RESULTADOS Y DISCUSIÓN

8. Resultados y discusión

8.1. ¿Existe relación entre la enfermedad de Alzheimer y defectos en el DNA mitocondrial?

Revista de neurología 33(4):301-305; 2001

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Contribución del doctorando:

Extracción de DNA y realización del trabajo experimental de análisis de mutaciones y reordenamientos en las muestras de pacientes y controles mediante PCR-RFLP y *Southern blot*.

Escritura del manuscrito y confección de la tabla y figura del artículo.

¿Existe relación entre la enfermedad de Alzheimer y defectos en el ADN mitocondrial?

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IS THERE A RELATION BETWEEN ALZHEIMER'S DISEASE AND DEFECTS OF MITOCHONDRIAL DNA?

Summary. Introduction. *Various studies have related Alzheimer's disease (AD) with mitochrondrial defects. These defects may be structural, biochemical or genetic in type. Amongst the genetic defects the rearrangement and particular mutations described in mitochondrial DNA (mtDNA) are striking.* Objective. *To study the incidence of rearrangement and 4 particular mutations in the mtDNA of patients with AD, and determine the possible differences from persons taken as controls.* Patients and methods. *Necropsies of the cerebellum, frontal cortex and hippocampus of patients with AD and controls. We also used blood from living patients diagnosed as having EA and from controls. The samples were analysed using hybrid Southern blot with a mitochondrial probe. Particular mutations G3196A, A3397G, A4336G and G5460A/T were also analysed.* Results. *No differences were found between the patients and controls, in either brain tissue or blood on analysis using Southern. No association was found between the particular mutations analysed and the AD of our samples.* Conclusions. *The results obtained did not support the hypothesis of involvement of mitochondria in AD. Regarding rearrangement and the 4 particular mutations analysed in the mtDNA of our samples. However, this does not rule out the possible existence of other particular mutations which were not analysed and/or other mitochondrial defects which contribute to the development of AD. [REV NEUROL 2001; 33: 301-5]*

Key words. *Alzheimer's disease. Deletions. Mitochondria. Mitochondrial DNA. Particular mutations. Rearrangements. Respiratory chain.*

INTRODUCCIÓN

La enfermedad de Alzheimer (EA) es una de las formas más comunes de demencia senil y presenil. Se caracteriza por un progresivo declive cognitivo global y del comportamiento y se define histológicamente por la presencia de placas amiloides –depósitos extracelulares que consisten principalmente en agregaciones de péptido β-amiloide– y de los denominados ovillos neurofibrilares –depósitos intracelulares mayoritariamente formados por proteína *tau* hiperfosforilada– [1]. Las causas genéticas de la enfermedad parecen ser heterogéneas. La EA de inicio temprano puede asociarse con mutaciones en *loci* de los cromosomas 1, 14 y 21 [2-5]. La EA de inicio tardío parece diferir genéticamente de la temprana; un factor de riesgo identificado para la EA tardía es la presencia del alelo 4 de la apolipoproteína E [6,7].

Diferentes estudios indican que existen factores genéticos que desempeñan un papel importante en la etiología de la EA [8,9]. La incidencia de la enfermedad es mayor en mujeres [10], aunque este efecto puede ser consecuencia de su mayor longevidad relativa. El genoma mitocondrial es una molécula circular de doble cadena de ADN de 16,5 kilobases (kb), que se transmite exclusivamente a través de la línea materna. En los últimos años se han descrito diversas anomalías en el metabolismo mitocondrial asociadas a la EA [11-26]. Estos defectos en las mitocondrias incluyen alteraciones de tipo estructural [11], bioquímico [15-17] y genético [16-22]. Entre los defectos estructurales destacan los cambios observados en las mitocondrias de líneas celulares de cíbridos, que afectan a las membranas y a la matriz [11]. También se han observado fallos en el control homeostático del calcio [12], que a su vez está relacionado con la apoptosis [13,14], aumento en la presencia de radicales libres y especies reactivas del oxígeno que causan daño en el ADN mitocondrial (ADNmt), el ADN nuclear y las membranas [8,14]. La disfunción bioquímica se ha relacionado con una disminución en la actividad de la cadena respiratoria [15], especialmente en la citocromo c oxidasa [16]. Esto conlleva un desajuste en el metabolismo mitocondrial oxidativo [17]. El cerebro presenta una gran dependencia metabólica de la producción de ATP a través de la fosforilación oxidativa. En tejido cerebral de pacientes con EA se ha demostrado un desacoplamiento parcial de la fosforilación oxidativa [17]. En el aspecto genético se han observado variaciones en las proporciones de moléculas de ADNmt con la deleción común –tamaño de 4,9 kb–, según el área cerebral analizada [18,19], así como la presencia de mutaciones puntuales [20-23]. También se ha descrito una reducción en la expresión de algunos genes mitocondriales [24].

Las mutaciones puntuales y reordenamientos en el ADNmt constituyen atractivos candidatos para explicar algunos de los defectos hallados en la función mitocondrial. Mutaciones en el genoma mitocondrial pueden causar defectos graves en la cadena respiratoria y provocar una serie de fenotipos degenerativos graves, como sordera, ataxia, cardiomiopatía y demencia de tipo Alzheimer [27]. En diversos tejidos, entre ellos el cerebro, se ha observado que con la edad se produce un aumento de las mutaciones somáticas y de deleciones en el ADNmt [28,29]. Si estas mutaciones y deleciones alcanzan o no niveles suficientes para

Recibido: 08.02.01. Aceptado tras revisión externa sin modificaciones: 09.04.01.

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Agradecimientos. A los pacientes con EA y a los individuos que han participado en el estudio. A Pilar Viedma y Rafael Blesa, del Servicio de Neurología del Hospital Clínic de Barcelona, por facilitar las muestras. A Montse Gómez y Enric Doménech, por sus comentarios. Este trabajo contó con las ayudas de la Fundació La Marató de TV3 nº 2002/97 y 2102/97 (V. Nunes y J. Casademont) y de la CICYT: SAF 99-0079 a V. Nunes. Benjamín Rodríguez-Santiago es becario predoctoral de la Fundació La Marató de TV3.

tener efectos negativos en la célula aún no se ha podido confirmar claramente –dada la convivencia entre ADNmt normales y ADNmt mutados en una misma célula, debe haber un umbral a partir del cual los ADNmt mutados pueden provocar disfunciones en la mitocondria–, pero su acumulación se encuentra acelerada en el tejido cerebral de pacientes con EA [19]. Sin embargo, existen otros trabajos en los que no se han hallado pruebas de esta asociación entre la mitocondria y EA, cuestionando la relevancia de estas anomalías en el desarrollo de la enfermedad [30-35].

Como consecuencia de la disparidad en los resultados observados en la bibliografía, en lo referente a reordenamientos y mutaciones puntuales, decidimos evaluar el estado del ADNmt en muestras de varias regiones del cerebro (cerebelo, hipocampo, córtex frontal) y en sangre de pacientes con EA y de controles. Se estudió la incidencia de cuatro mutaciones puntuales distintas previamente asociadas a la EA: las variantes G3196A, A3397G, A4336G y G5460A/T [21-23] –localizadas en los genes ND1, 16SrARN, tARN^{Gln} y ND2, respectivamente–, mediante amplificación por PCR (reacción en cadena de la polimerasa) y digestión con enzimas de restricción. La existencia de reordenamientos en el ADNmt (deleciones y duplicaciones) se analizó mediante *Southern blot* e hibridación.

PACIENTES Y MÉTODOS

Muestras

Para el estudio se utilizaron muestras de cerebelo y córtex frontal de 12 pacientes afectos de EA y siete individuos control. También se obtuvieron muestras de hipocampo en 10 de los pacientes y cuatro de los controles. Estas muestras las proporcionó el Banco de Tejidos Neurológicos del Hospital Clínic-Universitat de Barcelona. En todos los casos se realizó un análisis histopatológico para confirmar la presencia de depósitos de proteína β-amiloide, que definen la EA. Las muestras control pertenecían a individuos sin demencia u otra enfermedad neurológica en los que, además, los análisis neuropatológicos excluyeron la presencia de placas seniles u ovillos neurofibrilares. En todas las muestras de tejido cerebral, el tiempo de congelación después de la muerte fue inferior a las 12 horas. El intervalo de edades fue 56-84 años en los pacientes de EA y 55-70 años en los individuos control. Además, se recogieron y analizaron muestras de sangre de 17 pacientes de EA vivos –intervalo de edades, 59-87–, diagnosticados mediante criterios comúnmente aceptados [36], y de 27 individuos control sin historial de enfermedad neurológica ni demencia –intervalo de edades, 58-94.

Todas las muestras de Alzheimer correspondían a formas esporádicas de la enfermedad. El consentimiento para el estudio se obtuvo de acuerdo con las normas del comité ético del centro, siguiendo la declaración de Helsinki.

Análisis del ADNmt

Preparación del ADN

Se procesaron 50 mg, aproximadamente, de cada muestra de región cerebral congelada y 15 ml de las muestras de sangre para la extracción de ADN total (genómico y mitocondrial), según procedimientos estándar modificados [37].

Análisis moleculares

Se digirieron 4 µg de ADN total de cada muestra con el enzima de restricción *Pvu* II (Gibco). Esta enzima corta el ADNmt por un solo punto y lo alinea. El ADN digerido se sometió a electroforesis en gel al 0,8% de agarosa y se transfirió a una membrana de nilón (Amersham), según el método de Ausubel et al [37].

Como sonda se utilizó ADNmt total a una concentración de 50 ng/µl marcado con α-^{32}P(dCTP), mediante la técnica de cebado aleatorio (del inglés, *random priming*) [38]. La hibridación del filtro con el ADNmt humano marcado y los subsiguientes lavados se llevaron a cabo siguiendo el protocolo descrito por el fabricante de la membrana, y se procedió a continuación a la autorradiografía del mismo.

PCR y análisis de las mutaciones puntuales

Cada mutación se analizó mediante amplificación por PCR de la región del ADNmt susceptible de presentar la mutación, seguida de una digestión con el enzima de restricción adecuado, tal y como se describe a continuación:

– *Mutaciones G3196A y A3397G*. A partir de 200 ng de ADN se amplificó un fragmento de 474 pb que contenía parte del gen ND1 y del gen 16SrRNA, bajo las siguientes condiciones: oligonucleótidos L·leu-1 –posición en la cadena *light* (L) del ADNmt 3164-3183– y H363 –nucleótidos (nt) 3618-3637 de la cadena *heavy* (H) a 1 mM, dNTPs a 200 mM y 1,5 unidades de *Taq* (Roche). Las condiciones de PCR fueron: 5 min a 95 °C, seguido de 35 ciclos compuestos de tres segmentos –35 s a 94 °C, 35 s a 56 °C y 35 s a 72 °C–, más una extensión final de 10 min a 72 °C. Una alícuota de la reacción se digirió con el enzima *Dde* I (Roche) para la mutación G3196A o con *Rsa* I (New England Biolabs) para la A3397G; los fragmentos se separaron en geles de acrilamida al 7%. La mutación A3397G crea una diana *Rsa* I, mientras que la G3196A anula una diana *Dde* I.

– *Mutación A4336G*. Un fragmento del gen tARN^{Gln} de 785 pb se amplificó de forma similar a lo explicado anteriormente, usando los oligonucleótidos L401 (nt 4013-4031) y H479 (nt 4797-4778). Las condiciones de PCR fueron: 5 min a 95 °C, 35 ciclos compuestos por 35 s a 94 °C, 35 s a 51 °C, 35 s a 72 °C, y finalmente una extensión de 10 min a 72 °C. La digestión se realizó con el enzima *Nla* III (New England Biolabs) y la electroforesis en un gel de acrilamida al 6%. La mutación A4336G crea una diana.

– *Mutación G5460A/T*. Los cambios en la posición 5460 se detectaron usando los oligonucleótidos L2 (nt 5440-5459) y H2 (nt 5898-5879) con las condiciones de PCR: 5 min a 95 °C, 35 ciclos compuestos por 35 s a 94 °C, 35 s a 59 °C, 35 s a 72 °C, y una extensión final de 10 min a 72 °C. L2 es un oligonucleótido de mutagénesis: en la posición que corresponde al nt 5458 de la cadena L del ADNmt se ha sustituido el nt normal (una T) por una G. Este cambio crea una diana *Hha* I (Amersham) en moléculas salvajes. Una A o una T en la posición 5460 anula esta diana; además, una T crea una diana para el enzima *Alu* I (Amersham).

Secuenciación

Para identificar los cambios en los patrones de bandas anómalos que no correspondían a las mutaciones a estudiar se realizó un análisis de secuencia: el producto de PCR del fragmento con patrón irregular se purificó mediante columna (Qiagen). La PCR de secuencia se realizó con el equipo *ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction* (Applied Biosystems), siguiendo las recomendaciones del fabricante. Los resultados se recogieron y analizaron con los programas de ordenador del equipo *ABI Prism* 377 *DNA Sequencer* (Applied Biosystems) y el programa *Sequencher* (Gene Codes Corporation).

RESULTADOS

Los análisis mediante *Southern blot* e hibridación de los ADNmt obtenidos de necropsias cerebrales y de sangre de casos de Alzheimer no mostraron presencia de deleciones u otros reordenamientos del ADNmt, tales como dimerizaciones o duplicaciones. En la figura se muestra una imagen típica del patrón encontrado. El ADN de las muestras, hibridado contra una sonda específica de ADNmt total, puso de manifiesto en todos los casos de EA la existencia de moléculas con una banda de 16,5 kb de tamaño, correspondiente a una población de moléculas de ADNmt íntegras. Para un mismo individuo tampoco se observaron diferencias en el patrón obtenido mediante *Southern blot*, en las muestras correspondientes a las diferentes regiones cerebrales analizadas.

Los resultados obtenidos en el estudio de mutaciones puntuales se encuentran resumidos en la tabla. Las mutaciones G3196A, A3397G y G5460A/T no estaban presentes en ninguna muestra, ya sea de tejidos o muestras de sangre, ni en pacientes o controles. La mutación A4336G se detectó en todas las regiones cerebrales de dos casos con EA y no apareció en ningún control de muestras de tejido. En muestras de sangre, la mutación A4336G apareció en un control, pero no se observó en ningún paciente.

Se detectaron patrones anómalos que no correspondían con las mutaciones estudiadas. Los cambios encontrados fueron los siguientes: el cambio A4136G se detectó en linfocitos de un control, en las tres regiones cerebrales de un control y de un paciente con EA; el cambio T4216C se observó en los

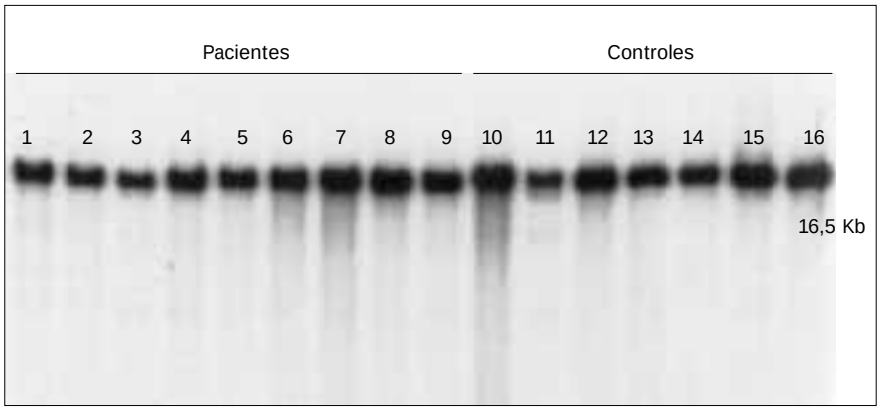

Figura. Ejemplo de imagen de *southern* de ADN extraído a partir de necropsias de cerebelo, digeridos con *Pvu* II e hibridados con una sonda de ADNmt total. En los *southern* de muestras de las otras regiones y de sangre se obtuvieron imágenes similares, con una banda única de 16,5 kb, correspondiente a ADNmt íntegro (carriles 1-9: pacientes con EA; carriles 10-16: controles).

Tabla. Frecuencia de mutaciones en el ADNmt de pacientes con EA y de controles.

Gen ADNmt	Variante	Cerebelo		Córtex frontal		Hipocampo		Sangre	
		C	P	C	P	C	P	C	P
16SrARN	G3196A	0/7	0/12	0/7	0/12	0/4	0/10	0/27	0/17
ND1	A3397G	0/7	0/12	0/7	0/12	0/4	0/10	0/27	0/17
$tARN^{Gln}$	A4336G	0/7	2/12	0/7	2/12	0/4	1/10	1/27	0/17
ND2	G5460A/T	0/7	0/12	0/7	0/12	0/4	0/10	0/27	0/17

C: controles; P: pacientes.

linfocitos de un control y de cuatro pacientes con EA, y también en las tres regiones de un caso con EA; por último, se detectaron el cambio G3423T en las tres regiones de seis individuos con EA y el cambio G4580A en otro individuo.

DISCUSIÓN

El presente estudio se realizó con el objetivo de observar el estado del ADNmt en cuanto a la posible presencia de mutaciones puntuales y reordenamientos en el tejido principalmente afectado en la EA, el cerebro. Para ello, se analizaron necropsias de cerebelo, hipocampo y córtex frontal tomadas de enfermos y se compararon con necropsias de individuos control.

La implicación mitocondrial en el desarrollo de la EA ha motivado diversas controversias. Se han descrito numerosas alteraciones en la función mitocondrial, pero aún está por aclarar si esas alteraciones desempeñan un papel primario o son un hecho secundario en la etiología de la enfermedad. Por tratarse de una enfermedad neurodegenerativa y progresiva, de años de duración, es fácil pensar que los defectos hallados en el metabolismo mitocondrial podrían ser consecuencia del progresivo deterioro al que se someten las principales áreas cerebrales afectadas. Diversos estudios han demostrado la presencia de factores perjudiciales para el ADNmt en enfermos de Alzheimer. Estos factores suponen un entorno potencialmente dañino para el metabolismo de la mitocondria: la presencia de proteína amiloide genera radicales libres y estrés oxidativo, con repercusiones sobre la cadena respiratoria y el ADNmt. A esto hay que añadir la práctica ausencia de mecanismos de reparación del ADNmt. Es por ello que resulta sorprendente no encontrar en las muestras analizadas –con confirmación histopatológica de EA– mayor presencia de mutaciones puntuales y reordenamientos en el ADNmt. No se puede descartar la existencia de moléculas delecionadas en cantidad inferior al 5% –nivel de resolución de la hibridación con sonda radioactiva. En cualquier caso, una proporción de este orden sería insuficiente para producir una disfunción de la mitocondria. Como se observa en la figura, la banda de 16,5 kb correspondiente a una población normal es muy abundante en todos los pacientes y muy similar a la de los individuos control. La cantidad de deleciones halladas en otros estudios varía según la zona cerebral analizada, siendo las áreas del córtex las más afectadas. En nuestro estudio, las tres regiones estudiadas no mostraron diferencias para un mismo individuo.

Trabajos previos han relacionado las mutaciones puntuales G3196A, A3397G, A4336G y G5460A/T con la EA [21-23]. Sin embargo, otros grupos no han encontrado esta relación [32-35]. Los datos de nuestro estudio tampoco aportan pruebas que apoyen la implicación de estas mutaciones en la enfermedad. No detectamos esas mutaciones en las muestras analizadas, con la excepción de la A4336G, descubierta en todas las áreas cerebrales de dos casos de EA. Esto parece sugerir que esta mutación puntual puede desempeñar algún papel en el cerebro de enfermos de Alzheimer. Para comprobar la relevancia de esta mutación en las áreas cerebrales sería preciso analizar más muestras, ya que los datos obtenidos no permiten establecer una significación estadística. Por otra parte, el hecho de hallar esta mutación en sangre de un control cuestiona su papel en la enfermedad y, eventualmente, podría tratarse de un polimorfismo. Como sugieren estudios previos [39], el cambio A4336G podría ser un marcador de la implicación del ADNmt en algunos casos de EA, pero es improbable que tenga un papel significativo en la mayoría de pacientes.

Aunque la sangre no es un tejido diana de la enfermedad y los defectos hallados en linfocitos pueden tener una importancia menor –si posteriormente no se confirman en tejido neurológico–, el hecho de que se hayan descrito algunas alteraciones mitocondriales en sangre de individuos con EA [25,26], unido a la posibilidad de disponer de muestras de pacientes vivos y a la idea de hallar un marcador para discriminar rápidamente a los pacientes, nos llevó a realizar los estudios con este tipo de muestras. Definitivamente, nuestros resultados no han permitido identificar ningún tipo de marcador para la enfermedad. Hay que indicar que las bandas observadas mediante hibridación correspondientes a ADNmt obtenido de sangre son más débiles que las procedentes de áreas de cerebro. Esto se explica sencillamente por la mayor presencia de mitocondrias en tejidos posmitóticos, como son el cerebro o el músculo esquelético, que presentan una mayor demanda energética de ATP.

Los cambios A4136G y T4216C encontrados –localizados en el gen mitocondrial ND1– se clasifican como polimorfismos en gran parte de la literatura, aunque algunos los consideran mutaciones LHON (del inglés, *Leber's Hereditary Optic Neuropathy*) de tipo secundario. Los cambios encontrados en las posiciones 3423 (gen mtND1) y 4580 (gen mtND2) se han descrito previamente como polimorfismos.

Los datos del presente artículo no permiten afirmar que en nuestras muestras con EA exista una alteración del genoma mitocondrial, en cuanto a la presencia de reordenamientos y de estas cuatro mutaciones puntuales. No obstante, otro estudio realizado con las mismas muestras apuntaba la posibilidad de que existieran diferencias en la cantidad de ADNmt (depleción), especialmente en la región del córtex frontal; en ésta, los pacientes de EA presentaban una reducción de hasta un 28% en su contenido de ADNmt, respecto a los controles [40]. Si las alteraciones descritas hasta el momento son primarias (patogénicas) o secundarias –consecuencia de la degeneración neuronal–, está por dilucidar. El estudio más exhaustivo de los defectos del ADNmt, junto a otros aspectos del metabolismo mitocondrial, incluida su relación con el genoma nuclear, debe servir para aclarar el papel que desempeñan las mitocondrias en las enfermedades neurodegenerativas y, en concreto, en la EA. En cualquier caso, el detenido conocimiento del estado mitocondrial deberá ayudar a comprender el delicado equilibrio que regula la pérdida selectiva de neuronas en la EA.

BIBLIOGRAFÍA

1. Dickson DW. The pathogenesis of senile plaques. J Neuropathol Exp Neurol 1997; 56: 321-39.
2. Chartier-Harlin MC, Crawford F, Houlden H, Warren A, Hughes D, Fidani L, et al. Early-onset Alzheimer's disease caused by mutations at codon 717 of the beta-amyloid precursor protein gene. Nature 1991; 353: 844-6.
3. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, et al. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 1991; 349: 704-10.
4. Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, et al. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science 1995; 269: 973-7.
5. Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, et al. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 1995; 375: 754-60.
6. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, et al. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 1993; 261: 921-3.
7. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, et al. Apolipoprotein E: high-avidity binding to beta-amyloid an increased frequency of type 4 allele in late-onset familial Alzheimer disease. Proc Natl Acad Sci U S A 1993; 90: 1977-81.
8. Christen Y. Oxidative stress and Alzheimer disease. Am J Clin Nutr 2000; 71 (Suppl): S621-9.
9. Price DL, Sisodia SS, Borchelt DR. Alzheimer disease-when and why? Nat Genet 1998; 19: 314-6.
10. Edland SD, Silverman JM, Peskind ER, Tsuang D, Wijsman E, Morris JC. Increased risk of dementia in mothers of Alzheimer's disease cases: evidence for maternal inheritance. Neurology 1996; 47: 254-6.
11. Trimmer PA, Swerdlow RH, Parks JK, Keeney P, Bennett JP Jr, Miller SW, et al. Abnormal mitochondrial morphology in sporadic Parkinson's and Alzheimer's disease cybrid cell lines. Exp Neurol 2000; 162: 37-50.
12. Sheehan JP, Swerdlow RH, Miller SW, Davis RE, Parks JK, Parker WD, et al. Calcium homeostasis and reactive oxygen species production in cells transformed by mitochondria from individuals with sporadic Alzheimer's disease. J Neurosci 1997; 17: 4612-22.
13. Suzuki A, Tsutomi Y, Yamamoto N, Shibutani T, Akahane K. Mitochondrial regulation of cell death: mitochondria are essential for procaspase 3-p21 complex formation to resist Fas-mediated cell death. Mol Cell Biol 1999; 19: 3842-7.
14. Deng G, Su JH, Ivins KJ, van Houten B, Cotman CW. Bcl-2 facilitates recovery from DNA damage after oxidative stress. Exp Neurol 1999; 159: 309-18.
15. Parker WD Jr, Parks J, Filley CM, Kleinschmidt-DeMasters BK. Electron transport chain defects in Alzheimer's disease brain. Neurology 1994; 44: 1090-6.
16. Mutisya EM, Bowling AC, Beal MF. Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. J Neurochem 1994; 63: 2179-84.
17. Redjems-Bennani N, Jeandel C, Lefebvre E, Blain H, Vidailhet M, Gueant JL. Abnormal substrate levels that depend upon mitochondrial function in cerebrospinal fluid from Alzheimer patients. Gerontology 1998; 44: 300-4.
18. Hamblet NS, Castora FJ. Elevated levels of the Kearns-Sayre syndrome mitochondrial DNA deletion in temporal cortex of Alzheimer's patients. Mutat Res 1997; 379: 253-62.
19. Corral-Debrinski M, Horton T, Lott MTDNA, et al. Marked changes in mitochondrial DNA deletion levels in Alzheimer brains. Genomics 1994; 23: 471-6.
20. Lin FH, Lin R, Wisniewski HM, Hwang YW, Grundke-Iqbal I, Healy-Louie G, et al. Detection of point mutations in codon 331 of mitochondrial NADH dehydrogenase subunit 2 in Alzheimer's brains. Biochem Biophys Res Commun 1992; 182: 238-46.
21. Shoffner JM, Brown MD, Torroni A, Lott MT, Cabell MF, Mirra SS, et al. Mitochondrial DNA variants observed in Alzheimer disease and Parkinson disease patients. Genomics 1993; 17: 171-84.
22. Hutchin T, Cortopassi G. A mitochondrial DNA clone is associated with increased risk for Alzheimer disease. Proc Natl Acad Sci U S A 1995; 92: 6892-5.
23. Egensperger R, Kosel S, Schnopp NM, Mehraein P, Graeber MB. Association of the mitochondrial tRNA(A4336G) mutation with Alzheimer's and Parkinson's diseases. Neuropathol Appl Neurobiol 1997; 23: 315-21.
24. Aksenov MY, Tucker HM, Nair P, Aksenova MV, Butterfield DA, Estus S, et al. The expression of several mitochondrial and nuclear genes encoding the subunits of electron transport chain enzyme complexes, cytochrome c oxidase, and NADH dehydrogenase, in different brain regions in Alzheimer's disease. Neurochem Res 1999; 24: 767-74.
25. Parker WD Jr. Cytochrome oxidase deficiency in Alzheimer's disease. Ann N Y Acad Sci 1991; 640: 59-64.
26. Mecocci P, Polidori MC, Ingegni T, Cherubini A, Chionne F, Cecchetti R, et al. Oxidative damage to mtDNA in lymphocytes from AD patients. Neurology 1998; 51: 1014-7.
27. DiMauro S, Wallace DC. Mitochondrial DNA in Human Pathology. New York: Raven Press; 1993.
28. Soong NW, Hinton DR, Cortopassi G, Arnheim N. Mosaicism for a specific somatic mitochondrial DNA mutation in adult human brain. Nat Genet 1992; 2: 318-23.
29. Corral-Debrinski M, Horton T, Lott MT, Shoffner JM, Beal MF, Wallace DC. Mitochondrial DNA deletions in human brain: regional variability and increase with advanced age. Nat Genet 1992; 2: 324-9.
30. Lezza AM, Mecocci P, Cormio A, Beal MF, Cherubini A, Cantatore P, et al. Mitochondrial DNA 4977 bp deletion and OH8dG levels correlate in the brain of aged subjects but not Alzheimer's disease patients. FASEB J 1999; 13: 1083-8.
31. Blanchard BJ, Park T, Fripp WJ, Lerman LS, Ingram VM. A mitochondrial DNA deletion in normally aging and in Alzheimer brain tissue. Neuroreport 1993; 4: 799-802.
32. Janetzky B, Schmid C, Bischof F, Frolich L, Gsell W, Kalaria RN, et al. Investigations on the point mutations at nt 5460 of the mtDNA in different neurodegenerative and neuromuscular diseases. Eur Neurol 1996; 36: 149-53.
33. Kosel S, Egensperger R, Mehraein P, Graeber MB. No association of mutations at nucleotide 5460 of mitochondrial NADH dehydrogenase with Alzheimer's disease. Biochem Biophys Res Commun 1994; 203: 745-9.
34. Wragg MA, Talbot CJ, Morris JC, Lendon CL, Goate AM. No association found between Alzheimer's disease and a mitochondrial tRNA glutamine gene variant. Neurosci Lett 1995; 201: 107-10.
35. Hutchin TP, Heath PR, Pearson RC, Sinclair AJ. Mitochondrial DNA mutations in Alzheimer's disease. Biochem Biophys Res Commun 1997; 241: 221-5.
36. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 1984; 34: 939-44.
37. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current protocols in Molecular Biology. New York: Willey; 1993.
38. Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 1983; 132: 6-13.
39. Bonilla E, Tanji K, Hirano M, Vu TH, DiMauro S, Schon EA. Mitochondrial involvement in Alzheimer's disease. Biochem Bioph Acta 1999; 1410: 171-82
40. Rodríguez-Santiago B, Casademont J, Nunes V. Is mitochondrial DNA depletion involved in Alzheimer's disease? Eur J Hum Genet 2001; 9: 279-85.

¿EXISTE RELACIÓN ENTRE LA ENFERMEDAD DE ALZHEIMER Y DEFECTOS EN EL ADN MITOCONDRIAL?

Resumen. Introducción. *Varios estudios han relacionado la enfermedad de Alzheimer (EA) con defectos mitocondriales. Tales defectos incluyen anomalías de tipo estructural, bioquímico y genético. Entre las de tipo genético destacan los reordenamientos y las mutaciones puntuales descritas en el DNA mitocondrial (ADNmt).* Objetivo. *Estudiar la incidencia de reordenamientos y cuatro mutaciones puntuales en el ADNmt de pacientes con EA y determinar las posibles diferencias respecto a individuos control.* Pacientes y métodos. *Necropsias de cerebelo, córtex frontal e hipocampo de pacientes con EA y controles. También se dispuso de sangre de enfermos vivos diagnosticados de EA y de controles. Las muestras se analizaron mediante Southern blot, hibridando con una sonda mitocondrial. También se analizaron las mutaciones puntuales G3196A, A3397G, A4336G y G5460A/T.* Resultados. *No se observaron diferencias entre pacientes y controles, ni en tejidos cerebrales ni en sangre en los análisis realizados mediante southern. No se halló asociación entre las mutaciones puntuales analizadas y la EA en nuestras muestras.* Conclusiones. *Los resultados obtenidos no apoyan la hipótesis de una implicación mitocondrial en la EA, en cuanto a reordenamientos y las cuatro mutaciones puntuales analizados en el ADNmt en nuestras muestras, lo cual no descarta la posible existencia de otras mutaciones puntuales no analizadas y/u otros defectos mitocondriales que contribuyan al desarrollo de la EA. [REV NEUROL 2001; 33: 301-5]*

Palabras clave. *Cadena respiratoria. Deleciones. Enfermedad de Alzheimer. Mitocondria. ADNmt. Mutaciones puntuales. Reordenamientos.*

EXISTIRÁ UMA RELAÇÃO ENTRE A DOENÇA DE ALZHEIMER E OS DEFEITOS NO ADN MITOCONDRIAL?

Resumo. Introdução. *Vários estudos relacionaram a doença de Alzheimer (DA) com defeitos mitocondriais. Esses defeitos incluem anomalias de tipo estrutural, bioquímico e genético. Entre as anomalias de tipo genético, destacam-se as reordenações e as mutações pontuais descritas no ADN mitocondrial (mtADN).* Objectivo. *Estudar a incidência de reordenação e 4 mutações pontuais no mtADN em doentes com DA, e determinar as possíveis diferenças em relação aos indivíduos de controlo.* Doentes e métodos. *Necropsias do cerebelo, córtex frontal e hipocampo de doentes com DA e controlos. Também se dispôs do sangue de doentes vivos diagnosticados com DA, e de controlos. As amostras foram analisadas por Southern blot utilizando uma sonda mitocondrial para hibridar. Foram analisadas as mutações pontuais G3196A, A3397G, A43365G e G5460A/T.* Resultados. *Não se observaram diferenças entre os doentes e os controlos, nas análises realizadas por Southern, no tecido cerebral, nem no sangue. Não se achou uma associação entre as mutações pontuais analisadas e a DA nas nossas amostras.* Conclusões. *Os resultados obtidos não sustentam a hipótese de um envolvimento mitocondrial na DA, quanto à reordenação e às 4 mutações pontuais analisadas no mtADN nas nossas amostras, o que não exclui a possível existência de outras mutações pontuais não analisadas e/ou de outros defeitos mitocondriais que contribuam para o desenvolvimento da DA. [REV NEUROL 2001; 33: 301-5]*

Palavras chave. *Cadeia respiratória. Delecções. Doença de Alzheimer. Mitocôndria. mtADN. Mutações pontuais. Reordenação.*

Experimentos adicionales

Nuestro grupo también ha analizado mediante secuenciación otras regiones del mtDNA diferentes a las descritas en el artículo. Una de estas regiones fue el fragmento entre las posiciones 111 y 1220 del mtDNA que incluye la región control (CR). También se analizó la secuencia de fragmentos del mtDNA en las regiones (3.330-3.6379); (15.701-16.039) y (13.473-13.970); en las que se ha descrito la presencia de variantes polimórficas que determinan el haplogrupo de mtDNA asociado a pacientes con EAE (http://www.mitomap.org). Los resultados obtenidos no proporcionaron diferencias significativas entre pacientes y controles, no detectando ninguna variante a destacar en los pacientes analizados.

También se determinó el genotipo APOE de las muestras disponibles mediante el producto "*LightCycler – Apo E Mutation Detection Kit*" (Roche). En la **tabla 10** se muestra el genotipo de APOE de las muestras analizadas procedentes de cerebro de individuos con EA.

Comentarios adicionales

La idea básica es que los productos de transcripción o de traducción sintetizados a partir de genes de mtDNA dañados oxidativamente o mutados pueden afectar negativamente la síntesis proteica y la función respiratoria de los enzimas. Una de las cuestiones que surgen habitualmente en los descubrimientos de mutaciones puntuales asociadas a la EA es la siguiente: ¿El aumento de mutaciones somáticas en el cerebro de pacientes con EA es simplemente un reflejo de envejecimiento acelerado o es un fenómeno patológico distinto? Es necesaria la realización de estudios adicionales que revelen si la acumulación de mtDNA mutante, incluso alcanzando proporciones altas, es capaz de producir un fenotipo clínico. Hay que recordar que las mutaciones puntuales del mtDNA descritas en enfermedades humanas son funcionalmente recesivas, en el sentido que es necesario por lo menos un 60% de dosis génica mutante para provocar un fenotipo discernible, al menos en modelos de laboratorio (Hayashi *et al*. 1991; Schon *et al*. 1997). En la mayoría de trabajos que describen mutaciones puntuales del mtDNA asociadas a la EA o bien no se aporta el grado de heteroplasmia de la mutación hallada, o bien no se correlaciona con el efecto producido sobre la CRM, en la mayoría de ocasiones no se hace ninguna de las dos cosas.

La contribución de mutaciones en el mtDNA, somáticas o heredadas, ha sido descrita en ciertos casos de EA, pero la determinación definitiva de su impacto debe ser corroborada con el estudio de la CRM. La asociación entre defectos de la CRM y mtDNA ha podido establecerse en diversos estudios *in vitro* con cíbridos elaborados con mtDNA de pacientes, pero el efecto de una mutación o cambio concreto y específico del mtDNA no ha sido evaluado. En nuestro caso, los resultados obtenidos no han justificado la práctica de estudios *in vitro* al no hallar alteraciones significativas en el mtDNA de pacientes con EA.

Otro aspecto que se ha debatido en la literatura es la relación entre determinadas variantes del mtDNA y el genotipo de APOE. Por ejemplo, Edland y colaboradores observaron que el polimorfismo A4336G del mtDNA era más frecuente en la sangre de individuos con EA portadores del alelo ε4 de

APOE (Edland *et al*. 2002). Esta observación no fue corroborada por otros grupos que estudiaron muestras procedentes de cerebro y de sangre (Zsurka *et al*. 1998; Chinnery *et al*. 2000). El estudio del genotipo APOE en las muestras analizadas por nuestro grupo mostraba que los dos pacientes portadores del polimorfismo A4336G también tenían un alelo ε4, lo que parece apoyar la observación de Edland y colaboradores, aunque se debería analizar un número mayor de individuos para obtener alguna conclusión más precisa sobre esta asociación. No se pudo establecer ninguna relación entre el alelo ε4 de APOE presente en 6 de los 21 individuos con EA y la variante A4336G en las muestras de sangre al no detectarse el polimorfismo en ningún caso.

TABLA 10. GENOTIPO APOE

Paciente EA	Genotipo APOE	A4336G
1	32	-
2	43	-
3	43	-
4	43	-
5	43	-
6	33	-
7	33	-
8	43	-
9	43	-
10	43	-
11	43	+
12	42	+

No parece que una mutación puntual en el mtDNA por si sola pueda influir sobre la aparición o el desarrollo de la EA. Otra posibilidad es que el linaje de mtDNA del individuo influya sobre la EA no debe obviarse a la vista de los estudios que indican un efecto protector de determinados haplogrupos sobre la EA y la EP que además estarían asociados a una longevidad mayor (Chagnon *et al*. 1999; Coskun *et al*. 2003; van der Walt *et al*. 2004).

Recientemente Coskun *et al*. han analizado mediante secuenciación la CR del mtDNA extraído de córtex frontal de pacientes con EA y de controles (Coskun *et al*. 2004). El análisis de este grupo revela la presencia de diversos cambios (en un grado de heteroplasmia cercano al 70% en algunos casos) en las muestras procedentes de pacientes, siendo el más destacado el cambio T414G. Este cambio afecta al lugar de unión del mtTFA del promotor de la cadena ligera (P_L, ver **figura 12**) y tiene como resultado una reducción de la cantidad de mRNA de ND6 y de mtDNA en los pacientes analizados. Según los autores estas reducciones podrían provocar la disminución de la actividad de la fosforilación oxidativa y por tanto pueden ser responsables de algunos de los defectos mitocondriales observados en la EA.

Esta región correspondiente a la CR del mtDNA también fue secuenciada en las muestras recogidas para los estudios recopilados en la presente memoria. En las secuencias realizadas no se detectó la presencia de cambios en la CR, ni en las muestras procedentes de necropsias de cerebro ni en las de sangre. Esta falta de cambios es consistente con el estudio de la expresión génica y transcripción recogidos en la presente memoria, en el que pacientes y controles mostraban resultados similares; y es consistente con el hallazgo de Coskun *et al.*, ya que este grupo ha podido correlacionar los cambios observados con cambios en la cantidad de tránscrito de ND6 y de mtDNA. Si se confirma el efecto sobre la actividad de la CRM, el análisis de la CR del mtDNA debería ser considerado de forma importante en el estudio de la EAE, teniendo en cuenta la relación entre el grado de heteroplasmia de la mutación y el impacto en la actividad de la CRM. El resultado obtenido en

nuestro laboratorio concuerda con otros trabajos (Chinnery *et al*. 2001; Simon *et al*. 2001). En ningún caso se encontraron evidencias significativas de que se produzca una acumulación de mutaciones somáticas en la CR del mtDNA de cerebro de personas de edad avanzada ni de individuos con EA, o con demencia con cuerpos de Lewy, o con EP, o en controles. Particularmente, la mutación T414G (el cambio más destacado en el trabajo de *Coskun et al*.) no fue detectada en el mtDNA procedente del cerebro de ninguno de los individuos analizados en nuestro laboratorio ni por los grupos citados (Chinnery *et al*. 2001; Simon *et al*. 2001). Con anterioridad, sí que se habían detectado mutaciones puntuales en la CR de mtDNA extraído de fibroblastos humanos de individuos de edad avanzada (Michikawa *et al*. 1999). Estas mutaciones no se detectaron en controles jóvenes y los estudios longitudinales mostraban que aparecían cuando la edad aumentaba. En concreto, la mutación T414G alcanzaba grados altos de heteroplasmia (por encima del 50%) y se detectó en fibroblastos de más de la mitad de los controles de edad avanzada. En este estudio las mutaciones no fueron asociadas a efectos fenotípicos obvios, pero el hecho de hallarlas en una región crucial para la replicación del mtDNA y la transcripción no descartaba la posibilidad de tener un efecto negativo en la función mitocondrial. Por este motivo y como los experimentos iniciales se llevaron a cabo en fibroblastos (en los que la proliferación celular continúa a lo largo de la vida) se impulsó el estudio de la CR en tejidos post-mitóticos para observar el significado de estas mutaciones en el cerebro de individuos con una enfermedad neurodegenerativa y con edad avanzada.

Las muestras analizadas en este trabajo se estudiaron inicialmente mediante la técnica de PCR larga para observar la integridad del mtDNA. Los resultados obtenidos con esta técnica mostraban la presencia de varias deleciones en muchos individuos con EA y también en algunos controles. La observación detallada de los geles en los que se separaban las moléculas de mtDNA amplificadas con la PCR larga revelaba una señal fuerte para la molécula de mtDNA íntegra de 16,5-kb en la mayoría de las muestras analizadas. Esta fuerte señal también se hallaba en muestras en las que se observaba alguna deleción. Esta observación por si sola cuestiona el posible efecto en el fenotipo de esas deleciones cuando coexisten con una abundante población de moléculas salvajes de mtDNA. La detección de esas moléculas delecionadas en la PCR larga no se confirmó mediante Southern. Es posible que en las muestras hubiera moléculas delecionadas y que no se detectaran quizás porque estaban en una cantidad tan pequeña que escapa al límite de resolución de la técnica de Southern blot (alrededor del 5% del total). Sin embargo, puesto que la señal correspondiente a la población de moléculas de mtDNA de 16,5-kb era tan fuerte (ver **figura** del artículo), no parece que esas moléculas delecionadas, en caso de existir, puedan ser responsables de manifestar ningún defecto bioquímico o clínico.

El reordenamiento que se ha descrito más frecuentemente en la EA ha sido la deleción común. La contribución de la deleción común es objeto de controversia por problemas metodológicos. El aumento de moléculas de mtDNA con la deleción común ha sido puesto en duda por algunos autores como signo de algún cambio patogénico (Lightowlers *et al*. 1999). La mayoría de los estudios en pacientes con EA se han realizado mediante técnicas basadas en la PCR (PCR semicuantitativa y PCR larga) obviando en general el análisis mediante la técnica de Southern o la secuenciación. El análisis

mediante Southern blot puede mostrar de forma directa el perfil cualitativo y cuantitativo de las moléculas de mtDNA presentes en una muestra de DNA. El incoveniente de la PCR larga es la frecuente aparición de bandas más pequeñas, a menudo artefactuales. Estas bandas pueden ser interpretadas equivocadamente como resultado de deleciones ya que, en general, posteriormente no se confirman al analizar mediante Southern o secuenciación. En algunos trabajos se recomienda que no se use la PCR larga para el diagnóstico (Kajander *et al*. 1999a) y se propone que, en todo caso, se amplifique mediante PCR larga diversas diluciones de la muestra y se confirme los resultados positivos mediante Southern blot siempre que sea posible (Kajander *et al*. 1999b). La aparición de artefactos y errores, tanto en PCR semicuantitativa como en PCR larga, es frecuente y por tanto la interpretación de los resultados debe ser hecha con precaución; la presencia de cantidades elevadas de moléculas delecionadas se puede aceptar plenamente si el estudio se apoya con otros métodos independientes o si se acompaña con muestras controles y de calibración rigurosas. Por lo tanto, el estudio del mtDNA mediante Southern, siempre que la cantidad de DNA disponible lo permita, debería ser un requisito previo para proceder con otros análisis cualitativos y cuantitativos mediante técnicas basadas en la PCR.

8.2. Is mitochondrial DNA depletion involved in Alzheimer's disease?

European Journal of Human Genetics (2001) 9, 279-285.

Benjamín Rodríguez-Santiago, Jordi Casademont, Virginia Nunes.

Contribución del doctorando:

Puesta a punto de un nuevo método de cuantificación del mtDNA mediante PCR en tiempo real para estudiar el fenómeno de la depleción. Diseño del experimento y obtención de rectas estándar de los genes mtND2 y el gen nuclear ribosómico 18S.

Trabajo experimental con las muestras de pacientes y controles para cuantificar relativamente el mtDNA. Cálculo de los ratios mtDNA/nDNA mediante las rectas patrón obtenidas a partir de los estándares.

Escritura del manuscrito, tabla 1 y confección de las figuras 1 y 2 del artículo.

European Journal of Human Genetics (2001) 9, 279–285

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ARTICLE

Is mitochondrial DNA depletion involved in Alzheimer's disease?

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Several studies have suggested that mitochondrial metabolism disturbances and mitochondrial DNA (mtDNA) abnormalities may contribute to the progression of the pathology of Alzheimer's disease (AD). In this study we have investigated whether the amount of mtDNA is modified in different brain regions (cerebellum, hippocampus and frontal cortex) of confirmed AD necropsies and in blood of living AD patients. We used a real-time PCR method to analyse the mtDNA relative abundance in brain regions from 12 AD and seven controls and from a group of blood samples (17 living AD patients and 11 controls). MtDNA from blood samples together with hippocampus and cerebellum brain areas did not show differences between controls and AD. However, AD patients showed a 28% decrease in the amount of mtDNA in the frontal cortex when compared to controls for this specific area. Since frontal cortex is a severely affected region in AD, our results support the hypothesis that mitochondrial defects may play a role in the pathogenesis of AD. *European Journal of Human Genetics* (2001) **9,** 279–285.

Keywords: mtDNA; depletion; mitochondria; Alzheimer's disease; brain

Introduction

Alzheimer's disease (AD) is one of the major causes of dementia. It is an age-related condition affecting 11% of the population over 65 years of age and 50% over age 85. AD is best characterised as a chronic brain disorder, having a lengthy pre-clinical phase followed by a malignant stage associated with neuronal degeneration, the loss of specific synaptic connections and the progressive erosion of higher cognitive functions.[1] AD has two major neuropathological hallmarks: an abundance of extracellular amyloid deposits, known as senile plaques, and fibrillar protein deposits within neurons, known as neurofibrillary tangles.[2]

The fundamental reason for the degeneration of nerve cells and synaptic connections that underlies the emergence of dementia has been elusive. It is widely considered that genetic factors, acting independently or in concert with other genetic and/or environmental factors, modify the risk of developing the disease.[3] Many genes associated with the disease have been identified. Some of these genes are involved in early onset forms of the disease and have a direct causal effect: The amyloid precursor protein (APP) gene located on chromosome 21 and presenilin genes 1 and 2 located on chromosome 14 and 1 respectively. The apolipoprotein E (apo E) gene (located on chromosome 19), the α_2-macroglobulin gene located on chromosome 12, and other unidentified genes may determine susceptibility in late-onset forms and sporadic cases.[4]

On the other hand, several studies have suggested that mitochondrial dysfunction may contribute to the progression of AD. Some evidence includes defects in the electron transport chain,[5] decreased cytochrome oxidase activity,[6] impairment of mitochondrial oxidative metabolism,[7] mtDNA point mutations,[8,9] rearrangements and deletions,[10,11] and decreased expression of genes.[12,13] Such defects could interfere with other features in which mitochondria are involved: Calcium homeostasis,[14] apoptotic pathways,[15,16] reactive oxygen species (ROS) generation and amyloid metabolism,[16] leading to a number of deleterious consequences. These features could form a vicious circle, with further impairment of the function of the respiratory machinery which, in turn, promotes the generation of more

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Received 25 September 2000; revised 9 December 2000; accepted 2 January 2001

free radicals, oxidative damage to membrane phospholipids and proteins, and more mtDNA mutations in a sort of *catastrophic* vicious cycle, leading to an increased neuronal susceptibility to cell death.

The reduction in mtDNA copy number (mtDNA depletion) has been described in several pediatric syndromes,[17–19] and in some toxic situations,[20,21] with an expanding spectrum of clinical presentations, including central nervous system involvement.[22] To our knowledge, mtDNA depletion has not been tested in neurodegenerative disorders, and specifically in AD, perhaps because up to now, the diagnosis of mtDNA depletion diseases has been based only on Southern blot analyses hybridising simultaneously with mtDNA and r18S probes.[17,22–24] Such a technique requires a relatively high amount of DNA, is difficult to standardise and is susceptible to misinterpretations. In the present work we have used a real time PCR quantification method based on Lightcycler technology (Roche Molecular Biochemicals, Germany) to investigate mtDNA in blood samples of 17 Alzheimer patients and 11 controls and in post-mortem samples from different brain regions of 12 Alzheimer patients and seven controls. Additionally, and although mtDNA depletion in blood has not been described in any disease, some authors have reported cytochrome oxidase deficiency[25] and oxidative damage to mtDNA in lymphocytes from AD patients.[26] Therefore, we have also performed the analysis in blood in order to look for possible defects in the levels of mtDNA in living AD patients.

Materials and methods

Subjects

Brain samples of cerebellum, hippocampus and frontal cortex from 12 histopathologically confirmed AD patients and from seven controls were obtained from the Neurologic Tissue Bank of the Hospital Clínic-Universitat de Barcelona. Areas finally analysed from each subject are summarised in Table 1. In all cases the time between death and frozen storage of neurological tissues was inferior to 12 h. The age range was 56–84 years for AD and 55–70 years for controls. Control

Table 1 MtND2/18S ratios and statistical data in blood and in the different brain areas

		Brain					
		MtND2/r18S ratio				*Blood*	
Subject	*Age*	*Cerebellum*	*Hippocampus*	*Cortex*	*Subject*	*Age*	*MtND2/r18S ratio*
Controls							
1	69	0.29	2.63	3.00	11	66	0.69
2	69	0.36	0.63	4.52	12	76	0.29
3	67	0.48	6.06	7.58	13	82	0.13
4	63	0.75	2.66	3.87	14	79	0.07
5	55	0.72		2.49	15	74	0.02
6	66	0.75		5.63	16	78	0.03
7	70	0.54		4.46	17	80	0.15
					18	77	0.04
					19	67	0.11
					20	61	0.06
					21	76	0.06
Mean	65.57	0.56	3.00	4.51		73.90	0.16
SD	5.22	0.19	2.25	1.71		6.63	0.20
Patients							
1	73	0.41	1.82	3.23	13	67	0.10
2	68	0.51	2.38	2.13	14	71	0.37
3	79	0.54	1.58	1.57	15	73	0.17
4	78	0.64	4.21	4.97	16	87	0.42
5	56	0.73	1.04	2.48	17	69	0.74
6	76	0.75	6.06	8.00	18	84	0.11
7	64	0.34	1.31	1.60	19	83	0.32
8	80	0.43	2.15	4.94	20	59	0.38
9	62	0.54	3.46	4.31	21	70	0.17
10	84	0.53	1.16	1.91	22	76	0.14
11	82	0.24		2.61	23	87	0.04
12	66	0.59		1.24	24	79	0.09
					25	83	0.09
					26	82	0.04
					27	78	0.03
					28	69	0.03
					29	71	0.05
Mean	72.33	0.54	2.61	3.25		75.36	0.20
SD	8.95	0.15	1.67	1.98		7.96	0.19

Each ratio results from the mean of two experiments (typical replicate CV was 1–12%) for each sample. (SD: standard deviation).

samples belonged to individuals without dementia or other neurological disease in whom the neuropathological analyses excluded the presence of senile plaques and neurofibrillary tangles.

Blood samples of 17 living AD patients (age range: 59 – 87) diagnosed by standard neurological criteria[27] and from 11 age-matched controls (age range: 61 – 80) were also analysed.

All AD patients were sporadic cases. The subjects' consent was obtained according to the declaration of Helsinki and following the rules of the Ethical Committee of the Centre.

DNA analyses

Total DNA was prepared from 50 mg of brain necropsy tissue and from 20 ml of blood following standard procedures based on phenol-chloroform extraction. A rapid and sensitive PCR real time method was used for quantification of mtDNA and nuclear DNA (represented by the ribosomal 18S genes). The method used a double-stranded DNA dye (SYBR Green I) to continuously monitor product formation and was able to quantify samples ranging up to 4 log units in concentration. The sensitivity of SYBR Green I detection is limited by non-specific product formation, which is monitored by fluorescence acquisition at temperatures at which only specific products are double-stranded.[28] The values found in each sample for the two genes (representative of mtDNA and nuclear DNA, respectively) allowed the calculation of a ratio mtDNA/nDNA to make comparisons between the different groups.

Measurement of mtND2 and r18S sequences by continuous monitoring of PCR

Real time PCR was performed in a fluorescence temperature cycler Lightcycler (Roche Molecular Biochemicals, Germany). Amplifications were carried out in a 20-μl final volume containing 3 mM $MgCl_2$ for mtND2 or 2 mM for r18S, 0.5 μM of each primer and 2 μl of the Lightcycler-FastStart DNA Master SYBR Green I mix. The oligonucleotide primers used to detect the mtND2 sequences were L456 (5′-GCCCTAGAAATAAACATGCTA-3′) and H479 (5′-GGGCTATTCCTAGTTTTATT-3′). The oligonucleotide primers used to detect the r18S sequences were 18SFOR (5′-ACGGACCAGAGCGAAAGCAT-3′) and 18SREV (5′-GGACATCTAAGGGCATCACAGAC-3′). The mtND2 PCR program included: initial denaturation at 95°C for 10 min followed by 40 amplification cycles consisting of heating at 20°C/s to 94°C with a 1-s hold, annealing at 20°C/s to 53°C with a 10-s hold and extension at 20°C/s to 72°C with a 10-s hold. The fluorescent product was detected at the last step of each cycle by single acquisition. After amplification, a melting curve was acquired by heating the product at 20°C/s to 95°C, cooling at 20°C/s to 65°C, and slowly heating it at 0.2°C/s to 94°C with continuous fluorescence collection. Melting curves were used to determine the specificity of the PCR products.[29] The r18S PCR program was performed in the same conditions as the ND2 program, except for: the annealing temperature (65°C), one melting curve acquisition step (cooling at 20°C/s to 72°C), and the number of cycles (45 in the r18S PCR program). Variations in sample loading or due to PCR tube-to-tube efficiency were corrected by performing two replicates for each standard and unknown sample.

Standard design

A known amount of human DNA (initial concentration of 15 ng/μl) from the Lightcycler control Kit was serially diluted and amplified for the mtND2 and the r18S regions to obtain two standard log-linear regression lines. The Lightcycler software first normalises each sample by background substraction. Then, a fluorescence threshold ($\cong$5% of full scale) is used to determine fractional cycle numbers that correlate inversely to the log of the initial template concentration. The least-squares best fit of the standards is used to calculate the amount of template initially present in the samples. Typical standard curves obtained for mtND2 and r18S are represented in Figure 1A,B.

Statistical analysis

The mean value of the two replicates (typical replicate coefficients of variation (CV) were 1 – 12%) for each sample was used to determine the values of mtDNA and r18S amount. The mtND2/18S mean ratios of each group were used for comparisons between them. An unpaired *t*-test previous verification that the samples had the same variance (Levene) was used to compare controls and AD patients.

Results

Amplification of mtND2 and r18S derived from amplifying fourfold dilutions of standard DNA provided two log-linear control standard lines (r^2=0.99 for both cases, see Figure 1C,D). DNA samples were amplified following the same standard conditions by continuously monitored PCR. The reason for choosing mtDNA primers in the ND2 gene was to avoid the common mtDNA deletion region. Furthermore, the ND2 gene is close to the light chain origin of replication (which is an area usually not involved in mtDNA deletions) and no nuclear pseudogene has been described in this region. The mtDNA/r18S quantification is found by determining the cycle number at which the product fluorescence becomes greater than a defined threshold. The method is based on the premise that the greater the quantity of initial DNA template in a sample, the fewer the PCR cycles required to reach this threshold,[28] as it can be seen in Figure 1. Also, the results of the two replicates of standard samples can be observed. The specificity of the products was assessed by monitoring the melting curve acquisition (Figure 2).

Since the quantification of the r18S housekeeping gene is used to standardise the results, the mtND2/r18S ratio can be used to standardise the relative mtDNA abundance in each sample such that the relative mtDNA contents of various

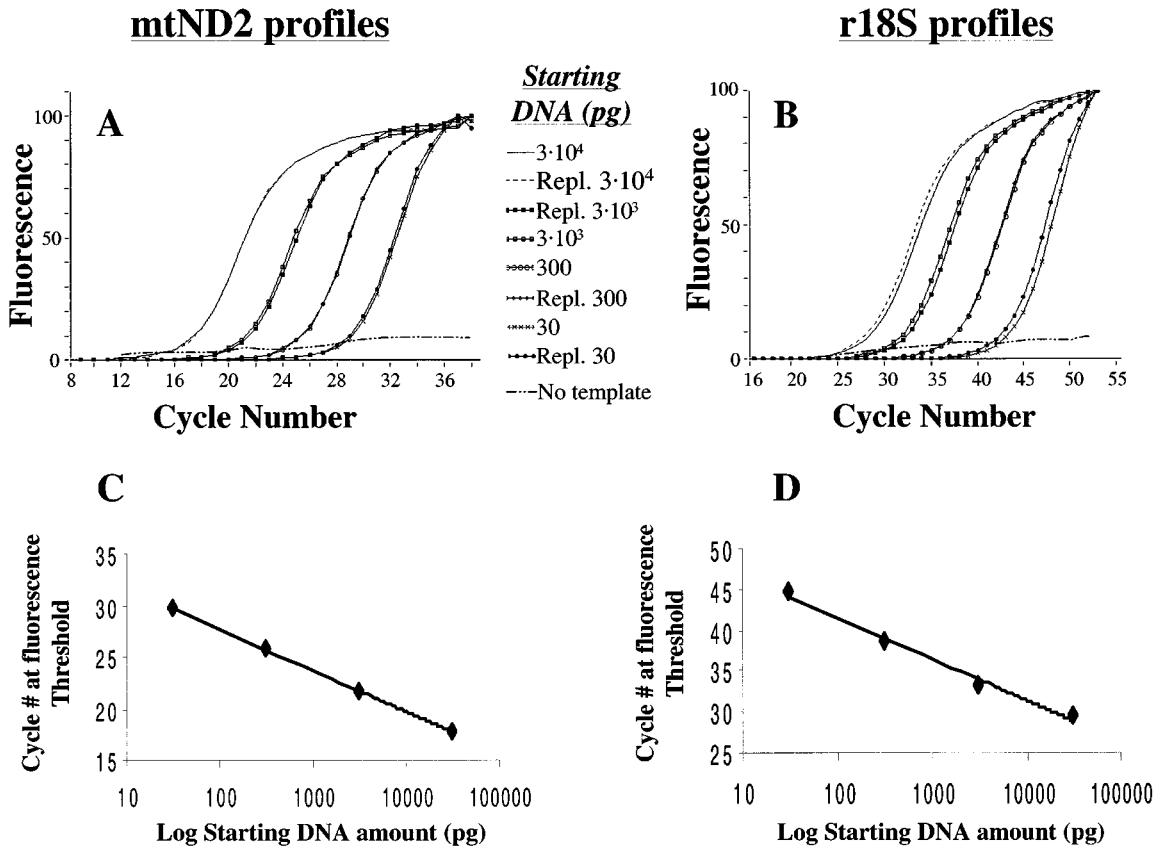

Figure 1 Fluorescent-amplification profiles and linear regression lines for mtND2 and r18S sequences. **A, B** Background-subtracted fluorescent emission of samples plotted as a function of cycle number. Serially dilluted samples of known DNA template were amplified for **A** mtND2 and **B** r18S sequences as described in Materials and methods. **C, D** These panels depict the fluorescence threshold for the samples presented in **A** and **B** plotted as a function of the starting DNA amount. The solid line indicates a linear regression fit to the data.

samples can be compared. The mtND2/r18S ratios calculated as the mean of two experiments for each sample are compiled in Table 1. When the groups were compared it could be observed that patients and controls presented similar amounts of mtDNA in cerebellum and hippocampus brain regions and in blood (Figure 3 and Table 1). On the other hand, AD patients showed a 28% reduction of their mtDNA amount in frontal cortex, although this difference did not reach statistical significance (P=0.18). No presence of large rearrangements were observed by Southern blot in any sample (data not shown).

Discussion

Although Alzheimer's disease is no longer the condition described in texts written some 10 years ago the aetiology of the disease remains enigmatic. The pathogenic pathways involved in the development of brain dysfunction in AD are much debated in the literature. Particularly, involvement of mitochondrial dysfunction remains controversial. Whereas it is clear that a primary mtDNA defect is not the substrate of sporadic cases of AD, the eventual contribution of mitochondrial dysfunction to worsen the clinical features is a possibility that can not be ruled out.

The objective of the present study was to assess whether mtDNA depletion, one increasingly recognised condition among the various molecular defects found in mitochondrial disorders, could be present in AD. Such studies can now rely on real-time quantification PCR technology which eludes a series of methodological and interpretational problems presented by the classical Southern blot analysis. In this way, the rapidity, reproducibility and specially the sensitivity of this PCR method makes it the technique of choice for detecting mtDNA depletion.

Although no depletion of mtDNA in lymphocytes has ever been described, previous studies revealing abnormalities in lymphocytes from AD patients[25,26] and the convenience of finding a parameter that could easily discriminate individuals prompted us to analyse the levels of mtDNA from lymphocytes of living patients. However, the results obtained

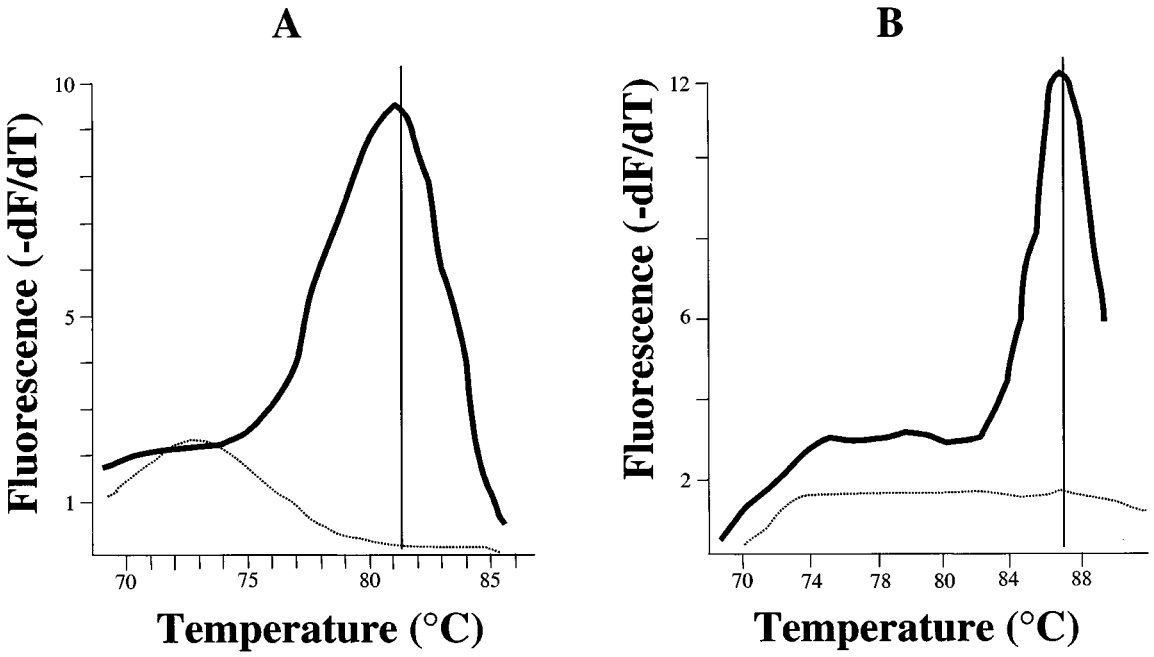

Figure 2 Melting curves for mtND2 and r18S. **A** mtND2 and **B** r18S PCR end products for samples containing DNA (solid line) or without template (light line). Following amplifications, dsDNA fluorescence was measured as the temperature was increased at 0.2°C/s from 65 (mtND2) or 72 (r18S) to 94°C. The rate of fluorescence change with changing temperature (-dF/dT) was plotted as a function of temperature. The mtND2 product T_m was 81.5°C and for r18S was 87°C.

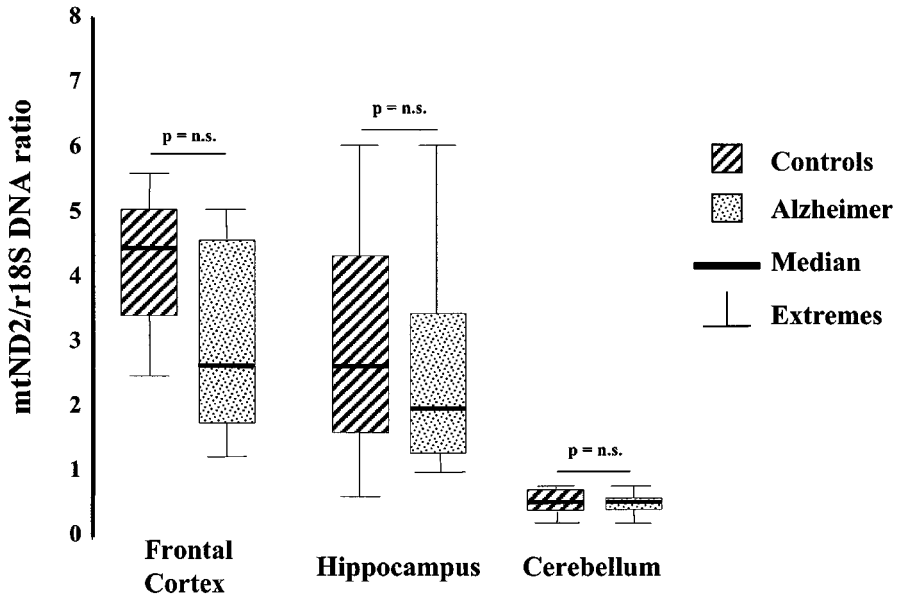

Figure 3 Comparison of Alzheimer and control mtND2/r18S ratios in the different brain areas analysed. Boxplots of the mitochondrial (mtND2)/nuclear (r18S) DNA ratio in the regions of the brain analysed. The numerical data for ages, medians and standard deviation values are presented in Table 1. (n.s: not significant).

indicate that there were no differences between AD patients and matched controls, so this parameter cannot be used for developing a diagnostic test for AD.

In the present study we have found a 28% reduction in mtDNA content in frontal cortex from patients with AD. Although statistically not significant, the data might suggest

mtDNA decrease in the frontal cortex of some patients. Mitochondrial studies in brain have to consider the differences in the relative abundance of mtDNA contents depending on the brain area analysed. Furthermore, it has to be noted that AD is a disease characterised by a very marked differential severity of the neuronal damage depending on the cortex area examined, as other authors have reported.[10] On the other hand, an age-related increase of mtDNA amount has been found in the frontal cortex of elderly individuals.[30] This increase in mtDNA content with age may be considered a gene dosage compensatory mechanism for mitochondria with altered capacity for substrate oxidation.

Once taken into account such considerations, the reduction of mtDNA circumscribed to frontal cortex in our group of AD patients is in accordance with the AD neuronal loss preferentially occurring in frontal cortex and less in cerebellum or hippocampus. The physiological increase in mtDNA of frontal cortex in normal individuals is completely overcomed in AD patients. This mtDNA defect seems to have, therefore, an anatomic specificity, and may contribute to impaired energy generation in AD reported by other authors.

The real contribution of such mtDNA defect in the pathophysiology of AD remains elusive. The precise balance between nuclear and mitochondrial genome, steady-state levels of mitochondrial RNA, mitochondrial respiratory chain function, calcium homeostasis, ROS production, amyloid metabolism, and cell signalling may provide the information necessary to complete our understanding of the mitochondrial role in neuronal loss in such devastating disease.

Acknowledgements

This work was supported by grants Fundació La Marató de TV3 2002/97 and 2102/97 and CICYT SAF 99-0079. B Rodríguez-Santiago is supported by Fundació La Marató de TV3. V Nunes is supported by Institut Català de la Salut. We thank the Neurologic Tissue Bank (Serveis Científico-Tècnics, Universitat de Barcelona) for providing the samples, Montse Gómez Zaera for comments on the manuscript, Carlos Manchado for technical support and Helena Kruyer for editorial help.

References

1 Vickers JC, Dickson TC, Adlard PA, Saunders HL, King CE, McCormack G: The cause of neuronal degeneration in Alzheimer's disease. *Prog Neurobiol* 2000; **60**: 139 – 165.
2 Dickson DW: The pathogenesis of senile plaques. *J Neuropathol Exp Neurol* 1997; **56**: 321 – 339.
3 Ray WJ, Ashall F, Goate AM: Molecular pathogenesis of sporadic and familial forms of Alzheimer's disease. *Mol Med Today* 1998; **4**: 151 – 157.
4 Christen Y: Oxidative stress and Alzheimer disease. *Am J Clin Nutr* 2000; **71**: 621S – 629S.
5 Parker Jr WD, Parks J, Filley CM, Kleinschmidt-DeMasters BK: Electron transport chain defects in Alzheimer's disease brain [see comments]. *Neurology* 1994; **44**: 1090 – 1096.
6 Mutisya EM, Bowling AC, Beal MF: Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. *J Neurochem* 1994; **63**: 2179 – 2184.
7 Redjems-Bennani N, Jeandel C, Lefebvre E, Blain H, Vidailhet M, Gueant JL: Abnormal substrate levels that depend upon mitochondrial function in cerebrospinal fluid from Alzheimer patients. *Gerontology* 1998; **44**: 300 – 304.
8 Egensperger R, Kosel S, Schnopp NM, Mehraein P, Graeber MB: Association of the mitochondrial tRNA(A4336G) mutation with Alzheimer's and Parkinson's diseases. *Neuropathol Appl Neurobiol* 1997; **23**: 315 – 321.
9 Hutchin T, Cortopassi G: A mitochondrial DNA clone is associated with increased risk for Alzheimer disease. *Proc Natl Acad Sci USA* 1995; **92**: 6892 – 6895.
10 Corral-Debrinski M, Horton T, Lott MT *et al*: Marked changes in mitochondrial DNA deletion levels in Alzheimer brains. *Genomics* 1994; **23**: 471 – 476.
11 Hamblet NS, Castora FJ: Elevated levels of the Kearns-Sayre syndrome mitochondrial DNA deletion in temporal cortex of Alzheimer's patients. *Mutat Res* 1997; **379**: 253 – 262.
12 Aksenov MY, Tucker HM, Nair P *et al*: The expression of several mitochondrial and nuclear genes encoding the subunits of electron transport chain enzyme complexes, cytochrome c oxidase, and NADH dehydrogenase, in different brain regions in Alzheimer's disease. *Neurochem Res* 1999; **24**: 767 – 774.
13 Chandrasekaran K, Hatanpaa K, Rapoport SI, Brady DR: Decreased expression of nuclear and mitochondrial DNA-encoded genes of oxidative phosphorylation in association neocortex in Alzheimer disease. *Brain Res Mol Brain Res* 1997; **44**: 99 – 104.
14 Sheehan JP, Swerdlow RH, Miller SW *et al*: Calcium homeostasis and reactive oxygen species production in cells transformed by mitochondria from individuals with sporadic Alzheimer's disease. *J Neurosci* 1997; **17**: 4612 – 4622.
15 Suzuki A, Tsutomi Y, Yamamoto N, Shibutani T, Akahane K: Mitochondrial regulation of cell death: mitochondria are essential for procaspase 3-p21 complex formation to resist Fas-mediated cell death. *Mol Cell Biol* 1999; **19**: 3842 – 3847.
16 Deng G, Su JH, Ivins KJ, Van Houten B, Cotman CW: Bcl-2 facilitates recovery from DNA damage after oxidative stress. *Exp Neurol* 1999; **159**: 309 – 318.
17 Moraes CT, Shanske S, Tritschler HJ *et al*: mtDNA depletion with variable tissue expression: a novel genetic abnormality in mitochondrial diseases. *Am J Hum Genet* 1991; **48**: 492 – 501.
18 Ricci E, Moraes CT, Servidei S, Tonali P, Bonilla E, DiMauro S: Disorders associated with depletion of mitochondrial DNA. *Brain Pathol* 1992; **2**: 141 – 147.
19 Poulton J, Sewry C, Potter CG *et al*: Variation in mitochondrial DNA levels in muscle from normal controls. Is depletion of mtDNA in patients with mitochondrial myopathy a distinct clinical syndrome. *J Inherit Metab Dis* 1995; **18**: 4 – 20.
20 Casademont J, Barrientos A, Grau JM *et al*: The effect of zidovudine on skeletal muscle mtDNA in HIV-1 infected patients with mild or no muscle dysfunction. *Brain* 1996; **119**: 1357 – 1364.
21 Dalakas MC, Illa I, Pezeshkpour GH, Laukaitis JP, Cohen B, Griffin JL: Mitochondrial myopathy caused by long-term zidovudine therapy [see comments]. *N Engl J Med* 1990; **322**: 1098 – 1105.
22 Vu TH, Sciacco M, Tanji K *et al*: Clinical manifestations of mitochondrial DNA depletion. *Neurology* 1998; **50**: 1783 – 1790.
23 Arnaudo E, Dalakas M, Shanske S, Moraes CT, DiMauro S, Schon EA: Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy. *Lancet* 1991; **337**: 508 – 510.
24 Campos Y, Martin MA, Garcia-Silva T *et al*: Clinical heterogeneity associated with mitochondrial DNA depletion in muscle. *Neuromuscul Disord* 1998; **8**: 568 – 573.
25 Parker Jr WD: Cytochrome oxidase deficiency in Alzheimer's disease. *Ann NY Acad Sci* 1991; **640**: 59 – 64.

26 Mecocci P, Polidori MC, Ingegni T *et al*: Oxidative damage to DNA in lymphocytes from AD patients. *Neurology* 1998; **51**: 1014–1017.
27 McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM: Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on *Alzheimer's Disease*. *Neurology* 1984; **34**: 939–944.
28 Morrison TB, Weis JJ, Wittwer CT: Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques* 1998; **24**: 954–958, 960, 962.
29 Ririe KM, Rasmussen RP, Wittwer CT: Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem* 1997; **245**: 154–160.
30 Barrientos A, Casademont J, Cardellach F, Estivill X, Urbano-Marquez A, Nunes V: Reduced steady-state levels of mitochondrial RNA and increased mitochondrial DNA amount in human brain with aging. *Brain Res Mol Brain Res* 1997; **52**: 284–289.

Comentarios adicionales

El fenómeno de la depleción es poco frecuente en comparación a otros defectos del mtDNA y se ha estudiado poco en enfermedades neurodegenerativas. En la EA por ejemplo, hasta el artículo presentado no se había analizado la cantidad de mtDNA de pacientes. La hipótesis es que una reducción del mtDNA en pacientes con EA puede estar relacionada con una menor síntesis de las subunidades que forman parte de los complejos de la CRM. La reducción en el mtDNA puede producir la disfunción de la CRM y provocar la disminución de la producción de ATP.

En este estudio la cantidad de mtDNA hallada era muy similar entre pacientes y controles. Después de cuantificar el mtDNA y el nDNA de tres regiones cerebrales y de linfocitos, el ratio mtDNA/nDNA no presentaba diferencias estadísticas cuando se comparaba individuos con EA y controles. El dato más destacado fue la reducción en la cantidad de mtDNA del 28% respecto a controles (mtDNA/nDNA = 4,51±1,71) observada en el córtex frontal de pacientes con EA (mtDNA/nDNA = 3,25±1,98). Quizás pueda explicarse que este resultado no alcanzase significación estadística por la heterogeneidad observada en las muestras de córtex frontal de los individuos con EA. Esta heterogeneidad se refleja en la elevada desviación estándard del grupo de pacientes. Esta alta heterogeneidad también se observó en el hipocampo (2,61±1,67 en los pacientes), pero en este tejido la diferencia entre pacientes y controles era mucho menor: un 13% de reducción en pacientes respecto a controles (3,00±2,25). En los tres tejidos de pacientes con EA se cuantificó una reducción en el contenido de mtDNA (28% en córtex frontal, 13% en hipocampo, 3,6% en cerebelo). Quizá este dato del 28% de reducción indique cierta implicación mitocondrial en los casos de EA estudiados, pero debería ser corroborado con el análisis de más muestras. Las diferencias entre tejidos pueden indicar o bien que el mtDNA se encuentra selectivamente alterado en función de la región cerebral e indicar que el daño mitocondrial es mayor en las regiones cerebrales más afectadas por la EA (córtex frontal e hipocampo, la cual es una especialización del córtex), o bien simplemente pueden ser el reflejo de la pérdida neuronal que ocurre en las diferentes regiones de un cerebro con EA, la cual es mayor en córtex frontal que en hipocampo o cerebelo.

La técnica basada en la PCR en tiempo real puesta a punto en este artículo constituyó uno de los primeros métodos descritos que sustituían al método clásico usado mayoritariamente para el estudio de la depleción del mtDNA. Tradicionalmente la técnica usada para obtener el ratio mtDNA/nDNA que sirve para determinar si hay o no una reducción en la cantidad de mtDNA ha sido la transferencia mediante Southern blot seguido de hibridación simultánea con dos sondas específicas (una mitocondrial y otra nuclear) y la cuantificación de las bandas mediante densitometría. Actualmente el método basado en la PCR en tiempo real es el más usado por la mayoría de grupos para estudiar la depleción del mtDNA.

La depleción se estudia de forma rutinaria mediante métodos basados en la PCR cuantitativa en tiempo real en enfermos con HIV para determinar el impacto de las terapias anti-retrovirales. Algunos de los fármacos usados en estos “cócteles” afectan a la replicación del mtDNA y generan

toxicidad mitocondrial. El método que se describe en el artículo ha sido aplicado en colaboración con el grupo del Dr. Miró en el hospital Clínic para el estudio de la toxicidad mitocondrial originada por los fármacos que toman enfermos de HIV. (Ver artículos del **Anexo**). Esta técnica también ha sido aplicada posteriormente en el estudio de la EA por otros investigadores (Coskun *et al*. 2004) basándose en el método descrito por primera vez en el artículo recopilado en la memoria.

8.3. Cholinesterase inhibitor rivastigmine enhance the mitochondrial electron transport chain in lymphocytes of patients with Alzheimer's disease.

Journal of the Neurological Sciences 206 (2003) 23– 26.

J. Casademont, O. Miró, B. Rodríguez-Santiago, P. Viedma, R. Blesa, F. Cardellach.

Contribución del doctorando:

Aislamiento de linfocitos a partir de muestras de sangre de pacientes y controles para medir la actividad de la CRM.

ELSEVIER

Journal of the Neurological Sciences 206 (2003) 23–26

Journal of the Neurological Sciences

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Cholinesterase inhibitor rivastigmine enhance the mitochondrial electron transport chain in lymphocytes of patients with Alzheimer's disease

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Received 14 March 2002; received in revised form 5 August 2002; accepted 6 August 2002

Abstract

Electron transport chain (ETC) dysfunction has been claimed to contribute to the expression of neurodegenerative disorders. We have investigated the effects of the treatment with rivastigmine, a commonly used cholinesterase inhibitor, on lymphocyte mitochondria of patients with Alzheimer's disease (AD). Increased enzymatic activities of diverse complexes and oxidative capacity of the ETC were found. Enhanced mitochondrial ETC function may contribute to the beneficial effects of rivastigmine on clinical manifestations of AD.

Keywords: Mitochondria; Electron transport chain; Respiratory chain; Rivastigmine; Neurodegenerative disorders; Dementia

1. Introduction

A growing body of evidence indicates that defects in mitochondrial metabolism, and particularly of the electron transport chain (ETC), may play an important role in the pathogenesis of Alzheimer's disease (AD). Decreased activity of cytochrome *c* oxidase (COX), either using direct enzymatic determinations or histochemical studies, has been the most frequent finding in AD brains. However, other complexes of the ETC, i.e. complexes I, II + III and III, as well as ATP-synthase, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, which are mitochondrial enzymes outside ETC also involved in energy metabolism, have also been found to be decreased in AD brains (for review, see Refs. [1,2]). Interestingly, some of the defects in ETC have also been described in non-neural tissues such as platelets and fibroblasts [3,4].

The importance of such findings is unclear. The general belief is that mitochondrial dysfunction could interfere, among others, with calcium homeostasis, amyloid metabolism and reactive oxygen species generation, enhancing the vulnerability of cholinergic neurons already compromised by the primary processes implicated in the etiology of AD [5]. Thus, in a hypothesis shared with other neurodegenerative disorders [6] and even with some theories on aging [7,8], mitochondrial dysfunction could be considered a secondary phenomenon, but with the ability to modulate the expression of the disease.

One of the most interesting advances in the treatment of AD has been the relatively recent introduction of cholinesterase inhibitors. Such drugs act favoring central cholinergic transmission [9]. Rivastigmine, one of the most commonly used in clinical practice, has proven to delay the progression of the neuropsychological symptoms of AD [10]. What, to our knowledge, has not been ascertained is whether such treatments have any effect on human mitochondrial ETC. If this was the case, the contribution of mitochondria in the expression of AD could, eventually, be better understood.

2. Patients and methods

Three groups of individuals were included in the present study: (a) 13 AD patients without any specific treatment (untr-AD), (b) 13 AD patients treated at least during the previous 6 months with 6–12 mg/day of rivastigmine (tr-

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PII: S0022-510X(02)00319-2

Table 1
Main characteristics of individuals included in the study, including citrate synthase activity

	C	untr-AD	tr-AD	*p*
N = 52	26	13	13	n.s.
Sex (M/F)	8/18	4/9	4/9	n.s.
Age in years	77.8 ± 8.6	76.9 ± 5.4	75.9 ± 7.4	n.s.
Evolution of AD in years	–	2.3 ± 0.7	2.5 ± 0.7	n.s.
Citrate synthase in nmol/min/mg protein	99.67 ± 25.58	110.09 ± 31.54	93.81 ± 49.04	n.s.

C = controls.
M/F = male/female.
untr-AD = untreated Alzheimer's disease patients.
tr-AD = rivastigmine-treated Alzheimer's disease patients.

AD), and (c) 26 control subjects matched by age and sex, without AD (C). AD was diagnosed following standard criteria and patients were mild to moderate according to the Mini-Mental State Examination. Exclusion criteria were active smoking habit or simultaneous treatment with other drugs. All patients or legal representatives gave informed consent to be included in the study. The Ethical Committee of our institution approved the protocol. A blood sample of 30 ml was obtained in all cases. Lymphocytes were obtained in a Ficoll's gradient and the protein concentration was quantified according to Bradford's method. Oxygen utilization was measured polarographically in intact cells, as well as after the permeabilization of lymphocytes with digitonin using pyruvate–malate, succinate and glycerol-3-phosphate as complexes I, II and III substrates, respectively. Enzyme activity of complexes II (succinate–ubiquinone reductase), III (ubiquinol–cytochrome *c* reductase) and IV (COX) were measured spectrophotometrically. Both, oxidative rates and enzyme activities, were corrected for citrate synthase activity, to control for mitochondrial content. Details are specified elsewhere [11,12].

2.1. Statistical analyses

Results are expressed as mean ± standard deviation. Comparisons between groups were performed by means of one-way analysis of variance (ANOVA). The threshold for determining statistical significance was $p < 0.05$. If ANOVA was significant, a Scheffé post hoc test was applied to determine between which pairs of groups the difference was.

3. Results

The main clinical characteristics of patients and controls included in the study are shown in Table 1. There were no differences among groups for sex, age, evolution of AD in years, or in mitochondrial content measured by citrate synthase activity. In general, a pattern of higher oxidative and enzymatic activities was seen in tr-AD patients when compared with C or untr-AD (Fig. 1). The differences reached statistical significance for oxidation of pyruvate–

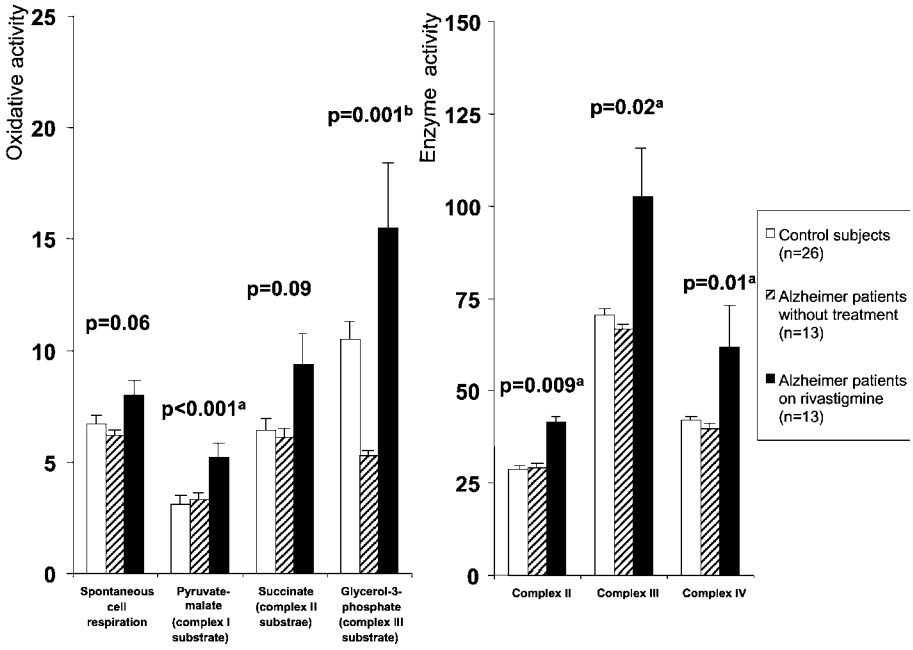

Fig. 1. Oxidative (left) and enzymatic (right) ETC activities. Oxidative activities are expressed as nmol of oxygen/min/mg protein × 100. Enzyme activities are expressed as nmol/min/mg protein. Results are corrected for citrate synthase activity to control for mitochondrial content. Bars represent means and error bars standard error of the mean. *p*'s denote the ANOVA test. When ANOVA was statistically significant, differences <0.05 on Scheffé post hoc test are indicated either by 'a', which indicates that the differences were between treated AD with respect to untreated AD and controls, or 'b', which indicates that the differences were between treated AD with respect to untreated AD patients. ETC: electron transport chain.

malate (substrate of complex I) and glycerol-3-phosphate (substrate of complex III), and for enzymatic activities of complexes II, III and IV. The differences were always present between tr-AD and untr-AD patients and, in most cases, between tr-AD and C (Fig. 1).

4. Discussion

The effect of cholinesterase inhibitors is directed towards increasing the cerebral concentrations of acetylcholine [9]. Herein we demonstrate that rivastigmine also exerts a profound effect on lymphocyte mitochondria. It enhances the mitochondrial ability to oxidize substrates for complexes I and III (respiratory capacity), and increases the enzymatic activity of ETC complexes II, III and IV. Although without reaching statistical significance, it also seems to stimulate spontaneous cell respiration and ability to oxidize succinate, a substrate for complex II (Fig. 1). It is interesting to note that all these effects are independent of the fact that lymphocytes do not express the deficiencies in ETC found in brain and other tissues in AD patients, as previously reported [13] and confirmed in the present study. Such changes, therefore, have to be attributed to treatment and not to underlying Alzheimer's disease.

It is important to stress that polarographic studies of ETC using different substrates reflect not only the activity of ETC complexes (more directly done by individual spectrophotometric measurements), but also analyze the integrity of diverse intermediary steps from the addition of a substrate to oxygen uptake, which is the ultimate parameter evaluated by polography. Thus, increase in oxidative rates, as for example with pyruvate–malate found in the present study, can be theoretically attributed to an increase of the activities of complexes I, III and IV through which electrons flow, but also to previous (pyruvate dehydrogenase or α-ketoglutarate complexes) or posterior (ATP synthase system, ADP/ATP transporters) steps. It is the combination of both polarographic and spectrophotometric approaches that allows us to localize the effect of rivastigmine on ETC, but we cannot exclude effects at other levels.

The mechanisms by which rivastigmine would stimulate ETC are uncertain. What has been proven is that cholinesterase inhibitors enhance cerebral glucose metabolism and blood flow [9]. In experimental models, acetylcholine administration has demonstrated to stimulate substrate-level phosphorylation [14]. Furthermore, acetylcholine, although rapidly hydrolyzed in body fluids, is detectable in blood [15], and acetylcholine receptors are present on the surface of blood cells [9] and specifically on lymphocytes [15]. All these points suggest that lymphocyte ETC stimulation is probably a drug class effect. To prove this hypothesis, other cholinesterase inhibitors should be studied, as well as other unrelated drugs useful for the treatment of Alzheimer's disease, such as *N*-methyl-D-aspartate receptor antagonist memantine.

The importance of the reported finding remains to be established, mainly because ETC stimulation does not necessarily mean an increased coupling to produce ATP. In addition, a cross-sectional study is not ideal to prove causality. A prospective, sequential study is certainly indicated from the present observations. However, if the herein demonstrated lymphocyte mitochondrial ETC stimulation is also present in the central nervous system, as one can reasonably presume, the possibility that this issue may contribute to the proven benefits of rivastigmine in AD offers new insights into the understanding of the contribution of mitochondria in the pathophysiology of neurodegeneration and may, eventually, provide new therapeutic strategies for this devastating disease.

Acknowledgements

We thank Mrs. Ana Beato for technical support. This study was funded by Fundació La Marató de TV3 (2102/97), and SGR 00279.

References

[1] Bonilla E, Tanji K, Hirano M, Vu TH, DiMauro S, Schon EA. Mitochondrial involvement in Alzheimer's disease. Biochim Biophys Acta 1999;1410:171–82.
[2] Manfredi G, Beal MF. The role of mitochondria in the pathogenesis of neurodegenerative diseases. Brain Pathol 2000;10:462–72.
[3] Parker Jr WD, Mahr NJ, Filley CM, et al. Reduced platelet cytochrome *c* oxidase activity in Alzheimer's disease. Neurology 1994;44:1086–90.
[4] Peterson C, Goldman JE. Alterations in calcium content and biochemical processes in cultured skin fibroblasts from aged and Alzheimer donors. Proc Natl Acad Sci 1986;83:2758–62.
[5] Blass JP. Brain metabolism and brain disease: is metabolic deficiency the proximate cause of Alzheimer disease? J Neurosci Res 2001;66:851–6.
[6] Cardellach F, Marti MJ, Fernandez-Sola J, et al. Mitochondrial respiratory chain activity in skeletal muscle from patients with Parkinson's disease. Neurology 1993;43:2258–62.
[7] Grau JM, Casademont J, Cardellach F, Fernandez-Sola J. Aging and muscle mitochondrial abnormalities. Ann Neurol 1995;38:273–4.
[8] Barrientos A, Casademont J, Rotig A, et al. Absence of relationship between the level of electron transport chain activities and aging in human skeletal muscle. Biochem Biophys Res Commun 1996;229:536–9.
[9] Weinstock M. Selectivity of cholinesterase inhibition: clinical implications for the treatment of Alzheimer's disease. CNS Drugs 1999;12:307–23.
[10] Birks J, Evans JG, Iakovidou V, Tsolaki M. Rivastigmine for Alzheimer's disease [Cochrane review]. The cochrane library, vol. 1. Oxford: Update Software; 2001.
[11] Rustin P, Chretien D, Bourgeron T, Gerard B, Rotig A, Saudubray JM, et al. Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta 1994;228:35–51.
[12] Miró O, Alonso JR, Jarreta D, Casademont J, Urbano-Márquez A, Cardellach F. Smoking disturbs mitochondrial respiratory chain function and enhances lipid peroxidation on human circulating lymphocytes. Carcinogenesis 1999;20:1331–6.

[13] Molina JA, de Bustos F, Jimenez-Jimenez FJ, et al. Respiratory chain enzyme activities in isolated mitochondria of lymphocytes from patients with Alzheimer's disease. Neurology 1997;48:636–8.

[14] Kondrashova MN, Doliba NM. Polarographic observation of substrate-level phosphorylation and its stimulation by acetylcholine. FEBS Lett 1989;243:153–5.

[15] Sato KZ, Fujii T, Watanabe Y, et al. Diversity of mRNA expression for muscarinic acetylcholine receptor subtypes and neuronal nicotinic acetylcholine receptor subunits in human mononuclear leukocytes and leukemic cell lines. Neurosci Lett 1999;266:17–20.

Comentarios adicionales

En este estudio se analizó el efecto sobre la CRM de un fármaco inhibidor de la colinesterasa usado comunmente en la EA: la rivastigmina. Las actividades enzimáticas de diversos complejos y la capacidad oxidativa estaban aumentadas en las mitocondrias de linfocitos de pacientes con EA tratados con rivastigmina. Esta estimulación de la CRM puede contribuir a los efectos beneficiosos descritos de la rivastigmina sobre las manifestaciones clínicas de la enfermedad.

La estimulación del metabolismo energético mediante rivastigmina mostrada en el estudio es un efecto diferente al que en un principio propició la introducción del medicamento para el tratamiento de la EA (aumentar la concentración de acetil colina en el cerebro). Los mecanismos que conducen a esta estimulación son todavía desconocidos. Este estudio fue hecho en linfocitos, ya que la obtención de mitocondrias de cerebro para este estudio sería cuando menos poco ética. No obstante, existen diversos trabajos previos que indican que las alteraciones halladas en tejidos periféricos de pacientes con EA pueden ocurrir también en el cerebro (Blass 2000). La existencia de defectos en el metabolismo energético en el cerebro con EA es un hecho suficientemente documentado, como se ha mostrado en la introducción. La magnitud del déficit en el metabolismo energético se correlaciona mejor con el grado de discapacidad clínica que la cantidad de cambios neuropatológicos observados en la autopsia. Esta observación ha hecho que Blass *et al*. propongan que la lesión cerebro-metabólica es una de las causas iniciales de la discapacidad clínica en la EA y a que se prueben terapias diseñadas a recuperar la capacidad energética en el cerebro (Blass 2001).

Las vías colinérgicas del cerebro son especialmente sensibles al deterioro del metabolismo cerebral. El trabajo presentado en la memoria indica que los inhibidores de colinesterasa pueden reforzar el metabolismo así como indirectamente actuar en la neurotransmisión. Sería estupendo si estos tratamientos mejoraran las anomalías observadas en el cerebro con EA mediante los dos mecanismos. La realización de estudios más extensos en humanos y animales puede suponer una ayuda valiosa para confirmar estas observaciones.

8.4. Mitochondrial respiratory chain in brain homogenates: Activities in different brain areas in patients with Alzheimer's disease.

Aging Clinical and Experimental Research, en prensa.

J. Casademont, B. Rodríguez-Santiago, O. Miro, A. Beato, S. López, V. Nunes, F. Cardellach.

Contribución del doctorando:

Homogenización de los tejidos de necropsia de pacientes y controles conjuntamente con Anna Beato para análisis posteriores de la actividad de la CRM. Medida de la peroxidación lípidica a partir de los homogenizados conjuntamente con Sònia López.

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Mitochondrial respiratory chain in brain homogenates: Activities in different brain areas in patients with Alzheimer's disease

Running head: Respiratory chain in Alzheimer's disease…

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Key words: Alzheimer's disease, dementia, free radicals, mitochondria, neurodegenerative disorders, respiratory chain.

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Abstract

Background: The potential influence of impaired oxidative metabolism in the modulation of sporadic Alzheimer's disease (AD) manifestations has attracted much attention in the last 50 years. Unfortunately, many clinical and experimental results to prove this hypothesis are still controversial.

Aims: To study the enzymatic activities of respiratory chain (RC) complexes I through V in three brain areas of a group of patients with definite AD and compare the results with a group of normal brains. We simultaneously assessed the lipid peroxidation of the samples as a measure of free radical damage.

Methods: The specific activity of the individual complexes of the RC was measured spectrophotometrically and the loss of *cis*-parinaric acid fluorescence was used to determine the chemical process of lipid peroxidation.

Results: We were not able to detect differences in any of the analyzed RC enzymatic activities or in the level of lipid peroxidation between patients with AD and controls. On the contrary, differences were found in the number of mitochondria and in the intrinsic enzymatic activities of complexes III and IV in different brain areas.

Conclusions: The spectrophotometric enzymatic analyses of respiratory complexes in brain homogenates do not support a primary contribution of a mitochondrial RC dysfunction in the pathogenesis of AD.

Introduction

Sporadic Alzheimer's disease (AD) is the most common neurodegenerative disease. It is clinically characterized by early impairment of recent memory followed by progressive intellectual dysfunction (1). Definite diagnosis relies on pathological studies, which disclose selective pyramidal neuronal death and accumulation of intraneuronal neurofibrillary tangles and extracellular fibrillar senile plaques (2).

Extensive data accumulated since the late 1940's have documented that the metabolic rate for oxygen is decreased in AD brains supporting the hypothesis that impaired oxidative metabolism has a role in the development of clinical disability (for a recent review see ref. 3). On the other hand, epidemiological data show a female-to-male ratio of 3.6-3.8:1 in the parental generation of probands suggesting that a maternally inherited genetic factor might be involved (4,5).

As mitochondria are the main source of energy in the cell and since mitochondrial DNA (mtDNA) is inherited via maternal line, many studies have been directed toward their study. The findings have been abundant but very variable (6-8). Initial work suggested that point mutations in the mitochondrial-encoded cytochrome *c* oxidase subunit genes segregated with AD patients (9), but more recent evidence has shown that the presumed mutations were polymorphisms in nuclear pseudogenes (10). Other mutations in mitochondrially encoded Complex I or tRNA genes (11,12) have not been confirmed by others (13,14).

In the absence of uniform mtDNA findings, and as this genome only codes for proteins of the mitochondrial respiratory chain (RC), a mitochondrial bioenergetic defect, and specifically a RC defect, could be the clue to impute a pathogenic role to mitochondria in AD. Again, diverse dysfunctions have been described, being a reduction of complex IV activity the most frequent finding. This reduction has been described in platelets and fibroblasts (15,16), although the finding has either not been confirmed by other investigators (17), or not further reported. Other extraneurological tissues such as lymphocytes disclosed normal mitochondrial function (18). In postmortem brain tissue the most frequent finding has also

been a reduction of complex IV enzyme activity (19-22), but other authors have found normal complex IV activity while activities of complexes II and III (23), or complex V (24) were decreased.

The reasons for the discrepancies are unclear, although they are likely to be explained by methodological factors such as variations in AD diagnostic criteria, methods in enzymatic studies, the influence of the age of patients, or delay in processing the samples after death.

In 1997 a cytochrome *c* oxidase defect was transferred from AD platelets to cybrids, and the ensuing cybrid cell lines showed increased free radical production (25). Such finding prompted to suggest that the variability in mitochondrial genetic and enzymatic findings could reflect secondary changes due to the result of the progressive accumulation of free radicals in mitochondria (26,27). In normal conditions, about 5% of the oxygen consumed by mitochondria is converted into free radicals as by-products of the RC (28). As they are highly reactive species, it is conceivable that free radicals cause damage near the site of their formation, i.e. mtDNA and RC itself. This would further impair the function of the respiratory machinery which, in turn, would promote the generation of more free radicals, oxidative damage of membrane phospholipids and proteins, and more mtDNA mutations in a sort of catastrophic vicious cycle (29). Similar mechanisms based on a free radical hypothesis have been proposed to modulate the expression of other neurodegenerative diseases, atherosclesosis and even aging (30-32). Until nowadays nonetheless, this possibility, although very attractive, remains speculative.

In an attempt to gain additional insight into this issue, we took advantage of the brain material stored at the Brain Bank, University of Barcelona-Hospital Clínic. In all cases the material was carefully classified from a neuropathological point of view, and the hypothetical influencing and confounding variables recorded. A complete RC analysis, together with an appraisal of the brain lipid peroxidation as a marker of free radical damage was undertaken.

Methods

The Brain Bank of the University of Barcelona-Hospital Clínic was created in 1990. It stores brains obtained from voluntary donations belonging either to patients with neurological illnesses, or to “normal” individuals -from a neurological point of view- died due to diverse circumstances. In all cases a standard recollection protocol is followed, including thorough neuropathological examinations to establish the final neuropathological diagnosis (33,34). Twelve brains that fulfilled the pathological criteria of definite AD with Braak’s staging V or VI were included in the present study. Eight normal brains were included as a control group. From each brain, a sample of approximately 250 mg was obtained from the frontal cortex, cerebellar cortex, and hippocampus. All biochemical and enzymatic studies were performed consecutively during a 6-week period of time.

Preparation of homogenates

Homogenates from frozen brain areas 3.5% (w/v) were prepared in 50mM of Tris-buffer (pH 7.5), 100 mM of potassium chloride, 5 mM of magnesium sulfate, and 1 mM of ethylene diaminetetraacetic acid. Tissues were disrupted by 7 strokes at 800 rpm in glass-Teflon homogenizer and filtered through two layers of cheesecloth. The homogenate was centrifuged at 2000 rpm for 3 min. The pellet was discarded, and the supernatant was used for biochemical studies. Protein content in the supernatant was measured according to Bradford’s protein-dye binding principle.

Mitochondrial respiratory chain enzyme assays

The measurement of the specific activity of the individual complexes of the respiratory chain was performed spectrophotometrically (UVIKON Spectrophotometer 922, KONTRON Instruments, Zurich, Switzerland). A total of 40-70 μg of homogenate protein was used to determine the activity of each complex, except for complex IV, for which we used 8-15 μg of protein (35). Measurements of complex I (Rotenone-sensitive NADH-Decylubiquinone Oxidoreductase), complex II (Succinate Decylubiquinone DCPIP Reductase), complex III (Ubiquinol Cytochrome *c* Reductase), complex IV (Cytochrome *c* Oxidase), and complex V (Oligomycin-sensitive ATP-synthase) were performed at 37 ºC in 1 mL of medium (36,37).

Measurement of citrate synthase (CS) was performed at 412 nm following the reduction of 2 mM 5,5'-dithio-bis(2-nitrobenzoic acid) in the presence of 0.1 mM acetyl-CoA and 12 mM oxalacetic acid in 1 mL of medium containing 10 mM KH_2PO_4 (pH 7.8, 37ºC), 2 mM EDTA, 1 mg/ml BSA and 0.1% Triton X-100. Enzyme activities were expressed as nmol of reduced or oxidized substrate · min^{-1} · mg of $protein^{-1}$.

Relative enzymatic activities

CS is a Krebs cycle enzyme outside the respiratory chain that can be used as a marker of the number of mitochondria present in homogenates. When activities of the RC complexes are normalized for CS, the *relative* activities are obtained, which better reflect the intrinsic activity of each enzyme in a given tissue.

Lipid membranes peroxidation

Brain homogenates (100 μg protein) were labeled with 5 μM *cis*-parinaric acid (Molecular Probes, Eugene, OR) in a cuvette containing 3 mL of nitrogenized PBS. Afterward, they were incubated in darkness at 37ºC, and fluorescence at 318-nm excitation and 410-nm emission was measured at 3 min intervals over 30 min as described (38). Measures were related to the first determination in each case and the loss of *cis*-parinaric acid fluorescence was used to measure the chemical process of peroxidation (the greater the loss, the higher the peroxidation).

Statistical analysis

Results are given as mean ± standard deviation (SD). The Fisher's exact test was used to compare qualitative variables. For quantitative variables, a Kolgomorov-Smirnov goodness-of-fit test was used to ascertain if the samples were normally distributed. A two-way ANOVA for repeated measures was used to test differences in enzymatic activities or peroxidation in different brain areas between AD patients and controls. This approach allows that, if there are no differences one way (for example between groups), from a statistical point of view the groups are considered together to test the other way (for example between areas), and *vice versa*, increasing the overall power of the analysis. A p-value less than 0.05 was considered

statistically significant. If ANOVA was significant, a Tukey *post-hoc* test was applied to determine between which pairs of groups the difference was.

Results

There were no differences when comparing the group of AD patients with controls regarding age, sex, duration of disease, smoking habit, psychoactive drugs taken at the time of death, and mean time elapsed from death to freezing of brain material (Table 1). The time elapsed after death to collecting and storing brain material ranged from 4.5 to 21.5 hours. During this period, there was a slight decrease for all the enzymatic activities analyzed, but in any case this decline reached statistical significance, either considering AD patients and controls as independent groups or taken together (Fig. 1).

When analyzing the enzymatic activities nothing opposed the assumption that the samples were normally distributed.

CS activity was not different between AD patients and controls. On the hand, there were significant differences among brain areas for CS activity, indicating that the number of mitochondria was higher in the cerebellum, followed by frontal cortex and hippocampus (Fig. 2).

When the activities of the respiratory chain complexes were normalized for CS, no differences were found between AD patients and controls (Fig. 3). On the contrary, there were differences regarding the activity for complex III and complex IV in different brain areas. In both cases, the maximum activity was in the frontal cortex, followed by the hippocampus and cerebellum (Fig. 3).

With respect to membrane lipid peroxidation studies we did not find any differences between AD patients and controls, or among the three areas of the brain analyzed (Fig. 4). The degree of lipid peroxidation did not correlate with the time elapsed after death (data not shown).

Discussion

In the present study we found that both, the RC enzymatic activities and the number of mitochondria, vary among the different brain areas under study. Once the activities of RC enzymes were corrected for the number of mitochondria present in homogenates, the intrinsic enzyme activities were different for complexes III and IV, the maximal activity being present in the frontal cortex, followed by the hippocampus and cerebellum. Apart from the biological interest of this observation, which confirms that the central nervous system is very heterogeneous in terms of mitochondrial bioenergetics (39), these variations are useful as an internal control to ensure that the sensitivity of enzymatic measurements undertaken in the present study are adequate enough to detect subtle variations in activity.

On the contrary, we were not able to detect differences in any of the analyzed RC enzymatic activities between AD patients and controls. Considering that our AD patients were in an advanced stage of disease at the time of death, and that control patients did not present any pathological criteria of AD, the lack of significant differences between the two groups argues against an inherent impairment in the ability of brain to oxidize substrates via the different respiratory chain complexes. Similarly, we found no differences in the level of membrane lipid peroxidation, as an indirect method to ascertain free radical damage. This leads to the conclusion that free radical damage has no particular relevant role in the modulation of clinical manifestations of AD, at least through damage to RC complexes.

A limitation of the present study is that enzymatic measurements were made in autopsy brain material. We herein demonstrate, nonetheless, that brain obtained at autopsy can be reliably used to study the activity of mitochondrial RC enzymatic activities provided that it is correctly frozen and stored within an interval of 4 to 21.5 hours after death. We base this conclusion on the fact that, although during this period there was a decrease in the activity of the individual complexes, including complex I, classically considered the most prone to lose activity (40), this decrease was not statistically significant (Fig. 1). The reliability of enzymatic studies in biological material obtained postmortem has generated many debates. This is

probably one of the reasons for the relatively low number of reports using this approach to evaluate mitochondria, despite the fact that abnormal function is essential to impute a pathogenic role to genetic, structural, or other defects. A conclusion similar to ours was previously obtained using mouse brains as an experimental model (41). The preservation of brain material for enzymatic analyses also seems to apply to lipid peroxidation studies, which could be performed in this period of time without a significant decrease in fluorimetry related to time postmortem. Experimental procedures support the suggestion that oxidative damage does not seem to play a role in these ranges of postmortem time (42).

A second limitation is the number of patients (12) and controls (8) analyzed due to the difficulty in finding appropriate material for these studies. It could be argued that the absence of enzymatic decreases in AD is due to a statistical error type 2, i.e., low power to detect significant differences due to a small sample. To overcome the difficulty in increasing the number of samples, we studied three brain areas for every individual using a two-way analysis of variance for comparisons. In fact, it is as if we studied 20 individuals (12 patients + 8 controls) to compare the differences between brain areas, and 60 individuals (20 frontal cortex + 20 cerebellar cortex + 20 hyppocampus) to compare AD patients versus controls, which, in terms of statistical power, are relatively large numbers.

Our findings are in apparent contradiction with previous reports demonstrating a reduction in oxidative/energy metabolism in AD dementia through diverse biochemical and imaging techniques (5). It should be taken into account that the activity of enzymes was herein measured under ideal “in vitro” situations that may not reflect what actually happens “in vivo”. There is always the question of causation: Is the reduction of brain metabolism a cause or a consequence of brain dysfunction? Activity of complexes may be down-regulated due to reduced neuronal activity. In this case, the reduced activity would probably not be detected by analyzing the intrinsic activities of individual RC complexes. Furthermore, oxidative capacity can be negatively influenced at levels other than respiratory complexes properly, i.e., pyruvate dehydrogenase or α-ketoglutarate complexes, ADP/ATP transporters, and

others. Polarographic studies of the oxidative capacity of intact mitochondria would be an ideal tool to analyze this issue because they reflect not only the activity of RC complexes, but also analyze the integrity of diverse intermediary steps from the addition of a substrate to oxygen uptake. Unfortunately, polarography can not be performed on frozen material. Finally, when studying tissue homogenates, the heterogeneous behavior of mitochondrial enzymes between neurons (43) is not taken into account and may contribute to these contradictory conclusions. Single neuron studies aimed at determining RC performance within individual neurons may be an eventual future approach to test if a defect in a small proportion of key neurons has any role in AD pathogenesis.

Conclusions

AD probably results from an interplay of various genetic, environmental and aging influences. From the present data obtained from the enzymatic analyses of RC complexes on brain homogenates, the putative role of a primary mitochondrial RC defect in AD cannot be confirmed.

Acknowledgements

We thank the Brain Bank, University of Barcelona-Hospital Clínic for their help in providing biological material and relevant information from the patients included in the study. This study was funded by Fundació La Marató de TV3 (2002-2102/97 and 1710/01), and SGR 00279 and 00399.

References

1. Honig LS, Mayeux R. Natural history of Alzheimer's disease. Aging Clin Exp Res [Aging (Milano)] 2001; 13: 171-82.

2. Katzman R. Alzheimer's disease. N Engl J Med 1986; 314:964-73.

3. Blass JP. Brain metabolism and brain disease: Is metabolic deficiency the proximate cause of Alzheimer dementia? J Neurosci Res 2001; 66:851-6.

4. Duara R, Lopez-Alberola RF, Barker WW, Loewenstein DA, Zatinsky M, Eisdorfer CE, Weinberg GB. A comparison of familial and sporadic Alzheimer's disease. Neurology 1993; 43:1377-84.

5. Edland SD, Silverman JM, Peskind ER, Tsuang D, Wijsman E, Morris JC. Increased risk of dementia in mothers of Alzheimer's disease cases: evidence for maternal inheritance. Neurology 1996; 47:254-6.

6. Bonilla E, Tanji K, Hirano M, Vu TH, DiMauro S, Schon EA. Mitochondrial involvement in Alzheimer's disease. Biochim Biophys Acta 1999; 1410:171-82.

7. Hutchin TP, Heath PR, Pearson RC, Sinclair AJ, Mitochondrial DNA mutations in Alzheimer's disease. Biochem Biophys Res Commun 1997; 241:221-5.

8. Manfredi G, Beal MF. The role of mitochondria in the pathogenesis of neurodegenerative diseases. Brain Pathol 2000; 10:462-72.

9. Davis RE, Miller S, Herrnstadt C, Ghosh SS, Fahy E, Shinobu LA, Galasko D, Thal LJ, Beal MF, Howell N, Parker WD Jr. Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer disease. Proc Natl Acad Sci USA 1997; 94:4526-31 .

10. Hirano M, Shtilbans A, Mayeux R, Davidson MM, DiMauro S, Knowles JA, Schon EA. Apparent mtDNA heteroplasmy in Alzheimer's disease patients and in normals due to PCR amplification of nucleus-embedded mtDNA pseudogenes. Proc Natl Acad Sci USA 1997; 94:14894-9.

11. Lin FH, Lin R, Wisniewski HM, Hwang YW, Grundke-Iqbal I, Healy-Louie G, Iqbal K. Detection of point mutations in codon 331 of mitochondrial NADH dehydrogenase subunit 2 in Alzheimer's brains. Biochem Biophys Res Commun 1992; 182:238-46.

12. Shoffner JM, Brown MD, Torroni A, Lott MT, Cabell MF, Mirra SS, Beal MF, Yang CC, Gearing M, Salvo R. Mitochondrial DNA variants observed in Alzheimer disease and Parkinson disease patients. Genomics 1993; 17:171-84.

13. Petruzzella V, Chen X, Schon EA. Is a point mutation in the mitochondrial ND2 gene associated with Alzheimer's disease. Biochem Biophys Res Commun 1992; 186:491-7.

14. Wragg MA, Talbot CJ, Morris JC, Lendon CL, Goate AM. No association found between Alzheimer's disease and a mitochondrial tRNA glutamine gene variant. Neurosci Lett 1995; 201:107-10.

15. Parker WD Jr, Filley CM, Parks JK. Cytochrome oxidase deficiency in Alzheimer's disease. Neurology 1990; 40:1302-3.

16. Curti D, Rognoni F, Gasparini L, Cattaneo A, Paolillo M, Racchi M, et al. Oxidative metabolism in cultured fibroblasts derived from sporadic Alzheimer's disease (AD) patients Neurosci Lett 1997; 236:13-6.

17. Van Zuylen AJ, Bosman GJ, Ruitenbeek W, Van Kalmthout PJ, De Grip WJ. No evidence for reduced thrombocyte cytochrome oxidase activity in Alzheimer's disease. Neurology 1992; 42:1246-7.

18. Molina JA, de Bustos F, Jimenez-Jimenez FJ, Benito-Leon J, Gasalla T, Orti-Pareja M, Vela L, Bermejo F, Martin MA, Campos Y, Arenas J. Respiratory chain enzyme activities in isolated mitochondria of lymphocytes from patients with Alzheimer's disease. Neurology 1997; 48:636-8.

19. Chandrasekaran K, Giordano T, Brady DR, Stoll J, Martin LJ, Rapoport SI. Impairment in mitochondrial cytochrome oxidase gene expression in Alzheimer disease. Mol Brain Research 1994; 24:336-40.

20. Kish SJ, Bergeron C, Rajput A, Dozic S, Mastrogiacomo F, Chang LJ, Wilson JM, DiStefano LM, Nobrega JN. Brain cytochrome oxidase in Alzheimer's disease. J Neurochem 1992; 59:776-9.

21. Mutisya EM, Bowling AC, Beal MF. Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. J Neurochem 1994; 63:2179-84.

22. Parker WD Jr, Parks J, Filley CM, Kleinschmidt-DeMasters BK. Electron transport chain defects in Alzheimer's disease brain. Neurology 1994; 44:1090-6.

23. Reichmann H, Florke S, Hebenstreit G, Schrubar H, Riederer P. Analyses of energy metabolism and mitochondrial genome in post-mortem brain from patients with Alzheimer's disease. J Neurol 1993 240:377-80.

24. Schagger H, Ohm TG. Human diseases with defects in oxidative phosphorylation. 2. F1F0 ATP-synthase defects in Alzheimer disease revealed by blue native polyacrylamide gel electrophoresis. Eur J Biochem 1995; 227:916-21.

25. Swerdlow RH, Parks JK, Cassarino DS, Maguire DJ, Maguire RS, Bennett JP Jr, Davis RE, Parker WD Jr. Cybrids in Alzheimer's disease: a cellular model of the disease? Neurology 1997; 49:918-25.

26. Hensley K, Butterfield DA, Hall N, Cole P, Subramaniam R, Mark R, Mattson MP, Markesbery WR, Harris ME, Aksenov M. Reactive oxygen species as causal agents in the neurotoxicity of the Alzheimer's disease-associated amyloid beta peptide. Ann N Y Acad Sci 1996; 786:120-34.

27. Mecocci P, Cherubini A, Polidori MC, Cecchetti R, Chionne F, Senin U. Oxidative stress and dementia: new perspectives in AD pathogenesis. Aging Clin Exp Res 1997; 9 (Suppl): 51-2.

28. Lee HC, Wei YH. Role in mitochondrial in human aging. J Biomed Sci 1997; 4:319-326.

29. Wallace DC, Melov S. Radicals r'aging. Nature Genet 1998; 19:105-6.

30. Corral-Debrinski M, Shoffner JM, Lott MT, Wallace DC. Association of mitochondrial DNA damage with aging and coronary atherosclerotic heart disease. Mutat Res 1992; 275:169-80.

31. Knight JA. Free radicals: their history and current status in aging and disease. Ann Clin Lab Sci 1998; 28:331-46.

32. Trounce I, Byrne E, Marzuki S. Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. Lancet 1989; 1:637-9.

33. Mirra SS, Hart MN, Terry RD. Making the Diagnosis of Alzheimer´s Disease. Arch Pathol Lab Med 1993; 117:132-44.

34. Braak H, Braak E. Neuropathological stageing of Alheimer-related changes. Acta Neuropathol 1991; 82: 239-59.

35. Miró O, Cardellach F, Barrientos A, Casademont J, Rötig A, Rustin P. Citochrme c oxidase assay in minute amounts of human skeletal muscle using single wavelength spectro-photometers. J Neurosci Meth 1998; 80:107-11.

36. Jarreta D, Orús J, Barrientos A, Miró O, Roig E, Heras M, Moraes CT, Cardellach F, Casademont J. Mitochondrial function in heart muscle from patients with idiopathic dilated cardiomyopathy. Cardiovasc Res 2000; 45:860-5.

37. Rustin P, Chretien D, Bourgeon T, et al. Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta 1994; 228:35-51.

38. Hedley D, Chow S. Flow cytometric measurement of lipid peroxidation in vital cells using parinaric acid. Cytometry 1992; 13:686-92.

39. Battino M, Bertoli E, Formiggini G, Sassi S, Gorini A, Villa RF, Lenaz G. Structural and functional aspects of the respiratory chain of synaptic and nonsynaptic mitochondria derived from selected brain regions. J Bioenerget Biomembr 1991; 23:345-63.

40. Mizuno Y, Suzuki K, Ohta S. Postmortem changes in mitochondrial respiratory enzymes in brain and a preliminary observation in Parkinson's disease. J Neurol Sci 1990; 96:49-57.

41. Scheuerle A, Pavenstaedt I, Schlenk R, Melzner I, Rodel G, Haferkamp O. In situ autolysis of mouse brain: ultrastructure of mitochondria and the function of oxidative phosphorylation and mitochondrial DNA. Virchows Archiv. B. Cell Pathol 1993; 63:331-4.

42. Te Koppele JM, Lucassen PJ, Sakkee AN, Van Asten JG, Ravid R, Swaab DF, Van Bezooijen CF. 8OHdG levels in brain do not indicate oxidative DNA damage in Alzheimer's disease. Neurobiol Aging 1996; 17:819-26.

43. Nagy Z, Esiri MM, LeGris M, Matthews PM. Mitochondrial enzyme expression in the hippocampus in relation to Alzheimer-type pathology. Acta Neuropath 1999; 97:346-54.

Figure Legends

Figure 1.- Activities of respiratory chain complexes related to time after death. In no case did the tendency to decrease reach statistical significance in this period of time. Note that for complex V, the three regression lines are superposed so that there appears to be only one line.

Figure 2.- Citrate synthase activity as an indicator of the number of mitochondria in brain homogenates. There were no differences between AD patients and controls, but the differences were statistically significant when comparing different brain areas. The Tukey *post-hoc* test indicated that the differences were between cerebellum and either the frontal cortex or hippocampus (^{a}). Bars represent means and error bars SD.

Figure 3.- When enzymatic activities of RC complexes were corrected by CS in order to normalize for mitochondrial content, there were no differences when comparing AD versus control brains for all the areas taken together (left). On the contrary, the differences between areas were present for complexes III and IV (right). p's denote the ANOVA test. The Tukey *post-hoc* test disclosed that the differences were between the frontal cortex and cerebellum (^{c}). Bars represent means and error bars SD.

Figure 4.- Membrane lipid peroxidation assessed as loss of *cis*-parinaric acid fluorescence (the greater the loss, the higher the peroxidation). There were no differences either comparing AD patients versus controls (left) or comparing different brain areas (right).

FIGURE 1

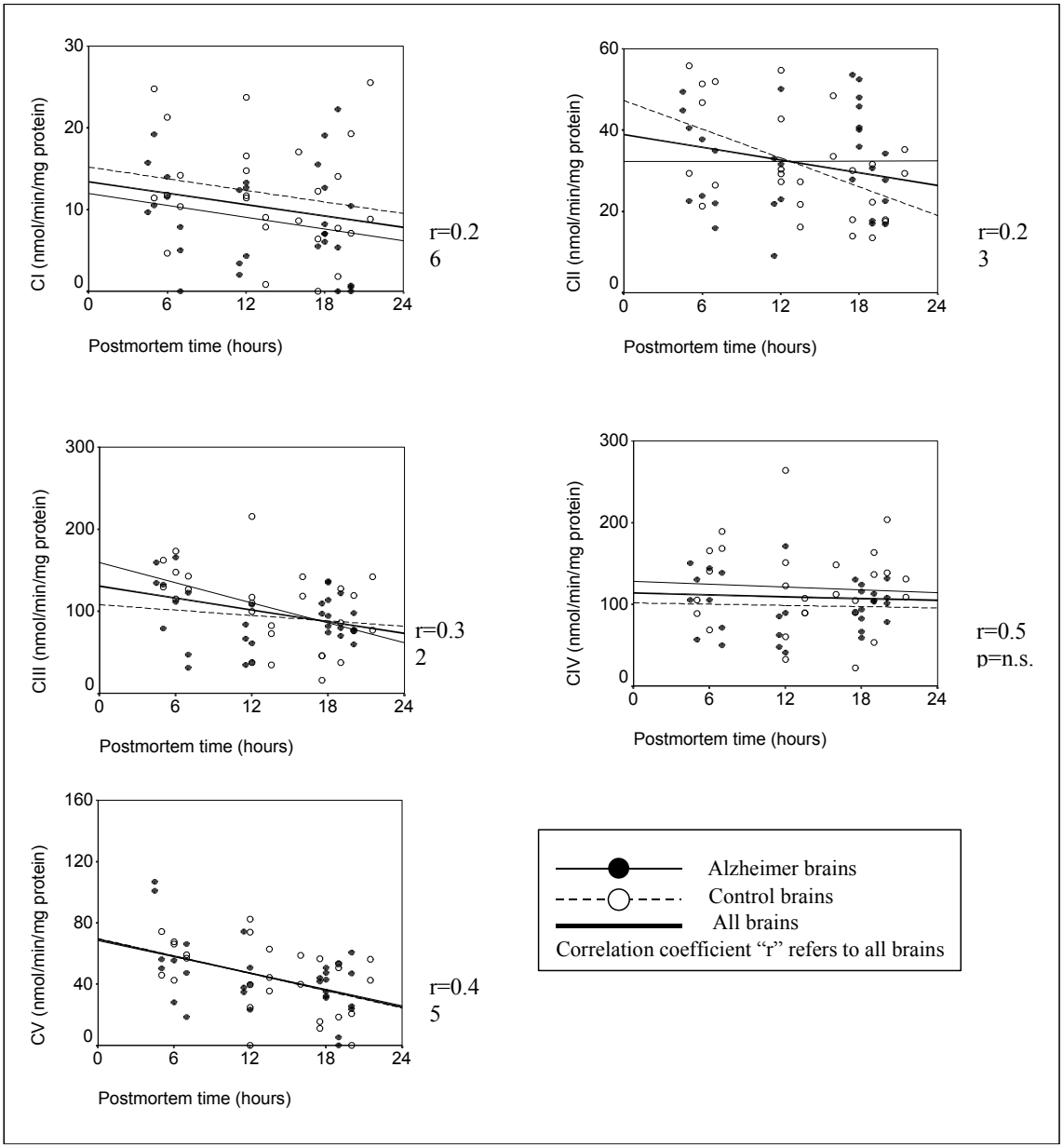
CI (nmol/min/mg protein)
Postmortem time (hours)
r=0.26
CII (nmol/min/mg protein)
Postmortem time (hours)
r=0.23
CIII (nmol/min/mg protein)
Postmortem time (hours)
r=0.32
CIV (nmol/min/mg protein)
Postmortem time (hours)
r=0.5
p=n.s.
CV (nmol/min/mg protein)
Postmortem time (hours)
r=0.45
Alzheimer brains
Control brains
All brains
Correlation coefficient "r" refers to all brains

FIGURE 2

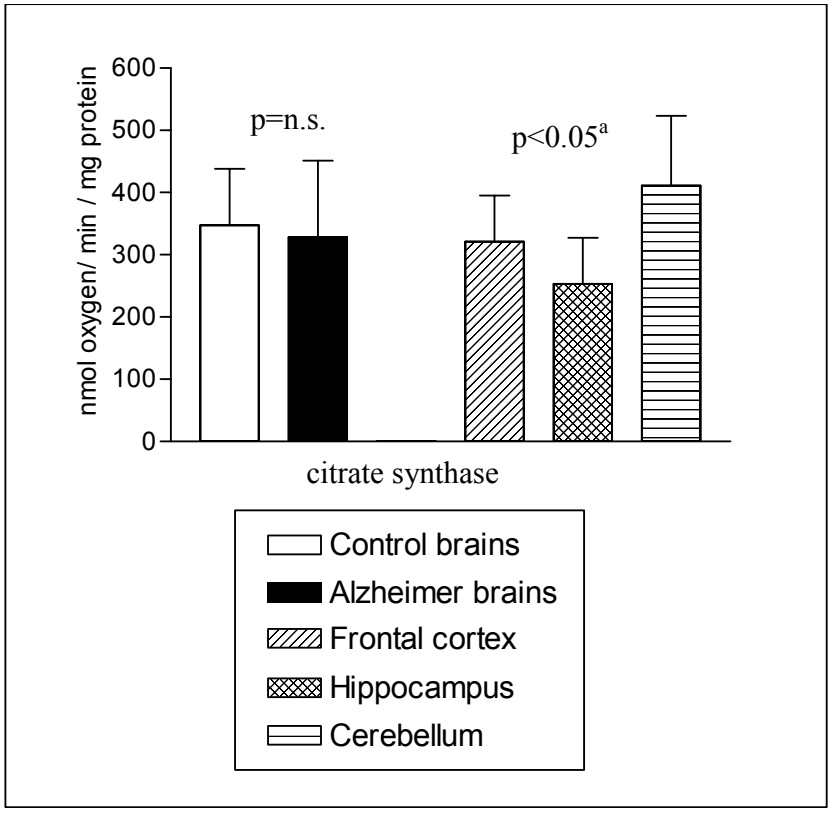
nmol oxygen/ min / mg protein
600
500
400
300
200
100
0
p=n.s.
p<0.05[a]
citrate synthase
Control brains
Alzheimer brains
Frontal cortex
Hippocampus
Cerebellum

FIGURE 3

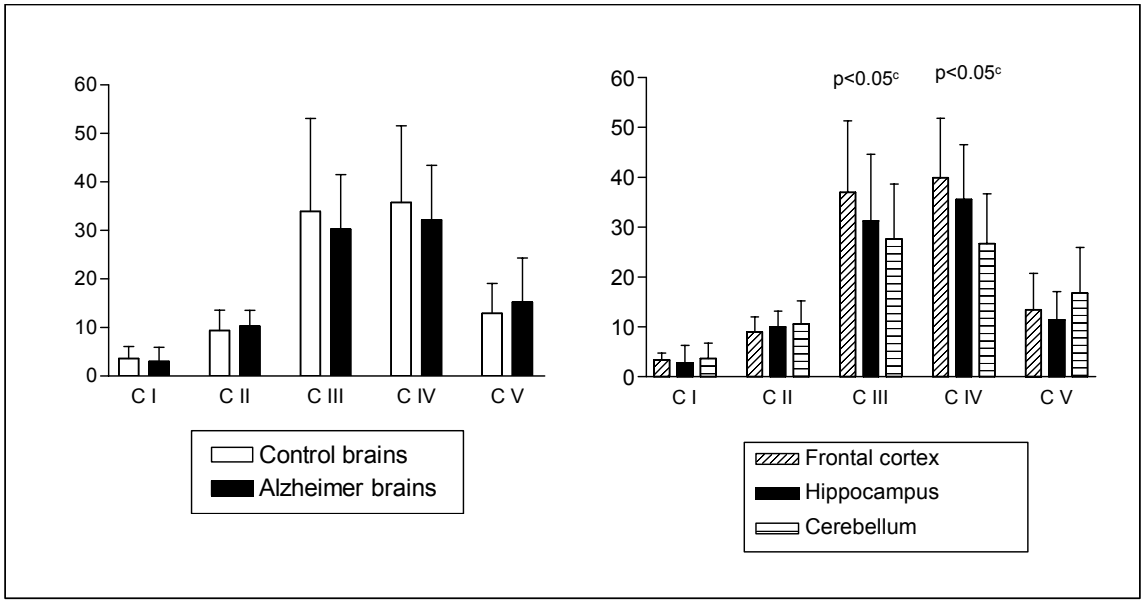
60
50
40
30
20
10
0
C I
C II
C III
C IV
C V
Control brains
Alzheimer brains
p<0.05c
p<0.05c
Frontal cortex
Hippocampus
Cerebellum

FIGURE 4

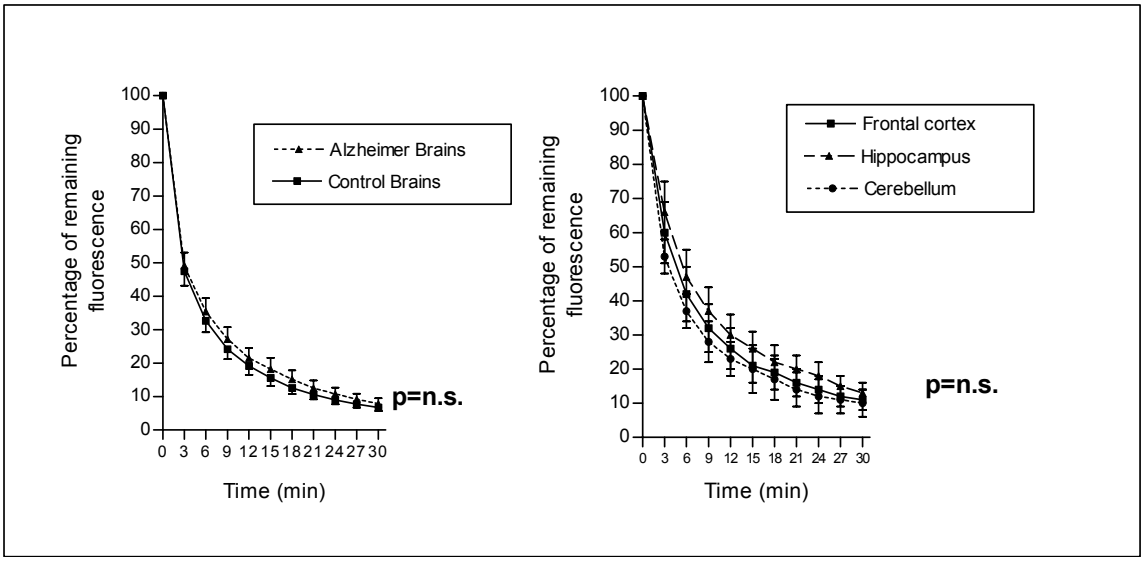
Percentage of remaining fluorescence
100
90
80
70
60
50
40
30
20
10
0
0 3 6 9 12 15 18 21 24 27 30
Time (min)
Alzheimer Brains
Control Brains
p=n.s.
Percentage of remaining fluorescence
Time (min)
Frontal cortex
Hippocampus
Cerebellum
p=n.s.

Table 1. Main clinical characteristics of patients and controls.

	Age	Sex (F/M)	Duration of disease	Smoking habit	Drugs	Time after death
AD patients	76.2±6.9	6/6	4.8±3.7	5	neuroleptics 4	13.2±6.2
n=12					benzodiacepines 3	
Controls	70.4±6.0	3/5	--	4	benzodiacepines 1	14.1±5.8
n=8						
	p=n.s.	p=n.s.	--	p=n.s.	p=n.s.	p=n.s.

Age and duration of disease is expressed in years ± SD.

Time from death refers to the delay in processing the samples from death. It is expressed in hours ± SD.

Comentarios adicionales

Este artículo recopila los resultados derivados del análisis de las actividades enzimáticas de la CRM y de la peroxidación lipídica de las muestras de cerebro disponibles. El comportamiento enzimático de los diferentes complejos analizados fue muy similar entre pacientes con EA y controles en las tres regiones cerebrales analizadas, no hallando ninguna diferencia estadística. Para algunos autores los defectos observados en la CRM de pacientes con EA son resultado de defectos nucleares o del daño adquirido en los componentes de la CRM. Se ha sugerido también que la disminución más frecuente hallada en el cerebro de pacientes (en la actividad de la citocromo c oxidasa o complejo IV) se relaciona más bien con el descenso global en la actividad mitocondrial que se manifiesta al haber un descenso en el número de mitocondrias (Ojaimi *et al*. 2001).

La medida del proceso químico de la peroxidación lipídica de membranas permite tener una idea del estado oxidativo de las muestras. El análisis de la peroxidación mediante la pérdida de fluorescencia del ácido cis-parinárico no reveló diferencias entre pacientes y controles en ninguna región cerebral. El aumento en la peroxidación lipídica descrito previamente en la EA por otros grupos se cree que es causado por radicales libres producidos por la disfunción de la CRM. En las muestras estudiadas en el artículo se da la correlación inversa: no se encontró un aumento de la peroxidación en los pacientes y por lo tanto este dato es coherente con la actividad de la CRM normal hallada. Nuestros datos indican que la CRM de los pacientes con EAE no se encuentra alterada significativamente y esto contradice los resultados descritos previamente por otros grupos. Las mínimas diferencias o la falta de ellas entre pacientes y controles parece indicar que los pacientes con EA estudiados no muestran un comportamiento muy diferente al que se ha descrito asociado al envejecimiento. Existen trabajos que describen actividades similares de algunos complejos de la CRM entre pacientes y controles de edad (Van Zuylen *et al*. 1992; Reichmann *et al*. 1993; Schagger *et al*. 1995). Se ha descrito la reducción de la actividad de la COX asociada al envejecimiento en cuatro regiones cerebrales diferentes (Ojaimi *et al*. 1999). También se ha observado que la peroxidación lipídica resultado del daño oxidativo puede ser un fenómeno que aumenta con la edad sin estar asociado a alguna enfermedad en concreto (Floyd *et al*. 2002). No obstante, existen abundantes evidencias de que en el cerebro de un paciente con EA se produce un aumento en la peroxidación lipídica como consecuencia del aumento en el estrés oxidativo (Marcus *et al*. 1998; Pratico *et al*. 2001; Pratico *et al*. 2004) pero la cuestión de si este aumento del estrés oxidativo está producido por una disfunción mitocondrial no se ha aclarado por completo. Algunos autores han indicado que la peroxidación lipídica puede ser una diana para el desarrollo de nuevas estrategias terapéuticas en fases tempranas de la EA. La medida de moléculas de isoprostano en el CSF puede contribuir a valorar experimentalmente terapias anti-oxidantes y el diagnóstico en el laboratorio de la EA (Montine *et al*. 2004).

Después de comprobar que no había diferencias entre los grupos de pacientes y de controles, se agruparon los datos por región cerebral y se compararon las actividades enzimáticas entre los tres tejidos cerebrales. Esta aproximación permitió observar que el número de mitocondrias y las

actividades enzimáticas de los complejos III y IV eran diferentes entre las tres regiones cerebrales. La dependencia del metabolismo energético varía según el órgano o tejido, por ejemplo los tejidos post-mitóticos son más dependientes y presentan actividades de la ETC mayores. En el cerebro puede darse también esta variabilidad en función de las necesidades energéticas de cada una de las regiones que forman parte de él, lo que explicaría las diferencias en las actividades enzimáticas de las diferentes regiones estudiadas por nuestro grupo.

8.5. Expression of mitochondrial genes and transcription estimation in different brain areas in Alzheimer's disease patients.

Neurobiology of disease (en prensa)

Benjamín Rodríguez-Santiago, Virginia Nunes.

Contribución del doctorando:

Extracción de RNA de las muestras de necropsia cerebral y de sangre de pacientes y controles y reacciones de transcripción inversa.

Puesta a punto del método de cuantificación de cDNA mediante PCR en tiempo real de cinco genes mitocondriales (cit b, ND4, 12S, COX II, ND6) y uno nuclear (18S). Trabajo experimental con las muestras de pacientes y controles. Cálculos matemáticos y estadísticos. Preparación y escritura del manuscrito, tablas 1 a 6 y figuras 1 y 2.

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Fecha: Tue, 12 Oct 2004 19:58:30 +0100
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Ms. No.: NBD-04-199R1
Title: EXPRESSION OF MITOCHONDRIAL GENES AND TRANSCRIPTION ESTIMATION IN DIFFERENT BRAIN AREAS IN ALZHEIMER'S DISEASE PATIENTS
Corresponding Author: Dr. Virginia Nunes
Authors: Benjamin Rodriguez-Santiago, BSC;

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ARTICLE IN PRESS
YNBDI-00904; No. of pages: 9; 4C: 4

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Neurobiology of Disease

www.elsevier.com/locate/ynbdi
Neurobiology of Disease xx (2004) xxx–xxx

Expression of mitochondrial genes and transcription estimation in different brain areas in Alzheimer's disease patients

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Received 28 July 2004; revised 16 September 2004; accepted 13 October 2004

Accumulation of mitochondrial defects is hypothesised to play a role in the complex pathophysiology of the sporadic form of Alzheimer's disease (SAD). Changes in expression of mtDNA encoded genes have been reported in SAD. However no conclusive results were obtained by using different methodologies and analysing distinct transcripts in a variety of brain areas. Here, we measured the expression of five mitochondrial-encoded genes in three brain areas and in lymphocytes from patients and controls by performing reverse transcription followed by quantitative real-time PCR. The analysis of gene expression was also performed by carrying out classic dot-blot experiments to evaluate the two techniques. SAD and control samples showed similar gene expression levels. Estimation of the transcription rate was also measured. No differences were observed when comparing patients and controls. Selective differences in transcription rates were observed only when samples were pooled by tissue, lymphocytes being the tissue with the highest rates.

Keywords: Alzheimer's disease; Aging; Neurodegeneration; Oxidative stress; Mitochondria; mtDNA; Gene expression; Transcription; Real-time PCR

Introduction

Although specific mutations in amyloid precursor protein and presenilin genes have been associated with the rare forms of familial Alzheimer's disease (AD), the causes of the much more commonly occurring sporadic AD that accounts for 90–95% of cases remain elusive. Sporadic forms of AD present a complex aetiology due to interactions between environmental conditions and the genetic background of each individual (Rocchi et al., 2003). Several studies have suggested that mitochondrial dysfunction may contribute to AD aetiology (Bonilla et al., 1999; Castellani et al., 2002). Structural (Hirai et al., 2001), biochemical (Sayre et al., 2001) and genetic (Lin et al., 2002) mitochondrial abnormalities have been described providing some evidence that mitochondria may play a role in the pathogenesis of AD. However, there are also several works reporting no mitochondrial association with AD (Chinnery et al., 2001; Davis and Parker, 1998; Janetzky et al., 1996), therefore, the role of mitochondrial disturbances is still controversial.

One of the mitochondrial abnormalities described is the change in the expression of mitochondrial and nuclear genes, encoding polypeptidic subunits of the cytochrome *c* oxidase and NADH dehydrogenase enzyme complexes integrated in the electron transport chain (ETC). The precise relationship between defects in the mitochondrial ETC and AD pathogenesis is still unknown, but it has been proposed that a decrease in mRNA levels of the ETC complexes subunits in affected brain regions may impair the oxidative metabolism affecting ATP and reactive oxygen species (ROS) production. These features could lead to an increase in neuronal susceptibility to cell death in AD. To understand the role of mitochondrial abnormalities of ETC in AD aetiology, several studies have investigated mRNA expression of mitochondrial-encoded genes in complexes I and IV (Aksenov et al., 1999; Chandrasekaran et al., 1994, 1997; Simonian and Hyman, 1994) and of nuclear-encoded mitochondrial genes in complexes IV and V (Aksenov et al., 1999; Chandrasekaran et al., 1994, 1997). Another recent study has analysed the expression of 11 mitochondrial-encoded genes (Manczak et al., 2004). Due to the disparity of the results, the methodologies used and the variety of brain regions analysed a definitive conclusion has not been established. In order to further investigate this point, we have evaluated mitochondrial gene expression in AD samples. These samples were previously analysed for several mtDNA point mutations and rearrangements and our results did not reveal a definitive conclusion (Rodriguez-Santiago et al., 2001a,b). Nonetheless, frontal cortex from AD patients showed a 28% reduction in their mtDNA contents when mtDNA depletion was analysed by real-time PCR (Rodriguez-Santiago et al., 2001b).

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Available online on ScienceDirect (www.sciencedirect.com.)

0969-9961/$ - see front matter © 2004 Published by Elsevier Inc.
doi:10.1016/j.nbd.2004.10.004

The relative expression of five mitochondrial genes (Cytochrome b, 12 S, ND4, COX II encoded by the mtDNA heavy chain and ND6 encoded by the mtDNA light chain) and one reference gene (nuclear ribosomal 18 S gene) have been determined in blood and in three brain areas (cerebellum, hippocampus and frontal cortex) from AD patients and controls. Dot-blot experiments have also been performed in order to compare the two relative quantification techniques. Due to the relationship between mtDNA amount, transcription and gene expression in the mitochondria, we have also estimated the transcription rates of the genes analysed in an attempt to provide a comprehensive picture of the mitochondrial genetics underlying AD.

Materials and methods

All AD patients were sporadic cases. Informed consent was obtained following the declaration of Helsinki and following the rules of the Ethical Committee of the Hospital Clínic-UB.

Brain samples

For relative gene expression and transcription rate studies, samples of cerebellum, hippocampus and frontal cortex from 12 histopathologically confirmed AD patients and from 7 controls were obtained from the Neurologic Tissue Bank of the Hospital Clínic-Universitat de Barcelona. The time between death and frozen storage of neurological tissues was always inferior to 12 h. This post-mortem delay was found not to influence in mRNA expression in similar studies, demonstrating that RNA can be obtained from AD brain even with a 24-h post-mortem interval and can be used for gene expression studies (Inoue et al., 2002; Johnson et al., 1986). The age range was 56–84 years for AD and 55–70 years for controls. Controls were individuals without dementia or other neurological disease in whom the neuropathological analyses excluded the presence of senile plaques and neurofibrillary tangles. Mitochondrial DNA from these brain samples had been qualitatively and quantitatively studied by our group in previous works (Rodriguez-Santiago et al., 2001a,b).

Blood samples

Blood samples from 25 living AD patients and from 16 age-matched controls were also analysed for gene expression studies. Seventeen of these AD patients and eight controls had been previously examined (Rodriguez-Santiago et al., 2001a,b). Unfortunately, both RNA and DNA for performing transcriptional analysis were only available from eight controls and 11 AD subjects. The age range for AD patients and controls was 59–87 years and 58–94 years, respectively. Living AD patients were diagnosed according to standard neurological criteria (McKhann et al., 1984).

RNA extraction and reverse transcription

Total RNA extraction was performed with the TriPure Isolation Reagent (Roche). Isolated total RNA integrity was electrophoretically verified by ethidium bromide staining and checked spectrophotometrically by scanning from 320 to 260 nm. First Strand cDNA Synthesis Kit (Amersham Biosciences) was used for RT. Two replicate RT reactions were performed for each isolated RNA. After RT, an aliquot of each sample was prepared at 37.5 ng cDNA per μl.

Real-time RT-PCR

Primer sequences used for detection and amplification of the mitochondrial and the reference 18 S target genes are summarised in Table 1. Real-time PCR mastermix consisted of (to the indicated end-concentration): 1.6 μl $MgCl_2$ (3 mM) for ND4, 12 S, COX II and ND6 reactions or 0.8 μl (2 mM) for Cyt b and 18 S; 0.5 μl forward primer (0.25 μM), 0.5 μl reverse primer (0.25 μM); 2 μl LightCycler Fast Start DNA Master SYBR Green I (Roche Molecular Biochemicals) and water up to 18 μl final volume. Seventy-five nanograms of cDNA were added as PCR template. The products of the two RT reactions were amplified two and

t1.1 Table 1
t1.2 Forward and reverse primers and PCR programs used to amplify and detect mitochondrial and reference genes

t1.3 Primers	Amplification	Melting
t1.4 ND4 (Complex I)	2 s 94°C	0 s 95°C
t1.5 5′-AAG TCA TCA AAA AGC TAT TA-3	5 s 50°C	30 s 73°C
t1.6 5′-CTT ACA TCC TCA TTA CTA TTC-3′	6 s 72°C*	0 s 90°C**
t1.7 Cyt b (Complex III)	2 s 94°C	6 s 95°C
t1.8 5′-GGG GCC ACA GTA ATT ACA AA-3′	5 s 57°C	40 s 63°C
t1.9 5′-GGG GGT TGT TTG ATC CCG TTT-3′	9 s 72°C*	0 s 92°C**
t1.10 12S (rRNA)	5 s 94°C	5 s 95°C
t1.11 5′-CCT CCC CAA TAA AGC TAA AA-3	5 s 50°C	30 s 72°C
t1.12 5′-GCT ATT GTG TGT TCA GAT AT-3′	5 s 72°C*	0 s 86°C**
t1.13 COX II (Complex IV)	2 s 94°C	0 s 95°C
t1.14 5′-CTG AAC CTA CGA GTA CAC CG-3′	5 s 55°C	30 s 79°C
t1.15 5′-TTA ATT CTA GGA CGA TGG GC-3′	12 s 72°C*	0 s 91°C**
t1.16 ND6 (Complex I)	2 s 94°C	30 s 94°C
t1.17 5′-ACA CTC AAC AAG ACC TCA ACC-3′	6 s 57°C	60 s 78°C
t1.18 5′-TAG TTT TTT TAA TTT ATT TAG GGG GAC T-3′	7 s 72°C*	0 s 88°C**
t1.19 18S (Reference)	4 s 94°C	10 s 94°C
t1.20 5′-GCG AAA GCA TTT GCC AAG AA-3′	10 s 58°C	45 s 78°C
t1.21 5′-CAT CAC AGA CCT GTT ATT GC-3′	15 s 72°C*	0 s 95°C**

t1.22 * Single fluorescence measurement.
t1.23 ** Continuous measurement increasing temperature at 0.1°C/s.

three times, respectively, to ensure the best accuracy and reproducibility.

The experimental PCR run protocol included: initial denaturation program at 95°C for 10 min followed by amplification–quantification (40 cycles) and melting curve programs. Details regarding temperatures and incubation times are shown in Table 1. The fluorescent product was detected at the last step of each amplification cycle by single acquisition. The Second Derivate Maximum Method was performed for crossing point (CP) determination using LightCycler Software 3.5 (Roche).

Relative expression software tool

After real-time PCR experiments, the CP mean value resulting from five replicates performed for each sample was introduced to a software tool. We have used the REST application (Pfaffl et al., 2002) developed on the basis of a mathematical model (Pfaffl, 2001) to perform the relative expression analysis. Real-time amplification PCR efficiencies were calculated with the equation: $E = 10^{(-1/\text{slope})}$ (Bustin, 2000) using REST software. Efficiency rates of the six transcripts investigated for every tissue studied were calculated using a range of dilutions (60 pg and 3, 15, 75 and 375 ng) repeated six times, three times for each of the two replicate RT pools of available cDNAs (Pfaffl et al., 2002), with high linearity (Pearson correlation coefficient >0.98). Therefore, efficiency corrected quantification is calculated automatically by REST. The statistical model included with REST software is a Pair Wise Fixed Reallocation Randomisation Test and was used as described (Pfaffl et al., 2002) to find differences in expression between AD patients and controls assessed in group means.

Dot-blot

Classic dot-blot and hybridisation techniques were performed to analyse mitochondrial gene expression of ND4, Cyt b, 12 S and COX II normalised with 18 S reference gene. Two dot-blots for each brain tissue (cerebellum, hippocampus, frontal cortex) with 1 μg of total RNA from each AD patient and control were performed. Blots were hybridised using specific [α-^{32}P]dCTP labelled probes for each mitochondrial and reference gene. Densitometric values were obtained with Quantity One software (Bio-Rad Laboratories, USA) after scanning the hybridised blots with Personal Molecular Imager FX System (Bio-Rad Laboratories). The mean value of the two blots was used to calculate the normalised levels of mitochondrial transcripts.

Transcription rate estimation

The mtRNA/mtDNA ratio has been used to estimate the transcription rate (Barrientos et al., 1997). Using the results from the mtDNA quantification previously reported (which were obtained by using real-time PCR quantification after extracting DNA through standard procedures (Rodriguez-Santiago et al., 2001b), two ratios were calculated for the estimation of the transcription rate. First, nDNA/nRNA ratio for 18 S was calculated for every AD and control subject in each tissue, the nRNA value being the result of applying the formula $(\text{Efficiency}_{18S})^{CP18s}$ of described models (Pfaffl, 2001; Soong et al., 2000). Likewise, a second mtRNA/mtDNA ratio was calculated for every subject of this study by dividing the mtRNA/nRNA expression ratio (obtained by applying the formula $\{(\text{Efficiency}_{\text{target}})^{CPtarget}/(\text{Efficiency}_{18S})^{CP18S}$ for each transcript and specific tissue} by the mtDNA/nDNA ratio previously obtained (Rodriguez-Santiago et al., 2001b). Then, mean ratios of each group were estimated for comparisons between them.

Statistical analysis

Results are given as mean ± standard deviation (SD). The SPSS software was used for statistical analysis excepting for gene expression, which was analysed by REST (see subsection above). Using SPSS, an unpaired *t* test previous verification that the samples had the same variance (Levene) was applied to compare AD patients and controls. A Kolgomorov–Smirnov goodness-of-fit test was used to ascertain if the samples were normally distributed. A two-way ANOVA for repeated measures was used to test differences in the calculated ratios in different brain areas and in lymphocytes between AD patients and controls. This approach allows that, if there are no differences one-way (for example between groups), from a statistical point of view, the groups are considered together to test the other way (for example between areas), and vice versa, increasing the overall power of the analysis. A *P* value of less than 0.05 was considered statistically significant. If ANOVA was significant, a Tukey post hoc test was applied to determine between which pairs of groups the difference lay.

Results

Relative expression of mitochondrial transcripts

Confirmation of primer specificity was assessed by agarose gel electrophoresis and resulted in a single product with the expected length for each RT-PCR transcript (Fig. 1). Melting curve analysis resulted in a single product-specific melting temperature, as is shown in Fig. 1. The real-time PCR efficiency rates of the six transcripts investigated for every tissue studied are summarised in Table 2. Using REST application relative expression ratios were calculated on the basis of group means for target mitochondrial genes versus the reference gene 18 S, and group ratio results were tested for significance. The expression of 18 S in every tissue has to be similar in patients and in controls (as can be seen in Table 2, the mean values were similar between AD and controls) to determine that expression of the nuclear reference gene used is not modulated by the disease. Then, 18 S can be used to normalise the expression of mitochondrial genes. Relative expression results were obtained and compared (Table 2). After performing two, or in some cases three replicates, for each cDNA proceeding from two different RT products (five replicates for each sample), the CP mean value ± SD remained very similar between AD patients and controls for the five mitochondrial genes and for the reference gene investigated. REST gave us the relative expression ratio of the five mitochondrial genes revealing that its gene expression was similar between AD and controls in the three brain areas and in lymphocytes. Fig. 2 illustrates the factor of regulation for the mitochondrial target genes analysed and if this up- or down-regulation is significantly different or not in each brain area. Although a global down-regulation of all genes was observed in cerebellum and hippocampus of AD, with several genes

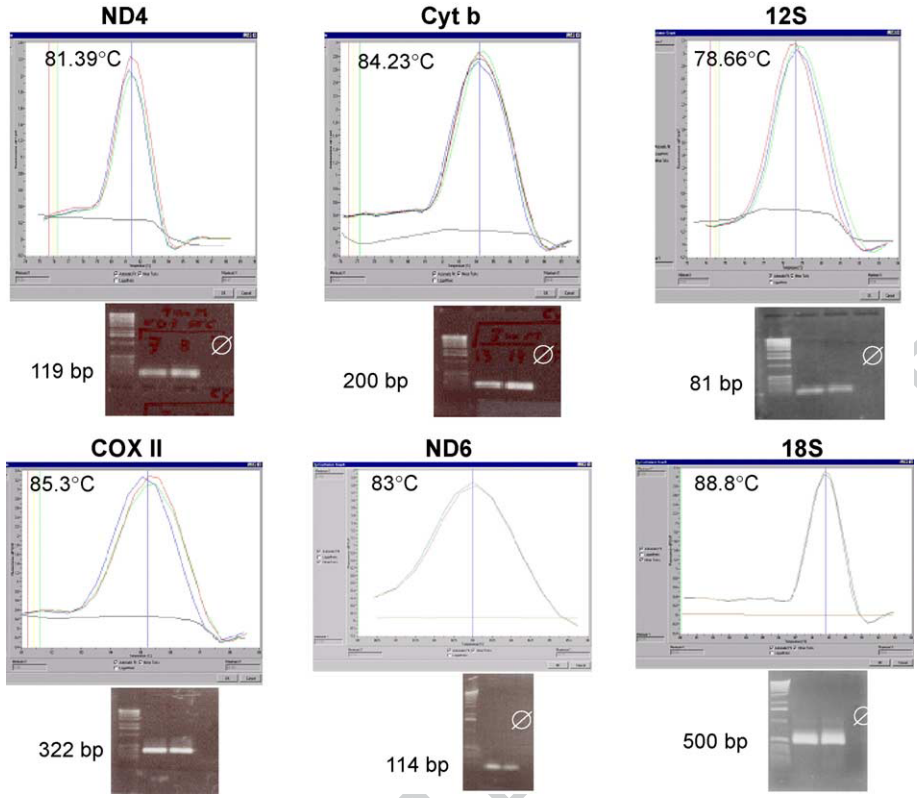

Fig. 1. Primer specificity confirmation for mitochondrial and nuclear reference gene amplifications. Melting curve analysis showing that each of the primer pairs used amplified a single predominant product with a distinct melting temperature (Tm). Once the predicted length of each product had been confirmed by agarose gel electrophoresis, the Tm was used to identify specific products in subsequent analysis.

showing >2.5-fold reduction, in no case did these data reach statistical significance.

Dot-blot experiments

Dot-blot experiments confirmed the obtained results by real-time PCR. After data management of densitometric results, the statistical analysis demonstrated that the mitochondrial gene expression was similar in patients and controls (Table 3).

Estimation of transcription rates

Previous to estimating the transcription rate values, we calculated the nDNA/nRNA ratio in every tissue to ascertain whether this coefficient was similar in patients and controls (Table 4). As the nDNA/nRNA ratio remained unchanged, it was considered a constant, and the estimation of the mitochondrial transcription rate was performed, dividing the mtRNA/nRNA ratio by the mtDNA/nDNA ratio.

The estimation of the transcription rates for the five mitochondrial genes in every tissue is shown in Table 5. Statistical analysis between AD and controls did not disclose significant differences, demonstrating similar transcription rates between AD and control groups for the genes analysed. Differences in transcription rates were only obvious when comparisons were performed grouping samples by tissue (Table 6). Lymphocyte was the tissue with a statistically significant increased rate for the five genes analysed.

Discussion

The sequence-independent detection of cDNA with real-time PCR based on SYBR Green I chemistry allowed the quantification of five mitochondrial cDNAs and one reference nuclear cDNA using gene-specific primers in a rapid and flexible method. In the present work, we performed a relative quantification analysis of different mitochondrial RNAs as representatives of the three mitochondrial polycistronic transcripts to investigate a possible alteration in mitochondrial gene expression in AD patients. The classic technique for performing relative quantification has been based mainly on Northern, slot or dot-blot followed by specific probe hybridisation. Blot techniques require a relatively high amount of RNA, are difficult to standardise and are susceptible to misinterpretations due to the densitometrically analysed bands often being saturated which can lead to an underestimation of the peaks. We have used reverse transcription (RT) followed by real-time PCR, which represents a sensitive and powerful tool for analysing RNA. While it has tremendous potential for quantitative applications, a comprehensive knowledge of its technical aspects is required. Careful considerations of the assay design, template preparation, mathematical evaluation and analysis of data generated, calculation of final results and statistical methods are essential for precise gene quantification (Freeman et al., 1999). Our real-time-PCR experiments of gene expression revealed results equivalent to those obtained with dot-blot experiments, but unlike dot-blot analysis, which requires 5–10 μg

t2.1 Table 2
t2.2 Mitochondrial relative gene expression by real-time PCR

t2.3		RT-PCR Efficiency	Control mean CP ± SD*	Alzheimer mean CP ± SD*	Normalised expression ratio
t2.4	Cerebellum		$n = 7$	$n = 12$	
t2.5	ND4	1.42	31.30 ± 2.94	31.81 ± 2.44	0.85
t2.6	12S	1.69	18.36 ± 1.69	19.02 ± 1.36	0.72
t2.7	Cyt b	1.61	17.47 ± 1.96	17.82 ± 1.26	0.86
t2.8	COX II	1.44	22.78 ± 4.89	23.68 ± 4.30	0.75
t2.9	ND6	1.78	21.87 ± 2.42	22.22 ± 1.57	0.83
t2.10	18S	1.76	19.55 ± 3.24	19.58 ± 2.62	
t2.11	Hippocampus		$n = 4$	$n = 9$	
t2.12	ND4	1.46	34.13 ± 1.66	34.05 ± 2.52	0.35
t2.13	12S	1.57	19.78 ± 1.61	19.46 ± 1.66	0.39
t2.14	Cyt b	1.75	18.98 ± 0.55	18.59 ± 1.22	0.42
t2.15	COX II	1.56	21.83 ± 1.06	21.42 ± 1.68	0.39
t2.16	ND6	1.79	23.70 ± 1.44	22.93 ± 1.85	0.53
t2.17	18S	1.80	21.26 ± 3.63	19.57 ± 3.04	
t2.18	Frontal Cortex		$n = 7$	$n = 12$	
t2.19	ND4	1.47	33.32 ± 2.28	33.37 ± 1.48	1.33
t2.20	12S	1.70	18.46 ± 2.00	19.41 ± 1.92	0.81
t2.21	Cyt b	1.73	18.39 ± 1.17	18.43 ± 0.61	1.36
t2.22	COX II	1.51	23.89 ± 4.26	24.24 ± 4.31	1.15
t2.23	ND6	1.67	22.80 ± 1.89	22.83 ± 1.48	1.31
t2.24	18S	1.77	19.91 ± 2.82	20.41 ± 2.87	
t2.25	Lymphocytes		$n = 25$	$n = 16$	
t2.26	ND4	1.51	32.58 ± 2.72	32.83 ± 2.96	0.87
t2.27	12S	1.70	19.69 ± 1.22	19.62 ± 1.47	1.03
t2.28	Cyt b	1.74	20.42 ± 1.68	20.43 ± 1.49	0.99
t2.29	COX II	1.53	24.88 ± 3.02	24.86 ± 1.87	1.00
t2.30	ND6	1.67	24.41 ± 2.24	24.33 ± 2.33	0.98
t2.31	18S	1.83	18.03 ± 2.04	18.02 ± 1.94	

t2.32 The relative expression ratio was computed by REST, based on its real-time PCR efficiency and the crossing point (CP) difference of AD patients samples versus control samples. P value > 0.05; not significant in all mean group normalised ratio comparisons.
t2.33 * Represents mean CP and SD resulting from three and two LightCycler runs performed, respectively, with the two cDNAs obtained from the same RNA sample.

of total RNA, real-time PCR techniques require only 1 μg for RT-PCR reactions. Moreover, real-time RT-PCR based on SYBR Green I chemistry in combination with the REST (Pfaffl et al., 2002) strategy provides sensitive, specific and reproducible quantification of RNA levels, allows for high throughput and reduces post-PCR manipulations.

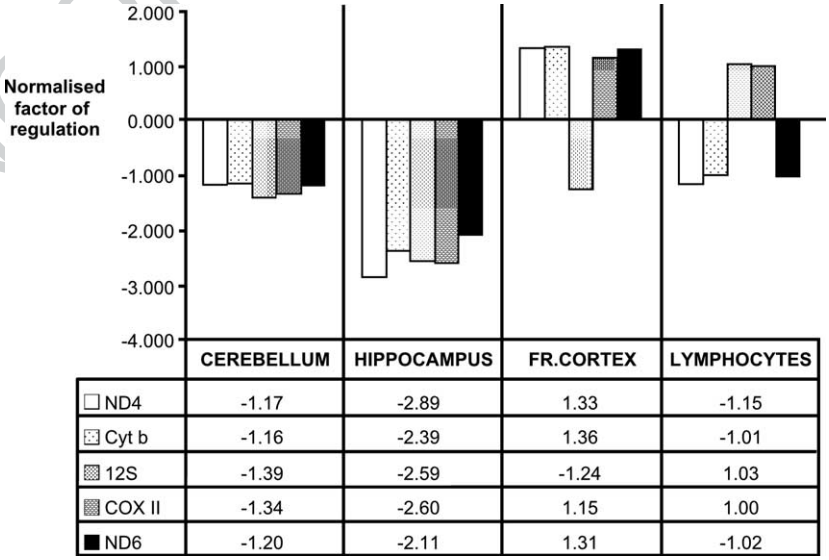

Fig. 2. Factor of regulation for the mitochondrial target genes analysed in different brain tissues and lymphocytes from Alzheimer's disease patients. When up-regulation occurs the factor of regulation is equal to the given value as Normalised Relative Expression Ratio of Table 2. In the case of down-regulation, the regulation factor is illustrated as a reciprocal value (1/expression ratio). P value >0.05 in all cases, all genes showed similar gene expression in AD patients and controls for every tissue analysed (brain regions and lymphocytes).

Table 3
Mitochondrial gene expression by dot-blot

	ND4	Cyt b	12S	COX II
Cerebellum				
AD (n = 7)	0.03 ± 0.01	0.11 ± 0.02	0.04 ± 0.01	0.20 ± 0.03
Controls (n = 12)	0.04 ± 0.01	0.16 ± 0.08	0.05 ± 0.01	0.24 ± 0.08
Hippocampus				
AD (n = 4)	0.03 ± 0.01	0.09 ± 0.01	0.05 ± 0.01	0.18 ± 0.03
Controls (n = 9)	0.03 ± 0.01	0.12 ± 0.03	0.04 ± 0.01	0.20 ± 0.06
Frontal cortex				
AD (n = 7)	0.03 ± 0.02	0.12 ± 0.07	0.06 ± 0.02	0.23 ± 0.16
Controls (n = 12)	0.03 ± 0.01	0.08 ± 0.01	0.05 ± 0.01	0.18 ± 0.03

Gene expression ratios normalised with the 18S nuclear gene. The mean value of two blots was used to calculate the normalised levels of mitochondrial transcripts. No differences were observed (P value >0.05; not significant).

A large number of studies have described mitochondrial defects in AD, and it has been hypothesised that a defective mitochondrial metabolism might set up a cascade of pathological events contributing to selective neuronal vulnerability in AD. However, there are several studies reporting contradictory evidence about the role of mitochondrial defects in AD (Chinnery et al., 2001; Davis and Parker, 1998; Janetzky et al., 1996). Nonetheless, the eventual contribution of mitochondrial dysfunction to worsen the clinical features of AD is a possibility that cannot be ruled out in view of the currently available data (Bonilla et al., 1999; Castellani et al., 2002; Hirai et al., 2001; Lin et al., 2002; Sayre et al., 2001). One of the possible factors leading to an impairment in energy production through mitochondrial metabolism could be a reduced expression of the genes that encode protein subunits of mitochondrial respiratory chain (RC) enzyme complexes, resulting in a decrease of complex activities.

We have found no evidence for a decrease in gene expression in the AD samples analysed. Reductions in COX I, COX III, ND1 and ND4 mRNA levels have been reported (Chandrasekaran et al., 1994, 1997) in the middle temporal association cortex region of five AD and five control brain samples by Northern blot analysis. Analysis of mitochondrial 12 S RNA in hippocampus from the same individuals did not reveal alterations in gene expression, which is in agreement with our findings in the same brain region. In these studies, the primary motor cortex region was also analysed but mitochondrial gene expression in AD was normal when compared to controls. Other authors found a decrease in mRNA levels of COX II and a reduced activity of cytochrome oxidase complex when performing in situ hybridisation experiments in hippocampus (Simonian and Hyman, 1994). In contrast to these studies, in the cortex region analysed in the present work (frontal cortex) with the real-time PCR quantification strategy, the mRNA levels of COX II and ND4 were similar in AD patients and controls. Recently, 11 mitochondrial-encoded genes have been analysed in frontal cortex samples from six early and six definite AD patients by using real-time PCR strategy and the comparative cycle threshold (CT) method for analysing results (Manczak et al.,

Table 4
mtDNA quantification and 18S expression (nDNA/nRNA) ratios

	Control	Alzheimer
Cerebellum	n = 7	n = 12
mtND2/18S*	0.56 ± 0.19	0.54 ± 0.15
$ndna_{18s}/nRNA_{18S}$	4.00 ± 3.99	2.071 ± 1.40
Hippocampus	n = 4	n = 9
mtNd2/18S*	3.00 ± 2.25	2.61 ± 1.67
$nDNA_{18s}/nRNA_{18S}$	0.68 ± 1.11	1.04 ± 1.46
Frontal cortex	n = 7	n = 12
mtND2/18S*	4.51 ± 1.71	3.25 ± 1.98
$nDNA_{18S}/nRNA_{18S}$	0.58 ± 0.46	0.43 ± 0.37
Lymphocytes	n = 8	n = 11
mtND2/18S	0.17 ± 0.22	0.21 ± 0.21
$nDNA_{18S}/nRNA_{18S}$	1.715 ± 1.791	1.156 ± 0.782

mtND2 used as representative of the whole mtDNA for quantification analysis.
Data previously published by our group (Rodriguez-Santiago et al., 2001b).
P value >0.05 in all cases.

Table 5
Estimation of transcription rates in different brain tissues and lymphocytes from AD patients and controls

	Control	Alzheimer
	Mean ± SD	Mean ± SD
Cerebellum	n = 7	n = 12
ND4	3.34 ± 6.67	3.21 ± 2.96
12S	0.70 ± 0.64	0.95 ± 0.94
Cyt b	0.24 ± 0.27	0.21 ± 0.20
COX II	0.24 ± 0.27	0.26 ± 0.28
ND6	18.18 ± 16.98	17.51 ± 17.50
Hippocampus	n = 4	n = 9
ND4	6.11 ± 11.75	10.75 ± 27.44
12S	0.06 ± 0.006	0.06 ± 0.09
Cyt b	0.50 ± 0.03	0.48 ± 1.00
COX II	0.21 ± 0.40	0.166 ± 0.30
ND6	1.73 ± 3.29	0.69 ± 0.55
Frontal cortex	n = 7	n = 12
ND4	1.33 ± 0.96	1.90 ± 1.76
12S	0.05 ± 0.03	0.12 ± 0.09
Cyt b	0.08 ± 0.05	0.12 ± 0.10
COX II	0.06 ± 0.04	0.13 ± 0.14
ND6	0.40 ± 0.25	0.63 ± 0.59
Lymphocytes	n = 8	n = 11
ND4	300.79 ± 264.26	159.56 ± 133.40
12S	14.39 ± 13.15	6.39 ± 6.10
Cyt b	36.62 ± 33.99	17.656 ± 13.89
COX II	11.20 ± 10.31	10.39 ± 9.94
ND6	138.89 ± 122.20	63.33 ± 54.51

Given are the mean group mtRNA/mtDNA value obtained by dividing the mtRNA/nRNA ratio) by the mtDNA/nDNA ratio shown in Table 4. SPSS statistical software was used to test for differences, P value >0.05; not significant in all mean group comparisons.

t6.1 Table 6
t6.2 Estimation of transcription rates by tissue

t6.3		Cerebellum	Hippocampus	Frontal cortex	Lymphocytes
t6.4		$n = 19$	$n = 13$	$n = 19$	$n = 19$
t6.5	ND4	3.25 ± 3.14	9.32 ± 23.27	1.69 ± 1.51	219.03 ± 205.55*
t6.6	12S	0.86 ± 0.83	0.06 ± 0.09	0.10 ± 0.08	9.76 ± 10.22*
t6.7	Cyt b	0.22 ± 0.22	0.49 ± 0.95	0.11 ± 0.09	25.64 ± 25.48*
t6.8	COX II	0.25 ± 0.27	0.18 ± 0.32	0.10 ± 0.12	10.73 ± 9.82*
t6.9	ND6	17.76 ± 17.93	1.01 ± 1.78	0.54 ± 0.50	95.14 ± 94.48*

t6.10 Given are means ± SD of AD and control samples grouped by tissue.
t6.11 * P value <0.05, significantly different as compared to brain tissues.

2004). The authors found a down-regulation in mitochondrial genes for complex I in AD patients and increased mRNA levels for complex III and IV mitochondrial genes in AD. This comparative CT method involved averaging duplicate samples taken as the CT values for the mitochondrial gene and the reference gene. The ΔCT value was obtained by subtracting the average reference CT value from the average CT value of mitochondrial genes. The average ΔCT of six control subjects was used as the calibrator. This study showed gene expression results as fold changes, calculated according to the formula $2^{-(\Delta\Delta CT)}$, where ΔΔCT was the difference between ΔCT and the ΔCT calibrator value. Unfortunately, statistical conclusions cannot be reached with this type of analysis (and other previous reports of gene expression in AD) between AD patients and controls, in contrast to the approach used in the present study which allowed us to perform statistical comparisons of the gene expression between groups.

Another study (Aksenov et al., 1999) analysed two mitochondrial and two nuclear genes encoding subunits of the cytochrome *c* oxidase and the NADH dehydrogenase in the hippocampus, inferior parietal lobule and cerebellum of 10 AD and 10 age-matched control subjects. The authors reported an altered proportion between COX II and COX IV mRNAs in the AD brain. Changes in the proportion of COX II and COX IV transcripts and a coordinated decrease of ND4 and ND15 mRNAs were found in the AD hippocampus and inferior parietal lobule, but not in cerebellum. The authors hypothesised that the decrease of ND4 gene expression could lead to the inhibition of the normal ubiquinone oxidoreductase activity of NADH dehydrogenase (Aksenov et al., 1999). Our results in cerebellum and hippocampus for ND4 and COX II gene expression did not show any changes and thus do not confirm previously reported results.

The authors of the aforementioned studies speculate that a decrease in mRNA levels in affected brain regions may contribute to alterations of oxidative metabolism in AD (Aksenov et al., 1999; Chandrasekaran et al., 1994, 1997; Simonian and Hyman, 1994). Our results disagree in part with this hypothesis because if there are alterations in oxidative metabolism in our AD patients, this would not be related with a reduction in mitochondrial RNA levels since we have not detected any. Besides, we have determined enzymatic activities and peroxidation of membranes (as a marker of oxidative stress) in the same tissues analysed in the present work, and the results did not reveal significant differences (Casademont et al., in press), suggesting that the brain samples studied had not suffered important mitochondrial alterations. Nevertheless, this is at variance with existing literature. There is some evidence reporting an increase of lipid peroxidation when measuring F2-isoprostane molecules (Montine et al., 2002; Pratico and Sung, 2004) and ETC defects in AD brain, reduction of complex IV activity being the most frequent finding (Kish et al., 1992; Mutisya et al., 1994; Parker et al., 1994). Other authors have found normal complex IV activity while activities of complexes II and III (Reichmann et al., 1993), or complex V (Schagger et al., 1995) were decreased. The reasons for the discrepancies described in lipid peroxidation as well as ETC activities are unclear, although they are likely to be explained by methodological factors such as variations in AD diagnostic criteria, methods in enzymatic studies, the influence of the age of patients or delay in processing samples after death. It must also be borne in mind that the DNA and RNA isolation techniques used in the present and other previous gene expression studies have been performed with tissue homogenates. This approach might not reveal profound individual cellular differences as were reported by Hirai et al. (2001) and could account for the heterogeneous state of mitochondrial transcripts and ETC enzymes in AD, contributing to these contradictory results. Single neuron studies aimed at determining the relationship between ETC alterations and mitochondrial genetic changes within individual neurons may be an eventual future approach to test if a defect in a small proportion of key neurons plays a role in AD pathogenesis.

The estimation of transcription rates for each transcript were similar between AD and controls in a given region, with considerably elevated SD values. The high SD value might be caused by the heterogeneity and influence of multiple genetic and environmental factors in AD patients. Differences in transcription rates between different brain areas and lymphocytes are likely to be detected because every cell type may have particular and specific rates depending on its energy requirements. We have observed that lymphocytes had the highest transcription rates and the lower mtDNA content, suggesting that increased activity of transcription is a cellular response to reach similar gene expression levels of that observed in brain regions.

mtDNA transcription and mtDNA replication are interrelated processes in mitochondria. The transcription of mtDNA-encoded genes is initiated at two different promoters: one on the heavy chain and another on the light strand of the molecule. The resultant polycistronic transcripts are processed to produce mature rRNAs, tRNAs or mRNAs (Clayton, 1991). A transcript generated from the light-strand promoter is also necessary to prime the replication of the mtDNA molecule, functionally coupling mitochondrial gene expression with genome maintenance (Shadel and Clayton, 1997). The replication and the transcription of the mitochondrial genome depend exclusively on nuclear-gene products (Shoubridge, 2002). One of these products, the mitochondrial transcription factor A (TFAM), plays a complex role in the regulation of both processes: it is required for mtDNA maintenance and, together with two other factors, TFB1 and TFB2, stimulates mitochondrial transcription (Falkenberg et al., 2002). The mtDNA reduction (not statistically

significant) in the frontal cortex region of AD patients (Rodriguez-Santiago et al., 2001b) does not seem to be associated with a down-regulation in RNA expression levels. This fact suggests the existence of a mechanism that compensates the reduction in mtDNA content, so that the transcriptional activity remains equivalent to controls to reach stable RNA steady-state levels in AD. Recently, Coskun et al. (2004) have analysed the mtDNA control region (CR) from the frontal cortex of AD patients and controls. The authors reported that AD brains exhibited a striking increase in mtDNA CR mutations located in elements known to be involved in mtDNA L-strand transcription and/or H-strand replication, and they are associated with reductions in the mtDNA L-strand ND6 mRNA and in the mtDNA copy number. Consequently, the authors propose that somatic mtDNA CR mutations could account for the sporadic appearance and mitochondrial defects seen in late-onset AD and thus may contribute to the etiology of this disease (Coskun et al., 2004). This CR was also analysed by sequencing in the AD brain samples studied in the present work by our group and no significant changes were detected in the CR (data not shown). Our data are consistent with the studies performed by others (Chinnery et al., 2001; Simon et al., 2001). These authors found no evidence that these somatic CR mtDNA point mutations accumulate either in the brains of normal elderly individuals or in the brains of individuals with AD. In the case of the samples analysed by our group, the gene expression of the mitochondrial-encoded ND6 gene was normal when compared to controls, so the relationship between these CR mutations and gene expression changes in the samples analysed could not be established since no alteration and point mutation were detected. The CR mutations associated to reduction in ND6 gene expression and mtDNA copy number observed by Coskun et al. is an attractive finding, but it needs further investigation. The analysis of the ETC activity or mitochondrial function will be of interest to establish a potential effect of these CR mutations on phenotype. An impairment between mtDNA replication and transcription systems has been described in other conditions, the aging process being the best known (Dinardo et al., 2003; Shoubridge, 2002). Besides, recent studies suggest the existence of tissue-specific differences in the regulation of mitochondrial gene expression. The existence of several mtDNA transcription factors and CR point mutations may allow flexible regulation of mtDNA gene expression in response to the complex physiological demands of mammalian metabolism (Shoubridge, 2002). This regulation seems especially interesting in affected brain regions in AD and aging. In view of our results, it appears that transcriptional activity in the frontal cortex of our AD patients could produce a number of mitochondrial transcripts similar to that in controls in spite of a reduced mtDNA content.

A complex dynamic relationship exists between mtDNA content, CR point mutations, mitochondrial RNA levels, mtDNA replication and gene expression, the mitochondrial function being dependent on its equilibrium. The characterisation of the regulation and stoichiometry of the different mitochondrial transcription factors in the brain areas might provide important clues for the understanding of the pathogenic mechanisms in AD. The functional implications of mitochondrial gene expression abnormalities in AD patients described in the literature remains elusive. We think that a better understanding of the mitochondrial role in the complex AD aetiology may be achieved through a multiple approach that combines the characterisation of mitochondrial genetics and the potential biochemical defects in sporadic AD patients.

Uncited reference

Schagger and Ohm, 1994

Acknowledgments

The authors thank Montse Gómez and Jordi Casademont for their critical and helpful comments on the manuscript and Helena Kruyer for editorial help. We thank the Neurologic Tissue Bank (*Serveis Científico-Tècnics*, *Universitat de Barcelona*) for providing the brain samples and all the subjects who have collaborated in this study. This work was supported by the Spanish MCYT (SAF99-0079 and SAF2002-02668), by the *Instituto de Salud Carlos III* (*Redes* C03/07 and G03/054) and grant 2001 SGR 00399 from the *Generalitat de Catalunya*, Spain.

References

Aksenov, M.Y., Tucker, H.M., Nair, P., Aksenova, M.V., Butterfield, D.A., Estus, S., Markesbery, W.R., 1999. The expression of several mitochondrial and nuclear genes encoding the subunits of electron transport chain enzyme complexes, cytochrome *c* oxidase, and NADH dehydrogenase, in different brain regions in Alzheimer's disease. Neurochem. Res. 24, 767–774.

Barrientos, A., Casademont, J., Cardellach, F., Estivill, X., Urbano-Marquez, A., Nunes, V., 1997. Reduced steady-state levels of mitochondrial RNA and increased mitochondrial DNA amount in human brain with aging. Brain Res., Mol. Brain Res. 52, 284–289.

Bonilla, E., Tanji, K., Hirano, M., Vu, T.H., DiMauro, S., Schon, E.A., 1999. Mitochondrial involvement in Alzheimer's disease. Biochim. Biophys. Acta 1410, 171–182.

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25, 169–193.

Casademont, J., Rodríguez-Santiago, B., Miro, O., Beato, A., S., L., Nunes, V. Cardellach, F., Mitochondrial respiratory chain in brain homogenates: Activities in different brain areas in patients with Alzheimer's disease. Aging Clinical and Experimental Research, in press.

Castellani, R., Hirai, K., Aliev, G., Drew, K.L., Nunomura, A., Takeda, A., Cash, A.D., Obrenovich, M.E., Perry, G., Smith, M.A., 2002. Role of mitochondrial dysfunction in Alzheimer's disease. J. Neurosci. Res. 70, 357–360.

Chandrasekaran, K., Giordano, T., Brady, D.R., Stoll, J., Martin, L.J., Rapoport, S.I., 1994. Impairment in mitochondrial cytochrome oxidase gene expression in Alzheimer disease. Brain Res., Mol. Brain Res. 24, 336–340.

Chandrasekaran, K., Hatanpaa, K., Rapoport, S.I., Brady, D.R., 1997. Decreased expression of nuclear and mitochondrial DNA-encoded genes of oxidative phosphorylation in association neocortex in Alzheimer disease. Brain Res., Mol. Brain Res. 44, 99–104.

Chinnery, P.F., Taylor, G.A., Howell, N., Brown, D.T., Parsons, T.J., Turnbull, D.M., 2001. Point mutations of the mtDNA control region in normal and neurodegenerative human brains. Am. J. Hum. Genet. 68, 529–532.

Clayton, D.A., 1991. Replication and transcription of vertebrate mitochondrial DNA. Annu. Rev. Cell Biol. 7, 453–478.

Coskun, P.E., Beal, M.F., Wallace, D.C., 2004. Alzheimer's brains harbor somatic mtDNA control-region mutations that suppress mitochondrial transcription and replication. Proc. Natl. Acad. Sci. U. S. A. 101, 10726–10731.

Davis II, J.N., Parker Jr., W.D., 1998. Evidence that two reports of mtDNA cytochrome *c* oxidase "mutations" in Alzheimer's disease are based on

nDNA pseudogenes of recent evolutionary origin. Biochem. Biophys. Res. Commun. 244, 877–883.

Dinardo, M.M., Musicco, C., Fracasso, F., Milella, F., Gadaleta, M.N., Gadaleta, G., Cantatore, P., 2003. Acetylation and level of mitochondrial transcription factor A in several organs of young and old rats. Biochem. Biophys. Res. Commun. 301, 187–191.

Falkenberg, M., Gaspari, M., Rantanen, A., Trifunovic, A., Larsson, N.G., Gustafsson, C.M., 2002. Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. Nat. Genet. 31, 289–294.

Freeman, W.M., Walker, S.J., Vrana, K.E., 1999. Quantitative RT-PCR: pitfalls and potential. BioTechniques 26 (112–122), 124–125.

Hirai, K., Aliev, G., Nunomura, A., Fujioka, H., Russell, R.L., Atwood, C.S., Johnson, A.B., Kress, Y., Vinters, H.V., Tabaton, M., Shimohama, S., Cash, A.D., Siedlak, S.L., Harris, P.L., Jones, P.K., Petersen, R.B., Perry, G., Smith, M.A., 2001. Mitochondrial abnormalities in Alzheimer's disease. J. Neurosci. 21, 3017–3023.

Inoue, H., Kimura, A., Tuji, T., 2002. Degradation profile of mRNA in a dead rat body: basic semi-quantification study. Forensic Sci. Int. 130, 127–132.

Janetzky, B., Schmid, C., Bischof, F., Frolich, L., Gsell, W., Kalaria, R.N., Riederer, P., Reichmann, H., 1996. Investigations on the point mutations at nt 5460 of the mtDNA in different neurodegenerative and neuromuscular diseases. Eur. Neurol. 36, 149–153.

Johnson, S.A., Morgan, D.G., Finch, C.E., 1986. Extensive postmortem stability of RNA from rat and human brain. J. Neurosci. Res. 16, 267–280.

Kish, S.J., Bergeron, C., Rajput, A., Dozic, S., Mastrogiacomo, F., Chang, L.J., Wilson, J.M., DiStefano, L.M., Nobrega, J.N., 1992. Brain cytochrome oxidase in Alzheimer's disease. J. Neurochem. 59, 776–779.

Lin, M.T., Simon, D.K., Ahn, C.H., Kim, L.M., Beal, M.F., 2002. High aggregate burden of somatic mtDNA point mutations in aging and Alzheimer's disease brain. Hum. Mol. Genet. 11, 133–145.

Manczak, M., Park, B.S., Jung, Y., Reddy, P.H., 2004. Differential expression of oxidative phosphorylation genes in patients with Alzheimer's disease: implications for early mitochondrial dysfunction and oxidative damage. Neuromol. Med. 5, 147–162.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., Stadlan, E.M., 1984. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. Neurology 34, 939–944.

Montine, T.J., Neely, M.D., Quinn, J.F., Beal, M.F., Markesbery, W.R., Roberts, L.J., Morrow, J.D., 2002. Lipid peroxidation in aging brain and Alzheimer's disease. Free Radical Biol. Med. 33, 620–626.

Mutisya, E.M., Bowling, A.C., Beal, M.F., 1994. Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. J. Neurochem. 63, 2179–2184.

Parker Jr., W.D., Parks, J., Filley, C.M., Kleinschmidt-DeMasters, B.K., 1994. Electron transport chain defects in Alzheimer's disease brain. Neurology 44, 1090–1096.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Pratico, D., Sung, S., 2004. Lipid peroxidation and oxidative imbalance: early functional events in Alzheimer's disease. J. Alzheimer's Dis. 6, 171–175.

Reichmann, H., Florke, S., Hebenstreit, G., Schrubar, H., Riederer, P., 1993. Analyses of energy metabolism and mitochondrial genome in post-mortem brain from patients with Alzheimer's disease. J. Neurol. 240, 377–380.

Rocchi, A., Pellegrini, S., Siciliano, G., Murri, L., 2003. Causative and susceptibility genes for Alzheimer's disease: a review. Brain Res. Bull. 61, 1–24.

Rodriguez Santiago, B., Casademont, J., Nunes, V., 2001a. Is there a relation between Alzheimer's disease and defects of mitochondrial DNA? Rev. Neurol. 33, 301–305.

Rodriguez-Santiago, B., Casademont, J., Nunes, V., 2001. Is mitochondrial DNA depletion involved in Alzheimer's disease? Eur. J. Hum. Genet. 9, 279–285.

Sayre, L.M., Smith, M.A., Perry, G., 2001. Chemistry and biochemistry of oxidative stress in neurodegenerative disease. Curr. Med. Chem. 8, 721–738.

Schagger, H., Ohm, T.G., 1995. Human diseases with defects in oxidative phosphorylation. 2. F1F0 ATP-synthase defects in Alzheimer disease revealed by blue native polyacrylamide gel electrophoresis. Eur. J. Biochem. 227, 916–921.

Shadel, G.S., Clayton, D.A., 1997. Mitochondrial DNA maintenance in vertebrates. Annu. Rev. Biochem. 66, 409–435.

Shoubridge, E.A., 2002. The ABCs of mitochondrial transcription. Nat. Genet. 31, 227–228.

Simon, D.K., Lin, M.T., Ahn, C.H., Liu, G.J., Gibson, G.E., Beal, M.F., Johns, D.R., 2001. Low mutational burden of individual acquired mitochondrial DNA mutations in brain. Genomics 73, 113–116.

Simonian, N.A., Hyman, B.T., 1994. Functional alterations in Alzheimer's disease: selective loss of mitochondrial-encoded cytochrome oxidase mRNA in the hippocampal formation. J. Neuropathol. Exp. Neurol. 53, 508–512.

Soong, R., Ruschoff, J., Tabiti, K., 2000. Detection of Colorectal Micrometastasis by Quantitative RT-PCR of Cytokeratin 20 mRNA. Roche Diagnostics internal publication.

Comentarios adicionales

La estrecha relación que existe entre los fenómenos de la replicacióny la expresión génica en el genoma mitocondrial nos llevó al estudio del estado de diversos RNA codificados por el mtDNA. La intención era determinar si había alguna alteración en la cantidad relativa de algún RNA mitocondrial y correlacionarla con las observaciones realizadas en el trabajo de cuantificación del mtDNA presentado en el apartado anterior. También se pretendió aclarar la discrepancia existente entre los diversos estudios previos sobre la expresión de genes mitocondriales en la EA. Estos estudios previos eran muy heterogéneos en cuanto a las técnicas usadas y el tejido analizado y quizá por eso los resultados publicados son tan dispares.

En el análisis realizado en nuestro laboratorio mediante RT-PCR cuantitativa en tiempo real se tuvieron en cuenta diferentes aspectos para asegurar la reproducibilidad y validez del método de cuantificación. Entre estos aspectos se incluyen el cálculo de las eficiencias de cada pareja de cebadores en cada tejido analizado, el uso de réplicas de la RT y de réplicas de la PCR y el uso de una herramienta informática que permitió establecer comparaciones estadísticas entre los diferentes grupos. La comparación entre pacientes con EA y controles no demostró diferencias significativas en la expresión de los 5 genes mitocondriales estudiados en ninguna de las tres regiones cerebrales ni en linfocitos. En el cerebelo y sobretodo en el hipocampo de los pacientes con EA se aprecia una disminución global de la expresión de todos los genes, mientras que en córtex frontal sólo el gen 12S presentaba una expresión disminuida. En linfocitos se apreció una ligera disminución de la expresión en los genes ND4, cit b y ND6. Quizá el resultado más sorprendente sea la disminución en el hipocampo, en el que todos los genes estaban al menos expresados dos veces menos que en los individuos control de esa región. Sin embargo, las pruebas estadísticas no evidenciaron diferencias significativas entre los dos grupos de individuos comparados. Dada la importancia de la función del hipocampo en los procesos cognitivos el dato mencionado merecería ser estudiado en una población mayor de pacientes con EA.

8.6. MITOCHONDRIAL GENETICS AND ALZHEIMER'S DISEASE

Capítulo del libro "*New frontiers in mitochondrial biogenesis and disease*", editor: F.Villarroya. Research Signpost Editors (2005), en prensa.

Benjamín Rodríguez-Santiago, Virginia Nunes

Contribución del doctorando

Escritura del manuscrito conjuntamente con la Dra. Virginia Nunes. Elaboración de las tablas 1 y 2 y las figuras 1, 3, 4, 5 y 6. Confección de la figura 2 a partir de una imagen cedida por la Dra. Montse Gómez.

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HAGO CONSTAR en calidad de editor del libro "New frontiers in mitochondrial biogenesis and disease" que será publicado por Research Signpost en el 2004, que el capítulo "Mitochondrial genetics and Alzheimer disease" que tiene por autores a Benjamín Rodríguez y Virginia Nunes, ha sido aceptado para su publicación en el mencionado libro.

A Barcelona, el 9 de noviembre del 2004

Firmado: Francesc Villarroya Gombau
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MITOCHONDRIAL GENETICS AND ALZHEIMER'S DISEASE

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Book: New frontiers in mitochondrial biogenesis and disease

Running Title: mtDNA and Alzheimer's disease

ABSTRACT

The causes of most neurodegenerative disease, including sporadic Alzheimer's disease (AD), remain enigmatic. There is, however, mounting evidence implicating mitochondrial dysfunction in AD patients. It has been suggested that the most consistent mitochondrial electron transport chain (ETC) defect reported in AD, a deficit in cytochrome c oxidase, could be determined by the mitochondrial genome. The issue of whether mitochondrial genetic alterations, inherited or acquired, underlie the disordered energy metabolism in AD has been analysed through multiple works studying several mitochondrial DNA (mtDNA) related defects. Specifically, mtDNA point mutations, presence of rearrangements, reduction in mtDNA copy number and changes in expression of mtDNA-encoded genes had been associated with AD etiology by several authors, but there are also other works reporting contradictory results or not confirming those previously reported. The mitochondrial involvement in AD etiology is still controversial. This chapter summarises the main mtDNA abnormalities reported in AD literature and the contribution of our group to the topic of mitochondrial genetic involvement in AD by performing diverse genetic and biochemical studies.

INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia in the elderly population [1]. AD is best characterised as a chronic brain disorder, having a lengthy pre-clinical phase followed by a malignant stage associated with neuronal degeneration, the loss of specific synaptic connections and the progressive erosion of higher cognitive functions [2]. AD has two major neuropathological hallmarks: an abundance of extracellular amyloid deposits, known as senile plaques, and fibrillar protein deposits within neurons, known as neurofibrillary tangles. Three genes have been identified as responsible for the rare early-onset familial form of the disease: the amyloid precursor protein gene, the presenilin 1 gene and the presenilin 2 gene. Mutations in these genes, however, account for less than 5% of the total number of AD cases. Mutations in these genes have not been identified in the remaining 95% of late-onset, sporadic AD cases, with a complex aetiology due to interactions between environmental conditions and genetic background of each individual [1].The molecular basis of the defects in these patients is the subject of several hypotheses.

Mitochondria carry out a number of important cellular functions, including apoptosis and essential pathways of intermediate metabolism: amino acid biosynthesis, fatty acid oxidation, steroid metabolism. Of key importance here is the role of mitochondria in oxidative energy metabolism. Mitochondrial oxidative phosphorylation (OXPHOS) generates most of the cell's ATP, and any impairment of the organelle's ability to produce energy cause catastrophic consequences, not only due to the primary loss of ATP, but also due to indirect impairment of "downstream" functions, such as the maintenance of organelle and cell calcium homeostasis. Moreover, deficient mitochondrial metabolism may generate reactive oxygen species (ROS) that can cause damage to several cell components. It is for these reasons that mitochondrial dysfunction is such an attractive candidate for an "executioner's" role in neuronal degeneration.

One hypothesis for the etiology of late-onset, sporadic AD is that it is caused by defects in mitochondrial OXPHOS. Structurally abnormal mitochondria have been observed in AD [3,4] brains, and deficiencies in mitochondrial OXPHOS enzymes, such as cytochrome oxidase (COX or complex IV), have been repeatedly reported in the brain and other tissues of AD patients [5,6]. Furthermore, mitochondrial OXPHOS defects have been recovered in cultured human cell cybrids by fusion of AD patient blood platelets to human cell lines that lack mtDNA (ρ^o cells) [5-13]. The suggested mechanism by which OXPHOS defects contribute to AD etiology is associated with the inhibition of ATP production and also an increase in

mitochondrial ROS production which, in turn, can activate the mitochondrial permeability transition pore (mtPTP). Electrons in the initial steps of the mitochondrial ETC can be transferred directly to molecular oxygen (O^2) to generate superoxide anion $O_2^{\cdot-}$. This superoxide anion is converted to hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutase, and the hydrogen peroxide is converted to hydroxyl radical $OH^{\cdot}$ by electron transfer from reduced transition metals. The resulting ROS (O^2, H_2O_2 and $OH^{\cdot}$) damage mitochondrial proteins and membranes and mutagenize mtDNA. This inhibits the ETC, causing the electron carriers to become reduced, further stimulating mitochondrial ROS production. The ultimate result is the activation of the mtPTP and destruction of the cell by apoptosis. Consistent with this scenario, the brains of late-onset, sporadic AD patients have been observed to have increased oxidative damage [14,15], increased activated caspase activity [16], and increased numbers of terminal-deoxynucleotidyltransferase-mediated-dUTP-nick-end-labeling-positive cells [5].

The origin of OXPHOS defects in AD and whether they are cause or consequence is unclear. It has been suggested that inhibition of mitochondrial OXPHOS leading to neurodegeneration could result from environmental intoxicants or from acquired or inherited mtDNA mutations [17]. Accumulation of mtDNA defects resulting from endogenous mitochondrial ROS damage could be a significant factor in the development of late-onset, sporadic AD [18]. Several groups have described several mtDNA alterations that could generate abnormal or a reduced number of components of the respiratory chain/OXPHOS system. The reported mtDNA alterations (which include, among others, point mutations, rearrangements, reduction in mtDNA copy number or gene expression changes) could account for some of the mitochondrial defects seen in AD mentioned above as is showed in **figure 1**. The study of mitochondrial genetics in AD patients has been performed with the idea that any mtDNA defect has potential consequences in ETC, contributing to mitochondrial dysfunction through ATP production impairment, which could interfere among others, with calcium homeostasis, amyloid metabolism and reactive oxygen species generation, enhancing the susceptibility of neurons to cell death. However, some of the mtDNA defects and mitochondrial abnormalities described have been also observed in the normal process of aging [19].

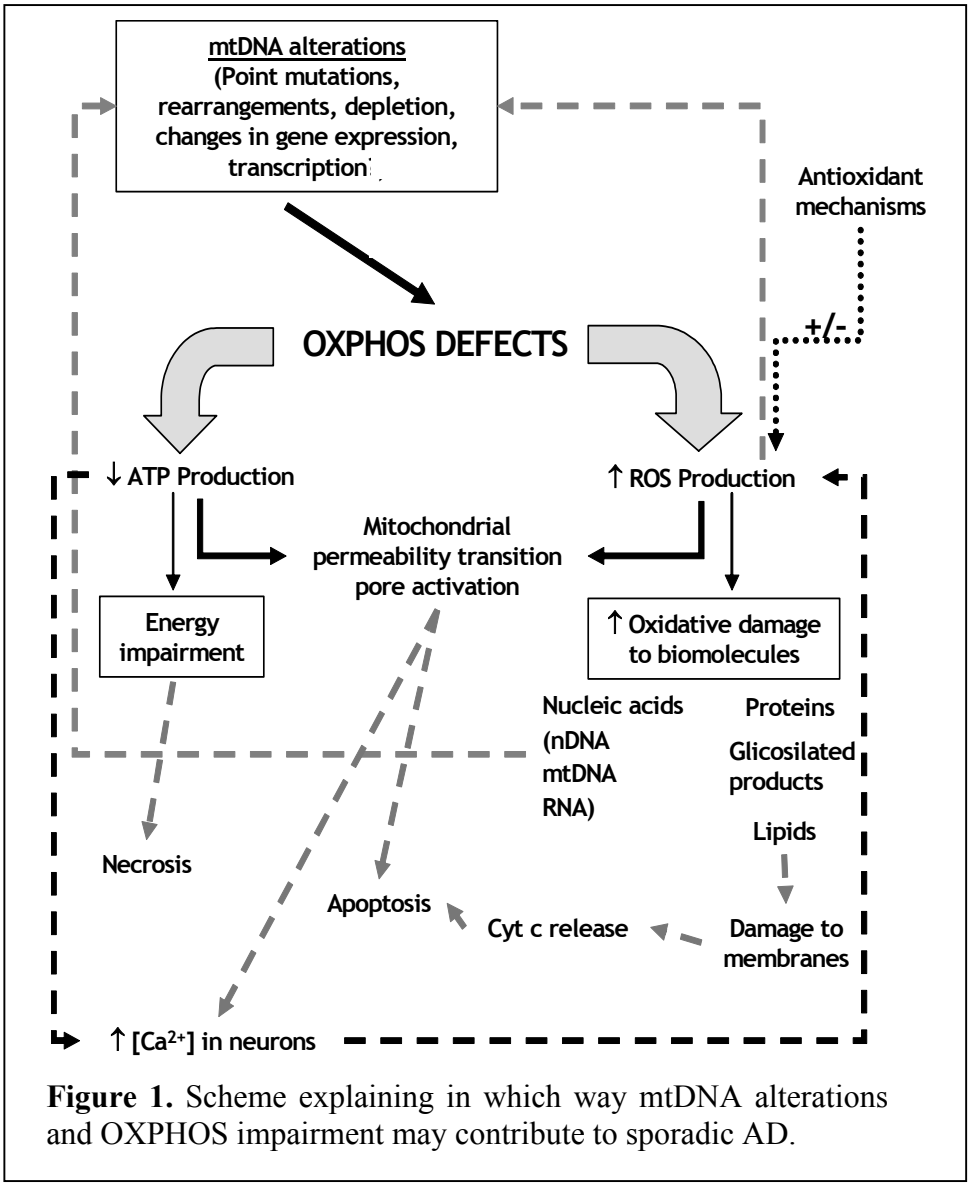


Figure 1. Scheme explaining in which way mtDNA alterations and OXPHOS impairment may contribute to sporadic AD.

FEATURES OF MITOCHONDRIAL GENETICS

A mitochondrial related disease is generally taken to mean a disorder of the respiratory chain, which is the final common pathway for oxidative metabolism. The respiratory chain is composed of over 70 different polypeptide subunits that form five enzyme complexes situated in the inner mitochondrial membrane. Most of these subunits are synthesised within the cytosol and are targeted to the inner mitochondrial matrix by a short peptide presequence, but 13 polypeptide subunits are synthesised within the mitochondrial matrix from mtDNA. A complex system of nuclear-encoded proteins is involved in the assembly of both nuclear and mtDNA subunits to form a functional respiratory chain. Each mitochondrion contains multiple copies (2-10) of a 16.5 kb circle of double stranded DNA that encodes for 37 genes: 13 essential OXPHOS polypeptides, 22 tRNA genes, a 12S, and a16S rRNA genes, necessary for protein synthesis within the mitochondrion (**figure 2**).

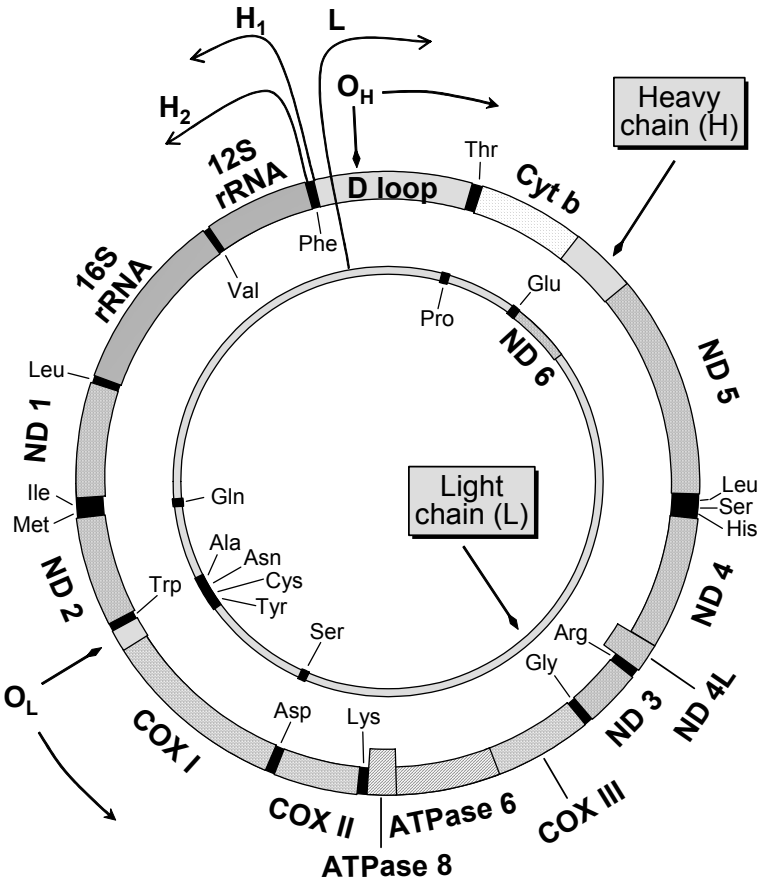


Figure 2. Scheme of the mitochondrial DNA genome.
tRNA genes are named following the three letter aminoacid code. Cyt b: cytochrome b; ND: NADH dehydrogenase subunits; COX: cytochrome c oxidase subunits. O_H and O_L are the origin of mtDNA heavy and light chain replication, respectively. Arrows indicate the synthesis direction. H_1, H_2 and L show the starting point and the direction of transcription of the heavy and light chain, respectively.

The genetics of mtDNA presents several specific features that are relevant for understanding some of the results obtained when mtDNA studies are performed:

- Maternal inheritance: The inheritance of mtDNA is mainly maternal, no significant influence of father's mtDNA has been described in the offspring.
- Bottleneck: The mitochondrial genetic bottleneck is the restriction in the amount of mtDNA within a single cell that occurs early during oogenesis. It is thought to be the main reason why heteroplasmic females transmit different percentages of mutant mtDNA to different offspring.
- Heteroplasmy: In humans, each human cell contains thousands of copies of mtDNA. Patients with defects in mtDNA often have a mixture of varying proportions of mutant and wild-type (normal) mtDNA, termed heteroplasmy.
- Relaxed replication: mtDNA replicates continuously, even in nondividing (postmitotic) cells. It is termed 'relaxed' because, unlike replication of nuclear DNA, it is not linked to the cell cycle.
- Threshold effect: Studies *in vitro* and *in vivo* have shown that the level of mutant mtDNA must exceed a critical threshold level before a biochemical defect of the mitochondrial respiratory chain becomes apparent in the cell. This is thought to arise through functional complementation by wild-type genomes at subthreshold levels of mutant mtDNA.

- Replicative segregation: When a cell divides, the mitochondria and mtDNA it contains are divided between the two daughter cells. These can receive different proportions of mutant and wild-type mtDNA.

mtDNA POINT MUTATIONS

There is increasing evidence that mitochondrial functions may be impaired in AD, but the pathogenic role of mtDNA mutations is still controversial. The first candidate mutation was reported in 1992, a G5460A transition in the ND2 gene [20]. It was found in 10 of 19 AD brains, but was absent in all 11 controls studied. These results could not be confirmed neither by others [21-24] nor by our group [25], and it appears that the G5460A mutation is almost certainly a neutral polymorphism. The presence of several mtDNA variants has been reported in AD but without establishing in any case a definite conclusion. **Table 1** summarises the most studied mtDNA variants found in AD patients. The cohort of AD patients and controls analysed in our laboratory did not present any of these mtDNA variants neither in AD nor in control brain and blood samples.

Table 1. Several mtDNA point mutations have been reported in AD

%AD	%Controls	%Normal population	Analysed tissue	Reference
		A3397G in ND1		
1.5 (2/135)	0 (0/20)	0 (0/248)	Brain	[26]
0 (0/65)	0 (0/76)	-	Brain	[27]
0 (0/70)	0 (0/80)	-	Blood	[28]
0 (0/92)	0 (0/59)	-	Blood-brain	[29][1]
0 (0/34)	0 (0/18)	-	Three brain areas	[25]
0(0/21)	0 (0/29)	-	Blood	
		G5460A/T in ND2		
52.6 (10/19)	0 (0/11)	-	Brain	[20]
53.3 (8/15)	0 (0/5)	-	Brain	[30][2]
0 (0/15)	3.6 (1/28)	-	Brain	[22]
0 (0/63)	3 (2/67)	-	Brain – Blood	[24]
3,1 (2/65)	2.6 (2/76)	2.8 (3/106)	Brain	[27]
2.5 (6/236)	2.9 (7/328)	-	Blood	[31]
0 (0/34)	0 (0/18)	-	Three brain areas	[25]
0(0/21)	0 (0/29)	-	Blood	
		G3196A in 16S rRNA		
0.7 (1/135)	-	0 (0/119)	Brain	[26]
0 (0/66)	0 (0/76)	-	Brain	[27]
0 (0/92)	0 (0/59)	-	Blood-brain	[29][1]
0 (0/34)	0 (0/18)	-	Three brain areas	[25]
0(0/21)	0 (0/29)	-	Blood	

1 Japanese population

2 Petruzzellla and colleagues were not able to detect this mutation in the same samples[21].

While a number of inherited and acquired mitochondrial diseases resulting from mutation are well known [32], the studies in AD have been hampered by the presence of ancient mitochondrial DNA fragments incorporated into the nuclear genome, or nuclear pseudogenes. In 1997 Davis and collaborators described the identification of six novel mtDNA point mutations in both AD patients and in control individuals, and furthermore, they reported that

the proportion of these polymorphisms was significantly higher in platelet-enriched DNA from the AD patients [33,34]. Three of the putative mutations were in subunit I of COX (G6366A, C6483T, and A7146G) while the other three were in the subunit II gene of COX (C7650T, C7868T, and A8021G). However, in subsequent studies, these putative mtDNA mutations were identified as artefacts [35] derived from PCR amplification of nuclear DNA, specifically, from nucleus-embedded mtDNA pseudogenes [36,37]. Lin *et al* have assessed the aggregate burden of mtDNA point mutations in a 1,197-bp fragment of the COX I gene in AD, elderly, and control subjects. The method used was a PCR-cloning-sequencing strategy which ensures that obtained results are not due to PCR error, nucleus-embedded mitochondrial pseudogenes or *ex vivo* oxidation [38]. They found no difference in average aggregate mutational burden between AD and elderly control subjects in types of point substitutions, distribution within the three codon positions or frequency of amino acid changes [38]. The mean aggregate mutational burden in AD and elderly subjects was twice the level found in younger controls, suggesting that point mutations accumulate or are acquired with aging. COX activity was also measured in that study resulting in a 20% decrease in elderly and AD subjects from the mean for younger controls [38].

Other germ-line mtDNA mutations have also been associated with late-onset AD, the most important being the A4336G variant. **Table 2** shows the main results concerning the analysis of this nucleotide pair (np) variant. The A4336G mutation in the $tRNA^{Gln}$ gene has been observed in about 5% of late-onset AD patients [26,43], and this association has been supported in three of four independent European studies [39,40,44,45]. The study of this point mutation in our lab by performing PCR-RFLP did not disclose significant conclusions since the point mutation was only detected in homoplasmy only in the three brain areas analysed from one AD patient and in the two available brain areas from another AD patient [25].

Although several mtDNA polymorphisms have been identified in AD patients (**Table 1 and 2**) none of them was common to all of the patients and all could have been due to the normal variation in human mtDNA. Thus, the pathogenic role of those polymorphisms could not be established. In summary, while these results are at least intriguing, it has been suggested that the presence of these variants, and specifically the A4336G variant, might be a marker for mtDNA involvement in some cases of AD, but they are unlikely to play a significant role in the vast majority of patients.

Studies analysing the association of mtDNA polymorphisms with APOE genotype revealed also contradictory results. Edland and colleagues reported that the prevalence of the mtDNA A4336G polymorphism was higher in AD subjects carriers of allele ε4 of the APOE gene [31], suggesting that APOE may be an important modifier of the A4336G effect and potentially explaining variable findings across previous studies. However, there are also studies that do not further confirm these findings [28,41].

Different works suggested that the European mtDNA haplogroups (which are defined by the presence of several specific mtDNA polymorphisms) J and Uk are protective of AD and Parkinson's disease [46,47] and are also associated with increased longevity [16]. Both European subhaplogroups J1 and Uk harbor the same cytocrome b mutation at np 14798, whereas subhaplogroup J2 harbors a different cytocrome b mutation at np 15257. Both of these cytocrhome b mutations alter conserved amino acids in the two coenzyme Q_{10} binding sites, and thus could affect the efficiency of proton pumping by the Q cycle of complex III. Because uncoupling mutations that partially depolarize the mitochondrial inner membrane proton gradient would keep the ETC carriers oxidized, these mutants would also reduce mitochondrial ROS production. This would reduce brain oxidative damage and neuronal somatic DNA mutations, explaining the protective effect of these mtDNA lineages for AD and Parkinson's disease [16,48].

Table 2. mtDNA A4336G tRNA^{Gln} point mutation in AD				
%AD	**%Controls**	**%Normal population**	**Analysed tissue**	**Reference**
3.7 (5/135)	0 (0/17)	0.7 (12/1691)	Brain	[26]
4.1 (8/194)	0.3 (1/296)	-	Brain	[39]
0.6 (1/155)	3.8 (4/105)	-	Blood	[40]
0 (0/65)	1.7 (1/59)	2.2 (2/92)	Brain	[27]
1.4 (1/70)	2.5 (2/80)	-	Blood	[28][1]
0 (0/92)	0 (0/59)	-	Blood - Brain	[29][2]
4.3 (8/185)	3.3 (6/179)	-	Brain	[41][1]
0.6 (1/161)	1.3 (1/78)	1.4 (2/144)	Blood	[42]
2.5 (6/236)	2.9 (7/238)	-	Blood	[31][1b]
16.6 (2/12)	0 (0/7)	-	Cerebellum	Partially previously published in [25]
10 (1/10)	0 (0/4)	-	Hippocampus	
16.6 (2/12)	0 (0/7)	-	Frontal Cortex	
0 (0/21)	0 (0/29)	-	Blood	
1 No association between APOE4 and A4336G 1b A43336G asociated with APOE4: 5/139 AD; 0 of 82 controls 2 Japanese population				

Examining the frequency distribution of mtDNA haplogroups and APOE, Carrieri and collaborators found differences between allele ε4carriers and non-carriers. For these authors, the APOE/mtDNA interaction is restricted to AD and may affect susceptibility to the disease. In particular, some mtDNA haplogroups (K and U) seem to neutralize the harmful effect of the APOE epsilon4 allele, lowering the epsilon4 odds ratio from statistically significant to non-significant values [49].

Recently Coskun and colleagues have analysed the mtDNA control region (CR), by cloning and sequencing, from the frontal cortex of AD patients and controls [50]. This CR includes the light and heavy strand transcription promoters, their mitochondrial transcription factor A (mtTFA) binding sites, the downstream conserved block sequences (CSB) I, II and III, and the origins of the heavy strand replication [51]. The authors reported that AD brains exhibited a striking increase in mtDNA CR mutations located in elements known to be involved in mtDNA light strand transcription and/or the heavy strand replication, and they are associated with reductions in the mtDNA light strand ND6 mRNA and in the mtDNA copy number [50]. AD brains had an average 63% increase in heteroplasmic CR mutations and certain AD samples harbored the disease-specific CR mutations T414G, T414C and T477C, and several AD brains between 74 and 83 years of age harbored the CR mutations T477C, T416C, and T195C, at levels of up to 70-80% heteroplasmy. The presence of the T414G mutation was confirmed in 65% of AD samples and it was absent in all controls. For these authors the fact that the T414G, T414C, and T477C mutations were only found in AD brains might suggest that AD is a distinct pathological phenomenon from aging [50]. However, these three mutations presented some particularities. The T414G mutation found in most AD brains was present in only a small percentage of mtDNA molecules with a sensitive PNA-clamping PCR method. This mutation could not be detected in other studies by different PCR and sequencing strategies [52,53]. Moreover, Coskun *et al* found that while the T414C and T477C were in a higher proportion of mtDNA molecules in some AD brains, not all AD brains harboured high levels of these mutations. After that the authors conclude that T414G could be a valuable marker for AD but that is the total mtDNA CR mutation load that contributes to the brain pathology and that this may include mutations such as T414C and T477C [50].

This CR was also analysed in the AD samples studied by our group [25,54] by sequencing and no changes were detected in the CR of the AD brain samples (unpublished data). This lack of changes is consistent with the studies performed by others [52,53], in which the authors found no evidence that these somatic CR mtDNA point mutations accumulate either in the brains of normal elderly individuals or in the brains of individuals with AD. In that aforementioned studies none of the subjects analysed harboured the CR T414G point mutation, reported to be specific of AD patients [50].

REARRANGEMENTS

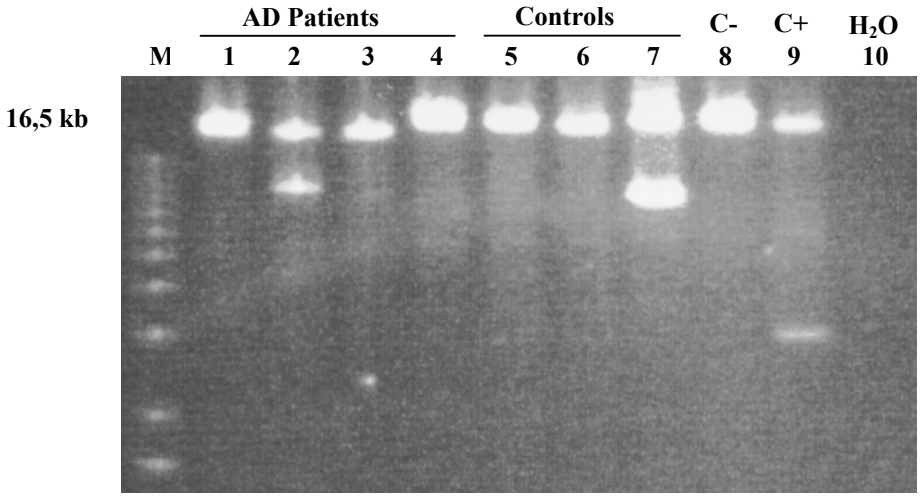
Somatic mtDNA rearrangement mutations have been observed to be increased in AD brains, the most frequent being the common mtDNA delta 4977 rearrangement, which has been reported to be elevated about 15-fold in AD patient brains up to age 75 years [55] or elevated 6.5-fold in temporal cortex of AD subjects [56]. In contrast, levels of this commonly studied rearrangement were not elevated in AD samples analysed by other groups [57-59]. The study of different rearrangements has also been performed; Gu and colleagues demonstrated that both the number and variety of mtDNA deletions/rearrangements were selectively increased in the substantia nigra of Parkinson's disease patients compared to patients with other movement disorders as well as patients with AD and age-matched controls [60].

Most of the supporting data for the accumulation of the common rearrangement has relied upon a derivative of the PCR method (semi-quantitative PCR, long PCR) rather than the more direct analyses based on Southern blotting or DNA sequencing. Particularly Southern blot can give qualitative and quantitative profiles of the integrity and proportion of wild type mtDNA to deleted mtDNA molecules, avoiding the problems and artefacts associated to PCR techniques. In the case of long PCR, it has been reported by several groups that this technique gives strong indications that a multitude of deletions can be found in aged individuals and in certain disease states [61]. The appearance of such products must, therefore, be regarded with caution and should only be accepted as evidence for the presence of an elevated level of deleted mtDNA if supported by an independent method, or if accompanied by rigorous controls and calibration.

The examination of mtDNA rearrangements in the AD samples was analysed by our group initially by long PCR. The data obtained revealed a variety of deletions in certain AD cases and controls. **Figure 3** illustrates the mtDNA profile of some cerebellum samples when long PCR was performed. The analysis by Southern blot confirmed that the samples studied reveal a very strong signal for the apparently full-length, intact, mtDNA in the three areas analysed of AD subjects [25]. The presence of deleted molecules in AD samples could not be detected by Southern; perhaps they could not reach the resolution level of this technique (~5%), but in view of the strong signal for the intact mtDNA, as shown in **figure 4**, it seems that the deleted molecules, if any, are unlikely to manifest any biochemical or clinical defect. This lack of effect on phenotype was demonstrated when analysis of ETC activities were performed by our group, showing normal ETC complexes values when compared to controls [62].

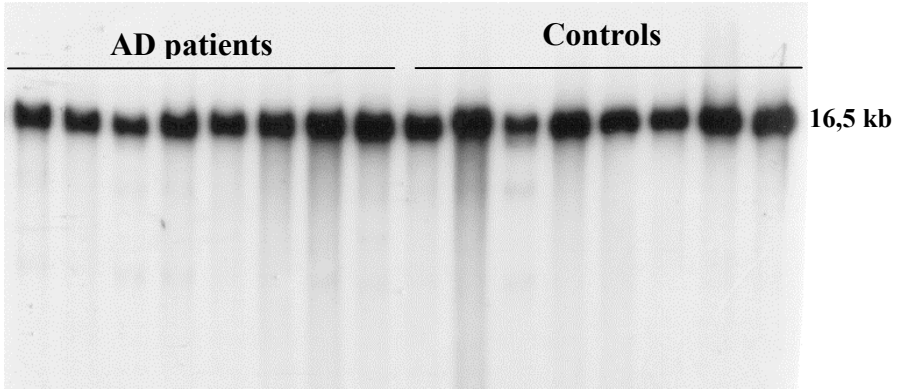
A recent quantification study of mtDNA delta 4977common deletion by using real-time PCR in various brain regions from neurodegenerative disorders and controls reported high levels of delta 4977 in affected regions in frontotemporal dementia, Parkinson's disease, and dementia with Lewy bodies, but not in Alzheimer's disease [63]. However, the significance of that study remains uncertain since only one AD patient was analysed.

Figure 3. Analysis of mtDNA in cerebellum brain necropsies by long PCR.



C-: negative control for rearrangements, DNA from normal individual.
C+: positive control for rearrangements, DNA from individual who showed mtDNA deletion when analysing by Southern blot.

Figure 4. *Pvu* II Southern blot analysis of cerebellum brain necropsies probed with whole mtDNA.



Similar results (16.5-kb band corresponding to a normal mtDNA population) were obtained when the DNA from hippocampus and frontal cortex brain areas and from blood samples were analysed [25].

mtDNA DEPLETION

The reduction in mtDNA copy number (mtDNA depletion) is a mtDNA defect that could account for some mitochondrial abnormalities observed in late-onset AD and thus may contribute to the etiology of the disease. Depletion of mtDNA would diminish the activities of ETC complexes I, III, IV and V because the mitochondrial genome encodes core subunits for each of these complexes [4,64]. Impairment of ETC and inhibition of OXPHOS would increase ROS production, sensitize the mtPTP, and enhance the apoptotic loss of synaptic connections between neurons [65]. The first work studying mtDNA depletion in AD brain was reported by our group describing a new method of analysis [54]. Before that the classical method for depletion studies was based on Southern blot followed by simultaneous hybridization with nuclear and mitochondrial probes to obtain a mtDNA/nDNA ratio. Blot techniques require a relatively high amount of DNA, are difficult to standardise and are susceptible to misinterpretations due to the densitometrically analysed bands often being

saturated which can lead to an underestimation of the peaks. The new strategy for mtDNA/nDNA ratio calculation was based on a real-time fluorescent PCR technique that allowed quantification of mitochondrial and nuclear genomes [54]. In this way, the rapidity, reproducibility and specially the sensitivity of this PCR method makes it the technique of choice for detecting mtDNA depletion. The analysis performed by our group did not show statistical differences when the mtDNA/nDNA ratio was compared between AD patients and controls although the three brain regions of AD patients analysed showed an apparent mtDNA reduction (**figure 5**). Samples from cerebellum gave an average ratio of 0.54±0.15 for AD patients and 0.56±0.19 for controls, a 3.5% reduction in mtDNA amount. In hippocampus AD patients showed an average ratio 2.61±1.67 while controls gave 3.00±2.25, resulting in a 13.8% reduction. Frontal cortex of AD patients showed a 28% reduction in mtDNA content (AD: 3.25±1.28; controls: 4.51±1.71). The lack of significance for these data could be an effect of the vast heterogeneity observed in AD patients analysed, but no definite conclusion could be established in view of our results.

The contribution of such mtDNA defect in the pathophysiology of AD has been also studied recently in the paper by Coskun *et al*. The analysis of the mtDNA/nDNA ratio by this group with similar methodology in frontal cortex samples showed an average ratio of 12±6.9 for 9 AD brains, but 22±18 for 17 control brains, which means a 50% reduction in mtDNA copy number (P = 0.03) [50]. This mtDNA reduction was associated to mtDNA CR sequence variations, specifically mutations in the light chain transcription promoter and the downstream CSBI and the heavy strand origins of replication elements could account for the observed reduction, because the light strand transcript, processed at CSB I, has been proposed to provide the primer for initiating mtDNA heavy strand synthesis [66]. For Coskun *et al.* the accumulation of somatic mtDNA CR mutations leading to reduced mtDNA copy number could enhance OXPHOS defects and provides an explanation for the neurological loss and resulting dementia associated with late-onset AD [50].

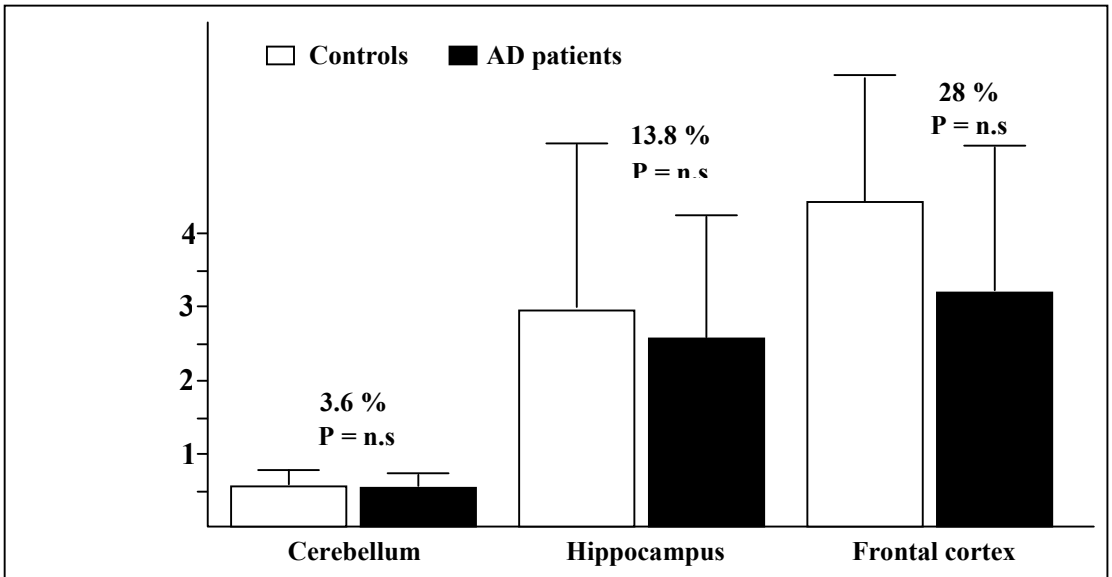


Figure 5. Mean mtDNA/nDNA ratios used for comparisons between AD and control subjects in different brain areas. The percentage reflects the difference in the mtDNA amount in AD patients respect to the mean ratio of controls. n.s: not significant. Adapted from [54].

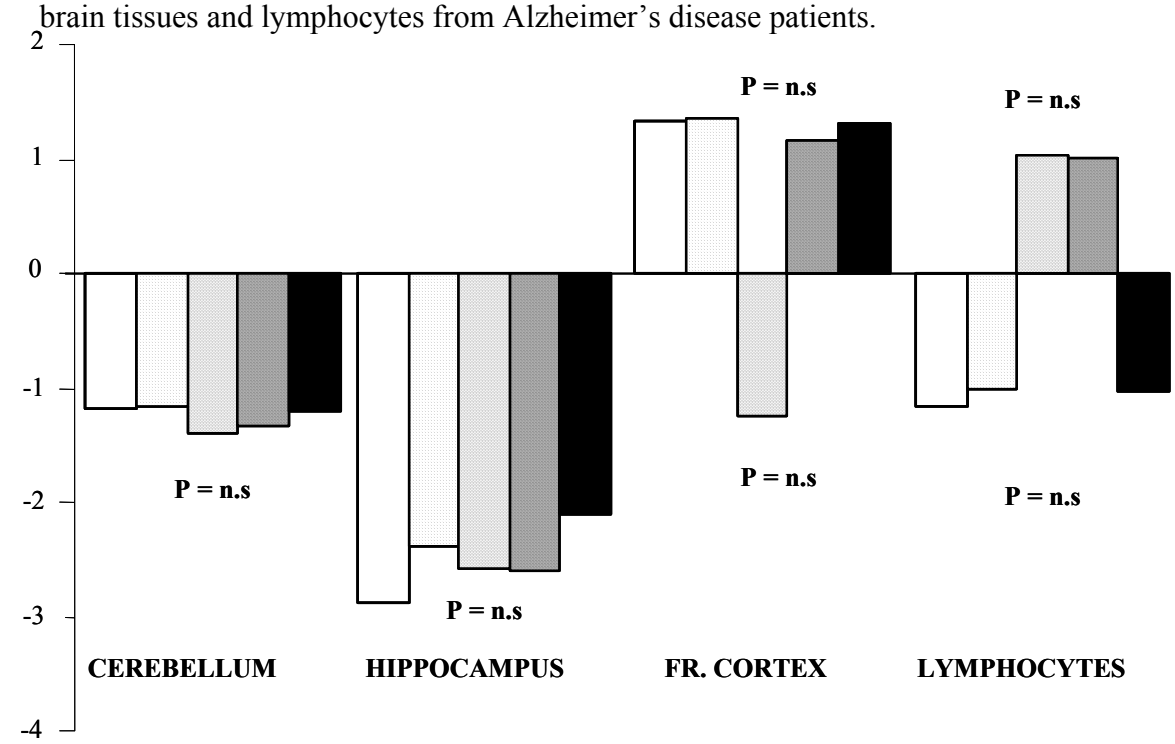
ANALYSIS OF GENE EXPRESSION

One of the mitochondrial abnormalities described in AD is the change in the expression of mitochondrial and nuclear genes, encoding polypeptidic subunits of the cytochrome c oxidase and NADH dehydrogenase enzyme complexes integrated in the ETC. It has been proposed that a decrease in mRNA levels of the ETC complexes subunits in affected brain regions may impair the oxidative metabolism affecting ATP and reactive ROS production. Several groups have investigated mRNA expression of mitochondrial-encoded genes in a variety of brain regions using different methodologies with diverse results [50,64,67-70]. In view of the disparity of these studies no definite conclusion could be established about the role of mitochondrial gene expression changes.

We have measured the expression of five mitochondrial-encoded genes (Cytochrome b, 12S, ND4, COX II encoded by the mtDNA heavy chain and ND6 encoded by the mtDNA light chain) and one reference nuclear gene (ribosomal 18S gene) in blood and in three brain areas (cerebellum, hippocampus and frontal cortex) from AD patients and controls by performing reverse transcription followed by quantitative real-time PCR [71]. **Figure 6** shows the main gene expression results obtained. Our group has found no evidence for a decrease in gene expression in the AD samples analysed. Reductions in COX I, COX III, ND1 and ND4 mRNA levels have been reported in the middle temporal association cortex region of 5 AD and 5 control brain samples by Northern blot analysis [64,68]. Analysis of mitochondrial 12S RNA in hippocampus from the same individuals did not reveal alterations in gene expression, which is in agreement with our findings in the same brain region. In these studies the primary motor cortex region was also analysed but mitochondrial gene expression in AD was normal when compared to controls. Other authors found a decrease in mRNA levels of COX II and a reduced activity of cytochrome oxidase complex when performing *in situ* hybridisation experiments in hippocampus [69]. In contrast to these studies, in the cortex region analysed by our group (frontal cortex) with the real-time PCR quantification strategy, the mRNA levels of COX II and ND4 were similar in AD patients and controls. Recently, 11 mitochondrial-encoded genes have been analysed in frontal cortex samples from 6 early and 6 definite AD patients by using real-time PCR strategy and the comparative cycle threshold (CT) method for analysing results [70]. The authors found a downregulation in mitochondrial genes for complex I in AD patients and increased mRNA levels for complex III and IV mitochondrial genes in AD. This comparative CT method involved averaging duplicate samples taken as the CT values for the mitochondrial gene and the reference gene. Unfortunately, statistical conclusions can not be reached with this type of analysis (as other previous reports of gene expression in AD) between AD patients and controls, in contrast to the approach used by our group which allowed us to perform statistical comparisons of the gene expression between groups. Another study [67] analysed two mitochondrial and two nuclear genes encoding subunits of the cytochrome c oxidase and the NADH dehydrogenase in the hippocampus, inferior parietal lobule, and cerebellum of 10 AD and 10 age-matched control subjects. The authors reported an altered proportion between COX II and COX IV mRNAs in the AD brain. Changes in the proportion of COX II and COX IV transcripts and a coordinated decrease of ND4 and ND15 mRNAs were found in the AD hippocampus and inferior parietal lobule, but not in cerebellum. The authors hypothesised that the decrease of ND4 gene expression could lead to the inhibition of the normal ubiquinone oxidoreductase activity of NADH dehydrogenase [67]. Our results in cerebellum and hippocampus for ND4 and COX II gene expression did not show any changes and thus do not confirm previously reported results [71].

The authors of the aforementioned studies speculate that a decrease in mRNA levels in affected brain regions may contribute to alterations of oxidative metabolism in AD. Our results disagree in part with this hypothesis, because if there are alterations in oxidative metabolism in our AD patients, this would not be related with a reduction in mitochondrial

Figure 6. Factor of regulation for the mitochondrial target genes analysed in different brain tissues and lymphocytes from Alzheimer's disease patients.



	CEREBELLUM	HIPPOCAMPUS	FR. CORTEX	LYMPHOCYTES
ND4	-1.173	-2.889	1.333	-1.151
Cyt b	-1.164	-2.394	1.363	-1.011
12S	-1.391	-2.588	-1.240	1.033
COX II	-1.341	-2.600	1.155	1.004
ND6	-1.204	-2.112	1.314	-1.022

In the case of down-regulation, the regulation factor is illustrated as a reciprocal value (1/expression ratio). p-Value > 0.05 in all cases. Data from [71].

RNA levels since we have not detected any. Besides, we have determined enzymatic activities and peroxidation of membranes (as a marker of oxidative stress) in the same tissues analysed in the present work, and the results did not reveal significant differences [62], suggesting that the brain samples studied had not suffered important mitochondrial alterations. Nevertheless, this is at variance with existing literature. There is some evidence reporting an increase of lipid peroxidation when measuring F2-isoprostane molecules [72,73] and ETC defects in AD brain, reduction of complex IV activity being the most frequent finding [9,74,75]. Other authors have found normal complex IV activity while activities of complexes II and III [76], or complex V [77] were decreased. The reasons for the discrepancies described in lipid peroxidation as well as ETC activities are unclear, although they are likely to be explained by methodological factors such as variations in AD diagnostic criteria, methods in enzymatic studies, the influence of the age of patients, or delay in processing samples after death. It must also be borne in mind that the DNA and RNA isolation techniques used in the present and other previous gene expression studies have been performed with tissue homogenates. This approach might not reveal profound individual cellular differences as were reported by Hirai

et al [78] and could account for the heterogeneous state of mitochondrial transcripts and ETC enzymes in AD, contributing to these contradictory results. Single neuron studies aimed at determining the relationship between ETC alterations and mitochondrial genetic changes within individual neurons may be an eventual future approach to test if a defect in a small proportion of key neurons plays a role in AD pathogenesis.

Mitochondrial DNA transcription and mtDNA replication are interrelated processes in mitochondria. The transcription of mtDNA-encoded genes is initiated at two different promoters: one on the heavy chain and another on the light strand of the molecule. The resultant polycistronic transcripts are processed to produce mature rRNAs, tRNAs or mRNAs [79]. A transcript generated from the light-strand promoter is also necessary to prime the replication of the mtDNA molecule, functionally coupling mitochondrial gene expression with genome maintenance [51]. The replication and the transcription of the mitochondrial genome depend exclusively on nuclear-gene product [80]. One of these products, the mitochondrial transcription factor A (TFAM) plays a complex role in the regulation of both processes: it is required for mtDNA maintenance and, together with two other factors, TFB1 and TFB2, stimulates mitochondrial transcription [81]. The mtDNA reduction (not statistically significant) in the frontal cortex region of AD patients observed by our group [54] does not seem to be associated with a down-regulation in RNA expression levels [71]. This fact suggests the existence of a mechanism that compensates for the reduction in mtDNA content, such that the transcriptional activity remains equivalent to controls to reach stable RNA steady-state levels in AD.

Interestingly, the increase in mtDNA CR mutations located in elements known to be involved in mtDNA light strand transcription and/or heavy strand replication reported by Coskun and collaborators (mentioned throughout this chapter) were associated with reductions in the mtDNA light strand ND6 mRNA [50]. AD patients showed a two-fold reduction when the ratio of the light strand ND6 mRNA versus the heavy strand ND2 mRNA determined by quantitative RT-PCR was compared to controls ($P = 0.01$). It appears that the method used for RNA quantification by Coskun *et al.,* was the same as that used for mtDNA quantification, being that reported by our group [54]. However, RNA quantification by RT followed by real-time PCR requires careful considerations that are essential for precise gene quantification. The assay design, template preparation, mathematical evaluation and analysis of data generated, calculation of final results and statistical methods, can not be performed equivalent to DNA quantification experiments [71,82].

A reduction in the ND6 mRNA would preferentially inhibit complex I, because ND6 is essential for complex I assembly [83,84]. In the case of the samples analysed by our group the gene expression of the mitochondrial-encoded ND6 gene was normal when compared to controls in all brain regions analysed, so the relationship between these CR mutations and gene expression changes in the samples analysed could not be established since no alteration and point mutation were detected. The CR mutations associated to reduction in ND6 gene expression and mtDNA copy number observed by Coskun and colleagues is an attractive finding, but it needs further investigation. The analysis of the ETC activity or mitochondrial function will be of interest to establish a potential effect of these CR mutations on phenotype. The existence of several mtDNA transcription factors and CR point mutations may allow flexible regulation of mtDNA gene expression in response to the complex physiological demands of mammalian metabolism [80]. This regulation seems especially interesting in affected brain regions in AD and aging, two conditions affected by global neuronal activity decline. In view of our results, it appears that transcriptional activity in the frontal cortex of our AD patients could produce a number of mitochondrial transcripts similar to that in controls in spite of reduced mtDNA content.

Because the disparity of previous results and ours analysing the expression of mtDNA-encoded genes and other mtDNA related defects found in AD, a reasonable explanation for placing the mitochondrial genetic defects reported by several groups in sporadic AD etiology requires further investigation. In the absence of uniform mtDNA findings (because it is not present or because it could not be detected with the molecular analysis performed), a mitochondrial bioenergetic defect due to alterations in ETC complexes activities could be the link between mitochondrial dysfunction and AD. Even when a mtDNA defect has been found, the abnormal function of ETC is essential to impute a pathogenic role to mitochondrial genetic defects.

ELECTRON TRANSPORT CHAIN ACTIVITY

Electron transport chain dysfunction may arise from defects in mitochondrial genetic, nuclear genetic or from toxic etiologies. Experiments using cytoplasmic hybrid (cybrid) systems can help distinguish which of these possibilities is the cause by facilitating expression of suspect mtDNA within a nuclear and environmentally controlled context. Perpetuation of ETC dysfunction in cybrids is consistent with an mtDNA pathogenesis while defect correction is not. Cybrid technology, which involves the transfer of mitochondria from living patients to cell lines deficient in mitochondria, has facilitated the study of energy metabolism in AD [85]. The cell lines are initially depleted of mtDNA by exposing them to low concentrations of ethidium bromide, which preferentially inhibits DNA replication. The exposed cells lose their mtDNA and assume an anaerobic phenotype. Studies using this technique have demonstrated that the deficits in COX in AD platelets could be transferred to ρ^o cells, which retain the COX deficit [86]. Additionally, the resulting cybrid cells showed markedly increased free radical production, impaired intracellular calcium buffering, elevated basal cytosolic calcium concentration, and enhanced sensitivity to inositol 1,4,5-triphosphate.mediated calcium release [87]. On the other hand, Ito *et al* have also performed biochemical analyses showing that all cybrid clones with mtDNA imported from platelets or brain tissues of patients with AD restored mitochondrial respiration activity to almost the same levels as those of cybrid clones with mtDNA from age-matched normal controls, suggesting functional integrity of mtDNA in both platelets and brain tissues of elderly patients with AD [88]. Furthermore, several methodological issues have been raised regarding the suitability of these cell lines in ascribing the cause for the observed defects to authentic mtDNA mutations [89].

Enzymatic activities of ETC complexes have been determined by our group in cerebellum, hippocampus and frontal cortex brain regions and results did not reveal significant differences between AD and elderly control subjects [62], so no correlation could be established between mtDNA reductions or gene expression changes observed in AD patients analysed by our group. However, there is some evidence reporting ETC defects in AD brain, reduction of complex IV activity being the most frequent finding [9,74,75]. Other authors have found normal complex IV activity while activities of complexes II and III [76], or complex V [77] were decreased. The reasons for the discrepancies described in as ETC activities are unclear, although they are likely to be explained by methodological factors such as variations in AD diagnostic criteria, methods in enzymatic studies, the influence of the age of patients, or delay in processing samples after death.

In another independent study we have investigated the effects of treatment with rivastigmine, a commonly used cholinesterase inhibitor, on lymphocyte mitochondria of patients with AD. Increased enzymatic activities of diverse complexes and oxidative capacity of the ETC were found in treated patients suggesting that enhanced mitochondrial ETC function may contribute to the beneficial effects of rivastigmine on clinical manifestations of AD [90]. This work raises the intriguing possibility that cholinesterase inhibitors stimulate

energy metabolism as well as inhibiting cholinesterase. The mechanism for that stimulation is not yet known. The study was performed in lymphocytes, for the obvious reason that brain biopsies to obtain mitochondria for this study would have been appallingly unethical. However, a large number of previous studies indicate that mitochondrial alterations found in peripheral tissues in AD patients are very likely to occur in the brain as well [91]. The existence of a lesion in energy metabolism in AD brain is well established [91,92]. The magnitude of the deficit in energy metabolism correlated much better with the degree of clinical disability than does the amount of neuropathological change at autopsy [92]. This observation has led to the proposal that the cerebrometabolic lesion is the proximate cause of the clinical disability in AD [92], and to test therapies for AD designed to improve the deficiency in brain energy metabolism.

CONCLUDING REMARKS

The causes of the sporadic appearance of late-onset AD remain enigmatic. There is increasing evidence implicating mitochondrial dysfunction mainly resulting from OXPHOS defects impairing ATP and ROS production. It is not definitely established where the observed mitochondrial changes are placed in the AD cascade. For some authors it is more likely that the ETC defects observed in AD brains derive from nuclear gene defects or from acquired damage to respiratory chain components. It has been suggested that the most frequent finding in AD brains (the decrease in cytochrome c oxidase activity) is more likely to be related to a global decline in mitochondrial activity manifested by downregulation in mitochondrial number [19]. At this point a cautionary message should be sent regarding functional studies. The massive amounts of cell death in the AD brain means that the cells postulated to be dying due to mitochondrial dysfunction (e.g cholinergic neurons in AD) are, in the final analysis, unavailable for examination, especially in the terminal stages of the disease and in autopsy tissue. Many, if not all, studies have focused on the function in a skewed population of surviving cells, such as glia, that are likely to be unrepresentative of the true pathogenetic course of events [93].

The role of mtDNA defects leading to OXPHOS impairment in AD etiology is still controversial at best in view of the conflicting studies mentioned above. Although cybrid studies strongly support the role of the mitochondrial genome in AD pathogenesis they have several limitations, not least of which is the absence of interactions among entire populations and subpopulations of cells that define the behaviour of a vascularized tissue *in vivo*. One of the remaining questions is whether the increase in somatic mtDNA defects observed in AD brain is simply a reflection of accelerated aging, or is it a distinct pathological event? Specific mtDNA mutations responsible for the defects seen in aging and AD have yet to be identified. Large mtDNA deletions have consistently been reported to increase with age [55,94-96], but the absolute level of any particular mutation is generally low, below the threshold of 50% thought necessary to cause dysfunction [97,98]. A variety of individual point mutations have been reported to increase with age [99] and AD [20,26,27,33,45], but have not been consistently reproduced by other laboratories [21,22,28,29,35-37,40,41,44,100,101] or are single unconfirmed reports [31,58]. Thus, single individual mutations appear unlikely to account for mitochondrial decline in AD. It has been proposed that it may be the aggregate burden of multiple mutations, each of which may be individually rare, that impairs mitochondrial function [102].

The aggregate burden of mtDNA mutations, particularly point mutations, has not been systematically quantified in aging or neurodegenerative disorders until recent years. Lin *et al* have shown that the aggregate level of multiple distinct, individually rare point mutations is higher in brain mtDNA from AD and elderly subjects than in brain mtDNA from younger controls [38]. This increasing burden of mitochondrial DNA point mutations with age was

negatively correlated to COX activity. Coskun *et al*, have discovered that AD brains harbour a high frequency of heteroplasmic mutations in the non-coding mtDNA CR, mainly in key elements that regulate mtDNA light strand transcription and heavy strand replication. As a consequence of the location of these mutations, AD brains showed a marked reduction in the light strand ND6 mRNA levels and in the cellular mtDNA copy number [50]. It seems that mitochondrial mutations increase with age [38] with certain variants specifically affecting mtDNA CR only in AD brains [50]. However, the significance and relationship of these mtDNA abnormalities in neurodegeneration remains uncertain since studies of ETC activity demonstrated no pathogenic role of increased mtDNA mutations [38]. Activities of ETC complexes related to CR mutations have not yet been reported [50].

No definitive conclusion of the mitochondrial gene expression incidence has been established in AD due to the disparity of the results, the methodologies used and the variety of brain regions reported. Most of the previous works on that topic did not perform ETC analysis, so the significance of the reduced expression of several mitochondrial-encoded genes previously reported remains enigmatic. Our results did not confirm the possible relationship between reductions in mitochondrial gene expression and mitochondrial dysfunction, because if there are alterations in oxidative metabolism in our AD patients, this would not be related with a reduction in mitochondrial RNA levels since we have not detected any [71]. Besides, our enzymatic activities and peroxidation of membranes (as a marker of oxidative stress) did not reveal significant differences [62]. It must be borne in mind that the DNA and RNA isolation techniques and ETC analysis used by us and by other groups have been performed with tissue homogenates. It has been mentioned that this approach might not reveal profound individual cellular differences and could account for the heterogeneous state of mitochondrial transcripts and ETC enzymes in AD, contributing to the contradictory results [78]. Single neuron studies aimed at determining the relationship between ETC alterations and mitochondrial genetic changes within individual neurons may be an eventual future approach to test if a defect in a small proportion of key neurons plays a role in AD pathogenesis.

AD probably results from interplay of multiple genetic, environmental and aging influence factors. It has been suggested that mutations in nuclear DNA may play a role in the age-related decrease in mitochondrial ETC activity [103]. Other aging-related factors, such as oxidative stress, might adversely affect both mutational burden and COX activity by independent mechanisms, and such factors may also play a role in explaining the correlation that both have with aging and therefore with each other. The fact that Lin *et a.l* have found no difference in aggregate mutational burden between AD and elderly control subjects is consistent with their previous functional study, in which AD cybrids showed no functional deficits when compared to elderly control cybrids [88]. Nonetheless, this is in contradiction to other studies which showed functional abnormalities in AD cybrids [86,104]. Mutations in the non-coding mtDNA CR recently reported [50] must be further investigated to ascertain its association and effects on mitochondrial function in AD. The effect of these CR mutations on mtDNA replication and transcription could be a possible link between mtDNA defects and mitochondrial OXPHOS defects leading to neurodegeneration in some cases of sporadic AD. Perhaps the transfer of the most important CR mutations to cybrid lines would be helpful to determine functional implications.

These features reflect the truly difficult nature of elucidating the pathogenesis of diseases as complex as sporadic AD. A mechanistic relationship between neurodegeneration and mitochondrial dysfunction still awaits a direct, clear, and unequivocal demonstration. This does not mean, however, that mitochondrial defects play no role in late-onset AD. Even if OXPHOS defects leading to mitochondrial dysfunction are secondary to other initiating causes, they are deleterious and part of a cascade of events that can lead to neuron death. Furthermore, the mitochondrion is considered nowadays a therapeutic target for future

medications directed toward AD patients [105]. A better understanding of the mitochondrial role in the complex AD aetiology may be achieved through a multiple approach that combines the characterisation of mitochondrial genetics and the potential bioenergetic defects in sporadic AD patients.

ACKNOWLEDGEMENTS

The authors thank Montse Gómez and Jordi Casademont for their critical and helpful contributions to the performed studies in our laboratory. We also thank Helena Kruyer for editorial help and the Neurologic Tissue Bank (Serveis Científico-Tècnics, Universitat de Barcelona) for providing the brain samples and all the subjects who have collaborated in our studies. This work was funded by Fundació La Marató de TV3 (2002-2102/97 and 1710/01), by the Spanish MCYT (SAF99-0079 and SAF2002-02668), by the Instituto de Salud Carlos III (Redes C03/07 and G03/054) and grant 2001 SGR 00399 from the Generalitat de Catalunya, Spain.

REFERENCES

1. Rocchi, A., Pellegrini, S., Siciliano, G., and Murri, L. 2003, Brain Res Bull, 61, 1-24.
2. Vickers, J.C., Dickson, T.C., Adlard, P.A., Saunders, H.L., King, C.E., and McCormack, G. 2000, Prog Neurobiol, 60, 139-165.
3. Harman, D. 2002, Ann N Y Acad Sci, 959, 384-395; discussion 463-385.
4. Castellani, R., Hirai, K., Aliev, G., Drew, K.L., Nunomura, A., Takeda, A., Cash, A.D., Obrenovich, M.E., Perry, G., and Smith, M.A. 2002, J Neurosci Res, 70, 357-360.
5. Cottrell, D.A., Borthwick, G.M., Johnson, M.A., Ince, P.G., and Turnbull, D.M. 2002, Neuropathol Appl Neurobiol, 28, 390-396.
6. Bosetti, F., Brizzi, F., Barogi, S., Mancuso, M., Siciliano, G., Tendi, E.A., Murri, L., Rapoport, S.I., and Solaini, G. 2002, Neurobiol Aging, 23, 371-376.
7. Valla, J., Berndt, J.D., and Gonzalez-Lima, F. 2001, J Neurosci, 21, 4923-4930.
8. Verwer, R.W., Jansen, K.A., Sluiter, A.A., Pool, C.W., Kamphorst, W., and Swaab, D.F. 2000, Exp Neurol, 163, 440-451.
9. Mutisya, E.M., Bowling, A.C., and Beal, M.F. 1994, J Neurochem, 63, 2179-2184.
10. Cardoso, S.M., Proenca, M.T., Santos, S., Santana, I., and Oliveira, C.R. 2004, Neurobiol Aging, 25, 105-110.
11. Trimmer, P.A., Keeney, P.M., Borland, M.K., Simon, F.A., Almeida, J., Swerdlow, R.H., Parks, J.P., Parker, W.D., Jr., and Bennett, J.P., Jr. 2004, Neurobiol Dis, 15, 29-39.
12. Kish, S.J., Mastrogiacomo, F., Guttman, M., Furukawa, Y., Taanman, J.W., Dozic, S., Pandolfo, M., Lamarche, J., DiStefano, L., and Chang, L.J. 1999, J Neurochem, 72, 700-707.
13. Nagy, Z., Esiri, M.M., LeGris, M., and Matthews, P.M. 1999, Acta Neuropathol (Berl), 97, 346-354.
14. Pratico, D. 2002, Biochem Pharmacol, 63, 563-567.
15. Gibson, G.E., and Huang, H.M. 2002, Front Biosci, 7, d1007-1015.
16. Coskun, P.E., Ruiz-Pesini, E., and Wallace, D.C. 2003, Proc Natl Acad Sci U S A, 100, 2174-2176.
17. Wallace, D.C. 2001, Novartis Found Symp, 235, 247-263; discussion 263-246.
18. de la Monte, S.M., Luong, T., Neely, T.R., Robinson, D., and Wands, J.R. 2000, Lab Invest, 80, 1323-1335.
19. Ojaimi, J., and Byrne, E. 2001, Biol Signals Recept, 10, 254-262.
20. Lin, F.H., Lin, R., Wisniewski, H.M., Hwang, Y.W., Grundke-Iqbal, I., Healy-Louie, G., and Iqbal, K. 1992, Biochem Biophys Res Commun, 182, 238-246.
21. Petruzzella, V., Chen, X., and Schon, E.A. 1992, Biochem Biophys Res Commun, 186, 491-497.

22. Kosel, S., Egensperger, R., Mehraein, P., and Graeber, M.B. 1994, Biochem Biophys Res Commun, 203, 745-749.
23. Schnopp, N.M., Kosel, S., Egensperger, R., and Graeber, M.B. 1996, Clin Neuropathol, 15, 348-352.
24. Janetzky, B., Schmid, C., Bischof, F., Frolich, L., Gsell, W., Kalaria, R.N., Riederer, P., and Reichmann, H. 1996, Eur Neurol, 36, 149-153.
25. Rodriguez Santiago, B., Casademont, J., and Nunes, V. 2001, Rev Neurol, 33, 301-305.
26. Shoffner, J.M., Brown, M.D., Torroni, A., Lott, M.T., Cabell, M.F., Mirra, S.S., Beal, M.F., Yang, C.C., Gearing, M., Salvo, R., and et al. 1993, Genomics, 17, 171-184.
27. Hutchin, T.P., Heath, P.R., Pearson, R.C., and Sinclair, A.J. 1997, Biochem Biophys Res Commun, 241, 221-225.
28. Zsurka, G., Kalman, J., Csaszar, A., Rasko, I., Janka, Z., and Venetianer, P. 1998, Biol Psychiatry, 44, 371-373.
29. Tanno, Y., Okuizumi, K., and Tsuji, S. 1998, Neurobiol Aging, 19, S47-51.
30. Torroni, A., Schurr, T.G., Yang, C.C., Szathmary, E.J., Williams, R.C., Schanfield, M.S., Troup, G.A., Knowler, W.C., Lawrence, D.N., Weiss, K.M., and et al. 1992, Genetics, 130, 153-162.
31. Edland, S.D., Tobe, V.O., Rieder, M.J., Bowen, J.D., McCormick, W., Teri, L., Schellenberg, G.D., Larson, E.B., Nickerson, D.A., and Kukull, W.A. 2002, Alzheimer Dis Assoc Disord, 16, 1-7.
32. Chinnery, P.F., and Turnbull, D.M. 2000, Mol Med Today, 6, 425-432.
33. Davis, R.E., Miller, S., Herrnstadt, C., Ghosh, S.S., Fahy, E., Shinobu, L.A., Galasko, D., Thal, L.J., Beal, M.F., Howell, N., and Parker, W.D., Jr. 1997, Proc Natl Acad Sci U S A, 94, 4526-4531.
34. Fahy, E., Nazarbaghi, R., Zomorrodi, M., Herrnstadt, C., Parker, W.D., Davis, R.E., and Ghosh, S.S. 1997, Nucleic Acids Res, 25, 3102-3109.
35. Hirano, M., Shtilbans, A., Mayeux, R., Davidson, M.M., DiMauro, S., Knowles, J.A., and Schon, E.A. 1997, Proc Natl Acad Sci U S A, 94, 14894-14899.
36. Davis, J.N., 2nd, and Parker, W.D., Jr. 1998, Biochem Biophys Res Commun, 244, 877-883.
37. Wallace, D.C., Stugard, C., Murdock, D., Schurr, T., and Brown, M.D. 1997, Proc Natl Acad Sci U S A, 94, 14900-14905.
38. Lin, M.T., Simon, D.K., Ahn, C.H., Kim, L.M., and Beal, M.F. 2002, Hum Mol Genet, 11, 133-145.
39. Hutchin, T., and Cortopassi, G. 1995, Proc Natl Acad Sci U S A, 92, 6892-6895.
40. Wragg, M.A., Talbot, C.J., Morris, J.C., Lendon, C.L., and Goate, A.M. 1995, Neurosci Lett, 201, 107-110.
41. Chinnery, P.F., Taylor, G.A., Howell, N., Andrews, R.M., Morris, C.M., Taylor, R.W., McKeith, I.G., Perry, R.H., Edwardson, J.A., and Turnbull, D.M. 2000, Neurology, 55, 302-304.
42. Garcia-Lozano, J.R., Mir, P., Alberca, R., Aguilera, I., Gil Neciga, E., Fernandez-Lopez, O., Cayuela, A., and Nunez-Roldan, A. 2002, Eur Neurol, 48, 34-36.
43. Brown, M.D., Shoffner, J.M., Kim, Y.L., Jun, A.S., Graham, B.H., Cabell, M.F., Gurley, D.S., and Wallace, D.C. 1996, Am J Med Genet, 61, 283-289.
44. Tysoe, C., Robinson, D., Brayne, C., Dening, T., Paykel, E.S., Huppert, F.A., and Rubinsztein, D.C. 1996, J Med Genet, 33, 1002-1006.
45. Egensperger, R., Kosel, S., Schnopp, N.M., Mehraein, P., and Graeber, M.B. 1997, Neuropathol Appl Neurobiol, 23, 315-321.
46. Chagnon, P., Gee, M., Filion, M., Robitaille, Y., Belouchi, M., and Gauvreau, D. 1999, Am J Med Genet, 85, 20-30.

47. van der Walt, J.M., Dementieva, Y.A., Martin, E.R., Scott, W.K., Nicodemus, K.K., Kroner, C.C., Welsh-Bohmer, K.A., Saunders, A.M., Roses, A.D., Small, G.W., Schmechel, D.E., Murali Doraiswamy, P., Gilbert, J.R., Haines, J.L., Vance, J.M., and Pericak-Vance, M.A. 2004, Neurosci Lett, 365, 28-32.
48. Ruiz-Pesini, E., Mishmar, D., Brandon, M., Procaccio, V., and Wallace, D.C. 2004, Science, 303, 223-226.
49. Carrieri, G., Bonafe, M., De Luca, M., Rose, G., Varcasia, O., Bruni, A., Maletta, R., Nacmias, B., Sorbi, S., Corsonello, F., Feraco, E., Andreev, K.F., Yashin, A.I., Franceschi, C., and De Benedictis, G. 2001, Hum Genet, 108, 194-198.
50. Coskun, P.E., Beal, M.F., and Wallace, D.C. 2004, Proc Natl Acad Sci U S A, 101, 10726-10731.
51. Shadel, G.S., and Clayton, D.A. 1997, Annu Rev Biochem, 66, 409-435.
52. Chinnery, P.F., Taylor, G.A., Howell, N., Brown, D.T., Parsons, T.J., and Turnbull, D.M. 2001, Am J Hum Genet, 68, 529-532.
53. Simon, D.K., Lin, M.T., Ahn, C.H., Liu, G.J., Gibson, G.E., Beal, M.F., and Johns, D.R. 2001, Genomics, 73, 113-116.
54. Rodriguez-Santiago, B., Casademont, J., and Nunes, V. 2001, Eur J Hum Genet, 9, 279-285.
55. Corral-Debrinski, M., Horton, T., Lott, M.T., Shoffner, J.M., McKee, A.C., Beal, M.F., Graham, B.H., and Wallace, D.C. 1994, Genomics, 23, 471-476.
56. Hamblet, N.S., and Castora, F.J. 1997, Mutat Res, 379, 253-262.
57. Blanchard, B.J., Park, T., Fripp, W.J., Lerman, L.S., and Ingram, V.M. 1993, Neuroreport, 4, 799-802.
58. Chang, S.W., Zhang, D., Chung, H.D., and Zassenhaus, H.P. 2000, Biochem Biophys Res Commun, 273, 203-208.
59. Lezza, A.M., Mecocci, P., Cormio, A., Beal, M.F., Cherubini, A., Cantatore, P., Senin, U., and Gadaleta, M.N. 1999, Faseb J, 13, 1083-1088.
60. Gu, G., Reyes, P.E., Golden, G.T., Woltjer, R.L., Hulette, C., Montine, T.J., and Zhang, J. 2002, J Neuropathol Exp Neurol, 61, 634-639.
61. Lightowlers, R.N., Jacobs, H.T., and Kajander, O.A. 1999, Trends Genet, 15, 91-93.
62. Casademont, J., Rodríguez-Santiago, B., Miro, O., Beato, A., S., L., Nunes, V., and Cardellach, F. Aging Clinical and Experimental Research, in press.
63. Mawrin, C., Kirches, E., Krause, G., Schneider-Stock, R., Bogerts, B., Vorwerk, C.K., and Dietzmann, K. 2004, Neurosci Lett, 357, 111-114.
64. Chandrasekaran, K., Hatanpaa, K., Rapoport, S.I., and Brady, D.R. 1997, Brain Res Mol Brain Res, 44, 99-104.
65. Rapoport, S.I. 2003, Neurotox Res, 5, 385-398.
66. Zhang, J., Asin-Cayuela, J., Fish, J., Michikawa, Y., Bonafe, M., Olivieri, F., Passarino, G., De Benedictis, G., Franceschi, C., and Attardi, G. 2003, Proc Natl Acad Sci U S A, 100, 1116-1121.
67. Aksenov, M.Y., Tucker, H.M., Nair, P., Aksenova, M.V., Butterfield, D.A., Estus, S., and Markesbery, W.R. 1999, Neurochem Res, 24, 767-774.
68. Chandrasekaran, K., Giordano, T., Brady, D.R., Stoll, J., Martin, L.J., and Rapoport, S.I. 1994, Brain Res Mol Brain Res, 24, 336-340.
69. Simonian, N.A., and Hyman, B.T. 1994, J Neuropathol Exp Neurol, 53, 508-512.
70. Manczak, M., Park, B.S., Jung, Y., and Reddy, P.H. 2004, Neuromolecular Med, 5, 147-162.
71. Rodriguez-Santiago, B., and Nunes, V. Neurobiol Dis, in press.
72. Montine, T.J., Neely, M.D., Quinn, J.F., Beal, M.F., Markesbery, W.R., Roberts, L.J., and Morrow, J.D. 2002, Free Radic Biol Med, 33, 620-626.

73. Pratico, D., and Sung, S. 2004, J Alzheimers Dis, 6, 171-175.
74. Parker, W.D., Jr., Parks, J., Filley, C.M., and Kleinschmidt-DeMasters, B.K. 1994, Neurology, 44, 1090-1096.
75. Kish, S.J., Bergeron, C., Rajput, A., Dozic, S., Mastrogiacomo, F., Chang, L.J., Wilson, J.M., DiStefano, L.M., and Nobrega, J.N. 1992, J Neurochem, 59, 776-779.
76. Reichmann, H., Florke, S., Hebenstreit, G., Schrubar, H., and Riederer, P. 1993, J Neurol, 240, 377-380.
77. Schagger, H., and Ohm, T.G. 1995, Eur J Biochem, 227, 916-921.
78. Hirai, K., Aliev, G., Nunomura, A., Fujioka, H., Russell, R.L., Atwood, C.S., Johnson, A.B., Kress, Y., Vinters, H.V., Tabaton, M., Shimohama, S., Cash, A.D., Siedlak, S.L., Harris, P.L., Jones, P.K., Petersen, R.B., Perry, G., and Smith, M.A. 2001, J Neurosci, 21, 3017-3023.
79. Clayton, D.A. 1991, Annu Rev Cell Biol, 7, 453-478.
80. Shoubridge, E.A. 2002, Nat Genet, 31, 227-228.
81. Falkenberg, M., Gaspari, M., Rantanen, A., Trifunovic, A., Larsson, N.G., and Gustafsson, C.M. 2002, Nat Genet, 31, 289-294.
82. Freeman, W.M., Walker, S.J., and Vrana, K.E. 1999, Biotechniques, 26, 112-122, 124-115.
83. Bai, Y., and Attardi, G. 1998, Embo J, 17, 4848-4858.
84. Ugalde, C., Janssen, R.J., van den Heuvel, L.P., Smeitink, J.A., and Nijtmans, L.G. 2004, Hum Mol Genet, 13, 659-667.
85. King, M.P., and Attardi, G. 1989, Science, 246, 500-503.
86. Swerdlow, R.H., Parks, J.K., Cassarino, D.S., Maguire, D.J., Maguire, R.S., Bennett, J.P., Jr., Davis, R.E., and Parker, W.D., Jr. 1997, Neurology, 49, 918-925.
87. Sheehan, J.P., Swerdlow, R.H., Miller, S.W., Davis, R.E., Parks, J.K., Parker, W.D., and Tuttle, J.B. 1997, J Neurosci, 17, 4612-4622.
88. Ito, S., Ohta, S., Nishimaki, K., Kagawa, Y., Soma, R., Kuno, S.Y., Komatsuzaki, Y., Mizusawa, H., and Hayashi, J. 1999, Proc Natl Acad Sci U S A, 96, 2099-2103.
89. Schon, E.A., Shoubridge, E.A., and Moraes, C.T. 1998, Neurology, 51, 326-327.
90. Casademont, J., Miro, O., Rodriguez-Santiago, B., Viedma, P., Blesa, R., and Cardellach, F. 2003, J Neurol Sci, 206, 23-26.
91. Blass, J.P., Sheu, R.K., and Gibson, G.E. 2000, Ann N Y Acad Sci, 903, 204-221.
92. Blass, J.P. 2001, J Neurosci Res, 66, 851-856.
93. Schon, E.A., and Manfredi, G. 2003, J Clin Invest, 111, 303-312.
94. Cooper, J.M., Mann, V.M., and Schapira, A.H. 1992, J Neurol Sci, 113, 91-98.
95. Cortopassi, G.A., Shibata, D., Soong, N.W., and Arnheim, N. 1992, Proc Natl Acad Sci U S A, 89, 7370-7374.
96. Lee, H.C., Pang, C.Y., Hsu, H.S., and Wei, Y.H. 1994, Biochim Biophys Acta, 1226, 37-43.
97. Porteous, W.K., James, A.M., Sheard, P.W., Porteous, C.M., Packer, M.A., Hyslop, S.J., Melton, J.V., Pang, C.Y., Wei, Y.H., and Murphy, M.P. 1998, Eur J Biochem, 257, 192-201.
98. Hayashi, J., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y., and Nonaka, I. 1991, Proc Natl Acad Sci U S A, 88, 10614-10618.
99. Zhang, C., Liu, V.W., Addessi, C.L., Sheffield, D.A., Linnane, A.W., and Nagley, P. 1998, Hum Mutat, 11, 360-371.
100. Pallotti, F., Chen, X., Bonilla, E., and Schon, E.A. 1996, Am J Hum Genet, 59, 591-602.
101. Lin, F.H., and Lin, R. 1992, Biochem Biophys Res Commun, 189, 1202-1206.
102. Arnheim, N., and Cortopassi, G. 1992, Mutat Res, 275, 157-167.
103. Isobe, K., Ito, S., Hosaka, H., Iwamura, Y., Kondo, H., Kagawa, Y., and Hayashi, J.I. 1998, J Biol Chem, 273, 4601-4606.

104. Miller, S.W., Trimmer, P.A., Parker, W.D., Jr., and Davis, R.E. 1996, J Neurochem, 67, 1897-1907.
105. Allain, H., Bentue-Ferrer, D., Tribut, O., Gauthier, S., Michel, B.F., and Drieu-La Rochelle, C. 2003, Fundam Clin Pharmacol, 17, 419-428.

Comentarios adicionales

Este capítulo está invitado a participar en el libro *New frontiers in mitochondrial biogenesis and disease* que aparecerá en 2005. El capítulo escrito por nuestro grupo pretende ser una revisión de la literatura existente hasta el momento sobre las principales alteraciones del mtDNA halladas en la EA. Además, de forma más breve, se trata la relación de esas alteraciones con los cambios en la actividad de la CRM en la enfermedad. También incluye las aportaciones realizadas por nuestro grupo y supone un resumen y discusión general, a pequeña escala, de todos los trabajos publicados por nuestro grupo de DNA mitocondrial en este campo. En la siguiente sección de la tesis (la Discusión General) se trata de forma más extensa los puntos tratados en este trabajo.