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1 FINAL VERSION [4584 words]

2 **Cerebral deficiency of vitamin B5 (D-pantothenic acid; pantothenate) as a potentially-**
3 **reversible cause of neurodegeneration and dementia in sporadic Alzheimer's disease**

4 Jingshu Xu ^{1,2}, Stefano Patassini ^{1,2}, Paul Begley ¹, Stephanie Church ¹, Henry J. Waldvogel ³,
5 Richard L. M. Faull ³, Richard D. Unwin ¹, and Garth J. S. Cooper ^{1,2,3,*}

6

7 ¹ Division of Cardiovascular Sciences, School of Medical Sciences, Faculty of Biology,
8 Medicine and Health, The University of Manchester, Manchester, M13 9WL and Centre for
9 Advanced Discovery and Experimental Therapeutics (CADET), Central Manchester University
10 Hospitals NHS Foundation Trust, Manchester Academic Health Sciences Centre, Manchester,
11 UK

12 ² School of Biological Sciences, Faculty of Science, University of Auckland, New Zealand.

13 ³ Centre for Brain Research, Faculty of Medical and Health Sciences, University of Auckland,
14 Auckland, New Zealand.

15

16 *Corresponding Author; e-mail garth.cooper@manchester.ac.uk.

17 Address for correspondence: Garth J S Cooper, Centre for Advanced Discovery and
18 Experimental Therapeutics, Division of Cardiovascular Sciences, School of Medical Sciences,
19 Faculty of Biology, Medicine & Health, The University of Manchester M13 9NT

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Abstract

Alzheimer's disease (AD) is the most common cause of age-related neurodegeneration and dementia, and there are no available treatments with proven disease-modifying actions. It is therefore appropriate to study hitherto-unknown aspects of brain structure/function in AD to seek alternative disease-related mechanisms that might be targeted by new therapeutic interventions with disease-modifying actions. During hypothesis-generating metabolomic studies of brain, we identified apparent differences in levels of vitamin B5 between AD cases and controls. We therefore developed a method based on gas chromatography-mass spectrometry by which we quantitated vitamin B5 concentrations in seven brain regions from nine AD cases and nine controls. We found that widespread, severe cerebral deficiency of vitamin B5 occurs in AD. This deficiency was worse in those regions known to undergo severe damage, including the hippocampus, entorhinal cortex, and middle temporal gyrus. Vitamin B5 is the obligate precursor of CoA/acetyl-CoA (acetyl-coenzyme A), which plays myriad key roles in the metabolism of all organs, including the brain. In brain, acetyl-CoA is the obligate precursor of the neurotransmitter acetylcholine, and the complex fatty-acyl groups that mediate the essential insulator role of myelin, both processes being defective in AD; moreover, the large cerebral vitamin B5 concentrations co-localize almost entirely to white matter. Vitamin B5 is well tolerated when administered orally to humans and other mammals. We conclude that cerebral vitamin B5 deficiency may well cause neurodegeneration and dementia in AD, which might be preventable or even reversible in its early stages, by treatment with suitable oral doses of vitamin B5.

43 Keywords: Alzheimer's disease; Human brain; Vitamin B5 (pantothenic acid; pantothenate);
44 Age-related neurodegeneration; Acetyl-CoA; Metabolic brain disease
45

46 Abbreviations: AD, Alzheimer's disease; CoA, coenzyme A; GC-MS, gas chromatography-
47 mass spectrometry; PANK, pantothenate kinase; PDH, pyruvate dehydrogenase; PKAN,
48 pantothenate kinase-associated neurodegeneration; TCA, tricarboxylic acid

49

1. Introduction

Alzheimer's disease (AD) is characterised by neuropathological hallmarks of senile plaques and neurofibrillary tangles in the brain, and clinically-insidious onset with progressive decline of neurocognitive function. AD is the most common cause of age-related neurodegeneration and dementia and represents the largest unmet medical need in neurology [1, 2].

The pathogenesis of AD is complex, and metabolic abnormalities in the brain lie at the core of its molecular pathology [3]. Well-documented metabolic disturbances associated with AD-evoked neurodegeneration include: abnormal A β and τ metabolism with the formation of protein aggregates implicated in tissue damage; mitochondrial dysfunction; oxidative stress; and impaired cerebral glucose uptake and energy metabolism. More recently, marked elevations in glucose levels of AD brain have been reported by three independent studies that employed different methodologies [4-6], and the evident global perturbation of metabolites supports the notion of AD as a pervasive metabolic disorder [7].

Our group's prior investigations have indicated that enzymes and metabolites involved in glycolysis, the pentose-phosphate pathway, the polyol pathway, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation are consistently affected in the AD brain when compared with controls [7, 8]. Acetyl-CoA plays a central role in metabolism, functioning as the necessary molecule through which glycolysis-derived pyruvate enters the tricarboxylic acid (TCA) cycle through pyruvate dehydrogenase (PDH)-mediated catalysis [9]. CoA itself not only forms the major integral aspect of the acetyl-CoA molecule but is also a cofactor for numerous pathways such as oxidative respiration and lipid metabolism, and plays a regulatory role in brain health through its involvement in the synthesis of neurotransmitters such as acetylcholine [10], and in cerebral myelin biosynthesis [11]. For the biosynthesis of CoA, vitamin B5 is essential as both a primary substrate and a rate-limiting metabolite, whose utilization is catalysed by the enzyme pantothenate kinase (PANK) [17]. Mutations in the gene *PANK2*, which encodes mitochondria-localized PANK2 (one of four human pantothenate kinases encoded in the human genome [12, 13]), lead to PANK-associated neurodegeneration (PKAN), which strongly suggests the critical importance of vitamin B5 for maintaining neural health and integrity; this view is further supported by its known obligate roles in the biosynthesis of acetylcholine and of myelin [11].

Here we applied quantitative gas chromatography-mass spectrometry (GC-MS) to determine putative alterations in vitamin B5 concentrations in brain tissue from AD cases and matched

83 controls. We analysed 18 human brains with short *post-mortem* delay wherein seven
84 anatomically- and functionally-distinct brain regions were examined in each: three of these
85 are known to undergo severe neuronal damage in AD (hippocampus, entorhinal cortex, and
86 middle temporal gyrus); three to be moderately affected (cingulate gyrus, sensory cortex
87 and motor cortex); and one (cerebellum) thought to be relatively spared [14-16].

88

2. Methods

2.1. Acquisition of human brains

All experiments were performed in accordance with relevant guidelines and regulations. This case-control study of post-mortem brain was approved by the University of Auckland Human Participants Ethics Committee with informed consent from all families.

Human brains were obtained from the Neurological Foundation Human Brain Bank, University of Auckland, as detailed in the Suppl. Methods 2.1.

2.2. Dissection of human brains

Human brains were dissected as described in the Suppl. Methods 2.2.

2.3. Metabolite extraction for GC-MS

Metabolites were extracted for GC-MS as detailed in the Suppl. Methods 2.3.

2.4. Sample preparation

Samples were prepared for GC-MS as detailed in the Suppl. Methods 2.4.

2.5. GC-MS

GC-MS was performed as detailed in the Suppl. Methods 2.5.

2.6. Data analysis

GC-MS data were analysed as described in the Suppl. Methods 2.6.

2.7. Cerebral vitamin B5 concentrations measured by GC-MS

The reproducibility and accuracy of our targeted vitamin B5 assay have been reported elsewhere [17]. Briefly, to determine vitamin B5 concentration by GC-MS, we generated a calibration curve using known concentrations of authentic synthetic standards (Sigma-Aldrich) in human-brain extract as matrix. We calculated vitamin B5 levels in individual brain samples using the calibration curve, and data have been presented here as $\mu\text{mol/kg}$ of fresh (wet) brain tissue. Calculation of metabolite abundance was performed on internal-standard ratios rather than raw peak areas in order to improve reproducibility.

2.8. Statistical analysis

Data were analysed using an in-house bioinformatic work-flow developed in R v3.5.1. Statistical analysis and data visualization were performed using in-house scripts implemented in the following R packages: tidyr v0.8.1; ggplot2 v3.0.0; ROCR v1.0-7; nnet v7.3-12; MASS v7.3-50; lattice v0.20-35; and magrittr v1.5. A receiver-operating

characteristic (ROC) curve was generated by combining all measurements in this study and applying ROCR v1.0-7; nnet v7.3-12 was used to fit multinomial log-linear models via neural networks.

To ensure the reproducibility of our assay, we first assessed the QC replicate samples and evaluated the % coefficient-of-variation (%CV) of vitamin B5 measurements in each experimental batch. We set the %CV for QC samples of 15% as the cut-off for acceptable reproducibility.

For each brain region, effects of age, *post-mortem* delay (PMD), and brain-weight on the vitamin B5 concentrations in human brain were investigated by Spearman's correlation analysis. A correlation was considered to be significant only if the correlation coefficient was >0.8 or <-0.8 , and the corresponding P-value was <0.01 .

3. Results and discussion

We measured vitamin B5 concentrations in each of 7 brain regions from 9 patients with AD and 9 controls, with matching of cases and controls for age, sex, and *post-mortem* delay (Table 1). Average brain weight of the AD group was significantly lower than that of the control group; the observed decline in brain weight is in line with the severity of neurodegeneration. All AD cases studied had Braak stages of IV or above; one control had Braak stage II, which was retained within the control group for analysis (Suppl. Table 1) as this finding is consistent with the known frequency of asymptomatic AD in similarly-aged groups in the study population [18].

Table 1

Case-control study of *post-mortem* human brain in AD: group characteristics

Variable	Control	Alzheimer's disease
Number	9	9
Age	70.1 (6.7)	70.3 (7.1)
Male sex, n (%)	5 (55.6)	5 (55.6)
PMD (h)	9 (5.5-13.0)	7 (4.0-12.0)
Brain-weight (g)	1260 (1094-1461)	1062 (831-1355)*
Plaques, n (%)	1 (11)	9 (100)**
Tangles, n (%)	1 (11)	9 (100)**

Values are: age, mean (SD); PMD and brain-weights, median (range):

*P = 0.005, **P < 0.0001 compared with corresponding Control value; all other differences are non-significant. Abbreviation: PMD, *post-mortem* delay

To quantify vitamin B5 levels in human brain tissue, we generated a calibration curve by constituting different concentrations of synthetic vitamin B5 as pure standard solutions (Fig. 1a) and also in human brain-derived background matrices (Fig. 1b). Vitamin B5 showed stable linearity ($R^2 > 0.99$) within the range at which we measured brain concentrations; the standard curve shown in Fig. 1b was used to interpolate the regional concentrations of vitamin B5 reported here.

We observed a global reduction in vitamin B5 levels across all 7 brain regions in cases compared with controls (Fig. 2a). Control brains showed greater variation in vitamin B5 levels in general, and regions corresponding to those heavily affected in AD (hippocampus, entorhinal cortex, and middle temporal gyrus) showed lower mean vitamin B5 values when compared to regions generally considered to be less severely affected (sensory cortex, motor cortex, and cerebellum). The QC-replicate samples ($N = 8$ per region per condition) measured in this study showed low %CV values across all brain regions (max = 12%) (Fig. 2b), demonstrating the high degree of reproducibility of our assay.

The decrease in cerebral vitamin B5 levels in AD cases was observed in all brain regions studied, which are representative of the majority of brain mass (Fig. 2). The range of mean vitamin B5 concentrations in control brains was between 33.7 (in cingulate gyrus) and 55.4 (cerebellum) $\mu\text{mol/kg}$ of fresh brain tissue, and between 15.2 (entorhinal cortex) and 25.4 (cerebellum) in AD. These data reflect copious amounts of vitamin B5 within the normal brain. Fold-changes in vitamin B5 were similar across all brain regions examined, showing an average of 60% reduction with statistical significance typically being $P < 0.01$ (Fig. 2c).

Thus similar levels of vitamin B5 depletion are pervasive in the AD brain. This finding is potentially consistent with comparable degrees of damage in cerebellar and cortical structures, which have also been reported by studies using other methods [19]. This observation raises the question of whether vitamin B5 depletion might be cause or consequence of the underlying neuropathological process. We interpret concomitant elevations in metabolites including glucose, glucose-6-phosphate, urea, and the polyol pathway intermediates sorbitol and fructose, along with impaired glucose uptake into the AD brain [4, 7, 20], taken together with similar perturbations in Huntington's disease brain [17, 21-23], to support the hypothesis that vitamin B5

deficiency may be an early perturbation in the development of AD-evoked neurodegeneration. The evident metabolic defects in the polyol and glycolytic pathways could be linked to cerebral deficiency of vitamin B5, which could cause defective formation of CoA and acetyl-CoA and uptake of acetyl-CoA into the TCA cycle, along with downstream alterations in cerebral urea metabolism.

A ROC curve with AUC of above 0.9 (Fig. 2d) indicates high discriminatory power of vitamin B5 concentration in classifying AD vs Control tissues. An optimal cutoff value can be chosen based on the clinical requirements for sensitivity/specificity or accuracy. In our data, the optimal cutoff value was 0.52 when given equal weight to sensitivity and specificity (true positive rate = 0.86, false positive rate = 0.19). The optimal cutoff value was 0.37 when accuracy was prioritized.

Vitamin B5 levels were not significantly correlated with age, PMD, or brain weight (data not shown); Braak stage and amyloid load served as surrogates for the diagnosis of AD (Suppl. Table 1), so their relationship to AD was not analysed further.

Fig.1

Figure 1. Calibration curve used for the determination of vitamin B5 concentrations in human brain. Calibration curves were generated by measurements of known concentrations of synthetic Vitamin B5 standards in a) aqueous medium, or b) in matrix generated from human-brain extract.

Fig.2

195

196 **Figure 2. Vitamin B5 levels in human brain in cases with AD and matched**
197 **controls.** a) Vitamin B5 concentrations measured in individual brain samples (N =
198 18/region = 126 in all); and b) in QC replicate samples; c) mean (±95% CI) values of
199 vitamin B5 concentration for each brain region in controls and AD cases, where
200 statistical significance was calculated using multiple two-tailed *t*-tests; d) receiver-
201 operating characteristic curve for vitamin B5 among all brain samples. Abbreviations:
202 HP, hippocampus; ENT, entorhinal cortex; MTG, middle temporal gyrus; CG,
203 cingulate gyrus; SCx, sensory cortex; MCx, motor cortex; CB, cerebellum.

204

Deficiency of several other B-group vitamins is known to associate with elevated risk of dementia or other psychiatric disorders [24]: those B vitamins hitherto implicated include thiamine (vitamin B1) [25]; niacin (vitamin B3) [26]; vitamin B6 (pyridoxal phosphate; pyridoxamine; pyridoxine) [24]; folate (vitamin B9) [24]; and cyanocobalamin (vitamin B12) [27, 28].

Here we hypothesize that severe, global deficiency of vitamin B5 in AD brain, as observed in our current study, could contribute to defects in a number of molecular pathways known to be implicated in the pathogenesis of neurodegeneration in AD, in particular those involving the metabolism of CoA and all those downstream pathways that use CoA or its reduced form CoASH as an essential cofactor or biosynthetic intermediate [29]: the latter roles include the cerebral biosynthesis of acetylcholine and of myelin, both of which are deficient in AD [11, 30-33]. Therefore, cerebral vitamin B5 deficiency is likely to be linked to the pathogenesis of dementia.

Notably, in our initial metabolomic study, vitamin B5 was the only B-vitamin whose brain concentrations were measurable by the approach taken, and whose brain levels were shown to be substantively deficient in AD dementia. More work will be required to quantitate (putative) cerebral levels of the other B-vitamins in AD to further characterise their possible relationship with the common age-related dementias.

The gene *PANK2* encodes the enzyme PANK2 and regulates the utilization of vitamin B5 through the pathway leading to the biosynthesis of CoA and of acetyl-CoA [34]. Mutations in *PANK2* and associated PANK2 deficiency can cause PKAN through impairing vitamin B5 metabolism and mitochondrial function, in turn leading to mitochondrial CoA deficiency-associated oxidative stress. This chain of events suggests that brain regions with higher metabolic demand could be particularly susceptible to vitamin B5 deficiency.

Interestingly, brain regions corresponding to those heavily affected in AD also showed relatively lower vitamin B5 levels in control brains. According to our measurements in control brains, vitamin B5 levels ranged between 34 and 38 $\mu\text{mol/kg}$ of fresh tissue in those regions (hippocampus, entorhinal cortex, middle temporal gyrus, and cingulate gyrus) that are heavily affected in AD; between 43 to 44 $\mu\text{mol/kg}$ in less affected brain regions (motor cortex and sensory cortex); and finally, 55

μmol/kg in the relatively-spared cerebellar region. Further study will be required to understand the mechanism and implications of these observations.

Vitamin B5 is the obligate precursor of acetyl-CoA, which is the substrate of the TCA cycle generated by action of PDH. Reduced PDH complex and TCA-cycle activities have previously been reported in AD brain [35, 36] and could be directly associated with severe depletion of this acetyl-CoA precursor.

Acetyl-CoA plays numerous essential roles in cerebral metabolism. It is not only required for energy production in mitochondria and diverse synthetic pathways in various extra-mitochondrial compartments, but is also of particular importance for cholinergic neurons in the brain: cholinergic neurons require additional amounts of acetyl-CoA for acetylcholine synthesis in their cytoplasmic compartment to maintain their transmitter functions [33]. Interestingly, the lowered PDH activity in AD brain coincided with low choline acetyltransferase (CAT) activity [35]. Therefore it has been suggested that deficits in acetyl-CoA supply may be more harmful for the cholinergic neurons than for non-cholinergic ones in neurodegenerative diseases such as AD [33]. Acetyl-CoA also plays key roles in biochemical pathways that underpin the metabolism of fatty acids, porphyrins, polyamines, amino acids, proteins, RNA, and histones; it is possible that alterations in any of the steps of the CoA biosynthetic pathway might influence the proper functioning of any or all of such dependent processes.

B-group vitamins are generally thought not to be stored in the brain; rather, specific transporters are said to maintain the proper brain levels of each of these vitamins as required by transferring them from the EC fluid. That said, vitamin B5 would seem to be an exception, since large concentrations are present throughout the brain, where it is localized largely with myelin in the white matter [11]. There is evidence that the transporter SLC5A6, which is expressed in human-brain microvessels, is a major contributor to luminal uptake of vitamin B5 and biotin at the human blood-brain barrier and provides the main route for their uptake into brain [37]. In this study, brain vitamin B5 levels in AD were lowered to 30-50% of control values, so substantive residual cerebral uptake via SLC5A6 must remain in AD-affected tissues; by contrast, our hypothesis-generating metabolomic method did not produce a biotin-specific signal. Vitamin B5 is ubiquitous in the diet, so dietary deficiency is not a plausible

explanation for the observed cerebral deficiency in AD [10]. Vitamin B5 is well tolerated when administered to humans in tablet form. Therefore, given the retention of residual cerebral uptake, we expect it could be possible to restore brain vitamin B5 levels to normal by chronic administration, potentially reversing this abnormality and, perhaps, the pathogenesis of AD.

In conclusion, there are evidently copious amounts of vitamin B5 throughout the normal human brain, and AD dementia is accompanied by widespread deficiency thereof. Vitamin B5 deficiency is perhaps more severe in those brain regions known to be more severely damaged in AD. Vitamin B5 dysregulation could lie at the centre of disturbed brain energetics, including the previously reported polyol-pathway activation and concomitant impairment of glycolysis in AD brain [4]. We conclude that cerebral vitamin B5 deficiency could cause neurodegeneration and dementia in AD, which might be preventable, or even reversible in its early stages, by treatment with oral doses of vitamin B5 sufficient to normalize brain levels.

Duality of interest statement

The authors state that they have no competing interests with respect to this work. Sponsors had no role in the study design; the collection, analysis, and interpretation of data; the writing of the manuscript, or the decision to submit the article for publication. This work was generated during previous employment of JX and SP, and not during their current employment.

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