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Altered Mitochondrial DNA Methylation Pattern in Alzheimer Disease—Related Pathology and in Parkinson Disease

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Mitochondrial dysfunction is linked with the etiopathogenesis of Alzheimer disease and Parkinson disease. Mitochondria are intracellular organelles essential for cell viability and are characterized by the presence of the mitochondrial (mt)DNA. DNA methylation is a well-known epigenetic mechanism that regulates nuclear gene transcription. However, mtDNA methylation is not the subject of the same research attention. The present study shows the presence of mitochondrial 5-methylcytosine in CpG and non-CpG sites in the entorhinal cortex and substantia nigra of control human postmortem brains, using the 454 GS FLX Titanium pyrosequencer. Moreover, increased mitochondrial 5-methylcytosine levels are found in the D-loop region of mtDNA in the entorhinal cortex in brain samples with Alzheimer disease-related pathology (stages I to II and stages III to IV of Braak and Braak; $n = 8$) with respect to control cases. Interestingly, this region shows a dynamic pattern in the content of mitochondrial 5-methylcytosine in amyloid precursor protein/presenilin 1 mice along with Alzheimer disease pathology progression (3, 6, and 12 months of age). Finally, a loss of mitochondrial 5-methylcytosine levels in the D-loop region is found in the substantia nigra in Parkinson disease ($n = 10$) with respect to control cases. In summary, the present findings suggest mtDNA epigenetic modulation in human brain is vulnerable to neurodegenerative disease states. (*Am J Pathol* 2016, 186: 385–397; <http://dx.doi.org/10.1016/j.ajpath.2015.10.004>)

Alzheimer disease (AD) and Parkinson disease (PD) are human neurodegenerative diseases characterized by intracellular aggregation of hyperphosphorylated tau that forms neurofibrillary tangles and extracellular accumulation of β -amyloid peptide in the form of extracellular senile plaques and by the occurrence of α -synuclein-containing intraneuronal inclusions called Lewy bodies and aberrant neurites in AD and PD, respectively.^{1–4} Moreover, several studies have reported altered mitochondrial function in both diseases.^{3,5–9}

Mitochondria are intracellular organelles that contain their own DNA, the mitochondrial DNA (mtDNA), which is a circular double-stranded DNA with a length of 16,569 bp.¹⁰ It codifies essential molecules for the biogenesis of this organelle: 22 tRNAs, 2 rRNAs, and 13 mitochondrial proteins of the respiratory chain [complex I: NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5, and 6 (ND1, ND2, ND3,

ND4, ND4L, ND5, and ND6); complex III: cytochrome b; complex IV: cytochrome oxidase I to III; and complex V: ATPase 6 and 8]. The rest of the mitochondrial proteins are codified in the nucleus. Moreover, mtDNA is devoid of intronic regions, and the only noncoding sequence is known as the control region or D-loop, which regulates mitochondrial transcription and replication.¹¹ Interestingly, reduced

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content and activity of complex I and IV subunits of the respiratory chain, such as ND4 in AD brains^{12–15} and ND6 in the substantia nigra in PD, have been reported.^{16–18} In addition, genetic studies have identified somatic mutations in several mitochondrially encoded cytochrome c oxidase genes and in the D-loop, probably altering mitochondrial transcription and replication, and mtDNA deletions in AD brains^{19–22} and in the substantia nigra in PD.^{23–25}

The transcriptional control of mtDNA is exerted by nuclear-encoded proteins such as mitochondrial transcription factor A, mitochondrial transcription factor B1, and mitochondrial transcription factor B2.^{26,27} In turn, polymorphisms in *TFAM* were also associated with a moderate risk factor for AD and PD.^{28–31} For the epigenetic control of the mitochondrial genome, there is an old debate about the existence or not of 5-methylcytosine (5mC) in this small circular genome.^{32–41} Recently, the first comprehensive map of 5mC in the mtDNA was published after analysis of 39 data sets available in the public domain.⁴² 5-Hydroxymethylcytosine (5hmC) was reported in cell lines and mouse brain in relation with the presence of the ten-eleven-translocation enzymes 1 to 3 family, which is involved in the conversion of 5mC into 5hmC.^{41,43} Moreover, the presence of 5hmC in the D-loop region in human and murine peripheral blood was shown by using methylated-DNA immunoprecipitation assays.³²

There are no data about the existence of mitochondrial 5mC levels in the entorhinal cortex and substantia nigra in the human brain and practically nothing about neurodegenerative diseases. To our knowledge, only one study revealed high global mitochondrial 5mC levels with immunohistochemistry in the motor neurons of amyotrophic lateral sclerosis patients.⁴⁴ For this reason, the present study was aimed at assessing the presence and extent of mtDNA 5mC levels in selected regions in the human brain, including CpG and non-CpG sites, because methylation in nuclear non-CpG sites was reported to be present in human postmortem brains but absent in other human somatic cell types.^{45,46}

To this end, the present study has analyzed the entorhinal cortex in control brains and in cases at the first stages of AD-related pathology because this is the first cerebral region affected by neurofibrillary tangle pathology in AD, and the substantia nigra in controls and PD cases because this is the paradigmatic region vulnerable to PD. In parallel, amyloid precursor protein/presenilin 1 (APP/PS1) mice, which are a model of familial AD, were also studied at 3, 6, and 12 months of age. Mitochondrial 5mC levels were measured with a deep sequencing technique with the 454 GS FLX Titanium pyrosequencer and mitochondrial 5hmC levels with hydroxymethylated DNA immunoprecipitation (hMeDIP) assay. This study provides the following new concepts to the mitoeptigenetics field: i) existence of mitochondrial 5mC levels in CpG and non-CpG sites in human and mouse brains, ii) a differential mtDNA methylation profile in AD-related pathology and PD with their control samples, iii) a dynamic mtDNA methylation along disease progression in APP/PS1 mice, and iv) presence of mitochondrial 5hmC levels in human brain.

Materials and Methods

Human Brain Samples

Tissue samples were provided by the Neurological Tissue Bank, University of Barcelona — Hospital Clínic de Barcelona and the Institute of Neuropathology, HUB-ICO-IDIBELL Brain Bank. The donation and procurement of samples were regulated by the ethics committee of both institutions. Half of each brain was maintained in 4% buffered formalin for structural and immunohistochemical study, whereas the other half was processed in coronal sections to be frozen at -80°C and made available for biochemical study. The neuropathologic examination was performed in all control and diseased cases on 30 standardized sections of the cerebrum, cerebellum, and brainstem, which were stained with hematoxylin and eosin, and Klüver Barrera, or processed for immunohistochemistry to glial fibrillary acidic protein, microglial markers, β -amyloid, phosphorylated tau (antibody AT8), α -synuclein, α B-crystallin, ubiquitin, and TDP-43. Cases with AD-related pathology and PD cases were staged according to current neuropathologic criteria.^{47–50} Cases with mixed pathology (including vascular lesions) were excluded from the present study. Control brains were from individuals without neurologic manifestations and with no lesions in the neuropathologic study. All cases analyzed are summarized in [Supplemental Table S1](#).

Animals

Male APP/PS1 transgenic mice and corresponding wild-type (C57BL/6J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The APP/PS1 model expresses a chimeric mouse/human APP (Mo/HuAPP695swe: APP Swedish mutation) and a mutant human PS1 (PS1-dE9), both directed to central nervous system neurons. Mice were maintained under standard animal housing conditions in a 12-hour dark-light cycle with free access to food and water. Animal handling was conducted in accordance with ethical guidelines (European Communities Council Directive 86/609/EEC) and were approved by the local ethics committee.

Total DNA Extraction

Total DNA was isolated from the human entorhinal cortex and substantia nigra, and the mouse neocortex with the use of the DNeasy Blood and Tissue Kit (Qiagen, Madrid, Spain) according to the manufacturer's instructions.

Bisulfite Treatment

DNA (300 ng) was treated with bisulfite with the use of the EZ DNA Methylation Kit (Zymo Research, Ecogen, Barcelona, Spain) according to the supplier's instructions. The human and murine bisulfite-treated DNA was

Table 1 Primer Sequences

Primer name	Forward	Reverse
Human D-loop	5'-TAGGGGTTTTTTGATTATTATTTT-3'	5'-ACAAACATTCAATTATTATTATATATCCT-3'
Human MT-ND1	5'-ATGGTTAATTTTTTATTTTTTATTGTATTT-3'	5'-TAATTTAAATTTAATACTCACCTAATCAA-3'
Human MT-ND6	5'-TTTAAGTTTTTTTTTATTTATGGGGG-3'	5'-CAAACAATATTCAACCAATAACTACTAC-3'
Murine D-loop	5'-AGGATATATTTGTGTATTTGATATATATT-3'	5'-AACTATATACTATCCTTTTCATACCTTAAC-3'

Specific primers for human regions amplified (D-loop: nt.16386-256; MT-ND1: nt.3313-3686; MT-ND6: nt.14190-14613, complementary reverse) which were based on the nucleotide position in NC_012920 GenBank sequence and murine D-loop (nt.15599-15979) based on GenBank DQ106412 number (<http://www.ncbi.nlm.nih.gov/genbank>).

ND, NADH dehydrogenase subunit.

resuspended in 30 μ L and 15 μ L, respectively. All samples were bisulfite-treated in parallel, using the same bisulfite stock to avoid differences in bisulfite conversion rate among different commercial batches.

FLX Primers Design for D-Loop, MT-ND1, and MT-ND6

The primers for the FLX experiment were designed according to Roche's (Sant Cugat del Vallès, Spain) Technical Bulletin Genome Sequencer FLX System: Amplicon Fusion Primer Design Guidelines for GS FLX Titanium Series Lib-A Chemistry. The amplicon fusion primers designed contained a directional GS FLX Titanium Primer A or Primer B sequence (including a four-base library key sequence) at the 5' portion of the oligonucleotide in addition to the template-specific sequence at the 3' end. Moreover, a multiplex identifier (MID) sequence was added between the Primer A (or Primer B) and the specific sequence for automated software identification of samples after pooling/multiplexing and sequencing. The primers used have the following components: forward primer (Primer A-Key-MID-template-specific sequence), 5'-CGTATCGCCTCCCTCGCGCCA-TCAG-MID-template-specific sequence-3'; reverse primer (Primer B-Key-MID-template-specific sequence): 5'-CTATGCGCCTTGCCAGCCCGC-TCAG-MID-template-specific sequence-3'. The primers used in this study were designed to avoid CpG sites. The template-specific sequences for each amplicon are indicated in Table 1. The specific sequence of MID for each patient and mouse is indicated in Tables 2 and 3, respectively.

Amplicon Library Preparation

The FLX PCRs for D-loop, MT-ND1, and MT-ND6 were performed according to the Amplicon Library Preparation Method Manual (GS FLX Titanium Series) of Roche. Total bisulfite-treated DNA (20 ng) per sample was used for FLX PCRs. Amplification of the bisulfite-converted DNA was performed in a reaction volume of 25 μ L. Each PCR reaction mixture consisted of a final concentration of 1 $\times$ FastStart 10 $\times$ Buffer 2, 0.05 U/ μ L FastStart HiFi Polymerase (Roche), 200 nmol/L each dNTP, and 200 nmol/L each specific primer forward and reverse. The primers were synthesized with a high-performance liquid chromatography purification quality

(Sigma-Aldrich, Madrid, Spain). Amplifications were performed in a Verity Applied Biosystems Thermocycler (Applied Biosystems, Madrid, Spain) with the use of the following conditions: 94°C for 3 minutes and then 36 cycles at 94°C for 15 seconds, specific annealing temperature for each amplicon (61°C for MT-ND1, 62°C for D-loop and MT-ND6, and 56°C for murine D-loop) for 45 seconds and 72°C for 1 minute, followed by a final extension step at 72°C for 8 minutes and a last hold at 4°C. Two microliters of each PCR product was checked on 1.5% agarose gel stained with SybrSafe DNA Gel Stain (Invitrogen, Madrid, Spain).

PCR Purification

PCR product was purified with the Agencourt AMPure XP PCR Purification (Beckman Coulter, Madrid, Spain)

Table 2 MID Sequences for Each Patient Analyzed

MID number	MID sequence
1	5'-ACGAGTGCCT-3'
2	5'-ACGCTCGACA-3'
3	5'-AGACGCACTC-3'
4	5'-AGCACTGTAG-3'
5	5'-ATCAGACACG-3'
6	5'-ATATCGCGAG-3'
7	5'-CGTGTCTCTA-3'
8	5'-CTCGCGTGTG-3'
9	5'-TAGTATCAGC-3'
10	5'-TCTCTATGCG-3'
11	5'-TGATACGTCT-3'
12	5'-TACTGAGCTA-3'
13	5'-CATAGTAGTG-3'
14	5'-CGAGAGATAC-3'
15	5'-ATACGACGTA-3'
16	5'-TCACGTACTA-3'
17	5'-CGTCTAGTAC-3'
18	5'-TCTACGTAGC-3'
19	5'-TGTACTACTC-3'
20	5'-ACGACTACAG-3'
21	5'-CGTAGACTAG-3'
22	5'-TACGAGTATG-3'
23	5'-TACTCTCGTG-3'
24	5'-TAGAGACGAG-3'

Forward and reverse primers contain the MID sequence (a barcode for each case).

MID, multiplex identifier.

Table 3 MID Sequences for Each Mouse Analyzed

Mouse phenotype	MID number	MID sequence
WT, 3 months	25	5'-TCGTCGCTCG-3'
APP/PS1, 3 months	26	5'-ACATACGCGT-3'
APP/PS1, 3 months	27	5'-ACGCGAGTAT-3'
APP/PS1, 3 months	28	5'-ACTACTATGT-3'
WT, 3 months	29	5'-ACTGTACAGT-3'
WT, 3 months	30	5'-AGACTATACT-3'
WT, 3 months	31	5'-AGCGTCGTCT-3'
APP/PS1, 3 months	32	5'-AGTACGCTAT-3'
APP/PS1, 6 months	33	5'-ATAGAGTACT-3'
APP/PS1, 6 months	34	5'-CACGCTACGT-3'
APP/PS1, 6 months	35	5'-CAGTAGACGT-3'
WT, 6 months	36	5'-CGACGTGACT-3'
APP/PS1, 6 months	37	5'-TACACACAGT-3'
WT, 6 months	38	5'-TACACGTGAT-3'
WT, 6 months	39	5'-TACAGATCGT-3'
WT, 6 months	40	5'-TACGCTGTCT-3'
WT, 12 months	41	5'-TAGTGTAGAT-3'
WT, 12 months	42	5'-TCGATCACGT-3'
APP/PS1, 12 months	43	5'-TCGCACTAGT-3'
APP/PS1, 12 months	44	5'-TCTAGCGACT-3'
APP/PS1, 12 months	45	5'-TCTATACTAT-3'
WT, 12 months	46	5'-TGACGTATGT-3'
APP/PS1, 12 months	47	5'-TGTGAGTAGT-3'
APP/PS1, 12 months	48	5'-ACAGTATATA-3'

Forward and reverse primers contain the MID sequence (a barcode for each case).

APP/PS1, amyloid precursor protein/presenilin 1; MID, multiplex identifier; WT, wild-type.

according to the instructions in Roche's Amplicon Library Preparation Method Manual (GS FLX Titanium Series).

Library Quantitation and FLX Sequencing

The quantitation and quality control of the libraries and the rest of the FLX sequencing protocol were performed by the Core Facilities-Genomic Platform of the Vall d'Hebron Institut de Recerca (Barcelona, Spain). The analysis of murine D-loop was performed by the MacroGen platform (Seoul, South Korea).

Selection of Differentially Methylated Sites

The sample size was determined statically before experimentation. Alignment and identification of CpG, CHG, and CHH sites and bisulfite conversion rates were performed with BiQ Analyzer HT software (Max Planck Institut für Informatik, Saabrücken, Germany).⁵¹ Quality control of raw data and all of the statistical analysis were performed with the free statistical language R (<http://www.r-project.org>, last accessed September 30, 2015) and bioconductor (<http://www.bioconductor.org>, last accessed September 30, 2015).

The selection of differentially methylated sites was based on computing a Fisher's Exact Test statistic and

calling differentially methylated those sites with an adjusted $P < 0.05$ with the method of Benjamini and Hochberg.⁵²

The β -value represented in Heatmaps is the ratio of methylated reads per site and the overall sum of methylated and unmethylated reads per site⁵³; that is, $\beta_{ij} = M/(M + U)$, where M is the number of methylated reads in site (i) and MID (j), and U is the number of unmethylated reads in site (i) and MID (j).

hMeDIP Assay

Chromatin shearing (between 200 and 500 bp) was obtained from human entorhinal cortex and substantia nigra genomic DNA, using the Bioruptor from Diagenode (Liege, Belgium). The resultant human DNA (500 ng) was immunoprecipitated with the hMeDIP kit (Diagenode) according to the manufacturer's instructions. As a negative control, immunoprecipitation was performed with rabbit serum provided with the kit. Moreover, the kit also provided three spikes or internal immunoprecipitation controls: an unmethylated DNA, a methylated DNA, and a hydroxymethylated DNA which were added to all samples analyzed. In parallel, an aliquot of chromatin sheared from each sample was separated as a loading control of the experiment (Input).

The analysis of MeDIPs was performed in 5 μ L SyBr Green reaction (Promega, Madrid, Spain): 1 μ L hMeDIP or input was mixed with 0.4 μ L of both primers (5 μ mol/L) and 2.5 μ L of master mix. Every experimental condition was performed in triplicate in 384-well optical plates with the use of the LightCycler 480 System (Roche). The reactions were performed with the following variables: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. PCR products were evaluated with SYBR green melting curve analyses. The primers used were the following: MT-ND1 forward, 5'-GCAGTAGCC-CAAACAATCTC-3'; MT-ND1 reverse, 5'-GTGTTCTT-GTGTGTGATAAGG-3'; MT-ND6 forward, 5'-CCA-AAGACAACCATCATTCC-3'; MT-ND6 reverse, 5'-ATT-GATTGTAGCGGTGTGG-3'; D-loop forward, 5'-CCT-CACCCACTAGGATACCAAC-3'; D-loop reverse, 5'-CAC-GGAGGATGGTGGTCAAG-3'. The primers for the three spikes were included with the kit. The value of the percentage of input was calculated according to the instructions provided with the hMeDIP kit (Diagenode).

RNA Purification

The RNA from human entorhinal samples was purified with RNeasy Midi kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. The concentration of each sample was obtained from A₂₆₀ measurements with Nanodrop 1000. RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA).

Retrotranscription Reaction

The retrotranscriptase reaction was performed with the High-Capacity cDNA Archive kit (Applied Biosystems, Madrid, Spain) according to the protocol provided by the supplier. Parallel reactions for each RNA sample were run in the absence of MultiScribe Reverse Transcriptase to assess the degree of contaminating genomic DNA.

TaqMan PCR

TaqMan PCR assays for every gene were performed in duplicate on cDNA samples in 384-well optical plates with the use of an ABI Prism 7900 Sequence Detection system (Applied Biosystems). For each 5 μ L TaqMan reaction, 2.5 μ L cDNA (dilution 1:100, which corresponds approximately to the cDNA from 12.5 ng of RNA) was mixed with 0.25 μ L 20 $\times$ TaqMan Gene Expression Assays and 2.25 μ L of 2 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems). Parallel assays for each sample were performed with primers and probe for X-prolyl aminopeptidase 1 (*XPNPEP1*) for normalization. Standard curves were prepared for every gene analyzed with the use of serial dilutions of a control entorhinal case. Finally, all TaqMan PCR data were captured with the Sequence Detector Software version 1.9 (Applied Biosystems). The identification numbers for MT-*ND1* and XPNPEP1 TaqMan probes were Hs02596873_s1 and Hs00958026_m1, respectively.

Statistical and mRNA/hMeDIP Data Analysis

Data were analyzed with GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). The MT-*ND1* mRNA expression levels and hMeDIP assays were evaluated with one-way analysis of variance (least significant difference post hoc test). Differences between mean values were considered statistically significant at $P < 0.05$.

Results

The study was performed on 44 samples that included AD-related pathology, PD, and control cases. Samples were processed in a single plate that consisted of two lanes. Samples of the entorhinal cortex of cases with AD-related

Figure 1 Heatmaps of β -values of CpG, CHG, and CHH sites associated with amplicon D-loop in the entorhinal cortex of AD-related pathology cases. Methylation heatmaps represent the extracted methylation patterns of the bisulfite reads graphically for all human cases analyzed (MIDs) in lane 1 (Table 2 and Supplemental Table S1). Columns are formed by the methylation sites found in the reference sequence that matches the analyzed methylation context, whereas rows correspond to the sequence reads. Horizontal lanes in the top of Heatmaps indicate the control group (red), AD I to II stages of Braak (green), and AD III to IV stages of Braak (blue) cases. A scale is shown, whereby positive (red) and negative (blue) values correspond, respectively, to higher and lower centered and scaled β -value (z-score) per site. AD, Alzheimer disease; MID, multiplex identifier.

Figure 2 Log₂(OR) plots for CpG (A), CHG (B), and CHH (C) sites in amplicon D-loop in the entorhinal cortex of AD-related pathology cases. On the x axis are each site sorted from 5' to 3'. Sites tagged with red diamonds are differentially methylated (FDR < 0.05). Blue dots are the OR estimate values, one per site, and cyan band is the union of all 95% CIs. AD, Alzheimer disease; C, control samples; FDR, false discovery rate.

pathology and corresponding control cases were analyzed for amplicons D-loop and MT-ND1 and were allocated in lane 1 (Supplemental Table S1). Samples of the substantia nigra of PD cases and controls were analyzed for amplicons D-loop and MT-ND6 and were allocated in lane 2 (Supplemental Table S1). Each patient was identified with a MID sequence in the oligos used (Table 2 and Supplemental Table S1). The methylation of CpG sites and non-CpG sites (CHG and CHH, where H = A, T, or C) was assessed with a 454 GS FLX Titanium pyrosequencer which generated 569,684 reads in the first lane and 513,579 in lane 2. Alignments of reads for each MID, amplicon, and lane to the respective reference sequences were scored and their percentages of identity were close to 100% (Supplemental Figures S1 and S2). The bisulfite means $\pm$ SD and

median conversion rates for each locus and MID analyzed are shown in Supplemental Table S2.

The number of unmethylated reads was higher than the number of methylated reads per identified site, and few sites of methylation were missing (Supplemental Figures S3 and S4). Those reads that showed at least one missing site in the methylation pattern after alignment were removed from the analysis to avoid a bias at the time of quantification. This approach eluded the analysis of putative mitochondrial pseudogenes, whereas amplicons had 100% identity with mtDNA when entered into the National Center for Biotechnology Information Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/blast>). In addition, specificity for mtDNA target sequences of primers used was also checked on DNA

Figure 3 Log₂(OR) plots for CpG (A), CHG (B), and CHH (C) sites in amplicon D-loop in the cerebral cortex of WT and APP/PS1 mice at different ages (3, 6, and 12 months old). On the x axis are each site sorted from 5' to 3'. Sites tagged with red diamonds are differentially methylated (FDR < 0.05). Blue dots are the OR estimated values, one per site, and cyan band is the sum of all 95% CIs. APP/PS1, amyloid precursor protein/presenilin 1; FDR, false discovery rate; TG, transgenic APP/PS1 mice; WT, wild-type mice.

extracted from osteosarcoma 143B.TK⁻ Rho⁰ cells, which are devoid of mtDNA (Supplemental Figure S5). Most of the identified sites in CpG, CHG, and CHH sites were unmethylated (Supplemental Figure S3). However, differential methylated sites were also found (Supplemental Table S3).

DNA Methylation Increases in D-Loop and Reduces in MT-ND1 Gene in Cases with Early-Stage AD-Related Pathology

Increased DNA methylation of CpG and non-CpG sites (CHG and CHH) in the D-loop region was observed in cases with AD-related pathology, stages I/II and III/IV of Braak (Figure 1). The degree of methylation was higher in cases with AD-related pathology for control samples and higher in stages I/II than in stages III/IV as represented in log₂(OR) plots (Figure 2). However, no differences were found in the

methylation of CHH sites between controls and cases with AD-related pathology at stages III/IV. This is further represented in Pearl-Necklace diagrams, which summarize methylation information for the whole set of filtered reads in each CpG site (Supplemental Figure S6). The plots show that the increased methylation in CpG sites in AD-related pathology samples represents a small population of mtDNA.

MT-ND1 analysis revealed that a few number of CpG and CHG sites were less methylated in cases with AD-related pathology at stages I/II and III/IV than in control samples, whereas no differences were found for CHH sites (Supplemental Figure S7). Next, we investigated whether these methylation changes showed an inverse relation with MT-ND1 mRNA levels. Only samples with an RNA integrity number greater than six were included in this analysis, which corresponds to a new set of human entorhinal cases (Supplemental Table S4). As Supplemental Figure S8 shows, an increase in MT-ND1 mRNA levels

was found in AD-related pathology at stages V/VI for control cases and AD-related pathology at stages I/II.

Dynamic DNA Methylation Pattern in the D-Loop in the Cerebral Cortex of APP/PS1 Mice along AD Pathology Progression

On the basis of human results, we decided to check the 5mC levels in the murine D-loop, using an AD mouse model at different ages (3, 6, and 12 months). Therefore, a new run of pyrosequencing was performed. Each mouse was identified with an MID sequence in the oligos used (Table 3). The 454 GS FLX Titanium pyrosequencer generated 865,185 reads. The bisulfite means $\pm$ SD and median conversion rates for each MID analyzed are shown in Supplemental Table S5. Differentially, 5mC levels were found between transgenic and wild-type mice along AD pathology progression in CpG sites (Figure 3A), CHG sites (Figure 3B), and to a lesser extent in CHH sites (Figure 3C). As shown in human brain, the 5mC levels represent a small population of total mtDNA (Supplemental Figure S9).

DNA Methylation Is Reduced in D-Loop and Maintained in MT-ND6 Gene in the Substantia Nigra in PD

In contrast to observations in the entorhinal cortex in AD, the D-loop region showed a loss of methylation in nearly all CpG and non-CpG sites in the substantia nigra in PD cases for control samples (Figures 4 and 5A). However, as in AD, the percentage of DNA methylation represented a small part of the total mtDNA (Supplemental Figure S10). No differences in methylation of CpG and non-CpG sites were found in MT-ND6 amplicon, except an increase in methylation in two CHH sites, in the substantia nigra in PD cases (Figure 5B).

Levels of Mitochondrial 5hmC Remain Unchanged in the D-Loop, MT-ND1, and MT-ND6

Finally, we also checked the levels of mitochondrial 5hmC with the use of the hMeDIP assay. To test the efficiency of the assay, we determined the 5hmC levels of an hmC spike added to an entorhinal cortex sample and to Rho⁰ cells. The anti-hmC antibody immunoprecipitated the hmC spike in both samples, whereas it did not recognize the other two

Figure 4 Heatmaps of β -values of CpG, CHG, and CHH sites associated with amplicon D-loop in substantia nigra of PD patients. Methylation heatmaps represent the extracted methylation patterns of the bisulfite reads graphically for all human cases analyzed in lane 2 (MIDs) (Table 2 and Supplemental Table S1). Columns are formed by the methylation sites found in the reference sequence matching the analyzed methylation context, whereas rows correspond to the sequence reads. Horizontal lanes in the top of Heatmaps indicate the control group (red) and PD patients (blue). A scale is shown, whereby positive (red) and negative (blue) values correspond, respectively, to higher and lower centered and scaled β -value (z-score) per site. MID, multiplex identifier; PD, Parkinson disease.

Figure 5 Log₂(OR) plots for CpG and non-CpG (CHG and CHH) sites in the substantia nigra of PD patients. Amplicons D-loop (A) and MT-ND6 (B). On x axis are each site sorted from 5' to 3'. Sites tagged with red diamonds are differentially methylated (FDR < 0.05). Blue dots are the OR estimate values, one per site, and cyan band is the union of all 95% CIs. C, control samples; FDR, false discovery rate; MT-ND, mitochondrial NADH dehydrogenase subunit. PD, Parkinson disease.

spikes also included in the tested samples (an unmethylated DNA and a methylated DNA) (Figure 6A). Moreover, the oligos used to determine the 5hmC levels in MT-ND1, MT-ND6, and D-loop did not amplify putative mitochondrial pseudogenes because no amplification was obtained in Rho⁰ cells. Therefore, the overall analysis performed in substantia nigra (Figure 6B) and entorhinal cortex (Figure 6C) samples revealed nonsignificant differences in 5hmC levels in the three loci studied.

Discussion

DNA methylation is a well-known epigenetic mechanism that regulates the expression of nuclear genes during development and in adulthood.^{54,55} Levels of 5mC are maintained in nuclear gene promoters and gene bodies

through the activity of DNA methyltransferases.⁵⁶ The existence of mitochondrial 5mC has been controversial for a long time.^{32–34,37,39–41} A study demonstrated for the first time human mtDNA methylation with the use of mass spectrometry analysis and mtDNA hypomethylation in Down syndrome.³⁶ Yet it was also suggested that CpG methylation does not occur in human mtDNA.³⁵ Importantly, the characterization of mitochondrial DNA methyltransferase 1⁴¹ has promoted growing interest in the study of epigenetic regulation of mtDNA.^{57,58}

In the present study, a high number of reads (>500,000) were used to analyze three mitochondrial loci, allowing the detection of a methylated mtDNA population in CpG and non-CpG sites at single base pair resolution. The overall study shows that methylated mtDNA represents only a small part of the total mtDNA. This aspect is in agreement with

Figure 6 Levels of hmC were measured with hMeDIP assay in the MT-ND1, MT-ND6, and D-loop. **A:** Analysis of anti-hmC antibody efficiency and oligos specificity. ER corresponding to MID3, lane 1, [Supplemental Table S1](#). **B:** Analysis of substantia nigra cases. **C:** Analysis of entorhinal cortex. All human samples studied are indicated in [Supplemental Table S1](#). Nonsignificant differences were found for all loci studied. Data are expressed as means $\pm$ SEM. ER, entorhinal cortex sample; hmC, hydroxymethylcytosine; hMeDIP, hydroxymethylated DNA immunoprecipitation; IP, immunoprecipitated; mDNA, methylated DNA; MID, multiplex identifier; MT-ND, mitochondrial NADH dehydrogenase subunit; Rho⁰, osteosarcoma 143B.TK⁻ Rho⁰ cells; uDNA, unmethylated DNA.

the predicted reduced percentage of methylation assigned to mtDNA.^{38–40} Note that the robustness of the present analysis showing the small but consistent methylation of mtDNA may explain the apparently controversial absence of 5mC reported in this small genome.^{34,35,37}

This study shows increased 5mC levels in the D-loop region in the entorhinal cortex of cases with AD-related pathology

stages I to II and III to IV of Braak and Braak with respect to control samples in most CpG and non-CpG sites. Interestingly, 5mC levels were higher in stages I/II than in stages III/IV. This altered mtDNA methylation profile was also replicated in the cerebral cortex of APP/PS1 mice, which present a memory decline and impaired learning at 6 months of age, whereas the first appearances of β -amyloid plaques and mitochondrial accumulation in aberrant neurites are at 3 months.⁵⁹ The increase in 5mC levels found in 6-month-old APP/PS1 mice is quite relevant, because it shows a similar behavior to human AD brains.

Because MT-ND1 methylation by mitochondrial DNA methyltransferase 1 overexpression was observed *in vitro*,⁴¹ 5mC levels in MT-ND1 were also measured in AD-related pathology cases. Reduced 5mC levels in MT-ND1 were detected in the entorhinal cortex in cases of AD-related pathology. Although our aim was to identify altered mitochondrial 5mC levels at early AD stages of Braak and Braak, we performed the analysis of MT-ND1 mRNA levels in a new cohort of cases, including advanced AD stages (AD V to VI). Our data suggest that changes in MT-ND1 methylation levels in early AD stages could promote an increase in mRNA expression levels at stages V to VI of Braak and Braak. Of note is the discrepancy between the D-loop and MT-ND1 methylation patterns, reminding us that in the dynamics of nuclear methylation 5mC gains in promoter genes are normally accompanied by 5mC losses in coding regions.⁵³

Oxidative stress damage to ATP synthase accompanied by reduced enzymatic activity was reported in AD entorhinal cortex at stages I to II of Braak.⁶⁰ Whether this change in the respiratory chain has any impact on the increase in 5mC levels in mtDNA is not known. The progression of AD stages is long, taking at least 16 years from stage I to stage II and about 14 years to pass from stage II to III.⁶¹ Therefore, considering such long periods of time, it is feasible that compensatory mechanisms may promote modifications in the methylation pattern in the D-loop region with disease progression and altered expression of MT-ND1 mRNA levels.

In contrast to D-loop data obtained in cases with AD-related pathology, reduced 5mC levels were found in the substantia nigra in PD cases compared with the substantia nigra in control samples. This might be attributable to neuronal loss in this cerebral region. However, methylation levels in MT-ND6 were maintained in PD cases compared with controls, thus supporting the notion that reduced 5mC levels in the D-loop were not because of reduced neuronal content. Therefore, a differential mtDNA methylation profile is found between AD-related pathology and PD cases.

It should be taken into account that this study was performed in total homogenates from the entorhinal cortex and substantia nigra, which impairs the identification of the cellular types affected by changes in mtDNA methylation levels. However, note that in AD-related pathology stages I to II, the entorhinal cortex does not show gliosis, lending support to the idea that the increased mitochondrial 5mC levels occur in neuronal cells.

We also checked the 5hmC levels in the three loci covered in this study. Interestingly, mitochondrial 5hmC levels were detected in both human cerebral regions analyzed with the hMeDIP assay, although nonsignificant differences were found in AD-related pathology and PD cases for control samples.

Several studies have reported mitochondrial impairment in AD and PD because of oxidative stress damage, mtDNA mutations/deletions, and mutations in mitochondrial genes codified in the nucleus.^{12–25,28–31} Our study provides new data for mitochondrial dysfunction, showing differential mtDNA methylation, mainly in the D-loop region, in the entorhinal cortex in cases with AD-related pathology and in the substantia nigra in PD. However, because mtDNA is transcribed as a polycistronic mRNA,⁶² further molecular and functional studies are required to elucidate the impact of mtDNA methylation in mitochondrial transcription both in normal conditions and in cases with AD-related pathology and PD. Moreover, another consideration is the consequence of mtDNA methylation in mtDNA replication. In this line, mtDNA replication is tightly linked to transcription, because mitochondrial DNA polymerase γ requires an RNA primer.⁶³ Indeed, the D-loop locus implicated in the processing of the RNA primer for mitochondrial replication was found to be methylated in human and murine peripheral blood, opening a door to the control of mitochondrial replication by mtDNA methylation.^{32,64}

Finally, it remains to be determined whether a minimal threshold in the percentage of mtDNA methylation is enough to alter mitochondrial homeostasis or nuclear epigenetics.^{65–67} Another important aspect to be elucidated is the unraveling of whether modifications in the mtDNA methylation are primary events or rather a consequence of disease-dependent oxidative stress and mitochondrial dysfunction in AD and PD.

In summary, the present report shows the presence of mitochondrial 5mC levels in CpG and non-CpG sites in human brain at single base pair resolution and an altered mtDNA methylation pattern in AD and PD. Therefore, these data support the existence of epigenetic mtDNA regulation.

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Supplemental Data

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