational Studies in Epidemiology (STROBE)" statement to ensure that the reporting of this study was consistent with best-practice

recommendations on what should be included in an accurate and complete report of an observational study, and to minimize the likelihood that important information is missing from the report or unclear in the way it is presented.[42]

Role of funders

The funders for this study did not have any role in the study design, data collection, data analyses, interpretation, writing of the report, or the decision to publish.

Results

ICP-MS analysis was performed on samples from four brain regions of *post-mortem* human tissue from six T2D cases and six controls with similar sex- and PMD-values to measure eight essential metals: sodium, magnesium, potassium, calcium, manganese, iron, copper, zinc, and the metalloid, selenium in order to probe elemental differences between T2D cases and controls. There were no significant differences in PMD between control and T2D groups (Tables 1 and 2). Contrastingly, T2D cases (70 y; 66–75 y [mean; range]) were slightly younger than controls (76 y; 69–79 y; $P = 0.011$).

In the first brain-metal analysis (Run 1), mean *post-mortem* T2D hippocampal levels of copper (394.8 µmol/kg dry weight [95% CI = 270.9–518.7]; $P = 0.005$), zinc (1172.4 µmol/kg dry weight [95% CI = 699.2–1645.7];$P = 0.040$), and potassium (346.1 mmol/kg dry weight [95% CI = 270.5–421.6]; $P = 0.019$) were significantly increased compared to controls (Fig. 1; Table S3; data were analysed using unpaired Welch's *t*-tests). T2D hippocampal copper (394.8 µmol/kg dry weight) displayed the highest mean-metal levels when compared across all regions, but the difference between control and T2D metal concentrations for potassium, copper, and zinc was also greatest in the hippocampus (Table S3). In addition, mean zinc levels were significantly lower in MN tissue only (Fig. 1; Table S3).

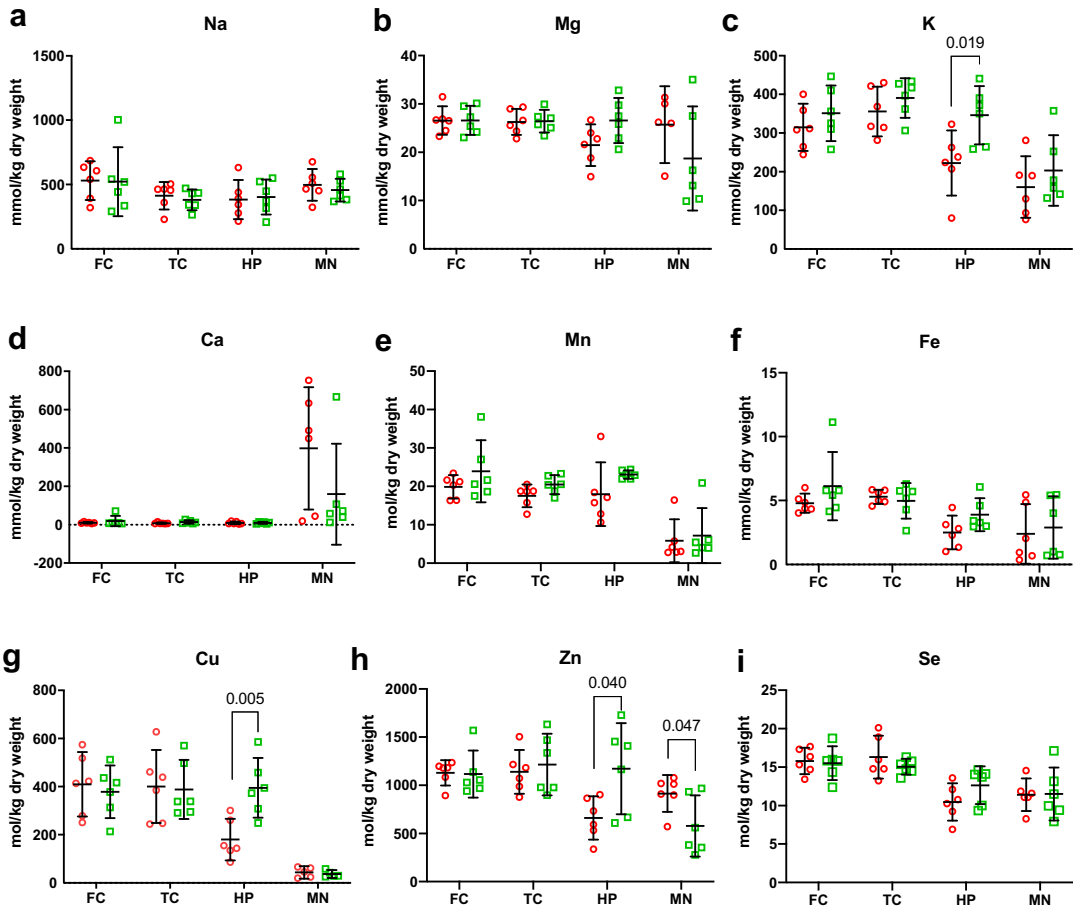

Fig. 1: **First run dry-weight concentrations of nine essential elements (a–i) in four brain regions compared between control (red) and T2D (green) human *post-mortem* tissue**. Data shown represents elemental means ± 95% CI (n = 12; measured using unpaired Welch's *t*-tests). Within the MN, a single outlier from both Mg and Cu were removed from the plot for clarity. FC, Frontal cortex; HP, Hippocampus; MN, Meninges; TC, Temporal cortex.

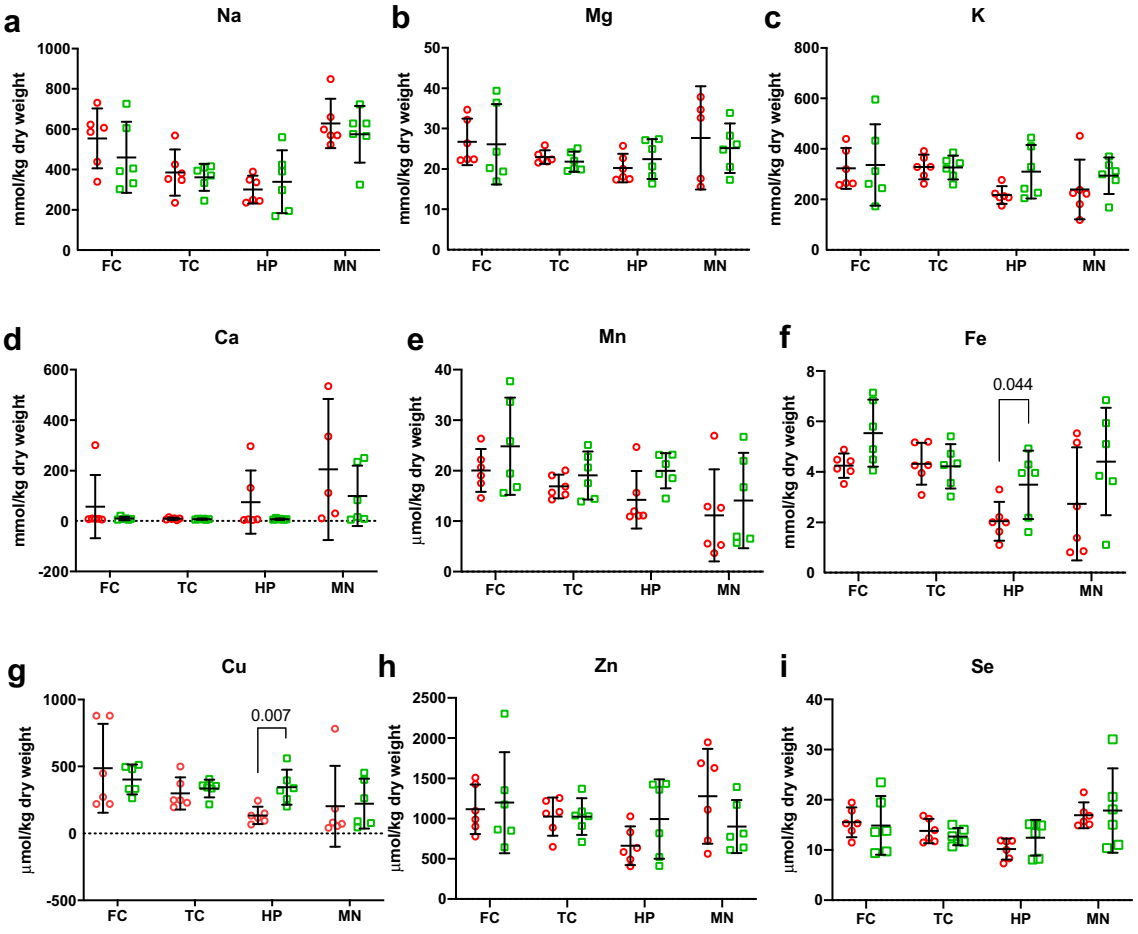

Fig. 2: **Complete technical replicate data of** Fig. 1 **showing repeat measurements in different tissue samples of dry-weight concentrations of nine essential elements (a–i) in four brain regions compared between control (red) and T2D (green) human *post-mortem* tissue**. Data shown represent elemental means ± 95% CI (n = 12; measured using unpaired Welch's *t*-tests). Within the MN, a single outlier from both Mg and Cu was removed from the plot for clarity. FC, Frontal cortex; HP, Hippocampus; MN, Meninges; TC, Temporal cortex.

In the second, replicate analysis (Run 2), which employed fresh tissue aliquots, only mean T2D *post-mortem* hippocampal levels of iron (3.49 mmol/kg dry weight [95% CI = 2.13–4.85]; $P = 0.044$) and copper (345.6 µmol/kg dry weight [95% CI = 214–477.1]; $P = 0.007$) were significantly increased compared to controls (Fig. 2; Table S4; data were analysed using unpaired Welch's *t*-tests). Similar to the first analysis, the hippocampus displayed the greatest difference between control- and T2D-copper concentrations. However, for iron, the largest change in metal levels was in the MN. None of the other physiological metals showed any statistically significant between-group differences.

The results of the sensitivity analysis were as follows. There were no identified protocol deviations in this study of *post-mortem* brain-metal values in cases of T2D and controls. The sensitivity analyses were restricted to the hippocampal copper data because our earlier analysis had excluded the presence of robust copper differences in the other brain regions studied; that is, the case–control differences in copper were localized solely to the hippocampus in this study of four brain regions. Therefore, elevated hippocampal copper co-localizes with the propensity to hippocampal damage that occurs in T2D. The copper datasets from each run were complete; that is, there were no missing copper values. Fitting of box plots excluded the presence of outliers in the case (n = 12) and control (n = 12) groups. Welch's independent sample *t*-tests provided robust evidence for case–control differences in copper values between run 1 ($P = 0.005$) and run 2 ($P = 0.007$). To further evaluate the between-group copper differences, non-parametric testing by applying exact Wilcoxon rank-sum tests was also performed (S-PLUS v8.2), which further supported the between-sample case–control differences in copper values in run 1 ($P = 0.009$) and run 2 ($P = 0.004$); thus, parametric, and non-parametric tests of the significance of between-group case–control

differences were mutually consistent, strengthening the robustness of these findings. Finally, in a further sensitivity test, single copper values were withdrawn sequentially from the case and control datasets from both runs in turn, and the between-group statistical analyses repeated (after extraction, each group now comprised n = 11 values, with 24 such groups tested in turn): this test showed that in no case did removal of a single value result in a loss of significance between cases and controls in either run, further supporting the sample size and emphasizing the robustness of the between-group copper differences (Tables S5 and S6).

Given the observation that hippocampal copper, zinc, and potassium were perturbed in T2D ICP-MS analysis, multivariate PCA using all nine essential-metal concentrations was implemented to further identify patterns within hippocampal *post-mortem* tissue from sAD (n = 9), T2D (n = 12), and controls (n = 20). The core methodology employed to measure brain-metal levels in the T2D cases and controls studied here, namely ICP-MS, was the same as that employed for the reported studies of sAD cases and controls. ICP-MS is a reference method that produces robust data of high quality and therefore strong evidentiary value.[10] Before PCA was performed, the suitability of the analytical methods was assessed. The Kaiser-Meyer-Olkin measure for sampling accuracy for the hippocampal dataset was 0.72, which provides robust evidence for the adequacy of these datasets for PCA. Bartlett's test of sphericity was statistically significant ($X^2(36) = 256.23$, $P < 0.001$), providing strong evidence for significant correlation between variables to support data reduction. Visual examination of the scree plots revealed that the first two components had eigenvalues >1 and together explained some 68% of the total variance, thus confirming their utility for two-dimensional PCA (Fig. S1). As PCA plots comparing both controls from sAD and T2D cohorts displayed substantial overlap and did not present any statistical differences in cohort characteristics (Table S7), both control cohorts were combined and thereafter used as a single control group for comparison against sAD and T2D, as we had specified *a priori*. When cohort characteristics were compared between sAD and T2D against pooled controls, PMD was found to be significantly lower in the sAD group compared to the pooled controls (Table S8). The hippocampal PCA plot revealed almost complete separation between sAD and T2D whereas there was a substantial overlap between T2D and control groups (Fig. 3a), providing robust evidence for differences in hippocampal metallomic profiles between sAD and T2D.

To enhance data discrimination, PLS-DA was performed on the same hippocampal dataset. The PLS-DA model showed a similar pattern to the PCA (Fig. 3b). Based on the PLS-DA model, VIP scores were generated which indicated the relative importance of each metal to the group separation. For both components 1 and 2, the VIP scores were highest for copper and manganese (Fig. 3c and d; Table S9). However, only copper from component 2 had a VIP score >1.5, thus fulfilling the criteria to acknowledge copper as a reliable discriminant within the present hippocampal dataset.

To allow for multi-regional comparisons between PCAs, TC, FC, and MN datasets were analysed using the same statistical approach as for the hippocampal dataset. However, cluster separation was not apparent in the remaining regions (Figs. S2 and S3).

Application of the STROBE process indicates that the reporting of this study is consistent with best-practice recommendations and that all necessary data have been included in the report, which provides an accurate and comprehensive statement of this observational study, and minimizes the likelihood that important information is missing from the report or that it is unclear in the way that it is presented.[42]

Discussion

Altered regulation of brain metals has been reported in several age-related neurodegenerative diseases including sAD and Parkinson's disease but, to our knowledge, no comparable brain-focussed investigation has been reported in T2D or type 1 diabetes (T1D).

Here, we report the concentrations of eight essential metals and selenium from four brain regions in *post-mortem* human T2D and controls. The main finding, which was unexpected, was that substantively elevated copper levels were present in the T2D hippocampus, approaching those reported in untreated cerebral WD. Sensitivity analyses provided evidence supporting the robustness of these data and of their interpretation in this manuscript (as described below). Parametric and non-parametric assessments of between-group differences yielded mutually supporting results consistent with substantively elevated values of hippocampal copper in T2D cases compared with controls. The sample size (n = 12) was supported by the retention of significance in paired case–control groups following sequential removal of single values (Table S5 and S6). Calculations supporting the sensitivity analysis are discussed below.

To further explore the implications of this dyshomeostasis of hippocampal-copper, we compared the levels measured here in T2D with corresponding values from our sAD dataset, where metals were measured using the same mass-spectrometry-based approach. Contrastingly, copper levels in the hippocampus of sAD patients were substantially lower than those in the controls, a reversal of the findings in T2D. Brain-copper levels in sAD have generally been measured in bulk tissue (as was done in our study), or in plaque-enriched material. Reported results have generally differed. That is, the predominant findings reported for bulk tissue in sAD are of widespread decreases in copper levels

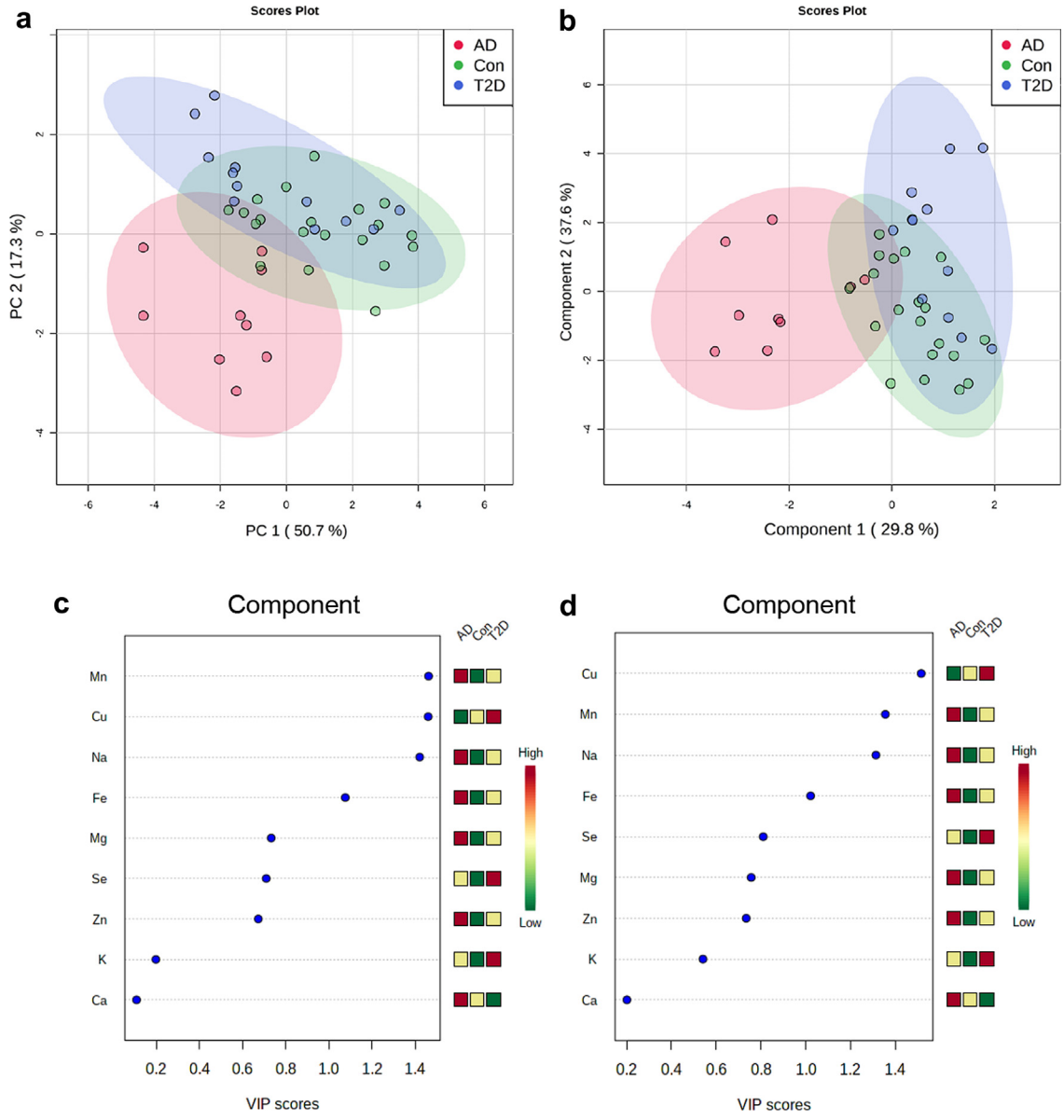

***Fig. 3*: Two-dimensional PCA and PLS-DA plots with VIP scores for human dry-weight hippocampal *post-mortem* tissue**. Data represents a) PCA and b) PLS-DA plots for sAD (red; n = 9), control (green; n = 20), and T2D (blue; n = 12) hippocampal tissue. The coloured ellipses for the PCA and PLS-DA plots represent 95% confidence regions. In the PCA plot, the first principal component (PC1) represents 50.7% of the total variance whereas the second (PC2) contributes 17.3%. The two diseases (sAD vs T2D) demonstrate almost complete separation whereas a substantial overlap is apparent between T2D and controls in both PCA and PLS-DA plots. The VIP score plots (bottom) show the relative contribution of each metal to the variance between sAD, T2D, and controls in c) component 1 and d) component 2. A larger VIP score indicates a greater contribution to the separation of groups. The coloured boxes to the right indicate whether metal concentrations are increased (red) or decreased (green) in the affected group. For both components, Cu and Mn achieved the top two highest VIP scores, with Cu in component 2 achieving the highest VIP score of 1.52. CON, Controls; PC, Principal component; PCA, Principal component analysis; PLS-DA, Partial least square-discriminant analysis; sAD, sporadic Alzheimer's disease; T2D, Type 2 diabetes; VIP, Variable importance for projection.

throughout most brain regions,[21,43–45] whereas by contrast, levels of copper as well as of iron and zinc, are evidently increased in plaque-enriched tissue extracts but less perturbed in bulk tissue.[44] We further applied multivariate statistical analysis with PCA and PLS-DA, which consistently revealed robust evidence for contrasting patterns of metal dysregulation between sAD and T2D.

In one of the replicate analytical runs, hippocampal levels of zinc and iron were significantly increased, and zinc levels in the meninges were lowered; none of these perturbations was replicated in the other analytical run, so the strength of evidence supporting the significance of the zinc and iron findings was considered to be modest. By contrast, there was strong evidence for perturbed hippocampal-copper homeostasis with *P*-values close to the three-sigma threshold in both runs.[46] However, in light of these findings, we would note that altered homeostasis of zinc and iron has previously been associated with sAD[10,47] and T2D[11] in some brain regions. Furthermore, statistical-power analysis revealed that copper was the only metal to achieve a power of >0.9 in this study (Table S10). Therefore, we conclude that increased hippocampal copper is the main metal perturbation present in this T2D dataset.

We also note that the control cohort is older (by 6 y) than the T2D but consider it unlikely that this age-difference exerted a significant effect on the outcome, since the levels of most metals in most regions in this study did not differ significantly between cases and controls. This conclusion is also supported by other studies of the ageing brain metallome that we have undertaken.[29–31] We will revisit this matter in the calculations of the sensitivity analysis presented below.

Copper is the third most abundant transition metal in the brain and is essential for cellular respiration and antioxidant mechanisms.[48] Copper dyshomeostasis plays a central role in the pathogenesis of WD (copper overload) and of Menkes' disease (copper deficiency), both of which diseases, when untreated, exert major deleterious impacts on the brain, including neurodegeneration.[49] Here, we found markedly increased copper levels in the hippocampal tissue of T2D cases, which approximated the brain-copper fold-change of hippocampus-adjacent regions previously reported in WD (Table 3). Direct comparisons of hippocampal-copper levels in WD determined using an ICP-MS-based approach such as that we employed here could not be performed because there are no available, comparable data from WD patients known to us.

Some rodent models of WD, for example the Long-Evans cinnamon rat, demonstrate lowered copper levels in several brain regions, particularly early in their disease development,[51] whereas the toxic milk mouse does not display dysregulation of brain copper.[52] Perhaps contrastingly, most corresponding brain regions show marked elevations in human cases of neurological WD.[50] Moreover, important differences exist between available mammalian models and human WD, particularly the absence of a convincing neurological phenotype in many models.[52] Care will therefore need to be taken in choosing an appropriate animal model of elevated hippocampal copper for evaluation of experimental-therapeutic interventions, for example an

Cummings, 1948 (Ref 50)

Brain region	Dry-weight copper concentrations (µmol/kg)		
	Controls	WD	Fold-change
Cortical white matter	524 (175–1302)	2037 (1730–2333)	3.89
Cortical grey matter	984 (381–1571)	2386 (381–1571)	2.42
Caudate	1121 (540–1492)	2947 (1603–5048)	2.63
Thalamus	937 (492–1968)	4471 (3286–5064)	4.77
Putamen	1476 (968–1905)	10,302 (9603–11,000)	6.98
Globus pallidus	2365 (1666–2984)	3773 (1333–6333)	1.60
Liver	1698 (587–2730)	13,270 (6250–24,830)	7.82

Philbert et al., 2022 ([a]this manuscript)

Brain Region	Dry-weight copper concentration (µmol/kg)		
	Controls	T2D	Fold-change
Hippocampus	180 (94–267)	395 (271–519)	2.19
Temporal cortex	401 (244–627)	389 (291–571)	−0.97
Frontal cortex	410 (251–627)	378 (213–512)	−0.92
Meninges	43.6 (19.7–58.3)	37.2 (25.9–58.3)	−0.85
Cerebral artery	55.5 (46.9–81.3)	47.7 (37.1–66.2)	−0.86
Vertebrobasilar artery	76.4 (40.9–151)	55.0 (39.0–64.9)	−0.72

Data are means (range) and fold-change of Cu concentrations taken from Cumings[50] and the present study. It should be noted that Cu measurements in Cummings's study were analysed using the sodium diethyldithio-carbamate method whose results are not quantitatively comparable with those from ICP-MS, a modern reference method. A measure of significance could not be obtained from Cumings's data as only mean Cu concentrations for controls, and not individual brain concentrations, were provided. Abbreviations: T2D, type 2 diabetes; WD, Wilson's disease. [a]Only the hippocampus was shown to have significantly different Cu levels between cases and controls in T2D (this study).

Table 3: **A comparison of copper concentrations and fold-changes between WD and T2D.**

Atp7b$^{-/-}$ mouse model that demonstrates an informative neurological phenotype.[53] With aging and disease progression, however, large amounts of copper from damaged hepatocytes can be released into the bloodstream. These alterations occur in late stages of the disease and allow the aberrant build-up of the metal in the brain of some WD models.[51,52] The evolution of copper imbalance in the WD scenario could reflect a similar process in T2D that is different depending on the stage of the disease. There is robust evidence for elevated plasma levels of non-Cp copper, also termed "free" copper, in both T1D[54] and T2D,[55] consistent with higher levels of urinary copper in both diseases.[11,54] Urinary copper is driven by circulating non-Cp-bound copper passing through the glomerular filter into the urine, so elevated non-Cp copper represents a shared feature among diabetes, WD, and sAD.

Hippocampal atrophy and cognitive impairment have both been shown to be prominent features of T2D, even during the early stages of disease.[17,18] However, at present, the mechanisms responsible for hippocampal atrophy in T2D remain unclear. Given the prior understanding of copper toxicity in disease, as documented in WD, the hippocampus-specific localisation of elevated copper observed in this study suggests a potential role of copper toxicity in the cerebral neuropathogenesis of T2D. It is proposed that there may be increased extracellular copper-labile pools, particularly in sAD,[56] despite a decrease in the tissue-copper content in some organs in both sAD and diabetes.[11,35,55,57] However, as tissues from diabetic cases with cognitive dysfunction were not examined in the current study owing to the unavailability of hippocampal tissue from such cases, the contribution of metal dysregulation to cognitive dysfunction in T2D remains to be established.

Copper plays central roles in the structure and function of cytochrome *C* oxidase (COX) in the mitochondrial electron transport chain via copper metalation of Cox subunit 1 (COX1) and 2 (COX2) at the Cu_A and Cu_B active sites of complex IV.[58] Copper plays important roles in the assembly of COX, and reduced COX activity has been observed in rats with copper overload,[59] and in rats with streptozotocin-induced diabetes.[60] In addition, measurement of the mitochondrial proteome in T2D-skeletal muscle indicated a downregulation of COX2.[61] While copper levels were not reported in any of these diabetes studies, mitochondrial dysfunction via reduced activity and/or downregulation of COX could represent a potential mechanism for the copper toxicity observed in T2D. However, as to our knowledge COX activity has not yet been reported in *post-mortem* hippocampal human T2D tissue, further research is needed to test the hypothesis of copper-mediated mitochondrial dysfunction in this context.

Despite copper's key roles in some antioxidant processes (e.g., those catalysed by superoxide dismutase 1 and by complex IV), it can also participate in the production of harmful reactive oxygen species (ROS) via the Fenton and Haber–Weiss reactions. There is evidence that Cu^{II} can bind to advanced glycation end-products (AGEs) whilst retaining its redox–active properties.[62–64] As protein glycation is significantly increased in T2D due to hyperglycaemia and polyol-pathway activation,[34] the elevated hippocampal copper measured in the present study has the potential to cause increased AGE-Cu^{II} complex formation. The formation of AGE-Cu^{II} complexes could thereby lead to increased Cu^{II}-mediated ROS production, and decreased copper bioavailability for antioxidative pathways due to the selective binding of extracellular Cu^{II} with AGEs. However, Cu^{II} is less abundant than Cu^{I}, so the role that AGE-Cu^{II} complexes might play in the pathogenesis of T2D remains to be clarified.

Given the unexpected identification of increased hippocampal copper in cases of T2D in this study, we employed two complementary multivariate methods, PCA and PLS-DA to analyse the pattern of brain-metal dyshomeostasis within our sAD dataset, which identified widespread, severe brain-copper deficiency.[10] The PCA analysis revealed almost complete separation between sAD and T2D, with a strong overlap between T2D and control hippocampal samples. To enhance discrimination between cases, PLS-DA modelling was employed, which displayed similar cluster separation to the PCA plot. These analyses identified distinct patterns of brain-metal dyshomeostasis between the two diseases. Only copper displayed a VIP score >1.5, thus showing it to be a reliable discriminant between hippocampal metals in T2D and sAD. Thus, copper is one of the key factors responsible for the separation of sAD and T2D clustering and adds strength to the hypothesis that copper perturbations could play a key role in the cerebral neuropathogenesis of T2D. The current findings support the potential existence of substantially different processes associated with neurodegeneration in these two diseases.

This study has limitations since it is potentially susceptible to several sources of bias: see the following for a comprehensive catalogue of biases (https://catalogofbias.org › biases). Here, the authors aimed to minimize susceptibility to bias in all aspects of the study design, implementation, data analysis, and formulation of conclusions, but some remaining sources of potential bias remain. First, the findings concerning substantively elevated hippocampal copper in T2D were unexpected and are potentially of considerable interest to the field, so this study is, by definition, susceptible to novelty bias.[65] A second important shortcoming is the small sample size ($n = 12 < 20$), which was driven by the marked lack of available human T2D-brain tissue, as well as the non-availability of such material from diabetic patients with impaired cognitive function or dementia accessible in existing brain banks. The sample size would ideally have been three-to-four times the

number of features (metals), which, for this study is calculated to be at least ~12 cases and 12 controls, so this is a further source of potential bias. When an overly small sample size is used, it often leads to chance findings.[66] Therefore, this study is potentially susceptible to the 'power failure' generated by small samples in underpowered case–control studies.[67] However, as previously stated, the availability of suitable brain samples with short PMD from sAD and T2D cases is very limited, but the importance of impaired cerebral function and dementia in T2D is considered to be high, so the authors determined on balance that accepting the risk entailed in reporting this study was nevertheless worthwhile. Selection bias is a further potential source of difficulty in this study. It occurs when individuals or groups in a study differ systematically from the population of interest leading to a systematic error in an association or outcome. Since the cases in this study all had T2D, which typically leads to frequent use of medical care, there is the clear potential for a systematic difference between cases and (non-diabetic) controls.

Given the small sample sizes in the present study (T2D vs control; n = 12), in order to assess risk further, we conducted statistical power tests for copper in hippocampal tissue to determine the likelihood of a type II error in this study as a necessary condition for publication. Across both runs, copper was found to have the highest power level of any element (>0.90; Table S10), whereas all other measured metals had power levels of <0.80. Furthermore, *a priori* analysis showed that only copper had a desired minimum sample size (n = 10) below that which was used in this study. Therefore, these power levels must be considered when interpreting the differences measured for hippocampal-metal levels in T2D. We would strongly recommend that in future, investigation of both T2D and sAD should be replicated in studies with increased sample numbers. Notably, such an objective may well require the development of one or more brain banks, or at least brain collections in different regions of the world, targeted towards impaired cognitive function and dementia in T2D.

In this study, cases and controls were identified with the objective of generating a cohort similar for sex, age, and PMD. In the event, possibly because of the low sample size, there was a difference (6 y) of uncertain significance in age, with the mean age of controls being 76 y and cases 70 y. This dissimilarity could generate bias, although the tests applied in the sensitivity analyses may somewhat ameliorate that risk.

Given the origin from the NDRI of the cases and controls, these findings are considered to reflect cases of similar age with and without T2D coming to *post-mortem* examination in the USA. The NDRI undertakes *post-mortem* examinations and systematic tissue collection in numerous sites in the USA, so there may be the potential for significant regional differences between brain-tissue samples acquired from different regions. On the other hand, the size and replicability of the case–control copper differences in this small study may reflect an important disease process that transcends bias from regional variation.

Available clinical data for each case and control participant are presented in Table S1. Unfortunately, these data are incomplete. For example, ethnicity was not available and so was not included in the statistical and sensitivity analyses.

Another limitation that can confound *post-mortem* analyses is the potential alteration in analyte levels due to excessive PMDs. However, although the duration of PMD is suggested to influence proteomic and metabolomic degradation, a recent study reported that brain-metal levels are unaffected by extended PMD (up to at least 72 h).[29] Thus, we are confident that PMDs in the present study did not affect brain-metal levels and conclusions based thereon.

In addition, some patients with myocardial infarction reportedly demonstrate elevated serum copper, perhaps due to efflux from ischaemic tissue.[68] Here, myocardial infarction was the certified cause of death in two of the six diabetic cases studied. We know of no published evidence that directly links myocardial infarction to elevated brain copper in human diabetes cases, but future studies seeking to replicate and extend the current investigation of brain copper in T2D should preferably avoid myocardial infarction as the cause of death in either cases or controls.

Taking the preceding factors together, a further potential cause of bias in this study may reflect differences in sampling caused by one or more of the processes outlined above that could in turn lead, for example, to ascertainment bias, which can happen when there is more intense surveillance or screening for outcomes among exposed individuals (here, those with T2D), than among unexposed individuals (controls).[69] Larger studies that are designed to address these shortcomings will be required to replicate and extend the current findings.

It is also worth noting that while the inconsistency of significant hippocampal-metal perturbations between runs might be attributable to insufficient statistical power, the sampling of certain regional subfields (e.g., Brodmann areas) may also have influenced the observed metal inconsistencies. Imaging studies investigating T2D have revealed various hemispheric discrepancies in hippocampal-volume subfields, such as the hippocampal tail.[18] Given the understanding that metal perturbations may be greater in more severely affected regions in T2D, it is plausible that the sampling of two different subfields may have occurred among samples. With this in mind, the investigation of metal concentrations in various cerebral subfields would be helpful for future studies examining brain-metal dyshomeostasis in T2D.

The following section outlines a sensitivity analysis for this case–control study of hippocampal copper in

T2D and its interpretation. Five-of-six cases and one-of-six controls had hippocampal-copper levels >266.5 μmol/kg, the upper limit of the 95% CI in the controls, which were considered to be 'normal' for purposes of this analysis. We calculated the risk ratio (RR; also called the relative risk), which compares the risk of a 'health event' (e.g., disease, injury, risk factor, death) among one group (comprising cases, the numerator) with the risk among the control group (the denominator).[41] RR was determined by dividing the risk of elevated hippocampal copper in the numerator group by that in the denominator group. Therefore, in this case, the RR = 5/6/1/6 = 83.3%/16.7% = 5, consistent with an increase in risk in the T2D group and providing evidence for the hypothesis that T2D causes build-up of copper in the hippocampus. Research findings are more likely true when RR values are ≥3.[66,70]

We also calculated the E-value, "which is related to the evidence for causality in observational studies that are potentially subject to confounding.[41] The E-value is defined as the minimum strength of association, on the risk–ratio scale, that an unmeasured confounder would need to have with both the treatment (in this case T2D) and the outcome (elevated hippocampal copper) to fully explain a specific treatment–outcome association, conditional on the measured covariates." The E-value was calculated by applying the following formula[41]:

$$\text{E-value} = RR + \text{sqrt}\{RR \times (RR - 1)\} = 5 + \text{sqrt}\{5 \times (5 - 1)\} = 9.5$$

The mean (±95% CI) of the six T2D cases were 394.8 (270.9–518.7) μmol/kg, and of the controls were 180.2 (94.0–266.5) μmol/kg. Five of the six T2D cases had hippocampal-copper levels > the upper limit of the 95% CI in the control group, and the effect size, for interpretation of the E-value, was (394.8–180.2)/82.2 = 2.61 (by Glass's delta), which is preferred here because of the significant difference between the standard deviations of cases and controls.[71,72] These values may support the potential presence of a causal effect in these data between T2D and elevated hippocampal copper. Neither BMI nor PMD differed significantly between cases and controls and so were unlikely to have affected the outcome; however, there was a difference between mean ages in the two groups (P = 0.011) which underwent further scrutiny. The estimated effect size for age was (75.8 – 70.0)/3.54 = 1.64 (by Glass's delta) and therefore age could theoretically have exerted an effect on the outcome, although we know of no plausible prior evidence for a mechanism that could drive age-related accumulation of copper in the T2D hippocampus.

However, since this is an observational case–control study, manifest caution is necessary concerning its interpretation, particularly when it comes to the question of causation vs association.[70,73] First, to be unequivocal, the sum of the new evidence produced in this study is insufficient to prove definitively (or for that matter, to disprove), the hypothesis that T2D causes accumulation of copper to toxic levels in the human hippocampus.

In addition, therefore, we employed multiple logistic regression to fit a generalized linear model (GLM) to these data, (viz. hippocampal-copper^{dichot} ~ {Age + Bmi + Pmd}, model = binomial; S-PLUS v8.2), in order to estimate the adjusted effects of age, BMI, and PMD on the dichotomous dependent variable, hippocampal-copper^{dichot}, that was generated by dichotomization at the value of 266.5 μmol/kg (the upper limit of the 95% CI for the control group) in order to generate a dichotomous variable ("hippocampal-copper ≤ 266.5", "hippocampal-copper > 266.5"). Analysis of deviance following application of the X^2 test showed no evidence that any of the adjusted variables, [namely those for age (P = 0.10), BMI (P = 0.73), and PMD (P = 0.94)], exerted a significant effect on the hippocampal-copper^{dichot} variable.

To further assess the evidence and to provide a rigorous assessment of the possible effect of the measured case–control age difference on hippocampal-copper, we additionally fitted a one-way ANOVA model with hippocampal-copper^{contin} as the *continuous dependent variable*, (T2D/control) as the dichotomous independent variable, and age as a continuous covariate (viz. hippocampal-copper^{contin} ~ {T2D/control + Age}). There is substantive evidence that the T2D/control variable evoked a significant effect on hippocampal copper in this model (P = 0.0038), consistent with our previous findings documented herein above, whereas age did not (P = 0.18); the lack of effect of age in this model mirrors the finding of the GLM. Therefore, the results from this ANOVA model reinforce those from the GLM.

Finally, another line of evidence concerning the potential for causality in an observational study may be derived by applying the approach of Bradford Hill, who provided nine viewpoints ('Bradford Hill Criteria') by which to determine whether causation might be deduced in an observational dataset. We applied these criteria to this T2D-case-control study and discuss the outcomes (see Supplemental Text). In brief, we found that, when the evidence derived from application of the Bradford Hill criteria is considered along with the other lines of evidence, it would appear to support a plausible case for T2D as a cause for defective copper homeostasis in the hippocampus, the main site of diabetes-elicited cerebral neurodegeneration.

In this study of hippocampal copper in T2D, both cases and controls demonstrated substantial numbers of co-morbidities (Table S1). Possible effects of myocardial infarction on systemic copper have been discussed above. Here, six-of-six controls and five-of-six T2D cases had documented evidence of cardiovascular disease, so rates were similar in both study-groups. Chronic T2D is frequently associated with cardiovascular disease, which plays a dominant role that largely accounts for its twofold increase in mortality.[74] These findings are consistent with the known relationship between

cardiovascular disease and T2D. Notably, however, there was a similar pattern of co-morbidity in both study-groups.

We conclude that, when these various lines of evidence are taken together, the data provide robust evidence for increased hippocampal-copper levels in cases of T2D, which approximate those in untreated WD. Based on these findings, we postulate that elevated hippocampal copper may contribute to the hippocampal atrophy in T2D, probably due to bioenergetic dysfunction and oxidative stress induced by elevated copper levels. Additionally, multivariate statistical modelling has revealed markedly contrasting patterns of hippocampal-copper levels between T2D, sAD, and non-diabetic controls, supporting the potential existence of different cerebral neurodegenerative processes in T2D and sAD. Further research is needed, in larger cohorts, to confirm and extend understanding of the significance of brain copper and its perturbed regulatory mechanisms in T2D. Subsequent metallomic investigations might have the potential to guide the development of new therapies for cognitive impairment in T2D, for example, the potential repurposing of copper chelators approved for clinical use in other indications such as WD.

Contributors

S.A.P. designed and performed experiments, analysed, and interpreted data, and wrote the manuscript; S.J.S. contributed to the research hypothesis, collected all the brain tissue, and revised the manuscript; J.X. performed experiments and revised the manuscript; S.J.C. performed experiments and analysed the data; R.D.U. designed experiments, interpreted the data, and revised the manuscript. G.J.S.C. designed the experiments, interpreted the data, revised the manuscript, and bears overall responsibility for the integrity of the manuscript and of the study. All authors have confirmed that they have read and approved the final version of the manuscript.

Data sharing statement

Raw metal data for the T2D/control study can be found in the supplementary material. Raw metal data for our previous sAD/control study[10] is available upon request.

Declaration of interests

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2022.104317.

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