Journal of Alzheimer's Disease xx (20xx) x–xx
DOI 10.3233/JAD-131715
IOS Press

Mitochondrial DNA Copy Numbers in Pyramidal Neurons are Decreased and Mitochondrial Biogenesis Transcriptome Signaling is Disrupted in Alzheimer's Disease Hippocampi

Ann C. Rice[a,b,*], Paula M. Keeney[a], Norah K. Algarzae[a], Amy C. Ladd[a], Ravindar R. Thomas[a] and James P. Bennett Jr.[a,b,c,d]
[a]*Parkinson's Disease Center, Virginia Commonwealth University, Richmond, VA, USA*
[b]*Department of Neurology, Virginia Commonwealth University, Richmond, VA, USA*
[c]*Department of Psychiatry, Virginia Commonwealth University, Richmond, VA, USA*
[d]*Department of Physiology and Biophysics, Virginia Commonwealth University, Richmond, VA, USA*

Accepted 24 November 2013

Abstract. Alzheimer's disease (AD) is the major cause of adult-onset dementia and is characterized in its pre-diagnostic stage by reduced cerebral cortical glucose metabolism and in later stages by reduced cortical oxygen uptake, implying reduced mitochondrial respiration. Using quantitative PCR we determined the mitochondrial DNA (mtDNA) gene copy numbers from multiple groups of 15 or 20 pyramidal neurons, GFAP (+) astrocytes and dentate granule neurons isolated using laser capture microdissection, and the relative expression of mitochondrial biogenesis (mitobiogenesis) genes in hippocampi from 10 AD and 9 control (CTL) cases. AD pyramidal but not dentate granule neurons had significantly reduced mtDNA copy numbers compared to CTL neurons. Pyramidal neuron mtDNA copy numbers in CTL, but not AD, positively correlated with cDNA levels of multiple mitobiogenesis genes. In CTL, but not in AD, hippocampal cDNA levels of PGC1α were positively correlated with multiple downstream mitobiogenesis factors. Mitochondrial DNA copy numbers in pyramidal neurons did not correlate with hippocampal $A\beta_{1\text{-}42}$ levels. After 48 h exposure of H9 human neural stem cells to the neurotoxic fragment $A\beta_{25\text{-}35}$, mtDNA copy numbers were not significantly altered. In summary, AD postmortem hippocampal pyramidal neurons have reduced mtDNA copy numbers. Mitochondrial biogenesis pathway signaling relationships are disrupted in AD, but are mostly preserved in CTL. Our findings implicate complex alterations of mitochondria-host cell relationships in AD.

Keywords: Laser capture microdissection, neural stem cells, PGC1 alpha, real-time PCR, TFAM

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease of adults and the major cause of age-related dementia. Memory loss first appears in the disorder known as "mild cognitive impairment" (amnestic MCI) that progresses into AD dementia at a rate of ∼15% per year. Biomarkers of MCI include reduced cerebral glucose accumulation with preserved oxygen uptake and increased brain tissue markers of oxidative stress [1, 2]. Progression into AD dementia is associated with further reductions of cortical glucose accumulation and reduced brain oxygen consumption

*Correspondence to: Ann C. Rice, Ph.D., Parkinson's Disease Center, Virginia Commonwealth University, PO Box 980599, Richmond, VA 23298-0599, USA. Tel.: +1 804 828 9664; Fax: +1 804 828 6373; E-mail: acrice@vcu.edu.

[1, 2]. These observations suggest that the AD brain may be "starving" metabolically [3].

Studies of postmortem tissue support impaired bioenergetic metabolism in AD that may play a role in the degenerative loss of hippocampal and cortical neurons. There is loss of activities of decarboxylase TCA enzyme complexes [2, 4–6] and reduced mitochondrial respiration, mitochondrial mass, and expression of mitochondrial biogenesis (mitobiogenesis) genes in postmortem AD brain tissue [5, 7]. Individual AD brain neurons have impaired cytochrome oxidase activity histochemically [6], are populated by deleted mitochondrial DNA (mtDNA) molecules [8], and have reduced expression of nuclear encoded respiratory genes [9]. Loss of cerebral glucose accumulation in MCI and later in AD dementia and direct indicators of impaired mitochondrial function in AD brains could occur from lowered glucose transport, impairment of glycolysis, reduced activities of mitochondrial oxidative decarboxylation in the TCA cycle, impaired mitochondrial respiration, or combinations of these processes.

Mitochondrial respiration involves many proteins encoded both from nuclear and mitochondrial DNA. A complex regulatory system ensures an appropriate coordinated expression from both genomes for both basal respiratory levels or stress-induced upregulation (for review, see [10–13]). Nuclear respiratory (transcription) factors 1 and 2 (NRF1 and NRF2) activate transcription of nuclear-encoded respiratory genes and transcription factor A from mitochondria (TFAM). TFAM initiates transcription of the mitochondrial-encoded respiratory genes and has a role in maintaining the mtDNA copy number and stabilizing the mtDNA. Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) is a coactivator for many mitobiogenesis-associated transcription factors including NRF1, NRF2, and estrogen-related receptor α (ERRα, associated with fatty acid oxidation). PGC1α plays a key role in responding to environmental changes, through both post-translational modifications and increased expression, to turn on mitobiogenesis [14]. Although the exact mechanism of how PGC1α upregulates expression of the nuclear transcription factors is not clear, increased expression of NRF1 and NRF2 and subsequent increased expression of TFAM are observed when PGC1α is overexpressed [15]. Additionally, PGC1α does bind with NRF1, NRF2, and ERRα and co-activates expression of their target genes resulting in significantly increased mitobiogenesis resulting in the label of the "master regulator".

The present study examines in more detail the status of mtDNA and the expression levels of select mitobiogenesis genes from postmortem AD and control (CTL) tissue. We used laser capture microdissection (LCM) to isolate hippocampal pyramidal neurons, GFAP(+) glia and dentate granule neurons and quantitative real-time multiplex PCR (qPCR) to demonstrate decreased mtDNA copy number in pyramidal neurons from AD compared to CTL cases. We show a loss of correlation of mitobiogenesis factors cDNAs in whole hippocampal tissue compared to mtDNA copy numbers in AD pyramidal neurons, which is preserved in CTL cases. In CTL hippocampal tissue, PGC1α expression levels significantly correlate or trend with expression of other mitobiogenesis genes, but this correlation is lost in AD tissue. Total hippocampal amyloid-β $(A\beta)_{1\text{-}42}$ peptide levels from postmortem tissue did not correlate with mtDNA copy numbers in any cell type in AD. Additionally, we could not induce the mtDNA copy number loss by exposing human H9 neural stem cells to the neurotoxic $A\beta_{25\text{-}35}$ fragment.

METHODS

Tissue/Samples

Human brain tissue was obtained following autopsy and flash frozen. Hippocampi were obtained from the Brain Resource Facility at the University of Virginia. Cases consisted of 10 AD by clinical and pathological diagnosis (mean age: 79.9 y; mean postmortem interval: 6 h; 6 female, 4 male) and 9 CTL clinically considered normal with no pathological abnormalities (mean age: 63.2 y; mean postmortem interval: 8.45 h; 6 female, 3 male). Tissue was embedded in Cryostat mounting media and sliced at 10 μm. Ten to twelve slices were processed for RNA extraction using the miRNeasy kit from Qiagen following the manufacturer's instructions. RNA integrity was evaluated using the BioRad Experion capillary electrophoresis system. RNA quality index (RQI) values were between 6.7 and 9.5 [16]. Representative electropherograms are presented in Supplementary Figure. 1. The RNA was converted to cDNA using BioRad i-script cDNA synthesis kit following the manufacturer's instructions. These samples are referred to as whole tissue cDNA. Approximately 15 slices were processed for protein isolation for sandwich ELISA for $A\beta_{1\text{-}42}$ using the kit from Invitrogen and following the manufacturer's instructions. Additional slices were melted on uncoated slides for laser capture microdissection of hippocampal pyramidal neurons, glia and dentate granule cells.

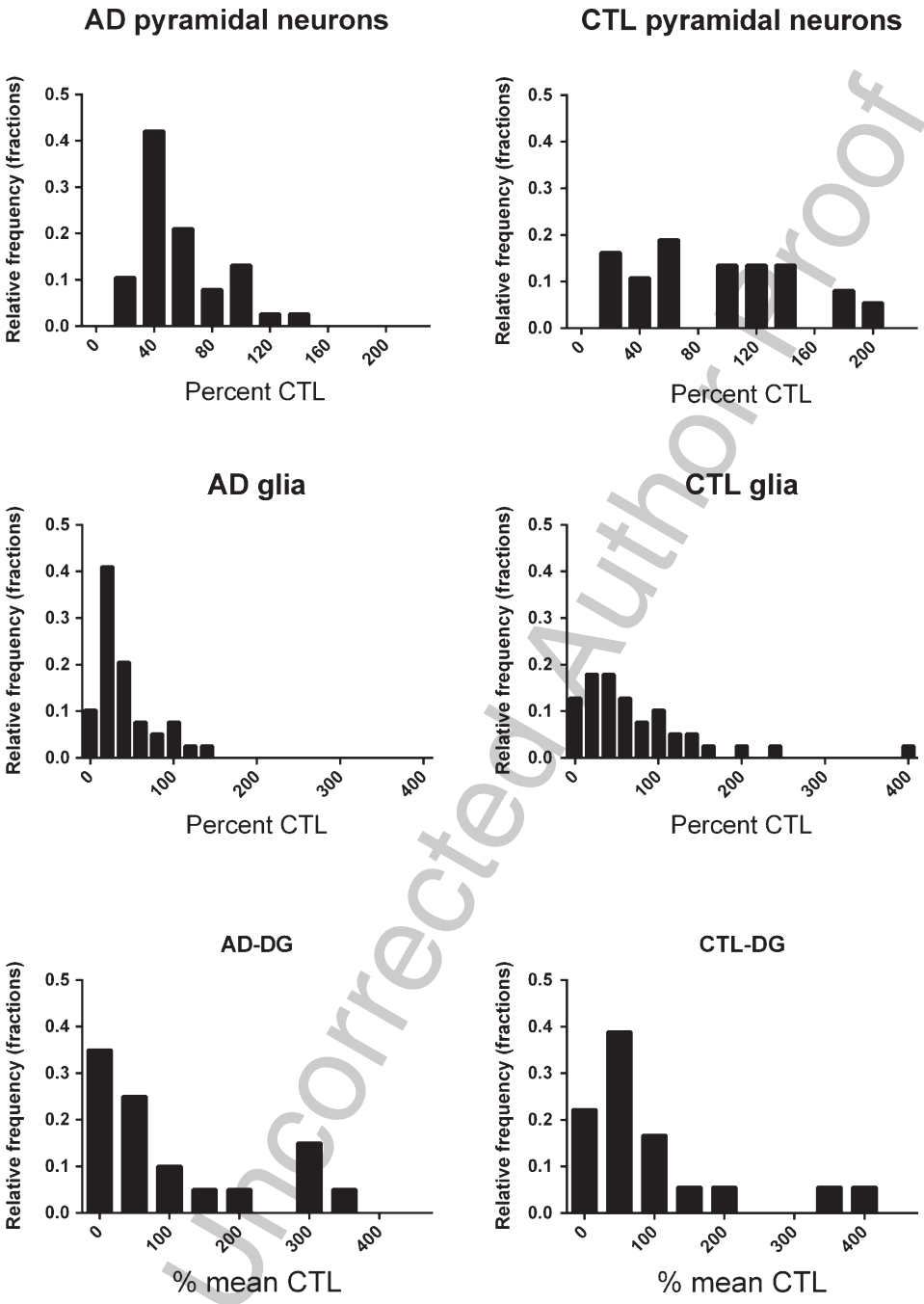

Fig. 1. Mitochondrial DNA copy numbers in genomic DNA isolated from groups of 20 pyramidal neurons, 20 GFAP(+) astrocytes, or 15 dentate gyrus granule cells from AD or CTL hippocampal sections. Shown are histogram distributions of mtDNA copy numbers (average of gene copy number of 12 S rRNA, ND2, COX III, and ND4) expressed as a % of mean CTL mtDNA copy number levels. Approximately 4 caps of 15–20 cells each were isolated/case.

Laser capture microdissection

Slices were fixed in 70% ethanol, hydrated, stained with methylene blue, dehydrated, and cleared in xylene for identifying and capturing hippocampal pyramidal neurons and dentate gyrus granule cells. Hippocampal glia were identified immunohistochemically using anti-GFAP (Millipore AB5804) at 1 : 100 followed by

Alexa-488-anti-rabbit at 1 : 100 for LCM (1 h each at room temperature). The Arcturus XT system was used for capturing specific cell types.

Quantitative real-time PCR

A BioRad CFX96 thermocycler was used for all qPCR experiments with BioRad power mix according to manufacturer's recommendations for each type of experiment. All samples were analyzed in triplicate.

mtDNA copy number from specific cell types (as done previously by our group [17])

From pilot studies we determined that capturing 20 cells/cap (pyramidal neurons and GFAP + glia) or 15 cells/cap (dentate gyrus granule cells) and four caps/case we could minimize the variability that occurs due to the 10 μm slice being in different planes of each cell (Supplementary Figure. 2). Caps were extracted overnight at 65°C with 50 μl tris buffered proteinase K for DNA extraction [17]. The proteinase K was heat inactivated and 200 μl TE was added. Multiplex qPCR was used to determine gene copy number for four genes around the mitochondrial genome (12 S rRNA, ND2, ND4, and COX III) from LCM isolated cells. The mtDNA copy number was determined/cap of 15 or 20 cells by comparing to a standard curve of circular human mtDNA run on the same plate. The mtDNA standards were prepared as described previously [18]. Since there was minimal variability between the four genes, the average of the four genes was used to estimate mtDNA copy number/LCM cap. Primer and probe sequences are listed in the Supplementary Table 1.

Relative gene expression from whole hippocampal tissue

Whole tissue cDNA was analyzed for expression of nuclear encoded mitobiogenesis genes. Relative expression of PGC-1α, NRF1, NRF2, TFAM, and ERRα were determined using Sofast EvaGreen (Bio-Rad) qPCR and primers we designed using Beacon designer software. A panel of six reference genes was assessed using Sofast EvaGreen qPCR to determine three with the least variability across all cases. GAPDH, 14-3-3z, and CYC-1 were selected with GeNorm analysis in qbase PLUS (Biogazelle) and their geometric means in each cDNA sample were used to normalize relative expression of the mitobiogenesis regulatory genes. All EvaGreen qPCR experiments included a human fetal brain cDNA standard curve for assessing relative expression of each gene. Melt curves were run with each qPCR to ensure only a single species was being amplified. Specific primer sequences used are in the Supplementary Table.

$A\beta_{1\text{-}42}$ ELISA

Slices were dissolved in 5 M guanidine, 50 mM Tris (pH 8.0) plus protease inhibitor cocktail (Calbiochem, set III). Protein concentrations were determined using the Pierce BCA protein assay with BSA as the standard. Samples were diluted with 0.1 M phosphate (pH 7.4) to achieve 1 mg/ml. Sandwich ELISAs were analyzed using 50 μl of each sample in duplicate following the manufacturer's protocol (Invitrogen). Most AD cases were much higher than the CTLs, so they were re-analyzed shortening the time of the horseradish peroxidase visualization step so the high end of the standard curve and those samples had absorbance values within the detection limits of the spectrophotometer.

Cell culture H9 neural stem cells

Human neural stem cells (H9-derived) (H9 NSCs) were purchased from Gibco (Life Technologies) and cultured according to the supplier's instructions. Briefly, cells were grown in Knock Out DMEM/F12 with 2 mM GlutaMax-I, 20 ng/ml basic Fibroblast Growth Factor, 20 ng/ml Epidermal Growth Factor, and 2% StemPro Neural Supplement in CellStart coated vessels. All medium components were purchased from Life Technologies. Medium was changed every 2 to 3 days and cells were kept at 37°C in a humidified CO_2 incubator. H9 NSC cultures were maintained between 50 and 90% confluence. $A\beta_{25\text{-}35}$ and $A\beta_{35\text{-}25}$ (Bachem, Torrance, CA) were diluted to 2 mM in water and aliquots stored frozen. 72 h before treatment, aliquots were placed at 37°C to aggregate [19]. 35 mm dishes of 90% confluent H9 NSCs were treated with 10 μM aggregated $A\beta_{25\text{-}35}$ or $A\beta_{35\text{-}25}$ in growth medium. After 48 h cells were lifted with Accutase, washed, and each pellet sonicated in 350 μl RLT Plus (Qiagen) with 1% 2-mercaptoethanol. DNA and RNA were isolated from triplicate samples for each treatment using an AllPrep DNA/RNA Mini Kit (Qiagen). Genomic DNA was quantified using a NanoDrop 2000c.

Statistical analysis

All analyses were performed using the statistical software by GraphPad Prism.

Frequency distribution of mtDNA copy numbers

Frequency distribution of mtDNA copy numbers determined for each LCM cap from the qPCR experiments were divided by the average mtDNA copy number/cap of the control cases for each cell type. A frequency distribution of each cap as a percent of CTL was analyzed using Kolmogorov-Smirnov test for comparing cumulative distributions after outliers ($\pm 2 \times$SD) were excluded. Between AD and CTL for each cell type a Mann Whitney non-parametric *t*-test was used to obtain *p*-values. $p < 0.0167$ was considered statistically significant.

Correlation analyses

Correlation analyses were performed between relative expression of the mitobiogenesis genes as determined by obtaining the relative expression of each gene normalized to the average of the three reference genes used for each case. A non-parametric Kruskal-Wallis analysis was performed to assess the correlation between PGC1α and the other mitobiogenesis genes from AD versus CTL cases, between the average mtDNA copy number/case from hippocampal pyramidal neurons and the normalized relative expression of the mitobiogenesis genes and between the $A\beta_{42}$ levels/case and the average mtDNA copy numbers/case.

RESULTS

mtDNA gene content is reduced in AD hippocampal pyramidal neurons

To determine if the mtDNA copy number was reduced in hippocampal pyramidal neurons, GFAP(+) astrocytes or dentate gyrus granule cells in AD cases compared to CTL cases, we isolated specific cell types using LCM and analyzed for mtDNA gene copy number for four genes around the mitochondrial genome (12 S rRNA, ND2, ND4, COIII; see Methods). Ratios of mtDNA protein coding genes to 12 S rRNA were all ~1.0 (Supplementary Figure. 3), did not suggest any significant level of mtDNA deletions in our populations and demonstrated that we were only assessing DNA (not RNA) from our extraction. The mtDNA copy number reported is the average copy number of these four mitochondrial genes/LCM cap. Mitochondrial DNA copy number values were divided by the mean of the CTL cases for each cell type and presented as a percentage of mean CTL values. Figure 1 depicts a histogram of the fraction frequency (in bins of 20) of mtDNA copy number for each LCM cap as the percentage of the mean of the CTL group for each cell type. The AD pyramidal neurons (Fig. 1A) are shifted to a larger proportion being less than 100% compared to CTL (Fig. 1B) indicating significantly decreased mtDNA copy numbers in hippocampal pyramidal neurons from AD cases ($p = 0.0086$, non-parametric Kolmogorov-Smirnov test). Mitochondrial DNA copy numbers in glia from AD (Fig. 1C) were not statistically significantly different from CTL (Fig. 1D), although there was a trend toward significance ($p = 0.0899$, non-parametric Kolmogorov-Smirnov test). Mitochondrial DNA copy numbers in dentate granule cells were not significantly different in AD compared to CTL (Fig. 1 E, F). A *t*-test of the raw values of the mtDNA copy numbers from hippocampal pyramidal neurons also indicated a statistically significant decrease of 17% ($p = 0.0475$) in AD compared to CTL (data not shown).

mtDNA copy number in CTL but not in AD pyramidal neurons correlates with cDNA levels of mitobiogenesis factors NRF2, ERRα, NRF1, and TFAM

To determine if the decreased mtDNA copy numbers in AD pyramidal neurons were related to the relative expression of the mitobiogenesis genes, we extracted total RNA from frozen sections of the same tissue block used for the specific cell isolation and generated cDNA using random hexamer priming. The relative expression of each gene was normalized to reference genes with the least variability across all samples (See Methods). Figure 2 shows the relatively linear relationships among levels of pyramidal neuron mtDNA gene copy numbers (average/case) and the reference-gene normalized expressions of the mitobiogenesis factors NRF2, NRF1, ERRα, and TFAM in CTL (Fig. 2B, D, F, H), but not AD pyramidal neurons (Fig. 2 A, C, E, G). PGC1α showed a trend toward a linear correlation, but did not reach statistical significance (Supplementary Figure. 4). These experiments indicate that in postmortem hippocampal tissue from CTL cases the relationship between mitobiogenesis factors and mtDNA copy number in pyramidal neurons is preserved, whereas this relationship is lost in AD cases.

Mitobiogenesis signaling relationships are preserved in CTL but not AD hippocampi

To determine if the relationship between PGC1α, the "master" upstream co-activator of mitobiogenesis signaling factors, and expressions of NRF1, NRF2, TFAM, and ERRα were preserved in each case, we

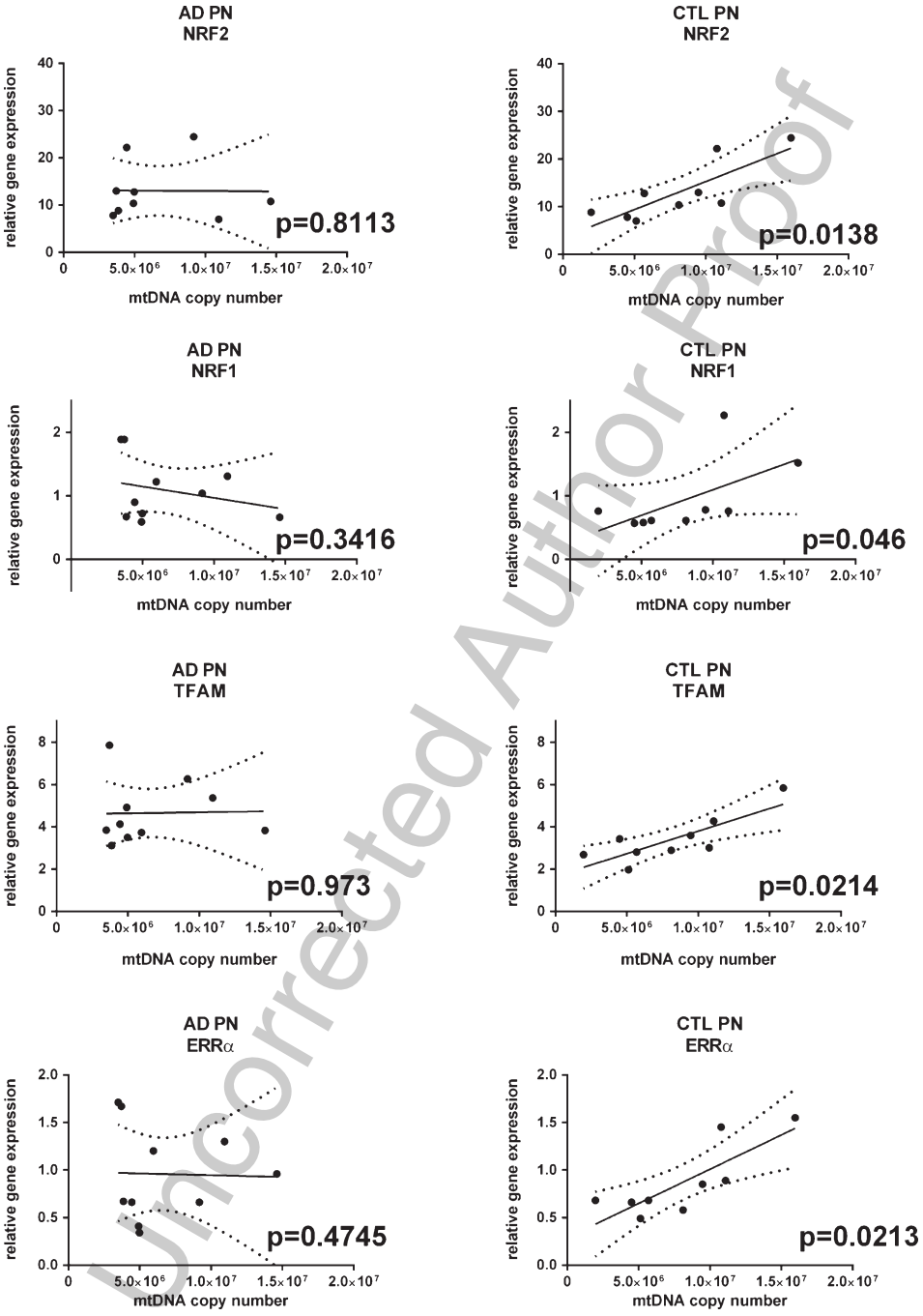

Fig. 2. Relationships among mtDNA copy numbers in genomic DNA isolated from groups of 20 hippocampal pyramidal neurons isolated from AD or CTL and expression of NRF2, NRF1, TFAM, and ERRα. cDNA in whole hippocampal tissue samples from same tissues. Linear regression lines and 95% confidence intervals are shown on each graph.

evaluated the correlation between the relative expression of PGC1α and each of the other genes/case. Figure 3 depicts the significant correlation between PGC 1α and NRF2 and ERRα in CTL cases (Fig. 3B & H, $p = 0.017$ and 0.021, respectively), but not in AD cases (Fig. 3A & G). NRF1 also showed a positive trend in CTL cases (Fig. 3D), but did not reach statistical significance ($p = 0.085$). In both AD and CTL, TFAM trended toward a correlation with PGC1α ($p \approx 0.2$). These experiments indicate the correlation between the

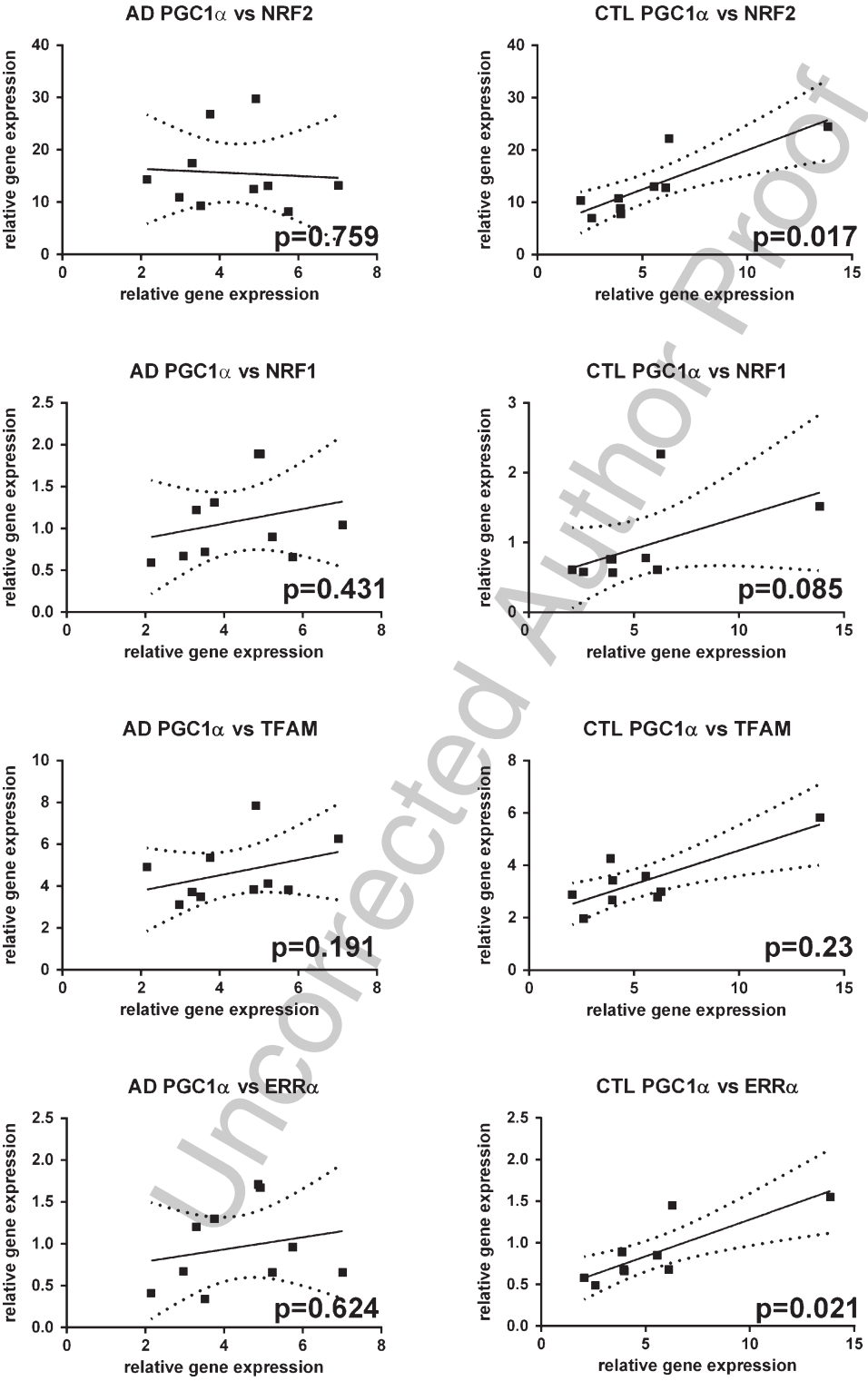

Fig. 3. Relationships among expression levels of PGC1α and the expression of other mitobiogenesis factors NRF 2, NRF 1, TFAM, and ERRα from AD or CTL hippocampal tissue samples. Linear regression lines and 95% confidence intervals are shown on each graph.

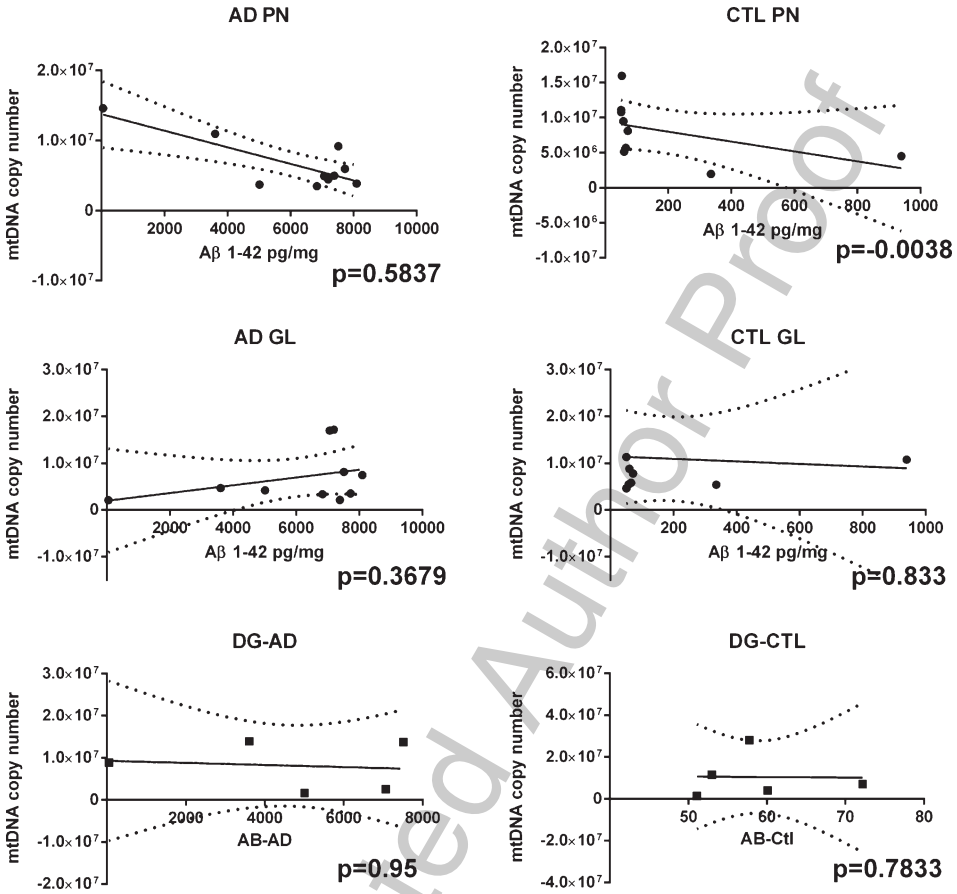

Fig. 4. Relationships between $A\beta_{1\text{-}42}$ peptide levels in hippocampal tissue and mtDNA copy numbers in pyramidal neurons, GFAP(+) astrocytes, and dentate gyrus granule cells from AD or CTL hippocampi. Linear regression lines and 95% confidence intervals are shown on each graph.

master regulator PGC 1α and the downstream mitobiogenesis factors in postmortem tissue is preserved in CTL cases, but lost in AD cases.

mtDNA copy number does not correlate to $A\beta_{1\text{-}42}$ levels

To determine if $A\beta_{1\text{-}42}$ levels were related to the decreased mtDNA copy numbers in AD pyramidal neurons, we prepared whole tissue homogenates from the same tissue blocks used to isolate specific cell types and analyzed for $A\beta_{1\text{-}42}$ levels using a sandwich ELISA. Figure 4 shows the lack of correlation of mtDNA gene levels in isolated pyramidal neurons, GFAP (+) astrocytes and dentate gyrus granule cells compared to $A\beta_{1\text{-}42}$ levels in AD. Interestingly, CTL hippocampal $A\beta_{1\text{-}42}$ levels positively correlated with the pyramidal neuron mtDNA copy number from each case ($p = 0.0038$; two cases had moderate $A\beta_{1\text{-}42}$ levels). This correlation must be viewed in the context that it is being driven by the two CTL cases with moderately elevated $A\beta_{1\text{-}42}$ levels. Additional correlations of $A\beta_{1\text{-}42}$ levels with mitobiogenesis gene expression levels were also not significant in AD cases. However CTL cases showed significant correlations with $A\beta_{1\text{-}42}$ and NRF1 and ERRα and close to significance ($p = 0.055$) for NRF2 (Supplementary Figure. 5). The lack of correlations in the AD cases may be skewed by the one AD case with low $A\beta_{1\text{-}42}$ levels.

Exposure of human H9 neural precursor cells to neurotoxic $A\beta_{25\text{-}35}$ does not reduce mtDNA gene copy number

To determine if we could induce the decreased mtDNA copy number we observed in postmortem AD tissue, we attempted to replicate in a non-tumor, human neural stem cell model the loss of mtDNA copy number observed in LCM-isolated AD hippocampal pyramidal neurons. Human H9 neural stem cells (derived from a

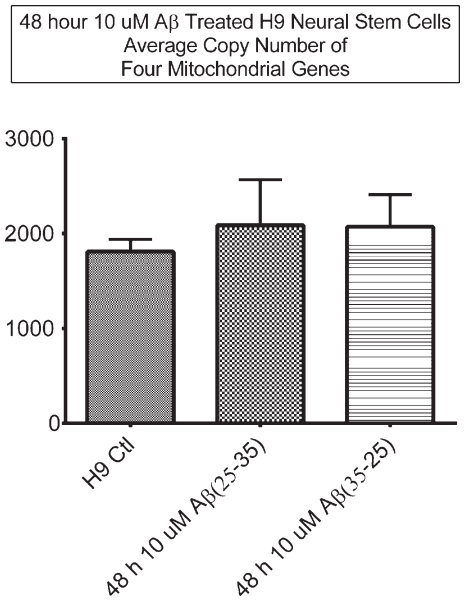

Fig. 5. Effect of 48 h of treatment of human neural precursor cells with vehicle, 10 μM $A\beta_{25\text{-}35}$, or 10μM $A\beta_{35\text{-}25}$ on mtDNA copy number. Values are ± SEM.

hESC line) were exposed to 10 μM neurotoxic $A\beta_{25\text{-}35}$ fragment for 48 h and ∼50% loss of cells was observed. Figure 5 shows that this treatment did not significantly alter mtDNA copy numbers when compared to vehicle treated cells or cells treated with the inverse sequence $A\beta_{35\text{-}25}$.

DISCUSSION

In our postmortem AD hippocampi we found that mtDNA gene copy numbers were significantly reduced in surviving pyramidal neurons compared to identical cell types in CTL hippocampi. This is consistent with the observation of reduced mtDNA copy numbers in whole hippocampi [7] and cortex [20] from AD cases. However, Hirai et al. [21] using *in situ* hybridization detected increased mtDNA in hippocampal pyramidal neurons from AD cases compared to CTL; from EM photos they determined many of these were in lipofuscin vacuoles and are likely from degrading mitochondria. Additionally, they did not detect mtDNA differences in the glia or granule cells by *in situ* hybridization, which is consistent with our observations in those cell types. They also comment that using PCR techniques, they did not detect an overall difference in mtDNA in AD compared to control tissue. Our cells were isolated by laser capture and only intact cells were selected, which could explain the difference between our study and their study. Another factor that could be contributing to the decreased mtDNA copy number in AD in our study is that the control cases were younger (∼16 y) than the AD cases. Our group has previously demonstrated dramatically decreased (∼90% loss) mtDNA copy numbers in whole brain tissue from 21 month old mice compared to 5 month old mice [22]. Since our samples were isolated from selected intact cells, we would not expect as dramatic a difference due to age as is observed in whole tissue studies. A comparison of mtDNA copy number/LCM cap or mitobiogenesis genes versus age revealed no correlation in either AD cases or CTL cases in our cohort of samples (Supplementary Figure. 6). Additionally, unknown factors about the individual's pre-mortem status, such as medications they may have been taking, could also have affected the mtDNA copy number values we determined.

Recently, cell-free mtDNA copy numbers in cerebrospinal fluid (CSF) from AD subjects, asymptomatic at risk subjects, and pre-symptomatic subjects with a pathogenic PSEN1 mutation have been shown to be markedly reduced compared to age-matched control subjects, non-pathogenic PSEN1 mutation family members, or individuals with a different dementia pathology (FTLD). These findings indicate that the reduced mtDNA copy numbers both occur prior to clinical symptoms appearing in AD and appear to be selective for AD dementia [23]. The same authors reported that levels of mtDNA were reduced in cortical neurons derived from a transgenic (AβPP/PS1) mouse model of familial AD. They conclude that reduced CSF mtDNA levels appear to be a robust biomarker for pre-AD dementia. They also suggest that the significant loss of mtDNA copy numbers we observed in hippocampal pyramidal neurons and nearly significant loss of mtDNA in GFAP(+) astrocytes have clinical correlates in CSF and may represent early pathogenic events in AD.

Previous studies from our group have demonstrated decreased mRNA expression of mitochondrial encoded genes in cortical AD tissue homogenates from the same tissue sources used in the present study [5]. Decreased mRNA expression of many of the mitobiogenesis genes (PGC1α, NRF1, TFAM, POLγ, and PPRC1) were also detected in the cortical AD tissue homogenates compared to CTL [5]. In hippocampal tissue homogenates from AD cases, Sheng et al. [7] observed decreased mitobiogenesis protein levels compared to control tissue (PGC1α, NRF1, NRF2α, NRF2β, and TFAM). Subsequently, when they stably knocked down PGC1α in a cell culture line, they

observed the expected decreases in protein levels as well as reduced mtDNA levels relative to nuclear DNA levels by about 50% [7], supporting the regulatory hierarchy of PGC1α upregulating NRF1 and NRF2 leading to increased levels of TFAM and subsequently increased mtDNA levels. Our decrease in mtDNA copy number in AD cases was not quantitated the same way as Sheng et al. [7] and our decreased AD pyramidal neuron mtDNA copy number was not as dramatic as in their cell culture. Our results, obtained from laser-captured, postmortem AD pyramidal neurons complement those Sheng et al. [7] obtained in cell culture.

We also observed a trend toward decreased mtDNA copy number in laser-captured GFAP(+) glia. Even though it only approached statistical significance, it does demonstrate that the glia, while not thought to be affected in AD, may in fact also be metabolically impaired and contribute to the deficits other groups observe in whole tissue homogenates. Additionally, we observed no difference in mtDNA copy number in dentate gyrus granule cells, suggesting that they may not be bioenergetically altered in AD, and adding cell specificity to our findings in pyramidal neurons. However, we cannot confirm bioenergetic status in individual postmortem cells isolated by LCM. Additionally, since we only selected healthy appearing cells, we cannot speculate what may be occurring in unhealthy or dying cells or what may have led to the death of cells no longer available for sampling.

We uncovered a more generalized defect in AD hippocampi, that of a dysregulation of mitochondrial biogenesis signaling. Using pyramidal neuron mtDNA copy number as an indicator of mitobiogenesis, we found in CTL hippocampi generally linear relationships between expression of upstream mitobiogenesis genes (NRF2, NRF1, TFAM, and ERRα) and mtDNA gene copy numbers. The relationship between CTL pyramidal neuron mtDNA copy numbers and PGC1α expression levels trended toward significance. Since PGC1α activity can also be modulated through post-translational modification, its mRNA levels represent only one level of regulation (transcriptional) that may have limited correlation with the expression levels of the other mitobiogenesis genes or with the mtDNA copy numbers. These data indicate that in control tissue from autopsy cases the hierarchy of mitobiogenesis regulation appears to be intact. However, in AD tissue this correlation was missing, supporting the idea of impaired mitobiogenesis in AD as purported by others [5, 7]. Microarray analysis of LCM isolated hippocampal pyramidal neurons from AD cases has shown decreased expression of nuclear encoded respiratory genes, which is also consistent with impaired mitobiogenesis [9].

We also found in CTL, but not AD, hippocampal cDNA significant linear relationships among expression of the "master" mitobiogenesis regulator PGC1α and multiple downstream mitobiogenesis factors it co-activates (NRF2 and ERRα) and a trend toward significance in others (NRF1) [11, 12]. Due to the heterogeneity of human samples, had our n values been larger, we may have achieved better correlations between all the mitobiogenesis genes expression levels. These studies also support an intact mitobiogenesis hierarchy in CTL autopsy tissue that is lost in AD tissue. Future studies will examine this relationship specifically in pyramidal neurons.

A significant correlation between PGC1α and two key metabolism related proteins (pyruvate dehydrogenase A1 and complex III subunit UQCRH) has been demonstrated in skeletal muscle biopsies from diabetics (type II), and those with and without a family history of diabetes mellitus [24]. In a model of muscle disuse atrophy, Cassano and colleagues [25] demonstrated the expression level of PGC1α significantly correlated with both NRF1 and TFAM levels in the muscle atrophy acetyl-L-carnitine–treated animals, but only with TFAM in the non-treated control group. Thus, mitobiogenesis factors seem to maintain their hierarchal correlation in other diseased tissues, while in AD hippocampal tissue it does not.

The origins of this substantial dysregulation of mitobiogenesis signaling in AD are not clear from our experiments, likely are multifactorial and may have different molecular origins among the AD cases. PGC1α mRNA and protein have been reported to be decreased in AD brain, suggesting that dysfunction of mitobiogenesis may be the origin of mitochondrial dysfunction observed by several groups [5, 7]. Speculation as to potential mechanisms of mitobiogenesis disruption would incorporate many potential etiologies and is beyond the scope of this discussion. Future studies will investigate the potential role epigenetic regulation may be playing.

While some studies correlate $A\beta_{1\text{-}42}$ levels with AD pathology, we found no correlation of $A\beta_{1\text{-}42}$ levels to the mtDNA copy number reductions in hippocampal pyramidal neurons, glia, or dentate granule cells from AD cases, which is similar to what our group found previously in cortical tissue homogenates [5]. Our limited experiments with exposure of H9 neural stem cells to $A\beta_{25\text{-}35}$ peptide do not support a primary role for Aβ peptide mediating loss of mtDNA

gene copy numbers acutely. However, the limitations of this experiment include using a more neurotoxic fragment and not $A\beta_{1\text{-}42}$ peptide itself, and short incubation times that were necessary given the degree of cell death produced in 48 h by $A\beta_{25\text{-}35}$ peptide. Longer term exposure of human neurons to $A\beta_{1\text{-}42}$ peptide, particularly oligomers at low concentrations, will help test the hypothesis that $A\beta_{1\text{-}42}$ peptide is a pathogenic factor in loss of mtDNA genes. Future studies will examine this relationship in these human neural stem cells after they have been differentiated into neurons.

Our findings show that maintenance of normal mtDNA gene levels within individual pyramidal neurons or astrocytes is defective in AD, and that mitobiogenesis signaling at the transcriptome level is preserved in CTL but not AD hippocampi. Whether mitobiogenesis signaling is also disrupted in vulnerable AD pyramidal neurons remains to be studied by LCM approaches. Because postmortem studies can only find correlations and not test causal mechanisms, our findings support but do not prove the concept that abnormal mitobiogenesis signaling and disrupted downstream responses contribute to metabolic deficiencies observed in AD and may be primarily pathogenic. Furthermore, our cohort of samples is small and displays considerable variability, therefore we caution against generalizing our results to the sizable population of AD sufferers.

ACKNOWLEDGMENTS

This research was supported by the Parkinson's and Movement Disorders Center at Virginia Commonwealth University through the Medical College of Virginia Foundation.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=2030).

SUPPLEMENTARY MATERIAL

Supplementary tables and figures are available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-131715.

REFERENCES

[1] Mosconi L, Mistur R, Switalski R, Tsui WH, Glodzik L, Li Y, Pirraglia E, De Santi S, Reisberg B, Wisniewski T, de Leon MJ (2009) FDG-PET changes in brain glucose metabolism from normal cognition to pathologically verified Alzheimer's disease. *Eur J Nucl Med Mol Imaging* **36**, 811-822.

[2] Gibson GE, Shi Q (2010) A mitocentric view of Alzheimer's disease suggests multi-faceted treatments. *J Alzheimers Dis* **20**(Suppl 2), S591-S607.

[3] Mamelak M (2012) Sporadic Alzheimer's disease: The starving brain. *J Alzheimers Dis* **31**, 459-474.

[4] Manczak M, Park BS, Jung Y, Reddy PH (2004) Differential expression of oxidative phosphorylation genes in patients with Alzheimer's disease: Implications for early mitochondrial dysfunction and oxidative damage. *Neuromolecular Med* **5**, 147-162.

[5] Young-Collier KJ, McArdle M, Bennett JP (2012) The dying of the light: Mitochondrial failure in Alzheimer's disease. *J Alzheimers Dis* **28**, 771-781.

[6] Cottrell DA, Borthwick GM, Johnson MA, Ince PG, Turnbull DM (2002) The role of cytochrome c oxidase deficient hippocampal neurones in Alzheimer's disease. *Neuropathol Appl Neurobiol* **28**, 390-396.

[7] Sheng B, Wang X, Su B, Lee HG, Casadesus G, Perry G, Zhu X (2012) Impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in Alzheimer's disease. *J Neurochem* **120**, 419-429.

[8] Krishnan KJ, Ratnaike TE, De Gruyter HL, Jaros E, Turnbull DM (2012) Mitochondrial DNA deletions cause the biochemical defect observed in Alzheimer's disease. *Neurobiol Aging* **33**, 2210-2214.

[9] Liang WS, Reiman EM, Valla J, Dunckley T, Beach TG, Grover A, Niedzielko TL, Schneider LE, Mastroeni D, Caselli R, Kukull W, Morris JC, Hulette CM, Schmechel D, Rogers J, Stephan DA (2008) Alzheimer's disease is associated with reduced expression of energy metabolism genes in posterior cingulate neurons. *Proc Natl Acad Sci U S A* **105**, 4441-4446.

[10] Scarpulla RC (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiol Rev* **88**, 611-638.

[11] Scarpulla RC (2011) Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. *Biochim Biophys Acta* **1813**, 1269-1278.

[12] Scarpulla RC, Vega RB, Kelly DP (2012) Transcriptional integration of mitochondrial biogenesis. *Trends Endocrinol Metab* **23**, 459-466.

[13] Hock MB, Kralli A (2009) Transcriptional control of mitochondrial biogenesis and function. *Annu Rev Physiol* **71**, 177-203.

[14] Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98**, 115-124.

[15] Wareski P, Vaarmann A, Choubey V, Safiulina D, Liiv J, Kuum M, Kaasik A (2009) PGC-1{alpha} and PGC-1{beta} regulate mitochondrial density in neurons. *J Biol Chem* **284**, 21379-21385.

[16] Fleige S, Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. *Mol Aspects Med* **27**, 126-139.

[17] Keeney PM, Bennett JP Jr. (2010) ALS spinal neurons show varied and reduced mtDNA gene copy numbers and increased mtDNA gene deletions. *Mol Neurodegener* **5**, 21.

[18] Keeney PM, Quigley CK, Dunham LD, Papageorge CM, Iyer S, Thomas RR, Schwarz KM, Trimmer PA, Khan SM, Portell FR, Bergquist KE, Bennett JP Jr. (2009) Mitochondrial gene therapy augments mitochondrial physiology in a Parkinson's disease cell model. *Hum Gene Ther* **20**, 897-907.

[19] Cho SO, Ban JY, Kim JY, Jeong HY, Lee IS, Song K-S, Bae K, Seong YH (2009) Aralia cordata protects against amyloid

β protein (25–35)–induced neurotoxicity in cultured neurons and has antidementia activities in mice. *J Pharmacol Sci* **111**, 22-32.

[20] Coskun PE, Wyrembak J, Derbereva O, Melkonian G, Doran E, Lott IT, Head E, Cotman CW, Wallace DC (2010) Systemic mitochondrial dysfunction and the etiology of Alzheimer's disease and down syndrome dementia. *J Alzheimers Dis* **20**(Suppl 2), S293-S310.

[21] Hirai K, Aliev G, Nunomura A, Fujioka H, Russell RL, Atwood CS, Johnson AB, Kress Y, Vinters HV, Tabaton M, Shimohama S, Cash AD, Siedlak SL, Harris PL, Jones PK, Petersen RB, Perry G, Smith MA (2001) Mitochondrial abnormalities in Alzheimer's disease. *J Neurosci* **21**, 3017-3023.

[22] Thomas RR, Khan SM, Smigrodzki RM, Onyango IG, Dennis J, Khan OM, Portelli FR, Bennett JP Jr. (2012) RhTFAM treatment stimulates mitochondrial oxidative metabolism and improves memory in aged mice. *Aging (Albany NY)* **4**, 620-635.

[23] Podlesniy P, Figueiro-Silva J, Llado A, Antonell A, Sanchez-Valle R, Alcolea D, Lleo A, Molinuevo JL, Serra N, Trullas R (2013) Low CSF concentration of mitochondrial DNA in preclinical Alzheimer's disease. *Ann Neurol* **74**, 655-668.

[24] Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A* **100**, 8466-8471.

[25] Cassano P, Sciancalepore AG, Pesce V, Fluck M, Hoppeler H, Calvani M, Mosconi L, Cantatore P, Gadaleta MN (2006) Acetyl-L-carnitine feeding to unloaded rats triggers in soleus muscle the coordinated expression of genes involved in mitochondrial biogenesis. *Biochim Biophys Acta* **1757**, 1421-1428.