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Reduced Posterior Cingulate Mitochondrial Activity in Expired Young Adult Carriers of the *APOE* ϵ 4 Allele, the Major Late-Onset Alzheimer's Susceptibility Gene

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Abstract

*In vivo*PET imaging studies of young-adult carriers of the apolipoprotein E ϵ 4 allele (*APOE* ϵ 4), the major Alzheimer's disease (AD) susceptibility gene, have demonstrated declines in glucose metabolism in brain areas later vulnerable to AD, such as posterior cingulate cortex, decades before the possible onset of symptoms. We have previously shown in postmortem studies that such metabolic declines in AD are associated with brain regional mitochondrial dysfunction. To determine whether young adult at-risk individuals demonstrate similar mitochondrial functional decline, we histochemically assessed postmortem tissues from the posterior cingulate cortex of young-adult carriers and noncarriers of *APOE* ϵ 4. At-risk ϵ 4 carriers had lower mitochondrial cytochrome oxidase activity than noncarriers in posterior cingulate cortex, particularly within the superficial cortical lamina, a pattern similar to that seen in AD patients. Except for one 34 year-old ϵ 4 homozygote, the ϵ 4 carriers did not have increased soluble amyloid- β , histologic amyloid- β , or tau pathology in this same region. This functional biomarker may prove useful in early detection and tracking of AD and indicates that mitochondrial mechanisms may contribute to the predisposition to AD before any evidence of amyloid or tau pathology.

Keywords

Alzheimer's etiology; bioenergetics; biomarkers; cytochrome c oxidase; differential vulnerability; neocortex

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Introduction

What are the earliest brain changes associated with predisposition to late-onset Alzheimer's disease (AD)? While it has been suggested that accumulation of histopathology precedes clinical onset by decades in people at risk for AD [1], brain imaging studies in young-adult carriers and non-carriers of the apolipoprotein E (*APOE*) $\epsilon 4$ allele suggest other early changes [2]. Alterations in imaging [3–8], cytochrome oxidase (CO) histochemistry [9], neuronal transcriptomic [10], and genetic association [11] studies have raised the possibility that mitochondrial processes may be involved in the pathogenic cascade.

Fluorodeoxyglucose positron emission tomography (FDG PET) studies have shown reduced cerebral metabolic rate for glucose (CMRglu) in posterior cingulate-precuneus (PCC), parieto-temporal and prefrontal regions in AD [2–8]. Our examination of PCC in postmortem AD cases revealed reduced mitochondrial CO activity [9], a measure of oxidative energy metabolism. These reductions were most pronounced in superficial cortical lamina and correlated with clinical disease duration. In another study, using laser-capture micro-dissected neurons from PCC and other AD-affected regions, we found widely reduced expression of nuclear genes encoding mitochondrial proteins, including CO, in AD [10], indicating mitochondrial changes may be secondary to other pathology. However, using FDG PET, we compared regional CMRglu in 18–40 year-old subjects (mean age 31 ± 5) with and without one copy of the *APOE* $\epsilon 4$ allele. Young-adult $\epsilon 4$ carriers had reduced CMRglu in PCC and other AD-affected regions, several decades before possible onset of dementia [2]. These early reductions appear to anticipate progressive CMRglu declines and some of the earliest fibrillar $A\beta$ deposition in later stages of AD [12], but it has not been established whether mitochondrial functional changes accompanied reductions in FDG PET and whether those alterations might be related to early $A\beta$ or tau pathology. Here, we demonstrate that young-adult *APOE* $\epsilon 4$ carriers do show significant mitochondrial CO declines in PCC. Like AD patients, these declines are most pronounced in the superficial cortical lamina, and they do not appear to be related to $A\beta$ or tau pathology.

Materials and Methods

Frozen cerebellar and separate frozen and formal-dehyde-fixed samples of PCC tissue were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD). Age at death, postmortem interval (PMI), gender, race, and cause of death (COD) were provided for each subject (Table 1); investigators were blind to this data and genotype. COD was further classified into unnatural (accident, suicide, homicide, toxicity) or natural (disease process) manner of death. DNA was isolated from frozen cerebellar tissue using the Qiagen Puregene DNA Isolation Kit and *APOE* genotyped [13].

Each sample was assessed for the presence of AD-associated histopathology by both ELISA and histology. Formalin-fixed tissue was cryoprotected in 15% sucrose in 0.1 M phosphate buffer, pH 7.2, and cut at 40 μ m. Free-floating sections were stained for $A\beta$ (6E10 clone, Chemicon) and phosphorylated tau (AT8 clone, Chemicon) as previously described [14]. Other sections were stained with Campbell-Switzer silver stain or thioflavine S. For ELISA analysis of $A\beta$ levels, PCC was prepared by homogenizing in 6 volumes 20 mM Trizma base, pH 7.8, with 5 mM EDTA and protease inhibitor cocktail (Roche). Homogenates were centrifuged at 540,000 $\times$ g for 20 min at 4°C. Initial supernatants were saved as soluble $A\beta$ fractions. Pellets were re-homogenized in 500 μ l of 5 M guanidine hydrochloride prepared in 50 mM Trizma base, pH 8.0. Homogenates were rotated for 3 h at 4°C and then centrifuged at 540,000 $\times$ g for 20 min at 4°C. These supernatants were collected as insoluble $A\beta$. Total protein/fraction was determined by BCA protein assay (Pierce). ELISA kits for

$A\beta_{1-40}$ and $A\beta_{1-42}$, obtained from Invitrogen and Innogenetics (high sensitivity method) respectively, were carried out following manufacturer instructions.

To assess laminar mitochondrial function, we stained the tissue histochemically for CO, Complex IV of the mitochondrial electron transport chain. It is important to emphasize that utilization of the stain in this way provides for the quantification of enzyme substrate turnover and not merely enzyme localization. For CO histochemistry and densitometric imaging, frozen PCC samples were sectioned at 40 μm perpendicular to the lamina, verified by Nissl staining. PCC localization was confirmed by cell typing and distribution, and sections were stored at -20°C . CO activity staining and densitometric imaging proceeded as previously described [9,15]. Sections from all subjects were included in a single staining batch to remove interbatch variability as a confound, and the batch included brain homogenate tissue standards of varying thicknesses (10–80 μm) prepared from mice to provide for the relative quantitation of CO activity. CO-stained sections were captured on a backlit fiber optic lightbox with a Photometrics Sensys high-resolution CCD camera and macro lens, digitized, and imported into Optimas Image Analysis software (Media Cybernetics). The light level was set and held constant across each session, and optical distortions were corrected by subtracting the background lightpath from each captured image. Nissl images were used to draw overlays delineating the 6 cortical layers, which were matched to fiduciary marks and surface features of CO-stained sections. Optical densities were averaged within each layer using eight square sampling windows of size dependent upon layer width, with their dimensions encompassing approximately three-quarters of each layer width; thusly, two sections from each subject were measured and averaged to provide laminar scores. All measurements were initially taken in OD units, converted subsequently to CO activity units based on the measurement of the tissue standards: a 40 μm -thick standard was assigned a CO activity value of 200 nmol cyt c oxidized/min/mg tissue, and assigned activity for each other tissue standard thickness was proportionally adjusted.

Primary statistical analysis proceeded using separate-variance t-tests, $\alpha = 0.05$, and utilizing a Bonferroni correction for multiple comparisons between the cortical laminae. Age and PMI were slightly but statistically significantly different (separate-variance t, no correction for multiple comparisons), and thus we performed independent analyses of covariance (ANCO-VA; Systat 9, SPSS, Inc.) across each layer, holding age and PMI constant, and also COD (natural vs. unnatural). Separate variance t-test P values were also calculated under two other conditions, independently: excluding an APOE $\epsilon 4$ homozygote with significant pathology and excluding a noncarrier with unusually high CO in Layer I (441 units). P values of the overall CO difference increased to 0.068 and 0.056, respectively; however, Bonferroni-corrected P values of CO differences within Layers I and II remained very similar to reported values and thus statistically significant, indicating that these cases were not driving the laminar differences.

Results

The 15 APOE $\epsilon 4$ carriers included 8 females, 7 males, 31.7 ± 6.9 (mean $\pm$ stdev) years old with PMI of 16.9 ± 4.3 h. The 25 $\epsilon 4$ non-carriers included 9 females, 16 males, were 25.4 ± 6.3 years old with PMI of 11.5 ± 4.3 h. With one notable exception, histologic $A\beta$ deposition, neurofibrillary tangles, or soluble $A\beta_{1-42}$ were not detected, and soluble and insoluble $A\beta_{1-40}$ and insoluble $A\beta_{1-42}$ were detected at similar levels in carriers and noncarriers (Table 2). The exceptional subject was a 34 year-old $\epsilon 4$ homozygote with frequent $A\beta$ -immunoreactive plaques, rare AT8-immunoreactive neurofibrillary tangles, and extremely high $A\beta_{1-42}$ levels (soluble: 141.4 pg/mg total protein; insoluble: 88.8×10^3 pg/mg total protein).

In contrast to histopathological measures, CO histochemistry showed significant differences between groups. Young-adult $\epsilon 4$ carriers had significantly lower PCC CO activity than non-carriers, and, in lamina-specific analyses, significantly lower CO activity in superficial layers I and II (Table 2). Lamina-specific differences were apparent even after excluding the above $\epsilon 4$ homozygote ($p = 0.017$ and $p = 0.009$, layers I and II respectively), and after individually controlling for carriers' slightly older age, slightly longer PMI, and attribution of natural/unnatural COD using ANCOVA ($p \leq 0.05$ for layers I-II correcting for age, layers I-III correcting for PMI or COD). Further, unbiased elimination of $\epsilon 4$ noncarriers with the lowest age (18–20 years) eliminated the significant effect of this variable ($p = 0.23$) while retaining highly significant differences in the laminar CO measures (Layer I: $p = 0.0059$; Layer II: $p = 0.0050$; Layer III: $p = 0.0342$); similarly, eliminating $\epsilon 4$ noncarriers with the lowest PMI (6–10 h) resulted in $p = 0.16$ while retaining significant CO differences (Layer I: $p = 0.0087$; Layer II: $p = 0.0014$, Layer III: $p = 0.0066$; separate-variance t, no correction for multiple comparisons). When a post hoc analysis was restricted to the comparison between the 19 young adults with the APOE $\epsilon 3/\epsilon 3$ genotype ($n = 19$) and the 13 young adults with the $\epsilon 3/\epsilon 4$ ($n = 13$) genotypes, the APOE $\epsilon 4$ carrier group continued to demonstrate significantly lower CO activity in layers I and II ($p = 0.007$ and $p = 0.008$, respectively).

Discussion

This study directly compared brain measurements in young adults at differential risk for late-onset AD in a brain region affected early. The laminar pattern of metabolic vulnerability is reminiscent of that seen in our study of AD patients [9]. Thus, functional mitochondrial changes may be the earliest indicator of AD-related risk detected to date, consistent with earlier brain imaging [4], histochemical [9], and neurotranscriptomic [10] studies in AD patients and tissues, as well as FDG PET imaging studies in similarly-aged young adults [2]. Considering that CO activity is primarily neuronal rather than glial [16], we demonstrate mitochondrial declines within PCC neurons themselves, prior to an increase in soluble or fibrillar $A\beta$ levels. However, the number of subjects assessed was relatively small, and we cannot exclude all potentially confounding effects on CO activity; additional studies in larger samples are needed to further address the potentially confounding effects of COD and agonal state, for instance. Age and PMI differed significantly across groups, but our post hoc analyses demonstrated that the differences were not influenced by these variables. As in our previous PET studies, there is significant overlap between groups in individual measurements, as both groups fall within the likely “normal” range of activity. Thus, overall effect size is small, although statistically significant. It is not possible to know how many carriers and non-carriers would have developed AD, and we do not know whether the biological alterations reflect very early age-related brain changes or abnormalities in neurological development. Additional studies are needed to determine whether the CO reduction is limited to PCC, and we cannot exclude the possibility of $A\beta$ or tau alterations in other regions of the brain impacting functional activity in the cingulate.

Specific AD-associated deficits in CO function have been reported in both postmortem brain tissues (e.g. [9, 17,18]) as well as peripheral cells, prominently including circulating platelets [18–20], indicating a systemic mitochondrial defect. We previously reported that a significant platelet CO deficit could be detected in subjects with mild cognitive impairment (MCI) as well [20], supporting theories positing primacy of mitochondrial dysfunction in AD [21]. The mechanism underlying the reduction in CO activity in AD is not known; it may be directly related to amyloid- β protein precursor [22] or amyloid- β expression [23]. Further, several *in vivo* [24,25] and *in vitro* [26,27] studies have supported a direct role for apoE $\epsilon 4$ fragments in cellular and mitochondrial dysfunction (reviewed in [28]), including direct binding to electron transport complex III and IV (CO) subunits resulting in decreased enzyme activities [27].

A recent study from Roses and colleagues [11] reported an association between longer poly-T polymorphisms in the TOMM40 gene and younger symptomatic AD onset, even after stratifying subjects by their APOE genotype. If this finding is confirmed in independent studies, the TOMM40 gene may account for additional cases of late-onset AD in APOE ϵ 4 non-carriers. This gene encodes the major pore-forming subunit of the translocase of the outer mitochondrial membrane, which most nuclear-encoded mitochondrial proteins must traverse. AD-associated amyloid- β protein precursor has been shown to arrest in this pore [22], and further, neuronal apoE ϵ 4 peptide fragments known to cause mitochondrial dysfunction interact at the outer mitochondrial membrane [26]. These interactions may already be operating to induce the subclinical mitochondrial decline reported here. The TOMM40 variable-length poly-T polymorphism has not yet been characterized in these subjects, but the sample size is likely too small to distinguish the relationship between TOMM40 and posterior cingulate measurements after controlling for APOE genotype.

PCC resides within the tonically active “default mode” brain network [29]. This network activity has been linked to memory retrieval, shows regionally-selective brain imaging declines in pre-symptomatic and symptomatic stages of AD, and is associated with A β deposition, leading to speculation that conducive metabolic conditions in this network (e.g., lifelong tonic activity) may lead to regionally-specific amyloid deposition in later life [30], a concept supported by amyloid secretion dependence on synaptic activity [31]. Blood oxygen level-dependent (BOLD) fMRI in young adult (age 20-35) APOE ϵ 4 carriers has shown that carriers have significantly *higher* regional co-activation in this network than noncarriers [32], and other earlier studies have indicated widespread differences in task-based activation patterns in similarly young ϵ 4 carriers versus noncarriers with H₂¹⁵O PET [33]. It remains to be shown how the FDG PET and baseline perfusion measurements, baseline BOLD co-activation patterns, task-dependent BOLD and H₂¹⁵O changes, and CO activity are related to APOE ϵ 4 carrier status, other risk factors, and each other, at different pre-symptomatic stages of AD.

Alternatively, CO activity changes could be a biomarker of pathophysiology not assessed in the current study, including disturbances in neuronal energy metabolism due to hemodynamic alterations: reported declines here may be early signs of chronic arterial disease that appears to accompany AD [34]. Or, they could stem from a decrement in efferent input resulting from pathology in an interconnected region. Further study will elucidate a number of possible alternatives. CO activity measures have proven to be useful biomarkers of regional, subregional, and laminar brain changes in AD and AD models [15], as well as in non-neural tissues [20]. Our findings provide a foundation for further investigating the role of mitochondrial and other neuronal terminal processes in the predisposition to AD, its earliest pathogenic stages, and discovery of novel pre-symptomatic treatments.

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Table 1

Characteristics of the subjects analyzed

Subject	Gender	Age	Race	PMI (h)	APOE	Cause of Death
84 Noncarriers						
1133	M	38	C	9	2/2	cocaine intoxication
1486	F	22	C	10	2/3	MVA
1502	M	29	C	19	2/3	MVA
1571	F	18	C	8	2/3	MVA
1584	F	18	C	15	2/3	MVA
4542	M	22	C	8	2/3	MVA
1027	M	22	C	9	3/3	MVA
1029	M	29	H	12	3/3	MVA
1037	M	19	C	11	3/3	narcotic intoxication
1101	F	19	C	7	3/3	MVA
1104	M	35	C	12	3/3	MVA
1136	F	33	C	19	3/3	ASCVD
1285	M	23	AA	8	3/3	cardiomyopathy
1347	F	19	C	16	3/3	MVA
1429	M	18	C	9	3/3	MVA
1541	F	20	C	19	3/3	MVA
1544	M	32	C	12	3/3	MVA
1573	F	32	C	12	3/3	MVA
1710	F	26	C	12	3/3	cardiac tamponade
1712	M	20	C	8	3/3	suicide (gunshot)
1859	M	30	C	20	3/3	MVA
1862	M	20	C	6	3/3	multiple injuries
1905	M	33	AA	7	3/3	coronary artery thrombosis
4545	M	31	C	13	3/3	MVA-ASCVD
4726	M	28	C	6	3/3	MVA
84 Carriers						
1028	M	39	C	14	3/4	MVA-compression asphyxia
1064	F	40	C	19	3/4	acute narcotic intoxication

Subject	Gender	Age	Race	PMI (h)	APOE	Cause of Death
1209	F	39	C	17	3/4	MVA
1259	M	24	C	8	3/4	compression asphyxia
1277	M	35	C	20	3/4	ASCVD
1364	F	33	AA	20	3/4	MVA
1365	M	28	C	17	3/4	MVA
1376	M	37	AA	12	3/4	ASCVD
1405	M	21	AA	13	3/4	drowning
1536	F	25	AA	24	3/4	multiple injuries
1579	F	21	AA	22	3/4	asthma
1715	F	33	C	17	3/4	asthma
1909	M	40	C	20	3/4	HASCVD
1577	F	34	AA	12	4/4	asthma
1614	F	27	C	18	4/4	suicide (gunshot)

AA, African American; C, Caucasian; H, Hispanic; ASCVD, arteriosclerotic cardiovascular disease; HASCVD, hypertensive arteriosclerotic cardiovascular disease; MVA, motor vehicle accident.

Table 2
Posterior cingulate measurements in young-adult APOE $\epsilon 4$ carriers and non-carriers

	Non-Carriers <i>n</i> = 25	Carriers ⁺ <i>n</i> = 15	
Number subjects with non-zero pathology score:			
A β Immunohistochemistry	0	1	
Thioflavin S	0	1	
AT8 Immunohistochemistry	6 (24%)	3 (20%)	
Campbell-Switzer	0	1	
Amyloid ELISA (pg/mg total protein), mean $\pm$ stdev:			P
Soluble A β_{1-40}	6.0 $\pm$ 6.9	4.5 $\pm$ 3.7	0.36
Insoluble A β_{1-40}	3.5 $\pm$ 6.2	6.8 $\pm$ 10.3	0.29
Soluble A β_{1-42}	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	–
Insoluble A β_{1-42}	63.3 $\pm$ 89.6	71.8 $\pm$ 49.1	0.71
CO Activity (nmol cyt c/min/mg), mean $\pm$ stdev:			
Overall	361 $\pm$ 14	352 $\pm$ 15	0.036 [*]
Superficial Layer I	360 $\pm$ 23	340 $\pm$ 14	0.011 ^{**}
External Granular Layer II	364 $\pm$ 16	349 $\pm$ 10	0.004 ^{**}
External Pyramidal Layer III	371 $\pm$ 16	359 $\pm$ 12	0.053
Internal Granular Layer IV	364 $\pm$ 14	358 $\pm$ 14	NS
Internal Pyramidal Layer V	359 $\pm$ 16	354 $\pm$ 18	NS
Multiform Layer VI	347 $\pm$ 18	350 $\pm$ 26	NS

⁺The APOE $\epsilon 4$ carriers included 2 homozygotes and 13 heterozygotes.

APOE $\epsilon 4$ homozygote with significant pathology excluded (all remain nonsignificant with inclusion, $p > 0.05$).

^{*} 2-tailed separate-variance t-test, $p < 0.05$.

^{**} 2-tailed separate-variance t-test, $p < 0.05$, reported P Bonferroni-corrected for multiple comparisons.