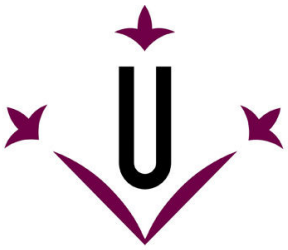
UNIVERSIDAD DE LLEIDA
FACULTAD DE MEDICINA
DEPARTAMENTO DE MEDICINA EXPERIMENTAL



Universitat de Lleida

"MOLECULAR MECHANISMS OF AGEING IN NEURODEGENERATION: ROLE OF OXIDATIVE AND ENDOPLASMIC RETICULUM STRESS IN DIFFERENT HUMAN DISEASES"

DOCTORAL THESIS

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Lleida, May 2009

Departamento de Medicina Experimental
Universidad de Lleida, Spain

Dr. MANUEL PORTERO-OTÍN, Professor at University of Lleida,
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declare and confirm that they have supervised and guided the PhD thesis entiteled:

MOLECULAR MECHANISMS OF AGEING IN NEURODEGENERATION: ROLE OF OXIDATIVE AND ENDOPLASMIC RETICULUM STRESS IN DIFFERENT HUMAN DISEASES, presented by **Ekaterina Vasileva Ilieva.** They hereby assert that this thesis fulfils the requirements to be defended for the Degree of Doctor.

Signature,

Dr. Manuel Portero-Otín,
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Lleida, 2009

A mis padres.

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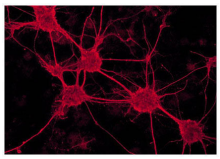

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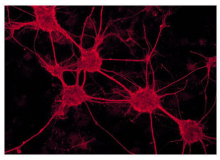
LIST OF ABBREVIATIONS

NDDs	Neurodegenerative diseases
CNS	central nervous system
AD	Alzheimer's disease
AGD	Argyrophilic grain disease
PD	Parkinson's diseases
PiD	Pick's disease
HD	Huntington's disease
PrD	Prion diseases
PSP	progressive supranuclear palsy
CJD	Creutzfeldt-Jakob disease
CBD	Corticobasal degeneration
FTD	Fronto-temporal dementia
FTDP-17	Parkinsonism linked to chromosome 17
DLB	Dementia with Lewy bodies
ALS	Amyotrophic Lateral Sclerosis
fALS	familial Amyotrophic Lateral Sclerosis
sALS	sporadic Amyotrophic Lateral Sclerosis
SC	spinal cord
Aβ	beta-amyloid
APP	Amyloid precursor protein
NFT	neurofibrillary tangles
LB	Lewy bodies
DLBD	Diffuse Lewy Body Disease
LBD	Lewy body disease
PGD	Polyglutamine diseases
MND	Motor neuron disease
TDP-43	TAR DNA binding protein 43
MN	motor neurons
SP	senile plaques
PB	Pick bodies
PC	Pick cells
AGs	argyrophilic grains
ApoE	Apolipoprotein E
BN	ballooned neurons
BC	brain cortex
OC	occipital cortex
FC	frontal cortex
HC	hippocampus
SOD1	cytosolic Cu/Zn superoxide dismutase
SOD2	mitochondrial Mn superoxide dismutase
mSOD1	mutant SOD1
wtSOD1	wild type SOD1
CB	coiled bodies
OS	oxidative stress
ER	endoplasmic reticulum
GFAP	glial fibrillary acidic protein
AGE	advanced glycation end products
UBB	ubiquitin-B
BBs	Bunina bodies
SETX	senataxin
VAMP	vesicle-associated membrane protein
VAPB	synaptobrevin/VAMP-associated protein B

GC/MS	Gas chromatography-mass spectrometry
ROS	reactive oxygen species
RNS	reactive nitrogen species
ATP	adenosine triphosphate
AIF	Apoptosis-inducing factor
HSPs	heat-shock protein
UCPs	uncoupling proteins
PGC1α	peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α
SIRT1	sirtuin 1
Nrf	nuclear factor-erythroid 2 p45-related respiratory
TFAM	mitochondrial transcription factor A
Mfn	mitochondrial fusion proteins
RIP140	transcriptional corepressor
GSA	glutamic semialdehyde
AASA	aminoadipic semialdehyde
DNP	2,4-dinitrophenylhydrazine
PUFA	polyunsaturated fatty acids
UFAs	unsaturated fatty acids
GO	glyoxal
MGO	methylglyoxal
MDA	malondialdehyde
4-HNE	4-hydroxynonenal
AGE	Advanced Glycation Endproducts
ALE	Advanced Lipoxidation Endproducts
NKTLs	Neuroketals
MDA-lys	N^{ε}-(malondialdehyde)lysine
CML	N^{ε}-(carboxymethyl)lysine
CEL	N^{ε}-(carboxyethyl)lysine
RCS	reactive carbonyl species
FA	fatty acid
DHA	docosahexaenoic acid
ARA	arachidonic acid
PI	Peroxidizability Index
DBI	double bond index
SFA	saturated fatty acids
MUFA	monounsaturated fatty acids
GSH	glutathione
GSSG	oxidized glutathione
UPS	ubiquitin proteasome system
UPR	unfolded protein response
UPs	unfolded proteins
CMA	chaperone-mediated autophagy
LAMP	lysosome-associated membrane protein
IRE1	inositol-requiring kinase 1
PERK	double-stranded RNA-activated protein kinase-like ER kinase
ATF6	activating transcription factor 6
XBP1	x-box bindin protein 1
eiF-2α	eukaryotic translation initiation factor 2 subunit alpha
p-eiF-2α	phosphorylated eiF-2α
Grp78/BiP	glucose-regulated proteins 78
Grp94	glucose-regulated proteins 94
PDI	disulfide isomerase
KDEL	C-terminal Lys-Asp-Glu-Leu sequence

PP	protein phosphatase
S1P	site-1 protease
S2P	site-2 protease
ERSE	ER stress element
ERAD	ER associated degradation
CSP	caspase
ASK1	apoptosis-signalling kinase 1
JIK	Jun N-terminal inhibitory kinase



SUMMARY

SUMMARY

It is hypothesized that the neurodegenerative diseases (NDDs) could be an accelerated form of aging selective for nervous tissue in specific anatomic locations. Accordingly, the processes observed in the biological basis of aging (oxidative stress, accumulation of highly modified protein aggregates, mitochondrial dysfunction) and the ensuing processes that it triggers are more intense and premature in these cell populations.

The aim of this work was to investigate the potential interplay between oxidative and endoplasmic reticulum (ER) stress and the underling signalling pathways, as a potential mechanism involved in the pathogenesis of the neurodegenerative disorders affecting different locations, and characterized by protein aggregates. We characterized protein oxidative damage, its major contributors and its pathophysiological consequences in the sporadic form of amyotrophic lateral sclerosis (ALS) patients with lumbar onset disease, in the frontotemporal tauopathy Pick's disease (PiD) and in the argyrophilic grain disease (AGD) patients. The results of ALS samples were compared with *in vitro* models of the disease.

After extensive pathological characterization, samples from spinal cords (SC) and frontal cortex (FC) from ALS patients, FC and occipital cortex (OC) from PiD patients, and hippocampus (HC) from AGD patients were analyzed in comparison with age-matched control samples. The concentration of markers for specific pathways of protein oxidative damage (direct oxidation, glycoxidation and lipoxidation) and fatty acid composition were assessed by mass spectrometry. Contributors to protein oxidation (mitochondrial respiratory complexes, antioxidant defence and proteolysis) and its consequences (endoplasmic reticulum stress and/or unfolded protein response (UPR)) were evaluated by western-blot of specific markers. Furthermore, the mitochondrial biogenesis system was assessed by measuring by western blot the levels of key factors.

ALS was associated to increased direct oxidative, glycoxidative and lipoxidative damage in SC and, to a lower extent, in FC samples. This was associated to increased lipid peroxidizability, and to impaired neuroprotective responses because of decreased docosahexaenoic content as well as alterations of the mitochondrial respiratory complexes and proteasomal impairment. Endoplasmic reticulum stress was evidenced in SC, but not in FC. Therefore, it could be concluded that sporadic ALS leads to increased oxidative damage in proteins and to ER stress in SC, while FC is less affected, but not preserved.

In samples from FC, but not in OC of PiD, there were evidences of ER stress such as activated UPR, associated to specific depletion in ER chaperones. Those findings are related to increased ubiquitination compatible with alteration in ubiquitin-proteasome system. In the same location, evidences for increased direct oxidative and lipoxidative damages targeting antioxidant enzymes were found, with decreased amount of glycoxidation markers. Strinkingly, increases in most of the examined parameters of oxidative stress in morphologically preserved OC of PiD patients were detected as well. The changes registered in PiD could be associated with disturbances in mitochondrial respiratory complexes compatible with diminished mitochondrial biogenesis and lack of antioxidant defence, combined with depletion in the contents of the neuroprotective docosahexaenoic acid observed in FC. In this line, the content of the transcription factors related to antioxidant responses and mitochondrial biogenesis showed significant changes in FC but less marked in OC. In contrast, while OC showed increased oxidative damage, mitochondrial respiratory chain and biogenesis were preserved, a finding associated to increased docosahexaenoic content, suggesting an appropriate response to the generated increase in oxidative stress.

Analysis of various oxidative stress biomarkers in HC of AGD revealed significantly decreased levels of the markers of glycoxidation, similarly to PiD, which is compatible with defects in glycolytic potential in this location. Those defects have been previously reported in other NDDs and may be associated to oxidative modifications of glycolytic enzymes also evidenced here. There were no changes in the concentrations of direct protein oxidation markers. This suggests a preferential role of other forms of oxidative damage, such as lipoxidation, as evidenced by increased malondialdehyde-lysine levels in this disease. Western blot measurements also revealed increased protein reactive carbonyl groups further supporting elevated oxidative damage in HC of AGD samples, which can be attributed to the mitochondrial dysfunction evidenced by disturbance in the respiratory chain function and reduced mitochondria number. Furthermore, the key molecules critically involved in UPR were found activated, which caused elevation in ER chaperones. Most importantly, despite the reduced number of mitochondria, transcription factors for their biogenesis were not increased, suggesting that impaired mitochondria biogenesis may be implicated in AGD pathogenesis.

The described results indicate the implication of oxidative and endoplasmic reticulum stress in sporadic ALS, PiD and AGD suggesting a possible interplay between them through proteolysis dysfunction, with a predominant role of mitochondrial impairment leading to the neurodegenerative process.

RESUMEN

La hipótesis a contrastar en esta tesis es que las enfermedades neurodegenerativas (ENDs) pueden ser una forma acelerada del envejecimiento, selectiva para determinadas localizaciones anatómicas del tejido nervioso. Consiguientemente, aquellos procesos subyacentes en las bases biológicas del envejecimiento (estrés oxidativo, acumulación de proteínas con alto grado de agregación, disfunción mitocondrial) y sus consecuencias son más intensas y prematuras en estas poblaciones celulares.

El objetivo de este trabajo es investigar la posible interrelación entre estrés oxidativo y de retículo (ER) y vías de señalización iniciadas por ambos procesos, como mecanismo patogénico en el desarrollo de ENDs de diferentes localizaciones y caracterizadas por la presencia de agregados proteicos. Así, hemos caracterizado la modificación oxidativa proteica, sus causantes más importantes y sus consecuencias fisiopatológicas en la forma esporádica de esclerosis lateral amiotrófica (ELA) con inicio lumbar, en la taupatía frontotemporal enfermedad de Pick (EdP) y en la enfermedad de granos argirofílicos (EGA). Los resultados de los análisis de muestras de pacientes con ELA se ha comparado con los obtenidos en modelos *in vitro* de la enfermedad.

Tras caracterización anatomopatógica exhaustiva, las muestras de médula espinal (ME) y de córtex frontal (CF) de pacientes de ELA; de córtex occipital (CO) y CF de enfermos de EdP, y de hipocampo (HC) de pacientes con EGA, se analizaron en comparación con muestras de individuos sanos con edades comparables. La concentración de marcadores de vías específica de modificación oxidativa proteica (oxidación directa, glicoxidación y lipoxidación), así como la composición en ácidos grasos se analizaron mediante espectrometría de masas combinada con cromatografía. Como factores reguladores de oxidación proteica se tomaron la cantidad de complejos respiratorios mitocondriales, sistemas de defensa antioxidante y sistemas proteolíticos, estimados mediante análisis de wester-blot. Además, se estableció mediante la misma metodología, las consecuencias en forma de ER y de respuesta a proteínas desplegadas (RPD). Asimismo, se estimó la biogenésis mitocondrial mediante análisis de la cantidad de factores reguladores de la misma, mediante western-blot.

Las muestras con ELA mostraron incrementos en los marcadores de oxidación directa, glicoxidación y lipoxidación en ME, y, de forma menor cuantitativamente, en muestras de CF. Ello se asoció a un incremento en la peroxidizabilidad lipídica, y a una disminución de respuestas neuroprotectoras debido a la disminución en el

contenido de ácido docosahexaenoico y a alteraciones del proteasoma y en el contenido de complejos respiratorios mitocondriales. Se evidenció estrés de retículo en ME, pero no en CF. Consiguientemente, se concluyó que la ELA esporádica conlleva incremento en lesión oxidativa proteica y a estrés de retículo en médula espinal, mientras que el CF, muestra menor afectación, pero no esta indemne.

Por otro lado, en muestras de CF, pero no en CO de EdP, se detectaron evidencias de estrés de retículo, como RPD, asociadas a pérdida de chaperonas de retículo. Estos hallazgos se relacionan con un incremento en la ubiquitinización compatible con alteraciones en la actividad proteasomal. En esta localización (CF), se hallaron incrementos en lesión oxidativa directa y lipoxidación, dirigidas a enzimas antioxidantes, con disminución en la concentración de marcadores de glicoxidación. Sorprendentemente, se demostraron incrementos en la mayoría de marcadores de lesión oxidativa en CO, localización morfológicamente preservada en EdP. Los cambios presentes en esta enfermedad se asociaron a cambios en la dotación de complejos respiratorios mitocondriales, compatibles con pérdida de biogénesis mitocondrial y de defensa antioxiante, combinados con depleción del ácido docosahexaenoico, considerado como neuroprotector, en CF. En este contexto, el contenido de los factores de transcripción relacionados con respuestas antioxidantes y con biogénesis mitocondrial, mostraron cambios significativos en CF y menos marcados en CO. En contraste, mientras que en CO se observó incremento en lesión oxidativo en EdP, las cadenas respiratorias mitocondriales y la biogénesis podrían estar preservadas, de forma conjunta con un incremento de ácido docosahexaenoico, sugiriendo una respuesta apropiada al estrés oxidativo.

El análisis de los marcadores de estrés oxidativo en HC de EGA revelaron disminuciones significativas en marcadores de glicoxidación, de modo similar a EdP, hallazgos compatibles con un déficit de glicolisis en esta situación. Estos defectos se han descrito previamente en otras ENDs y pueden asociarse a modificaciones oxidativas de enzimas glicolíticos, tambien evidenciados aquí. Ello sugiere un papel preferencial de otros modos de lesión oxidativa, como la lipoxidación, como evidencian los incrementos en concentración de malondialdehido-lisina en esta enfermedad. La medición, mediante western-blot, de los carbonilos proteicos reactivos reforzó la existencia de estrés oxidativo en HC, atribuible a la disfunción mitocondrial evidenciable por cambios en la función respiratoria y en su número. Asimismo, diversas moléculas clave en la RPD mostraron incrementos en las muestras procedentes de enfermos, causando incrementos en chaperonas de retículo endoplasmatico. De forma remarcable, a pesar del número reducido de mitocondrias, los factores transcripcionales implicados en su biogénesis no se elevaron, sugiriendo

que un defecto en biogénesis mitocondrial puede estar implicado en la patogénesis de EGA.

Los resultados descritos en esta memoria de tesis indican la interrelación entre estrés oxidativo y de retículo endoplasmatico, en ELA, EdP y EGA sugiriendo su relación recíproca a través de disfunción proteolítica, y un papel clave de la función mitocondrial, conduciendo al proceso neurodegenerativo.

RESUM

La hipòtesi de treball d'aquesta tesi es que les malalties neurodegeneratives poden considerarse formes accelerades de l'envelliment, selectives per a determinades localitzacions anatòmiques del teixit nerviós. D'acord a això, aquells processos subjacents a les bases biològiques de l'envelliment (estrès oxidatiu, accumulació de proteïnes agregades, disfunció mitocondrial) i les seves conseqüències son més intenses i prematures en aquestes poblacions cel.lulars.

L'objectiu d'aquest treball es investigar la possible relació entre estrès oxidatiu i de retícle (ER) i les vies de senyalització iniciades per ambdós processos, com a mecanisme patogènic en el desenvolupament de les malalties neurodegeneratives, de diferents localitzacions i caracteritzades per la presència d'agregats proteics. Així, hem caracteritzat la modificació oxidativa proteica, els seus causants més importants i les seves conseqüències fisiopatològiques en la forma esporàdica de l'esclerosi lateral amiotròfica (ELA) amb inici lumbar, en la taupatia frontotemporal malaltia de Pick (MdP) i en la malaltia de granuls argirofílics (MGA). Els resultats dels analisis de mostres de pacients amb ELA s'han comparat amb els obtinguts en models in vitro de la malaltia.

Després de la caracterització anatomopatològica exhaustiva, les mostres de mèdul.la espinal (ME) i d'escorça frontal (EF) de malalts amb ELA, d'escorça occipital (EO) i EF de malalts amb MdP, i d'hipocamp (HC) de malalts amb MGA, es varen analtizar en comparació amb mostres d'individus sans amb edats comparables. La concentració de marcadors de vies específiques de modificació oxidativa proteica (oxidació directa, glicoxidació i lipoxidació), així com la composició en àcids grasos es va analitzar mitjançant espectrometria de masses combinada amb separació cromotogràfica. Com a factors reguladors d'oxidació proteica es varen emprar la quantitat de complexes respiratoris mitocondrials, sistemes de defensa antioxidant i sistemes proteolíticis, estimats mitjançant western-blot. A més s'establí, emprant la mateixa metodologia, les conseqüències en forma d'ER i de resposta a proteïnes desplegades (RPD). Així mateix, s'estimà la biogènesi mitcondrial mitjançant anàlisi de la quantitat de factors reguladors de la mateixa, mitjançant western-blot.

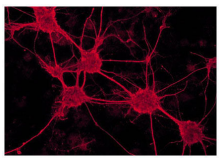
Les mostres dels pacients amb ELA mostraren increments en els marcadors d'oxidació directa, glicoxidació i lipoxidació en ME, i, de forma menys important, en mostres d'EF. Això s'associava a un increment en peroxidizabilitat lipídica, i a una disminució de respostes neuroprotectores, degut a la disminució en el contingut d'àcid docosahexaenoic, i a alteracions proteasomals i en el contingut de complexes respiratoris mitocondrials. Es va evidenciar estrés de reticle en ME, però no a l'EF.

Així, es va concloure que l'ELA esporàdica duu a increments en lesió oxidativa proteica i a estrés de reticle en ME, mentres que a EF, hi ha menys afectació, bo i no estar indemne.

D'altra banda, en mostres de EF, però no en EO de MdP, es varen detectar evidències compatibles amb estrés de reticle, com la RPD, associada a deplecció de xaperones de reticle. Aquestes troballes es relacionen amb un increment en la ubiquitinització, compatible amb alteracions en l'activitat proteasomal. En EF, es varen trobar increments en lesió oxidativa directa i lipoxidació, afectant a enzims antioxidants, amb disminució en la concentració de marcadors de glicoxidació. Sorprenentment, es varen evidenciar increments a la majoria de marcadors de lesió oxidativa en EO, localització morfològicament preservada a la MdP. Els canvis presents en aquesta malaltia s'associaven a canvis en la dotació de complexes respiratoris mitocondrials, compatibles amb pèrdues de biogenesi mitocondrial i de defensa antioxidant, combinats amb deplecció de l'àcid docosahexaenoic, considerat un neuroprotector, en EF. En aquest context, el contingut dels factors de transcripció relacionat amb respostes antioxidants i amb biogènesi mitocondrial, mostraren disminucions significatives en EF i de forma menys marcada, a EO. En contrast, mentres que a EO es va veure increment d'estrés oxidatiu, les cadenes respiratories mitocondrials i la biogenesi no semblaven afectades, de forma conjunta amb un increment d'àcid docosahexaenoic, suggerint una resposta apropiada a l'estrés oxidatiu.

L'anàlisi dels marcadors d'estrés oxidatiu en HC de MGA revelaren disminucions significatives en marcadors de glicoxidació, de manera similar a MdP, troballes compatibles amb un dèficit glicolitic. Aquests defectes glicolítics, s'han descrit previament en altres malalties neurodegeneratives i podrien associarse a modificacions oxidatives d'enzims glicolítics, també evidenciats aquí. Això suggerreix un paper preferencial d'altres modalitats de lesió oxidativa, com la lipoxidació, com evidencien els increments en concentració de malondialdehid-lisina en aquesta malaltia. La medició dels carbonils proteics va reforçar l'existència d'estrés oxidatiu a HC, atribuible a la disfunció mitocondrial, evidenciada per canvis en complexes i en el nombre de mitocondris. Així mateix, diverses molecules clau en la RPD varen mostrar increments a les mostres procedents de malalts de MGA, causant increments a les xaperones de reticul endoplasmàtic. De forma remarcable, a pesar de les troballes compatibles amb la reducció mitocondrial, els factors transcripcionals implicats en la seva biogènesi no s'elevaren, suggerint que un defecte en biogènesi mitocondrial podria estar implicat a la patogenesi de la MGA.

Els resultats descrits a la present memòria de tesi, indiquen la relació entre estrés oxidatiu i de retícul endoplasmàtic, en ELA, MdP i MGA, suggerint la seva relació recíproca, a través de disfunció proteolítica, i un paper clau de la funció mitocondrial, o la seva pèrdua, conduint al procès neurodegeneratiu.



INTRODUCTION

INTRODUCTION

1. Neurodegenerative diseases

Neurodegenerative diseases (NDDs) are a heterogeneous group of pathologies which includes complex multifactorial diseases, monogenic disorders and disorders for which are known inherited, sporadic and transmissible forms [Coppede 2006]. NDDs as diverse as Alzheimer's disease (AD), Parkinson's diseases (PD), Huntington's disease (HD), Prion diseases (PrD), Fronto-temporal dementia (FTD), Amyotrophic Lateral Sclerosis (ALS) and many others, are relentlessly progressive disorders of the central nervous system (CNS) characterized by loss of its functional and structural integrity. The clinical heterogeneity that consists of cognitive, motor, and/or behavioural dysfunction is attributable to pathological variability and selective vulnerability of populations of brain and spinal cord (SC) cells to the disease process. In many cases, these conditions arise sporadically and the causes are unknown [Terry 1994]. Factors associated with predisposition and vulnerability to NDDs may be described within the context of gene–environment interplay (Fig. 1). The genetic factors include: in AD, mutations in 3 genes (*APP*, *PSEN1*, and *PSEN2*); in PD, mutations in α-synuclein (PARK1), parkin (PARK2), ubiquitin carboxy-terminal hydrolase (PARK5), and at least five other linkage loci (PARK 3, 4, 6, 7, and 8), indicating additional contributing genes; In HD, polyglutamine expansions in huntingtin; in familial ALS (fALS) mutations in the SOD1 gene [Coppede 2006; Bertram 2005]. However, the majority of neurodegenerative pathologies can be described as age-related disorders. Ageing is the single most important risk factor for degenerative disease of the CNS, whereas the genes and the environmental factors modulate the age on-set and progression of disease [Outeiro 2008]. As the lifespan of humans continues to increase, an increasing burden of degenerative diseases is emerging.

Numerous studies have demonstrated that the pathogenesis of NDDs includes broad changes and involvement of multiple biochemical pathways. These common biochemical and cellular processes include protein misfolding; oligomerization and aggregation; impaired proteolysis; protein chaperone insufficiency; post-translational modifications; macromolecular oxidation; mitochondrial disturbance; activation of cellular stresses (oxidative, mitochondrial, endoplasmic reticulum stress); inflammation and activation of pro-apoptotic responses (Fig. 1). Misfolded proteins are prone to aggregation. Therefore, when the protein quality controls fail, many proteins tend to accumulate in excessive quantity in the affected cell. Thus, besides those NDDs occur from gradual neuronal death in various brain regions and may have different symptoms, most diseases share a common feature-aggregation and deposition of

abnormal protein. Abnormal protein aggregation in the form of either extracellular plaques or intracellular inclusions, within the cytoplasm or the nucleus of brain cells, represents an important pathological feature of the majority of NDDs, suggesting that the disorders are products of a common pathological process [Ross 2004; Kudo 2007].

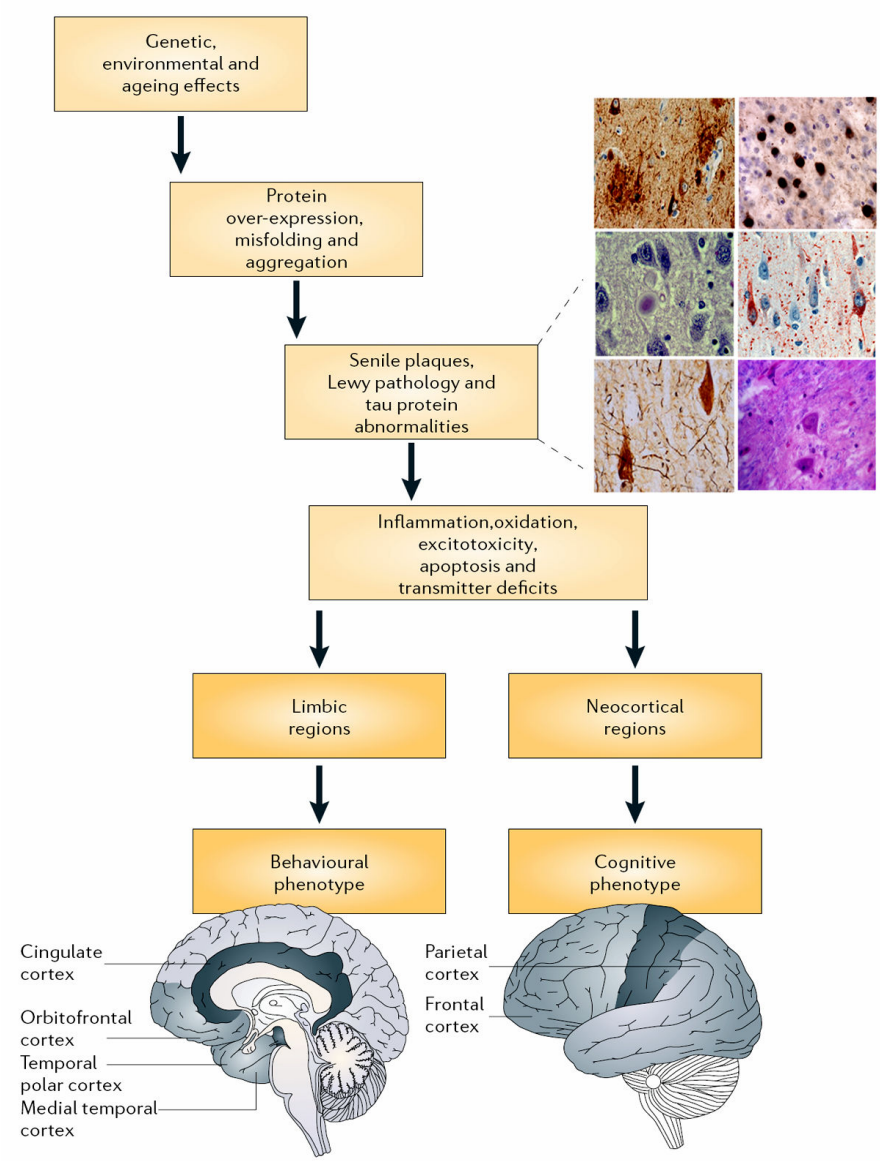


Figure 1: *Gene–environment and age-related risk factors for degenerative diseases of the CNS* [modified from Cummings et al., Nature Reviews Drug Discovery (2006) 5: 64-74]

The combination of two independent lines of research has made possible the recent progress in understanding of some of the most common NDDs. On the one hand, the biochemical study of the neuropathological lesions that define these diseases led to the identification of their molecular components. And on the other hand, the study of familial forms of disease led to the identification of gene defects that cause the inherited variants of the different diseases. Thus, the study of common

pathways between those two independent lines can be useful to show the pathogenic scenario of a given disease.

1.1. Abnormal protein aggregation as unifing feature of neurodegenerative diseases. Classification of NDDs according to the accumulated proteins.

A broad range of NDDs is characterized by neuronal damage that may be caused by toxic, aggregation-prone proteins. Neurons are particularly vulnerable to the toxic effects of mutant or misfolded protein. One or more specific proteins have been identified to accumulate in each NDD. Whether the cellular inclusions are truly pathogenic or represent markers of neurodegeneration remains debatable.

Chemical analysis of brain lesions show that they have a complex and varied composition (Table 1).

Lesion	Primary constituent	Associated constituents
Aβ deposit	*Aβ*	Apo E, Apo D, PrPsc, Tau, MAC, Amyloid-P, bFGF, Neuropeptide-Y, α2-macroglobulin, Clusterin, HSPG, Vibronectin, Maillard reaction products, PHF antigens, NF protein, Ubiquitin, Somatostatin
Prion protein deposits	*PrPsc*	Apo E, CgB, Aβ, Laminin, Clusterin
Neurofibrillary tangles	*3R/4R Tau*	Apo E, HSPG, Ubiquitin, NF protein, Synaptophysin, Aβ, MAC, GFAP, bFGF, Amyloid-P
Lewy bodies	*α-synuclein*	Tau, Ubiquitin, Tubulin, MAP5, cdk5, IF proteins, αB-crystallin, NF protein
Pick bodies	*3R Tau*	Apo E, bFGF, AGE, CgB, Clusterin, CD59, Clathrin, NF protein, Synaptophysin, GFAP, Ubiquitin
CBD inclusions	*4R Tau*	GFAP, Leu-7
PSP inclusions	*4R Tau*	GFAP, PHF antigens, Ubiquitin
Glial cytoplasmic inclusions	*α-synuclein*	Tau, MAP2, αB-crystallin, Tubulin, Ubiquitin, cdk5, NAPK, Rabaptin 5
NIFID inclusions	*α-internexin*	Ubiquitin, IF proteins
Ubiquitin inclusions (FTLD-U, ALS)	*TDP-43*	Ubiquitin

Table 1: *Molecular composition of the major brain lesions.* MAC, Membrane attack complex; bFGF, Basic fibroblast growth factor; HSPG, Heparan sulfate proteoglycan; PHF, Paired helical filament; NF, neurofilament; Chromogranin B; MAP5, Microtubule associated proteins; cdk5, Cyclin-dependent kinase-5; IF, Intermediate filament; CD59, Membrane complement inhibitor; Leu-7, Marker for killer lymphocytes [modified from Armstrong et al., Neuropathology (2008) 28: 351–365]

AD, the most common degenerative dementia, is characterized by senile plaques and neurofibrillary tangles consisting of beta-amyloid (Aβ) and highly phosphorylated tau proteins, respectively, in neural tissues. Amyloid precursor protein (APP) is an essential protein lodged in the surface of neurons. When unusually high levels of APP fragments known as Aβ, are present, the Aβ fragments can clump together to form plaques. These plaques are thought to disrupt the signalling between cells and eventually result in massive cell death [Hardy 2002]. Subsequent steps in the disease process include neurofibrillary tangles (NFT) of tau proteins, classifying the disease as a tauopathy. Tauopathy refers to a large group of disorders, in which NFT (Fig. 2) consisting of highly phosphorylated tau are found in neurons or glia. To this category also belong FTD, Corticobasal degeneration (CBD), Pick's disease (PiD), Argyrophilic grain disease (AGD), and progressive supranuclear palsy (PSP).

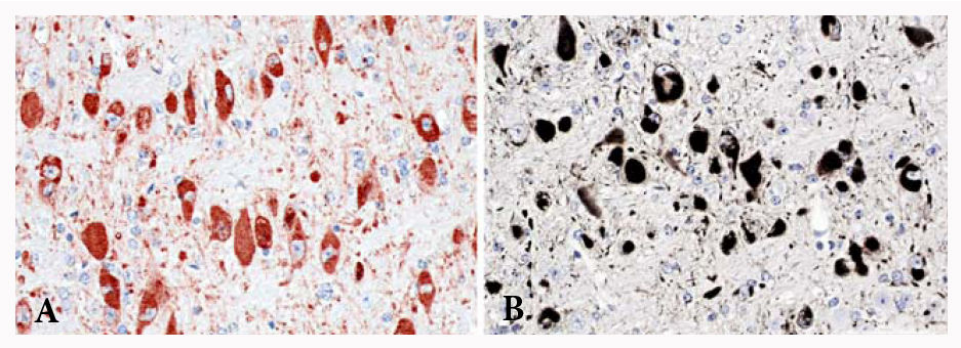


Figure 2: *Neurofibrillary tangles: (A) stained with AT8, (B) stained with Gallyas* [Frank et al., Acta Neuropathol (2008) 115: 39–53]

In synucleinopathies, Lewy bodies (LB) consisting of α-synuclein are observed in neurons (Fig. 3). This category comprises among others Diffuse Lewy Body Disease (DLBD) and PD.

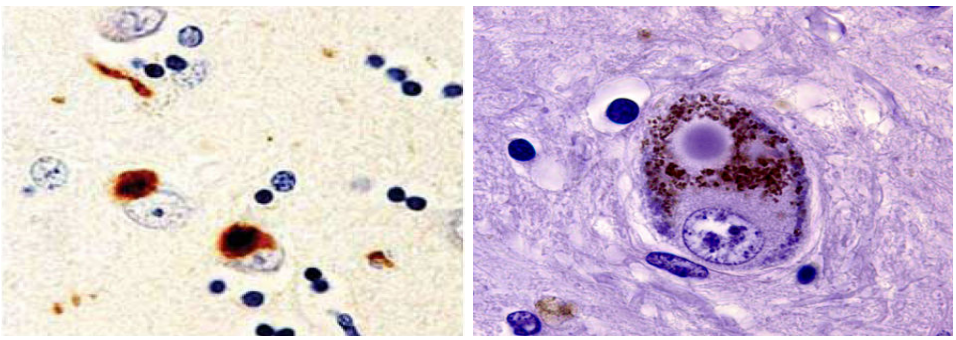


Figure 3: *Lewy body inclusions* [Popescu et al., Arch Neurol. (2004) 61: 1915-1919]

Polyglutamine diseases (PGD), such as HD, Kennedy's disease, and several forms of spinocerebellar ataxia, are disorders with abnormally extended polyglutamine chains in intraneuronal inclusions due to expansion of the CAG repeat in a causal gene [Zoghbi 2000]. In each of these diseases, expansion of the polyglutamine tract

beyond the normal range results in adult-onset, slowly progressive neurodegeneration. Longer expansions correlate with earlier onset and more severe disease. Each of the PGD is characterized by a different pattern of neurodegeneration and thus different clinical manifestations.

The TAR DNA binding protein 43 (TDP-43) has been shown as the major pathological protein present in neuronal inclusions in sporadic ALS (sALS), whereas there is no evidence of pathological TDP-43 in ALS cases with SOD1 mutations [Mackenzie 2007]. Thus, it has been suggested that TDP-43 may be a specific marker for sALS, althought it has been also detected in some forms of FTD. TDP-43 may be useful in characterization of motor neuron disorders (MNDs) and leads to two broad classes of disorders-TDP-43-positive and TDP-43-negative [Dickson 2007] (Fig. 4).

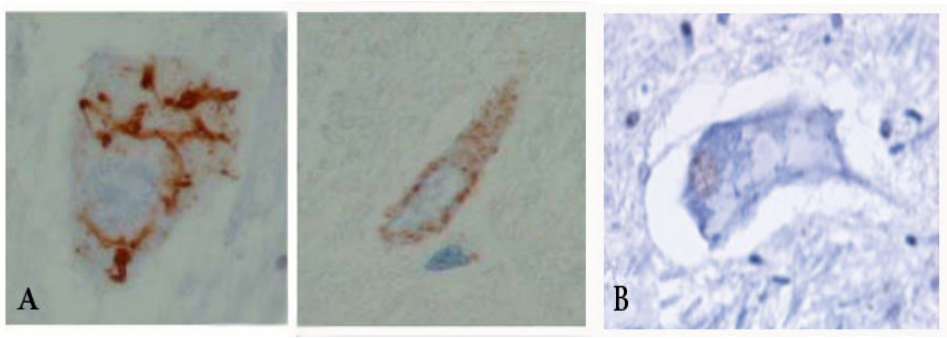


Figure 4: *TDP-43-immunoreactive neuronal inclusions: (A) TDP-43 positive and (B) TDP-43 negative* [Dickson and Josephs, Acta Neuropathol (2007) 114: 71–79]

Generally, inclusion bodies seen in NDDs are composed of unfolded and insoluble proteins that are highly ubiquitinated (Fig. 5). Considering this fact, the proteins that should have been metabolized in the ubiquitin proteasome system (UPS), in NDDs accumulate for some unknown reason to form inclusion bodies [Kudo 2007].

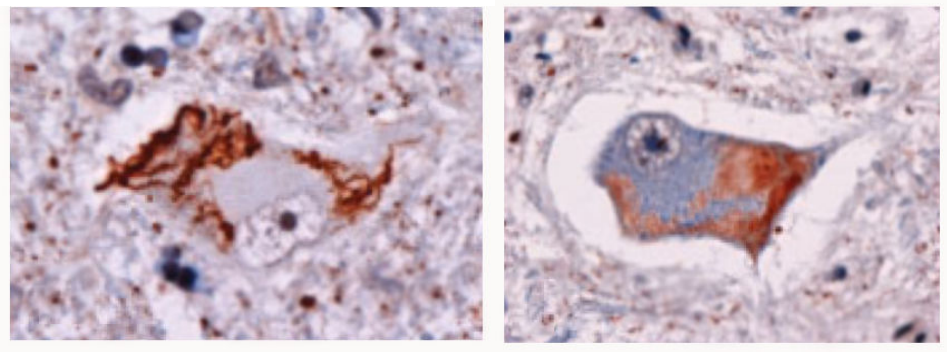


Figure 5: *Ubiquitin-immunoreactive neuronal cytoplasmic inclusion* [Mackenzie et al., Ann Neurol (2007) 61: 427–434]

The major molecular constituents of these lesions, namely Aβ, tau, α-synuclein, huntingtin (HTT) and TDP-43, play a defining role in the pathological diagnosis and

classification of disease based on the presence or absence of distinct brain lesions [Armstrong 2008]. These lesions are of two basic types. First, extracellular protein deposits that develop as senile plaques (SP) such as the Aβ deposits in AD and Down's syndrome [Glenner 1984], and deposits of the protease resistant form of prion protein (PrPSc) found in Creutzfeldt-Jakob disease (CJD) [Schulz-Schaeffer 1996]. And second, intracellular protein aggregates form inclusions within the cell bodies, nuclei, and processes of neurons. These include the various neuronal cytoplasmic inclusions such as neurofibrillary tangles (NFT) in AD, LB in PD and dementia with Lewy bodies (DLB) [McKeith 1996], Pick bodies (PB) in PiD [Love 1988], argyrophilic grains (AGs) in AGD [Braak 1987], TDP-43 in ALS [Dickson 2007] and tau positive neurons in CBD [Ikeda 1997].

The classification of NDDs is increasingly becoming based on the identity of the protein which accumulates forming microscopically-visible cellular deposits called "inclusion bodies" [Woulfe 2008] (Table 2).

Tauopathies	***Polyglutaminopathies***
Alzheimer's disease Amyotrophic lateral sclerosis/Parkinson-dementia complex Argyrophilic grain disease Corticobasal degeneration Dementia pugilistica Diffuse neurofibrillary tangles with calcification Down's syndrome Frontotemporal dementia/parkinsonism linked to chromosome 17 Gerstmann–Straussler–Scheinker disease Guadeloupian parkinsonism Myotonic dystrophy Niemann-Pick disease type C Pick's disease Postencephalitic parkinsonism Progressive subcortical gliosis Progressive supranuclear palsy Subacute sclerosing panencephalitis Tangle only dementia	Huntington's disease Dentatorubropallidoluysian atrophy Kennedy's disease Spinocerebellar ataxia 1, 2, 3, 6, 7, and 17 ***Prion Proteinipathies*** Creutzfeld–Jakob disease Fatal insomnia (familial and sporadic) Gerstmann–Straussler–Scheinker disease Kuru ***TDP-43 proteinopathies*** Frontotemporal lobar degeneration with ubiquitin-only inclusions Amyotrophic lateral sclerosis ***RNA-mediated diseases*** Fragile X-associated tremor ataxia syndrome Myotonic dystrophy Spinocerebellar ataxias 8, 10, 12 Huntington's disease-like type 2 ***Others*** Neuronal intermediate filament inclusion body disease Neuronal intranuclear inclusion disease Neuroferritinopathy TDP-43-negative FTLD-U
Synucleinopathies Parkinson's disease Dementia with Lewy bodies Multiple system atrophy Neurodegeneration with brain iron accumulation Pure autonomic failure Meige's syndrome	

Table 2: *Protein-based classification of neurodegenerative diseases* [modified from Woulfe et al., Biochimica et Biophysica Acta (2008) 1783: 2195–2206]

Following these pathogenic lines, advances in molecular neuropathology have allowed a classification system of NDDs based on the protein accumulation as (i) tauopathies and (ii) non tauopathies [Armstrong 2005].

1.2. Tauopathies

Taupathy is the commonest group of NDDs [Tolnay 1999]. All these diseases have in common the presence of aberrant tau aggregates. Tau was first implicated as a protein involved in the pathogenesis of AD when it was discovered to be a major component of the NFT [Lovestone 2002]. Subsequently, the occurrence of NFT in a wide range of conditions led to the suggestion that tau deposition may be an incidental nonspecific finding associated with cell death or cellular dysfunction. Later, the discovery that multiple mutations in the gene encoding tau are associated with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) provided strong evidence that abnormal forms of tau protein contribute to NDDs and dementia [Cole 1999; Spillantini 2000; Martin 2001].

Tau proteins are low molecular weight, normally cytosolic, microtubule-associated proteins that are abundant in the central and peripheral nervous system although nonneuronal cells usually have trace amounts. They are expressed predominantly in axons, but in the tauopathies they are redistributed to the cell body and dendrites [Binder 1985]. Mainly in pathological conditions tau proteins can be also expressed in glial cells [Chin 1996]. Physiologically, tau promotes the polymerization of Tubulin into microtubules and stabilizes them [Hasegawa 2006]. Human tau proteins are encoded by a single gene located on the long arm of chromosome 17 at band position 17q21 that spans over 110 kb. The tau primary transcript contains 16 exons. The CNS isoforms are generated by alternative splicing involving these exons (Fig. 6) [Neve 1986; Goedert 1988]. Exons 4A, 6 and 8 are constitutively skipped. Inclusion of exon 3 is associated with that of exon 2, whereas exon 2 can be included alone [Sergeant 2005]. In the CNS, the alternative splicing of exons 2, 3 and 10 generates six isoforms of tau protein. These six isoforms differ by the presence or absence of a 31 amino acid insert in the C-terminal region, encoded by exon 10, in conjunction with the presence or absence of a 29 or 58 amino acids inserts in the N-terminal domain, coded by exon 2 or exons 2 and 3, respectively [Andreadis 1992].

The repeat sequences in the C-terminal region of tau bind to tubulin and promote microtubule assembly. Deletion or insertion of the cassette exon 10 produces tau isoforms with three binding repeats (3R tau) or four binding repeats (4R tau), respectively. The 4R form of tau binds more strongly to microtubules and tends to

aggregate more rapidly than the 3R form. The 4R isoform promotes microtubule assembly at a rate about 2.5-fold higher than the 3R tau isoform [Hasegawa 2006]. In the normal adult human brain, there are similar proportions of 3R-tau to 4R-tau isoforms, about 50% each one. Thus, a correct ratio of 3R to 4R tau isoforms may be relevant to preventing neurodegeneration and dementia in mid-life.

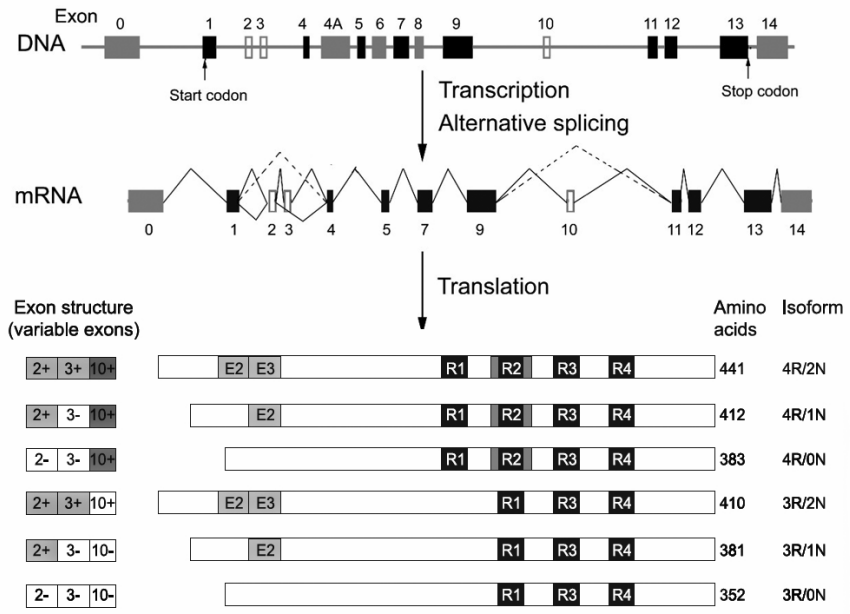


Figure 6: *Tau gene structure, the pre-mRNA alternative splicing in CNS and the six human tau isoforms.* [modified from Sergeant and Brandt, Biochimica et Biophysica Acta (2005) 1739: 179-197 and 331-354]

Mutations in the six messenger RNA transcripts for tau often result in multiple disease entities under the clinical union of FTDs. Tau mutations are either missense, deletion or silent mutations in the coding region, or intronic mutations located close to the splice-donor site of the intron following the alternatively spliced exon 10 (Fig. 6). Functionally, they fall into two largely non-overlapping categories-those whose primary effect is at the protein level and those that influence the alternative splicing of tau pre-mRNA. Most of these mutations lead to the formation of nerve cell inclusions that consist of filaments made of all six tau isoforms.

Comparative biochemistry of abnormally and hyperphosphorylated tau protein aggregates shows that they differ in both phosphorylation and content of tau isoforms, which enable a molecular classification of tauopathies. Five classes of tauopathies have been defined depending on the type of tau aggregates that constitute the “Bar Code” for NDDs (Fig. 7):

- **Class 0** - frontal lobe degeneration without tau aggregates
- **Class I** - a major tau triplet at 60, 64, 69 kDa

- **Class II** - a major tau doublet at 64 and 69 kDa and the concept of 4R tauopathies
- **Class III** - a major tau doublet at 60 and 64 kDa and the concept of 3R tauopathies
- **Class IV** - a major tau 60

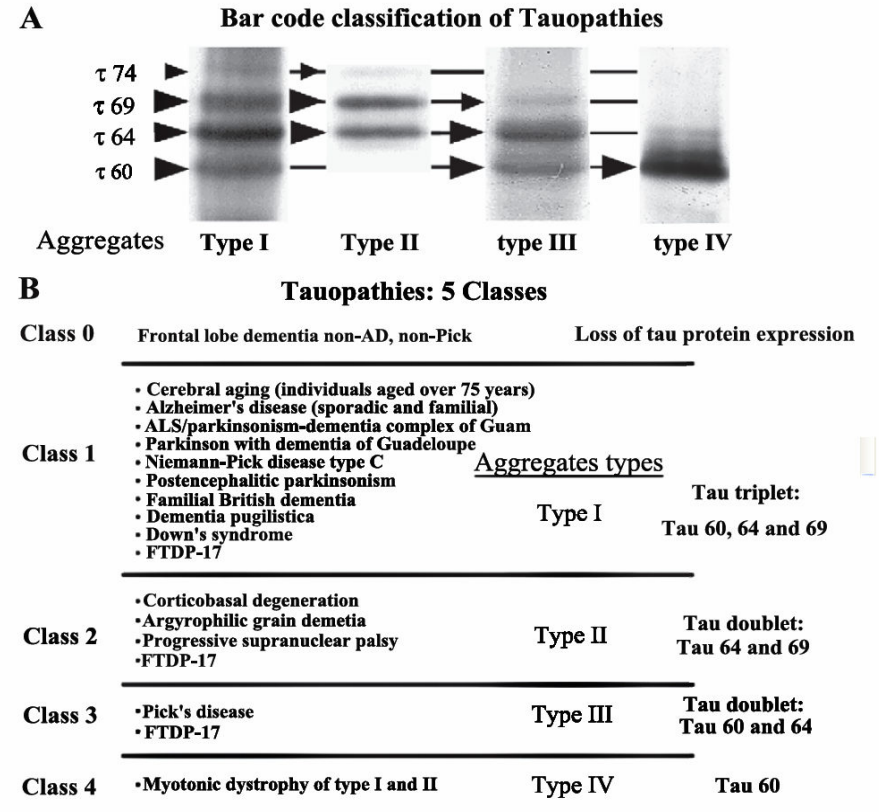


Figure 7: *The bar code of tauopathies and their classification. (A) Four different electrophoretic patterns of pathological tau proteins revealed by WB with phospho-dependent tau antibody in human brain tissue from patients affected by different tauopathies. Tau bands at 60, 64, 69 and 74 kDa correspond to pathological tau found in aggregates. These four main patterns of pathological tau thus represent a bar code of tauopathies. (B) Five classes of tauopathies has been defined and classified according to the bar code in neurological disorders* [Sergeant et al., Biochimica et Biophysica Acta (2005) 1739: 179– 197]

1.2.1. Alzheimer's disease

1.2.1.1. AD-overview

Alois Alzheimer, a German psychiatrist and neuropathologist, described for the first time a case of "presenile dementia", which later was identified by Kraepelin as Alzheimer's disease. The term AD was formally adopted in medical nomenclature to describe individuals of all ages with a characteristic common symptom pattern,

disease course, and neuropathology [Amaducci 1986]. AD is an insidious and progressive NDD that accounts for the vast majority of age-related dementia [Tanzi 1999], characterized by the presence of two simultaneous degenerating processes frequently observed in aging: amyloidosis and neurofibrillary degeneration [Delacourte 2003]. Consequently, AD is the most common dementia, which cause and progression are not well understood. Studies have shown that AD is more common among women than men by a ratio of 1.2 to 1.5 [Gao 1998]. The disease is characterized by global cognitive decline and the accumulation of Aβ deposits and NFT in the brain (Fig. 8).

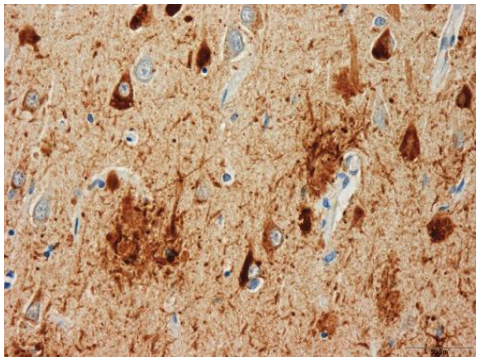


Figure 8: *NFT and Aβ staind for tau* [Kauwe et al., Proceedings of the National Academy of Sciences (2008) 105 (23): 8050-8054]

AD is also considered as the most common tauopathy (3R and 4R) due to abnormal aggregation of the tau protein [Tolnay 1999]. Each individual experiences the symptoms of AD in unique ways. The duration of the disease is between 5 and 10 years. The incidence of AD is less than 1% in people aged 60-65 years. This possibility doubles every 4 years and becomes more than 6% in people older than 85 [Kawas 2001].

1.2.1.2. Clinical and pathological features

The disease can develop many years before it is eventually diagnosed. In its early stages, episodic memory loss, shown as a difficulty to remember recently learned facts, is the most common symptom, although it is often initially misdiagnosed as age-related memory-loss or stress [Waldemar 2007]. As the disease advances, symptoms include confusion, anger, mood swings, language breakdown, long-term memory loss, and the general withdrawal of the sufferer as his or her senses decline [Tabert 2005].

Neuropathologicaly AD is characterised by loss of neurons and synapses in the cerebral cortex and certain subcortical regions. This results in atrophy of the affected regions, including degeneration in the temporal lobe and parietal lobe, and parts of the

frontal cortex and cingulate gyrus [Wenk 2003]. Both amyloid plaques and NFT are clearly visible by microscopy in AD brains [Tiraboschi 2004].

Plaques are dense, mostly insoluble deposits of Aβ protein and cellular material outside and around neurons. As described above, Aβ is a short peptide that is a proteolytic byproduct of the transmembrane protein APP, whose function is unclear but thought to be involved in neuronal development. Although Aβ monomers are soluble and harmless, they may undergo a conformational change and then aggregate to form amyloid fibrils that deposit outside neurons in dense formations known as senile plaques or neuritic plaques, or in less dense aggregates as diffuse plaques, and sometimes in the walls of small blood vessels in the brain [Ohnishi 2004].

Tangles are insoluble twisted fibers that build up inside the nerve cell. Although many older people develop some plaques and tangles, the brains of AD patients have them to a much greater extent and in different brain locations [Bouras 1994]. In AD patients, hyperphosphorylated tau accumulates as paired helical filaments [Goedert 2006] that in turn aggregate into masses inside nerve cell bodies known as NFTs and as dystrophic neurites associated with amyloid plaques.

1.2.1.3. Genetic predisposition and risk factors

Genetic and environmental factors can determine the personal risk of AD and both playing an important role in the development of the disease.

The vast majority of cases of AD are sporadic and they do not associate in families. Nevertheless, molecular genetic analyses suggest that there are some genes associated with increase of the susceptibility for developing AD. Although it is predominantly a disease of late life, there are families in which AD is inherited as an autosomal dominant disorder of mid-life. Three genes have been described as implicated in the inherited form of the disease: the APP gene [Citron 1992], which encodes the Aβ peptide; and the presenilin protein genes (PS1 and PS2), which encode transmembrane proteins Presenilins that participate in the cleavage of the APP protein within its Aβ region [Sherrington 1995; Levy-Lahad 1995]. Late onset AD is influenced by the genetic risk-factor Apolipoprotein E (ApoE) [McMurray 2000]. There are three alleles that encode three different isoforms of ApoE-ε2, ε3 and ε4. Individuals in which the ε4 isoform is produced, the risk of developing AD are increased [Pietrzik 2004]. There are evidences that ε4 enhances Aβ aggregation and reduce Aβ clearance. It has been suggested that ε4 might increase the risk of AD by enhancing amyloidogenic processing of APP, promoting cerebrovascular pathology, increasing OS and impairing neuronal plasticity. Recently mutations in SORL1 gene encoding for Sortilin were proposed to be risky for late onset AD [Rogaeva 2007].

Epidemiological findings suggest that low educational level, history of head trauma, consumption of high-calorie, high-fat diets and a sedentary lifestyle may each increase the risk of AD [Ninomiya 1993]. Specific dietary components may affect the risk of AD. Individuals with low dietary folate intakes are at increased risk of AD.

Among environmental factors metals have been extensively studied for their posible contribution to neurodegeneration. The homeostasis of Zn, Cu and Fe are altered in the brain of AD individuals, and under mildly acidic conditions, such as those believed to occur in AD brain, Fe and Zn ions have been observed to induce Aβ aggregation [Cherny 1999]. Elevated levels of Fe, Zn and Se in the brain have been associated with AD [Cornett 1998]. The role of Cu in Aβ aggregation is still controversial. However, the strongest risk factor for AD is aging.

1.2.2. Pick's disease

1.2.2.1. PiD –overview

PiD is a relatively rare, degenerative brain illness that causes dementia. The first description of the disease was published in 1892 by Arnold Pick for whom Pick disease is named. During his tenure, he encountered several patients with unusual clinical manifestations of dementia and concomitant neuropathologic evidence of severe, circumscribed cerebral atrophy. A microscopic evaluation that would later serve as a defining feature of PiD was absent from his original presentations. This evaluation was originally conducted by Alois Alzheimer, who described rounded, microscopic intraneuronal inclusions, ballooned neurons (BN), and spongy cortical wasting within the previously noted circumscribed areas of gross atrophy [Case records 2000]. PiD has been little studied, and much less is known about it than about AD. The diagnosis of PiD is difficult during life, because its symptoms are so variable and because they overlap so much with AD. Until recently it was thought that PiD could not be distinguished from AD during life. PiD differs from AD in several ways. First, the two diseases produce different abnormalities in brain cells. Neither of PiD marked changes such as PBs and swollen neurons appear in AD, and the pathology of AD (plaques and tangles) is not found in PiD. Secondly, PiD tends to affect only certain areas of the brain, particularly the frontal and anterior temporal lobes, whereas the occipital lobes seem pathologically less affected, while AD can affect any part of the brain. In contrast to AD, in which early memory loss predominates, the first symptoms of PiD are personality changes. Whereas, AD brains show the 6 tau isoforms, PiD exhibits only 3 tau repeat isoforms of the microtubular binding domain and belond to the group of 3R tauopathies [Rossor 2001].

Epidemiologically, approximately 0.4% to 2% of all cases of dementia are attributable to PiD making it 3 to 5 times less common than AD [Armstrong 2000]. The onset of PiD is generally before age 65 years with discrete male predilection [Bergeron 2003]. The average course is about 7 years, but it ranges from 2-15 years [Kertesz 2002; McKhann 2001]. Neither the cause nor cure for PiD is known.

1.2.2.2. Clinical and pathological features

Clinically, PiD progresses rapidly with ensuing disability and death secondary to infection or multisystem organ failure. Patients with PiD may initially present with behavioural changes or language dysfunction and a decline in function at work and home. Behavioral changes are often marked by lack of inhibition, manifesting as inappropriate behaviour in social situations as well as impulsiveness. Patients may progressively become apathetic, additionally losing concern for their own personal appearance, with subsequent deterioration in personal hygiene. As the illness advances, difficulties with language become common. Language disturbances commonly manifest as difficulty with expression and word finding [McKhann 2001]. Patients become unusually quiet, and when they do speak it may be slowly, in brief sentences. They may labour to make the sounds of words and their speech may sound distorted. Some become extremely apathetic -they may sit for hours doing nothing at all unless prompted to do so by another, while others become extraordinarily restless, and may pace unceasingly. Executive functions, such as planning and organization, may become impaired, while memory, orientation, and visual-spatial function are preserved [Bird 1998].

Inspection of the postmortem brain typically reveals circumscribed frontotemporal atrophy resulting in an average brain weight of 900 to 1000g in affected women and 1000 to 1200g in affected men. In 60% of cases atrophy is asymmetric, affecting the left hemisphere more than the right [Case records 2000]. This atrophy is due to a marked neuronal loss accompanied by proliferation of astrocytes in vulnerable regions, expressing glial fibrillary acidic protein (GFAP), together with hyperphosphorylated tau deposition in neurones. In addition, phospho-tau inclusions are also found in astrocytes and oligodendrocytes [Komori 1999; Bergeron 2003]. Microscopically, the characteristic features of PiD are histopathologic entities called PBs and Pick cells (PCs) [McKhann 2001; Kertesz 2004]. PBs are round, well circumscribed cytoplasmic inclusions that are weakly eosinophilic [Case records 2000] (Fig. 9A) and intensely argyrophilic with silver stains [Dickson 2001; Kertesz 2002] (Fig. 9B). The major constituent of PBs is the hyperphosphorlyated protein tau.

Antibodies against hyperphosphorylated tau, ubiquitin, advanced glycation end products (AGE) and many others have been reported to react positively with these inclusion bodies [Armstrong 2000]. PCs are described as large, swollen, ballooned, diffusely argyrophilic neurons with vacuolated cytoplasm lacking neuronal achromasia [Kertesz 2002].

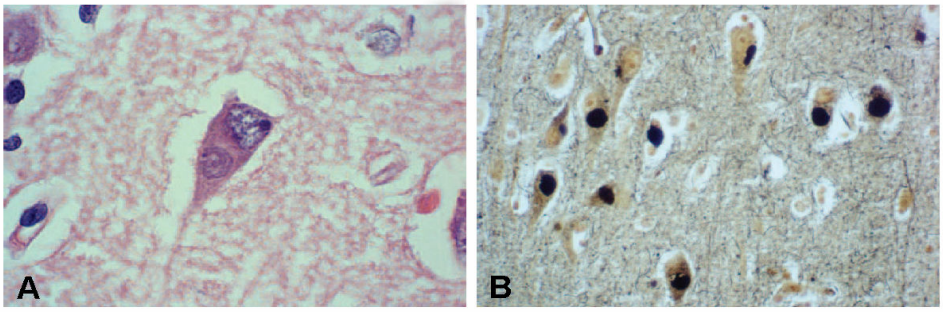


Figure 9: *Intracytoplasmic Pick body present within a neuron of the dentate gyrus highlighted with (A) hematoxylin-eosin and (B) Bielchowsky stain* [Frederick et al., Arch Pathol Lab Med (2006) 130]

Three types of PiD can be distinguished:

- Classic PiD, or **Type A** according to the classification scheme proposed by Tissot, Constantinidis and Richard, demonstrates both PBs and PCs in addition to prominent frontotemporal and limbic degeneration.
- In contrast, **Type B**, commonly referred to as CBD, or appropriate FTDP-17, is a disease where PBs are absent and PCs are numerous throughout the superior frontal and parietal lobes.
- **Type C**, the last subdivision within the classification schema, differs from Type A by lacking PBs and PCs as well as exhibiting either diffuse or circumscribed atrophy grossly and variable involvement of gray matter. This last type could be also categorized as dementia lacking distinctive histopathology or as one of the FTDs [Dickson 2001].

1.2.2.3. Genetic predisposition and risk factors

Little is known about the genetics of PiD. Most cases are thought to be sporadic with no other family member affected. However, a few studies suggest that PiD may have a genetic component. There have been several reports of families with what appears to be inherited familial cases of PiD. Hereditary PiD as a clinicopathological phenotype of FTDP-17 has been associated with the G272V mutation in the tau gene [Heutink 1997; Spillantini 1998]. Neuronal and glial tau inclusions can vary in morphology and tau isoform composition depending on the type

and localization of the mutation in the tau gene. In these families PBs are observed in patients with most exon 9 mutations and several mutations outside exon 10 and flanking regions: K257T, L266V, L315R, S320F, Q336R, G342V, K369I, G389R, [Pickering-Brown 2000; Spillantini 2000; van Swieten 2007]. Currently, 32 different mutations have been identified in over 100 families. About half of the known mutations have their primary effect at the protein level. They reduce the ability of tau protein to interact with microtubules and increase its propensity to assemble into abnormal filaments. The other mutations have their primary effect at the RNA level and perturb the normal ratio of 3R to 4R tau isoforms. This resulted in a relative overproduction of tau protein with four microtubule-binding domains in the brain. Individual tau mutations give rise to diseases that resemble PSP, CBD or PiD [Goedert 2005].

Although those data, there is no evidence that classical PiD pathology can run in families or has a genetic cause. Other risk factors, besides ageing, are unknown.

1.2.3. Argyrophilic grain disease

1.2.3.1. AGD-overview

AGD, first described by Braak and Braak [Braak 1987], is a sporadic degenerative disorder of the human brain, which becomes increasingly prevalent with advancing age and cause severe brain dysfunction. AGD was first reported as an adult-onset dementia, but recent studies have emphasized personality change, emotional imbalance, and memory problems as clinical features of AGD [Togo 2002]. The disease mainly affects limbic structures and results from cytoskeletal degeneration in only a few neuronal types and in oligodendrocytes. AGD is characterized by the presence of AGs constituting the most important histopathological hallmark of AGD [Ghebremedhin 1998]. Abnormally phosphosphorylated tau is the main protein constituent of AGs and tau is hyperphosphorylated in up to 80% of nerve cells in areas rich in AGs [Probst 2002]. AGD exhibits only four tau repeat isoforms of the microtubular binding domain and thus belongs to the group of 4R tauopathies [Togo 2002; Saito 2002; Zhukareva 2002]. AGD classification among dementia disorders is still unclear because most of the reported AGD cases are associated with neurofibrillary lesions (e.g. NFT) which are also typical for AD. Morphological, immunohistochemical, biochemical and genetic studies strongly support the view that AGD is separate from AD, although there might be difficulties in distinguishing the two disorders clinically [Tolnay 2004]. Some studies support the hypothesis that dementia in AGD correlates more with the density and distribution of AGs than with associated lesions of the Alzheimer-type [Tolnay 1999]. Then the term diffuse AGD has been

proposed as a subgroup of AGD to differentiate these cases from the most common limbic AGD [Maurage 2003].

The mean age of disease onset is about 75–80 years, its duration is between 4 and 8 years and account for about 1 to 5% of all demented patients [Braak and Braak 1998]. The cause of AGD is not known. Since the genetic studies have failed to discover a direct relation between AGD and a particular gene locus, the disease appears to be sporadic [Ferrer 2008].

1.2.3.2. Clinical and pathological features

Only little is known about the clinical presentation of AGD patients, especially the early symptoms. As a common initial manifestation of AGD some authores describe mild amnestic cognitive impairment [Jicha 2006; Petersen 2006].

Morphologically, AGD is characterized by the presence of neuronal AGs (Fig. 10A), which contain straight filaments or tubules, and by coiled bodies (CBs) (Fig. 10B), a common additional findings composed of accumulations of fibrils. AGs are small spindle shaped, comma-like, rod-like, button-like or round bodies localized in the neuropil of CA1 area of the hippocampus, presubiculum, the entorhinal and transentorhinal cortex, neighbouring temporal cortex, orbitofrontal cortex, insular cortex, basolateral nuclei of the amygdala and hypothalamic lateral tuberal nucleus [Braak 1989; Schultz 1998; Itagaki 1989].

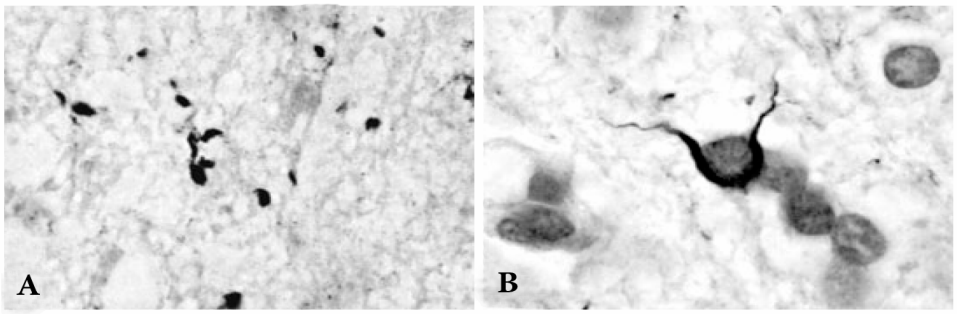


Figure 10: *Gallyas stain diffuse argyrophilic grains in the neuropil of hippocampal sector CA1 (A) and oligodendroglial coiled bodies (B)* [Tolnay and Clavaguera, Neuropathology (2004) 24: 269–283]

AGs are preferentially localized in dendrites and dendritic branches [Ikeda 1995], but also have been reported in axons [Tolnay 2004]. AGs might or might not be associated with a cognitive decline. Series of epidemiology studies directed to the conclucion that the presence of AGs in human brain is not necessarily associated with a cognitive decline [Tolnay 2004]. In the original paper by Braak and Braak [Braak 1989], all demented patients were reported to suffer from adult-onset progressive

mental deterioration. The most often symptoms accompanying AGD are behaviour abnormalities, inconstant mood, episodic memory loss, personality and emotional changes [Braak and Braak 1998; Ikeda 2000]. During the course of the disease, patients became anxious, restless, often depressed and emotionally shallow. Many of them showed incontinence and cachexia. AGD may also be manifested with cognitive decline and dementia in some cases, whereas a small number of patients present aggression or ill temper [Tolnay 2001, 2004; Saito 2002; Togo 2002]. The involvement of structures similar to those affected in patients with mild cognitive impairment and AD makes it difficult to distinguish AD from AGD on the basis of neuroradiological data. Biological assays also do not permit a clinical diagnosis of AGD during live [Ferrer 2008].

CBs are conspicuous, curvaceous, whip-like and often branched oligodendroglial inclusions, which are located in the vicinity to the cell nucleus [Tolnay 2004]. Both filamentous lesions (AGs and CBs) consist of the microtubule-associated protein tau in an abnormally hyperphosphorylated state [Tolnay 1997]. About 50% of AGs and CBs are immunoreactive to anti-ubiquitin antibodies [Tolnay 2003] (table 3).

1. Core lesions of AgD
1.1. Essential for diagnosis – Argyrophilic grains (Gallyas-positive, Tau-positive) 1.2. Consistent features but not essential for diagnosis – Coiled bodies (Gallyas-positive, Tau-positive) – Abundant non-argyrophilic (Gallyas-negative), tau-positive limbic projection neurons (‘pretangle’ neurons). 1.3. Biochemical tau profile of AgD: – Tau doublet at 64 and 69 kDa. Pathological tau aggregates mainly made of four-repeat tau isoforms
2. AgD associated lesions – frequent findings but not essential for diagnosis
– Ballooned neurons (Gallyas-negative. Tau- and αB-crystallin-positive. Present in amygdala and layers V and VI of basal temporal neocortical areas) – Non-argyrophilic (Gallyas-negative), tau-positive astrocytes (present in amygdala and entorhinal and transentorhinal cortices) – Associated lesions of the Alzheimer-type – Neurofibrillary lesions (Braak stages I-III; frequent finding) – Senile plaques (few, mainly diffuse type; two thirds of cases)
3. AgD associated lesions – atypical findings
– Superficial laminar spongiosis (layers II-III of basal temporal neocortical areas) – Cortical and subcortical gliosis (entorhinal and transentorhinal cortices, posterior parahippocampal gyrus)

Table 3: *Neuropathological and biochemical features of AGD* [modified from Tolnay and Clavaguera, Neuropathology (2004) 24: 269–283]

Hyperphosphorylated tau also accumulates in limbic astrocytes. The presence of tau-containing astrocytes is variable from one case to another [Ferrer 2008]. Most tau-containing astrocytes in the limbic system are Gallyas negative [Tolnay 1997]. Other constant findings in AGD together with CBs are pre-tangle neurons (Fig. 11A-C). Their distribution is the same as that for AGs [Tolnay 2003 and 2004]. Variable numbers of tangles and neuropil threads may be present in the same regions as AGs and pre-tangle neurons. This adds difficulties to differentiate between AGD with a few tangles and AGD with associated AD [Cras 1991]. BNs in the limbic system are non-specific accompanying lesions encountered in many familial and sporadic tauopathies (CBD, PiD, PSP and AD) [Fujino 2004]. Whereas, Gallyas-negative BNs expressing αB-crystallin, commonly observed in the amygdala, have been proposed as a marker of AGD [Tolnay 1998; Togo 2002]. BNs are characterized by a swollen pale-staining cytoplasm and a large eccentrically located nucleus [Tolnay 2004] (Fig. 11D). BNs in AGD are strongly labeled with antibodies against αB-crystallin, a feature they share with BNs in other NDDs [Lowe 1992].

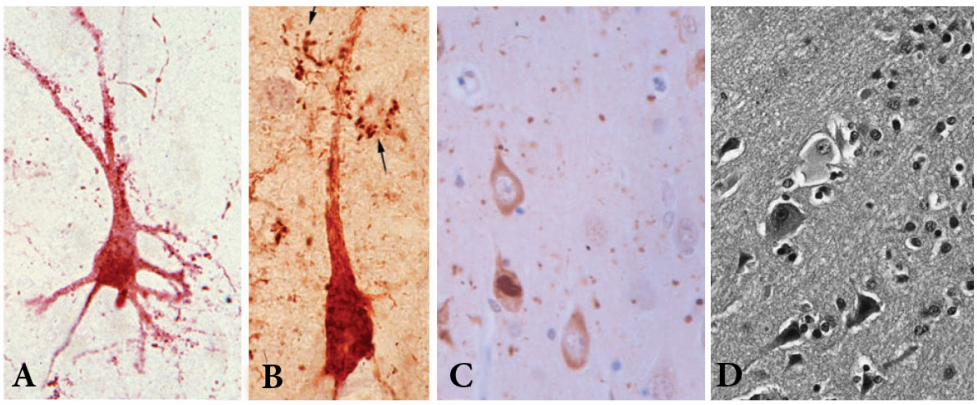


Figure 11: *Pre-tangle and ballooned neurones in AGD cases. Tau-immunostained pretangle neuron in the entorhinal cortex (A); Argyrophilic grains located within side branches of apical dendrites (arrows) in a pretangle neuron (B)* [Tolnay and Clavaguera, Neuropathology (2004) 24: 269–283]; *Ubiquitin immunoreactivity in pre-tangle neurons (C)* [Ferrer et al., Brain (2008) 131: 1416-32]; *Ballooned neurones (D)* [Ishihara et al., *Neuropathology* (2005) 25: 165–170]

The brains of AGD patients appear virtually unchanged or show only mild diffuse or frontotemporal cortical atrophy, without obvious atrophy of the hippocampus formation and/or the amygdaloid complex [Braak 1998; Jellinger 1998].

The presence of AGs cytoplasmic inclusions in AGD and the presence of AGs in other NDDs, including PSP [Masliah 1991; Togo 2002] CBD, MSA [Wakabayashi 1999] and MND [Tolnay 2001] suggests that AG may co-occur with other pathologies [Ikeda 1995]. Nonetheless, in a small percentage of dementia patients (usually older than 65 years of age), AGs are the overwhelmingly predominant neuropathological

lesions. As well, there are significant differences in the clinical presentation between these disorders, thereby suggesting that AGD is a distinct NDD [Tolnay 1997].

1.2.3.3. Genetic predisposition and risk factors

To date it has not been reported any familial form of AGD, and there is no reported mutation either on the tau gene or other causative gene. In 1998 was described an association between the apolipoprotein E (ApoE) ε2 allele and AGD. Individuals afflicted with AGD reveal a significantly higher frequency of the ApoE ε2 allele compared to AD and controls [Ghebremedhin 1998]. Other studies demonstrated a lack of relationship between AGD and ApoE ε4. The frequency of ApoE ε4 allele in AGD is similar to that of the general population [Tolnay 1998; Togo 2002].

Recently, it has been discovered a molecular misreading in some human genes linked to AD and Down syndrome and expressed during aging, such as the gene encoding an ubiquitin-B (UBB) [van Leeuwen 2000; 2002]. Molecular misreading in UBB mRNA resulting in dinucleotide deletion generates a mutant protein called UBB^{+1}. UBB^{+1} protein accumulation has been found in ubiquitin containing neuropathological hallmark lesions of PGD [de Pril 2004] and several tauopathies, among them PiD, FTDP-17, PSP and AGD, whereas they have not been found in ubiquitinated lesions of α-synucleinopathies [Fisher 2003]. Therefore, the accumulation of UBB^{+1} protein was suggested as a specific marker of proteasomal dysfunction in the diseased brain, specifically in neurodegenerative tauopathies, including AGD. UBB^{+1} is also expressed in neurons with tangles and in AGs in AGD [Fisher 2003], whereas very low levels are noticed in pre-tangle neurons. Since the inhibition of the proteasome activity by UBB^{+1} is dose dependent [van Tijn 2007], it was suggested that low levels of UBB^{+1} in pre-tangle neurons would not impede proteasomal function, while high levels of UBB^{+1} in tangles and grains make these structures resistent to degradation via the UPS [Ferrer 2008].

At present, as in the case of PiD, age can be considered as the only risk factor for AGD [Tolnay 2004].

1.3. Non tauopathies

1.3.1. Synucleopathies

α-synuclein is the major molecular component of Lewy bodies (LB) and Lewy neurites (LN). It is a highly conserved 140 amino acid phosphoprotein of unknown function localized in the presynaptic terminal of the neuron [Wakabayashi 2000;

McLean 2000] and implicated in synapses plasticity [Jakes 1994]. In LB and LN, α-synuclein accumulates as fibrillar aggregates. Mutations on the α-synuclein gene were discovered in two autosomal familial dominant PD strongly suggesting that α-synuclein is the major etiological agent. Thus, neurological disorders with α-synuclein aggregation were named synucleopathies.

DLB is the second cause of neurodegenerative dementia after AD and it is essentially sporadic [McKeith 1999]. The major brain lesions are LB and LN. Other neurological disorders share these neuropathological entities such as the substantia nigra in PD and glial cytoplasmic inclusions in multisystemic atrophy and are also often observed in AD [Dickson, 2001].

Oxidative stress (OS) may be a main contributory factor in the pathogenesis of PD [Markesberry 2001; Jenner 2003]. The pathological hallmark of PD is the deposition of cytoplasmic LB inclusions, composed largely of α-synuclein, within dopaminergic neurons. Dopaminergic neurons may be more sensitive to the disease process than other neurons because they sustain more protein damage through OS induced by dopamine metabolism. Rare autosomal dominant forms are caused by mutations of α-synuclein, others by mutations of parkin, and other genes of the ubiquitin-proteasomal pathway.

1.3.2. Prion disease

The most common human PrD is sporadic CJD. PrD are distinct from other NDDs because of their transmissibility. Although they share a common molecular aetiology, the PrD vary greatly in their clinical manifestations, which may include dementia, comportamental disturbances, disordered movement, ataxia, and insomnia. The pathology of PrD shows varying degrees of spongioform vacuolation, gliosis, and neuronal loss. One consistent pathological feature of the PrD is the accumulation of amyloid material that is immunopositive for prion protein (PrP). Prions consist of an abnormal isoform of PrP [Colling 2001]. Structural analysis indicates that normal cellular PrP (designated PrPC) is a soluble protein. In contrast, PrP extracted from the brains of affected individuals (designated PrPSc) is highly aggregated and detergent insoluble. The polypeptide chains for PrPC and PrPSc are identical in amino acid composition, differing only in their 3D conformation. Aggregation and deposition of PrPSc may be a consequence of a rare, conformational change leading to sporadic cases.

1.3.3. Polyglutamine diseases

At least nine inherited adult-onset neurological disorders are caused by trinucleotide (CAG) repeat expansion, including HD [Zoghbi 2000]. The genes responsible for these diseases appear to be functionally unrelated. The only known common feature is a CAG trinucleotide repeat in each gene's coding region, resulting in a polyglutamine tract in the disease protein. In animal models of the PGD and in the CNS of patients with these disorders expanded polyglutamine forms neuronal intranuclear inclusions [Ross 1997]. These inclusions consist of accumulations of insoluble aggregated polyglutamine-containing fragments, in association with other proteins. It has been proposed that proteins with long polyglutamine tracts misfold and aggregate [Perutz 1994]. HD is a considerable variability in clinical manifestations and age at which symptoms first appear. The cause is a gene mutation in chromosome 4 with a CAG trinucleotide repeat of 35 or greater [Huntington Disease Collaborative Research Group 1993].

1.3.4. Motor neuron diseases

MNDs are clinically and pathologically heterogeneous. Some forms of MNDs affect primarily motor neurons (MNs) in the SC or brainstem, while others affect MNs at all levels of the neuraxis. They can be classified into those that affect primarily upper MNs, lower MNs or both. The most common disorder to affect both upper and lower MNs is ALS [Swash 2003; Talbot 2006].

1.3.4.1. Amyotrophic lateral sclerosis

1.3.4.1.1. ALS-ovreview

Human ALS (hALS) also known in America as Lou Gehrig's disease and in the UK as MND was described in 1869 by the French neurologist Jean-Martin Charcot. ALS is one of the major NDDs alongside AD and PD, and is the most common adult-onset MND [Kurtzke 1982]. It is characterized by degeneration of upper and lower MNs, generalized weakness and muscle atrophy. Sometimes ALS is seen to overlap with other NDDs, some patients have associated FTD [Neary 2000]. The course of the disorder is progressive, with 50% of patients dying within 3 years of onset. Death occurr within 1–5 years of onset, usually resulting from respiratory failure, often with associated pneumonia or choking, and general malnutrition may also contribute. About 20% of people with ALS live five years or more and up to 10% will survive more than ten years and 5% will live 20 years. From those cases with onset in the swallowing muscles, only 5% survive 5 years. Survival is somewhat better for those with limb

muscle onset, of whom 15% survive 5 years. ALS appears with worldwide incidence of up to 2 cases per 100 000 of the population each year. Individuals with sALS have an average age of onset of 55 years [Pasinelli 2006], compared with 46 years for fALS with SOD1 mutations [Andersen 2006]. Males are twice as frequently affected as females (ratio about 1,6:1) [Donaghy 1999, 2002].

Three forms of ALS have been described: **Sporadic** - the most common form of ALS; **Familial** - accounting for a very small number of cases; and **Guamanian** – a local form of ALS that occurs with an extremely high incidence in Guam and the Trust Territories of the Pacific in the 1950's.

Most cases (90%) are classified as sALS, as they are not associated with a documented family history. The remainder 10% of all cases are inherited with a Mendelian autosomal pattern of inheritance, earlier onset and referred to as fALS. Some of them result from mutations in the gene encoding the antioxidant enzyme Cu/Zn superoxide dismutase (SOD1). Several other mutated genes have also been found to predispose to ALS. Historically, the discovery of Cu/Zn-SOD1 mutations led to the generation of the first animal models of ALS. Several mouse lines were generated that overexpress ubiquitously mutant SOD1 (mSOD1) at levels sufficient to induce a MND closely resembling hALS [Gurney 1994; Ripps 1995; Wong 1995]. Sporadic and familial forms of ALS are clinically and pathologically similar, suggesting a common pathogenesis [Bruijn 2004]. There is variability of disease presentation and course in fALS patients with different SOD1 mutations similar to the variability seen in sALS. It may be envisioned that ALS is not a single disease but rather a syndrome and might turn out to be a common end-stage phenotype of diverse causes [de Aguilar 2007].

The findings of recent genetic polymorphism studies in ALS suggest that the influence of genetic risk factors for the disease may vary by ethnicity. The incidence of ALS may be lower among African, Asian, and Hispanic ethnicities than among whites [Cronin 2007]. Areas with an apparently higher prevalence of an ALS-like disease compared with other regions are around the Pacific rim (e.g, Guam island). The traditional diet of the Chamorro population, who live on the Pacific island of Guam, includes the fruit bat, which feeds on cycad seeds and has been reported to bioaccumulate methylaminoalanine. Scientific research suggests a cyanobacterial origin for methylaminoalanine in cycad tissue with resultant biomagnification in the food chain, so the role of methylaminoalanine as a MND inductor is of particular interest [Ince 2005].

1.3.4.1.2. Clinical and pathological features

The clinical features of ALS are indicative of the loss of neurons at all levels of the motor system-from the cortex to the anterior horn of the SC. Physical signs of this disorder encompass both upper and lower MN findings. The clinical features can be considered in relation to neurological regions or levels: bulbar, cervical, and lumbar [The ALS Association].

- Bulbar-onset patients present slurring of speech, swallowing difficulty, or both.
- Cervical-onset ALS patients present unilateral or bilateral upperlimb symptoms. Proximal weakness can present as difficulty with tasks associated with shoulder abduction (e.g, hair washing, combing, etc), and distal weakness can manifest with impairment of activities requiring pincer grip. Upper limb signs might also be upper MN, lower MN, or both. The arm can be strikingly wasted with profuse fasciculation and brisk reflexes.
- Lumbar onset implies degeneration of the anterior horn cells of the lumbar enlargement and is associated with lower MN symptoms and signs in the legs, such as a tendency to trip or difficulty on stairs.

At the onset of ALS the symptoms may be so slight that they are frequently overlooked. The initial symptoms or patterns of progression of ALS can be quite varied among different individuals. But, progressive muscle weakness and paralysis are universally experienced. In most patients, that muscle region which was first affected usually continues to be the worst affected. Since ALS attacks only MNs, the sense of sight, touch, hearing, taste and smell are not affected. Intellectual function remains unaffected, and there is no involvement of skin sensation, or of the control of the bladder or bowel. The striated muscles which control eye movements are not affected, as well. Patients with advanced forms of ALS may become breathless when the diaphragm and intercostal muscles get affected from the atrophy that spreads all over the body, or may experience potentially fatal choking attacks because the throat weakness allows food and liquid to be inhaled rather than swallowed. Apart from these respiratory complications of ALS, malnutrition due to impaired swallowing is the other major complication affecting general health [Donaghy 1999, 2002]. ALS is remarkably similar whether it is inherited or appears spontaneously in a person with no family history of the disease.

ALS causes degeneration both of the lower MNs in the SC and brainstem, which directly control voluntary muscles, and of the upper MNs in the motor areas of the cerebral cortex, which direct and integrate the activity of these lower MNs. Pathological examination of the SC and brain of patients who have died from ALS

shows extensive loss of lower MNs, and also of the upper MNs from the motor cerebral cortex.

The neuropathology of ALS is characterized by the abnormal accumulation of insoluble proteins (phosphorylated neurofilaments, Bunina bodies (BBs) and LB-like inclusions) in the cytoplasm of degenerating MNs [Leigh 1991; Lowe 1994]. BBs are small eosinophilic intraneuronal granular inclusions in the remaining lower MNs, generally considered to be a specific pathologic hallmark of ALS (Fig. 12) [Pasinelli 2006].

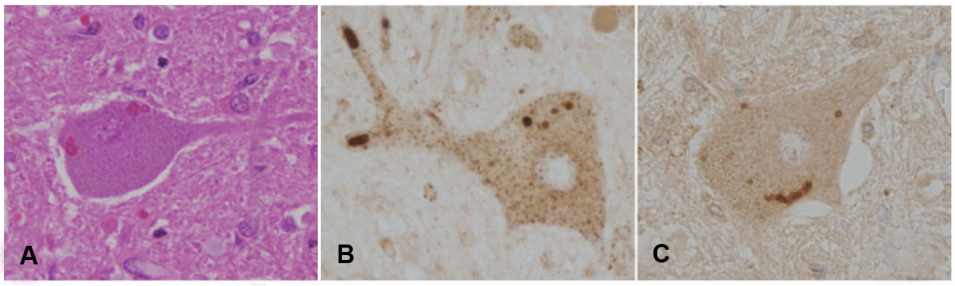


Figure 12: *Bunina bodies in the anterior horn cells in the lumbar cord of ALS. (A) Bunina bodies visualized with phosphotungstic acid-hematoxylin and eosin staining as bright pink, small, oval eosinophilic intraneuronal inclusions; (B) cystatin C immunoreactivities in the Bunina bodies in an anterior horn cell and its dendrites; (C) transferrin immunoreactivities in the Bunina bodies.* [Okamoto et al., Neuropathology *(*2008) 28: 109–115]

BBs' number varies in each neuron and they are seen not only in the cytoplasm but also in the dendrites, but they have not been found within the axoplasm [Kuroda 1990]. They appear either singly or in a group, and sometimes arranged in small beaded chains. BBs are mainly distributed in the MNs in the SC and of the brain stem. The bodies are absent in a subset of fALS with posterior column involvement and in MND with basophilic inclusions, but are present in almost all patients with sALS. Immunohistochemical studies have shown only two proteins to be present in BBs, one is cystatin C and the other is transferrin [Okamoto 2008]. Other antibodies against neurofilament, tau, α- and β-tubulin, microtubule-associated proteins, actin, myosin, desmin, synaptophysin, APP, GFAP, α-synuclein [Sasaki 2006] and p62 [Mizuno 2006] failed to demonstrate BBs immunoreactity. The nature and significance of the BBs in sALS are not yet clear, and the fact that BBs appeared not only in the degenerating neurons but also in the normal-looking neurons suggests that BBs are an initial change or a reaction of the MNs.

Ubiquitin-positive inclusions such as skein-like inclusions or LB-like round inclusions are another hallmark of ALS. Usually, BBs were negative for ubiquitin [Leigh 1991], however, a few researchers described that a small percentage of BBs show

positive immunoreactivites for ubiquitin [Lowe 1988; Murayama 1990]. Murayama et al. described that antiubiquitin antibody recognized an ill-defined structure in or around some BBs (Bunina body-related structure).

Recently, the TAR DNA-binding protein of 43 kDa (TDP-43), a nuclear protein involved in transcriptional repression and alternative splicing, was identified as a major component of neuronal intracytoplasmic inclusions in MNs in ALS [Dickson 2007; Mackenzie 2007], incompatible with its physiological location in nuclei. Various studies, suggest that this protein is directly involved in the pathogenesis of sALS [Neumann 2006]. In contrast, no evidence of pathological TDP-43 was found in familiar cases with the most common SOD1 mutations [Mackenzie 2007]. The presence of TDP-43–positive inclusions in glia suggests that disruption of some protective or supportive role of these cells may contribute to pathogenesis [Boillee 2006]. Skein-like and LB-like round inclusions in ALS are positive for TDP-43, however, BBs are negative [Tan 2007].

In addition, the activation and proliferation of astrocytes and microglia are also common in ALS [Ince 2000]. Although those data the cause of sALS at present is unknown.

1.3.4.1.3. Genetic predisposition and risk factors

Mutations in more than 50 human genes have been associated with MN pathology. Defects in several mutated Mendelian genes (Table 4) encoding cytosolic SOD1 [Rosen 1993], alsin [Hadano 2001; Yang 2001], dynactin [Puls 2003; Munch 2004], angiogenin [Greenway 2006], senataxin (SETX) [Chen 2004] and VAPB (synaptobrevin/VAMP (vesicle-associated membrane protein)-associated protein B) [Nishimura 2004] have been reported to cause or predispose to fALS [Pasinelli 2006; Gros-Louis 2006].

SOD1 is a cytosolic Cu/Zn-binding protein involved in antioxidant resistance that represents 1–2% of total cellular protein. Further strengthening the complex nature of the disease is that the same sod1 mutation does not necessarily cause a homogenous phenotype, but is rather able to induce different clinical presentations within the same family [Orrell 1997; Mase 2001; Rezania 2003]. SOD1 consists of 153 amino-acids that functions as a homodimer. Each subunit of SOD1 binds one Zn and one Cu atom. Through cyclical reduction and oxidation (dismutation) of Cu, SOD1 converts the superoxide anion, a by-product of oxidative phosphorylation in the mitochondrion, to hydrogen peroxide. About 20–25% of all fALS cases arise because of mutations in SOD1. More than 125 mutations have been identified, spanning all five exons of SOD1. 114 cause disease, whereas six silent mutations and five intronic

variants do not. Although most mutations are missense, 12 are nonsense or deletion mutations that produce a truncated protein [Andersen 2003].

ALS disease type	Gene	Chromosome	Inheritance	Clinical features
Mendelian genes				
ALS1	*SOD1*	21q22	AD	Typical ALS
ALS2	*ALS2*	2q33	AR	Juvenile onset, slowly progressive, predominantly corticospinal
ALS4	*SETX*	9q34	AD	Adult onset, slowly progressive
ALS8	*VAPB*	20q13	AD	Typical ALS
ALS	Dynactin	2p13	AD	Adult onset, slowly progressive, early vocal cord paralysis
Mendelian loci				
ALS5	?	15q15–21	AR	Juvenile onset, slowly progressive
ALS6	?	16q12	AD	Typical ALS
ALS7	?	20p13	AD	Typical ALS
ALS-FTD	?	9q21–22	AD	ALS, frontotemporal dementia
ALS-FTD	?	9p	AD	ALS, frontotemporal dementia
ALS-X	?	Xcen	XD	Typical ALS
Mitochondrial genes				
ALS-M	*COX1*	mtDNA	Maternal	Single case, predominantly corticospinal
ALS-M	*IARS2*	mtDNA	Maternal	Single case, predominantly lower motor neuron

Table 4: *Gene mutations in fALS* [Pasinelli and Brown, Nature Publishing Group (2006) 7: 710-23]

The als2 gene encodes a 184 kDa protein alsin [Hadano 2001]. Alsin is ubiquitously expressed and is abundant in neurons, where it localizes to the cytosolic portion of the endosomal membrane. The function of alsin is not fully understood. Alsin suppresses mutant SOD1-mediated toxicity in immortalized MN cell lines (NSC 34) by binding to SOD1 [Kanekura 2004]. Multiple different mutations have been identified in als2, among them several were shown to be linked to a rare juvenile form of ALS primarily characterized by upper MN involvement. Most mutations are predicted to truncate the protein. Alsin truncation varying with phenotype and in patients with milder phenotypes alsin is less truncated [Hadano 2001; Yang 2001]. The findings that all alsin mutants are unstable and that most patients are homozygous for the mutations indicate that this form of ALS is caused by a loss of function of alsin. Loss of alsin in mice does not trigger MN degeneration and disease, but does predispose to OS [Cai 2005], and causes age-dependent neurological defects [Hadano 2006].

A subset of familial and sporadic forms of ALS has been recently associated with mutations in the gene encoding dynactin, an activator of the major molecular axonal retrograde motor protein dynein [Puls 2003, 2005; Munch 2004]. The dynein/dynactin complex has multiple basic cellular functions, including vesicular transport and cell division. Dynein is also involved in neuron-specific activities such as retrograde transport in the axon [Levy and Holzbaur 2006]. Dynein/dynactin perturbations trigger MND. Similarly, motoneuronal overexpression of an ALS-linked

mutant dynactin is also sufficient to induce MN degeneration. These findings suggest that an alteration of the dynein/dynactin complex of motoneuronal origin is enough to induce MND [Hafezparast 2003]. Dynein/dynactin-linked ALS is considered as a 'pure' MN form [de Aguilar 2007]. Studies on mice had reported that MN-restricted overexpression of dynamitin, another subunit of the dynein complex, induces disassembling of dynactin and hence dynein dysfunction. These transgenic mice show a MND reminiscent of ALS, with decreased strength and endurance, muscle denervation and MN loss [LaMonte 2002].

More recently, mutations in ang, which encodes the potent inducer of neovascularization angiogenin, were found to be linked to several fALS and sALS cases [Greenway 2006]. The data concerning SETX and VAPB is still scarce.

1.3.4.1.4. ALS hypothesis

The pathogenic mechanisms leading individually or in concert to MN degeneration, neuron death and finally ALS are largely uncertain. Many causal and pathogenetic hypotheses for ALS have been proposed over the years, ranging from heavy-metal toxic effects [Mitchell 1987] to environmental and occupational exposures [Armon 2003]. However, despite of extensive research the disorder remains poorly understood, the precise cause is still unknown, and there is no effective remedy to stop the course of the disease.

Our understanding of the pathobiology of ALS is predicated largely on studies of ALS-associated gene mutations. Since the clinical and pathological profiles of sALS and fALS are similar, it was predicted that insights from studies of ALS-causing gene mutations also apply to sALS. Most data on ALS pathogenesis are derived from studies of cell death initiated by mutant SOD1 protein. The realized investigations from the last two decades hypothesized diverse processes that may interplay in the selective lesions of MNs in ALS. A mutant gene of antioxidant enzyme and exitotoxicity, mediated through glutamate excess, may lead to OS, mitochondrial dysfunction and the formation of damaging free radicals, which can harm all the nerve cell's macromolecules. The free radicals also may injure neurofilaments from the citoskeleton and thus to disrupt the axoplasmic transport. On the other hand mitochondrial abnormalities can lead to Ca^{2+} dysbalance that may drive to ER disturbance. In addition, neuroinflamation and autoimmunity may also be involved in harming neurons. Viral hypotheses drawing from the role of poliovirus in poliomyelitis have been pursued extensively without positive evidence emerging. One of the first clues that ALS might involve an environmental factor was obtained on the Guam island in the Pacific. Suspects have included trace metals or their lack in the soil, and

dietary factors. Some aspects of lifestyle that can interact with genes to cause or contribute to disease are suspect in ALS. Candidates as a causative factor in ALS include heavy metals, organic solvents, radiation, and electromagnetic fields but no conclusive proof exists for any toxin. As well as the Chamorro of Guam, U.S. veterans of World War II, the Korean War, the War in Vietnam and the Gulf War are another group of people who appear to develop ALS more often than the general population. One idea that researchers offer is that soldiers on active duty are engaged in strenuous physical labor or they are exposed to chemical toxins which could play a role in ALS.

2. Oxidative stress

2.1. Biochemistry of oxidative stress

OS is the condition arising from the imbalance between the physiological production of toxic reactive oxygen species (ROS) and reactive nitrogen species (RNS), and the physiological scavenging activities. Both ROS (superoxide, hydrogen peroxide and hydroxyl radical) and RNS (nitric oxide, peroxynitrite) react with cellular components and thereby damage all sorts of biomolecules such as lipids, proteins and DNA. Nervous tissue is particularly sensitive to OS. OS represents a significant pathway that leads to the destruction of neuronal and non-neuronal cells in the CNS, as they suffer in several interconnected ways, including direct oxidative damage to crucial molecular species, increase in intracellular free Ca^{2+}, ATP depletion and release of excitatory amino acids [Carrì 2003]. The production of ROS can also lead to cell injury through the peroxidation of cellular membrane lipids [Siu 2002], peroxidation of docosahexaenoic acid, a precursor of neuroprotective docosanoids [Mukherjee 2004], the cleavage of DNA during the hydroxylation of guanine and methylation of cytosine [Lee 2002], and the oxidation of proteins that yield protein carbonyl derivatives and nitrotyrosine [Adams 2001]. Several of these reactive species are produced at low levels during normal physiological conditions and are scavenged by endogenous antioxidant systems that include superoxide dismutase, glutathione peroxidase, catalase, and small molecule substances such as Vitamins C and E. Superoxide radical is the most commonly occurring oxygen free radical that produces hydrogen peroxide by dismutation. Hydroxyl radical is the most active oxygen free radical and is generated from hydrogen peroxide through the Haber–Weiss reaction in the presence of Fe ions. Hydroxyl radical alternatively may be formed through an interaction between superoxide radical and nitric oxide [Fubini 2003]. Nitric oxide interacts with superoxide radical to form peroxynitrite that can further lead to the

generation of peroxynitrous acid. Hydroxyl radical is produced from the spontaneous decomposition of peroxynitrous acid. Nitric oxide itself and peroxynitrite are also recognized as active oxygen free radicals. In addition to directly altering cellular function, nitric oxide may work through peroxynitrite that is potentially considered a more potent radical than nitric oxide itself [Pfeiffer 2001].

In CNS, there are many different sources by which the ROS are generated. They can be generated at various cellular sites (mitochondria, peroxisomes) and under various conditions including enzymatic reactions (e.g. the membrane NADPH oxidase, lipoxygenases, cyclooxygenases, and peroxidases). Most ROS come from the endogenous sources as by-products of normal and essential metabolic reactions, such as energy generation from mitochondria or the detoxification reactions involving the hepatic cytochrome P-450 enzyme system. Exogenous sources include exposure to cigarette smoke, environmental pollutants, excess consumption of alcohol, asbestos, exposure to ionizing radiation, and bacterial, fungal or viral infections. However, in healthy postmitotic tissues under physiological conditions, most free radicals are generated in mitochondrial respiratory chain.

2.2. Mitochondria - the main source of ROS in the cell

Mitochondria are important organelles in all cell types, but they are particularly important in the nervous system, helping neurons to meet the high energy demands of proper neuronal function. About 85 to 90 per cent of oxygen in aerobic organisms is used by the mitochondria; these organelles are the major source of energy (as adenosine triphosphate (ATP) molecule). Electrons from reduced substrates move from complexes I and complex II of the electron transport chain (ETC) (Fig. 13) through complexes III and complex IV to oxygen, forming water and causing protons to be pumped across the mitochondrial inner membrane. The proton motive force set up by proton pumping drives protons back through the ATP synthase in the inner membrane, forming ATP from their precursors ADP (adenosine diphosphate) and phosphate. There are two major relevant side reactions related to OS: 1) electrons leak from the respiratory chain react with oxygen to form free radicals; and 2) pumped protons leak back across the inner membrane, diverting the conserved energy away from ATP biosynthesis into heat production. The finding that the percentage of total electron flow directed to free radical generation in mitochondria is not constant in different tissues suggests that ROS generation is not a simple byproduct of mitochondrial respiration. Oxygen radical generation at the respiratory chain has been classically attributed to complex III semiquinone [Boveris 2000]. However, complex I also contain an important ROS generator in intact functional heart and brain

mitochondria [Herrero 1997; Barja 1998; Genova 2001]. Inhibition of complex I causes leakage of ROS from the respiratory chain, which in turn further deactivate the respiratory chain, thus linking mitochondrial damage and OS. Concerning the identity of the ROS generator inside complex I, some studies suggest that the source of ROS might be the complex I FeS clusters [Herrero and Barja 2000]. Because all FeS clusters of complex I are situated in the hydrophilic matrix domain of the complex, ROS arising from them will damage targets situated in the mitochondrial matrix such as mtDNA. In contrast, complex III ROS generation seems to be directed to the cytosolic side [St-Pierre et al., 2002].

One of the most common methods to measure protein oxidation is the detection of protein carbonyls. In studies, investigating the subcellular distribution of protein carbonyls it became clear, that the main ROS generating structures, like mitochondria, are co-localized with the highest amounts of protein carbonyls [Bizzozero 2006]. Furthermore, mitochondria-mediated apoptosis and OS are strictly linked. Thus, mitochondria have increasingly gained popularity in the field of NDDs [Beal 2004].

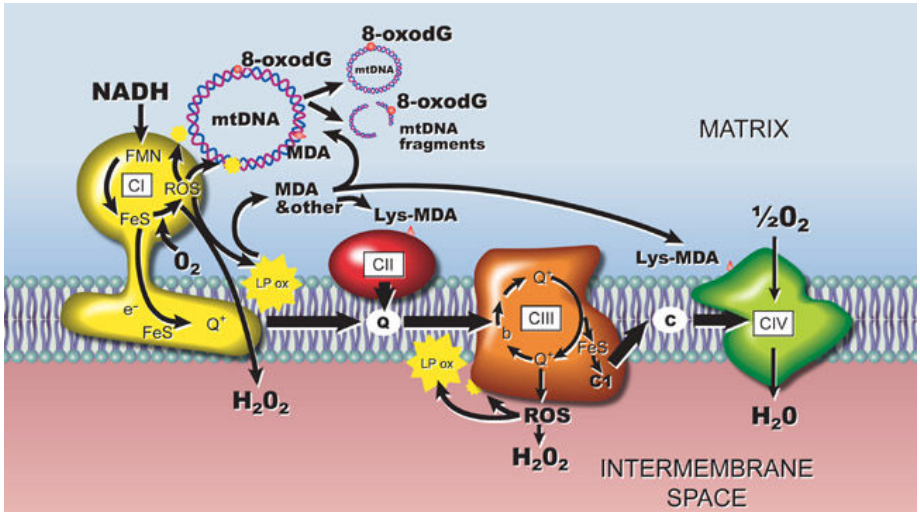


Figure 13. *Production of superoxide by the mitochondrial electron-transport chain* [Hulbwert et al, Physiological reviews (2007) Physiol Rev. 87(4):1175-213]

Mitochondria being one of the main sources of energy providing the ATP in the cell and possessing a significant capacity for continuous free radical production, also play a pivotal role in intermediate metabolism, in maintaining cellular calcium homeostasis, and are central players in the intrinsic pathway of apoptotic cell death. Since, the mitochondria have many fundamental functions in the cell, which are strictly linked with each other, mitochondrial dysfunction may easily shift the delicate balance between life and death causing cell degeneration.

2.2.1. Mitochondrial proteins related to OS

Together with the mitochondrial respiratory chain complexes various mitochondrial proteins are related to the OS as well.

Recently, **Apoptosis-inducing factor (AIF)**, besides its function related to the apoptosis, has been associated with OS. AIF is a ubiquitously expressed 67 kDa flavoprotein with NADH oxidase activity, normally anchored in the mitochondrial intermembrane space or loosely associated with the inner mitochondrial membrane (62 kDa). Upon apoptosis induction, AIF is cleaved and translocates from mitochondria to the nucleus (57 kDa) [Susin 1999; Modjtahedi 2006]. The nuclear translocation of AIF can be inhibited by overexpression of heat-shock protein 70 (HSP70), which can intercept AIF in the cytosol [Ravagnan 2001]. Increased neuronal cell death in response to OS was found in Harlequin mice, where the expression of AIF is reduced to 10–20% of the normal, leading to the speculation that AIF might act as an antioxidant component [Lipton 2002]. AIF absence compromise the composition and function of the respiratory chain, targeting mostly complex I. It was also suggested that AIF is required for the correct assembly and/or for the maintenance of the respiratory chain complex I presumably, because complex I is also constitutive of a I/III/IV 'supercomplex' called respirasome, and AIF is required for neuronal cell survival and normal mitochondrial respiration in neurons.

Other mitochondrial proteins related to the OS are **mitochondrial uncoupling proteins (UCPs)**. UCPs are a proton transporter family that reside in the mitochondrial inner membrane and promote a proton leak across the membrane, thereby decreasing oxidative phosphorylation and ROS production. Five molecules have been identified as members of the UCP family [Andrews 2005]. UCP1 is the classical UCP form in brown adipose tissue. UCP2 is distributed in many tissues including in selected neurones, whereas UCP3 is expressed preferentially in muscle, and UCP4 and UCP5 mRNAs are expressed only in the CNS. UCP2, 4 and 5 can be activated by free radicals and free fatty acids, and their activity has a profound influence on neuronal function. Increased mitochondrial uncoupling through UCP decreases ROS production and consequent OS. The UCP4-mediated shift in energy metabolism reduces ROS production and increases the resistance of neurons to oxidative and mitochondrial stress, thus UCP4 can protect neurons against OS and calcium overload [Liu 2006]. By regulating mitochondrial biogenesis, calcium flux, free radical production and local temperature, neuronal UCPs can also directly influence neurotransmission, synaptic plasticity and neurodegenerative processes. Mitochondrial uncoupling mediated by UCPs allows controlled proton leak back into the mitochondrial matrix, thereby

reducing the membrane potential. In this ways neuronal UCPs might reduce neurodegenerative pathology.

Prohibitins comprise an evolutionary conserved and ubiquitously expressed family of membrane proteins. Various roles in different cellular compartments (mitochondria, nuceus, and plasma membrane) have been proposed for prohibitin proteins. Large assemblies of two homologous prohibitin subunits, PHB1 and PHB2, have been identified in the inner membrane of mitochondria as the physiologically active structure. Mitochondrial prohibitin complexes control cell proliferation, cristae morphogenesis and the functional integrity of mitochondria [Merkwirth 2008]. Its best described function is as a chaperone protein involved in the stabilization of newly synthesized subunits of mitochondrial respiratory enzymes. In the nucleus, it may serve as a modulator of transcriptional activity. In non neuronal cells, prohibitin was suggested to fulfil a cellular defence against OS. Prohibitin deficiency in C.elegans is associated with inhibition of mitochondrial biogenesis and senescence [Theiss 2007].

2.2.2. Mitochondrial biogenesis

Besides their individual characteristics, another level of regulation of mitochondrial function is constituted by mitochondrial number. Among the molecules implicated in the mitochondrial biogenesis (MB) three proteins have been the focus of study in this work as they play key roles in this process: the MB activator **peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α (PGC1α)**, together with one of its upstream regulator **sirtuin 1 (SIRT1)**, and the MB corepressor **RIP140**. PGC1α is a potent stimulator of MB and respiration. PGC1α stimulates MB in concert with the increased expression of nuclear-encoded ETC components, metabolic enzymes, uncoupling proteins, and integrating the action of several transcription factors (Nrf1, Nrf2) [Hood 2006]. PGC1α as a transcriptional coactivator functions through direct physical interaction with transcription factors directly bound to DNA promoter regions. PGC1α orchestrates a program of MB in part by serving as a trans-activator of nuclear respiratory factor 1 (Nrf1) target gene leading to the expression of mitochondrial transcription factor A (TFAM) and mitochondrial fusion proteins (Mfn1 and 2).

Human nuclear-encoded **mitochondrial transcription factor A** (hmtTFA or **TFAM**), a 25-kDa protein encoded by a nuclear gene, is involved in the control of replication and transcription of mtDNA. TFAM is a member of the high mobility group (HMG)-box family of nucleus-encoded factors (TFAM, TFB1M, TFB2M, mTERF), where the HMG-box domain is responsible for DNA binding ability. TFAM is abundant

in mitochondria and contributes to the stabilization and maintenance of the mitochondrial chromosome. Human TFAM is one of the major regulatory factors of MB. In1994 Virbasius and Scarpulla noted that the TFAM contains potential binding sites for Nrf1, Nrf2 within its promoter region and its gene expression is under the direct regulation of Nrf1. They determined that Nrf1 has a more robust effect on TFAM promoter activity than Nrf2, so that activation by Nrf2 required the presence of a functional Nrf1-binding site [Virbasius 1994]. Homozygous TFAM knockout mice exhibit embryonic lethality and depletion of mtDNA, confirming an essential role of that protein in mtDNA maintenance in mammals [Larsson 1998].

Mitochondrial fusion protein Mfn1 and Mfn2 are large GTPases that are localized to the mitochondrial outer membrane and that direct mitochondrial fusion. As individual mitochondria are subject to injury and dysfunction, it is likely that mitochondrial fusion serves as a protective mechanism, by preventing these deficiencies from damaging the entire neuron while maintaining an adequate level of bioenergetic capacity. As single knockout cells have residual fusion activity, either Mfn1 or Mfn2 can support mitochondrial fusion whereas double knockout Mfn1/Mfn2-/- cells lack any detectable fusion *in vivo*. However, cells containing only Mfn2 (Mfn1-/-) appear to have less fusion activity in comparison to Mfn2-/- cells [Hoppins 2007]. It has been shown that in brain tissues Mfn1 expression is predominat and therefore is more related to NDDs [Eura 2003].

Furthermore, **PGC1α** is powerfully induced by ROS and plays a key role in the ROS homeostatic cycle, regulating a cellular antioxidant defence system through activating transcription factor Nrf2. Since the mitochondrial ETC is the main producer of ROS in cell, PGC1α also activates genes that encode enzymes involved in ROS detoxification, including the expression of the genes encoding Cu/Zn-SOD (SOD1), Mn SOD (SOD2), catalase, and glutathione peroxidase (GPx1).

Increasing PGC1α levels dramatically protects neural cells in culture from oxidative stressor-mediated death. PGC1α function in the brain is consistent with a major role in neuroprotection [Kelly 2004]. Moreover, PGC1α has been found in association with the GCN5 acetyl transferase transcriptional complex. GCN5 directly acetylates PGC1α at multiple lysine residues and negatively regulates its transcriptional activity, at least in part, through nuclear sublocalization. To terminate gene expression, GCN5 acetylate PGC1α resulting in relocalization to repressive subnuclear foci where PGC1α has been shown to co-localize with the transcriptional repressor RIP140 [Lerin 2006]. Conversely, SIRT1 activation will maintain PGC1α in a deacetylated active form bound to the chromatin and increasing rates of transcription (Fig. 14) [Gerhart-Hines 2007].

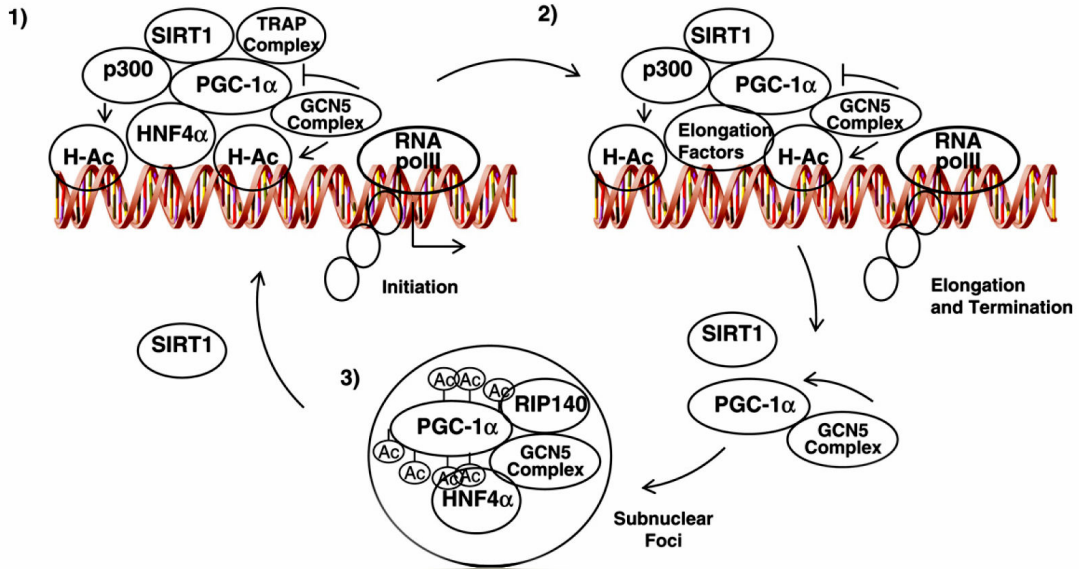


Figure 14: *Model for PGC1α transcriptional gene expression activity. (1) PGC1α is part of multiprotein complexes that contain histone acetyl transferase activity that open and remodels chromatin to allow the transcription factor to bind DNA. In the transcriptional initiation complex SIRT1 would maintain deacetylated PGC1α. (2) PGC1α and its associated proteins will move with RNA processing, elongation factors and RNA polII to transcribe the mRNA. (3) After the mRNA processing, GCN5 would acetylate PGC1α localizing the whole complex to a RIP140 containing subnuclear repressive foci. To initiate another cycle, SIRT1 would deacetylate PGC1α freeing it from the repressive foci, allowing it to become incorporated into protein complexes at promoter regions.* [Rodgers et al., FEBS Letters (2008) 582: 46–53]

Sirtuins have originally been defined as a family of NAD-dependent enzymes that deacetylate Lys residue on various proteins. The mammalian sirtuins **SIRT1**–SIRT7 are implicated in a variety of cellular functions ranging from gene silencing, over the control of the cell cycle and apoptosis, to energy homeostasis [Yamamoto 2007]. OS as well as calorie restriction has been identified to modulate SIRT1 protein levels. The SIRT1 molecular mechanisms of function have been related to regulation of gene expression. In some cases, SIRT1 represses transcription and it is present in histone deacetylases protein complexes. In other cases SIRT1 can act either positive or negative depending on the type of target genes. SIRT1 acts positively on activation of other genes through direct deacetylation of PGC1α in at least 13 lysine residues in different domains of the protein. In this context, PGC1α and SIRT1 protein complex controls ROS formation and are important to maintain survival [Rodgers 2008].

	Localization	Activity	Targets	Biological Function
SIRT1	Nucleus (nuclei)	Deacetylase	PGC-1α, FOXOs, NFκB	Metabolism/inflammation/ neurodegeneration
SIRT2	Cytoplasm	Deacetylase	H4, α-tubulin	Cell cycle/tumorigenesis
SIRT3	Nucleus and mitochondria	Deacetylase	AceCS2	Metabolism
SIRT4	Mitochondria (matrix)	ADP-ribosyl transferase	GDH	Insulin secretion
SIRT5	Mitochondria	Deacetylase	Unknown	Unknown
SIRT6	Nucleus (heterochromatic region)	ADP-ribosyl transferase	DNA polymerase β	DNA repair
SIRT7	Nucleus (nucleoli)	Unknown	RNA polymerase I	rDNA transcription

Table 5: *Main characteristic of mammalian Sirtuins* [modified from Yamamoto et al., Molecular Endocrinology (2007) 21(8): 1745–1755]

RIP140, via its interplay with other coregulators, plays a fundamental role in determining both the normal and pathogenic physiological state of mitochondrial number. Several studies have demonstrated that RIP140 can repress the activity of many, if not all nuclear receptors [Treuter 1998]. RIP140 seems to function primarily as a scaffold protein that links nuclear receptors to chromatin remodelling enzymes involved in chromatin condensation and transcriptional repression. The repressive function of RIP140 can be modulated by post-translational modifications. RIP140 null mice are viable indicating that its expression is not essential for development, however, a variety of different phenotypic changes occur in specific tissues that result in major physiological consequences. Analysis of genes ascribed to specific metabolic pathways demonstrated that 33% of genes were upregulated and only 4% were downregulated in the absence of RIP140, consistent with the function of RIP140 as a corepressor [White 2008]. Genes downregulated in the absence of RIP140 were involved in anabolic pathways, in particular fatty acid (FA) and triglyceride synthesis. The vast majority of upregulated genes were involved in catabolic pathways including FA oxidation, oxidative phosphorylation, and glycolysis. In the absence of RIP140 there is also increased expression of genes involved in MB and activity (for example the gene coding for the mitochondrial uncoupler UCP) [Ström 2008]. These changes also are accompanied by increased mitochondrial number, suggesting that RIP140 functions to repress oxidative metabolism and mitochondrial function. The alternative roles for RIP140 and PGC1α in the regulation of expression of specific genes together with the identification of similar nuclear receptors as potential targets suggests an

important functional interplay between these coregulators (Fig. 14). Therefore, it is probable that via their ability to repress or activate anabolic and catabolic functions, may act as important elements in the disease progression [Christian 2005].

Despite all these data, it remains to be described whether those processes are impaired or involved in NDD, especially in those diseases where mitochondrial changes have been observed.

2.3. Protein oxidation

Once formed, either from mitochonria or from other sources ROS are known to oxidize all sorts of macromolecules, being proteins an important target. This is because proteins are major components of biological systems with high functional relevance and the age-related increase in oxidative damage to proteins could be important. Oxidative modifications of proteins have been suggested to play a key role in the causation of age-associated losses in physiological functions. Most of the oxidative modifications to proteins are irreversible, i.e., the only way known to repair the protein is through its turnover. However, some oxidative modifications such as methione sulfoxide and disulfide formations are reversible.

Proteins can be oxidized in different ways. Oxidation can occur at both the protein backbone and on the amino acid sidechains. The products of oxidation of amino acids (oxidized amino acids, modified amino acids and cross-links formed by a combination of enzymatic and nonenzymatic mechanisms) are indicators for modification of proteins in biological systems [Davies 1997; Dean 1997; Stadtman 1998; Hawkins 2001]. The hydroxyl radical-dependent abstraction of a hydrogen atom from the α-carbon of amino acids, from the protein polypeptide backbone and also from the aliphatic side chains of hydrophobic amino acid residues of proteins are initial sites of attack [Dean 1997].

Oxidative protein damage arises from direct exposure to reactive oxygen, chlorine, or nitrogen species generating oxidative products such as glutamic (GSA) and aminoadipic (AASA) semialdehydes. Protein oxidative modifications may also arise from reaction with low molecular weight reactive carbonyl compounds derived from amino acids, carbohydrates or polyunsaturated fatty acids (PUFA) such as glyoxal (GO), glycolaldehyde, methylglyoxal (MGO), malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), among other. These carbonyl compounds could react primarily with lysine, arginine and cysteine residues, leading to formation of Advanced Glycation/Lipoxidation Endproducts (AGE/ALE) in protein. Because the generation of carbonyl derivatives occurs by many different mechanisms, the level of carbonyl groups in proteins is widely used as a marker of oxidative protein damage. It was

reported that GSA and AASA constitute the majority of protein carbonyls generated by metal catalyzed oxidation (MCO) [Requena 2001]. Proteins modified indirectly by reactive carbonyl compounds formed during the autooxidation of PUFA, lead to the generation of advanced Maillard products like N^{ε}-(malondialdehyde)lysine (MDA-lys) and N^{ε}-(carboxymethyl)lysine (CML).

OS and ROS catalyzed the non-enzymatic chemical modification of proteins by Maillard reactions *in vivo* [Baynes 1991]. Reactive carbonyl species (RCS) formed on oxidation of carbohydrates, lipids and amino acids were identified as intermediates in the formation of irreversible, AGE/ALEs in protein.

The first stage in the classical Maillard reaction is the formation of a Schiff base and Amadori adducts between reducing sugars and free amino groups in protein [Baynes and Thorpe 1999] (Fig. 15). The Amadori adduct formed from glucose *in vivo* undergoes non-oxidative rearrangement and hydrolysis reactions, releasing 1- and 3-deoxyglucosones (1DG, 3DG) preserving the carbon skeleton of the sugar. The Schiff base and Amadori adduct also undergo facile oxidation, especially in the presence of transition metal ions, and fragment to yield shorter chain sugars and reactive intermediates, such as GO, MGO and MDA [Nagai 2002]. It should be recalled that most of MGO *in vivo* may arise from the spontatneous formation from dihydroxyacetone-phosphate or glyceraldehydes-3-phosphate. A protein-bound dicarbonyl compounds, described as intermediates formed during the second stage of the Maillard reaction, react with lysine and arginine (and other aminoacids) residues in protein to produce a wide range of protein-bound AGEs and cross-links during the third and final stage of the classical scheme of the reaction.

There are multiple pathways for formation of AGEs from reducing sugars (Fig. 15): some proceed from the Amadori compound [Hodge, 1953], while others proceed from the Schiff base [Hayashi and Namiki 1986] or by direct autooxidation of carbohydrates (autooxidative glycosylation) [Wolff and Dean 1987]. Some AGEs, such as the fluorescent vesperlysines and crosslines, retain the intact carbon structure of glucose and thus they appear to be directly derived from glucose. In contrast, formation of pentosidine, another AGE from glucose requires oxidative cleavage and loss of one carbon atom. Other AGEs, such as CML and CEL, require oxidative fragmentation of the carbon skeleton of glucose, but may also be formed from other hexoses, pentoses, glycolytic intermediates or ascorbic acid. The term, glycoxidation product [Baynes, 1991], was originally introduced to characterize products formed by sequential glycation and oxidation reactions.

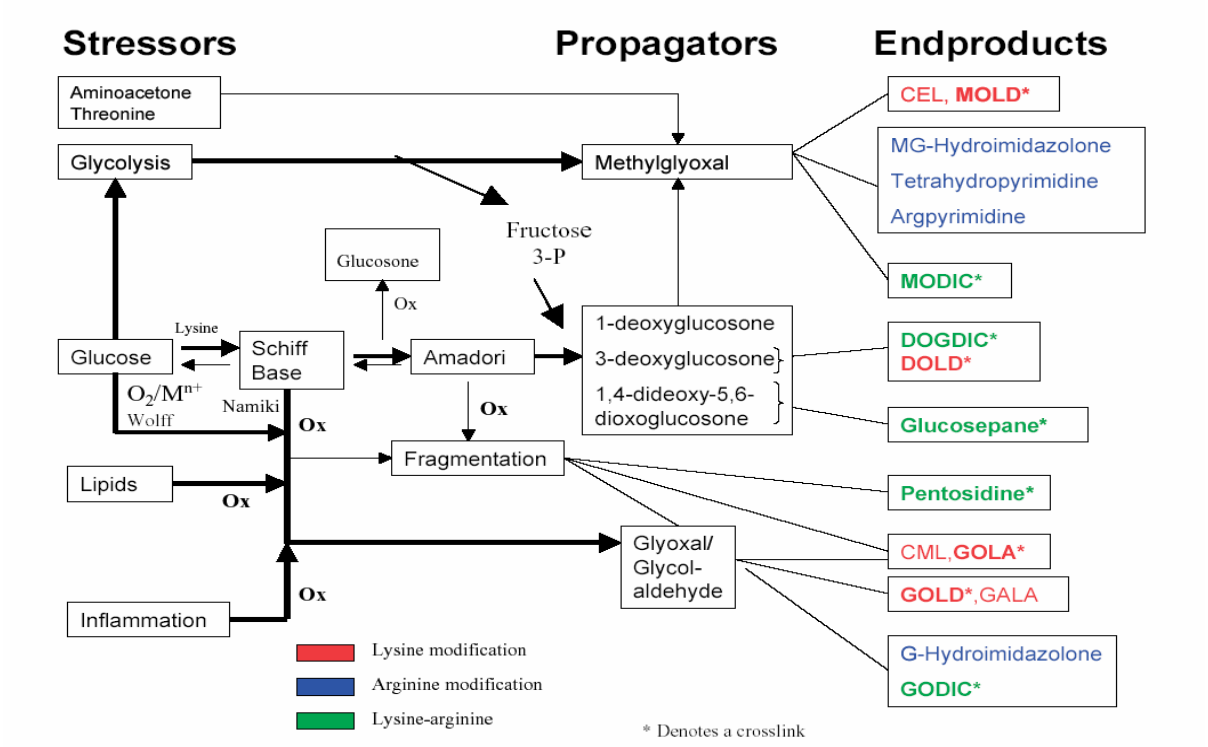


Figure 15: *Selected chemical pathways in Maillard reaction in vivo* [Monnier 2003].

However, some glucose-derived glycoxidation products may be derived from other precursors by non-oxidative routes, e.g. the formation of CEL from triose phosphates or MGO, which are products of anaerobic metabolism [Ahmed 1997]. CML may also be formed from a variety of non-carbohydrate sources, including lipid [Fu 1996] and amino acid [Anderson 1999] oxidation products. When CML is formed from lipids, it is described as an advanced lipoxidation end-product. In those cases where its origin is uncertain, CML is best described as AGE-ALEs. Thus, there is increasing evidence that lipids are as important as carbohydrates in the chemical modification of tissue proteins and development of pathologies [Baynes and Thorpe 1999]. The formation of AGEs, like the metabolism of sugars, may be non-oxidative or oxidative, while the formation of ALEs, like the metabolism of lipids, requires oxidative chemistry to form intermediates.

The nonenzymatic protein modifications are spontaneous, random, unprogrammed and uncatalyzed chemical reactions. All proteins can be the target of free radicals and RCS. For extracellular proteins, nonenzymatic protein modifications increase with aging. For intracellular proteins, the evidence is quite equivocal, and no such conclusion can be drawn.

2.4. Lipid peroxidation

Lipid peroxidation is an autocatalytic process in which free radicals attack double bonds in membrane lipids, resulting in structural damage to membranes and the liberation of toxic aldehydes such as 4-HNE, MDA and others. In addition to ROS production, another constitutive characteristic of this process is the degree of FA unsaturation of tissue cellular membranes. Unsaturated fatty acids (UFAs) are extremely sensitive to oxidation, their sensitivity to oxidation exponentially increasing as a function of the number of double bonds per FA molecule [Bielski 1983]. PUFAs are the cellular macromolecules most sensitive to ROS, making lipid peroxidation a major oxidative process in tissues affected by OS. The CNS is particularly vulnerable to lipid peroxidative damage because it has high energy requirements, high concentrations of PUFA, high oxygen consumption rates, and a relative deficit of antioxidant defence systems compared with other organs [Floyd 1999]. However, lipid peroxidation not only damages the lipids, because its final products like malondialdehyde (MDA), as well as secondary radicals generated during the process, can also alter tissue proteins and DNA. Oxidative decomposition of PUFAs initiates chain reactions that lead to the formation of specific ALEs such as MDA-Lys, HNE-Lys, and Nε-(hexanoyl)-lysine, among others.

MDA is the major oxidative breakdown product of PUFA. Its product with Lys (MDAL) is more sensitive to changes in FA unsaturation than other protein markers. MDAL is frequently determinate like indicator of lipid peroxidation and OS *in vivo*. Some studies show that levels of MDAL in proteins increase during aging in rat tissue [Lambert 2004] and suggest that the increase of MDAL content may be associated with protein properties including increased aggregation, sensitivity to degradation and secondary ROS generation.

Neuroketals (NKTLs) are isoprostanes, a class of prostaglandin-like compounds produced by free radical-induced peroxidation of docosahexaenoic acid (DHA) and arachidonic acid (ARA), which are highly enriched in the brain [Roberts 1998; Nourooz-Zadeh 1998]. NKTLs were found to be formed in abundance *in vitro* during oxidation of docosahexaenoic acid, and were shown to rapidly adduct to lysine, forming Schiff base adducts. Because DHA is highly concentrated in nervous system tissue these compounds were termed neuroprostanes. The fact that DHA is prone to free radical attack and free radicals have been implicated in a number of NDDs makes NKTLs a unique and prominent marker of oxidative injury in the brain.

As introduced above lipid peroxidation increases exponentially as a function of the number of FA double bonds. It was shown that protein oxidative modifications increase as a function of the degree of unsaturation of the PUFA present in the samples [Refsgaard 2000]. Conversely, a low degree of FA unsaturation decreases the amount of

peroxidizable lipid substrate. This low rate of lipid peroxidation should protect not only lipids, but also proteins from lipoxidation-dependent processes. Furthermore, MDAL levels were positively correlated with the double bond content across species.

Combining the susceptibilty of different FAs with the composition of the FA in the lipid membrane it is possible to calculate the so called “Peroxidizability Index (PI)”. The PI of one membrane, the susceptibilty of the lipids that contain to be oxidized, differs from the “index of of saturation” also called “double bond index (DBI)”, which is a measurement of the density of double bonds in the membrane. The calculation of the PI and the DBI requires previous analysis of the FA composition of the determinante membrane: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids from n-3 and n-6 series (PUFAn-3 and PUFAn-6). To characterise the lipid peroxidation of one membrane the following fatty acyl indices can be calculated:

Average chain length (ACL) = [(Σ%Total14 x 14) + (Σ%Total16 x 16) + (Σ%Total18 x 18) + (Σ%Total20 x 20) + (Σ%Total22 x 22)]/100;

Double bond index (DBI) = [(1 x Σmol% monoenoic) + (2 x Σmol% dienoic) + (3 x Σmol% trienoic) + (4 x Σmol% tetraenoic) + (5 x Σmol% pentaenoic) + (6 x Σmol% hexaenoic);

and Peroxidizability index (PI) = [(0.025 x Σmol% monoenoic) + (1 x Σmol% dienoic) + (2 x Σmol% trienoic) + (4 x Σmol% tetraenoic) + (6 x Σmol% pentaenoic) + (8 x Σmol% hexaenoic)].

2.5. DNA oxidative damage

Oxidative damage to DNA is a result of interaction of DNA with ROS, in particular the hydroxyl radical. Superoxide and hydrogen peroxide are normally not reactive towards DNA. However, in the presence of ferrous or cuprous ion (the Fenton reaction), both superoxide and hydrogen peroxide are converted to the highly reactive hydroxyl radical. Hydroxyl radical produces a multiplicity of modifications in DNA. Oxidative attack by OH radical on the deoxyribose moiety lead to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple abasic (AP) sites. One of the major types of damage generated by ROS is AP site, a site where a DNA base is lost.

AP sites are also formed from spontaneous depurination. Under physiological conditions at least 10,000 depurination events occur per cell per day. A similar amount of AP site is thought to be generated by normal aerobic respiration. In addition to AP site, a wide spectrum of oxidative base modification occurs with ROS. The C4-C5 double bond of pyrimidine is particularly sensitive to attack by OH radical, generating a

spectrum of oxidative pyrimidine damage including thymine glycol, uracil glycol, urea residue, 5-OHdU, 5-OHdC, and others. Similarly, interaction of OH radical with purines will generate 8-OHdG, 8-OHdA, formamidopyrimidines and other less characterized purine oxidative products. It has been estimated that endogenous ROS can result in about 200,000 base lesions per cell per day. The biological consequences of many of the oxidative products are known. Unrepaired thymine glycol is a block to DNA replication and is thus potentially lethal to cells. 8-oxoG, an abundant oxidative damage to dG, is readily bypassed by the DNA polymerase and is highly mutagenic. Unrepaired 8-oxoG mispair with dA, lead to an increase in G to T transition mutations [Garinis 2008].

The physical proximity of the mtDNA to the sites of ROS production in the inner mitochondrial membrane renders the mtDNA more vulnerable to being damaged by such species in comparison to nuclear DNA. Several groups have demonstrated that the levels of oxidized bases in mtDNA are 2–3 times greater than in nuclear DNA [de Souza-Pintoa 2008].

All organisms have evolved many different repair pathways to remove various types of DNA damage, resulting from either endogenous or external DNA reactive agents. Sometimes changes in DNA repair activities may play a role in premature aging and age-associated NDDs.

2.6. Antioxidant regulatory mechanisms of oxidative stress

2.6.1. Nrf1 and Nrf2 positive regulators of the cellular antioxidant capacity

The cellular response to ROS can be divided into two categories: basal and inducible regulation. Basal regulation refers to the ‘housekeeping’ response to ROS produced as metabolic by-products. In contrast, inducible regulation occurs during high stress conditions or under conditions in which basal regulation fails. A similar complement of genes is induced by varying oxidative insults, and in many cases, these genes contain cis-acting DNA sequences known as antioxidant response elements (AREs) (5’ (G/A)TGA(G/C)nnnCG(G/A) 3’) in their promoter regions. These genes encode proteins that function as antioxidants and enzymes involved in glutathione biosynthesis [Cullinan 2006]. Transcriptional activation through the ARE involves a family of transcription factors, the Nrfs, members of the CNC (Cap ‘n’ Collar) family of basic leucine zipper (bZip) including nuclear respiratory factor-1 to 3 (Nrf1-3) [Andrews 1993]. Nrf1-3 transcription factors that reside normally in the cytoplasm of the cells are ubiquitously expressed and respond to OS within cells (Fig. 16), besides their function in mitochondrial biogenesis.

Nrf1 acts on nuclear genes encoding respiratory subunits and components of the mitochondrial transcription and replication machinery. Analysis of Nrf1-deficient fibroblasts suggests that it is also involved in the OS response. Nrf1 has been shown to activate expression of both the catalytic and regulatory subunits of glutamyl-cysteine ligase genes, the first rate limiting enzyme of glutathione synthesis, through AREs in the promoters of these genes [Wang 2006]. Colocalization analysis indicates that Nrf1 is primarily a membrane bound protein localized in the ER. Deletion of the membrane targeting domain of the Nrf1 protein resulted in a predominantly nuclear localization of Nrf1 that significantly increased the activation of reporter gene expression. It have been suggested that ER stress may play a role in modulating Nrf1 function as a transcriptional activator [Wang 2006].

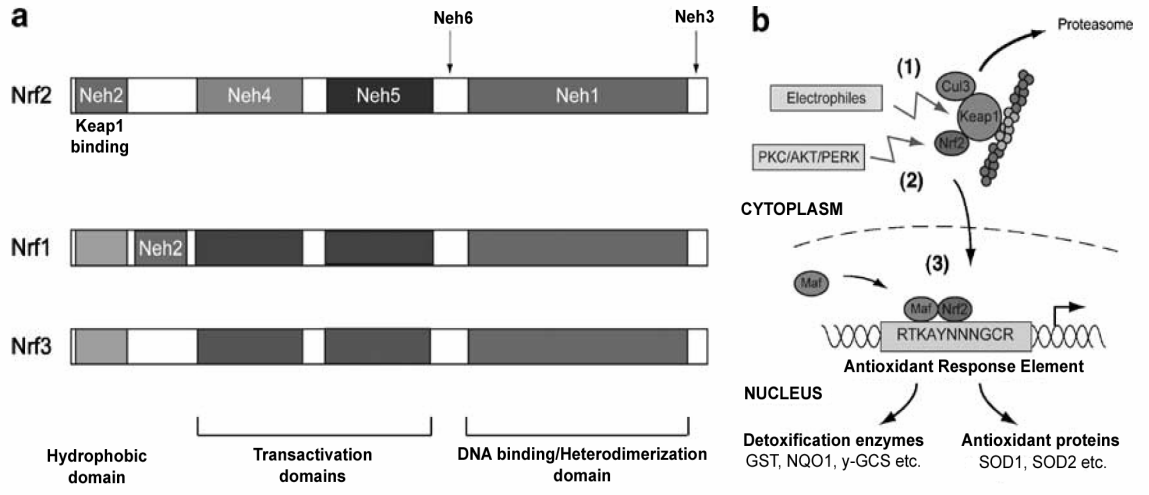


Figure 16: *(a) Domain composition of Nrf transcription factors. Neh1 comprises the CNC/bZip domain and the nuclear localization signal and is conserved in all three family members. It is responsible for protein–protein interaction and DNA binding. All Nrf transcription factors have two independent transactivation domains, conserved to a certain extent. The Neh2 domain is present in Nrf1 and Nrf2, and it is responsible for the cytoplasmic retention of Nrf2 and probably of Nrf1 by Keap1. (b) Schematic representation of the Nrf2/Keap1 system. In response to electrophiles, which directly react with Keap1 (1), or upon phosphorylation of Nrf2 by PKC, Akt kinase or PERK (2), Nrf2 is stabilized, liberated from Keap1 and translocates to the nucleus (3). Upon heterodimerization with a small Maf protein, Nrf2 binds to AREs in the promoters of its target genes, which encode – among others – ROS-detoxifying enzymes and antioxidant proteins* [Beyer et al., Cell Death and Differentiation (2007) 14: 1250–1254]

Controlling intracellular localization of transcription factors is an important mechanism for regulating gene expression. Nrf2 activity is regulated by Keap1-mediated compartmentalization in the cell. The Keap1-Nrf2 system is the major regulatory pathway of cytoprotective gene expression against OS. Under unstressed conditions Nrf2 is maintained as a latent cytoplasmic transcription factor through binding Keap1 and thus constitutively suppresses Nrf2 activity [Jaiswal 2004]. Upon exposure to OS oxidants provoke the repression of Keap1 activity, inducing the Nrf2

activation. Nrf2 escapes Keap1-mediated repression and translocates to the nucleus where it binds to the ARE in gene promoters and activates transcription of target genes. Nrf2 has been reported to constitute a key regulatory factor in the coordinate induction of endogenous cytoprotective genes, including those encoding for antioxidant proteins such as SOD1, SOD2 and enzymes involved in glutathione biosynthesis. Nrf2 also promotes the expression of several components of the proteasome as well as several genes involved in protein folding perhaps in an effort to decrease the amount of misfolded and/or aggregated proteins within the cell. In addition to genes encoding antioxidants, Nrf2 promotes the expression of genes implicated in cell growth, protein folding and cell survival [Kwak 2003; Cullinan 2006]. ARE-containing, Nrf2 target genes, including enzymes involved in glutathione biosynthesis and chemical detoxification, are induced during the UPR as well, suggesting that Nrf2 activation during ER stress conditions will be equivalent to activation during OS. In resume, OS-induced, Nrf2- dependent genes fall into several categories: antioxidants and detoxification enzymes, immune signalling, protein trafficking, protein degradation, cell growth and survival and the chaperone system. Nrf2 has been shown to be the strongest trans-activator among the CNC factors. However, the precise molecular mechanisms by which OS promotes the liberation of Nrf2 from Keap1 repression remain to be elucidated. One model suggests that Nrf2 is phosphorylated, following oxidative insult and that this modification disrupts Nrf2/Keap1-binding. Another model posits that Keap1 acts as a stress sensor protein in this system. It has been suggested that Nrf2 activation may precede oxidative damage, indicating that yet a different mechanism may contribute to Nrf2 activation. Other studies have also shown that Keap1 deficiency results in higher levels of Nrf2 protein, suggesting that Keap1 plays a role in Nrf2 degradation. Nrf2 is a short-lived protein, Keap1, in conjunction with Cullin3, directs Nrf2 poly-ubiquitination in unstressed cells and promotes the degradation of Nrf2 through ubiquitin-proteosome-mediated pathway [Cullinan 2004; Kobayashi 2002]. While the mechanism of Nrf2/Keap1 complex dissolution remains unresolved, Keap1 is the crucial mediator of Nrf2 activity and the protein complex an important regulator of cellular redox status.

While Nrf1 deficiency results in embryonic lethality, Nrf2 deficient (Nrf2 −/−) mice develop normally with basal expression of most ARE-containing genes remaining similar to that of wild type littermates. The loss of Nrf2, however, completely ablates the induction of Nrf2 target genes, rendering Nrf2−/− mice susceptible to high levels of OS with a modest decrease in life expectancy, resulting from improper ROS detoxification [Chan 1996, 1998]. The simultaneous loss of Nrf1 and Nrf2 results in early embryonic lethality [Leung 2003]. Expression of ARE-bearing genes is severely

impaired in fibroblasts deficient in Nrf1 and Nrf2, resulting in marked sensitivity to OS. These studies indicate that Nrf1 have overlapping functions with Nrf2 during development and in mediating ARE function in the OS response. Although the structural similarities between Nrf1 and Nrf2, it is not fully investigated whether Nrf1 activity is regulated similarly to Nrf2.

2.6.2. Antioxidants

Since cells continuously produce free radicals, their OS homoeostasis can only be maintained if endogenous cellular antioxidants are present. A large battery of antioxidant defences, both enzymatic and nonenzymatic, have been selected and conserved during the evolution. Superoxide dismutase (SOD) eliminates superoxide radical converting it to oxygen and H_2O_2. There are three different forms of this enzyme: a Cu/Zn form in the cytosol and in the intermembrane mitochondrial compartment; a Mn form in the mitochondrial matrix; and another form in the extracellular compartment. The mitochondrial enzyme is essential for life, its decline leading to mitochondrial dysfunction, pathology, or neonatal lethality depending on the level of depletion. Although SOD eliminates superoxide radical, could not be considered strictly as an antioxidant because, it produces another source of ROS, H_2O_2. Two main kinds of enzymes eliminate the H_2O_2 produced by SOD. Catalase decomposes H_2O_2 at high rates but shows low affinity for the peroxide and should be most useful during peaks of H_2O_2 production or accumulation [Goth 2000]. Glutathione peroxidases (GSH-Px), present in selenium- and nonselenium-dependent forms, are complementary to catalase, since they decompose H_2O_2 slowly but with higher affinity. Thus, they are most useful to decompose the small amounts of peroxide continuously produced inside cells. These enzymes use the reduced form of glutathione (GSH) to decompose the peroxide. In this process is generated oxidized glutathione (GSSG), which is highly toxic to cells. GSSG is then reduced back to GSH by another antioxidant enzyme-glutathione reductase (GSH-Red).

In addition to antioxidant enzymes, various types of endogenous non-enzymatic antioxidants are present in cells and tissues. Their low molecular weight can also be an advantage to eliminate ROS at sites not accessible to the much larger enzymes. The main low molecular weight hydrophilic non-enzymatic endogenous antioxidants are glutathione and ascorbate. The tripeptide glutathione is particularly abundant in many tissues [Sies 1999]. Its antioxidant activity is due to the reduced thiol group of its cysteine residue. Glutathione plays a central role in maintaining cellular redox balance [Cnubben 2001] since GSSG/GSH is the most abundant redox couple in the cell. Changes in GSSG/GSH ratio appear to influence the entrance to

different cellular states including proliferation, differentiation or apoptosis [Schafer & Buettner 2001]. Ascorbate is usually the other most abundant reduced non-enzymatic antioxidant in cells. Although for humans, other primates, guinea pigs and fruit-eating bats it is a vitamin (vitamin C), in most mammals and vertebrates it is endogenously synthesized and maintained at high levels in tissues. It reacts with ROS and thus is converted to oxidized forms which can be reduced back again by NADPH-dependent [Rose 1993] or GSH-dependent [Maellaro 1994; Wells 1994] dehydroascorbate reductases or by a NADH-dependent plasma membrane ascorbate free radical reductase [Navas 1994]. *In vivo* GSH and ascorbate interact cooperatively [Meister 1994].

There are also non-enzymatic antioxidants functioning optimally in the lipophilic membrane environment. Two important ones are tocopherols and carotenoids [Debier 2005]. Vitamin E is the main exogenous lipophilic antioxidant in animal cells, which antioxidant activity is due to the reducing capacity of the hydroxyl group of its chromanol ring. Its lipid solubility allows its direct access to lipid peroxyl groups, reducing them to hydroperoxides, thus inhibiting the propagation of lipid peroxidation in a chain reaction [Esterbauer 1991]. Vitamin E can also reduce lipid alkoxyl radicals to lipid alcohols. In both cases, vitamin E is oxidized to tocopheryl radicals, which must be recycled back to vitamin E, since the membrane tocopherol pool is small. This could possibly occur at the membrane cytosol interface due to reduction of tocopheryl radicals by ascorbate, which would explain the synergistic effects of the two vitamins. An alternative is the reduction of vitamin E radical back to vitamin E by ubiquinone [Landi 2001]. Ubiquinones (coenzyme Q) antioxidant activity is due to either direct scavenging of lipid radicals or indirectly to reduction of vitamin E radical. The antioxidant potency of coenzyme Q is also dependent on the regeneration of its reduced form by enzymes like NAD(P)H:(quinone-acceptor) oxidoreductase (DTdiaphorase) in the cytosolic compartment, or possibly NADH-coenzyme Q reductase in plasma membranes [Landi 2001]. Hundreds of different carotenoids have been described, although only some of them, like α- and β-carotene, lutein, licopene, zeaxanthine or criptoxanthine, are present at relevant concentrations in animal tissues and plasma [Krinski 1993]. Their antioxidant capacity can be related to their antiinflammatory [Schweigert 2001], antimutagenic and anticarcinogenic activity, although at high oxygen tensions like those present in the lungs paradoxical may occur procarcinogenic effects.

3. Protein turnover mechanisms

Mammalian cells possess several major pathways for general protein degradation including lysosomal proteases (autophagy), calcium-dependent proteases, the proteasomal system, and the mitochondrial Lon protease. Proteins that enter cells from the outside, as well as several intracellular proteins (especially long-lived ones or proteins from various organelles), are degraded within lysosomes solely in the cytosol. Soluble intracellular cytoplasmic and nuclear proteins are degraded by the intracellular proteasomal system in different compartment of the cell [Grune 2004].

3.1. Ubiquitin-proteasome system (UPS)

As described in the prior section, the formation of oxidized proteins is one of the highlights of OS. In order to avoid the accumulation of such proteins mammalian cells have developed potent degradation systems, which selectively degrade damaged and misfolded proteins. The major proteolytic system responsible for the removal of oxidized proteins is the proteasome. The proteasome is distributed throughout the cytosolic and nuclear compartment of mammalian cells as well as attached to the endoplasmic reticulum [Rivett 1998]. The eukaryotic proteasomal system consists of the so called 20S “core” proteasome and several regulatory components like the 11S and the 19S, which can bind to the 20S proteasome and modify the activity and the selectivity of the degradation process [Voss 2006]. The 20S proteasome is composed of 28 subunits arranged in four stacked rings that form a hollow barrel. Each ring itself consists out of seven subunits, the outer rings contain seven homologous α-, and the inner rings seven homologous β-subunits. Unfolded protein substrates enter the cylinder via the opening of the outer rings, and are degraded in the inner cavity of the cylindrical particle where are located the active centres [Coux 1996]. Binding of 11S or 19S regulators, on one or both sides of the core proteasome, lead to the formation of various proteasomal complexes. Combination of the proteasomal core with two 11S regulators leads to an enhanced peptidase activity making the degradation of peptides by the proteasome more efficient. The 26S proteasome consists of the 20S core with a 19S regulator on each side. It is mainly responsible for the recognition and ATP dependent degradation of polyubiquitinated proteins.

3.1.1. Ubiquitin and protein ubiquitination

In order to ensure specific recognition of the protein substrate the complex process of protein polyubiquitination involves ubiquitin and three enzymes (E1, E2 and E3). Ubiquitination is a cellular process by which short lived or damaged proteins are

conjugated with multimers of ubiquitin, marking them for degradation in the proteosome [Voss 2007]. Ubiquitin is an evolutionarily highly conserved 76 amino acid polypeptide that is abundant in all eukaryotic cells. Its main function is to mark other proteins for proteolysis. Ubiquitin can also mark transmembrane proteins (receptors) for removal from membranes and fulfill several signalling roles within the cell. At least four ubiquitin molecules attach to a lysine residue on the condemned protein, in a process called polyubiquitination, and the protein then moves to a proteasome. If the polyubiquitin chain is longer than 3 ubiquitin molecules, the tagged protein is rapidly degraded by the 26S-proteasome [Widmer 2006]. The ubiquitination process consists of a series of steps (Fig. 17):

1. The initial step is activation of ubiquitin. This step is ATP-dependent and involves the linkage of ubiquitin to a ubiquitin-activating enzyme E1, in a high energy thioester bond.

2. Ubiquitin is then transferred in a second thioester linkage to an ubiquitin conjugating enzyme (Ubc), or E2, which in turn catalyzes the transfer of ubiquitin to the substrate protein in a covalent bond.

3. The final step of the ubiquitination cascade generally requires the activity of an E3 ubiquitin-protein ligase. E3 enzymes function as the substrate recognition modules of the system and are capable of interaction with both E2 and substrate. Both E2 and E3 proteins exist as large families and it is thought that different combinations of E2s with different E3 proteins define the substrate specificity [Grune 1997].

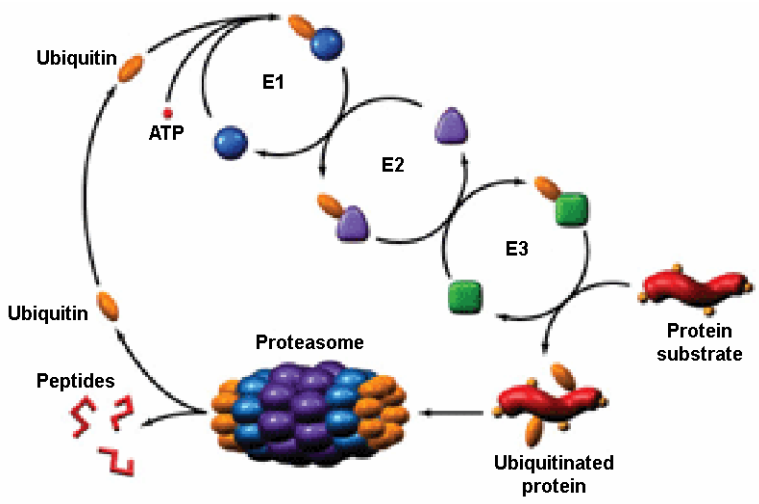


Figure 17: *The Ubiquitin-proteasome system: polyubiquitination of the substrate and its degradation in the 20S catalitic core of the 26S proteasome*

Finally, the marked protein is digested in the 26S-proteasome into small peptides. Ubiquitin moieties are cleaved off the protein by proteasome deubiquitinating enzymes and are recycled for further use [Jung 2007; Grune 2004].

3.1.2. Oxidative stress, proteasome and degradation of oxidatively modified proteins

Numerous studies have demonstrated the key role of the proteasomal system in the degradation of oxidized proteins. For most of the oxidative damaged proteins the rapid degradation is the only way to remove these proteins from the intracellular environment (Fig. 18). Formation of such oxidized amino acids within a protein requires degradation of the protein. Since one of the main functions of the proteasome is the removal of oxidatively damaged proteins [Pacifici 1989; Davies 2001; Shringarpure 2001; Mehlhase 2002], the proteasomal activity is regulated by OS. It has been shown that the proteasomal degradation rises due to mild oxidation of the substrate, whereas a stronger oxidative damage leads to the decrease of the proteasomal activity [Grune 1997; 2000]. A clear decline of 26S proteasome activity and other components of the ubiquitination system have been reported to occur after OS. No similar decline in the activity of the 20S proteasome after moderate OS has been detected [Grune 1997, 2000]. Furthermore, cells with a non-functional ubiquitination cascade are still able to remove oxidized proteins efficiently through 20S.

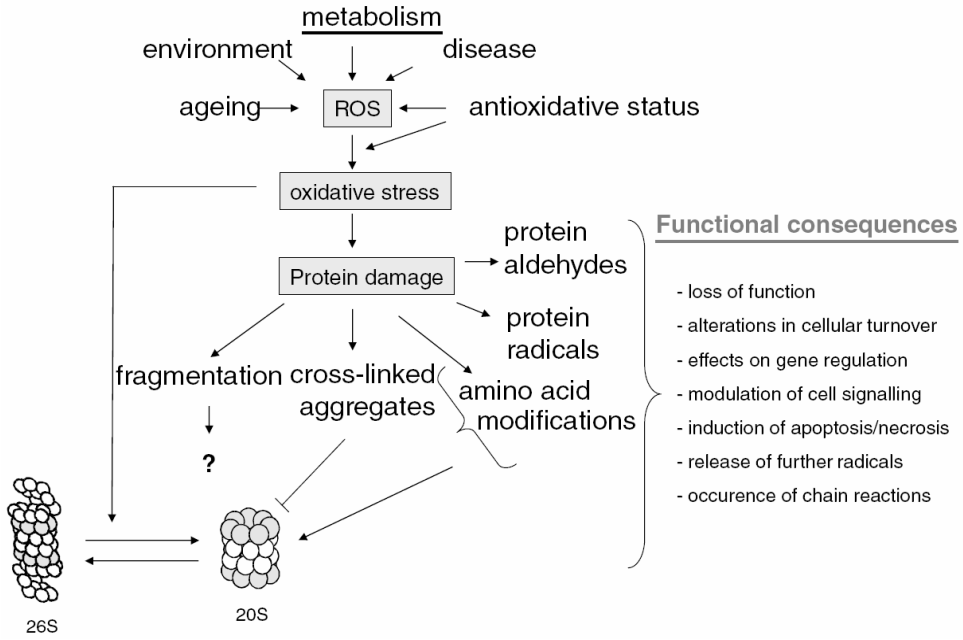


Figure 18: *Interaction of intracellular protein degradation and the removal of oxidized proteins in the cytosol and the nucleus of mammalian cells.* [Bader et al., Experimental Gerontology (2007) 42: 864–870]

The first step of a proteolytic process is the recognition of the substrate. In the case of oxidized and misfolded proteins, they expose more hydrophobic amino acid residues to the surface than native proteins do, these hydrophobic patches serve as first recognition-sites for the 20S proteasome [Pacifici 1993]. Damaged proteins are at least partially unfolded and the polypeptide chain enters the proteasome through the narrow opening of one α-ring. The two outer α-rings are responsible for regulator binding, whereas the inner β-subunit rings contain the proteolytically active centres of the proteasome. Three of the β-subunits in each ring bare the different catalytic activities so that the six catalytic active centers are hidden in a chamber inside the barrel between the two β-rings [Stadtman 1990]. Within the catalytic chamber all polypeptide chains are cut by the active centres (with three main peptidase activities: chymotrypsin-like, trypsin-like and peptidylglutamyl peptide hydrolyzing activities) into smaller polypeptides, which leave the protease through the opening of the opposite α-ring [Voss 2007]. In contrast to the 20S proteasome, which degrades proteins in an ATP and ubiquitin independent manner the 26S protease processes predominantly proteins that are marked for degradation by polyubiquitination [Braun 1999]. The resulting oligopeptides are further hydrolyzed by several intracellular peptidases to single amino acids.

The highest amount of oxidized proteins and proteasome in relation to the protein ratio is found near the cell membrane. Even if the relative proteasome amount is high in the area, the load of oxidized proteins per proteasome is still highest there. Therefore, it was concluded that the most active degradation of oxidized proteins is going on the periphery of the cell [Jung 2006], and that the proteasomal degradation of oxidized proteins is largely taking place in the cytosol, the area of the highest formation of oxidized proteins. On the other hand the nucleus is well protected from the formation of oxidized proteins and their accumulation, partially due to the high content of the proteasome there [Bader 2006; Grune 2003].

3.2. Autophagy

Autophagy (from Greek ‘self eating’) or lysosomal degradation is another major intracellular pathway for general protein degradation and recycling of long-lived proteins and cytoplasmic organelles. Defective autophagy is the underlying cause of a number of pathological conditions, including NDDs [Cuervo 2004; Cherra 2008]. Autophagy is mediated by formation of a large double membrane bound autophagic vacuole called the autophagosome. As autophagosomes engulf a portion of cytoplasm, autophagy is generally thought to be a nonselective degradation system used for bulk proteolysis, while the UPS is necessary for fine control of protein

degradation, which specifically recognizes only ubiquitinated proteins for proteasomal degradation. Autophagic vacuole formation is activated as an adaptive response to a variety of extracellular and intracellular stimuli, including nutrient deprivation, hormonal or therapeutic treatment, bacterial infection, aggregated and/or misfolded proteins and damaged organelles, such as those present in some NDD. Hence, autophagy has a greater variety of physiological and pathophysiological roles than expected, such as intracellular protein and organelle clearance, anti-aging, starvation adaptation and recycling of materials during starvation, development, elimination of microorganisms, cell death and tumor suppression [Mizushima 2005; 2007]. Autophagy complements the proteosomal pathway in routine turnover of long-lived cellular proteins, protein aggregates, and damaged organelles (e.g. mitochondria and ER). Once targeted for degradation, damaged mitochondria or aggregates of misfolded proteins are encircled by membrane and captured in newly-delineated autophagosome, which fusses with a lysosome resulting in the degradation of the content of the autophagosome. Because the degradation of molecules and organelles by autophagy results in the production of energy and amino acids for protein synthesis, it is a cellular protective pathway [Kelekar 2005; Martinez-Vicente 2007; Mizushima 2007; Kundu 2008].

Autophagy is subdivided into three major forms (Fig. 19): macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macro- and microautophagy are both highly conserved from yeast to mammals while chaperone-mediated autophagy appears confined to mammalian systems.

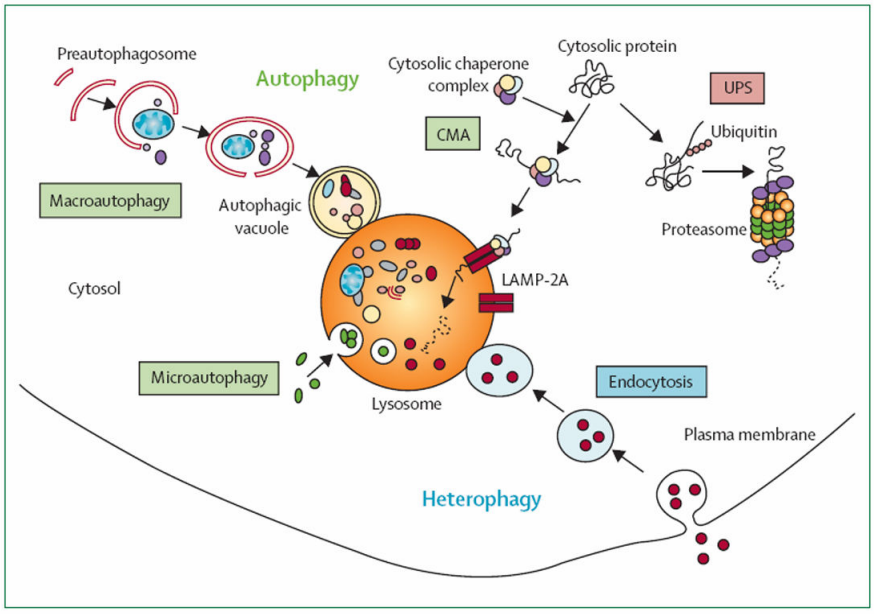


Figure 19: *Types of autophagy in mammalian cells* [Martinez-Vicente et al., Lancet Neurol (2007) 6: 352–61]

Macroautophagy (Fig. 19) is the bulk lysosomal degradation of larger cytoplasmic proteins and organelles such as mitochondria, fractured ER, and peroxisomes within a double-membrane vesicle of nonlysosomal origin that then fuses with lysosomes. Among the three types of autophagy, macroautophagy is the major catabolic pathway for energy generation and for the breakdown of macromolecules and damaged organelles into their essential constituents during periods of stress or nutrient deprivation [Kelekar 2005; Donati 2006]. Macroautophagy is a stress-induced form of autophagy. The different steps in macroautophagy (formation of the limiting membrain, elongation, maturation, lysosomal fusion and degradation) are mediated by a group of more than 20 proteins known as ATG proteins.

Less information is currently available about the mechanisms and molecular components that participate in **microautophagy** (Fig. 19). Unlike macroautophagy, there is no sequestering double-membrane formed in the cytoplasm. In this type of autophagy, cytosolic components are directly sequestered by the lysosome through invaginations or tubulations that “pinch off” from the membrane into the lysosomal lumen where they are rapidly degraded. Microautophagy participates in the continuous “basal” turnover of cellular components in normal cellular conditions [Martinez-Vicente 2007].

Chaperone-mediated autophagy (Fig.19) is a lysosomal pathway of proteolysis that is responsible for the degradation of 30% of cytosolic proteins. CMA involves formation of a complex between a cytosolic “substrate” protein and “chaperone” protein that interacts with a lysosomal membrane receptor for translocation into the lysosome. Molecular chaperones in the cytosol and in the lysosomal lumen stimulate this proteolytic pathway. The molecular chaperones in the cytosol unfold substrate proteins prior to their translocation across the lysosomal membrane, while the chaperone in the lysosomal lumen is required to pull the substrate protein across the lysosomal membrane [Massey 2004]. A critical component for CMA is a receptor in the lysosomal membrane, the lysosome-associated membrane protein 2A (LAMP-2A). CMA is activated by OS [Dice 2007]. Reduction of levels of LAMP-2 reduces CMA activity, but macroautophagy is activated as a result.

4. ER stress

4.1. ER stress-overview

The ER is an essential cellular compartment for protein synthesis and maturation. It also functions as a Ca^{2+} storage organelle and resource of calcium signals. The perturbation of ER functions can result in accumulation of unfolded or

misfolded proteins and the failure of the ER to cope with the excessive protein load. This leads to ER stress, which is defined as an imbalance between the cellular demand for ER function and ER capacity. To reduce the excessive protein loading, the cells trigger the unfolded-protein response (UPR), which signals transient attenuation of protein translation, degradation of malfolded proteins and the induction of molecular chaperones and folding enzymes to augment the ER capacity of protein folding and degradation. The ER contains a number of molecular chaperones physiologically involved in the posttranslational modification, disulfide bond formation, folding, assembly and quality control of newly synthesized proteins to preserve cellular homeostasis [Ni 2007]. Activation of the UPR transcriptionally upregulates an array of genes required for protein folding, ER expansion, ER-Golgi trafficking, and ER associated degradation (ERAD), which all act collectively to relieve the stress within the ER [Travers 2000]. To ensure proper processing of proteins within the ER, eukaryotic cells employ a quality control mechanism that recognizes and degrades aberrantly folded proteins to prevent the aggregation and/or delivery of potentially dysfunctional or cytotoxic proteins. Two simple adaptive mechanisms are employed to bring the folding capacity of the ER and its unfolded protein burden into line and return the ER to its normal physiological state: (1) upregulation of the folding capacity of the ER through induction of ER-resident molecular chaperones and foldases and an increase in the size of the ER [Ma 2004; Schröder 2005; Ni 2007; Ellgaard 2003], and (2) down-regulation of the biosynthetic load of the ER through shut-off of protein synthesis on a transcriptional [Wek 2007] and translational level and increased clearance of unfolded proteins from the ER through upregulation of ERAD [Haynes 2004; Schröder 2005]. However, when these mechanisms do not remedy the stress situation, apoptotic pathways are activated in the damaged cells of higher eukaryotic organisms [Kim 2006; Shiraishi 2006; Schröder 2005].

4.2. ER stress signalling

To cope with the ER stress, cells activate an intracellular signalling pathway – the UPR. The UPR is an integrated intracellular signalling pathway that transmits information about the protein folding status in the ER lumen to the cytoplasm and the nucleus (Fig. 20). The UPR includes transcriptional induction of UPR genes (red arrows), translational attenuation of global protein synthesis (black arrows) and ERAD (green arrows). These divergent outputs provide adaptive responses for survival. If the protein-folding defect is not corrected, cells undergo apoptosis (light-blue arrows). The most ER-proximal regulators of the UPR consist of a set of transmembrane ER-resident proteins [Liu 2003; Kaufman 2002; Schröder 2005]. The three major

transducers of the UPR are two protein kinases IRE1 (inositol-requiring kinase 1), and PERK (double-stranded RNA-activated protein kinase-like ER kinase), and the transcription factor ATF6 (activating transcription factor 6). The transducers sense ER stress through their luminal domain and activate downstream events. Signalling from these stress-sensing proteins protects the cell or, alternatively, promotes cell death.

PERK is an ER transmembrane serine/threonine protein kinase that mediates the translational control arm of the UPR by enhancing phosphorylation of α-subunit of translation initiation factor 2 (eIF-2α) and Nrf2 in response to ER stress [Cullinan 2006]. Phosphorylation of eIF-2α reduces the formation of translation initiation complexes, which leads to reduced recognition of AUG initiation codons and therefore general translational attenuation. Thus, phosphorylation of eIF-2α reduces global protein synthesis, preventing further overload of the secretory pathway [Wek 2007; Malhotra 2007]. Although phosphorylation of eIF-2α inhibits general translation initiation, it is required for the selective translation of several mRNAs. One fundamental transcription factor for which translation is activated on PERK-mediated phosphorylation of eIF-2α is the activating transcription factor 4 (ATF4). GADD34 transcription is induced by the UPR through ATF4, and the protein product recruits protein phosphatase 1 (PP1) to dephosphorylate p-eIF-2α and reverse the translational attenuation. Nrf2 phosphorylation promotes Nrf2 nuclear entry and the transcription of genes whose protein products promote redox homeostasis. While the phosphorylation of Nrf2 and eIF-2α occur independently, the two signalling pathways share amplifying PERK signalling [Cullinan 2006]. A role for Nrf2 activation during the UPR was established following the identification of Nrf2 as a PERK substrate. Similar to OS-inducing agents, ER stress induces Nrf2 nuclear translocation in a PERK-dependent manner. This activation does not require the accumulation of ROS or the phosphorylation of eIF-2α [Cullinan 2003]. Given the role of Nrf2 in combating OS, these findings suggested a cytoprotective role for Nrf2 signalling in response to ER stress as well, and for cell survival during the UPR [Schröder 2007].

ATF6 is an ER transmembrane activating transcription factor. Upon ER stress, in the absence of Grp78/BiP binding ATF6α and ATF6β transit to the Golgi compartment where they are cleaved by site-1 protease (S1P) and site-2 protease (S2P) to yield a cytosolic fragment. S1P, a serine protease, cleaves ATF6 in the luminal domain. The N-terminal membraneanchored half is cleaved by the metaloprotease S2P within the phospholipid bilayer [Schröder 2005]. These proteolytic reactions release the cytosolic bZIP domain of ATF6, which then translocates into the nucleus where ATF6 binds to the ATF/cAMP response element (CRE) and to the ER stress response elements (ERSE-I and -II). In the nucleus the free ATF6 fragment

activate transcription of many UPR target genes including Grp78/BiP and XBP1 [Okada 2002].

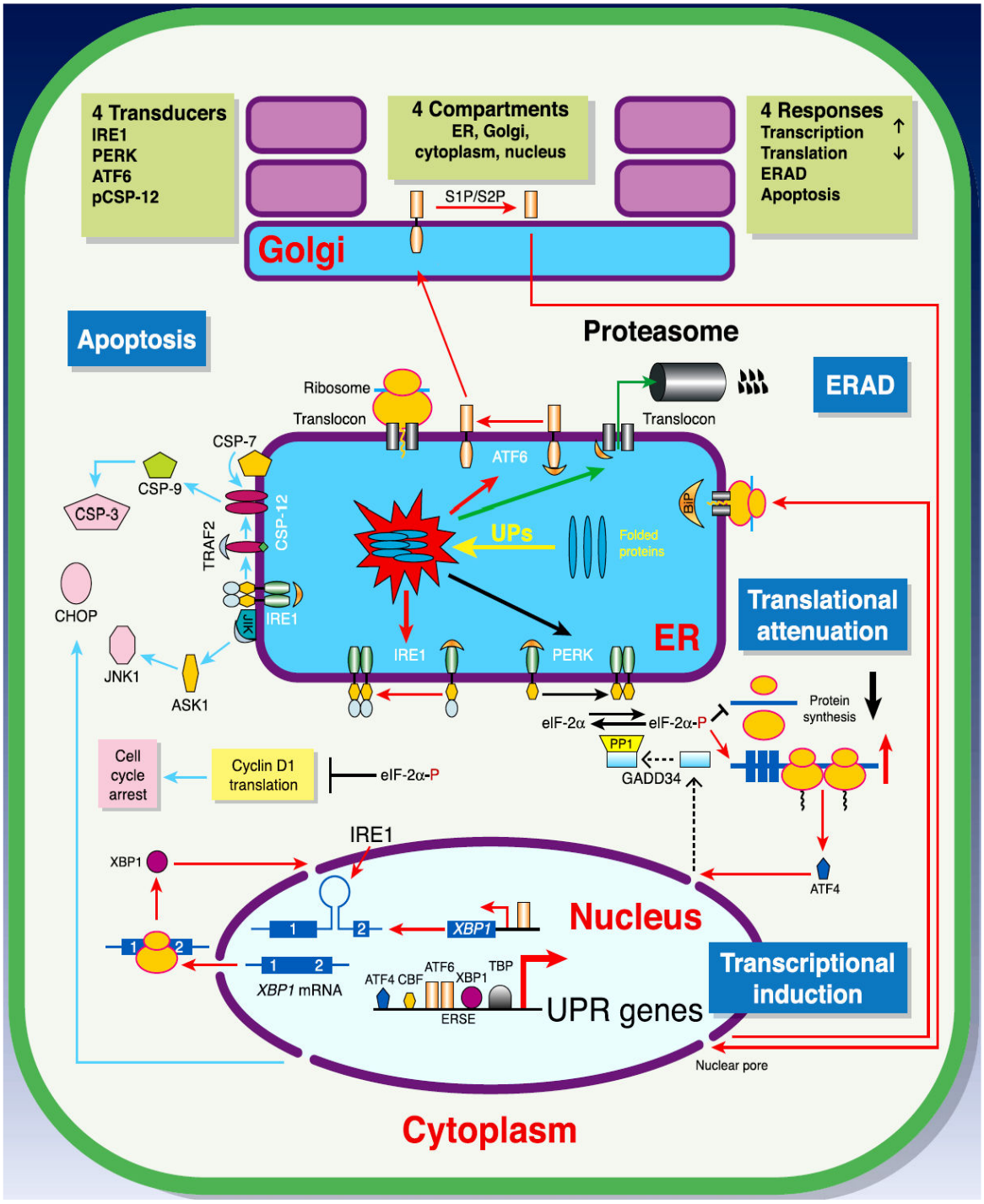


Figure 20: *Signalling the UPR in eukaryotes. Three proximal sensors, IRE1, PERK, and ATF6, coordinately regulate the UPR through their various signalling pathways. Whereas IRE1 and PERK are dispensable for many aspects of the response, ATF6 cleavage is required for UPR transcriptional induction and appears to be the most significant of these effectors in mammalian cells. Grp78/BiP negatively regulates these pathways* [Liu and Kaufman, Journal of Cell Science (2003) 116: 1861-1862]

IRE1 is an ER transmembrane glycoprotein that has both kinase and a site-specific endoribonuclease (RNase) activity in the cytoplasmic domain. ER stress leads to its homodimerization and the subsequent trans-autophosphorylation to activate its RNase activity. XBP1 mRNA is a substrate for the endoribonuclease activity of mammalian IRE1. XBP1 mRNA, encodes a basic leucine-zipper-containing transcription factor. The ATF6 pathway increases transcription of XBP1 mRNA, therefore the transcription factor XBP1 is produced upon splicing of its mRNA by IRE1 [Bernales 2006]. On activation of the UPR, IRE1 RNase activity cleaves XBP1 mRNA to remove a 26-nucleotide intron and to generate mature XBP1 mRNA. This splicing reaction creates a translational frameshift to produce a larger form of XBP1 that contains a novel transcriptional activation domain in its C-terminus. Spliced XBP1 is a transcriptional activator that plays a key role activation of wide variety of UPR target genes, including several ER-resident chaperones [Liu 2003; Lee 2003; Malhotra 2007]. Deletion of IRE1 or XBP1 in mice creates an embryonic lethality at e11.5-e14.

Whereas the ATF6 and PERK pathways are not conserved in lower eukaryotes, the IRE1 signalling pathway is conserved in all known eukaryotic cells. The signalling from downstream effectors of IRE1, PERK and ATF6 merges in the nucleus to activate transcription of UPR target genes. However, in certain cells, different stress conditions can selectively activate only one or two of the ER stress sensors. The mammalian ER stress element (ERSE) is present in the promoter regions of many, but not all, UPR target genes. XBP1 and ATF6 along with ATF4, all of which bind to ERSE, activate transcriptional induction of target genes. ATF6 also induces XBP1 transcription, providing a positive feedback for the UPR. Upregulation of molecular chaperones and folding catalysts increases the folding capacity of the ER, providing a protective effect for cell survival. In addition, activated Ire1p in yeast induces transcription of genes that mediate phospholipid biosynthesis to increase the ER volume [Malhotra 2007].

The UPR also induces transcription of genes encoding proteins that mediate ERAD. This important component of the UPR stimulates the degradation and clearance of unfolded proteins in the ER lumen. Several target genes appear to encode proteins that remodel the secretory pathway to decrease the concentration of UPs.

Grp78/BiP, an ER chaperone, is the master regulator of the activation of the three ER stress transducers – IRE1, PERK and ATF6 [Kaufman 2002; Liu 2003]. The three independent sensors are activated by a common stimulus, the accumulation of unfolded proteins in the ER lumen. All three transducers contain a lumenal domain that interacts with Grp78/BiP. Under normal conditions, Grp78/BiP serves as a

negative regulator of IRE1, PERK and ATF6 activation. Upon ER stress, the unfolded proteins bind Grp78/BiP and sequester it from interacting with IRE1, PERK, and ATF6 allowing Grp78/BiP release from the transducers and elicit their activation. In this manner, Grp78/BiP senses both the level of UPs and the energy (ATP) level in the cell in regulating the UPR. Grp78/BiP release from IRE1 and PERK permits their spontaneous homodimerization mediated by their lumenal domains and activation through phosphorylation by their endogenous kinase activities. Grp78/BiP interaction with ATF6 prevents trafficking of ATF6 to the Golgi compartment. ATF6 is also retained in the ER by interaction with the lectin calreticulin. For this reason, Grp78/BiP release from ATF6 permits its transport to the Golgi compartment where it gains access to S1P and S2P proteases for regulated intramembrane proteolysis [Liu 2003; Malhotra 2007; Kaufman 2002]. Thus, Grp78/BiP regulated activation provides a direct mechanism to sense the folding capacity of the ER. The increase in Grp78/BiP during the UPR would provide a negative feedback to turn off UPR signalling.

Prolonged UPR activation leads to apoptotic cell death, in which IRE1 serves a proapoptotic function. Activated IRE1 recruits Jun N-terminal inhibitory kinase (JIK) and TRAF2 to activate apoptosis-signalling kinase 1 (ASK1), which in turn activates JNK and mitochondria/Apaf1-dependent caspases. Procaspase-12 (pCSP-12) is an ER-associated proximal effector of apoptosis. TRAF2 release from pCSP-12 permits the clustering and activation of CSP-12. Activated CSP-12 activates CSP-9, which in turn activates CSP-3, leading to apoptosis. Upon ER stress, activated CSP-7 can cleave pCSP-12 to generate active CSP-12. In addition, UPR activation induces CHOP/GADD153 expression through the PERK and ATF4 pathways. CHOP is a proapoptotic transcription factor that potentiates apoptosis. In response to prolonged ER stress, attenuation of cyclin D1 translation through PERK leads to cell cycle arrest during G1 phase. This provides an ER checkpoint to prevent cells from progressing through the cell cycle [Kaufman 2002; Liu 2003].

4.3. ER chaperones, UPR and ERAD

The transcriptional up-regulation of ER chaperones is the hallmark of the ER stress response and occurs in all eukaryotic organisms [Ma 2004]. During stress ER chaperones play the same function in ERAD as under normal physiological conditions, only perhaps greater. There are two known chaperone systems in the ER, calnexin/calreticulin and Grp78/Grp94. The ER chaperones can be categorized into three groups: (a) chaperones of heat shock protein family including Grp78, Grp94 and the co-chaperones; (b), chaperone lectins like calnexin, calreticulin and EDEM; and (c)

substrate-specific chaperones such as Hsp47. Additionally, there are at least two groups of folding catalysts, namely thiol oxidoreductases of the protein disulfide isomerase (PDI) family such as PDI and GRP58/ERp57 and peptidyl prolyl isomerases (PPIs) [Ma 2004].

Calnexin is a 90 kDa type I ER membrane protein and calreticulin is a 60 kDa soluble ER lumen protein, both with a C-terminal KDEL signal [Helenius 1997]. When the newly synthesized polypeptides enter the ER, they are often modified by N-linked glycans (Glc3Man9GlcNAc2) and the glucoses are rapidly removed by glucosidases I and II. The nascent protein with monoglucosylated N-linked glycans (Glc1Man9GlcNAc2) is recognized by the calnexin/calreticulin system for subsequent folding and assembly steps. PDI is also an important component involved in the calnexin/calreticulin system. It contains thioredoxin motifs and acts as a thiol oxidoreductase to catalyze the disulfide bond formations of the loaded glycoproteins. If the glycoprotein cannot be correctly folded, another ER protein UGGT (UDP-glucose glycoprotein-glucosyltransferase) recognizes surface-exposed hydrophobic regions of the unfolded or misfolded glycoprotein and catalyzes the transfer of a glucose unit from UDP-glucose to a specific mannose residue within the N-glycan chain of the glycoprotein [Ni 2007; Ma 2004].

Grp78, also known as BiP, is the ER homologue of Hsp70 proteins [Hendershot 2004]. As a chaperone, Grp78 recognizes and binds to the proteins with hydrophobic residues in the unfolded regions. Therefore, some calnexin/calreticulin substrates can bind to Grp78 if the N-glycosylation is blocked. Grp78 is a member of a large multi-protein complex with a set of ER molecular chaperones, Grp94, PDI, ERp72, GRP170/ORP150, UGGT, CaBP1 (calcium binding protein), cyclophilin B and SDF2-L1, which forms an ER chaperoning network processing the unfolded protein substrates. In this complex, Grp94, an ER homologue of HSP90 protein, often functions as a dimmer providing a platform for the assembly or oligomerization of loaded protein cargo [Ellgaard 2003; Ni 2007].

During conditions of ER stress, alterations in the ER environment can profoundly affect the folding of many proteins. Normal chaperone function is a key factor for endogenous stress adaptation. A major function of the ER chaperones is to promote protein folding by preventing misfolding or aggregation. Although Grp78/BiP appears to be the sole chaperone that is monitored by the cell to sense ER stress, many of the chaperones are coordinately up-regulated via the presence of ERSEs in their promoters. Thus, the main function of the increased levels of ER chaperones is to bind to unfolded proteins, prevent them from aggregating, and to aid and monitor their refolding if normal physiological conditions are changed. Proteins that have ultimately

failed ER quality control are degraded to prevent their accumulation in the ER. The final step of the turnover mechanism termed ERAD is retrotranslocated of both malfolded and multimeric protein aggregates into the cytosol. This retro-translocation process usually coupled with ubiquitination, which occurs at the cytosolic surface of the ER membrane. This appears to be an important process in maintaining ER homeostatis during normal physiological conditions, since interfering with this process results in activation of the ER stress pathway [Ma 2004; Haynes 2004]. However, the upstream ERAD signals that help cells to select malfolded proteins and feed them into the downstream degradation machinery remain fairly unclear. One mechanism for identifying malfolded glycoproteins for ERAD involves ER chaperones calnexin, calreticulin, and calmegin [Cabral 2001]. The ERAD machinery depends on the up-regulation of ER-resident chaperones to deal with the increased load of malfolded ERAD substrates during ER stress. It was reported that both calnexin and Grp78/BiP are required for retro-translocation of ERAD substrates and may act sequentially to recognize and target proteins for degradation [Ma 2004]. If the stress is not resolved rapidly, many unfolded proteins will be targeted for ERAD as one way to decrease the load of malfolded proteins that accumulate in the ER. Last decade studies demonstrate that the UPR regulates not only ER-resident chaperones, but it also controls components of the ERAD system, as well as components of the proteasome system. While the UPR does not seem to be required for the normal degradation of ER proteins, deletion of ERAD components results in significant UPR activation, demonstrating that there is cross-talk in both directions between these two pathways [Ng 2000].

5. Neurodegenerative diseases an accelerated form of neuronal aging

Aging is a progressive deterioration of physiological function that impairs the ability of an organism to maintain homeostasis and consequently increases the organism's susceptibility to disease and death [Harman 1972]. Many theories of aging are based on the concept that molecular damage, both due to normal toxic by-products of metabolism or inefficient repair/defensive systems, accumulate throughout the entire lifespan and cause aging. Thus, it has been hypothesized that the NDDs could be an accelerated form of neuronal population aging, hence all the parameters observed in the general aging (oxidative stress, accumulation of highly modified protein aggregates, mitochondrial dysfunction) are more intensive and premature in these cells (Fig. 21).

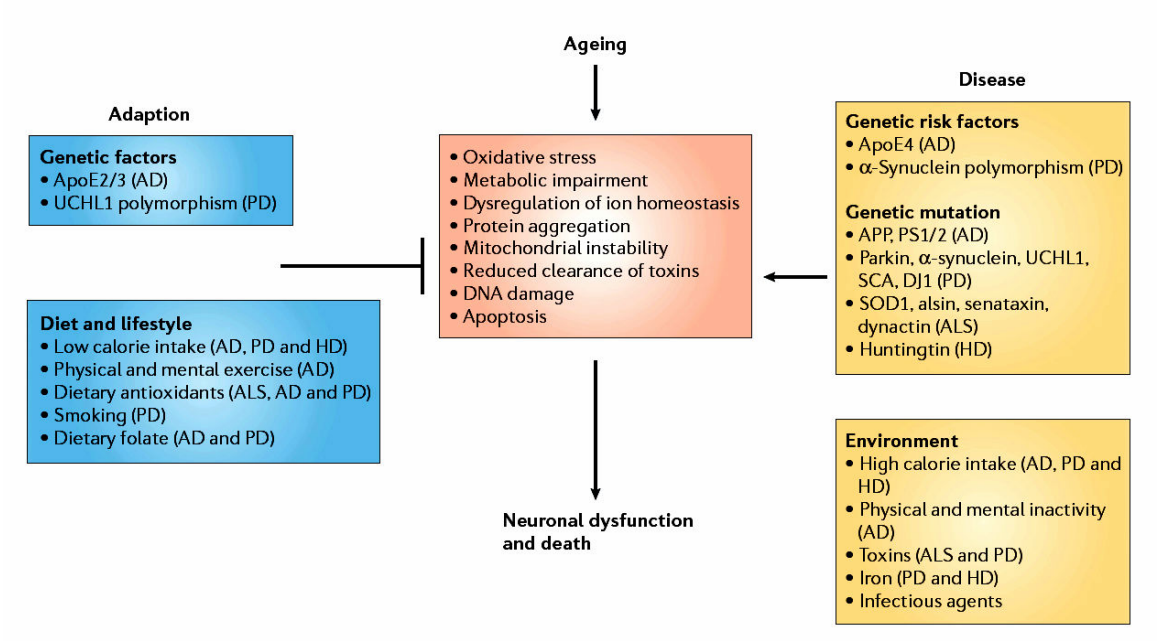


Figure 21: *Neuronal death in ageing and NDDs triggered by specific genetic mutations and/or environmental factors such as toxins or dietary components. Initiating factors promote cellular alterations, including increased ROS production, perturbed energy and calcium homeostasis, and activation of apoptotic cascades. Each factor cooperates with age-related increases in OS, metabolic compromise, DNA instability and ion homeostasis dysregulation to disrupt neuronal integrity, thereby resulting in synaptic dysfunction and cell death.* [Mattson and Magnus, Nature Neuroscience (2006) 7: 278-294]

OS, damaging all sorts of macromolecules, is considered as the general decline in cellular functions that are associated with many human diseases, as well as a determinant factor in the aging process [Portero-Otin 2001]. Accordingly, aging has been considered as a major risk factor for a variety of NDDs [Outeiro 2008].

5.1. Neurodegenerative diseases, aging and oxidative stress of mitochondrial origin

ROS can be produced in all cellular compartments and ultimately results in protein damage. Furthermore, the exposure of biologic systems to various conditions of OS leads to age-dependent increases in the cellular levels of oxidatively modified proteins, lipids, and nucleic acids, and subsequently predisposes to the development of age-related disorders that cause impaired cognitive function and metabolic integrity [Stadtman 2002]. OS could be related to aging through an increase in ROS generation. Accumulating evidence suggests that protein unfolding and production of ROS are closely linked events [Naudí 2007]. Because physiological oxidative protein folding occurs in the ER and perturbations in protein folding can cause deleterious consequences, alterations in redox status or generation of ROS could directly or indirectly (or both) affect ER homeostasis and protein folding. On the other hand, free radical

levels are higher in tissues that are more metabolically active. Thus, brain tissue is especially vulnerable to free radicals induced degeneration and the brain constitutes perhaps the most vulnerable tissue to OS. As described before, several cellular features of the brain suggest that this organ is highly sensitive to OS [Portero-Otin and Pamplona 2006]: (i) The brain is known to possess the highest oxygen metabolic rate of any organ in the body. The brain consumes approximately twenty percent of the total amount of oxygen in the body. This enhanced metabolic rate leads to an increased probability that excessive levels of ROS will be produced; (ii) In addition, the brain tissues contain increased amounts of PUFAs that are the tissue macromolecules that are most sensitive to oxidative damage; (iii) The brain contains high levels of iron which have been associated with free radical injury [Herbert 1994]; (iv) and finally, given the increased risk factors for the generation of elevated levels of ROS in the brain, the brain also may suffer from an inadequate defence system against OS, as quantitatively, it exhibits lower antioxidant capacity.

OS and protein misfolding play critical roles in the pathogenesis of a large class of conformational diseases associated with accumulation of abnormal protein aggregates composed of misfolded proteins in and around affected neurons such as AD, PD, HD, ALS. In vitro studies have shown that these aggregates are stabilized by oxidation [Malhotra 2007]. At the cellular level, neuronal death or apoptosis may be mediated by OS and ER stress or both. Recent studies indicate that oligomeric forms of polypeptides predisposed to β-sheet polymerization and fibril formation may be the toxic forms that cause neuronal death. The impact of these oligomeric, potentially toxic species on ER function and generation of ROS is presently not understood.

A group of NDDs including AD is characterized pathologically by the deposition of intracellular aggregates containing abnormally phosphorylated forms of the microtubule-binding protein tau. Using a model relevant to human NDDs, including AD, it was demonstrated that OS plays a casual role in neurotoxicity and promotes tau-phosphorylation [Lee 2001].

5.2. Neurodegenerative diseases, aging and mitochondrial dysfunction

The fact that mitochondria are responsible for a quantitatively relevant percentage of free radical production suggests that mitochondria could play a causal role in the progressive phenomenon that is aging. Decreases in mitochondrial function have often been associated with ageing in general, and ageing of the nervous system in particular [Barja 2004]. Measurements of the activities of mitochondrial enzyme in brain tissue samples revealed significant decreases in the activities of the pyruvate

dehydrogenase complex, isocitrate dehydrogenase and the α-ketoglutarate dehydrogenase complex in patients with AD compared with control subjects. Mitochondrial complex I activity declines in the brain during normal ageing, but much more so in PD [Smigrodzki 2004]. Patients with HD lose weight progressively, despite maintaining a high caloric intake, an abnormality that might result from impaired mitochondrial function [Seong 2005; Gil 2008]. Deficits in mitochondrial function might also occur early in the course of ALS, perhaps first in the axons and presynaptic terminals of the MNs [Mattson 2006; Wiedemann 2002]. Analyses of mitochondria isolated from different brain regions of young, middle-aged and old rat models of NDDs revealed that mitochondria from the cerebral cortex of old rats show enhanced ROS production and mitochondrial swelling in response to increasing Ca^{2+} loads compared with cortical mitochondria from younger rats. By contrast, the sensitivity of cerebellar mitochondria to Ca^{2+} is unaffected by ageing. The capacity of mitochondria to respond appropriately to excitation might be impaired during ageing. Moreover, ageing increases the vulnerability of mitochondria to toxins. In addition to alterations in mitochondria, neurons also show impaired glucose uptake during normal ageing, further compromising their ability to maintain ion homeostasis and other energy-dependent cellular processes. Many of the age-related deficits in energy metabolism might be a consequence of OS. Caloric restriction can preserve mitochondrial function during ageing, apparently by reducing ROS production, and can protect neurons from being killed by mitochondrial toxins [Mattson 2006]. Aβ has been shown to impair mitochondrial function, and studies of APP-mutant mice suggest a key role for an Aβ-binding alcohol dehydrogenase in this pathogenic action. Mitochondria in neurons of mutant presenilin 1-knockin mice show increased sensitivity to toxins and cellular Ca^{2+} overload. Huntingtin-mutant mice also manifest alterations in mitochondrial function and energy metabolism. Mitochondria from huntingtin-mutant mice maintain an abnormally low resting membrane potential and are hypersensitive to Ca^{2+}. Mutant huntingtin might also perturb energy metabolism indirectly by impairing the transport of mitochondria along axons [Trushina 2004]. ALS (Cu/Zn-SOD mutant) mice exhibit impaired mitochondrial function in SC neurons, and treatment of the mice with creatine, which enhances cellular energy metabolism, suppresses the neurodegenerative process and improves survival of these mice [Shults 2002].

Mitochondria actively communicate and interact with each other and with other cellular organelles, such as the ER, to satisfy the cell's changing energetic needs and protect it from excessive Ca^{2+} influx, oxidative damage and mitochondrial DNA (mtDNA) mutations - events that typically characterize aging and neurodegenerative processes [Knott 2008].

5.3. Neurodegenerative diseases, aging and protein turnover

The age-dependent decrease in the efficiency and failure of maintenance, repair and protein turnover pathways may be a major contributing factor to the accumulation of modified proteins during aging [Grune 2000]. Some observations reporting an age-related decrease in proteasome activity associated with an age-related increase in the levels of oxidized proteins suggest that the two events may be related. OS promotes a variety of protein modifications, incompatible with proper protein function [Stadtman 1998]. Dysfunction of the ubiquitin-proteasomal degradation system has increasingly been implicated in the process of neuronal degeneration as well. Most protein oxidative modifications are non-repairable, and have deleterious consequences on protein structure and function. The major fate of oxidized proteins is catabolism by proteosomal and lysosomal pathways, but some materials appear to be poorly degraded and accumulate within cells. Therefore, the observed age-related accumulation of oxidized cross-linked material may be the result of both increased protein oxidation followed by aggregation and/or declined protein breakdown and a malfunction of the proteasomal system.

Accumulation and aggregation of oxidatively damaged proteins involved in the aging process is known to be linked to some severe NDDs [Davies 2005; r. in Voss 2007]. In several studies analyzing the activity of the 20S ‘core’ proteasome it was reported that only the peptidylglutamyl hydrolyzing activity of the proteasome was severely affected during aging [Conconi 1997; Anselmi 1998]. Although the trypsin-like activity is also oxidation-dependent, it seems to be protected during aging due to the interaction of the proteasome with Hsp90 [Conconi 1997]. There are almost no studies investigating the role of the 26S proteasome during aging in the context with the accumulation of oxidized proteins.

Proteasomal function in susceptible cell populations—such as MNs or nigral dopaminergic neurones—may be overwhelmed, leading to decreased protein turnover and pathological aggregation. This idea is further supported by the finding that ubiquitinated and nitrated protein aggregates, present in NDDs, can occur as a direct result of proteasomal inhibition [Hyun 2003]. It is noteworthy that oxidative modifications of some proteins in age-related NDDs, especially those leading to generation of cross-linkages, lead to derivatives that are not only resistant to degradation by the proteasome, but inhibit the ability of the proteasome to degrade oxidized forms of other proteins [Davies 2000].

Autophagy has been also related to a number of NDDs and aging [Cherra 2008]. Reduced autophagic activity is associated with NDDs, such as PD, AD, HD and transmissible spongiform encephalopathy [Rajawat 2008]. There is experimental

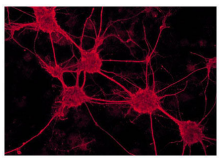
evidence that certain proteolytic activities, such as macroautophagy, decrease with age [Ward 2002; Cuervo 1998]. Recent reports indicate that, when injurious cellular stresses occur, cells protect themselves using macroautophagy to remove damaged mitochondria and mutated mtDNA [Donati 2006], as well as redundant, damaged or non-functional peroxisomes [Locci-Cubeddu 1985] and ER. The reduced CMA in aging can be explained by reduced LAMP2 in the lysosomal membrane. This reduction is caused by an age-related increased degradation of LAMP2 and an age-related reduced ability of LAMP2 to reinsert into the lysosomal membrane [Dice 2007].

5.4. Neurodegenerative diseases, aging and ER stress

ER dysfunction has been reported in age-related neuronal diseases, consequently damage to the ER and its subsequent impaired functionality may be involved in the process of aging [Paschen 2001]. There are several phenomena that suggest its involvement in the aging process, like (1) a preferential susceptibility to oxidation of ER resident proteins that are involved in protein folding and, (2) the upregulation of ER stress response chaperones, (3) impairment of maintaining the calcium homeostasis [van der Vlies 2003] during aging.

The age-dependent upregulation of ER stress response genes that process damaged or misfolded proteins may reflect the importance of protein oxidation for biological aging [Lee 2001] and a common defence mechanism against these effects in diverse species. Upregulation of these genes in aging may imply the presence of accumulated damaged and misfolded proteins in the ER caused by improper folding. Proper folding requires optimal functioning of folding proteins. As a consequence of oxidation of the ER Ca^{2+}-ATPases the required calcium concentration inside the lumen of the ER cannot be established, and cause an impaired functioning of the Ca^{2+}-binding chaperones [van der Vlies 2003]. Upregulation of ER stress markers has been demonstrated in post-mortem brain tissues and cell-culture models of many NDDs, including PD, AD, and expanded polyglutamine diseases such as HD and spinocerebellar ataxias [Malhotra 2007], but they were scarcely studied in other diseases, such as PiD, ALS and AGD. Whereas the ERAD of misfolded proteins is affected in PD, it is the UPR that is down-regulated in AD and the ER calcium homeostasis that is disturbed in ischemia. Several postmortem studies of primary human AD brain tissues have provided evidence of ER stress in the form of enhanced ER chaperone expression and immunohistochemical reactivity for specific markers of the UPR branch activity. Although the link between α-synuclein and ER stress is unclear, parkin is a ubiquitin-protein ligase (E3) involved in ERAD. Expression of

parkin is induced by ER stress, and neuronal cells overexpressing parkin are resistant to ER stress [Imai 2001]. These findings support the involvement of ER stress in PD. Several additional reports support the link between ER stress and PD. PD-like inducerss, such as 6-hydroxydopamine, specifically induce ER stress in neuronal cells. Expression of ER chaperones such as PDI is upregulated in the brain of PD patients as well, and PDI is accumulated in Lewy bodies. The identification of PDI family member PDIp in experimental PD and Lewy bodies suggests that oxidative protein folding in the ER may be perturbed in PD [Malhotra 2007].



OBJECTIVES

HYPOTHESIS AND OBJECTIVES

NDDs share some common features, such as deposition of aggregated proteins [Bence 2001], deficiency of the ubiquitin-proteasome system [Halliwell 2006b], impairment of mitochondrial functions [Lin and Beal 2006] and increased oxidative damage [Halliwell 2006a]. The development of ER stress in these conditions [Lindholm 2006] has been suggested to be involved in the pathophysiology of a variety of human NDDs including PD [Hoozemans 2007], AD [Hoozemans 2005], PrD [Ferreiro 2006], Pelizaeus-Merzbacher [Garbern 2007], PGD [Shastry 2003] and multiple sclerosis [Mhaille 2008].

The hypothesis based on these previous observations was established as follows: oxidative and ER stress interplay in the development of the neurodegeneration process in different disorders associated with deposition of aggregated proteins (in particular the most common MND-ALS, and two rare tauopathies-PiD and AGD) through variety of cellular mechanisms and signalling pathways, ultimatelly leading to neuronal death.

We hypothesize that an oxidative stress, directly related with a mitochondrial dysfunction, is implicated in ALS, PiD and AGD and through its interaction with cellular proteolysis, could provoke an inhibition of the proteasome, which would contribute to the generation of the deposits of aggregated, ubiquitinated proteins and could play a relevant role in the pathogenesis of theses diseases. A consequence of such impairment, secondary to OS, could be an ER stress. The cell could try to compensate with adaptive responses known as UPR, through: 1) Translational attenuation by phosphorylation of eukaryotic translation initiation factor 2 (subunit alpha) - eIF-2α; 2) Overexpression of ER chaperones, folding enzymes and ER membrane receptors; and 3) if these mechanisms do not lead to the correction of ER stress, the cell activates ER stress-derived apoptotic pathways, related with the mitochondrial pathways of apoptosis.

To evaluate this hypothesis the following objectives were planned:

1. To characterize the possible implication of OS in protein and DNA damage, in a case-control approach, in human samples from patients with ALS, PiD and AGD, including
   a) To analyze the presence and distribution of protein oxidative markers in proteins by means of western-blot technique and immunohistochemistry

   b) To identify the protein targets of oxidative damage through proteomic and wester-blot technique in human samples from the three diseases mentioned above (ALS, PiD, AGD)

2. To measure the concentrations of markers for direct protein oxidation (aminoadipic (AASA) and glutamic (GSA) semialdehydes), for lipoxidation (malondialdehyde-lysine (MDAL)) and for glycoxidation (carboxymethyl-lysine (CML) and carboxyethyl-lysine (CEL)) in proteins from human samples of ALS, PiD and AGD; and to characterize the composition of fatty acids and derived indexes by the means of gas chromatography coupled to mass spectrometry.
3. To assess the state of the mitochondria, and in particular the respiratory chain, as the major source in the production of free radicals in the cell; and to estimate mitochondrial biogenesis as its dysfunction could participate as a contributor to the pathogenesis of ALS, PiD and AGD.
4. To examine the role of the antioxidant defence system as a way to respond to the cellular stress related with the increase of the reactive carbonyls content derivatives from free radicals, in the pathogenesis of ALS, PiD and AGD through the analysis of specific biomarkers and transcriptional factors of the genes codifying antioxidant enzymes.
5. To examine the state of a major proteolytic system in the cell - the proteasome - in the pathogenesis of the three diseases (ALS, PiD and AGD) related to the accumulation of protein aggregates, assessing the proteasome activity by the means of specific fluorescently labeled peptides and by assessing the levels of the ubiquitinated proteins
6. To characterize the possible participation of the ER stress in the pathogenesis of ALS, PiD and AGD comparing the case samples with age-matched controls; and to examine the response to the ER stress using immunohistochemisty and western-blot techniques.
   a) To evaluate the signs of ER stress in human samples: 1) the translational attenuation through the phosphorylation of eIF-2α; and 2) the activation of the transducers of the ER stress IRE1 and ATF6 involved in the unfolded protein response during the stress.
   b) To verify the state of activation of the ER stress response, including the expression of XBP1, foldases and ER chaperons in the samples of the above mentioned human diseases.

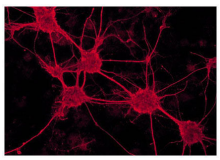
HIPÓTESIS Y OBJETIVOS

Diversos trabajos demuestran que las enfermedades neurodegenerativas comparten en su patogénesis varias características comunes, tales como agregados intracelulares de proteínas, el incremento de la lesión oxidativa , la alteración de la mitocondria y del proteasoma, con implicación del estrés de retículo endoplasmatico. Debido a estas razones, hipotetizamos que el estrés oxidativo, relacionado directamente con disfunciones mitocondriales puede provocar la inhibición del proteasoma. La alteración proteasomal, secundaria a un incremento del estrés oxidativo, puede conducir a estrés de retículo endoplasmático y a la activación de vías apoptóticas en la neurona, que intentaría compensar mediante la respuesta de adaptación denominada respuesta a proteínas desplegadas (UPR), mediante la represión transcripcional, la sobreeexpresión de chaperonas y, en caso que la célula no se recuperase, la inducción de muerte celular por diversas vías.

Para estudiar esta hipótesis se establecieron los siguientes objetivos:

1. Caracterizar la posible participación del estrés oxidativo proteico (y de ADN en el caso de la enfermedad de Pick) en una aproximación caso-control en muestras humanas de pacientes con la esclerosis lateral amiotrofica, enfermedad de Pick y enfermedad de granulos argirofiílicos.
   a) Analizar, por medio de técnicas de western-blot y inmunohistoquimica, la presencia y distribución de marcadores oxidativos en proteínas.
   b) Identificar las dianas proteicas de lesión oxidativa por medio de técnicas de proteómica y western en muestras humans de las tres enfermedades citadas (ELA, PiD y AGD)
2. Medir las concentraciones de marcadores de oxidación proteica directa (semialdehidos aminodípico (AASA) y glutámico (GSA)), de lipoxidación (malondialdehidolisina (MDAL)) y de glicoxidación (carboximetilisina (CML) y carboxietilisina (CEL)) en proteínas de las muestras de pacientes con ALS, PiD y AGD; y caracterizar la composición en ácidos grasos e índices derivados por medio de cromatografia de gases acoplada a espectrometria de masas.
3. Evaluar el estado de las mitocondrias y concretamente la cadena respiratoria como la mayor fuente de radicales libres en la célula; y el estado de alteraciones en la biogénesis mitocondrial que participaría en la disfunción mitocondrial como posible factor fisiopatológico en las tres enfermedades.

4. Examinar el sistema de defensa antioxidante en la patogénesis de la ALS, PiD y AGD, mediante el análisis de marcadores bioquímicos específicos y factores de trascripción de genes codificantes de enzimas antioxidantes.
5. Examinar el estado de uno de los principales sistemas proteolíticos celulares - el proteasoma - en la patogénesis de las tres enfermedades (ALS,PiD, AGD) propiedad relacionada con la acumulacion de agregados proteicos, evaluando si la actividad proteasomal esta alterada por medio de sustratos fluorescentes específicos y del establecimiento de los niveles de proteínas ubiquitinizadas, en las muestras humanas en una comparación caso-control.
6. Caracterizar la posible participación del estrés del retículo en la patogénesis de la ELA, PiD y AGD comparando las muestras de las enfermedades con las de casos control; examinando las respuestas a estrés de reticulo por medio de métodos immunohistoquímicos y de western-blot.
   a) evaluar los signos de estres de reticulo en muestras humanas: 1) la represión transcripcional a traves de la fosforilación de eIF-2α; y 2) la activación de los transductores de estrés de retículo endoplasmático IRE1 y ATF6 involucrados en la respuesta de las proteínas desplegadas durante el estrés de reticulo.
   b) Establecer el estado de activación de las respuestas a estrés de retículo endoplasmático, incluyendo la expresión de XBP1, foldasas y chaperonas de retículo, en las muestras de las enfermedades humanas anteriormente citadas.



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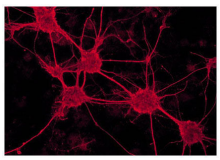

ARTICLE I

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Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis

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The occurrence of endoplasmic reticulum (ER) stress in the sporadic form of amyotrophic lateral sclerosis (ALS) is unknown, despite it has been recently documented in experimental models of the familial form. Here we show that spinal cord from patients with sporadic ALS showed signs of ER stress, such as increased levels of ER chaperones such as protein-disulfide isomerase, and increased phosphorylation of eukaryotic initiation factor 2α (eIF2α). Among the potential causes of such ER stress proteasomal impairment was confirmed in the same samples by demonstrating increased ubiquitin immunoreactivity and increased protein lipoxidative (125%), glycoxidative (55%) and direct oxidative damage (62%) over control values, as evidenced by mass-spectrometry and immunological methods. We found that protein oxidative damage was strongly associated to ALS-specific changes in fatty acid concentrations, specifically of *n*-3 series (as docosahexaenoic acid), and in the amount of mitochondrial components as respiratory complexes I and III, suggesting a mitochondrial dysfunction leading to increased free radical production. Oxidative stress was also evidenced in frontal cortex, suggesting that this region is affected early in ALS. As those events were partially reproduced by threohydroxyaspartate exposure in organotypic spinal cord cultures, we concluded that changes in fatty acid composition, mitochondrial function and proteasome activity, which may be driven by excitotoxicity, lead to oxidative stress and finally contribute to ER stress in sporadic ALS.

Keywords: Proteasome; glycation; lipoxidation; mitochondria; motor neuron

Abbreviations: ER = endoplasmic reticulum; GSA = glutamic semialdehyde; AASA = aminoadipic semialdehyde

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Introduction

Amyotrophic lateral sclerosis is a multifactorial disease whose pathophysiological mechanisms include decreased availability to neurotrophic factors, disturbances in calcium metabolism, increased neuroinflammatory status, cytoskeletal changes and oxidative stress (Dupuis *et al.*, 2004; Rao and Weiss, 2004; Strong *et al.*, 2005; Boillee *et al.*, 2006). Recent data indicate (Atkin *et al.*, 2006; Turner and Atkin, 2006) that endoplasmic reticulum (ER) stress may be also involved in the familial form of the disease. ER stress, a complex pattern involving highly specific signalling pathways, ensures through the so-called unfolded protein response that protein folding capacities of ER are not overwhelmed. However, prolonged ER stress could contribute to cell death, both by mitochondria-dependent and independent pathways (Lindholm *et al.*, 2006). In contrast with the familial form, no data was available on the occurrence of ER stress in the more common, sporadic form of the disease.

Some of the pathological hallmarks of ALS, such as increased ubiquitinated bodies, neuronal and astrocytic hyaline inclusions as well as axonal spheroids are protein aggregates that may be related to ER and oxidative stress. This fact is based on the relationship between protein oxidative damage and proteasomal activity following an inverted U shape, i.e. while moderate oxidative modifica-

tion of proteins increases their susceptibility for proteasome clearance, higher rates of oxidative modification actually inhibit proteasome activity (Grune *et al.*, 1996; Sitte *et al.*, 2000; Grune *et al.*, 2004). Such a decreased proteasomal activity has been previously recognized in other neurodegenerative processes, and it may explain ALS-characteristic increased ubiquitination and presence of proteinaceous aggregates. The disruption of the ER-associated degradation, a pathway which helps to clear misfolded protein species from ER, is a potential consequence of such proteasomal impairment, finally contributing to ER stress (Marciniak and Ron, 2006; Oyadomari *et al.*, 2006; Zhang and Kaufman, 2006).

Despite these data suggesting the importance of protein oxidative damage in ALS, its study by using selective, chemically characterized markers has not been reported, though immunohistochemical evidences support ALS-induced increased oxidative damage (Ferrante *et al.*, 1997; Pedersen *et al.*, 1998; Shibata *et al.*, 1999; Kato *et al.*, 2000; Kikuchi *et al.*, 2000; Shibata *et al.*, 2000; Kikuchi *et al.*, 2002). Such molecular dissection, allowing quantitative analyses of oxidative pathways should be useful for diagnostic and therapeutic approaches. The use of highly selective mass spectrometry-based techniques could help to further delineate the potential pathogenic role of oxidative stress in ALS. Several markers could be used, such as glutamic semialdehyde (GSA), which derives from the metal-catalysed oxidation of proline and arginine; or aminoadipic semialdehyde (AASA) which results from lysine oxidation (Requena *et al.*, 1997, 2001, 2003; Dalfo *et al.*, 2005). Besides these direct modifications of protein structures, the effects of free radical efflux in proteins could also involve third-party molecules which also give rise to increased damage, such as carbohydrates and/or lipids, in processes termed glycoxidation and lipoxidation, respectively (Requena *et al.*, 1997; Baynes, 2003). Both carbohydrates and polyunsaturated fatty acids, when reacting with free radicals generate highly reactive dicarbonyl compounds, such as glyoxal, methylglyoxal, 4-hydroxynonenal and malondialdehyde, among others. These reactive carbonyl compounds can generate specific non-enzymatic adducts when reacting with proteins, such as N^{ε}-carboxymethyl-lysine (CML), N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-malondialdehyde-lysine (MDAL) (Baynes and Thorpe, 2000). The high content of polyunsaturated fatty acid in central nervous system and its elevated oxygen consumption support the possible significance of lipid peroxidation-derived processes in neurodegeneration, including ALS. However, there is no chemical evidence for lipoxidative or glycoxidative damage of proteins in sporadic or familial ALS based on structural identification and supported by mass-spectrometry.

For these reasons, in this work we have studied the development of ER stress in sporadic ALS. We examined ER stress causal factors such as proteasome function, protein oxidative damage, fatty acid composition and potential disturbances in mitochondrial respiratory complexes (as the major sources of free radical efflux). These changes have been evaluated in human samples and in lumbar spinal cord organotypic cultures under chronic excitotoxicity, a well-supported model of the sporadic form of ALS (Rothstein *et al.*, 1993).

Patients and methods

Human spinal cord and brain specimens

Brain and spinal cord samples were obtained from the Institute of Neuropathology Brain bank following the guidelines of the local Ethics Committee. The brains and spinal cords of seven men and five age-matched controls (four men and one woman) were obtained from 3 to 6 h after death, and were immediately prepared for morphological and biochemical studies. The agonal state was short with no evidence of acidosis or prolonged hypoxia. The pH of the post-mortem brain was between 7 and 7.4. All ALS patients had suffered from clinical signs and symptoms of lower and upper motorneuron disease, finally involving motor nuclei of the medulla oblongata. Importantly, none of these patients had cognitive impairment or dementia. Although variable from one case to another, the terminal stage of the disease was characterized by predominant bulbar failure manifested as impaired swallowing and usually complicated by aspiration pneumonia, or by dominant respiratory insufficiency. Age-matched controls did not show clinical and neuropathological anomalies. Frozen samples of the spinal cord (lumbar enlargement) and frontal cortex area 8 were used for biochemical studies. Samples of control and diseased spinal cords and brains were processed in parallel. Summary of the main clinical and neuropathological aspects is shown in Table 1.

Organotypic cultures

Spinal cord cultures were prepared from lumbar spinal cord of postnatal day 8 rat pups as previously described (Rothstein *et al.*, 1993) and maintained in 50% minimal essential medium, 25 mM Hepes, 25% Hanks balanced salt solution with D-glucose 25.6 mg/l, 25% heat-inactivated horse serum, 2 mM L-glutamine. Incubation at DIV7 of the slices with the glutamate transport inhibitor D,L-threo-hydroxyaspartate (THA) at 200 μM injures motorneurons with a morphology typical of excitotoxic degeneration after several weeks of treatment. In selected experiments, slices were also incubated with the ER stress inducers thapsigargin (32 ng/ml) and tunicamycin (500 and 5 ng/ml). After 15 or 30 days of treatment, cultures were harvested and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C and processed for immunocytochemistry. For western-blot and mass-spectrometric measurements slices were washed with PBS containing 1 mM diethylenetriaminepentaacetic acid and 1 μM butylated hydroxyl toluene, harvested and frozen at −80°C. Experiments for each condition ($n = 30$ slices per experimental group) were repeated at least three times.

Immunohistochemistry

De-waxed sections 5-μm-thick of the spinal cord were processed for immunohistochemistry following the streptavidin LSAB method (Dako). After incubation with methanol and H_2O_2 in PBS and normal serum, the sections were incubated with anti-phosphorylated eukaryotic initiation factor 2α (eIF2α) (1:100, Abcam, UK). Following incubation with the primary antibody, the

Table 1 Summary of clinical and pathological data in the present series

Case	Age	Gender	Diagnosis	Duration	Cause of death	p-m delay
1	41	M	ALS	3	Dysphagia, bronchopneumonia	3
2	68	M	ALS	5	Respiratory failure	3
3	76	M	ALS	4	Dysphagia, bronchopneumonia	3
4	69	M	ALS	4	Dysphagia, bronchopneumonia	2
5	40	M	ALS	3	Respiratory failure	2
6	73	M	ALS	4	Dysphagia, bronchopneumonia	4
7	71	M	ALS	4	Respiratory failure	6
8	75	M	Control		Neoplasia	6
9	76	M	Control		Cardiac infarction	4
10	67	F	Control		Cardiac infarction	3
11	51	M	Control		Bronchopneumonia	4
12	68	M	Control		Neoplasia	6

Note: ALS: amyotrophic lateral sclerosis; p-m delay: post-mortem delay (in hours); age and duration are in years.

sections were incubated with LSAB for 15 min at room temperature. The peroxidase reaction was visualized with diaminobenzidine and H_2O_2. Control of the immunostaining included omission of the primary antibody; no signal was obtained following incubation with only the secondary antibody. Sections were slightly counterstained with haematoxylin.

Confocal immunocytochemistry

The antibodies used are listed in the supplementary information (Table S1). Fluorescein-conjugated *Bandeiraea simplicifolia* lectin (type I) was used as a label for microglia. Appropriate secondary antibodies: Alexa Fluor 488 F(ab)$_2$ fragment of goat anti-mouse IgG (1:500, Molecular Probes, USA) and Alexa Fluor 546 goat anti-rabbit IgG (1:500, Molecular Probes, USA) were used for immunofluorescence. Image analyses were carried out by a single investigator who was blinded to the experimental conditions. Large (>25 μm in diameter) SMI-32-immunopositive neurons were identified as motorneurons. Immunocytochemical controls were performed by the omission of the primary antibodies, resulting in a negative immunostaining in all cases studied. Mounted slices were examined under a FluoView 500 Olympus confocal laser scanning microscope (Hamburg, Germany).

Protein electrophoresis and western blot

Samples (spinal cord, frontal cortex or organotypic spinal cord cultures) were homogenized in a buffer containing 180 mM KCl, 5 mM 3-[*N*-morpholino]propanesulfonic acid, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM diethylenetriaminepentaacetic acid and 1 μM butylated hydroxyl toluene, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, pH 7.3 with a Potter–Eljeveim device at 4°C. After a brief centrifugation (500 × *g*, 5 min) to pellet cellular debris, protein concentrations were measured in the supernatants using the Lowry assay (BioRad Laboratories, München, Germany).

For detection of protein carbonyls, and prior to electrophoresis, samples were derivatized with 2,4-dinitrophenylhydrazine (DNP) as previously described (Pamplona *et al.*, 2005). Briefly, to 15 μl homogenates adjusted to 3.75 μg/μl protein SDS was added to a final concentration of 6%, and, after boiling for 3 min, 20 μl of 10 mM DNP in 10% trifluoroacetic acid were added. After 7 min at room temperature, 20 μl of a solution containing 2 M Tris base, 30% glycerol and 15% β-mercaptoethanol were added for neutralization and sample preparation for loading onto SDS–PAGE gels.

For immunodetection, after SDS–PAGE, proteins were transferred using a Mini Trans-Blot Transfer Cell (BioRad) to PVDF membranes (Immobilon-P Millipore, Bedford, MA). Immunodetection was performed using as primary antibodies those listed in supplementary information (Table S1): (i) for ER stress and proteasome function assessment: anti-KDEL, which recognizes KDEL-sequence containing proteins such as ER chaperones as protein disulfide isomerase (PDI), GRP78 and GRP94; anti-eIF2α anti-GRP78, anti-phosphorylated eIF2α (S52), anti-PDI and anti-ubiquitin; (ii) for protein oxidative damage: anti-DNP antibody, anti-CML, anti-neuroketal and anti-MDAL; and (iii) for mitochondrial studies: anti-NDUFA 9 antibody for respiratory complex I, anti-core II antibody for respiratory complex III and anti-apoptosis inducing factor (AIF) antibody. For detection of primary antibodies, the following peroxidase-coupled secondary antibodies were used: sheep anti-mouse (1:30 000, Amersham, USA); anti-rabbit (1:40 000, Pierce, USA) and anti-goat (1:7500, Abcam, Cambridge, UK) antibodies. Luminescence was recorded and quantified in a Lumi-Imager equipment (Boehringer, Mannheim, Germany), using the Lumianalyst software. Control experiments showed that omission of primary or secondary antibody addition produced blots with no detectable signal.

Measurement of specific, protein-oxidation-derived markers: GSA, AASA, CML, CEL and MDAL

GSA, AASA, CML, CEL and MDAL concentrations in total proteins from spinal cord, frontal cortex or organotypic culture homogenates were measured by isotope-dilution gas chromatography/mass spectrometry (GC/MS) as previously described (Pamplona *et al.*, 2005). Samples containing 500 μg of protein were delipidated using chloroform:methanol (2:1 v/v), and proteins were precipitated by adding 10% trichloroacetic acid (final concentration) and subsequent centrifugation. Protein samples were reduced overnight with 500 mM $NaBH_4$ (final concentration) in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-foam reagent. Proteins were then reprecipitated by adding 1 ml of 20% trichloroacetic acid and subsequent centrifugation. The following isotopically labelled internal standards were then added: [$^2H_8$]Lysine (*d8*-Lys; CDN Isotopes); [$^2H_4$]CML (d4-CML), [$^2H_4$]CEL (*d4*-CEL) and [$^2H_8$]MDAL (*d8*-MDAL); [$^2H_5$] 5-hydroxy-2-aminovaleric acid (for GSA analysis) and [$^2H_4$]6-hydroxy-2-aminocaproic acid (for AASA analysis). The samples were hydrolysed at 155°C for 30 min in 1 ml of 6 N HCl, and then dried *in vacuo*. The *N,O*-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared and GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30 m HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector (Agilent, Barcelona, Spain). The injection port was maintained at 275°C; the temperature program was 5 min at 110°C, then 2°C/min to 150°C, then 5°C/min to 240°C, then 25°C/min to 300°C and

finally hold at 300°C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analytes were detected by selected ion-monitoring GC/MS. The ions used were: lysine and *d8*-lysine, *m/z* 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and *d5*-5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), *m/z* 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and *d4*-6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), *m/z* 294 and 298, respectively; CML and *d4*-CML, *m/z* 392 and 396, respectively; CEL and *d4*- CEL, *m/z* 379 and 383, respectively; and MDAL and *d8*-MDAL, *m/z* 474 and 482, respectively. The amounts of products were expressed as the ratio μmol GSA, AASA, CML, CEL or MDAL/mol lysine.

Fatty acid analysis

Distributional analysis of fatty acids was performed as previously described (Dalfo *et al.*, 2005; Pamplona *et al.*, 2005). Total lipids from spinal cord, frontal cortex or organotypic cultures were extracted with chloroform:methanol (2:1, v/v) in the presence of 0.01% butylated hydroxytoluene to avoid artifactual oxidation. The chloroform phase was evaporated under nitrogen, and the fatty acids were transesterified by incubation in 2.5 ml of 5% methanolic HCl for 90 min at 75°C. The resulting fatty acid methyl esters were extracted by adding 2.5 ml of *n*-pentane and 1 ml of saturated NaCl solution. The *n*-pentane phase was separated, evaporated under nitrogen, re-dissolved in 75 μl of hexane and 1 μl was used for GC/MS analysis. Separation was performed in a SP2330 capillary column (30 m × 0.25 mm × 0.20 μm) in a Hewlett Packard 6890 Series II gas chromatograph (Agilent, Barcelona, Spain). A Hewlett Packard 5973A mass spectrometer was used as detector in the electron-impact mode. The injection port was maintained at 220°C, and the detector at 250°C; the temperature program was 2 min at 100°C, then 10°C/min to 200°C, then 5°C/min to 240°C and finally hold at 240°C for 10 min. Identification of fatty acid methyl esters was made by comparison with authentic standards and based on mass spectra. Results are expressed as mol%.

From fatty acid composition, the following indexes were calculated: saturated fatty acids (SFA) = ∑% of saturated fatty acids; unsaturated fatty acids (UFA) = ∑% unsaturated fatty acids; monounsaturated fatty acids (MUFA) = ∑% of monoenoic fatty acids; polyunsaturated *n*-3 fatty acids (PUFAn-3) = ∑% of polyunsaturated fatty acids *n*-3 series; Polyunsaturated n-6 Fatty Acids (PUFAn-6) = ∑% of polyunsaturated fatty acids *n*-6 series; average chain length (ACL) = $[(\sum\%\text{Total}_{14} \times 14) + \cdots + (\sum\%\ \text{Total}_{n} \times n)]/100$ (*n* = carbon atom number); peroxidizability index (PI) = [(∑ mol% Monoenoic × 0.025) + (∑ mol% Dienoic × 1) + (∑ mol% Trienoic × 2) + (∑ mol% Tetraenoic × 4) + (∑ mol% Pentaenoic × 6) + (∑ mol% Hexaenoic × 8)].

Statistical analyses

All statistics were performed using the SPSS software (SPSS Inc., Chicago, IL). Once normality of distribution was assessed by Kolmogorov–Smirnov test, differences between groups (ALS samples versus control; THA treated versus vehicle) were analysed by the Student's t-tests and correlations between variables were evaluated by the Pearson's statistic. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Results

Samples from ALS patients evidence signs of ER stress: proteasomal impairment as a potential mechanism

Since ER stress has been implied in ALS experimental models we examined signs of ER stress by western-blot and immunohistochemistry in samples from ALS patients. The results demonstrated ER stress in ALS samples (Fig. 1). Thus, increased expression of chaperones PDI and KDEL-containing proteins were found in spinal cord from ALS patients (Fig. 1A), but not in frontal cortex samples (not shown). This was accompanied by a marked increase in eIF2α phosphorylation (a sign of protein synthesis control after ER stress), both in spinal cord lysates (Fig. 1A) and in remaining neuronal bodies in spinal cord (Fig. 1B).

As proteasome is responsible for degradation of ER misfolded proteins, we examined whether its function was preserved. Accounting that ubiquitin-modified proteins are degraded by the proteasome, its function can be inferred by western-blot and immunohistochemical analyses of ubiquitin-modified proteins. The results of such approach showing increased protein ubiquitination in spinal cord from ALS patients, but not in frontal cortex, are compatible with compromised proteasomal function (Fig. 1C). It should be also considered that increased protein turnover due to massive protein degradation could saturate the ubiquitin-proteasome system in spite of proteasome preserved activity.

Proteins from spinal cord and frontal cortex present structurally characterized oxidation products and the amount of these modifications increases with ALS, favoured by changes in fatty acid composition

While moderate oxidative modification of proteins increases their susceptibility for proteasome clearance, higher rates of oxidative modification actually inhibit proteasome activity. Therefore, we evaluated protein oxidative damage in spinal cord and frontal cortex samples, to ascertain whether proteasomal dysfunction could be related to increased protein oxidative damage. GC/MS analyses demonstrated that those proteins contained oxidation-derived products resulting from metal-catalysed oxidation, glycoxidation and lipoxidation. The more abundant products were those derived from metal-catalysed oxidation, AASA and GSA assuming almost 95% of measured markers. GSA stood as the more frequent modification, with levels being 30-fold higher than of those of AASA. The concentrations of both GSA and AASA were significantly higher in proteins from spinal cord ($P < 0.001$) and frontal cortex ($P < 0.01$) samples of ALS patients than in control age-matched individuals (Fig. 2A). Similarly to specific oxidation products, the concentrations of CEL and CML, glycoxidation products, were also significantly higher in

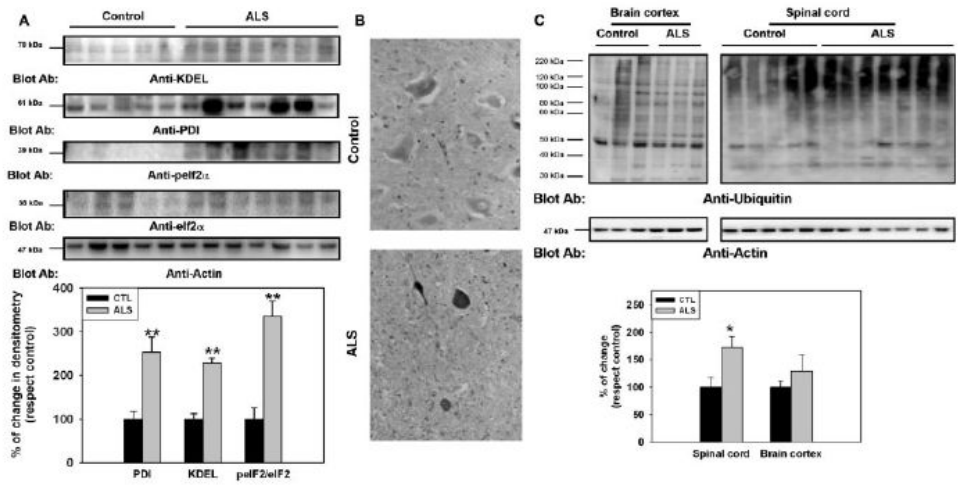

Fig. 1 ER stress and protein ubiquitination in spinal cords from ALS patients. (**A**) Representative western blot of spinal cord homogenates showing increased content of ER resident proteins containing KDEL motifs, PDI and phosphorylated eIF2α in samples from ALS patients. (**B**) Representative immunohistochemical image of phosphorylated eIF2α in the motor column of human lumbar spinal cords, showing increased staining in ALS samples. (**C**) Representative western blot of anti-ubiquitin, suggesting decreased ubiquitin degradation in spinal cord samples from ALS patients and control individuals, but not in frontal cortex. *Right numbers* of the blot indicate apparent molecular weight. Immunoblotting of actin is also shown. The *lower panels* shows the quantitation of those blots by densitometry, adjusted to actin content and differences were analysed respect to control group by Student's *t*-test being $^{*}P < 0.01$ and $^{**}P < 0.001$.

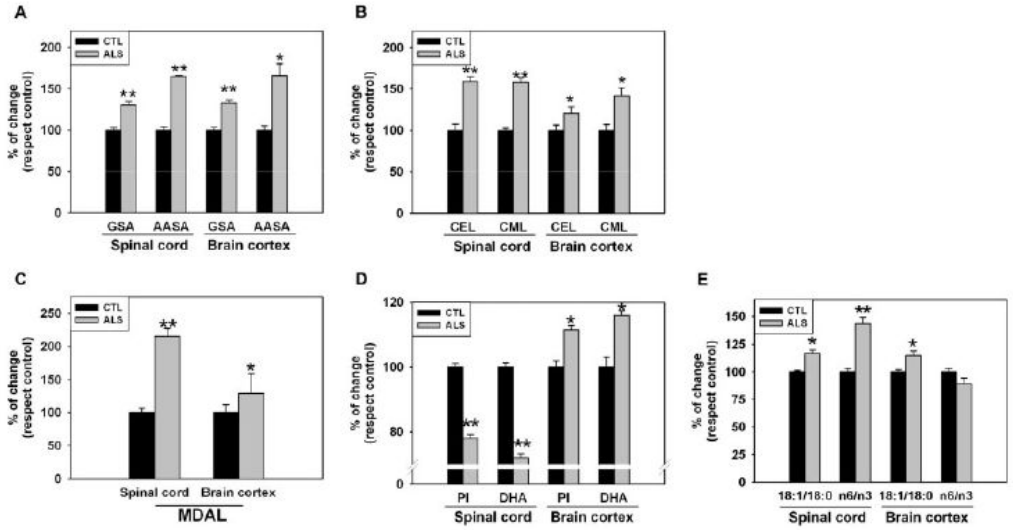

Fig. 2 Proteins from ALS samples show significant increases in the amounts of oxidation markers and changes in fatty acid composition. **A–C** show GC/MS analyses of GSA and AASA (markers of MCO), CML and CEL (arising from glycoxidation and lipoxidation) and MDAL, originated from lipoxidation. (**D**) Changes in PI and DHA levels associated with ALS in spinal cord differ from those present in frontal cortex. (**E**) ALS also leads to organ-specific changes in desaturation indexes (18:1/18:0) and *n*6/*n*3 ratios. Values shown are% changes of mean ± SE over values from control samples (in spinal cord GSA: 21498 ± 619 µmol/mol lysine; AASA: 102 ± 7 µmol/mol lysine; CEL: 385 ± 57 µmol/mol lysine; CML: 521 ± 30 µmol/mol lysine and MDAL: 258 ± 48 µmol/mol lysine; PI: 116.24 ± 4.23; DHA: 10.89 ± 0.20%; 18:1/18:0 ratio: 1.36 ± 0.025; *n*6/*n*3 ratio: 0.67 ± 0.04; in frontal cortex GSA: 20512 ± 638 µmol/mol lysine; AASA: 82 ± 4 µmol/mol lysine; CEL: 249 ± 16 µmol/mol lysine; CML: 522 ± 37 µmol/mol lysine MDAL: 197 ± 6 µmol/mol lysine; PI: 155.31 ± 1.55; DHA: 12.35 ± 0.17; 18:1/18:0 ratio: 0.75 ± 0.01; *n*6/*n*3 ratio: 1.1 ± 0.034). $^{*}P < 0.01$ and $^{**}P < 0.001$ respect to control group by Student's *t*-test.

Table 2 Fatty acid composition (mol%) of total lipids from spinal cord and frontal cortex

	Frontal cortex CTL	ALS	P<	Spinal cord CTL	ALS	P<
14:0	0.50 ± 0.03	0.59 ± 0.05	0.234	0.49 ± 0.04	0.58 ± 0.01	0.112
16:0	24.57 ± 0.48	23.97 ± 0.35	0.352	14.61 ± 0.25	15.47 ± 0.23	0.02
16:1*n*-7	1.63 ± 0.15	1.64 ± 0.25	0.980	1 ± 0.07	1.53 ± 0.15	0.005
18:0	25.20 ± 0.40	22.92 ± 0.39	0.007	23.95 ± 0.23	22.34 ± 0.68	0.027
18:1*n*-9	19.09 ± 0.15	19.90 ± 0.45	0.141	30.15 ± 0.25	32.82 ± 0.25	0.001
18:2*n*-6	1.02 ± 0.28	0.77 ± 0.13	0.458	0.33 ± 0.03	0.62 ± 0.05	0.001
18:3*n*-3	n.d	n.d	–	0.24 ± 0.02	0.27 ± 0.001	0.254
20:0	0.88 ± 0.20	0.57 ± 0.04	0.190	7.05 ± 0.43	6.28 ± 0.25	0.117
20:1	n.d	n.d	–	0.54 ± 0.03	0.78 ± 0.07	0.016
20:2*n*-6	0.47 ± 0.16	0.29 ± 0.07	0.345	0.31 ± 0.03	0.31 ± 0.05	0.997
20:3*n*-6	0.36 ± 0.04	0.25 ± 0.05	0.210	0.6 ± 0.08	0.98 ± 0.25	0.137
20:4*n*-6	7.39 ± 0.23	8.70 ± 0.22	0.007	2.9 ± 0.17	3.15 ± 0.11	0.508
22:4*n*-6	4.16 ± 0.22	3.78 ± 0.42	0.460	2.86 ± 0.05	3.05 ± 0.14	0.289
22:5*n*-6	0.72 ± 0.14	0.66 ± 0.18	0.794	0.27 ± 0.03	0.22 ± 0.03	0.173
22:5*n*-3	0.17 ± 0.01	0.20 ± 0.01	0.170	0.14 ± 0.02	0.19 ± 0.03	0.118
24:0	0.61 ± 0.07	0.54 ± 0.04	0.466	0.91 ± 0.13	0.81 ± 0.15	0.600
24:1*n*-9	0.47 ± 0.14	0.56 ± 0.05	0.557	2.26 ± 0.10	2.23 ± 0.10	0.793
ACL	18.41 ± 0.02	18.49 ± 0.02	0.055	18.67 ± 0.01	18.51 ± 0.01	0.001
SFA	51.78 ± 0.58	48.60 ± 0.56	0.008	46.21 ± 0.37	44.55 ± 0.65	0.001
UFA	48.21 ± 0.58	51.39 ± 0.56	0.008	52.59 ± 0.35	54.92 ± 0.45	0.001
MUFA	21.20 ± 0.04	22.11 ± 0.73	0.263	33.84 ± 0.31	37.6 ± 0.46	0.001
PUFA	27.00 ± 0.58	29.28 ± 0.60	0.037	18.7 ± 0.22	16.61 ± 0.4	0.002
PUFA *n*-6	14.13 ± 0.33	14.47 ± 0.69	0.676	7.46 ± 0.18	8.39 ± 0.19	0.005
PUFA *n*-3	12.87 ± 0.40	14.81 ± 0.22	0.006	11 ± 0.15	8.63 ± 0.12	0.001
DBI	152.18 ± 2.68	166.92 ± 1.70	0.004	127.82 ± 1.58	119.69 ± 1.59	0.003

Note: Values: mean ± SEM. *N* × group: for brain cortex ($n = 4$); for spinal cord ($n = 5$ for control and $n = 7$ for ALS). ACL, average chain length; SFA, saturated fatty acids; UFA, unsaturated fatty acids; PUFA *n*-6/*n*-3, polyunsaturated fatty acids *n*-6 or *n*-3 series; MUFA, monounsaturated fatty acids; DBI, double bond index; Docosahexaenoic acid levels are shown in Fig. 1.

proteins from spinal cord ($P < 0.001$) and frontal cortex ($P < 0.01$) of ALS patients than in control individuals (Fig. 2B). The concentration of MDAL, a lipoxidation product, was also increased in samples from ALS patients, both in spinal cord ($P < 0.001$) and in frontal cortex ($P < 0.01$) compared to control individuals (Fig. 2C). Nonetheless, the magnitude of difference between ALS and control samples was considerably higher in spinal cord (120%) than in frontal cortex (50%).

As fatty acid profile strongly influences membrane peroxidizability, and consequently protein lipoxidative damage, we analysed fatty acid content in ALS samples. Those analyses revealed significant differences associated with ALS in spinal cord and frontal cortex, both in individual fatty acids and in global indexes (Table 2). The most remarkable change involves the highly peroxidizable docosahexaenoic acid (DHA), which showed a significant decrease in spinal cord samples with ALS ($P < 0.01$), contrasting with the significant increase observed in frontal cortex ($P < 0.01$; Fig. 2D). With reference to the fatty-acid-derived indexes, spinal cords from ALS patients showed significant decreases in the content of PUFA of the *n*-3 family ($P < 0.001$; Table 2), while increases of this parameter were detected in frontal cortex ($P < 0.006$; Table 2). Changes in fatty acid profile led to significant changes in double bond index ($P < 0.004$; Table 2), PI ($P < 0.003$; Fig. 2D), Δ9-desaturase estimation ($P < 0.01$; Fig. 2E) and *n*6/*n*3 ratio ($P < 0.001$; Fig. 2E). Overall, these indexes could reflect the potential vulnerability of membranes to peroxidative damage. Since changes in double bond and PI in spinal cord were inverse to those observed in frontal cortex, these data suggest that spinal cord membranes are actively producing substrates for peroxidative modification of proteins, while as frontal cortex are not under this circumstance. Accordingly, analysis of MDAL/PI ratio, suggest that for a given PI, rates of MDAL formation are 3-fold higher in spinal cord than in frontal cortex from ALS patients.

Different kinds of protein oxidative damage are correlated together and are associated to changes in fatty acid content

After quantitations of protein oxidation and fatty acid analyses, several significant correlations were present among anatomically different locations (Fig. 3). GSA levels correlated significantly with AASA ($r = 0.918$; $P < 0.0001$; Fig. 3A), with MDAL ($r = 0.865$; $P < 0.0001$; Fig. 3B), with CEL ($r = 0.716$; $P < 0.002$) and with CML ($r = 0.878$; $P < 0.0001$). This suggests that protein carbonyl formation

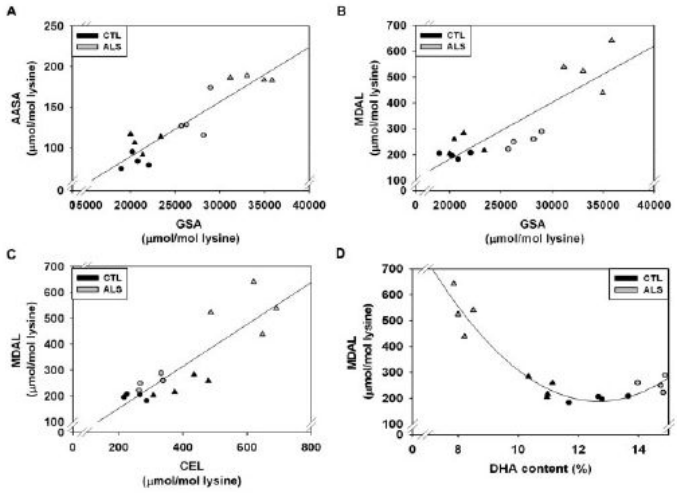

Fig. 3 Changes in protein oxidation indexes and fatty acid contents are strongly correlated. Indexes of protein carbonyl GSA and AASA are strongly correlated (upper panel, left; $r = 0.92$; $P < 0.0001$). Protein oxidative damage is also correlated with protein lipoxidative damage (upper panel, right; $r = 0.865$; $P < 0.0001$). Protein lipoxidative damage is correlated to glycoxidative modifications (lower panel, left; $r = 0.865$; $P < 0.0001$). DHA content shows a quadratic relationship with protein lipoxidative damage (lower panel, right; $r = 0.95$; $P < 0.0001$; model: $[MDAL] = 17.3^{*}[DHA]^{2} - 440.3^{*}[DHA] + 2977$). Triangles (%) represent values from spinal cord samples and circles (○) represent values from frontal cortex. Results are only shown for those samples were both frontal cortex and spinal cord were available ($n = 4$ for each group).

is also associated to glycoxidative and lipoxidative modifications in spinal cord and frontal cortex. Furthermore, peroxidizability index was inversely correlated with CEL ($r = -0.816$; $P < 0.0001$) and MDAL ($r = -0.685$; $P < 0.003$) concentrations, suggesting an association between lipid peroxidizability, glycoxidation and lipoxidation modifications. Interestingly, levels of MDAL display a second-order relationship with DHA levels ($r = 0.961$; $P < 0.0001$; Fig. 3D), suggesting an intimate interplay between these two factors.

Protein oxidative damage, showing preferential targets in ALS spinal cord proteins can be associated to mitochondrial disturbances

It is known that an important determinant for oxidative damage of proteins, besides fatty acid content, is the mitochondrial free radical production. Therefore, we analysed the expression of representative subunits of mitochondrial respiratory complexes I and III, whose activity is counted among the major sources for mitochondrial free radical production (Herrero and Barja, 2000; Chen *et al.*, 2003), as well as the levels of AIF, shown recently to enhance the functional stability of complex I (Vahsen *et al.*, 2004). These analyses demonstrated that both complex I and III concentrations are significantly decreased in spinal cord samples from ALS patients ($P < 0.01$; Fig. 4A) while AIF expression is not changed in ALS (Fig. 4A). Reinforcing an apparently different pace of ALS-induced changes between frontal cortex and spinal cord, these effects were not present in frontal cortex from ALS patients (data not shown).

Western-blot analyses of frontal cortex and spinal cord proteins showed differences in the distribution of oxidation (DNP-reactive), glycoxidation (anti-CML) and lipoxidation-derived (anti-neuroketal) protein modifications (Fig. 4B) supporting both diversity and specificity of protein oxidative damage. These findings agree with quantitative analyses by GC/MS as densitometric measurements revealed increased oxidative damage in ALS samples. Major targets of glycoxidation were proteins with apparent molecular weights ranging from 35 to 55 kDa, partially coincident with anti-DNP immunoreactivity (which was also evident for high-molecular weight bands). This pattern differs from targets of neuroketal formation (Fig. 4B), which showed more discrete targets (being targets of 40 and 60 kDa the more prominent). These differences were not present in frontal cortex samples (data not shown).

Chronic excitoxicity in organotypic spinal cord cultures leads ER stress, ubiquitin alterations, protein oxidative damage and changes in fatty acid profile

Chronic excitotoxicity has been implied in the pathogenesis of ALS (Rothstein *et al.*, 1993; Boillee *et al.*, 2006). This can be reproduced *in vitro* by treatment with the pre-synaptic

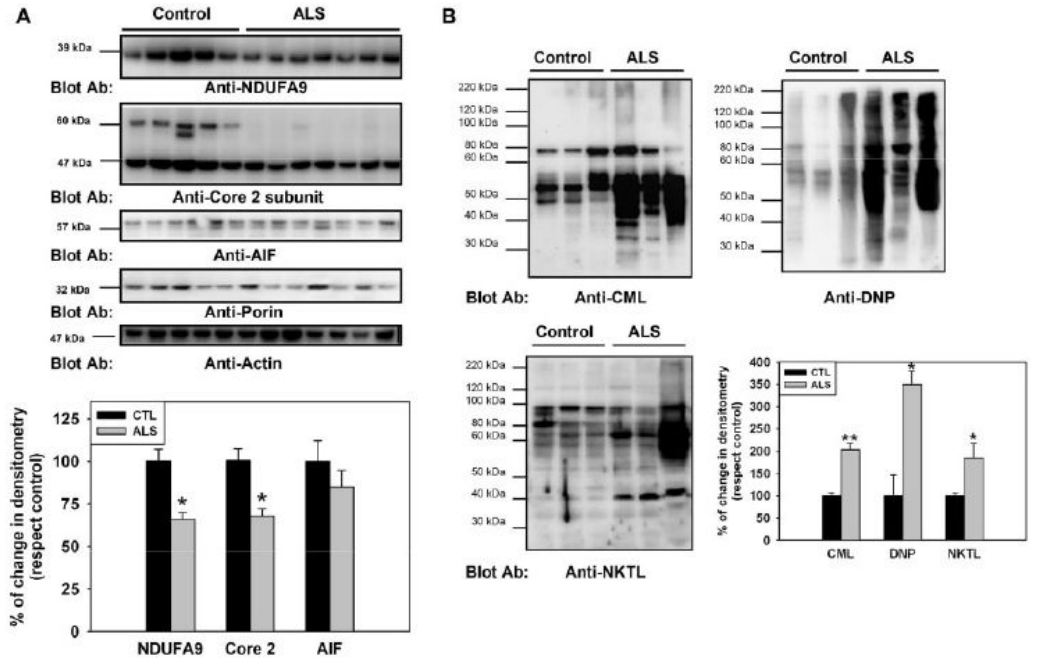

Fig 4 ALS-induced mitochondrial changes and protein-oxidation-specific targets in spinal cord. (**A**) Representative western blot of peptides NDUFA9 and core 2 subunits of mitochondrial respiratory chain complexes I and III, respectively, suggesting decreased complex III content in spinal cord samples from ALS patients compared to control individuals. This was not associated with increased amount of apoptosis-inducing factor (AIF). The *lower panel* shows the quantitation of these blots by densitometry, adjusted to porin density and differences were analysed respect to control group by Student's *t*-test being $^{*}P < 0.01$. (**B**) Western blot for mixed glycoxidation/lipoxidation (CML), MCO (DNP) and lipoxidation [neuroketal (NKTL)] revealed differential targets for each of these oxidative pathways. *Right numbers* of the blots indicate apparent molecular weight. The *lower panels* show the quantization of these blots by densitometry, after densities of bands (ranging between 30 and 220 kDa) or actin (not shown, for B), and differences were analysed respect to control group by Student's *t*-test being $^{*}P < 0.01$ and $^{**}P < 0.001$.

glutamate transport inhibitor THA, leading to losses in the number of motorneurons, as assessed by SMI-32 immunoreactivity (data not shown). In accordance to findings in ALS samples, signs of ER stress were also found in spinal cord slices under chronic excitotoxicity after a 30-day period (Figs. S1A and S2A). Although eIF2α phosphorylation was present in neuronal bodies of vehicle-treated slices, increased immunoreactivity was found along neuritic processes as well as in small cellular populations morphologically compatible with glia on THA-treated slices. Furthermore, demonstration of a markedly increased intracellular ubiquitin immunoreactivity excluding nucleus (Fig. S1B) suggest a role of proteasomal dysfunction in this phenomena at an earlier stage. Furthermore, incubation with the ER stress inducing agents tunicamycin and thapsigargin led to increases in chaperone expression (Fig. S2A) and to a marked decrease in the number of motorneurons, suggesting its preferential sensitivity to ER stress (Fig. S2B).

Since those analyses revealed ER stress and proteasome dysfunction in chronic excitotoxicity, we analysed whether protein oxidative damage was also increased in these conditions. The results demonstrated that protein oxidative damage is increased by excitotoxicity (Fig. S3). All measured markers of oxidation, glycoxidation and lipoxidation, increased significantly after THA treatment ($P < 0.001$, Fig. S3A). As in the ALS cases, these increases in protein oxidative modifications were associated to changes in fatty acid composition (Table S2, Fig. S3B). Noteworthy, THA-induced changes resembled those present in frontal cortex, with 2-fold increases in the DHA content ($P < 0.0001$, Fig. S3B), PI increases—150% respect vehicle-treated slices—($P < 0.0001$, Fig. S3B) and decreased $n6/n3$ ratios ($P < 0.0001$, Fig. S3B). Confocal microscopy of the most THA-sensitive marker, CEL, revealed that proteins modified with this product were present, in increased amounts, throughout glial and neuronal populations (Fig. S3C). Neuropil and star-shaped cells resembling microglia were major distribution sites of CEL immunoreactivity in THA-treated slices. Co-localization studies with *Bandairaea Simplicifolia* lectin supported the microglial origin of some of those cells (Fig. S3C).

Nevertheless, chronic excitotoxicity also led to increased AIF immunoreactivity in a non-nuclear distribution, suggesting common basis for mitochondrial dysfunction between sporadic ALS and chronic excitotoxicity both in neuronal and glial cells (Fig. S1C).

Discussion

Cells facing protein misfolding in the ER initiate the so-called unfolded protein response. As a part of this unfolded protein response, the initiation process in global protein synthesis is repressed by eIF2α phosphorylation via PERK kinase. There is also increased expression of ER chaperones and, if ER stress cause is not corrected, apoptosis is induced (Marciniak and Ron, 2006; Zhang and Kaufman, 2006). The results reported here, showing both increased phosphorylation of eIF2α and increased expression of ER chaperones (PDI and KDEL-containing proteins), strongly support the participation of ER stress in ALS pathogenesis. The relevance of this pathway was also evident in the chronic excitotoxicity paradigm, where both motorneurons and glial cells were stained for anti-phosphorylated eIF2α. Noteworthy, the importance of this pathway is also demonstrated by immunohistochemical analyses, where remaining neuronal bodies of spinal cord ventral horns in ALS show intense anti-phosphorylated eIF2α. Our data suggest that ER stress arises from oxidative stress and from a mitochondrial disturbance. Based on correlative data, we propose that this is a late phenomenon, when compared with protein oxidative damage, as we are able to detect increased protein oxidative damage, increased DHA amount and other fatty acid changes in brain cortex, without increased ubiquitination or any other noticeable change. Recent data demonstrates the occurrence of ER stress in familial ALS paradigms, associating PDI and mutated SOD in motorneurons (Atkin *et al.*, 2006; Furukawa *et al.*, 2006), showing also caspase-12 activation (Wootz *et al.*, 2004; Turner and Atkin, 2006). More interestingly, chronic excitotoxicity leads to protein aggregation in ER from motorneurons (Tarabal *et al.*, 2005), without inducing an upregulation of heat-shock proteins, as those cells have a characteristically high threshold for heat-shock proteins induction (Batulan *et al.*, 2003). This fact, when added to the increased chaperone expression and immunohistochemical data on organotypic cultures, strongly suggest that ER stress is also taking place in glial cells. Due to the important role of the proteasome in ER-directed disposal of misfolded proteins, ER stress could be caused by decreased ER-associated degradation (Bush *et al.*, 1997; Obeng *et al.*, 2006; Yamamuro *et al.*, 2006).

In agreement with our findings, previous data indicate a potential loss of proteasome activity in ALS (Urushitani *et al.*, 2002; Cheroni *et al.*, 2005; Ahtoniemi *et al.*, 2006; Basso *et al.*, 2006; Kabuta *et al.*, 2006; Koyama *et al.*, 2006; Mendonca *et al.*, 2006), specially in the familial forms of the disease. *In vitro* experiments and *in vivo* data show that mutated SOD is associated with decreased amount of specific proteasome subunits, particularly LMP7 (Allen *et al.*, 2003). Furthermore, proteasome inhibition leads to the reproduction of the abnormal solubility properties shown by mutated SOD *in vivo* (Koyama *et al.*, 2006). However, the role of proteasome in sporadic ALS has received less attention. Recent data demonstrate increased proteasome immunoreactivity both in glia and motorneurons from spinal cords in sporadic ALS (Mendonca *et al.*, 2006). Accordingly, findings reported here, demonstrating increased amount of ubiquitinated proteins in spinal cord samples, is compatible to such proteasomal involvement. It should be recalled that ubiquitinated lesions are prominent in ALS morphology. Noteworthy, this increased ubiquitination was also evident under chronic excitotoxicity, being present both in neuronal and non-neuronal populations. This agrees with the previous works, showing that proteasome inhibition in organotypic cultures induce selectivity damage to motorneurons, as chronic excitotoxicity does (Tsuji *et al.*, 2005). As chronic excitotoxicity leads to increased oxidative damage (Rao and Weiss, 2004; Rival *et al.*, 2004), our data suggest that this condition could contribute to proteasome inhibition. Relating differences with frontal cortex, western-blot analyses did not reveal such changes in this latter location. Once a given protein is modified by oxidation, it is degraded by 20S proteasome in an ATP and ubiquitin-independent fashion (Shringarpure *et al.*, 2003; Grune *et al.*, 2005), as recently shown for metal-free SOD (Di Noto *et al.*, 2005). Nevertheless, a high degree of oxidative modification could even decrease proteasome activity (Sitte *et al.*, 2000).

For these reasons we evaluated whether, by using novel mass spectrometry measurements, ALS samples presented increases in protein oxidative damage, both in spinal cord and in frontal cortex. Nonetheless, the magnitude of changes with reference to control was higher in spinal cord than in frontal cortex. These results suggest an early selective involvement of oxidative stress in spinal cord during ALS pathogenesis. In addition, the involvement of frontal cortex agrees with previous reported data in other neurodegenerative diseases, such as Alzheimer disease or Parkinson disease, where pathologically preserved locations of nervous system show incipient increases in protein oxidative damage, thought at a lower extent than classical targets of disease (Dalfo *et al.*, 2005; Pamplona *et al.*, 2005). Cognitive dysfunction and dementia have been reported as complications in a subgroup of patients with ALS, the majority of them presenting atrophy, neuronal loss and reactive gliosis in the frontal and temporal lobes (Kato *et al.*, 2003). Although the present study is not focused on ALS cases with frontotemporal dementia, these results point to the suggestion that frontal cortex is a vulnerable region to ALS. As a working hypothesis it can be proposed that modifications in the levels of certain lipids are predisposing factors to further cellular damage. Nevertheless, data presented here support the notion that

lipid peroxidation-associated processes seem to be the more sensible cellular oxidative phenomenon, based on MDAL values. Previous immunohistochemical evidences demonstrate increased lipid peroxidation in ALS (Hall *et al.*, 1998; Pedersen *et al.*, 1998). Furthermore, recent data showing accumulation of 4-oxo-2-nonenal DNA etheno adduct in brain cortex from ALS patients support the importance of lipid peroxidation-derived pathways (Shibata *et al.*, 2006). Interestingly, while PI is still increased in frontal cortex, basically due to increases in DHA content, a potential defensive response of nervous tissue (Akbar *et al.*, 2005), these indexes were decreased in spinal cord, suggesting a functional collapse and/or a lower content of neurons. Thus, while brain cortex could produce *n*-3-derived anti-inflammatory resolvins and docosatrienes (Hong *et al.*, 2003), spinal cord neurons would have decreased DHA availability due to increased lipoxidative consumption. Rather than being a general phenomena, the selectivity for changes in *n*-3 strongly suggest specific mechanisms of the disease depending on these fatty acids, maybe affecting biosynthetic pathways and/or membrane remodelling systems that deserve further studies. Nonetheless, long-chain unsaturated fatty acids contribute to the formation of cytotoxic aggregates of ALS-linked superoxide dismutase-1, thereby stressing the importance of fatty acid changes in ALS pathogenesis (Kim *et al.*, 2005). To shed further light on these issues we analysed a chronic excitotoxicity paradigm. The results demonstrated—at an early stage of the excitotoxic paradigm—changes partially resembling those present in frontal cortex, e.g. increases in all oxidative markers and increases in DHA content and PI. This was present at a time when motorneuron death is still not evident. In line with this, DHA induces resistance against excitotoxic degeneration of cholinergic neurones *in vivo*, leading to higher survival, lower dendritic involution and decreased axon degeneration (Hogyes *et al.*, 2003). Globally, these results suggest a pathological spectrum, driven by excitotoxicity, ranging between overt pathological manifestation (present in spinal cord) and more subtle changes, with variations in fatty acids as reactive changes and protein oxidative modifications as important signals of disease.

The significant correlations found between different types of protein oxidative damage suggest that ALS could influence general mechanisms determining protein oxidative modifications, such as free radical production (Pamplona and Barja, 2006). In most cells, the major sources of free radical production are mitochondrial respiratory complexes I and III (Herrero and Barja, 2000) although in brain some mitochondrial matrix enzymes also contribute to free radical production (Starkov *et al.*, 2004; Tretter and Adam-Vizi, 2004). The analyses performed in spinal cords suggest decreases in both respiratory complexes, as well to qualitative changes in complex III distribution. Due to the role of complex I and III as free radical generators (Chen *et al.*, 2003), the results may be compatible with increased free radical efflux by incorrect assembly of complexes or to metabolic reprogramming (Iuso *et al.*, 2006). These data complements previous reports demonstrating decreased activities of respiratory chain complexes I + III, II + III and IV, suggesting a loss of mitochondria in spinal cords from ALS patients (Wiedemann *et al.*, 2002). Moreover, specific decreases in the activities of complexes II are observed in a mutated SOD transgenic model (Jung *et al.*, 2002). Furthermore, losses of complex IV activity are present in the *Wobbler* mouse (Xu *et al.*, 2001). It may be suggested that ALS samples presented a defect in the assembly of mitochondrial respiratory complexes, that would lead to changes in free radical production (Rana *et al.*, 2000; Sellem *et al.*, 2005). It may be also hypothesized that this scenario could contribute to a more reduced state of Fe-S clusters, leading to increased free radical efflux (Herrero and Barja, 2000). In this line, the amount of AIF, a bifunctional flavoprotein with NADH oxidase activity involved in mitochondrial respiration and caspase-independent apoptosis, was unchanged in spinal cords. Besides an ALS-induced increased apoptotic rate in this tissue (Oh *et al.*, 2006), AIF could be also considered as a part of an adaptation response to increased free radical production (Zhu *et al.*, 2003; Cande *et al.*, 2004) or to decreased respiratory activities. Changes in AIF are not evident in ALS samples when compared to data in the *in vitro* paradigm: AIF presenting increased non-nuclear staining in THA-treated slices suggests an important role of this protein in the response to excitotoxicity, that may be focus of future studies. Therefore, it could be suggested that increased free radical production, arising from complex I and III suboptimal assembly, together with unchanged or even increased AIF expression, contributes to protein oxidative damage specifically in spinal cords, being those changes absent in frontal cortex. Despite such an increased mitochondrial leak could lead to potentially extensive protein modifications, western-blot analyses revealed that there are specific targets for oxidative, glycoxidative and lipoxidative damage that will be the focus of future studies.

To summarize, the present data demonstrate that there is increased protein oxidative modification in spinal cords and frontal cortex from ALS cases, together with changes in lipid composition. Concerning cellular targets of those phenomena, we have demonstrated a motorneuron involvement, but it should be remarked that glial cells are the major contributors to protein mass in spinal cords. Therefore, it is feasible to assume that many of the changes observed here respond to changes in both (or mainly) glial cell and neuronal populations. In this line, it should be recalled the importance of glial support and trophic environment for motorneurons, so any given change to glial population could contribute to motorneuron loss. In spinal cords, where loss of motorneurons was evident, build up of oxidatively damaged proteins was linked to changes in mitochondrial respiratory complexes and to increased ubiquitination, potentially linked to impaired

proteasome function. More importantly, this was associated to changes in ER proteins suggesting the occurrence of ER stress. Since those changes were reproducible by an excitotoxic paradigm at an early stage, it can be suggested that excitotoxicity leads to increased protein oxidation, proteasomal dysfunction and ER stress in neuronal and non-neuronal cells, potentially contributing to motor-neuron death in ALS.

Supplementary material

Supplementary material is available at *Brain* online.

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ADDITIONAL INFORMATION-ALS

Figure 1 AI-ALS:

Protein oxidative damage in sporadic ALS patients and spinal cord slices under chronic excitotoxicity

(**A**) Western blot for the glycoxidation marker CEL in brain cortex (BC) (left panel) and spinal cord (SC) (right panel) display increased glycoxidative damages in SC of sALS patients and significant differences between both areas SC and BC. Right numbers of the blots indicate apparent molecular weight. The lower panel displays the quantification of these blots by densitometry. β-actin (47 kDa) was used as a loading control. Differences were analysed respect to control group by Student's t-test being ** $p<0.001$.

(**B**) Lumbar SC slices were treated for 15 days with the inhibitor of glutamate uptake D,L threohydroxyaspartate (THA) at 200μM. GC/MS analyses of markers for protein oxidation pathways reveal significantly increased oxidative damage, especially relevant for CEL (upper panel). Fatty acid analyses disclose significantly increased PI and DHA content, and decreased n6/n3 and 18:1/18:0 ratios (lower panel). Values shown are % changes of mean ± S.E.M over values from vehicle-treated slices (GSA: 35608±246 μmol/mol lysine; AASA: 109±4 μmol/mol lysine; CEL: 232±12 μmol/mol lysine; CML: 1698±34 μmol/mol lysine and MDAL: 199±6 μmol/mol lysine; DHA: 5.97±0.22 %; PI: 99.45±3.8; 18:1/18:0 ratio:1.85±0.02; n6/n3 ratio: 1.22±0.03). ** $p<0,001$ respect to vehicle treated group by Student's t test. GC/MS analyses were performed on 15 slices for each condition, and the experiment was repeated twice.

Figure 1 AI-ALS:

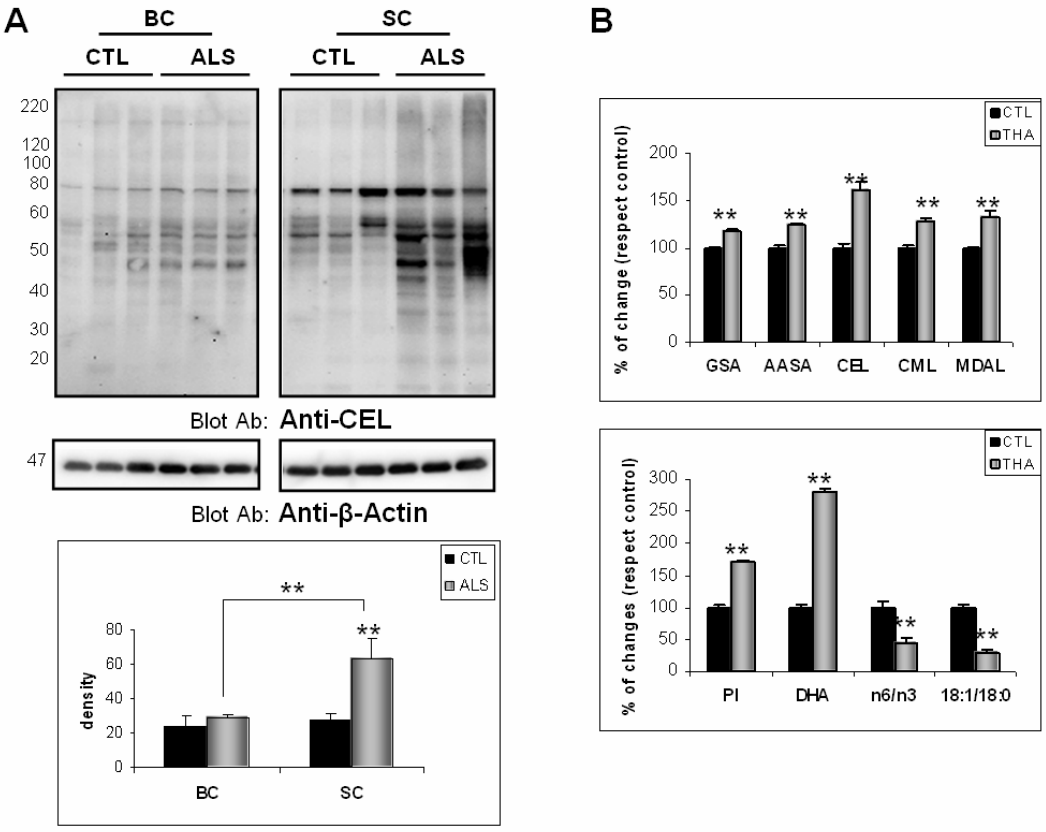


Figure 2 AI-ALS:

Confocal microscopy of spinal cord slices under chronic excitotoxicity
Representative confocal microscope images of fluorescence labelling for CEL (green) and GFAP (red) immunoreactivity, and DAPI staining for DNA (blue) in ventral horns of lumbar SC slices cultured for 15 days with 200 μM THA (**B**) or vehicle (**A**). Scale bar=50 μM. (**C**) The lower panel shows a detail from a slice under THA treatment, in which the two typical distributions of CEL immunoreactivity in these conditions are present: a neuronal cell displays CEL immunoreactivity filling the soma and neurite profiles (arrowhead) and several cells, with a microglial-compatible shape, show an intense CEL immunoreactivity (arrow). Scale bar= 10 μM. At least 30 hemislices from each condition were examined.

Figure 2 AI-ALS:

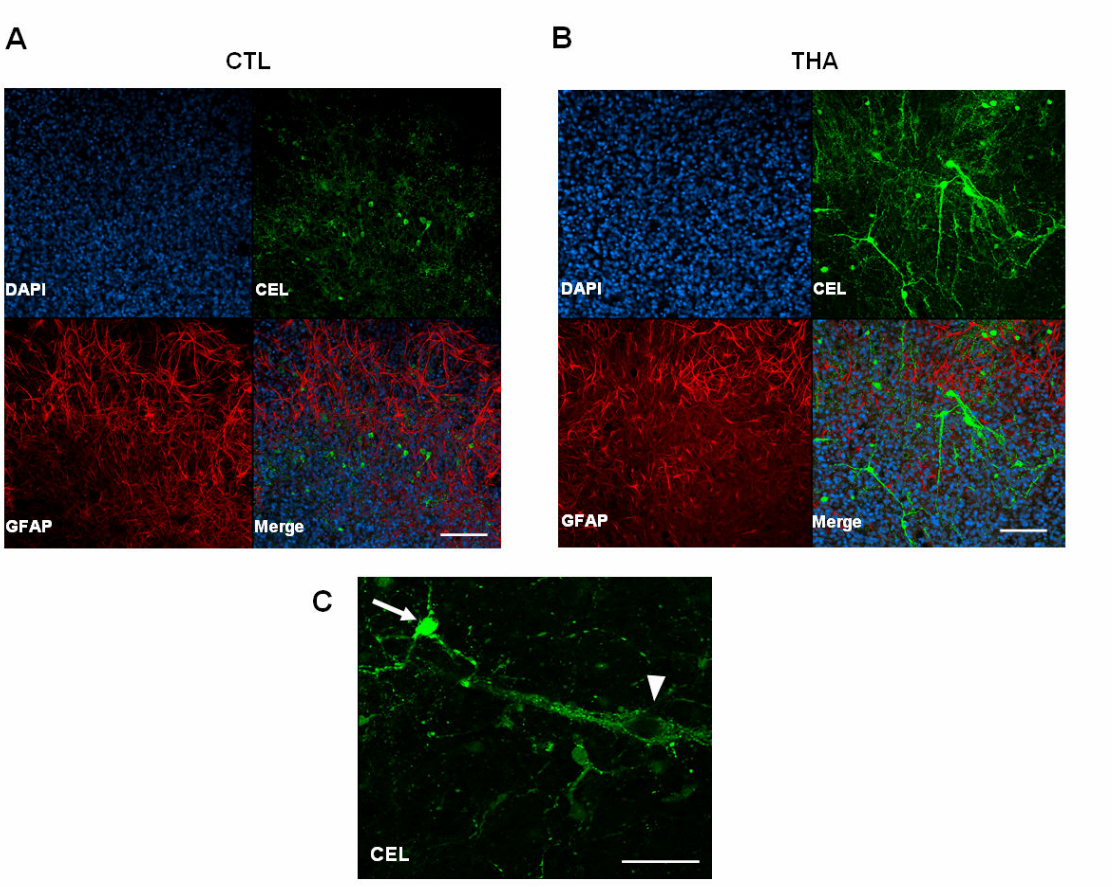


Figure 3 AI-ALS:

Mitochondrial changes and mitochondrial dysfunction in sporadic ALS and chronic excitotoxicity

(**A**) Representative western blots of peptides SDHA and MTCO1 subunits of mitochondrial respiratory chain complexes II and IV, respectively, suggest increased complex II and decreased complex IV content in SC samples from sporadic ALS patients compared to control individuals. This was associated with decreased amount of cytosolic (AIF 67 kDa) and mitochondrial (AIF 62 kDa) fraction of apoptosis-inducing factor (AIF) and decreased content of porin. Porin was used as a control of total mitochondrial mass. The lower panel shows the quantification of these blots by densitometry, adjusted to porin density (complex II and IV, AIF 62) when porin and AIF 67 were adjusted to actin (´). Differences were analysed respect to control group by Student's t-test being * $p<0.05$, ** $p<0.001$.

(**B**) Representative confocal microscope images of fluorescence labelling showi increased AIF immunoreactivity in whole mounts of slices cultured for 15 days with 200 μM THA (lower panel) or vehicle (upper panel). At least 30 hemislices from each condition were examined throughout the different experiments. Scale bar=25 μM.

Figure 3 AI-ALS:

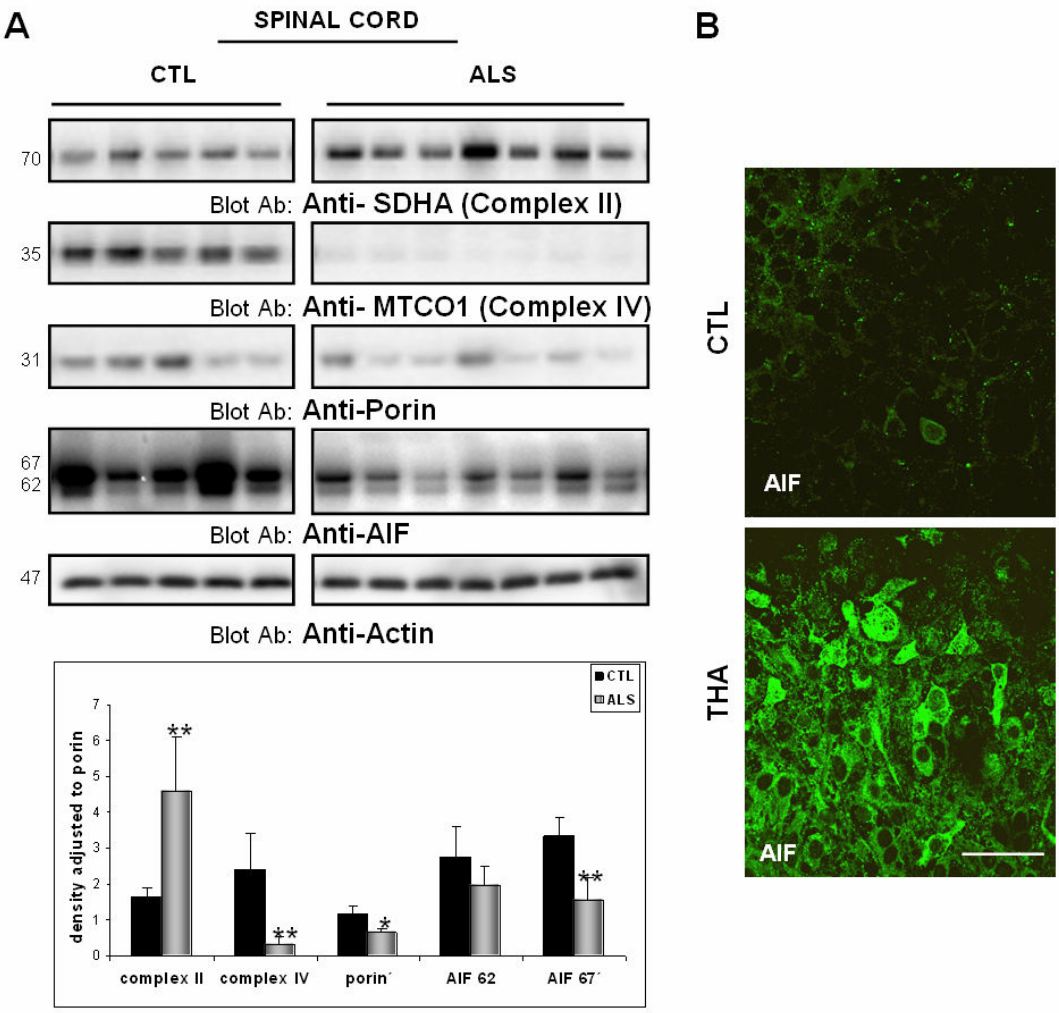
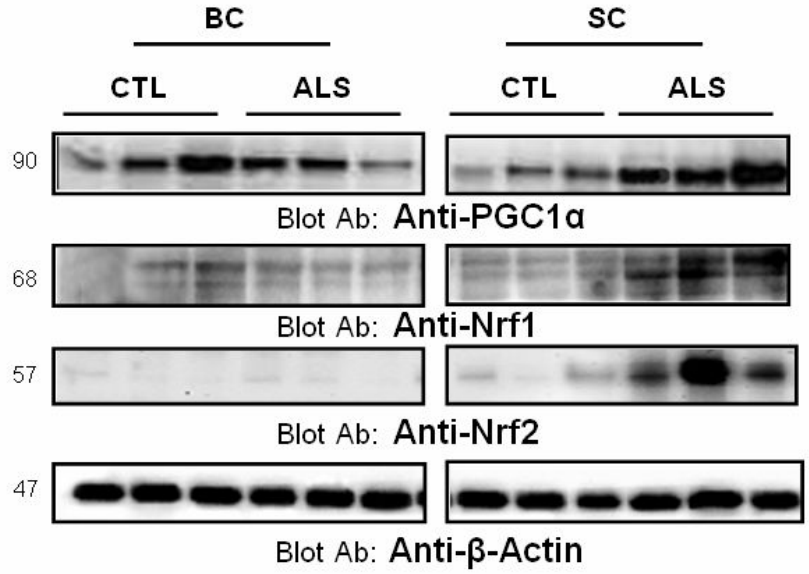


Figure 4 AI-ALS:

Antioxidant response in sporadic ALS

Representative western blots of the positive regulator of the cellular antioxidant response PGC1α and its downstream regulated transcription factors Nrf1 and Nrf2, demonstrate significantly increased levels in SC and significant difference between both areas SC and BC in sporadic ALS. These increases suggest a need of antioxidant response in SC of sALS patients. The lower panel represents the quantification of these blots by densitometry, after densities of bands were adjusted to β-actin content and differences were analyzed respect to control group by Student's t-test being ** $p<0.001$.

Figure 4 Al-ALS:



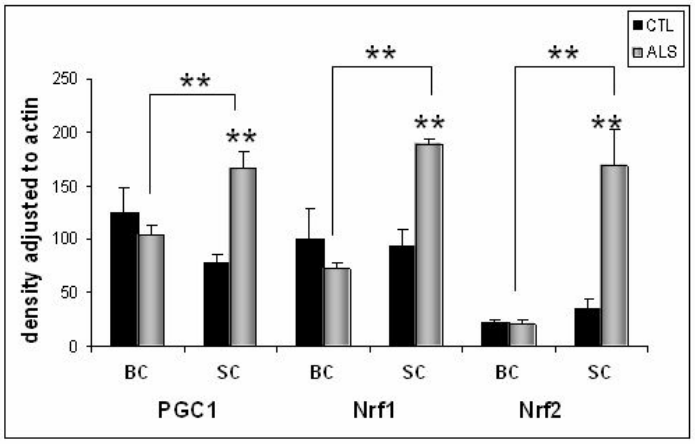


Figure 5 AI-ALS:

Immunoblotting of cellular inclusions in sporadic ALS and protein ubiquitination in chronic excitotoxicity

(**A**) Representative western blots of TDP-43, show increased levels in SC of ALS patients, suggesting accumulation of TDP-43 only at this anatomic area and display significant difference between both areas (SC and BC) in sALS. The lower panel displays the quantification of these blots by densitometry. β-actin (47 kDa) was used as a loading control. Differences were analysed respect to control group by Student's t-test being ** $p<0.001$.

(**B**) Representative confocal microscope images of fluorescence labelling show increased ubiquitination in whole mounts of slices cultured for 15 days with 200 μM THA (lower panel) compared with those incubated with vehicle (upper panel). Left panels scale bar=500 μM. Panels at right show a detail from these slices suggesting cytosolic and non neuronal ubiquitination. Right panels scale bar=50 μM.

Figure 5 AI-ALS:

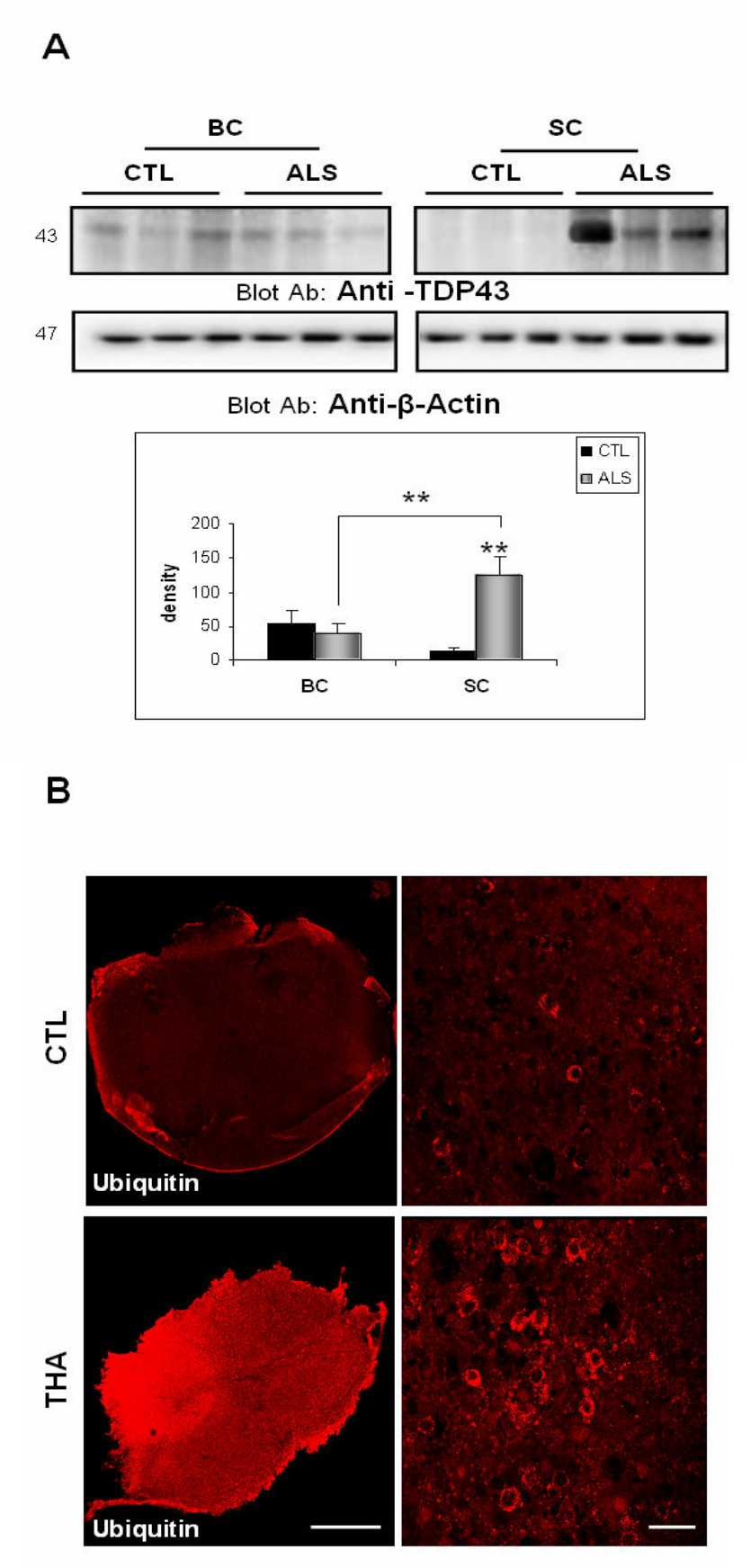


Figure 6 AI-ALS:

Endoplamic reticulum (ER) stress in patients with sporadic ALS and in a model of chronic exitotoxicity

(**A**) Representative western blots of BC and SC homogenates with findings compatible with ER stress, evidenced by increased expression of ER-resident chaperones Grp78/BiP, Grp94 and increased levels of the eIF-2α phosphorylating kinase PKR. The lower panel shows the quantification of the blots by densitometry, adjusted to actin content. Differences were analysed respect to control group by Student's t-test being * $p<0.05$, ** $p<0.001$.

(**B**) Representative confocal microscope images of fluorescence labelling show increased phosphorylation of eIF-2α at Ser 51 immunoreactivity in ventral horns of slices cultured for 15 days with 200 μM THA (lower panel) or vehicle (upper panel). Increased phosphorylated eIF-2α immunoreactivity in THA treated slices was especially evident for small cell population compatible with morphology of glial cells. Scale bar=50 μM.

Figure 6 AI-ALS:

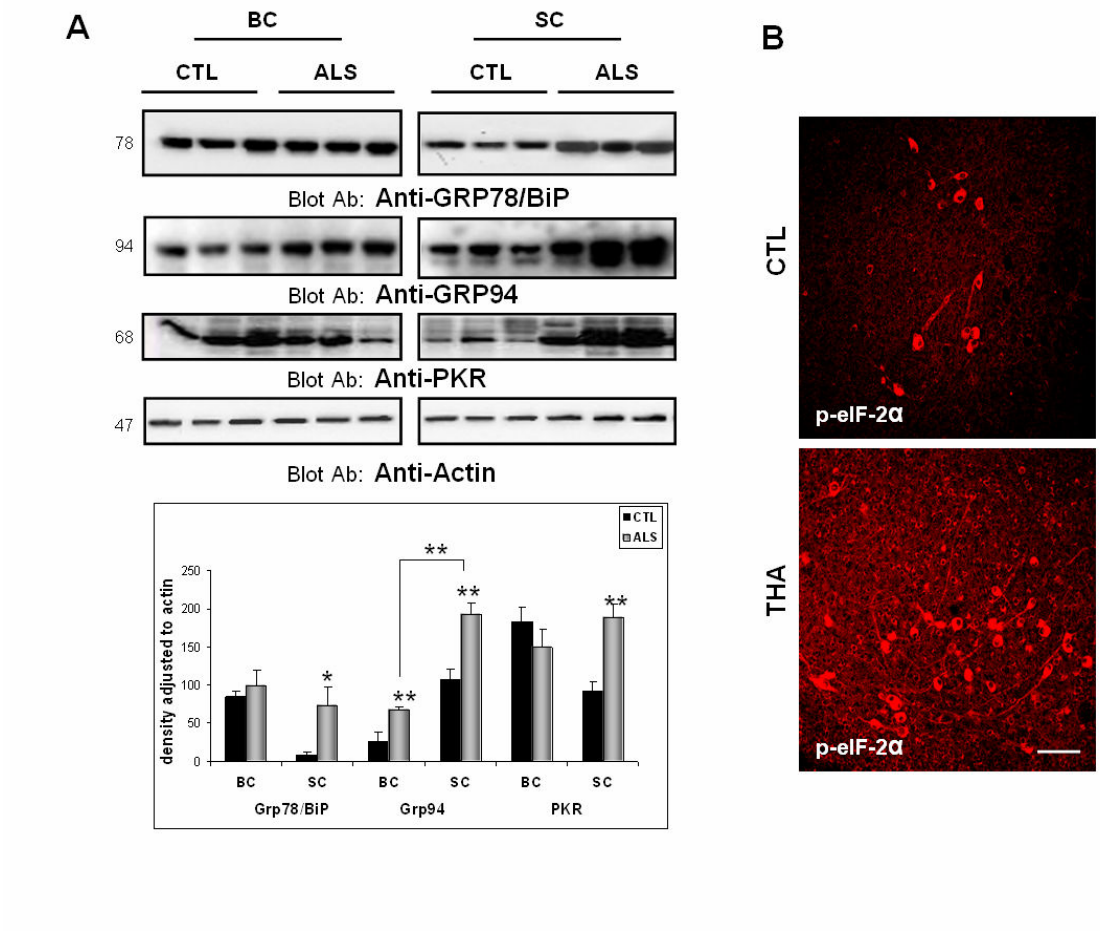


Figure 7 AI-ALS:

Cellular model of familiar ALS.

As described in the introduction section 1.3.4.1. 2% of all cases of familiar ALS are associated with mutations in the coding regions of the antioxidant enzyme SOD1. Most of these mutations do no impair the enzymatic activity of SOD1, they do not lead to the loss of function but to the gain of novel function(s) which is toxic to the motor neurones. We focused our investigation in the G93A mutation, a substitution of Glycin to Alanine at position 93.

We hypothesized that the aggregation-prone proteins such as mutant SOD1 could induce oxidative stress (OS) and inhibition of the ubiquitin-proteasome system. The OS through its interaction with the cellular proteolysis is directly related to the generation of aggregated, ubiquitinated proteins. During normal conditions physiologicaly these aggregates are cleaved by the proteasome. During the stress conditions the excess of substrate may inhibit directly or indirectly the proteasome activity. Since the proteasome activity is implicated in the elimination of oxidized proteins as well as in the regulation of the pathways for the ER stress response, a consequence of such proteasome impairment secondary to OS would be the disturbance in ERAD, leading to ER stress and activation of specific pathways of neuron death. To test this hypothesis the following experiments were designed.

Co-transfection of human wild type SOD1 (SOD-wt) and mutant SOD (SOD-G93A) with poteasome sensor vector (proSensor) in HEK 293 cells.

(**A**) Proteasome Sensor Vector encodes a GFP-like destabilized green fluorescent protein (ZsGreen) that is rapidly degraded by the proteasome. To convert ZsGreen into a proteasomal substrate its C-terminus is fused to a specific degradation motif that targets the fusion for removal by the 26S proteasome, but ZsGreen does not need to be modified by ubiquitin in order to be degraded. This protein becomes a proteasome target as soon as it is translated. When proteasome is inhibited, the fluorescent protein quickly accumulates to levels detectable by fluorescence microscopy, flow cytometry, or fluorometry [*Adams, J. (2002) Trends Mol. Med. 8(4 Suppl):S49–S54*].

(**B**) Fluorescente microscopy of HEK 293 cells 72h after transfection with human SOD-wt and human SOD-G93A, cloned in the pcDNA3.1 vector, co-transfected with proSensor, and 48h after the treatment with Epoxomicin, a selective and irreversible proteasome inhibitor. This experiment reveals significant difference in the fluorescence of SOD-G93A (lower panel) without treatment (left panel) and after the treatment with 1μM Epoxomicin (right panel) when compared with SOD-wt (upper panel). As well significant differences in the fluorescence where detected among both SOD-wt and SOD-G93A in the untreated and in treated with Epoxomicin conditions. The increased fluorescence could be associated with increased number of undegraded protein aggregates. The left panel (untreated) display that only the presence of mutant SOD-G93A is sufficient to inhibit the proteasome, whereas additional events, such as treatment with specific inhibitors, are required in the case of SOD-wt. Hoechst nuclear staining was used as an indicator of cell number.

(**C**) Graphical presentation of the fluorescence intensity quantified by image analyses. Values represent the mean ± SEM of three independent experiments. * $p < 0.05$; ** $p < 0.001$; Student's *t*-test.

Figure 7 AI-ALS:

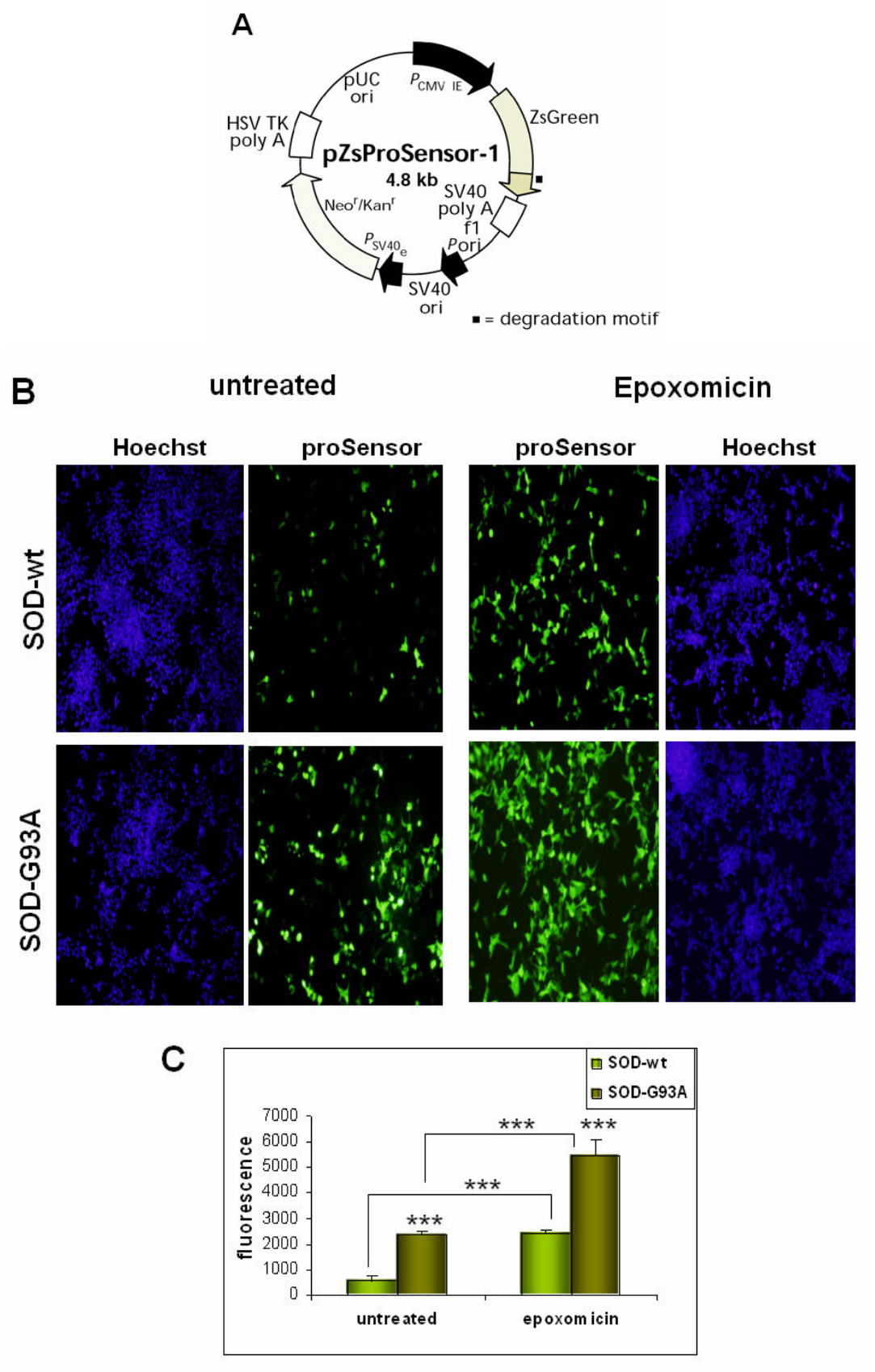


Figure 8 AI-ALS:

Confocal microscopy of HEK 293 cells transfected with SOD-wt, SOD-G93A and co-transfected with proSensor

(**A**) Representative confocal images of fluorescence with proSensor (green), SOD1 (red), and merged images show differences in the co-localization of proSensor and SOD1 and different destribution of SOD-wt and SOD-G93A in the cell. The left upper panel reveals lack of co-localization between proSensor and SOD1 in cells transfected with SOD-wt without treatment, whereas the right upper panel displays co-localization between proSensor and SOD1 when cells transfected with SOD-wt were treated with an inhibitor of the proteasome. The lower panels reveal co-localization of proSensor and SOD-G93A in both conditions without inhibitor and with inhibitor of the proteasom activity, suggesting that mutant SOD-G93A is sufficient to inhibit the proteasome, where as treatment with specific inhibitors, are required in the case of SOD-wt.

(**B**) Representative western blot of SOD1 shows forms compatible with dimeric and hexameric aggregates of SOD-G93A in both conditions without inhibitor and with inhibitor of the proteasom activity, whereas aggregates of SOD-wt were seen only when the cells were treated with Epoxomicin. The right panel represents the quantification of these blots by densitometry, after densities of bands were adjusted to β-actin (not shown) content and differences were analysed between SOD-wt and SOD-G93A by Student's t-test being ** $p<0.001$. Values represent the mean ± SEM of three independent experiments.

Figure 8 Al-ALS:

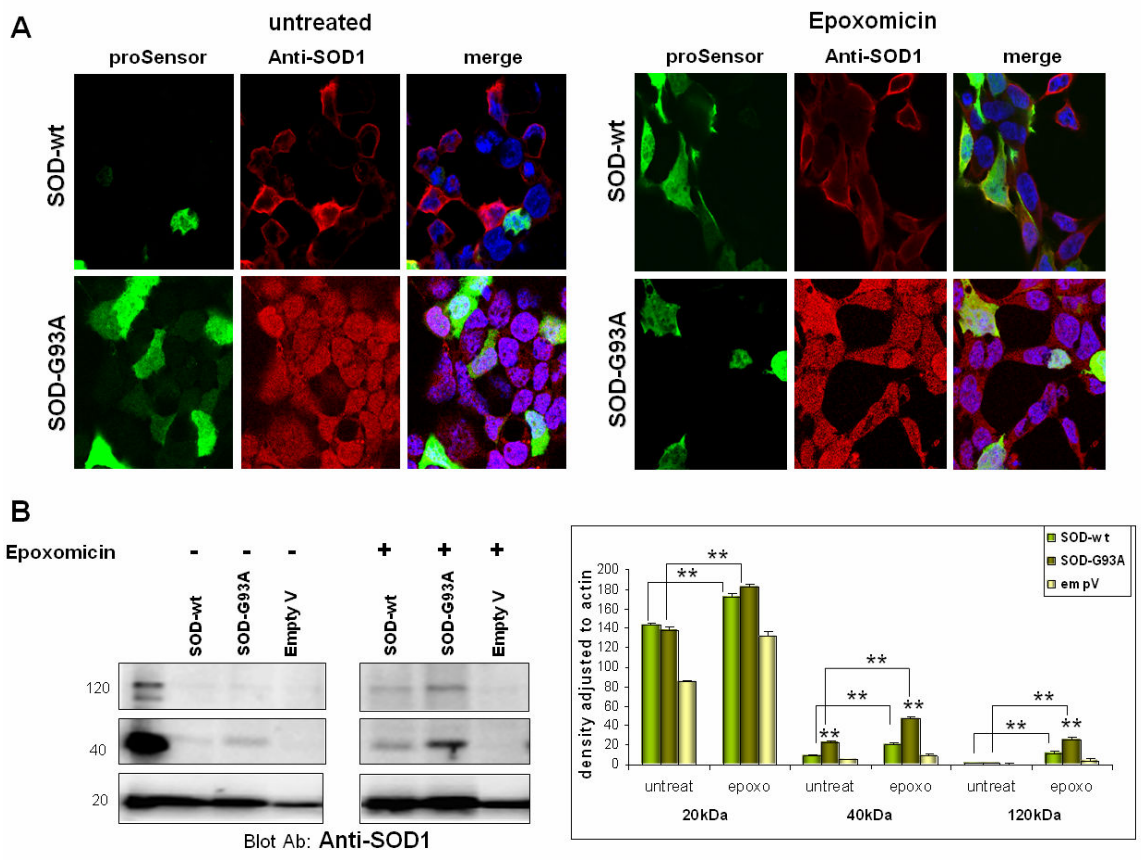


Figure 9 AI-ALS:

Immunoblotting and confocal microscopy of protein ubiquitination in cells transfected with SOD-wt and SOD-G93A

(**A**) Representative western blot of Ubiquitin shows highly ubiquitinated proteins in lysates of HEK 293 cells transfected with SOD-G93A without and with treatment with Epoxomicin in comparison with lysates of cells transfected with SOD-wt. Significant increase in the ubiquitination was detected for SOD-wt treated with Epoxomicin respect to SOD-wt without treatment. The lower panel represents the quantification of these blots by densitometry. Differences were analysed between SOD-wt and SOD-G93A by Student's t-test being *** $p<0.0001$. Values represent the mean ± SEM of three independent experiments.

(**B**) Representative confocal images of fluorescence labelling showing increased Ubiquitin immunoreactivity in SOD-G93A transfected untreated cells compared with untreated cells transfected with SOD-wt. A differential distribution of the ubiquitinated proteins was observed-perinuclear for SOD-G93A transfected cells and cytosolic for SOD-wt transfected cells.

Figure 9 AI-ALS:

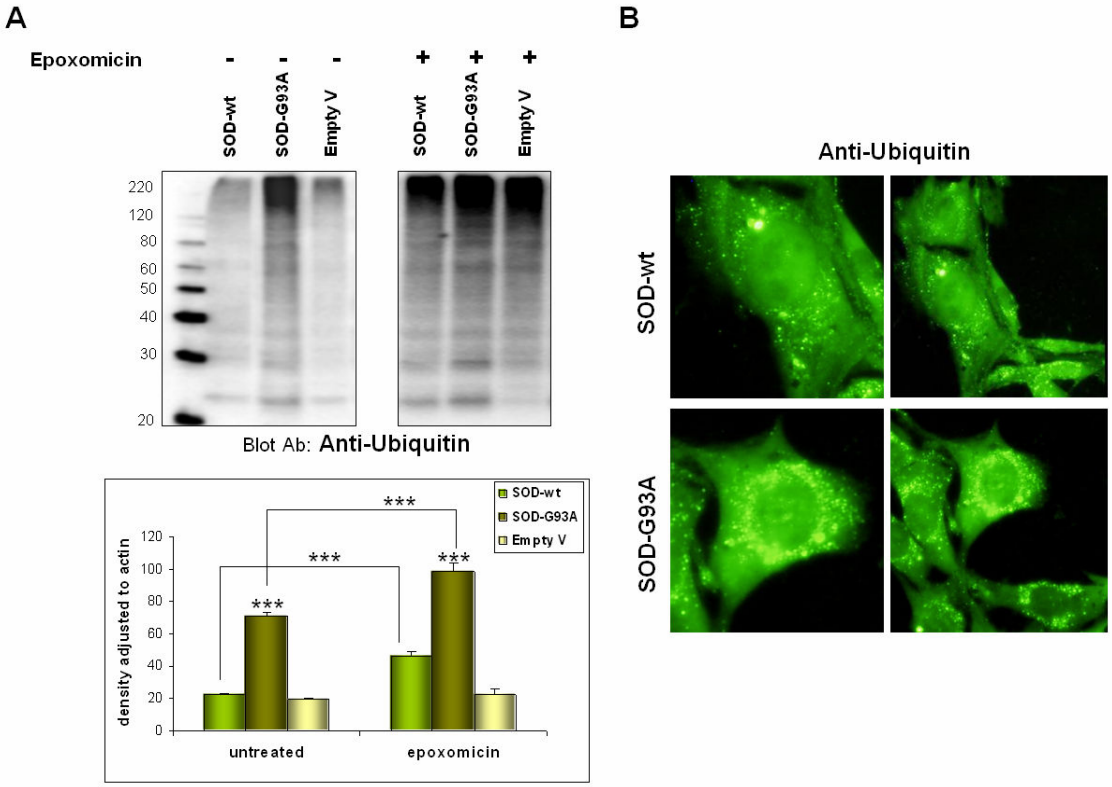


Figure 10 AI-ALS:

Oxidative stress evidenced by reactive carbonyl groups in proteins in a cellular model of familiar ALS

Representative western blot of DNP shows increased levels of reactive carbonyl groups in the proteins of cells transfected with SOD-G93A, untreated with inhibitors of proteasome activity, in comparison with lysates of cells transfected with SOD-wt, evidencing increased oxidative stress in SOD-G93A transfected cells respect to SOD-wt cells. Significaficant increase in the oxidative stress, similar to that for SOD-G93A, was detected for SOD-wt transfected cells treated with epoxomicin repect to SOD-wt cells without treatment. The lower panel represents the quantification of this blot by densitometry. Differences were analysed between SOD-wt and SOD-G93A by Student's t-test being *** $p<0.0001$. Values represent the mean ± SEM of three independent experiments.

Figure 10 AI-ALS:

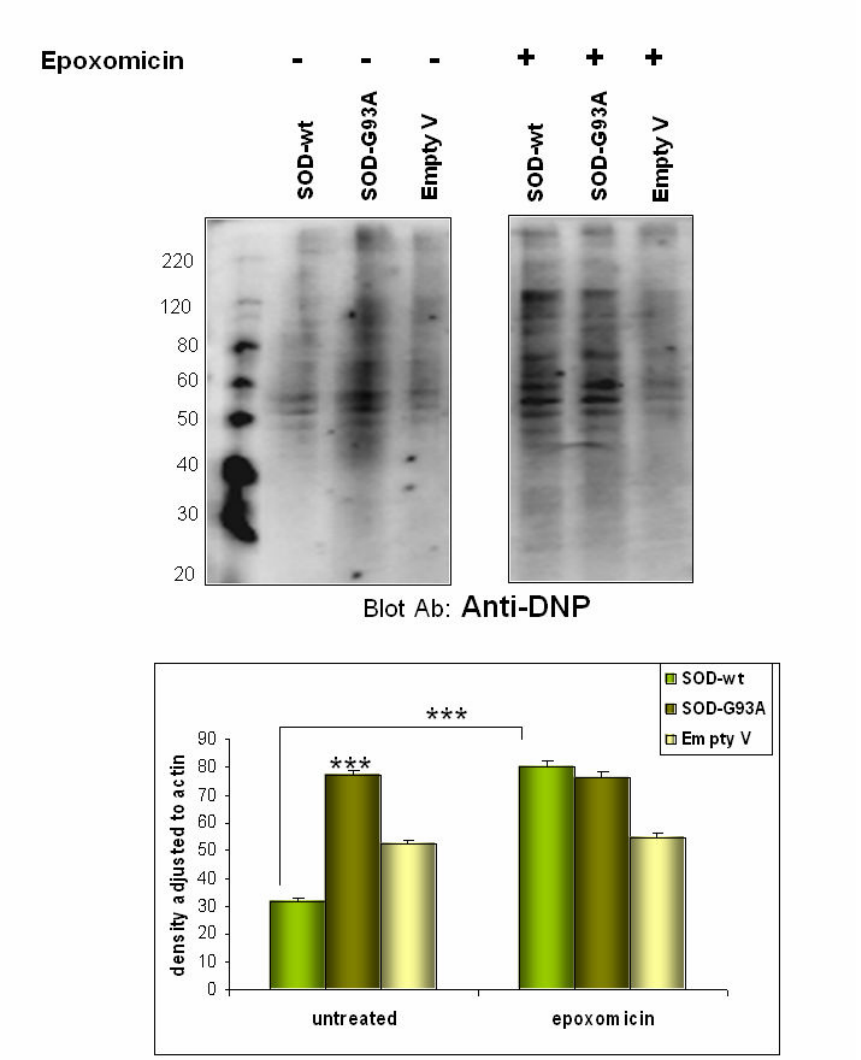


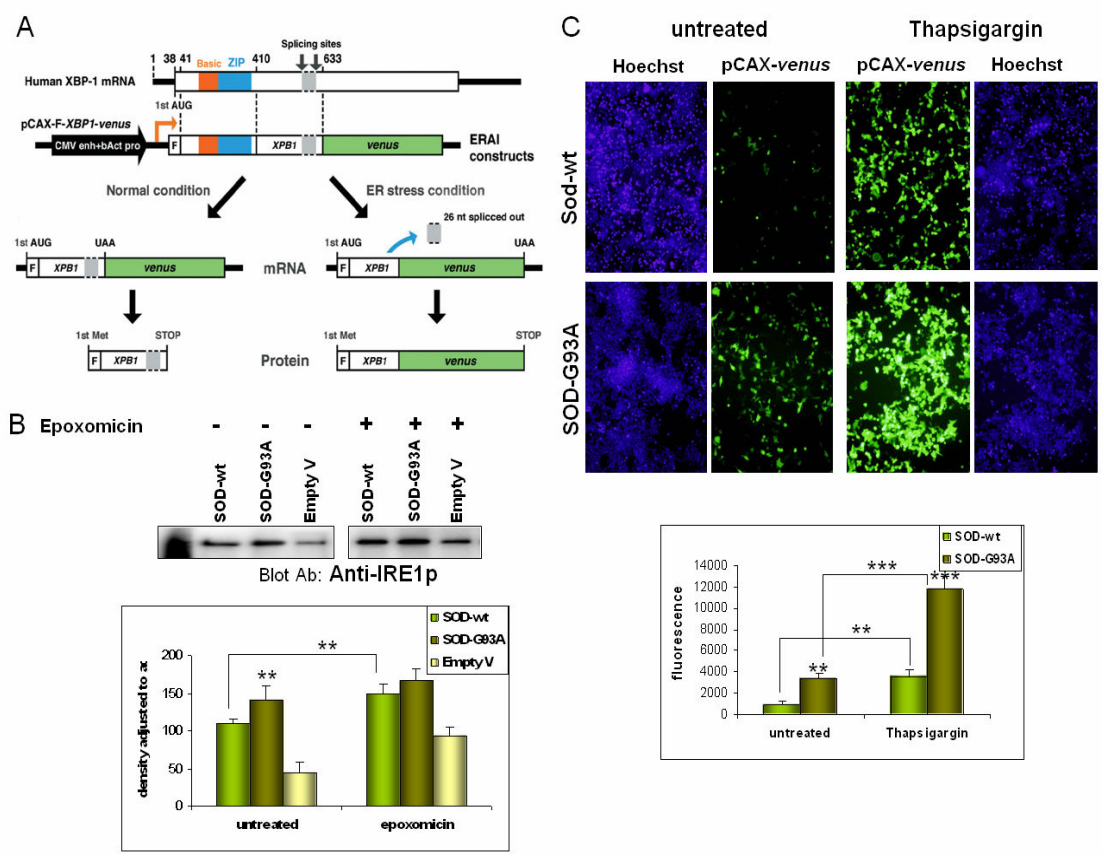
Figure 11 AI-ALS:

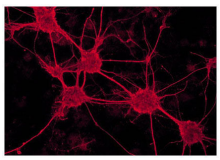
ER stress in a cellular model of familiar ALS
(**A**) pCAX-F-XBP1-*venus* is generated as ER-activated indicator (ERAI) construct. Venus is a variant of green fluorescent protein. Basic (orange) and ZIP (blue) indicate coding regions of basic and leucine zipper domains, respectively. Transcript from ERAI construct is not spliced under normal conditions. Unspliced mRNA is translated into a truncated XBP1 with a FLAG tag coding sequence at its N terminus. Under ER stress, the transcript is spliced, leading to a frameshift. The spliced mRNA is translated into an XBP1-venus fusion protein with a FLAG tag at its N terminus. Thus, the fluorescence is detectabel only in cells experiencing ER stress. ERAI works as a specific and sensitive indicator for ER stress in an IRE1-dependent manner [*Miura et al. Nature Medicine (2004) 10: 98-102*].

(**B**) Representative western blot of IRE1 shows increased levels for SOD-G93A, related to increased ER stress, in both conditions without inhibitor and with inhibitor of the proteasom activity, whereas increase for SOD-wt was seen only when the cells were treated with epoxomicin, suggesting that oxidative stress induced by mutant SOD probably leads to ER stress in the cellular model of familiar ALS through proteaome dysfunction. The lower panel represents the quantification of the blot by densitometry, after densities of bands were adjusted to β-actin (not shown) content and differences were analysed between SOD-wt and SOD-G93A by Student's t-test being ** $p<0.001$. Values represent the mean ± SEM of three independent experiments.

(**C**) Fluorescente microscopy of HEK 293 cells 72 h after transfection with human SOD-wt and human SOD-G93A, co-transfected with pCAX-F-XBP1-venus, and 48h after the treatment with Thapsigargin, an inhibitor of intracellular calcium (SERCA) pumps and inductor of ER stress, reveals significant difference evidenced by the image analyses (C) in the fluorescence intensity of SOD-G93A (lower panel) without treatment (left panel) and after the treatment with 2μM Thapsigargin (right panel) when compared with SOD-wt (upper panel). Significant differences in the fluorescence intensity were detected among both SOD-wt and SOD-G93A in the untreated and in treated with Thapsigargin conditions. The increased fluorescence could be assocciated with increased ER stress. The left panel (untreated) display that only the presence of mutant SOD-G93A is sufficient to induce ER stress, whereas additional treatment with specific inducers of ER stress is required in the case of SOD-wt. Hoechst nuclear staining was used as an indicator of cell number. The lower panel represents the fluorescence detected by fluorometry. Values represent the mean ± SEM of three independent experiments. Differences were analyzed between SOD-wt and SOD-G93A by Student's t-test being ** $p<0.001$, *** $p<0.0001$.

Figure 11 AI-ALS:





ARTICLE II

Maillard Reaction versus Other Nonenzymatic Modifications in Neurodegenerative Processes

REINALD PAMPLONA, EKATERINA ILIEVA, VICTORIA AYALA, MARIA JOSEP BELLMUNT, DANIEL CACABELOS, ESTHER DALFO, ISIDRE FERRER, AND MANUEL PORTERO-OTIN

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Nonenzymatic protein modifications are generated from direct oxidation of amino acid side chains and from reaction of the nucleophilic side chains of specific amino acids with reactive carbonyl species. These reactions give rise to specific markers that have been analyzed in different neurodegenerative diseases sharing protein aggregation, such as Alzheimer's disease, Pick's disease, Parkinson's disease, dementia with Lewy bodies, Creutzfeldt-Jakob disease, and amyotrophic lateral sclerosis. Collectively, available data demonstrate that oxidative stress homeostasis, mitochondrial function, and energy metabolism are key factors in determining the disease-specific pattern of protein molecular damage. In addition, these findings suggest the lack of a "gold marker of oxidative stress," and, consequently, they strengthen the need for a molecular dissection of the nonenzymatic reactions underlying neurodegenerative processes.

Key words: **advanced glycation end products; advanced lipoxidation end products; Alzheimer's disease; amyotrophic lateral sclerosis; Creutzfeldt-Jakob disease; energy metabolism; free radicals; mitochondria; oxidative stress; Parkinson's disease; Pick's disease; reactive carbonyl species**

Nonenzymatic Oxidative Protein Modification

As a rule, chemical reactions in living cells are under strict enzymatic control and conform to a tightly regulated metabolic program. One important factor implicit in evolution, from a biomolecular view, is the minimizing of unwanted side reactions. Nevertheless, uncontrolled and potentially deleterious reactions occur, even under physiological conditions. Free radicals (reactive oxygen species [ROS] and reactive nitrogen species [RNS]) are generated by both enzymatic and nonenzymatic sources and have been implicated in a multitude of physiological processes including aging and disease initiation and/or progression.[1] Oxidative stress occurs when the net flux of free radical production during normal aerobic metabolism exceeds the antioxidant defenses of the cell. Emerging evidence indicates that this stress causes specific protein modifications that may lead to a change in the structure and/or function of the oxidized protein.[2,3] Carbonylation is one of those changes, altering the conformation of the polypeptide chain and determining the partial or total inactivation of proteins. This can have a wide range of downstream functional consequences and may be the cause of subsequent cellular dysfunctions and tissue damage.

Structurally, carbonylation may arise from direct oxidation of amino acid side chains, mainly Pro, Arg, Lys, and Thr, resulting in the formation, among others, of glutamic semialdehyde (GSA) and aminoadipic semialdehyde (AASA), the main carbonyl products of metal-catalyzed oxidation of proteins. In addition, carbonyl groups may be introduced into proteins by secondary reaction of the nucleophilic side chains of Cys, His, and Lys residues with reactive carbonyl species (RCS) produced during lipid peroxidation (lipoxidation reactions) or generated as a consequence of the reaction with highly reducing sugars, such as glyoxal and methylglyoxal (an usual byproduct of glycolysis) or their oxidation products (glycation and glycoxidation reactions). Most of the biological effects of intermediate RCS are attributed to their capacity to react with the nucleophilic sites of proteins, forming advanced lipoxidation end products (ALEs) and advanced glycation end products (AGEs).[2] Compared to free radicals, RCS are stable and can diffuse within or even escape from the cell and attack targets far from the site of formation. Therefore, these soluble reactive intermediates are not only cytotoxic per se but

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also behave as mediators and propagators of oxidative stress and tissue damage, acting as second cytotoxic messengers.[4]

Oxidative decomposition of polyunsaturated fatty acids (PUFAs) initiates chain reactions that lead to the formation of a variety of RCS which, by reacting with nucleophilic sites in proteins, generate specific ALEs, such as MDA-Lys, HNE-Lys, and Nε-(hexanoyl)lysine, among others. These adducts have been detected by chemical and immunohistochemical methods in a broad range of tissues and species during physiological aging[4] and specific pathological states. The involvement of toxic RCS as products and propagators of oxidative damage in neurodegenerative diseases is currently under study.

The residual aldehyde group in some ALEs can further react to give protein cross-links and fluorescent products that are very similar to AGEs. Lipofuscin, the nondegradable intralysosomal fluorescent pigment that accumulates with age in postmitotic cells, is a recognized hallmark of aging.[4] Other important toxic products formed during nonenzymatic modification of proteins in aging and disease are referred to as "either advanced glycation or lipoxidation end products," so named because they may be formed from either carbohydrates or lipids. *N*ε-(carboxymethyl)lysine (CML) and *N*ε-(carboxyethyl)lysine (CEL) are, on a molar basis, the major modifications that have been measured in tissue proteins among these mixed-origin products, emphasizing the importance of the intersection between carbohydrate and lipid chemistry.[2]

Protein Damage and Neurodegenerative Diseases

The nervous system is potentially sensitive to oxidative modifications because i) the particular fatty acid composition of neuronal tissues that is rich in PUFA (hence easily peroxidizable); ii) the high O_2 consumption; and iii) the relatively poor expression antioxidant systems.[5] Also, nervous tissue is considered a postmitotic tissue and therefore highly susceptible to aging.[6] Cells in all regions of the nervous system are affected by aging, as indicated by the decline of sensory, motor, and cognitive functions with time. As this process is involved as a risk factor in most neurodegenerative diseases—there is a dramatic increase in the probability of developing a neurodegenerative disorder (e.g., Alzheimer's disease [AD], Parkinson's disease [PD], or amyotrophic lateral sclerosis [ALS], among others) during the sixth, seventh, and eighth decades of life—and oxidative modifications play a key role in aging, it is often accepted that these diseases should have increased oxidative damage.

Cells in the nervous system are affected by, and respond to, aging much as cells in other organ systems do, and so cells in the brain experience increased amounts of oxidative stress, impaired mitochondrial function and perturbed energy homeostasis, accumulation of damaged proteins, and lesions in their nucleic acids.[5,6] These changes during normal aging are exacerbated in vulnerable populations of neurons in neurodegenerative disorders. Therefore, some diseases might be viewed as a syndrome of accelerated aging in selected neurons.

The interest in the molecular dissection of each of these three pathways (i.e., direct oxidative modification, glycoxidation, and lipoxidation) clearly exceeds an academic context. An appropriate knowledge in this sense could help to rationally design therapeutic approaches aimed either at diminishing oxidative damage in a nonselective way (provided each oxidative pathway is increased in a similar extent) or at pinpointing those processes selectively increased. With this goal in mind, tissues from human tauopathies (AD and Pick's disease [PiD]), synucleopathies (PD and dementia with Lewy bodies [DLB]), and other neurodegenerative processes linked to protein misfolding and/or deposits (Creutzfeldt-Jakob disease [CJD] and ALS) were studied (TABLE 1). The concentration of selected markers of each pathway of protein oxidative damage was analyzed by gas chromatography coupled to mass spectrometry by using authentic deuterated internal standards, according to previously described procedures.[7] Tissues located in "target" zones of the diseases (showing pathological abnormalities) and "control" zones (without morphological changes) were evaluated in order to offer a biochemical correlate of the disease (TABLE 1). These diseases were chosen by the fact that all share accumulation or involvement of structurally modified protein deposits, which have been detected as modified with oxidation products using immunohistochemical procedures.

Tauopathies: Alzheimer's Disease and Pick's Disease

Alzheimer's disease is the more studied and prevalent neurodegenerative disease and is associated with β-amyloid deposits either in neurofibrillary tangles or hyaline bodies. The measurement of oxidative protein modifications[7] reveals that GSA and AASA contents are higher in the frontal cortex (area 8) of AD patients than in age- and sex-matched healthy controls. These increases (around 50% over control values) are larger than those present for well-known

TABLE 1. Changes in specific markers from oxidation-, glycoxidation-, and lipoxidation-derived reactions in human neurodegenerative diseases: a comparative molecular pathology approach

Disease	Number of cases	Location	GSA	AASA	CEL	CML	PI	DHA	MDAL
AD[7]	13	FC	↑	↑↑	↑	↑	↑	↑	↑↑
PiD[8] (1)	7	FC	↑	↑↑	↓	↓	↓	↓	↑
	7	OC	↑	↑	↑	↑	↑	↑	↑↑
PD[10]	7	SN	=	=	=	=	↓	↓	↑
	7	FC	=	=	↓	=	↑	↑↑	↑↑
DLB[10]	4	FC	=	=	↓	=	↑	↑	=
CJD (2)	10	FC	↑	↑	↑	↑	↓	↓	↑
ALS[12]	11	SC	↑	↑	↑	↑	↓	↓↓	↑↑
	11	FC	↑	↑	↑	↑	↑	↑	↑

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CJD, Creutzfeldt-Jakob disease; DLB, dementia with Lewy bodies; PD, Parkinson's disease; PiD, Pick's disease. Location: FC, frontal cortex; OC, occipital cortex; SC, spinal cord; SN, substantia nigra. (1) unpublished results; (2) Pamplona, Naudi, Gavin, *et al.*, University of Lleída, Lleída Spain AASA, aminoadipic semialdehyde; CEL, *N*ε-(carboxyethyl)lysine; CML, *N*ε-(carboxymethyl)lysine; DHA, docosahexaenoic acid; GSA, glutamic semialdehyde; MDAL, Nε-(malondialdehyde)lysine; PI, peroxidizability index.

AGE markers (CEL and CML), which roughly increase 15% over control values. As suggested by changes in peroxidizability, which is significantly increased in these AD patients (basically because of changed contents of docosahexaenoic acid [DHA]), the most affected marker of protein oxidative modification is Nε-(malondialdehyde)lysine (MDAL), which doubles its content in samples from AD patients. By using a combination of two-dimensional electrophoresis, Western blot, and peptide fingerprinting with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), several cytoesqueletal proteins, metabolic enzymes, and heat shock proteins were identified as modified by MDAL.[7]

To ascertain whether those phenomena are specific to AD, we analyzed PiD (unpublished results and Ref. 8). PiD is another tauopathy, characterized by the specific involvement of the frontotemporal cortex. In common with AD, significant increases were found in AASA, GSA, and MDAL, suggesting increased direct oxidative and lipoxidative damage, although at a lower extent than in AD. However, concentrations of lipoxidative and glycoxidative protein modifications were decreased (both CEL and CML), a fact that can be related to the loss of glycolytic potential, well described in this disease.

The occipital cortex is usually viewed as a location without morphological evidence of involvement of the disease, and hence morphological evidence serves as controls for measurements. In this case, we evidenced increased oxidative, glycoxidative, and lipoxidative damage in this location, supporting the fact that oxidative stress may be an early-stage change in the pathogenesis of this disease. Most interestingly, there was a direct and significant correlation between CEL concentration and DHA levels, suggesting that DHA is increased in response to neuronal stress. This would involve both oxidative stress and increased glycolysis, leading to increased CEL through potentially increased methylglyoxal efflux in the occipital cortex, whereas in the frontal cortex, because of neuronal loss and consequent decreased glycolysis, decreased values in both DHA and CEL content are present. Concerning the targets of oxidative damage, five different proteins exhibit increased anti-DNP staining: reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) carbonyl-reductase, glial fibrillary acidic protein, heat shock protein 70, cathepsin D precursor, and vesicle-fusing ATPase (unpublished results).

Synucleopathies: Parkinson's Disease and Dementia with Lewy Bodies

Parkinson's disease is the most prevalent synucleopathy. Despite previous results describing increased immunoreactivity to anti-AGE and oxidative damage adducts,[9] no chemical evidence of protein oxidative damage was available. When evaluating the content of the above-mentioned markers in substantia nigra from incidental DLB,[10] MDAL was the only marker that was significantly increased in PD (approximately 100%). Similar increases were also found in the frontal cortex and amygdala, suggesting the importance of lipoxidation. In clear contrast, CEL levels were significantly decreased in both the amygdala and the frontal cortex, a fact that can be in accordance with a described loss of glycolysis in PD.[11] Targets of lipoxidative damage comprise several antioxidant enzymes, proteasome components, α-synuclein, and other proteins not shared with AD or PiD. To shed further light

on the potential relationship of oxidative damage in synucleopathies, we analyzed cortex samples from patients with DLB, which showed a lack of increased MDAL content but, again, significant decreases in CEL content.[10]

Other Neurodegenerative Processes Linked to Protein Misfolding and/or Deposits

Creutzfeldt-Jakob disease is a neurodegenerative spongiform disease, linked to transmissible prionopathies. The protein oxidative profile in the frontal cortex shows similarities to AD: increased direct oxidative, glycoxidative, and lipoxidative damage, but in this case a decrease in n-3 fatty acids is present. (Pamplona, Naudi, Gavin, *et al.*, University of Lleída, Lleída, Spain) All increases are in the same range, suggesting a general change in the modified protein turnover. This would be compatible with reported alterations in proteasome present in related prion-induced diseases. Concerning targets of glycoxidative damage, we evidenced two key enzymes in glycolysis to be highly modified in CJD samples: glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-bisphosphate aldolase. As the activity of this latter enzyme involves the formation of a Schiff's base in its active site, we hypothesize that some of those bases may be transformed, under increased oxidative conditions, to CEL.

Amyotrophic lateral sclerosis is characterized by the selective loss of motor neurons in spinal cord and in brain cortex, associated with highly ubiquitinated deposits of proteinaceous material. Spinal cord lysates from ALS patients showed significant increases in direct oxidative, glycoxidative, and lipoxidative damage.[12] Analogous to samples from the brain cortex in AD, the more sensible marker was MDAL, suggesting the importance of lipoxidative modification in this context. Similar to substantia nigra samples in PD and frontal cortex samples in PiD, lipoxidative damage was accompanied by a strong loss in the content of DHA. As observed from other locations without morphological evidence of pathology, samples from the brain cortex of ALS patients also showed increased oxidation, glycoxidation, and lipoxidation, associated with reactive increases in the content of n-3 fatty acids, particularly DHA. All these features are reproducible *in vitro* by the generation of chronic excitotoxicity—a mechanism linked to selective neuronal loss by disturbed intracellular Ca^{++} homeostasis—in a spinal cord organotypic culture, supporting the involvement of this neurodegenerative pathway *in vivo*.[12]

Final Remarks

In conclusion to these analyses, no single marker of protein oxidative modification (among those used here) can be viewed as a gold standard for assessment of oxidative damage in neurodegenerative processes. The same applies to targets of oxidative damage that show disorder-specific differences. Moreover, in some cases CEL levels decreased, supporting CEL relationship with glycolysis potential. There are significant associations between changes in fatty acid composition (especially DHA) and protein oxidative damage. Most interestingly, when tissue from pathologically preserved locations was available, the tissue analyses indicated that protein oxidative modifications take place before potential morphological and clinical changes appear, suggesting an early involvement of protein oxidative damage in neurodegenerative process.

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Conflict of Interest

The authors declare no conflicts of interest.

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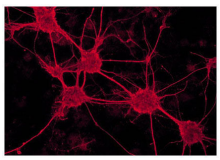

ARTICLE III

"LOSS OF THE STRESS TRANSDUCERS Nrf2 AND Grp78/BiP IN PICK'S DISEASE"

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ABSTRACT:

Pick's disease (PiD) is an extremely rare and fatal tauopathy. In this work was assessed the implication of endoplasmic reticulum (ER) stress associated with oxidative stress in PiD, as a potential mechanism involved in the pathogenesis of this neurodegenerative disorder. Both samples from morphologically affected frontal cortex (FC) and even from an apparently pathologically preserved occipital cortex (OC), showed region-dependent increases in different protein oxidative damage pathways. The oxidative modifications targeted antioxidant enzymes, proteases, HSP and synaptic proteins. This was associated with compromised proteasomal function and ER stress consisting with IRE1, ATF6, XBP1 activation and eIF-2α phosporylated in FC, with depletion in ER chaperones (glucose-regulated proteins Grp78/BiP and Grp94) and decreased levels of nuclear factor-erythroid 2 p45-related respiratory 2 (Nrf2), required for cell survival during stress conditions. The results presented in this work demonstrate increased, region-specific protein oxidative damage in PiD, with proteasomal dysfunction and atypical ER stress response probably caused by complete and specific depletion of Grp78/BiP that can contribute to the pathophysiology of this tauopathy.

Keywords: ER stress, oxidative stress, proteasome, UPR, proteomics, GC/MS

Abbreviations: PiD, Pick's disease; ER, endoplasmic reticulum; FC, frontal cortex; OC, occipital cortex; Nrf2, factor-erythroid 2 p45-related respiratory 2; HSP, heat shock proteins; GSA, glutamic semialdehyde; AASA, aminoadipic semialdehyde; UPR, unfolded protein response; Grp78/BiP, glucose-regulated protein 78/ immunoglobulin heavy chain binding protein; Grp94, glucose-regulated protein 94; ATF6, activating transcription factor 6; PERK, double-stranded RNA-activated protein kinase–like ER kinase; IRE1, inositol requiring 1; XBP1, x-box bindin protein 1; eiF-2α, eukaryotic translation initiation factor 2 alpha subunit; PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; ARA, Arachidonic acid; DBI, double bond index; PI, peroxidizability index

INTRODUCTION

Pick's disease (PiD) is an extremely rare, age-related neurodegenerative disorder (1, 2). Classical PiD is characterized macroscopically by severe frontal and temporal atrophy whereas occipital cortex (OC) appears preserved. Microscopically, there is marked neuron loss, accompanied by marked astrocytic gliosis, mainly in the frontal and temporal isocorcortex, anterior cingular and insular cortex, hippocampus and entorhinal cortex. Argentophilic tau- and ubiquitin-positive intracytoplasmic inclusions (Pick bodies) are the neuropathological markers of the disease. In addition, swollen achromatic cells (Pick cells), and tau-immunoreactive glial inclusions are accompanied features (1). Although described for the first time in 1896, the basic molecular mechanisms underlining PiD pathology are still not well characterized.

Both oxidative and endoplasmic reticulum (ER) stress have been associated with many different neurodegenerative diseases (3). Oxidative stress-induced molecular alterations, affect all sorts of biological molecules including especially sensitive amino acid residues on proteins, whose chemical modification results in the formation of specific markers such as glutamic (GSA) and aminoadipic semialdehyde (AASA) (4). Protein modifications also involve third-party molecules such as carbohydrates and/or lipids, through the generation of highly reactive carbonyl compounds in processes termed glyco- and lipoxidation respectively, leading to the formation of specific adducts, such as N^{ε}-carboxymethyl-lysine (CML), N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-malondialdehyde-lysine (MDAL) (5).

Some of the pathological hallmarks of PiD, e.g. tau- and ubiquitin- positive inclusions, are protein aggregates that may be related to oxidative stress, based on the relationship between protein oxidative damage and proteasomal activity. Moderate oxidative modification of proteins increases their susceptibility for proteasome clearance, while higher rates of oxidative modification actually inhibit proteasome activity and facilitate the protein aggregation (6). The disruption of the ER-associated degradation, which helps to clear misfolded protein species from ER, is a potential consequence of such proteasomal impairment, finally contributing to ER stress (7).

In response to ER stress, cell triggers an unfolded protein response (UPR) signalling pathway, aimed at its survival or to its apoptotic demise if stress is

not alleviated. The UPR changes the expressions of the hallmark markers for ER stress through nuclear factor-erythroid 2 p45-related respiratory 2 (Nrf2) (8). Nrf2 regulates the inducible expression of antioxidant response elements containing genes, encoding subset of antioxidants and detoxifying enzymes in response to oxidative stress and several chaperones in response to ER stress (9). The ER chaperone glucose-regulated protein 78 (Grp78/BiP) as master regulator, when chaperoning misfolded proteins for proteasome degradation, initiates UPR and inhibit protein synthesis to decrease the load within the ER through phosphorylation of eukaryotic translation initiation factor 2 (eiF-2α). Three molecules are critically involved in UPR processes: activating transcription factor 6 (ATF6α), inositol requiring 1 (IRE1) and double-stranded RNA-activated protein kinase–like ER kinase (PERK) (3). PERK signalling during the UPR consists in independent activating of eIF-2α and Nrf2 (9).

In this study, we examined region-specific changes in oxidative and in ER stress in human PiD, and the role of master regulators of both phenomena Grp78/BiP and Nrf2 as a contributing mechanism to the neuronal death implicated in the development of PiD.

PATIENTS AND METHODS

I. Human cases

Brain tissues were obtained from the Institute of Neuropathology and University of Barcelona Brain banks following the guidelines of the local ethics committees. The cases with clinical diagnosis of PiD were two men and two women aged 65, 68, 66 and 69 years, with no familiar history of neurological diseases, and who had suffered from progressive behavioural impairment followed by fronto-temporal dementia and accompanied by severe fronto-temporal lobar atrophy on neuroimaging studies (CT and MRI). The post-mortem delay between death and tissue processing was between 2 and 10 hours; corpses were maintained at 4º C. The fresh brain weights were 850, 1000, 900 and 900 g. Fifteen age-matched cases with no neurological disease were considered as putative controls. Cases with and without clinical neurological disease were processed in the same way following the same sampling and staining protocols. At autopsy, half of each brain was fixed in 10% buffered formalin, while the other half was cut in coronal sections 1 cm thick, frozen on dry ice and stored at −80 °C until use. In addition, 2-mm-thick samples of the frontal cortex (area 8) were fixed with 4% paraformaldehyde for 24 hours, cryoprotected with 30% sucrose, frozen on dry ice and stored at −80 °C until use.

The neuropathological study was carried out on formalin-fixed, paraffin-embedded samples of the frontal (area 8), primary motor, primary sensory, parietal, temporal superior, temporal inferior, anterior cingulate, anterior insular, and primary and associative visual cortices; entorhinal cortex and hippocampus; caudate, putamen and pallidum; medial and posterior thalamus; subthalamus; Meynert nucleus; amygdala; midbrain (two levels), pons and medulla oblongata; and cerebellar cortex and dentate nucleus. The sections were stained with haematoxylin and eosin, and with Klüver Barrera, or processed for immunohistochemistry following the EnVision + system peroxidase procedure (Dako, Barcelona, Spain). After incubation with methanol and normal serum, the sections were incubated with one of the primary antibodies at 4ºC overnight. Antibodies to glial fibrillary acidic protein (GFAP, Dako), β-amyloid (Boehringer, Barcelona, Spain) and ubiquitin (Dako) were used at dilutions of 1:250, 1:50, and 1:200, respectively. Antibodies to α-

synuclein (Chemicon, Barcelona, Spain) were used at a dilution of 1:3,000. Monoclonal anti-phospho-tau AT8 (Innogenetics, Gent, Belgium) was diluted 1:50: Phospho-specific tau rabbit polyclonal antibodies Thr181, Ser199, Ser202, Ser214, Ser231, Ser262, Ser396 and Ser422 (all of them from Calbiochem, Barcelona, Spain) were used at a dilution of 1:100, excepting anti-phospho-tauThr181, which was used at a dilution of 1:250. Antibodies to 3R and 4R tau (Upstate, Millipore, Barcelona, Spain) were used at dilutions 1:800 and 1:50, respectively.TDP-43 was examined by using two different antibodies: a mouse monoclonal antibody (Abnova, Tebu-Bio, Barcelona, Spain, H00023435-M01) raised against a full-length recombinant human TARDBP, used at a dilution of 1:1,000, and a rabbit polyclonal antibody (Abcam, Cambridge, UK, ab54502) raised against a synthetic peptide corresponding to C terminal (aa 350-414) of human TARDBP, used at a dilution of 1: 2,000. Phospho-TDP-43 was studied by using two different antibodies: a mouse monoclonal antibody directed to CMDSKS(p)S(p)GWGM,S(p), Ser409/410, used at a dilution of 1:5,000, and a rabbit polyclonal antibody raised against NGGFGS(p)S(p)MDSKC,S(p), Se403/404, used at a dilution of 1:2,500 (both from Cosmo Bio CO., LTD, Koto-ku, Japan). The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. Sections were counterstained with haematoxylin. Sections processed for phospho-tau immunohistochemistry were boiled in citrate buffer prior to incubation with the primary antibody. Sections processed for βA4-amyloid and α-synuclein were pre-treated with 95% formic acid. After neuropathological examination the four cases were categorized as Pick's disease following well-established neuropathological criteria (34,1). In no case, TDP-43 pathology was observed in the hippocampal complex and cerebral cortex. Seven cases from the control group had Alzheimer's disease-related pathology at stages II-III of Braak (35) and they were disregarded for the present study. Eight cases were selected as controls on the basis of lack of neurological symptoms and no abnormalities in the neuropathological examination.

II. Protein electrophoresis and western blot

Brain samples (200 mg) of the FC and OC from PiD and control cases were homogenized under antioxidant conditions as previously described (18).

Immunodetection was performed using as primary and secondary antibodies, those listed in supplementary information (Table S1). The monoclonal antibody to β-actin (Sigma, USA), diluted 1: 5000, was used to control protein loading. Luminescence was recorded and quantified in a Lumi-Imager equipment (Boehringer, Mannheim, Germany), using the Lumianalyst software. Protein bands were visualised with the chemiluminescence ECL method (Millipore Corporation, Billerica, MA, USA). The density of the immunoreactive bands was determined by densitometry analysis using a GS-800 Calibrated Densitometer (Bio-Rad).

III. Measurement of specific, protein-oxidation derived markers: GSA, AASA, CML, CEL and MDAL

GSA, AASA, CML, CEL and MDAL were determined as trifluoroacetic acid methyl esters derivatives in acid hydrolyzed delipidated and reduced protein samples by GC/MS using a HP6890 Series II gas chromatograph (Agilent, Barcelona, Spain) with a MSD5973A Series and a 7683 Series automatic injector, a HP-5MS column (30-m x 0.25-mm x 0.25-μm), and the described temperature programmme (18). Quantification was performed by internal and external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analyses were carried out by selected ion-monitoring GC/MS (SIM-GC/MS). The ions used were: lysine and [2H8]lysine, *m/z* 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and [2H5]5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), *m/z* 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and [2H4]6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), *m/z* 294 and 298, respectively; CML and [2H4]CML, *m/z* 392 and 396, respectively; CEL and [2H4]CEL, *m/z* 379 and 383, respectively; and MDAL and [2H8]MDAL, *m/z* 474 and 482, respectively. The amounts of product were expressed as the μmolar ratio of GSA, AASA, CML, CEL or MDAL/mol lysine.

IV. Fatty acid analysis

Fatty acyl groups of human FC lipids were analyzed as methyl esters derivatives by GC/MS as previously described (18). Separation was performed

in a SP2330 capillary column (30 m x 0.25 mm x 0.20 μm) in a GC Hewlett Packard 6890 Series II gas chromatograph (Agilent, Barcelona, Spain). A Hewlett Packard 5973A mass spectrometer was used as detector in the electron-impact mode. Identification of fatty acyl methyl esters was made by comparison with authentic standards and on the basis of mass spectra. Results are expressed as mol%. The following fatty acyl indexes were also calculated: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids from n-3 and n-6 series (PUFAn-3 and PUFAn-6); average chain length (ACL) = [(Σ%Total14 x 14) + (Σ%Total16 x 16) + (Σ%Total18 x 18) + (Σ%Total20 x 20) + (Σ%Total22 x 22)]/100]; double bond index (DBI) = [(1 x Σmol% monoenoic) + (2 x Σmol% dienoic) + (3 x Σmol% trienoic) + (4 x Σmol% tetraenoic) + (5 x Σmol% pentaenoic) + (6 x Σmol% hexaenoic)], and peroxidizability index (PI) = [(0.025 x Σmol% monoenoic) + (1 x Σmol% dienoic) + (2 x Σmol% trienoic) + (4 x Σmol% tetraenoic) + (6 x Σmol% pentaenoic) + (8 x Σmol% hexaenoic)].

V. Two-dimensional electrophoresis and mass spectrometry for protein identification

The bi-dimensional electrophoresis was performed as previously described (18). Polyclonal Anti-DNP antibody (1:2500, Sigma, USA), recognizing oxidation-derived carbonyls, was used as a primary antibody.

For gel staining, a MS-modified silver staining method (Amersham Biosciences) was used as described by the manufacturer. For membrane staining, a silver staining method based on the Gallyas intensifier was used according to previously described procedures.

Enzymatic digestion was performed with trypsin (Promega, Madison, WI) following conventional procedures**.** The MALDI-reTOF MS analysis of the samples was performed using a Voyager DE-PRO MALDI-reTOF mass spectrometer (Applied Biosystems, Foster City, CA). The instrument was run in reflectron mode with an average resolution of 12,000 full-width half-maximum at *m*/*z* 1500. A 5 mg/ml α-cyano-4-hydroxycinnamic solution was used as MALDI matrix.

VI. Image analysis

The gels and PVDF blots were scanned using a GS800 Calibrated Densitometer (Bio-Rad). PDQuest two dimensional analysis software (Bio-Rad) was used for matching and analysis of silver stained gels and membranes. The average mode of background subtraction was chosen to compare protein and DNP immunoreactivity content between cortex samples from PiD patients and control individuals.

VII. Data base search

The Protein Prospector software version 4.0.1 (University of California San Francisco, Mass Spectrometry Facility) was used to identify proteins from the peptide mass fingerprinting obtained from MALDI-reTOF MS. Swiss-Prot (European Bioinformatics Institute, Heidelberg, Germany) and GenBankTM (National Center for Biotechnology Information) data bases were used for the search.

VIII. Proteasome activity

Proteasome peptidase activities were evaluated by using fluorescently labeled peptides specific for chymotrypsin-like (CHTRP-LK) and trypsin-like (TRP-LK) activities in the presence of the proteasome specific inhibitor lactacystin as described (36).

IX. Statistical analyses

All statistics were performed using the SPSS software (SPSS Inc., Chicago, IL). Differences between groups (PiD samples versus control or FC versus OC) were analysed by the Student's t-tests and correlations between variables were evaluated by the Pearson's statistic. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

RESULTS

Protein oxidative damage in PiD

To delineate the potential pathogenic role of oxidative stress in PiD, we analized the steady state levels of specific markers of different kinds of oxidative damage to proteins, such as: glutamic (GSA) and aminoadipic semialdehyde (AASA) as direct protein oxidation markers, N^{ε}-carboxyethyl-lysine (CEL) as glycoxidation marker, N^{ε}-malondialdehyde-lysine (MDAL) as lipoxidation marker and N^{ε}-carboxymethyl-lysine (CML) as a mixed marker of glyco- and lipoxidation by highly selective mass spectrometry-based techniques. Isotope-dilution GC/MS analyses demonstrated that the more abundant products were those derived from metal catalyzed oxidation, AASA and GSA. The mean concentrations of both GSA and AASA were significantly higher in proteins from FC ($P<0.001$) and OC ($P<0.01$) samples of PiD patients than in control age-matched individuals (Fig. 1A). In contrast, the concentrations of CEL and CML were significantly lower in proteins from FC ($P<0.007$) and significantly higher in OC ($P<0.02$) of PiD patients compared to the controls (Fig. 1B). The concentration of MDAL was also significantly increased in samples from PiD patients, both in FC ($P<0.03$) and in OC ($P<0.001$) in comparison with control individuals (Fig. 1C).

Fatty acid profile in PiD

The high content of polyunsaturated fatty acid (PUFA) in central nervous system, its elevated oxygen consumption as well as the high lipoxidative damage observed (Fig.1C) support the possible significance of lipid peroxidation-derived processes in PiD neurodegeneration. Taking this into account we measured the fatty acid composition in the FC of PiD patients in front of OC and age-matched controls. Those analyses revealed significant differences associated with PiD in FC and OC, both in individual fatty acids and in global indexes (Table 1). Whereas, the 18:2n-6, 22:5n-6 and 24:0 fatty acids, exhibited 2-fold increases in their % content in PiD samples, PiD samples showed almost half of the % content of 20:0 fatty acids, compared with the control samples. The most remarkable changes involve the highly peroxidizable ω-3 docosahexaenoic acid (DHA) and ω-6 arachidonic acid (ARA). The DHA showed a significant decrease in FC samples with PiD ($P<0.001$), that

contrasts with the significant increase observed in OC (P<0.001; Fig. 1D). Furthermore, DHA levels correlated significantly with CEL (r=0,89; p<0.0001) (Fig. 1E). At the same time, the ARA presented a significant increase in FC (P<0.03) and unchanged levels in OC (Fig.1D). With reference to the fatty acid-derived indexes, FC from PiD patients showed significant decreases in the content of PUFA of the ω-3 family, with subsequent increases for monounsaturated fatty acids (P<0.001; Table 1). In contrast, increases of ω-3 content were detected in OC (P<0.001). Overall, changes in fatty acid profile led to significant changes in double bond index (DBI) (P<0.001), and peroxidizability index (PI) (P<0.001) (Fig.1C), favouring lipoxidative potential.

Correlation between protein oxidative damage and changes in fatty acid profile

After quantitation of protein oxidation and fatty acid analyses, several significant correlations were present among anatomically different locations. The most relevant were DHA levels, which correlated significantly with CEL (r=0,89; p<0.0001) (Fig. 1E). A highly significant correlation between CEL - which formation is directly related with the oxidation of glycolytic metabolites (10) - and DHA levels suggest that this marker, in this tissue, is more related to metabolic potential (e.g. glycolysis) of neuronal cells than to damage to proteins, as DHA modulation is usually viewed as a protective mechanism for these cells (11). Analysis of MDAL/PI ratio, suggested that, for a given PI in PiD patients, rates of MDAL formation are higher in FC than in OC (Fig.1C).

Protein oxidation targets

To identify proteins targets altered in PiD, we used 2D gel electrophoresis and MALDI mass peptide fingerprinting analysis to identify proteins differentially damaged in FC of the patients with PiD (Fig. 2). Targets of protein oxidative damage were identified as a vesicle-fusing ATPase, GFAP, cathepsin D precursor, carbonyl reductase NADPH1 isoforms, glyceraldehyde 3-phosphate dehydrogenase and Heat shock protein 70 kDa showing 2 to 8-fold increases in its oxidative modification (P <0.05) (Fig. 2).

Proteasomal dysfunction in PiD

The oxidative modifications of the proteins make them prone to proteolysis in the proteasome. Its function may be inferred by western blot analyses of ubiquitin-modified proteins. Our results showing increased protein ubiquitination in FC from PiD patients, but not in OC, are compatible with a compromise of proteasomal function in FC (Fig. 3A). In accordance with tau-induced saturation of the ubiquitin-proteasome system in spite of proteasome preserved activity (12), our results of peptide hydrolase activities confirmed increased trypsin-like activity in homogenates of FC of PiD samples, without changes in OC (Fig. 3B).

ER stress transducers in PiD

Perturbations of cellular physiology outside the ER, as oxidative stress and accumulation of modified proteins resistant to proteasomal degradation, such as tau protein, can be propagated to the ER to cause ER stress and activate the UPR. To explore whether the ER stress is implicated in PiD, we assessed ER stress and UPR markers in samples from PiD patients using Western blot analysis. The results displayed a set of evidences involving ER stress in PiD pathology (Fig. 4).

Thus, the phosphorylated α subunit of eukaryotic translation initiation factor 2 (p-eIF-2α), a UPR marker that controls the protein synthesis after ER stress, was markedly increased in FC of PiD cases confronted with control cases. In the same group of PiD patients, comparing FC with unaffected OC, in FC we detected a significant elevation in the levels of p-eIF-2α (Fig.4). In the brain samples from PiD patients, we also found the key UPR-related molecules IRE1, XBP1 and ATF6 to be activated in FC. XBP1, whose mRNA is processed by IRE1 in response to ER stress (13) was also highly expressed in both OC and FC from PiD patients (Fig. 4).

Depletion of Grp78/BiP and Nrf2 in PiD

Since ATF6, IRE1, as well as eIF-2α phosphorylation (via PERK) are dependent on Grp78/BiP chaperoning activity, and ATF6 and XBP1 - activated after ER stress - promote the transcription of Ca^{2+}-dependent molecular chaperones, such as Grp78/BiP, Grp94 and Calreticulin (14, 15), we measured its levels. The Grp78/BiP and Grp94 levels were likewise alterated, but in quite

unexpected manner (Fig. 5). In FC and even in OC both molecules showed an abnormal response of drastically diminished levels, while the foldases and other proteins residents of the ER such as Calreticulin, PDI and PKR did not present any significant change in FC. However in OC there were changes in the levels of PKR and PDI (Fig. 5). Although amounts of the UPR marker p-eIF-2α were increased in FC (Fig. 4), levels of the eIF-2α upstream molecule PKR were normal (Fig. 5), suggesting that elevated p-eIF-2α in FC is independent of PKR. The major eIF-2α phosphorylation regulator during the UPR is PERK that also leads to Nrf2 nuclear translocation. Nrf2, required for cell survival during UPR, favouring the expression of chaperones and antioxidant enzymes, shares PERK as a common upstream activation pathway with eIF-2α (8). Interestingly, the levels of the Nrf2 were significantly lower in FC of PiD when compared with controls (Fig. 5).

DISCUSSION

The results of the present work constitute the first evidence of the involvement of both oxidative stress and ER stress in the neurodegeneration of different locations in PiD (Fig. 6). ER stress, usually invoked as a homeostatic response to cope with an unbalanced equilibrium between protein synthesis and export in ER, leads to a concerted response characterized by transcriptional stop, increased expression of ER resident chaperones and, when those later are non effective, to a programmed cell death. Oxidative stress has been previously suggested in FC in PiD (17), but no data was available on its involvement in OC. Assuming that this disease is a tauopathy affecting different anatomic locations at a different pace, disturbances taking place in OC could shed some light on pathogenesis of the disease in the FC at earlier stages. Therefore, OC shows an increase in all types of protein oxidative damage, reacting with increased DHA levels, a potentially defensive response previously seen in pathologically preserved zones in Alzheimer disease (AD) and amyotrophic lateral sclerosis patients (18-20, 26). Furthermore, PiD in OC is associated with a decreased expression of a key chaperone in ER, Grp78/BiP, followed by an increased XBP1 formation. The physiological response to low Grp78/BiP would be increased Nrf2 nuclear translocation via PERK dimerization (21). As this response would generate an increase of glutathione concentration (22, 27), (i.e. therefore preventing build-up of oxidative damage), but this is not compatible with increased oxidative damage observed in this situation, it may be suggested that a failure of Nrf2-ER chaperone signalling axis is a key event in the disease. Previous evidence suggest that chemical dysfunction of PiD occur in advance of overt clinical manifestations of the disorder, and those disturbances are not centred exclusively in FC (28-30).

Assuming a continuum between OC and FC, advanced tauopathy (i.e. PiD in FC) is linked to increased protein lipoxidation and direct oxidation. This is also associated to more marked signs of increased ER stress, but despite transcriptional activators of ER resident chaperones were detected, no increased level of those was evident. As a matter of fact, the loss of Grp78/BiP observed in FC can be a major causal factor in ER stress in this location. Nevertheless, as this location is depleted of neurons, and those cells exhibit a high glycolytic rate, concentrations of glycolysis intermediates (e.g.

glyceraldehyde-3-phosphate) should be diminished. This is consistent with lower levels of glycoxidative damage.

Among the different causes of ER stress, it may be counted cellular redox disturbances, glucose deprivation, aberrant Ca^{2+} regulation in ER and impairment of proteasome function (3). Our data demonstrate a novel pathway of ER stress in neurodegeneration, the lack of Grp78/BiP. This protein acts as a checkpoint so when ER chaperoning function is overcome by accumulation of misfolded proteins, its release allows the activation of ER stress transducers, such as PERK, IRE1 and ATF6. Besides lack of Grp78/BiP, other pathways may explain the increased ER stress evidenced in PiD. As suggested in other neurodegenerative disorders, situations that lead to an interference in proteasome function can impair retrograde transport of misfolded proteins into the cytosol, thus contributing to ER stress (16). Accordingly we were able to detect increased general ubiquitination in proteins, findings compatible with a proteasomal dysfunction in FC. It is known that ubiquitin-rich structures have been detected as ribosome-like granular components in the neuronal cytoplasm and dendrites, related to the ER (31). In vitro tripsin-like activities of 20S proteasome were increased in FC, suggesting that this response is not effective *in vivo*, a finding compatible with a non covalent modification of proteasome. Nevertheless, as increased ubiquitin is not present in OC, despite this region shows XBP1 processing -i.e. ER stress-, it may be suggested that proteasome impairment is not a primary event in ER during PiD pathogenesis. Most strinkingly, recent data demonstrate that phosphorylated tau protein leads to decreased proteolysis, despite actually increase isolated proteasome activity (12).

When evaluating the targets of oxidative damage, we identified GFAP as previously described (17), glyceraldehyde-3-phosphate dehydrogenase but also some defensive proteins such as a 70 kDa HSP, a NADPH-dependent carbonyl reductase and cathepsin D. All these data suggest that both reduction of oxidative damage precursors (carbonyl reductase) as well as clearance systems (cathepsin D and HSP) can be impaired, contributing to a vicious circle increasing oxidative damage. Evidences from both human samples and experimental models indicate that carbonyl reductase has a physiological role for neuroprotection, by detoxifying reactive aldehydes derived from lipid

peroxidation thereby confering protection against oxidative stress-induced brain degeneration (23, 32). The potential modification of its activity by oxidation could contribute to a vicious cycle generating further oxidative damage. Concerning cathepsin D, besides its pathogenical role in AD, it is known that its deficit leads to high rate of neuronal death with accrual of undigested lipofuscin (24). In other taupathies, up-regulation of molecular chaperones -specifically HSP70- suppress formation of neurofibrillary tangles by directing tau into a productive folding pathway and thereby preventing tau aggregation (25, 33). Loss of glyceraldehyde-3-phosphate dehydrogenase may contribute to decreased glycolytic potential, as suggested by lower CEL values. Damage of vesicle-fusing ATPase may contribute to defects in synaptic function, which could contribute to clinical manifestations of PiD.

Collectively, data presented here reveal a novel pathogenic pathway for this tauopathy, consisting in defective Grp78/BiP-Nrf2 signalling after oxidative and ER stress (Fig. 6), both in frontal and occipital cortex, with damage on key enzymes in antioxidant defence, protein folding and synaptic proteins. This would suggest that combined therapeutic approaches (aiming both at protein chaperoning and oxidative stress) may be of potential use in the treatment of this disease.

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Figure 1. Protein oxidative modifications in PiD patients

Proteins from PiD samples show a significant increase in the amounts of direct oxidation markers (**A**) GSA and AASA suggesting increased free radical modification, as supported by increased MDAL levels (**C**), both in FC and in OC of PiD patients. In contrast, decreased levels of glycoxidative markers CEL and CML (**B**) in FC are present in PiD samples. OC exhibits increased oxidative damage (direct oxidation, lipoxidation and glycoxidation). Changes in DHA levels (**D**) are present both for FC and OC. DHA levels correlate significantly with CEL (**E**) (r=0,89; p<0,0001; [CEL] (µmol/mol lys)=31,9* [DHA] (%)-12,12)). ARA (arachidonic acid) levels (**D**), increased in FC, but not in OC. [a]-significant difference between the control and the PiD samples in the same area. [b]-significant difference between the FC and OC (p<0,01 by Student's t test). Values shown are % changes of mean ±S.E.M over values in FC samples of control individuals (GSA: 17414±1250 µmol/mol lys; AASA: 104±4 µmol/mol lys; CEL: 427±38 µmol/mol lys; CML: 756±39 µmol/mol lys; MDAL: 163±7 µmol/mol lys; see Table S2 for PI, DHA and ARA values)

Figure 1

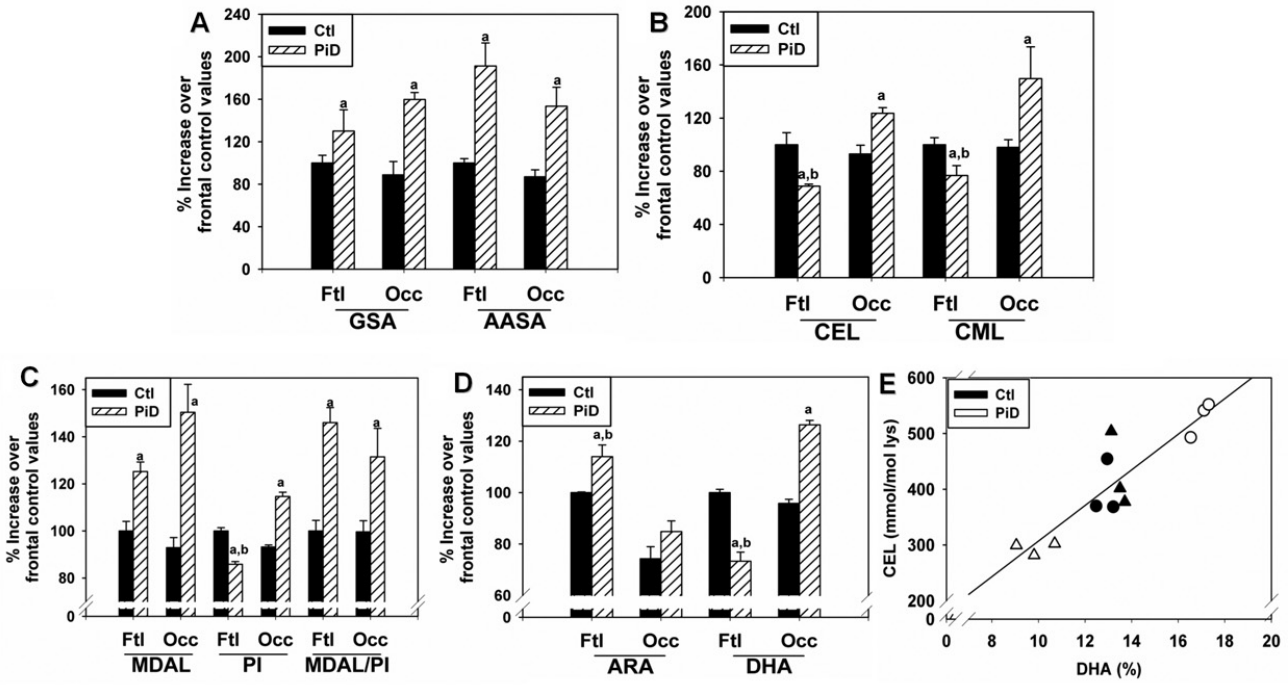

A
B
C
D
E
Ctl
PiD
% Increase over frontal control values
Ftl
Occ
GSA
AASA
CEL
CML
MDAL
PI
MDAL/PI
ARA
DHA
CEL (mmol/mol lys)
DHA (%)

Figure 2. Proteomic analysis of oxidized proteins in PiD patients

Bi-dimensional gel electrophoresis and western blotting of membranes stained with anti-DNP antibodies. The right upper panel displays a strong increase in the amount of oxidized spots in pH 3-->11 strips at about 30-55 kDa and pH 5-7 of the FC in PiD when compared with an age-matched control. Bi-dimensional gels processed in parallel and stained with silver are shown on the panel below for comparison. They were used to obtain the spots for mass spectrometry analysis. Dissection of the spots and their analysis revealed a vesicle-fusing ATPase (a) (access No P46459), GFAP (b) (access No P14136), two intracellular proteases Cathepsin D precursor isoforms (c, d) (access No P07339), antioxidante defence enzyme Carbonyl reductase NADPH1 isoforms (e, f) (access No P16152), glyceraldehyde 3-phosphate dehydrogenase (g) (access No P04406) and heat shok 70 kDa protein (k) (access No P08107).

Figure 2

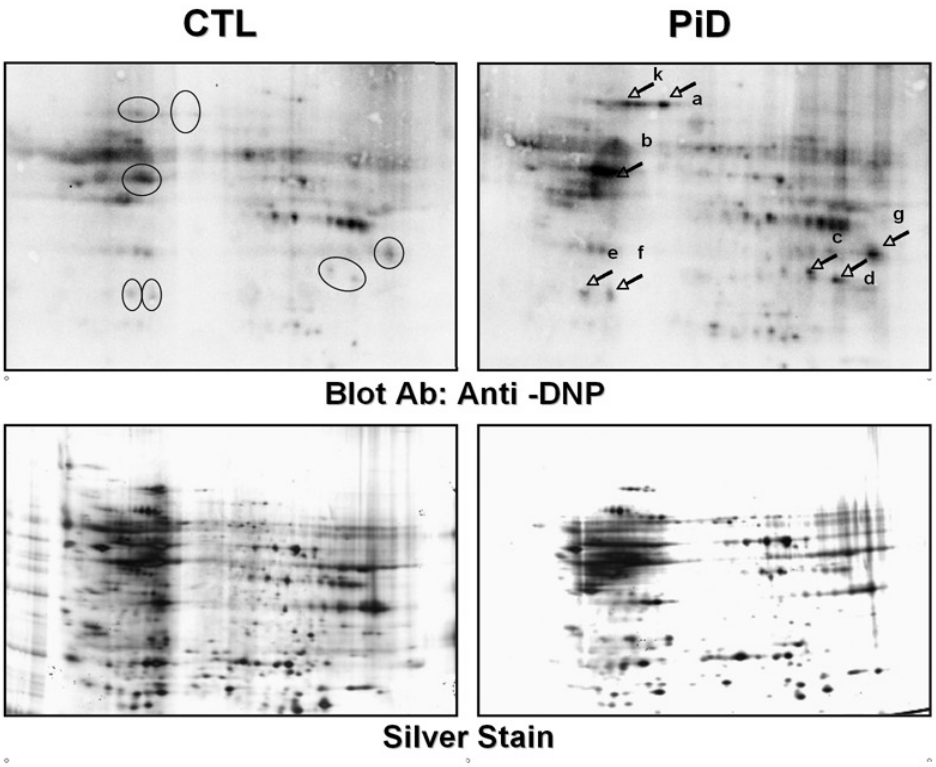
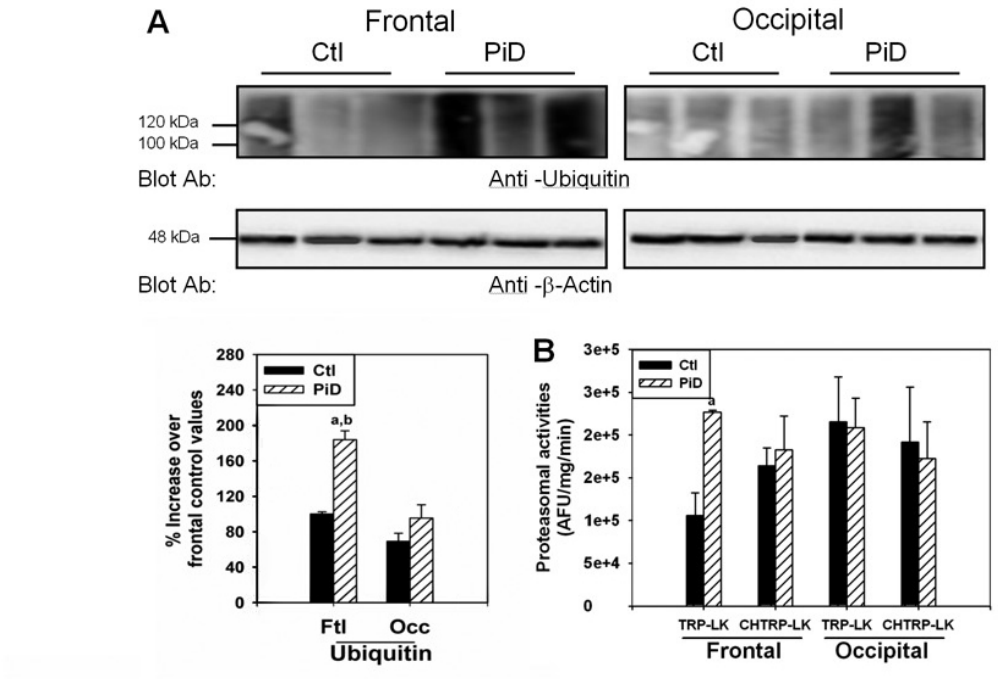


Figure 3. Proteasomal function assessment in PiD patients

Representative western blots with anti-Ubiquitin (**A**) reveal high molecular weight bands corresponding to polyubiquitinated proteins, suggesting decreased ubiquitin degradation in FC samples from PiD patients, but not in OC. Right numbers of the blot indicate apparent molecular weight. Immunoblotting of actin is also shown as a loading control. The lower panel indicates bars representing blot quantifications by densitometry, adjusted to actin content. [a]-significant difference between the control and the PiD samples in the same area. [b]-significant difference between the OC and FC ($p<0,001$ by Student's t test). Proteasome activity measurement (**B**) using fluorescently labeled peptides specific for chymotrypsin-like (CHTRP-LK) and trypsin-like (TRP-LK) activities shows an enhanced TRP-LK activity of the proteasome only in FC of PiD samples, without changes in OC. Changes in the CHTRP-LK activity were not detected either in FG or in OC.

Figure 3


A
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120 kDa
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Blot Ab:
Anti -Ubiquitin
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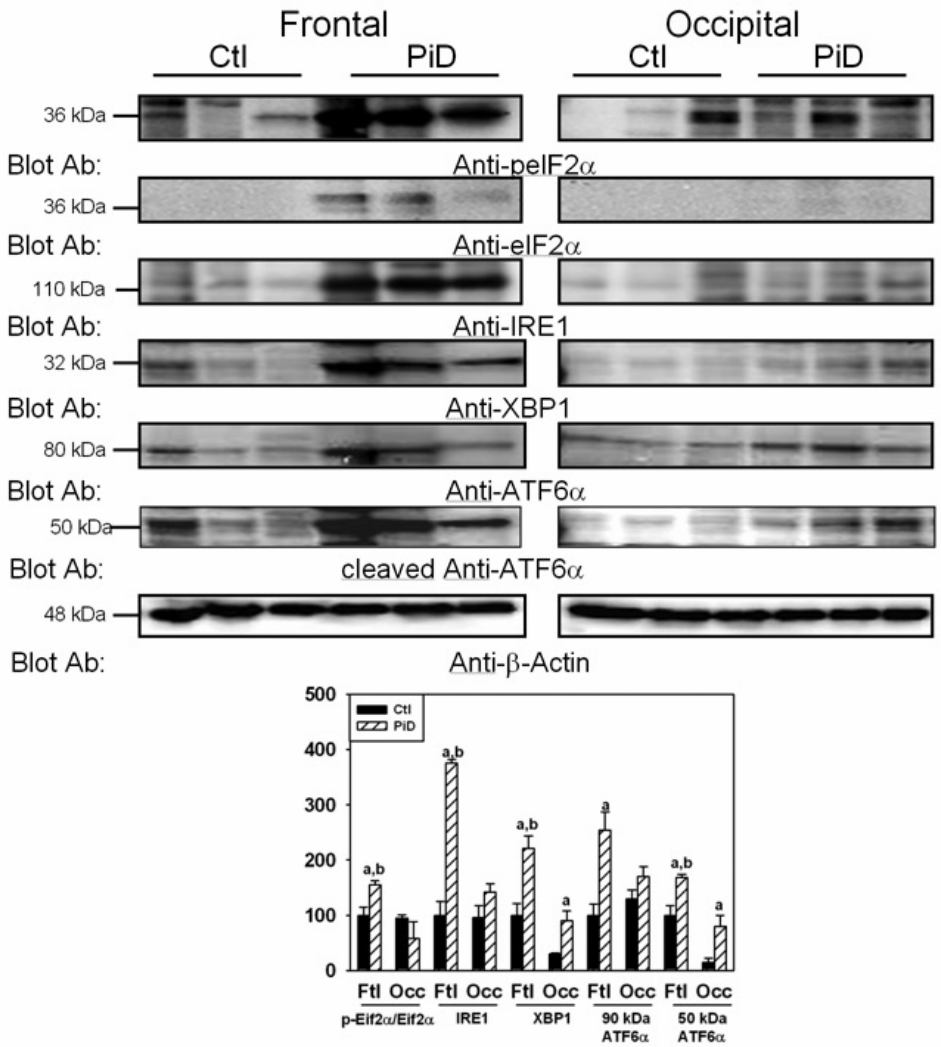
Blot Ab:
Anti -β-Actin
% Increase over frontal control values
280
240
200
160
120
80
40
0
Ctl
PiD
a,b
Ftl
Occ
Ubiquitin
B
Proteasomal activities (AFU/mg/min)
3e+5
3e+5
2e+5
2e+5
1e+5
5e+4
0
Ctl
PiD
a
TRP-LK
CHTRP-LK
TRP-LK
CHTRP-LK
Frontal
Occipital

Figure 4. Activated UPR in response to ER stress in PiD patients Representative western blots of FC and OC homogenates show signs of UPR such as increased eIF-2α phosphorylation and activation of the key UPR membrane transducers IRE1, XBP1 and ATF6 in FC, in contrast to OC. The lower panel shows the quantification of the blots by densitometry, adjusted to actin content. [a]-significant difference between the control and the PiD samples in the same area. [b]-significant difference between the OC and FC ($p<0,001$ by Student's t test).

Figure 4


Frontal
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Anti-peIF2α
36 kDa
Blot Ab:
Anti-eIF2α
110 kDa
Blot Ab:
Anti-IRE1
32 kDa
Blot Ab:
Anti-XBP1
80 kDa
Blot Ab:
Anti-ATF6α
50 kDa
Blot Ab:
cleaved Anti-ATF6α
48 kDa
Blot Ab:
Anti-β-Actin
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p-Eif2α/Eif2α
IRE1
XBP1
90 kDa ATF6α
50 kDa ATF6α

Figure 5. Lack of ER chaperones and Nrf2 depletion in frontal cortex of PiD patients

Representative western blots of frontal and occipital cortex homogenates show an abnormal response to ER stress, evidenced by lack of ER chaperones Grp94, Grp78/BiP and Nrf2 regulator, but not PDI and other proteins residents of the ER although they did not present any significant change in FC homogenates from PiD patients. The lower panels show the quantification of these blots by densitometry, adjusted to actin content. [a]-significant difference between the control and the PiD samples in the same area. [b]-significant difference between the OC and FC ($p<0,001$ by Student's t test).

Figure 5

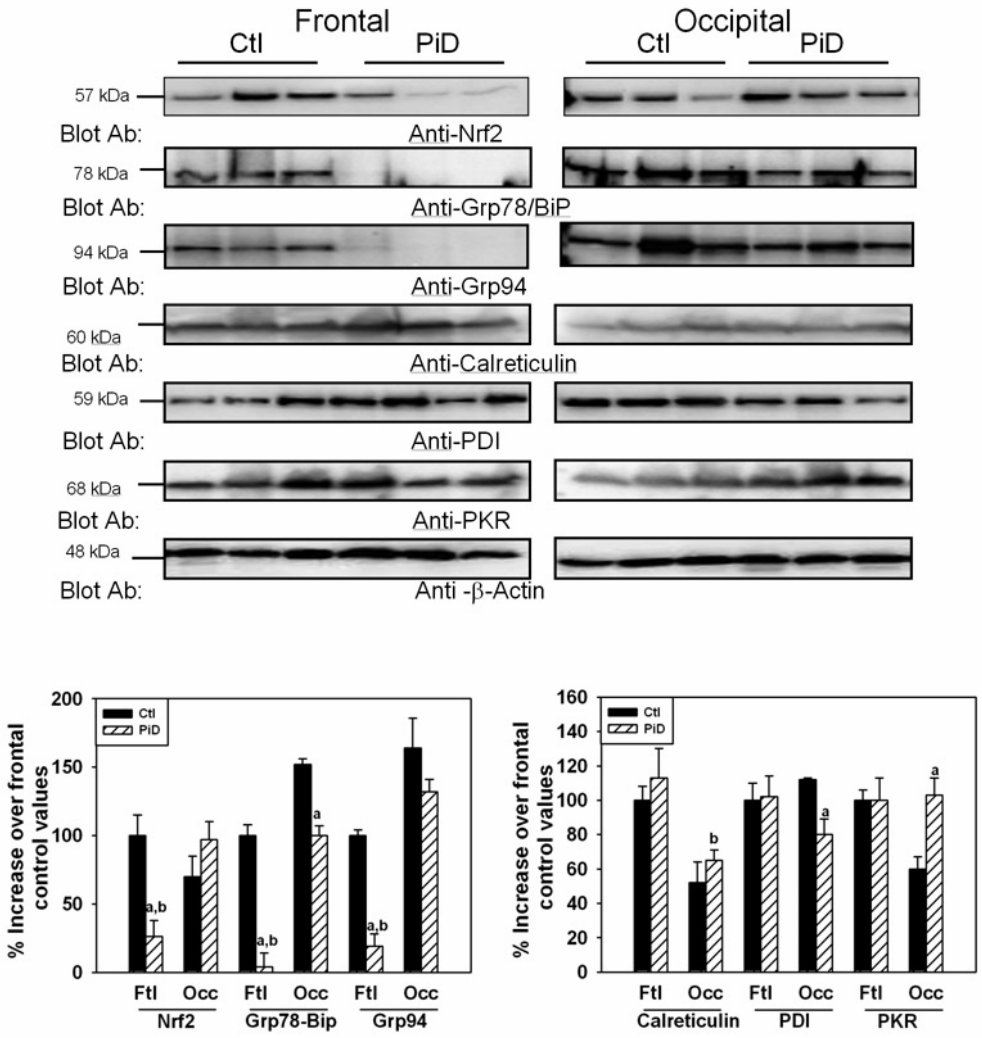

Frontal
Occipital
Ctl
PiD
Ctl
PiD
57 kDa
Blot Ab:
Anti-Nrf2
78 kDa
Blot Ab:
Anti-Grp78/BiP
94 kDa
Blot Ab:
Anti-Grp94
60 kDa
Blot Ab:
Anti-Calreticulin
59 kDa
Blot Ab:
Anti-PDI
68 kDa
Blot Ab:
Anti-PKR
48 kDa
Blot Ab:
Anti -β-Actin
% Increase over frontal control values
Ctl
PiD
Ftl
Occ
Nrf2
Grp78-Bip
Grp94
Calreticulin
PDI
PKR

Figure 6. Schematic representation of the intercommunication between oxidative and ER stress through the proteasome dysfunction

Oxidative stress-induced molecular alterations, affect all sorts of biological molecules (1). The potential oxidative modification of some defensive proteins such as carbonyl reductase, cathepsin D, HSP70 an others (2) could contribute to further generation of oxidative damage. The oxidative modifications of the proteins make them prone to proteolysis in the proteasome. Higher rates of oxidative modification actually inhibit the proteasome (3). Oxidative stress, accumulation of modified proteins resistant to proteasomal degradation, disruption of the ER-associated degradation (a potential consequence of such proteasomal impairment), finally contribute to ER stress (4) and activate the UPR (5). The ER chaperone Grp78/BiP as master regulator of the key UPR-related molecules IRE1, PERK and ATF6 initiates UPR activating them through their release. Phosphorylation of eIF-2α via PERK (6) leads to translational attenuation, thus inhibits protein synthesis to decrease the load within the ER. The major eIF-2α phosphorylation regulator during the UPR is PERK that also leads to Nrf2 nuclear translocation (7). Nrf2, required for cell survival during UPR, regulates the inducible expression of antioxidant response elements containing genes, encoding subset of antioxidants and detoxifying enzymes in response to oxidative stress and several chaperones in response to ER stress. Thus, the significantly lower levels of Nrf2 together with the depletion of Grp78/BiP are an abnormal response to the stress conditions that could contribute to the neuronal death (7) in PiD.

Figure 6

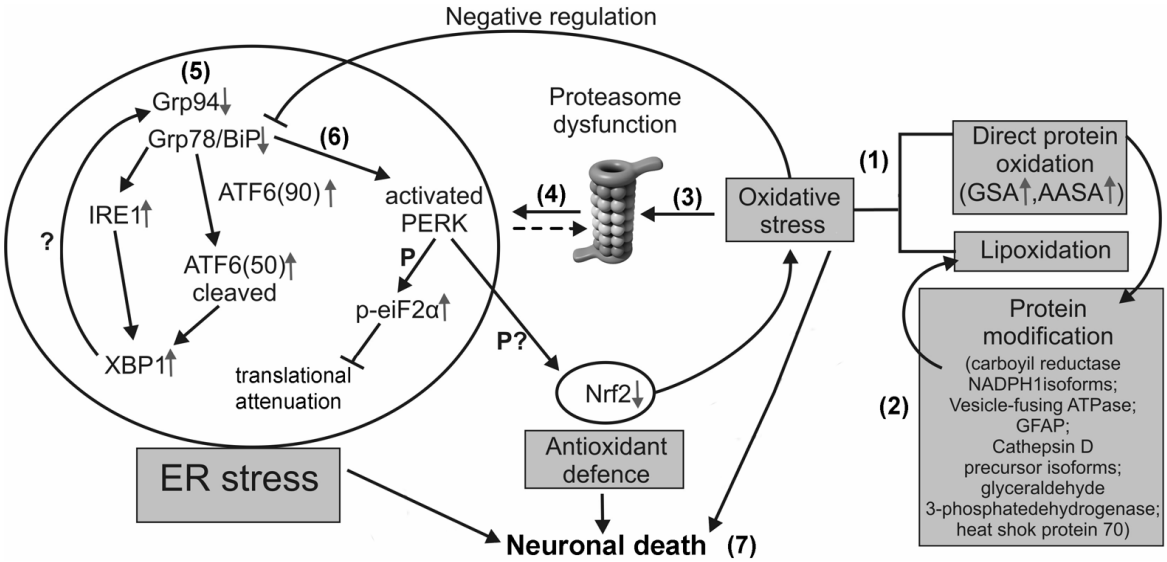

Negative regulation
(5)
Grp94↓
Grp78/BiP↓
(6)
IRE1↑
ATF6(90)↑
activated PERK
?
ATF6(50)↑ cleaved
P
p-eiF2α↑
XBP1↑
translational attenuation
P?
Proteasome dysfunction
(4)
(3)
Oxidative stress
(1)
Direct protein oxidation (GSA↑,AASA↑)
Lipoxidation
Protein modification
(carboyil reductase NADPH1isoforms; Vesicle-fusing ATPase; GFAP; Cathepsin D precursor isoforms; glyceraldehyde 3-phosphatedehydrogenase; heat shok protein 70)
(2)
Nrf2↓
Antioxidant defence
ER stress
Neuronal death
(7)

Table 1. Fatty acid composition (mol %) of total lipids from frontal and occipital cortex

	Frontal Cortex Ctl	**Frontal Cortex Pick**	**P<**	**Occipital Cortex Ctl**	**Occipital Cortex Pick**	**P<**
14:0	0.58±0.02	0.80±0.06	0.007	0.54±0.05	0.78±0.01	0.004
16:0	20.62±0.35	25.15±0.53	0.015	20.24±1.86	23.12±0.62	0.084
16:1n-7	0.89±0.09	1.04±0.11	0.460	0.75±0.19	0.58±0.11	0.376
18:0	18.55±1.80	17.40±0.23	0.527	19.13±0.23	18.73±1.61	0.822
18:1n-9	27.06±2.27	26.12±0.40	0.617	28.42±0.97	21.72±0.46	0.006
18:2n-6	0.53±0.21	0.88±0.07	0.084	0.57±0.07	0.71±0.07	0.438
18:3n-3	0.18±0.01	0.18±0.01	0.991	0.21±0.04	0.19±0.01	0.606
20:0	2.09±0.27	1.11±0.32	0.096	2.38±0.50	1.65±0.29	0.190
20:3n-6	0.53±0.05	0.80±0.01	0.002	0.63±0.03	0.64±0.03	0.959
20:4n-6	8.15±0.01	9.28±0.36	0.034	6.05±0.38	6.91±0.33	0.089
22:4n-6	4.80±0.09	3.86±0.44	0.145	5.03±0.64	4.75±0.22	0.641
22:5n-6	0.60±0.10	1.17±0.18	0.010	0.59±0.09	0.74±0.05	0.408
22:5n-3	0.14±0.02	0.14±0.03	0.986	0.18±0.02	0.16±0.01	0.378
22:6n-3	13.44±0.17	9.85±0.47	0.000	12.87±0.21	16.98±0.23	0.000
24:0	0.27±0.03	0.55±0.02	0.000	0.31±0.05	0.57±0.02	0.001
24:1n-9	1.50±0.30	1.58±0.39	0.852	2.00±0.32	1.72±0.16	0.541
ACL	18.62±0.01	18.39±0.03	0.006	18.62±0.07	18.72±0.01	0.160
SFA	42.12±2.05	45.03±0.46	0.148	42.62±1.10	44.86±0.96	0.253
UFA	57.87±2.05	54.96±0.46	0.148	57.37±1.10	55.13±0.96	0.253
MUFA	29.46±2.61	28.76±0.60	0.746	31.19±1.07	24.03±0.73	0.009
PUFA	28.40±0.55	26.20±0.23	0.010	26.18±0.21	31.10±0.66	0.000
PUFAn-6	14.63±0.46	16.01±0.51	0.066	12.89±0.11	13.76±0.59	0.218
PUFAn-3	13.77±0.13	10.18±0.43	0.000	13.28±0.19	17.33±0.23	0.000
DBI	168.92±0.70	151.83±0.58	0.000	160.46±1.41	181.04±2.69	0.000
PI	166.55±2.27	142.93±1.97	0.000	155.16±1.45	190.93±3.0	0.000

Table S1. Primary and secondary antibodies used for western blot immunodetection

Antigen	***Supplier***	***Use(dilution)***
1. Phosporylated (S51) eIF2α	Abcam Co/Cell Signaling	WB (1:750) / WB (1:1000)
2. eIF2α	Abcam Co/Cell Signalling	WB (1:500) / WB (1:1000)
3. IRE 1α	ProSci	WB (1:1000)
4. XBP1	ProSci	WB (1:1000)
5. ATF6α	ProSci	WB (1:1000)
6. Grp78/BiP	Stressgen	WB (1:1000)
7. Grp94	Santa Cruz	WB (1:250)
8. PDI	Abcam	WB (1:500)
9. PKR	Abcam	WB (1:500)
10. Calreticulin	Abcam	WB (1:250)
11. Nrf2	Santa Cruz	WB (1:750)
12. Ubiquitin	Sigma	WB (1:1000)
13. DNP	Sigma	WB (1:2500)
14. β-Actin	Sigma	WB (1:5000)
15. anti-mouse	Amersham	WB (1:30000)
16. anti-rabbit	Pierce	WB (1:100000)
17. anti-goat	Vector	WB (1:15000)

ADDITIONAL INFORMATION-PICK'S DISEASE

Figure 1 AI-PiD:

As stated in section 2.5., another macromolecule that can also be damaged as a consequence of oxidative stress besides proteins is DNA. Free radicals produce a multiplicity of modifications in DNA. Oxidative attack on the deoxyribose moiety leads to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple abasic-apurinic/apyrimidinic (AP)-sites. AP sites are one of the major types of DNA lesions formed during the course of base excision and repair of oxidized, deaminated or alkylated bases generated by ROS [Asaeda A. et al. Nucleosides & Nucleotides, 17, 503 (1998)]. The level of AP sites in cells is a good indicator of DNA lesion and repair against chemical damage and cell aging.

To evaluate the base DNA damage in PiD samples, we used a commercial DNA Damage Quantification Kit ((Ref.N: JM-K253-25), MBL, USA) based on quantification of abasic sites in DNA. The DNA Damage Quantification Method utilizes the Aldehyde Reactive Probe (ARP) reagent that reacts specifically with an aldehyde group which is the open ring form of the AP sites. After treating DNA containing AP sites with ARP reagents, AP sites are tagged with biotin residues, which can be quantified using avidin-biotin assay followed by a colorimetric detection measuring the O.D. at 650 nm.

DNA damage quantification in PiD. Determination of the number of abasic sites in genomic (left) and mitochondrial (right) DNA using the calibration curve and comparation between the numbers of AP sites in PiD-affected samples vs. control samples. The genomic DNA from PiD patients extracted from FC contain approximately 3,5 AP sites per 10^5 nucleotides compared with OC where 2,5 AP sites were detected per 10^5 nucleotides. In contrast, the mitochondrial DNA from PiD patients extracted from FC contain approximately 6,5 AP sites per 10^5 nucleotides compared with OC where 4 AP sites were detected per 10^5 nucleotides. Bars represent the mean ±S.E.M of AP sites, $p<0.05$ according to the Student t-test for genomic DNA damage and $p<0.001$ for mitochondrial DNA damage, respect controls.

Figure 1 Al-PiD:

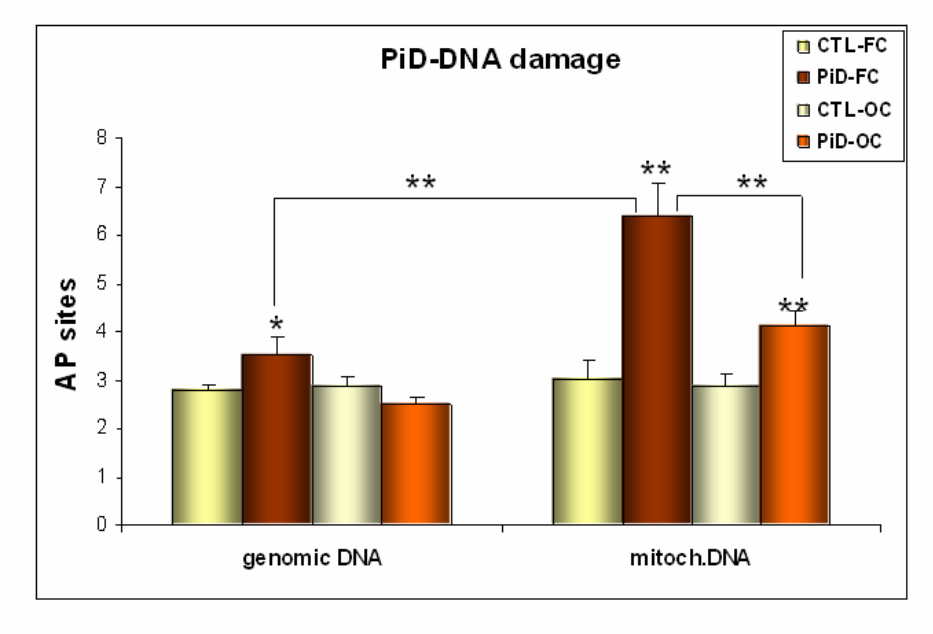


Figure 2 AI-PiD:

Neuroketals in FC and OC of patients with PiD

Representative western blots of the lipoxidation marker Neuroketals in FC (left panel) and OC (right panel) of PiD patients display differential targets of lipoxidation ranging between 30 and 70 kDa, suggesting significant increase in amount of lipoxidation products in FC but not in OC of PiD patients when compared with control cases.

Right numbers of the blot indicate apparent molecular weight. β-Actin (47 kDa) was used as a loading control. The lower panel shows the quantification of these blots by densitometry, and differences were analyzed respect to control group by Student's t-test being *$p<0.05$.

Figure 2 AI-PiD:

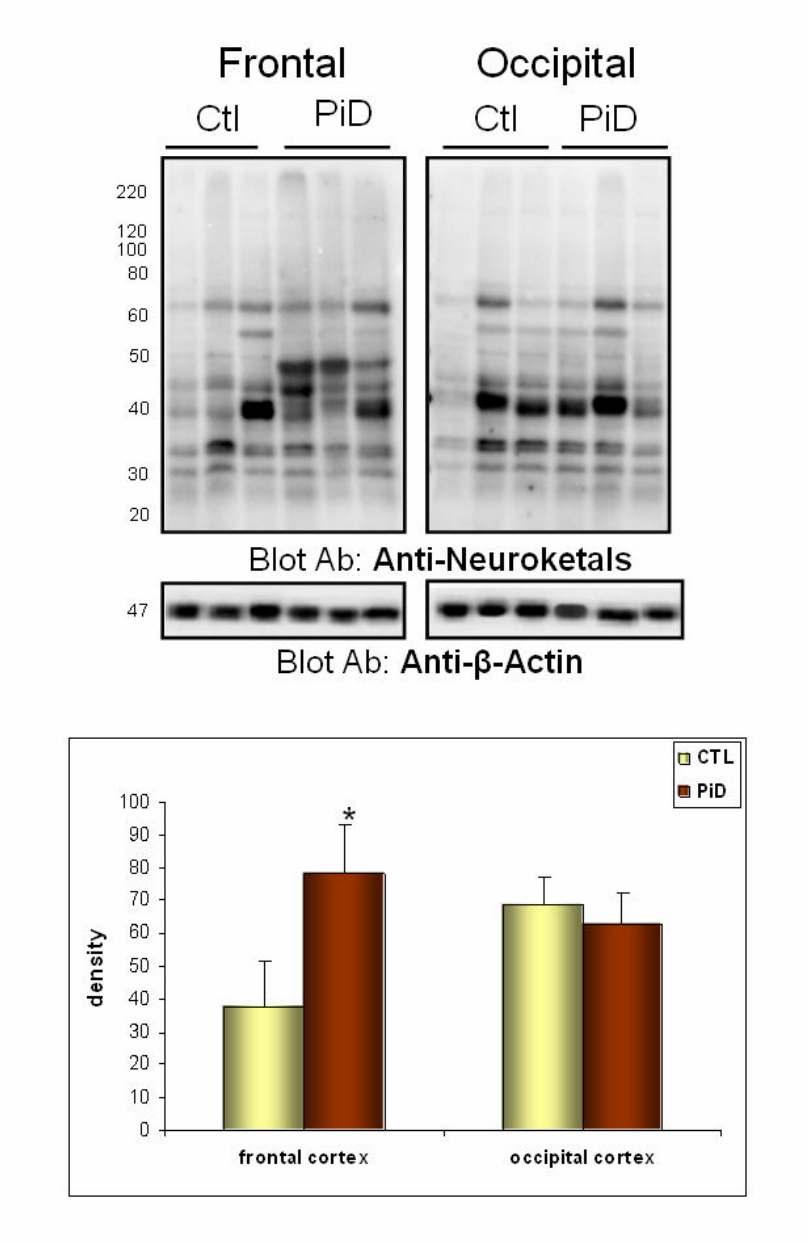


Figure 3 AI-PiD:

Mitochondrial changes in PiD

(**A**) Representative western blot of peptides NDUFA9, SDHA, core 2 and MTCO1 subunits of mitochondrial respiratory chain complexes I-IV, respectively, suggesting increased complex I and IV content in FC samples from PiD patients compared to control individuals. Porin was used as a control of total mitochondrial mass. The lower panel represents quantification of these blots by densitometry, adjusted to porin density except porin which was adjusted to β-actin. Differences were analyzed respect to control group by Student's t-test being minimum mean value *p<0.05, **p<0.001.

Complex IV activity in FC homogenates was measured in a high resolution Oxygraph 2k respirometer (Oroboros, Austria) as the KCN inhibitable cytochrome C oxidase dependent oxygen consumption in MiR05 medium in the presence of 2 mM sodium ascorbate, 500 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), 10 μM cytochrome C and 2.5 μM antimycin, with a final addition of 1 mM KCN [Justo R. et al. *Am J Physiol Cell Physiol* 289: 372-378, 2005]. Chemical background oxygen flux due to autooxidation of TMPD was corrected under experimental conditions over the entire oxygen range.

(**B**) Activity of complex IV is significantly increased in FC of PiD samples, as evidenced by oxygen consumption using the complex IV substrate TMPD-ascorbate, in the presence of specific inhibitors of complex I to III and cytochrome C excess. Differences were analyzed respect to control group by Student's t-test being minimum mean value *p<0.05.

Figure 3 AI-PiD:

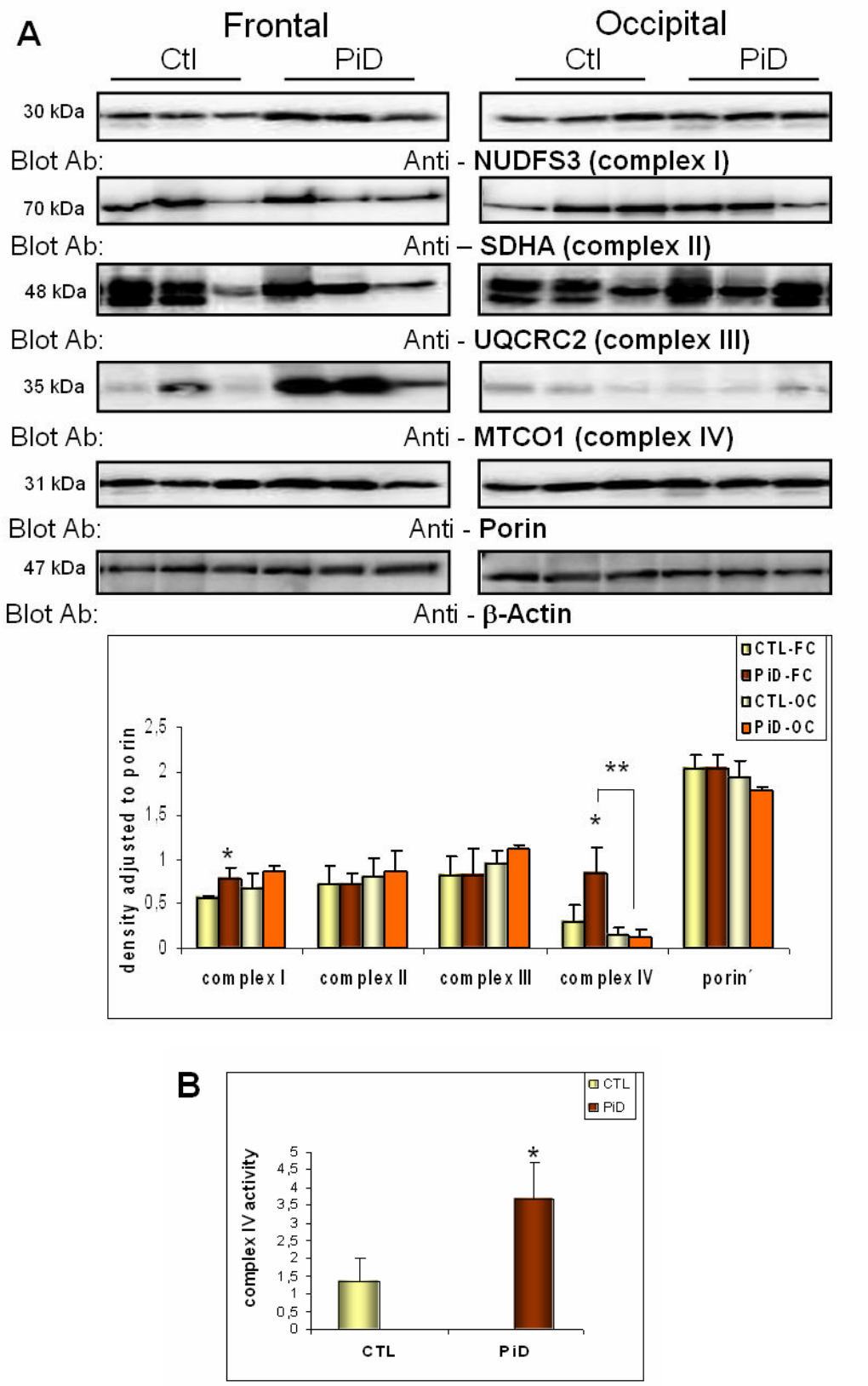


Figure 4 AI-PiD:

Mitochondrial changes in PiD

Representative western blots for cytosolic (AIF 67kDa), mitochondrial (AIF 62 kDa) and nuclear (AIF 57 kDa) fraction of AIF, UCP4 and Prohibitin show decreased content of UCP4 in FC compatible with increased ROS efflux from mitochondria, and increased mitochondrial fraction of AIF in both FC and OC of PiD as a potential compensatory mechanism of impaired control of mitochondrial free radical production, compatible with the changes observed in mitochondrial respiratory chain complexes. The unchanged levels of Prohibitin that constitutes a cellular defence against oxidative stress suggest inappropriate antioxidant responses in both areas. The lower panel shows the quantification of these blots by densitometry, adjusted to actin for AIF 67 and AIF 57, and adjusted to porin for AIF 62, UCP4 and Prohibitin. The differences were analyzed respect to control group by Student's t-test being *$p<0.05$, **$p<0.001$.

Figure 4 AI-PiD:

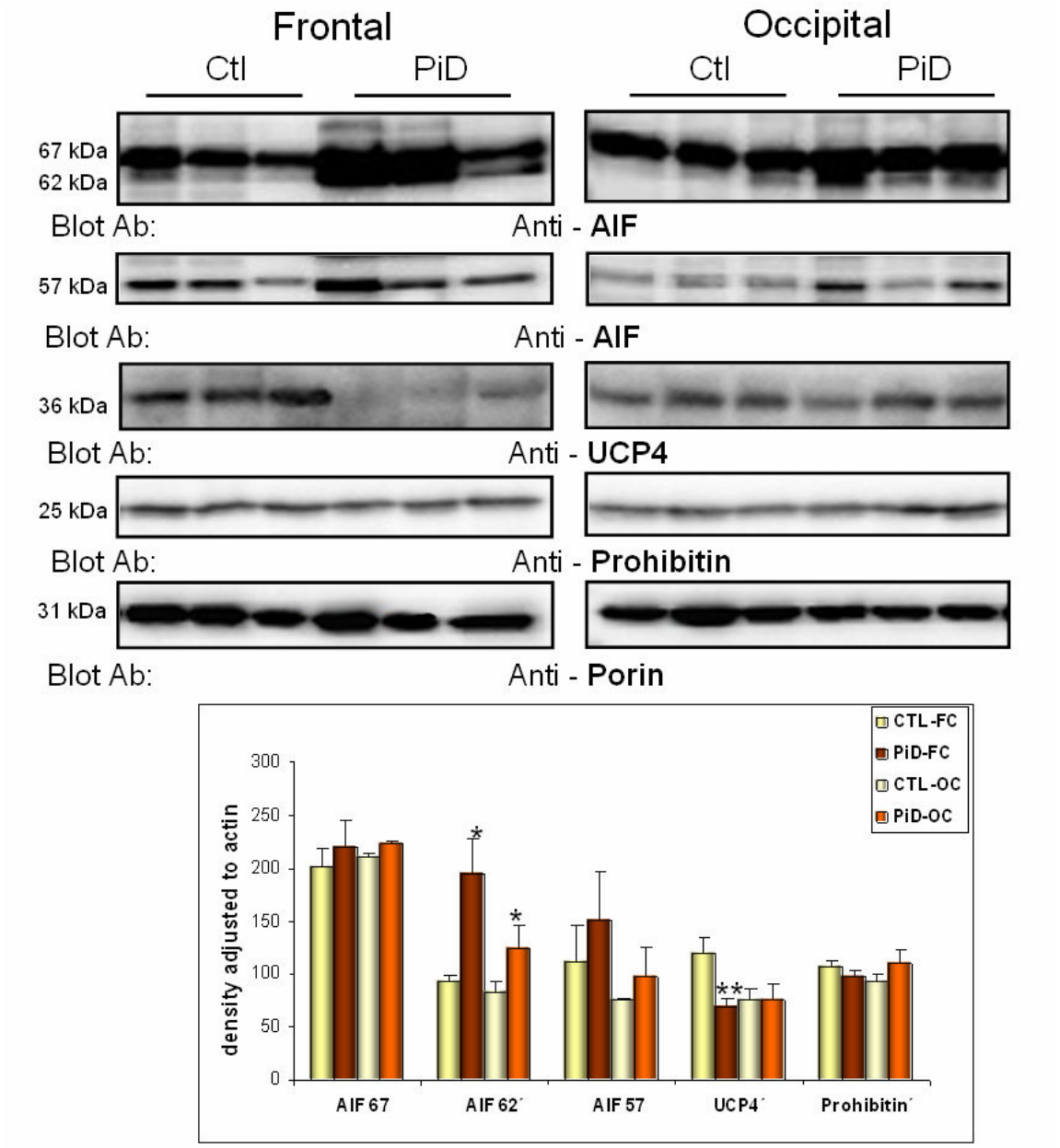


Figure 5 AI-PiD:

Defective antioxidant response in PiD

(**A**) Western blots of the regulator of ROS homeostatic cycle PGC1α, its upstream regulator Sirt1 and its downstream regulated transcription factors (Nrf1 and Nrf2) related to expression of antioxidant defence enzymes, demonstrate significantly decreased levels in FC and significant difference between both areas FC and OC in PiD. This decrease suggest defective antioxidant response evidenced by the significantly low levels of the antioxidant enzymes SOD1 and SOD2 (**B**) in FC of PiD patients respect control cases. The lower panel represents the quantification of these blots by densitometry, after densities of bands were adjusted to actin content and differences were analyzed respect to control group by Student's t-test being *$p<0.05$, **$p<0.001$.

Figure 5 AI-PiD:

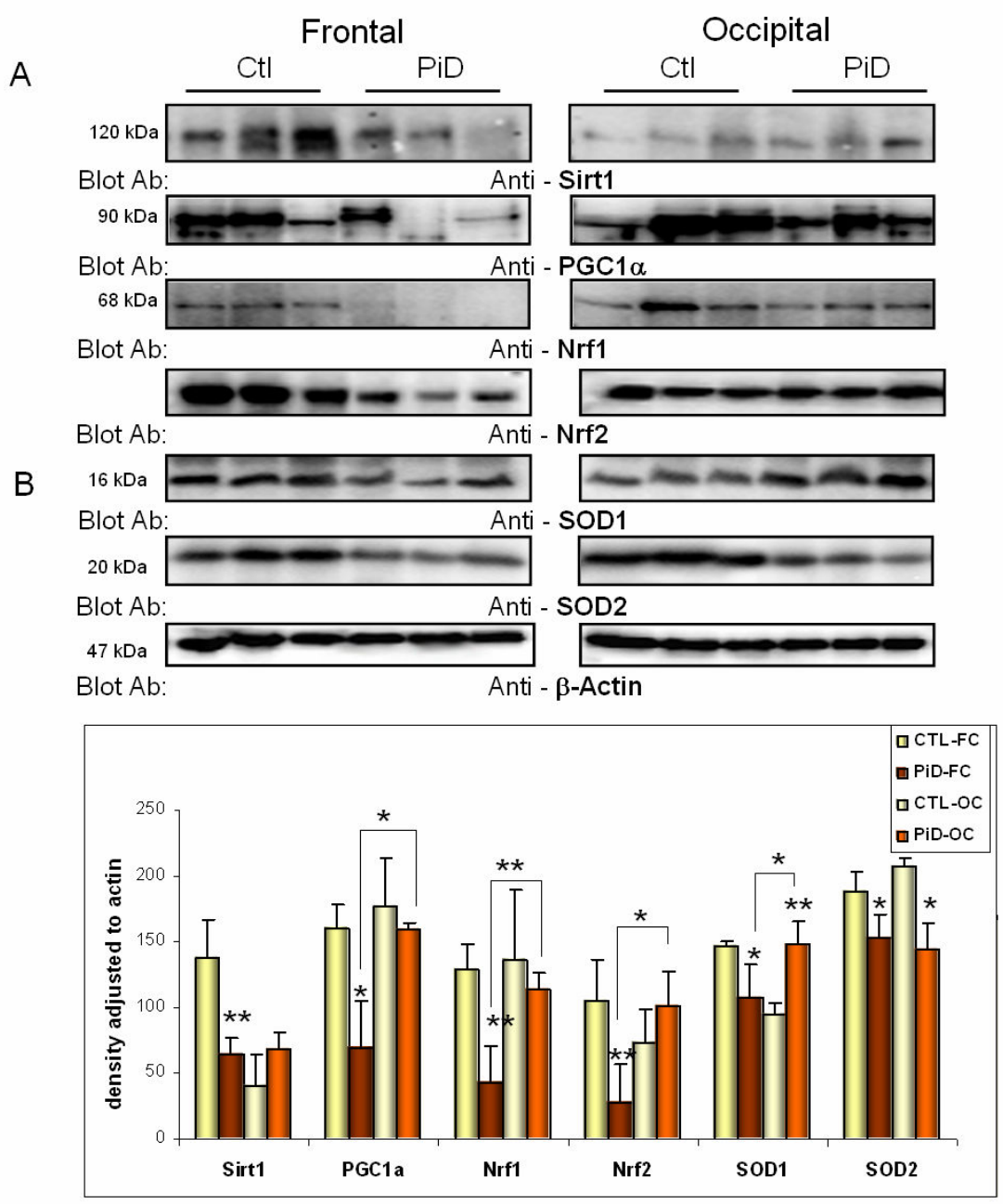


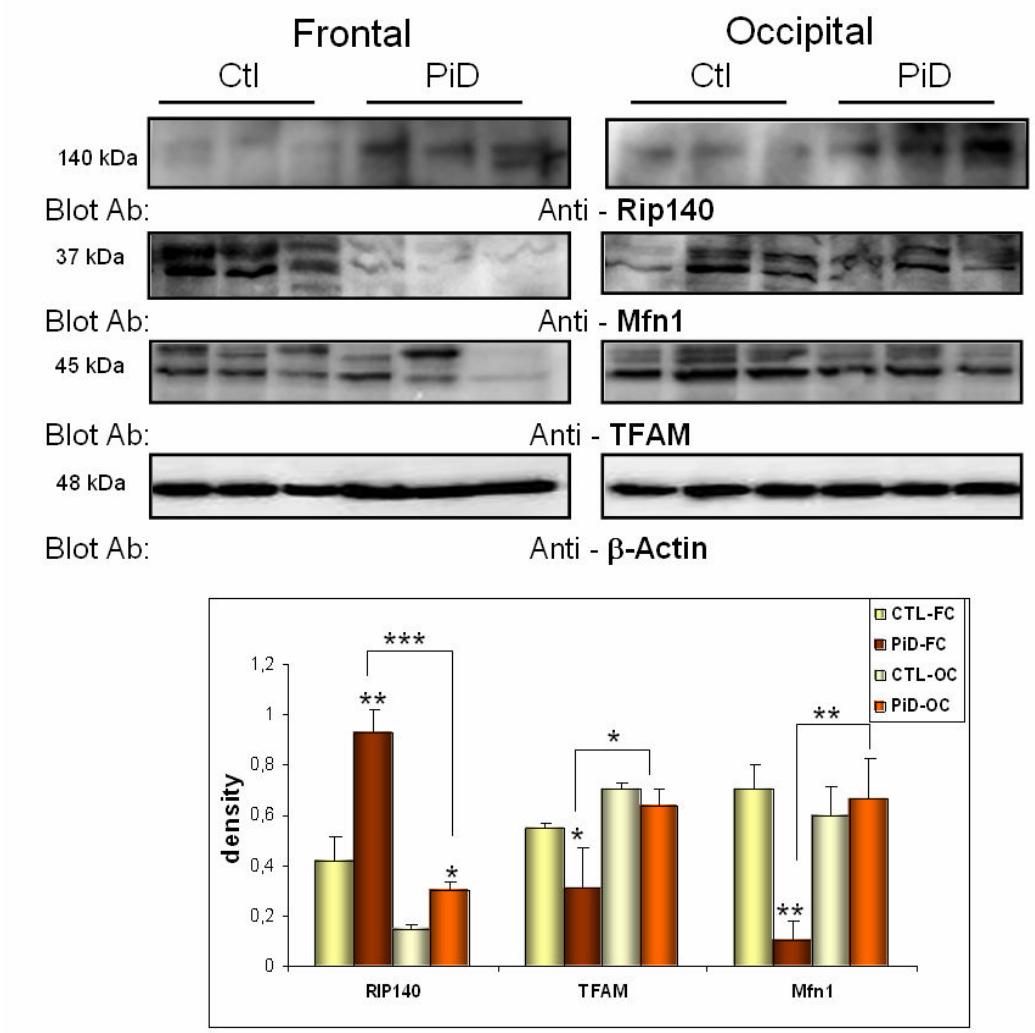
Figure 6 AI-PiD:

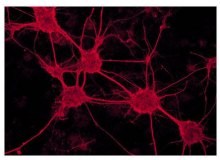
Mitochondrial biogenesis regulation in PiD

Representative western blots of mitochondrial biogenesis (MB) corepressor RIP140 and Nrf1 downstream regulated mitochondrial fusion protein (Mfn 1) and mitochondrial transcription factor A (TFAM) reveal decreased levels of the MB mediators TFAM and Mfn-1 in FC and increased amounts of RIP140 in both FC and OC, suggesting an important decrease in mitochondrial biogenesis. This could be associated with the lowered levels of the MB coactivator PGC1α and its positive regulator Sirt1.

The lower panel displays the densitometry analysis of the bands by Student's t-test, *$p<0.05$, **$p<0.001$, ***$p<0.0001$. The difference between the FC and OC is also significant for the three immunoblots. The density of RIP140 bands were adjusted to actin, and for Mfn1 and TFAM bands to porin content.

Figure 6 Al-PiD:





ARTICLE IV

"MITOCHONDRIAL DYSFUNCTION INDUCES BOTH OXIDATIVE AND ENDOPLASMIC RETICULUM STRESS IN ARGYROPHILIC GRAIN DISEASE"

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ABSTRACT:

Argyrophilic grain disease (AGD) is a recently described late-onset dementia. In this work have been studied the critical role of oxidative and endoplasmic reticulum (ER) stress, as well as a dysregulation of mitochondrial biogenesis as a novel mechanisms in AGD neurodegeneration. Thus, key molecules involved in unfolding protein response were found activated, leading to elevation in ER chaperones. Searching for a cause of ER stress, oxidative stress (OS) was evidenced by the significant increase of specific biomarkers targeting glycolytic enzymes in AGD. In turn, OS can be attributed to a mitochondrial dysfunction characterized both by disturbance in the respiratory chain complex distribution and by reduced mitochondrial mass, despite considerable augmentation in mitochondrial biogenesis regulators. Those findings could be useful in understanding the molecular mechanisms involved in the pathogenesis of AGD as in other neurodegenerative disorders.

Keywords: ER stress, oxidative stress, proteasome, proteomics, glycoxidation, lipoxidation, GC/MS, Argyrophilic grain disease.

INTRODUCTION

Argyrophilic grain disease (AGD) is a late-onset dementia with distinctive features among tauopathies. AGD is a four-repeat (4R) tauopathy, morphologically characterized by the presence of abundant hyper-phosphorylated tau protein in neuritic swellings known as argyrophilic grains (AGs), the most important histopathological hallmark of AGD, as well as in neurons with pre-tangles and in coiled bodies in oligodendrocytes, located predominantly in limbic regions of the brain[1, 2].

AGD shows a significant correlation with advancing age, and it is often associated with other neurodegenerative diseases (NDDs) as Alzheimer's disease (AD), other tauopathies and synucleinopathies[1, 3]. It is well documented that during the aging process cells in nervious system undergo oxidative stress (OS)[4], accumulation of damaged proteins[5, 6] and mitochondrial dysfunction. This latter[7] as well as decreased proteasomal activity[8, 9], associated with presence of intracellular protein aggregates, have been previously recognized as important contributors to a number of human pathologies. As a consequence of proteasomal impairment, it should be counted the disruption of the endoplasmic reticulum (ER)-associated degradation, a pathway which helps to clear misfolded protein species from ER induce ER stress[10, 11]. Expression of mutant ubiquitin resulting from mRNA misreading, and impaired proteasomal function have been observed in AGD[1].

Among several OS-derived damage, protein oxidation impacts on cellular homeostasis. Oxidative protein damage arises from direct exposure to reactive oxygen species (ROS) generating oxidative products such as glutamic and aminoadipic semialdehydes[12-14]. Protein modifications may also arise from reaction with low molecular weight and highly reactive carbonyl compounds derived from carbohydrates or polyunsaturated fatty acids (PUFA) oxidation in processes termed glyco- and lipoxidation respectively, leading to the formation of specific adducts, such as N^{ε}-carboxymethyl-lysine (CML), N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-malondialdehyde-lysine (MDAL)[15, 16].

Although the profound exploration of other tauopathies, AGD is still a poorly understood neurological disorder. Oxidative and ER stress play a critical role in the pathogenesis of Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Pick's disease (PiD) and other NDDs[17-20] but little is known about their

role in AGD. Indirect observations, as activation of stress kinases p38 and SAPK/JNK, involved in tau phosphorylation, pointed to the fact that oxidative stress may play a role in AGD pathogenesis[1, 21]. Aging is the most important determinant factor in AGD. Thus, the biological basis of aging such as OS may play a crucial role in the development of the disease. Furthermore, activation of an ER stress response, which is also involved in aging[22] could be implicated in AGD.

For these reasons in this study we amplify the vision about how the cellular stresses become involved in AGD.

MATERIALS AND METHODS

I. Human hippocampus specimens

Brain samples of the HC were obtained from the Institute of Neuropathology Brain Bank following the guidelines of the local ethics committee. The brains of eight patients with AGD and eight age-matched controls were obtained 1-13 h after death. The agonal state was short with no evidence of acidosis or prolonged hypoxia; the pH of the brain was between 6.8 and 7. Five AGD cases and five controls with a post-mortem delay between 1 and 9 h were selected for biochemical studies. The restricted post-mortem delay was within the range enabling the study of oxidative damage in the post-mortem brain[48]. Only pure cases of AGD with no associated pathology, including AD, other tauopathies and synuclenopathies were selected for the present study. All cases were categorized as stages 2 and 3[1] (Table S1).

II. Protein electrophoresis and western blot

Brain samples (200 mg) of the HC from AGD and control cases were treated as previously described[17].

Immunodetection was performed using as primary and secondary antibodies, those listed in supplementary information (Table S2). Protein bands were visualised with the chemiluminescence ECL method (Millipore Corporation, Billerica, MA, USA). The monoclonal antibody to β-actin (Sigma, USA), diluted 1: 5000, was used to control protein loading. The density of the immunoreactive bands was determined by densitometry analysis using a GS-800 Calibrated Densitometer (Bio-Rad).

III. Two-dimensional electrophoresis and mass spectrometry for protein Identification and image analysis

The two-dimensional electrophoresis was performed as previously described[17]. Immunoblotting was performed using an anti-CML polyclonal antibody as primary antibody.

For gel staining, a MS-modified silver staining method (Amersham Biosciences) was used as described by the manufacturer. For membrane staining, a silver staining method based on the Gallyas intensifier was used according to previously described procedures.

The gels and PVDF blots were scanned using a GS800 Calibrated Densitometer (Bio-Rad). PDQuest two dimensional analysis software (Bio-Rad) was used for matching and analysis of silver stained gels and membranes. The average mode of background subtraction was chosen to compare protein and CML immunoreactivity content between HC samples from AGD patients and control individuals.

Enzymatic digestion was performed with trypsin (Promega, Madison, WI) following conventional procedures as described[49]. The MALDI-reTOF MS analysis of the samples was performed using a Voyager DE-PRO MALDI-reTOF mass spectrometer (Applied Biosystems, Foster City, CA).

IV. Data base search

The Protein Prospector software version 4.0.1 (University of California San Francisco, Mass Spectrometry Facility) was used to identify proteins from the peptide mass fingerprinting obtained from MALDI-reTOF MS. Swiss-Prot (European Bioinformatics Institute, Heidelberg, Germany) and GenBankTM (National Center for Biotechnology Information) data bases were used for the search.

V. Immunohistochemistry and double labelling immunofluorescence and confocal microscopy

For immunohistochemistry, de-waxed sections, 5 microns thick were processed following the EnVision + system peroxidase procedure (Dako, Barcelona, Spain). After incubation with methanol and normal serum, the sections were incubated with one of the primary antibodies at 4ºC overnight. Primary antibodies were rabbit polyclonal mitochondrial porin (Abcam, Cambridge, UK, used at 1:2,000), mouse monoclonal cytochrome C oxidase subunit IV (COX; Molecular Probes, Leiden, Netherlands, used at a dilution of 1:1,000), phospho-tau (AT8; Inverness Medical, Hospitalet de Llobregat, Spain, dilution 1:50), polyclonal phospho-tau Thr181 (tauThr181; Calbiochem, San Diego, USA, dilution 1:500), goat polyclonal anti-SOD1 (Novocastra, Servicios Hospitalarios, Barcelona, dilution 1:100), rabbit polyclonal anti-SOD2 (Stressgen, Barcelona, diluted 1:500) and goat polyclonal anti-RAGE antibody (Santa Cruz, Quimigranel, Barcelona, diluted 1:300). After washing with PBS, the sections

were incubated in a cocktail of secondary antibodies in the same vehicle solution for 3 h at room temperature. The secondary antibodies were Alexa 488 and Alexa 546 anti-mouse or anti-rabbit (Molecular Probes), and they were used at a dilution of 1:400. Subsequently, the nuclei were stained using To-pro®-3-iodide (Molecular Probes) at a dilution of 1:1,000 for 20 min at room temperature. Sections were mounted with Fluorescent Mounting Medium (DakoCytomation), sealed and dried overnight at 4ºC. Sections were examined with a Leica TCS-SL confocal microscope. In order to rule out non-specific reactions, some sections were incubated only with the secondary antibodies.

VI. Measurement of specific, protein-oxidation derived markers: GSA, AASA, CML, CEL and MDAL, and fatty acid analysis

GSA, AASA, CML, CEL and MDAL were determined as trifluoroacetic acid methyl esters derivatives in acid hydrolyzed delipidated and reduced protein samples by GC/MS[17] using a HP6890 Series II gas chromatograph (Agilent, Barcelona, Spain) with a MSD5973A Series and a 7683 Series automatic injector, a HP-5MS column (30-m x 0.25-mm x 0.25-µm), and the described temperature program[17]. Quantification was performed by internal and external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. The amounts of product were expressed as the µmolar ratio of GSA, AASA, CML, CEL or MDAL/mol lysine.

Fatty acyl groups of human HC lipids were analyzed as methyl esters derivatives by GC/MS as previously described[17]. Separation was performed in a SP2330 capillary column (30 m x 0.25 mm x 0.20 µm) in a GC Hewlett Packard 6890 Series II gas chromatograph (Agilent, Barcelona, Spain). A Hewlett Packard 5973A MS was used as detector in the electron-impact mode. Identification of fatty acyl methyl esters was made by comparison with authentic standards and on the basis of mass spectra. Results are expressed as mol%.

VII. Statistical analysis

All statistics were performed using the SPSS software (SPSS Inc., Chicago, IL). Once normality of distribution was assessed by Kolmogorov–Smirnov test, differences between groups (Argyrophilic grain disease samples versus

controls) were analysed by the Student's t-tests and correlations between variables were evaluated by the Pearson's statistic. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

RESULTS

Unfolded protein response (UPR) and ER stress in AGD

NDDs associated to protein misfolding often induce ER stress. Thus, ER stress was examined in AGD, including ER stress transducers: PKR-like ER protein kinase (PERK) evaluated through phosphorylated eukaryotic translation initiation factor 2, α subunit (p-eIF-2α); inositol requiring 1 protein (IRE1); and activating transcription factor 6 (ATF6). The ratio p-eIF-2α/eIF-2α was increased 5.7-fold ($p<0.007$) in AGD cases (Fig. 1A). Increased intensity for the three ER stress signalling molecules IRE1, ATF6 and X-box binding protein 1 (XBP1) was detected in the AGD affected HC, which density exceeded 3.4-fold for IRE1 ($p<0.0002$), 2.5-fold for the entire ATF6 form ($p<0.002$) and 3.2-fold for the cleaved ATF6 form ($p<0.001$), and 3.7-fold for the IRE1 activated XBP1 ($p<0.001$) when compared to controls (Fig. 1A).

As chaperones are required during UPR resolution, WB analyses were performed to examine several ER chaperones and folding enzymes. Thus, 2 to 6-fold increased expression of chaperones Grp78/BiP ($p<0.001$), Grp94 ($p<0.002$) and KDEL-containing proteins ($p<0.0001$) were found in HC from AGD patients (Fig. 1B), consisting with UPR being active in AGD. This was accompanied by marked reduction of the foldase - protein disulphide isomerase (PDI) - in AGD cases ($p<0.004$; Fig.1B).

Since proteasome is responsible for degradation of ER misfolded proteins, its potential dysfunction could induce the observed ER stress. As ubiquitin-modified proteins are degraded by the proteasome, an indirect assessment of its function can be obtained by analysis of protein ubiquitination. Results, showing increased protein ubiquitination in AGD samples, are compatible with compromised proteasomal function ($p<0.0001$; Fig. S1).

Protein oxidative modifications in hippocampus of AGD

Both ER stress and proteasome impairment may be related to OS as previously evidenced in various neurodegenerative disorders[10], although the mechanisms linking both stress processes are currently not disentangled. To evaluate OS contribution to ER stress in AGD, protein oxidative damage markers were analyzed in samples from HC of AGD patients by WB. Protein carbonyl content resulted 1.5-fold increased in AGD–affected HC respect to

control group ($p<0.02$; Fig. 2A). The lipoxidation marker MDAL was 6.5-fold higher in AGD patients than in control individuals ($p<0.001$; Fig. 2A). There was no significant change in the levels of the glycoxidation marker CEL, as well as in the total levels of the AGE product CML (Fig. 2A). However, the latter showed a significant increase in the modifications for particular proteins between 30 and 50 kDa in patients with AGD. This was accompanied by a marked increase in immunohistological staining of receptor for AGE products (RAGE) in both HC and EC of AGD cases (Fig. S2 A, B).

To offer a more accurate quantitative measurement of oxidation in HC proteins, a highly selective mass spectrometry-based technique was applied. Results obtained reinforced findings from WB analysis, as there were significant differences neither for CEL and CML concentrations nor for the products derived from direct protein oxidation AASA and GSA, between samples of AGD patients and those from age-matched individuals (Fig. 2B). Only MDAL concentration was significantly increased in samples of HC from AGD patients compared to control individuals ($p<0.01$; Fig. 2B).

2D electrophoresis characterization of CML-modified proteins

WB with CML immunostaining demonstrated some specific damages for AGD, CML-modified proteins were identified after 2D electrophoresis with MALDI-TOF mass spectrometry revealing two isoforms of fructose-bisphosphate aldolase - A and C (increased in AGD), and phosphatidylethanolamine-binding protein (PEBP) (decreased in AGD) (Fig. S3).

Fatty acid composition and lipoxidative modification of proteins in AGD

The high content of PUFAs in central nervous system and its elevated oxygen consumption support the possible significance of lipid peroxidation-derived processes in neurodegeneration, including AGD. In order to assess whether fatty acid changes favours lipid peroxidizability the fatty acid profile in HC from AGD patients was compared with age-matched controls. Those analyses revealed significant differences associated with AGD in HC in some individual fatty acids but not in global indexes (Table S3). The 16:0 (SFA), 18:1n-9 (PUFA) and 22:4n-6 (PUFA) fatty acids exhibited significantly reduced proportions in their % content in AGD samples compared with the control

samples ($p<0.05$ for all of cases). With reference to the fatty acid-derived indexes no differences were observed either in double bond index or in peroxidizability indexes (Table S3).

Mitochondrial dysfunction and antioxidant defence in AGD

As mitochondria are a major free radical source, its components as well as key elements of antioxidant defence were evaluated by WB. The expression of the representative subunits of mitochondrial respiratory complexes I to IV, whose activity is counted among the major sources for ROS production in the cell[23, 24] was analysed. Complex I and complex IV, were significantly decreased, with 1.4-fold ($p<0.04$) and 1.9-fold ($p<0.02$) respectively, in the diseased cases in comparison with the controls. There was no significant change observed in the content of subunits from respiratory complexes II and complex III (Fig. 2C). Uncoupling protein 4 (UCP4), a mitochondrial protein specific for the brain tissue that modulate neuronal energy metabolism by increasing glucose uptake and shifting the mode of ATP production from mitochondrial respiration to glycolysis, can contribute to decreased free radical leak. UCP4 levels were found significantly decreased in AGD with reference to control cases ($p<0.01$; Fig. 2C). The apoptosis inducing factor (AIF), another multifunctional mitochondrial protein, involved in OS as an ROS scavenger[25], maintenance of mitochondrial complexes structure[26], and contribution to cell death, showed a 3.7-fold increase of the nuclear fraction (57 kDa) but reduction of the cytosilic and mitochondrial fraction levels (data not shown), in AGD-affected samples compared to the controls ($p<0.001$), compatible with the decreased content of complex I and increased oxidative stress (Fig. 2C). To further delineate the potential response to OS, the nuclear factor-erythroid 2 p45-related respiratory 1 and 2 (Nrf1 and Nrf2), both activating the stress-dependent expression of a set of genes such as SOD1 and SOD2, through an antioxidant responsive element (ARE)[27], were also analysed. Nrf1 and Nrf2 were increased correspondingly 2.2-fold ($p<0.01$) and 8.5-fold ($p<0.007$) in AGD (Fig. 3B). Immunohistological staining of antioxidants SOD1 and SOD2, Nrf2 regulated genes demonstrated a marked augmentation in CA1 of HC from AGD patients (Fig. 3C, 3D).

Besides qualitative mitochondrial changes leading to increased OS, quantitative changes in mitochondria could also explain OS. Thus, porin levels, adjusted to actin content, were decreased 1.2-fold ($p<0.04$) in AGD cases compared to the controls (Fig. 2C). To investigate whether mitochondrial biogenesis (MB) was affected in AGD, levels of mitochondrial transcriptional factor A (TFAM) and mitochondrial fusion protein-1 (Mfn1), were analyzed as MB markers, with no changes in AGD HC when compared to control cases (Fig. 3A), suggesting a lack of response over diminished mitochondrial content. Double-labelling immunofluorescence and confocal microscopy disclosed lack of porin and COX staining in grains, as revealed by combined immunostaining with anti-phophorylated tau antibodies (Fig. S4). This is in striking contrast with dystrophic neurites of senile plaques which contain huge numbers of degenerated mitochondria and increased expression of porin[28].

To shed further light in potential dysfunction of MB in AGD, were analyzed levels of sirtuin 1 (SIRT1) (positive MB regulator, actuating via peroxisome proliferator-activated receptor- coactivator-1 (PGC1α)) and levels of receptor interacting protein 140 (RIP140) (negative regulator of MB). The levels of SIRT1 ($p<0.01$; Fig. 3B) were found 2.4-fold decreased, whereas the levels of both PGC1α ($p<0.0001$; Fig. 3B) and RIP140 ($p<0.002$; Fig. 3A) were increased 3-fold and 2.7-fold, respectively, in AGD cases compared with control samples.

DISCUSSION

Although NDDs have distinct clinical manifestations they share common features, including the intra- and extracellular accumulation of misfolded proteins, compromised stress responses, mitochondrial dysfunction, and neuroinflammation. Most of these proceses are strongly influenced by the aging process, a predominant and unifying risk factor for NDDs. From the data obtained in the present study, AGD is not an exception.

Our findings demonstrate that OS, mitochondrial disfunction and ER stress interplay in AGD (Fig. 4), as it was reported in various NDDs like ALS[20], PD[29], and different tauopathies such as AD[17] and PiD [Ilieva et al., unpublished data], though a disease specific pattern can be diverse. Thus, the markers of direct protein oxidation AASA and GSA increased in AD[17], ALS[20] and PiD [Ilieva et al., unpublished data] and glycoxidation markers CEL and CML that were significantly diminished only in PiD [Ilieva et al.,unpublished data] in AGD there were not significantly different. AGE modification targeted fructose-bisphosphate aldolase, which catalyses the enzymatic cleavage of β-D-Fructose-1,6-bisphosphate leading to the formation of glyceraldehyde 3-phosphate, the major source of methylglyoxal formation in physiological systems[30]. Methylglyoxal is the main precursor of CEL, and a consequence of potential loss of fructose-bisphosphate aldolase would be diminished formation of methylglyoxal, and CEL. The other identified protein, PEBP, is an individual signalling protein, developmentally regulated in hippocampus[31]. As PEBP in AD acts as an inhibitor of the chymostrypsin-like activity of proteasome[32], diminished PEBP in AGD may be a response to impaired proteasome function.

Proteasomal dysfunction–induced by OS- leads to ER overloading with unfolded proteins, a state known as ER stress. The activation of ER transmembrane proteins IRE1, ATF6 and PERK comprises the UPR, a physiological response to ER stress, regulated by Grp78/BiP[33-35]. In AGD, ATF6 was found cleaved to a 50 kDa, suggesting its activation, which also explains increased XBP-1 levels. IRE1 levels were also found elevated in AGD, also leading to increased XBP-1. During OS conditions, XBP1 mRNA is upregulated in an Nrf2-dependent fashion[35]. The last key protein, PERK (PKR-like ER kinase), is considered the major eIF-2α phosphorylation regulator during the UPR, phosphorylating and inactivating eIF-2α, thus blocking the

translocational initiation of the proteins to avoid their accumulation in ER lumen. AGD samples also showed increased eIF-2α phosphorylation, suggesting increased PERK activity.

The resulting response to the ER stress provides protection from cell death induced by OS and Ca^{2+} disturbances, an adaptation reproduced by gene transfer–mediated overexpression of foldases and Ca^{2+}-dependent molecular chaperones, such as Grp78/BiP and Grp94. In addition, the oxidizing environment of the ER creates a constant demand for cellular PDI to catalyze and monitor disulfide bond formation in a regulated manner[33]. PDI levels were significantly decreased in AGD finding that is compatible with accumulation of unfolded proteins in ER. However, in AGD increased levels of Grp78/BiP, Grp94 and KDEL-containing proteins were detected, as also was shown in ALS and AD[20,36], suggesting the activation of adaptive mechanisms to restore homeostasis in response to ER stress in AGD.

ER stress and OS both trigger the release of Nrf2, a downstream target of PERK (Fig. 4), from Keap1-Cul3 cytoplasmic complex, resulting in the accumulation of Nrf2 in the nucleus and induction of transcription of Nrf2 target genes encoding antioxidants and detoxifying enzymes, such as SOD1 and SOD2, as well as ER chaperones.

Increased Nrf2 levels, together with the activated ATF6 and XBP1, as well as antioxidant response in AGD further demonstrate an active response to ER stress and OS.

Besides PERK, PGC1α is also an upstream signal for Nrf2 activation. Accordingly, there is considerable evidence that PGC1α has a neuroprotective function[37]. PGC1α is a master regulator of the antioxidant defence and orchestrate the MB stimulating the expression of Nrf1, Nrf2 and TFAM, which in turn activates expression of nuclear and mitochondrial genes encoding mitochondrial proteins[38].

A physiological response to increased PGC1α and Nrf1 levels in AGD would be increased levels of the proteins involved in MB, as TFAM and Mfn1, which was not the case. Lack of MB response would result in two main consequences that impact cellular survival: mitochondrial dysfunction and alterations in mitochondrial number. Most importantly, PGC1α transcriptional co-activator and the RIP140 co-repressor of MB promote opposing physiological

functions[39, 40]. Surprisingly, in AGD the levels of the RIP140 were found significantly increased as well as those of PGC1α. Therefore, the disturbed MB and the unchanged levels of TFAM and Mfn1 could be attributed to these abnormal levels of the MB repressor RIP140.

OS and presumably cell damage increase with age due to either diminished antioxidant defences or the increase in mitochondrial dysfunction[41]. The evidences of OS and disturbance of the MB in AGD directed to mitochondrial dysfunction. Since mitochondria are the primary producers of ROS, oxidative damage of mitochondrial proteins is likely to contribute to the mitochondrial dysfunction that is characteristic of many NDDs[42]. Different peptides of the mitochondrial complexes (from I to IV), in particular complex I and complex IV, revealed significantly reduced levels, suggesting a mitochondrial dysfunction in AGD.

The MB activator PGC1α as well regulates the expression of another crucial mitochondrial protein – UCP4, an important regulator of the mitochondrial homeostasis and ROS formation[44]. Increased mitochondrial membrane potential associated with increased production of ROS can be regulated by the activation of UCP[45,46]. By shifting energy metabolism to reduce ROS production and cellular reliance on mitochondrial respiration, UCP4 can protect neurons against OS and calcium overload[44]. Likewise the other proteins involved in MB UCP4 levels were not increased in AGD in spite of increased PGC1α. The depletion of UCP4 in the HC of AGD patients could be associated with elevated ROS production. RIP140 antagonize the function of PGC1α thereby suppressing UCP expression and ATP uncoupling[40]. In the absence of RIP140, altered expression of the PGC1α coactivator is not essential for UCP expression[39]. Thus, it can be hypothesized that upregulation of RIP140 suppress the expression of UCP4, thus probably contributing to increased production of ROS in AGD.

Furthermore, significantly decreased levels of porin were seen in AGD when compared to controls, which suggest that the mitochondria in AGD could not only be affected but also could have reduced number, probably through the repression of mitochondrial fusion by RIP140. In vivo studies show that Mfn-deficient neurones have impaired respiratory complex activity and defects in inner membrane structure characteristic of respiratory dysfunction[46].

Overexpression of RIP140 is predicted to impair mitochondrial function and this could be compensated by an increase in the activity of coregulators such as PGC1α[40], since its overexpression increases mitochondrial content[47]. The major upstream regulator of PGC1α, SIRT1, was depleted in AGD samples, a surprising finding in the context of increased PGC1α.

It is becoming increasingly clear that mitochondrial dysfunction leads to neurodegeneration and aging[46]. In the present work, we demonstrate that mitochondrial dysfunction could contribute to AGD leading to oxidative and ER stress. The AGD-linked mitochondrial dysfunction is characterized both by changes in respiratory chain complexes with lowered UCP4 expression - despite PGC1α-Nrf2 antioxidant axis seems highly active – and by findings compatible with compromised MB. Thus, while the antioxidant response arm of PGC1α-Nrf2 axis would be reacting over AGD-OS derived lipoxidative damage, the MB arm would show oversensitivity in front of RIP140 repressor role with lowered SIRT1 levels, evidenced by decreased porin content. Needless to say, more work is needed to further clarify the molecular basis of differential sensitivity of those two PCG1α-derived responses, and to discern causes of decreased SIRT1 levels and increased RIP140 functionality in this neurodegeneration towards the extension of those mechanisms as a novel pathogenic pathway in human neurodegenerative disease.

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Figure 1. ER stress in hippocampus from AGD patients

Representative western blots of HC homogenates show ER stress, evidenced both by increased eIF-2α (Ser 51) phosphorylation, IRE1 amount, ATF6 cleavage and XBP1 splicing (**A**), and by increased expression of ER-resident chaperones Grp78/BiP, Grp94and KDEL-motif containing proteins, but decreased PDI expression (**B**). The lower panels show the quantification of the blots by densitometry, adjusted to actin content, with differences shown between the control and the AGD samples (*p<0,05; **p<0.001 and ***p<0.0001 by Student's t test).

Figure 1

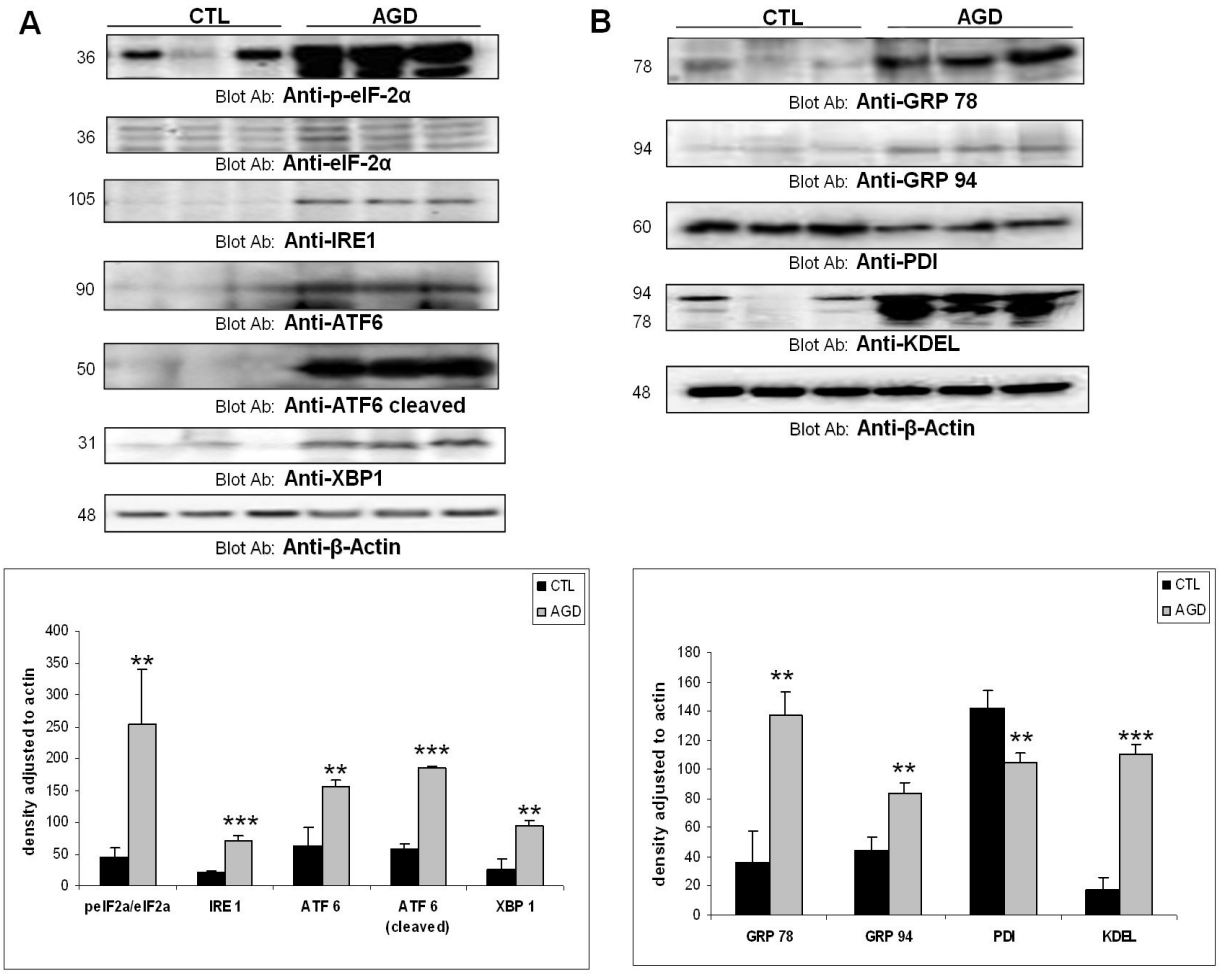

A
CTL
AGD
36
Blot Ab: Anti-p-eIF-2α
36
Blot Ab: Anti-eIF-2α
105
Blot Ab: Anti-IRE1
90
Blot Ab: Anti-ATF6
50
Blot Ab: Anti-ATF6 cleaved
31
Blot Ab: Anti-XBP1
48
Blot Ab: Anti-β-Actin
B
CTL
AGD
78
Blot Ab: Anti-GRP 78
94
Blot Ab: Anti-GRP 94
60
Blot Ab: Anti-PDI
94
78
Blot Ab: Anti-KDEL
48
Blot Ab: Anti-β-Actin
CTL
AGD
density adjusted to actin
peIF2a/eIF2a
IRE 1
ATF 6
ATF 6 (cleaved)
XBP 1
CTL
AGD
density adjusted to actin
GRP 78
GRP 94
PDI
KDEL

Figure 2. Mitochondrial changes and oxidatively modified proteins in hippocampus from AGD patients

(**A**) Representative western blots for protein reactive carbonyls (anti-DNP), glycoxidation (anti-CEL), mixed glycoxidation/lipoxidation (anti-CML), and lipoxidation (anti-MDAL) revealed differential targets for each of these oxidative pathways in AGD. Right numbers of the blots indicate apparent molecular weight. (**B**) Proteins from AGD samples show a significant increase in the amounts of lipoxidation marker MDAL, but not in direct oxidation markers GSA and AASA or in CEL and CML markers, measured by mass-spectrometry. Values shown are % changes of mean ±S.E.M over values in hippocampus of control individuals (GSA: 22175±1104 µmol/mol lys; AASA: 87±18 µmol/mol lys; CEL: 279±7 µmol/mol lys; CML: 1026±35 µmol/mol lys; MDAL: 248±9 µmol/mol lys). (**C**) Representative western blots of peptides NDUFS3, SDHA, core 2 UQCRC2 and MTCO1, subunits of mitochondrial respiratory chain complexes I, II, III and IV respectively, suggesting decreased complex I and IV contents in hippocampus from AGD patients and control individuals. This was associated with decreased contents of UCP4 and porin. The lower panels show the quantification of blots (A and C) by densitometry, after densities of bands were adjusted to actin content (for A and AIF 57kDa) or to porin content (for C). Differences were analyzed respect to control group by Student's t test being *p<0,05 and **p<0.001

Figure 2

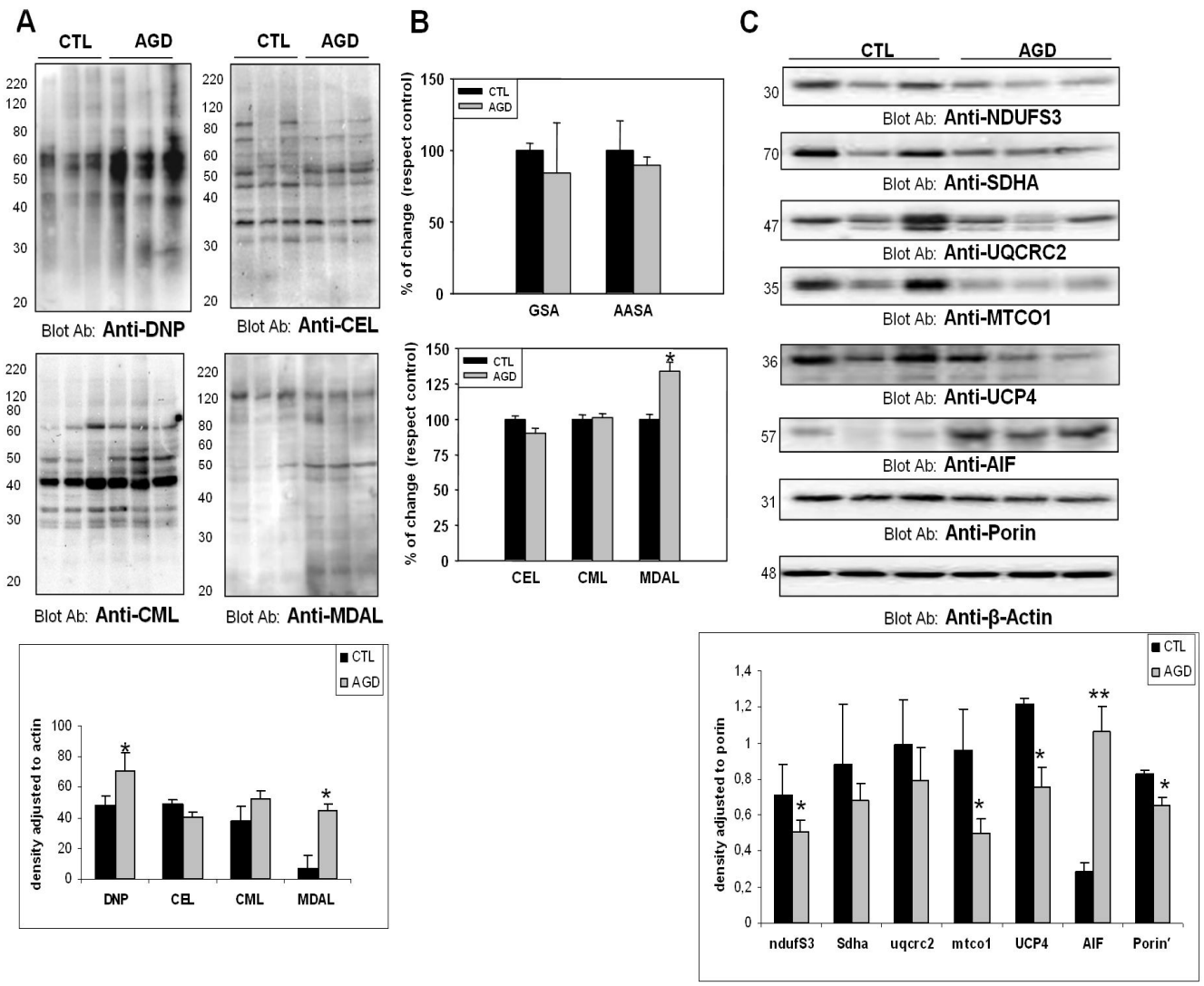

A
CTL
AGD
Blot Ab: Anti-DNP
Blot Ab: Anti-CEL
Blot Ab: Anti-CML
Blot Ab: Anti-MDAL
B
% of change (respect control)
GSA
AASA
CEL
CML
MDAL
C
Blot Ab: Anti-NDUFS3
Blot Ab: Anti-SDHA
Blot Ab: Anti-UQCRC2
Blot Ab: Anti-MTCO1
Blot Ab: Anti-UCP4
Blot Ab: Anti-AIF
Blot Ab: Anti-Porin
Blot Ab: Anti-β-Actin
density adjusted to actin
density adjusted to porin
ndufS3
Sdha
uqcrc2
mtco1
UCP4
AIF
Porin'

Figure 3. Antioxidant response and mitochondrial biogenesis dysfunction in hippocampus from AGD patients

Lack of effective mitochondrial biogenetic response evidenced by representative western blots revealing unchanged levels of MB mediators TFAM and Mfn-1 and increased amount of MB corepressor RIP140 (**A**). This is reinforced by lowered levels of MB positive regulator Sirt1, despite the levels of positive MB coactivator PGC1α as well as downstream antioxidant response components Nrf1 and Nrf2 were increased (**B**). The lower panel shows the quantification of blots by densitometry, after densities of bands were adjusted to actin content. Differences were analyzed respect to control group by Student's t test being *p<0,05 and **p<0.001. Immunohistochemistry for antioxidant response enzymes related to Nrf2, such as SOD1(**C**) and SOD2 (**D**) in CA1 area of the hippocampus. Bar = 25 microns

Figure 3

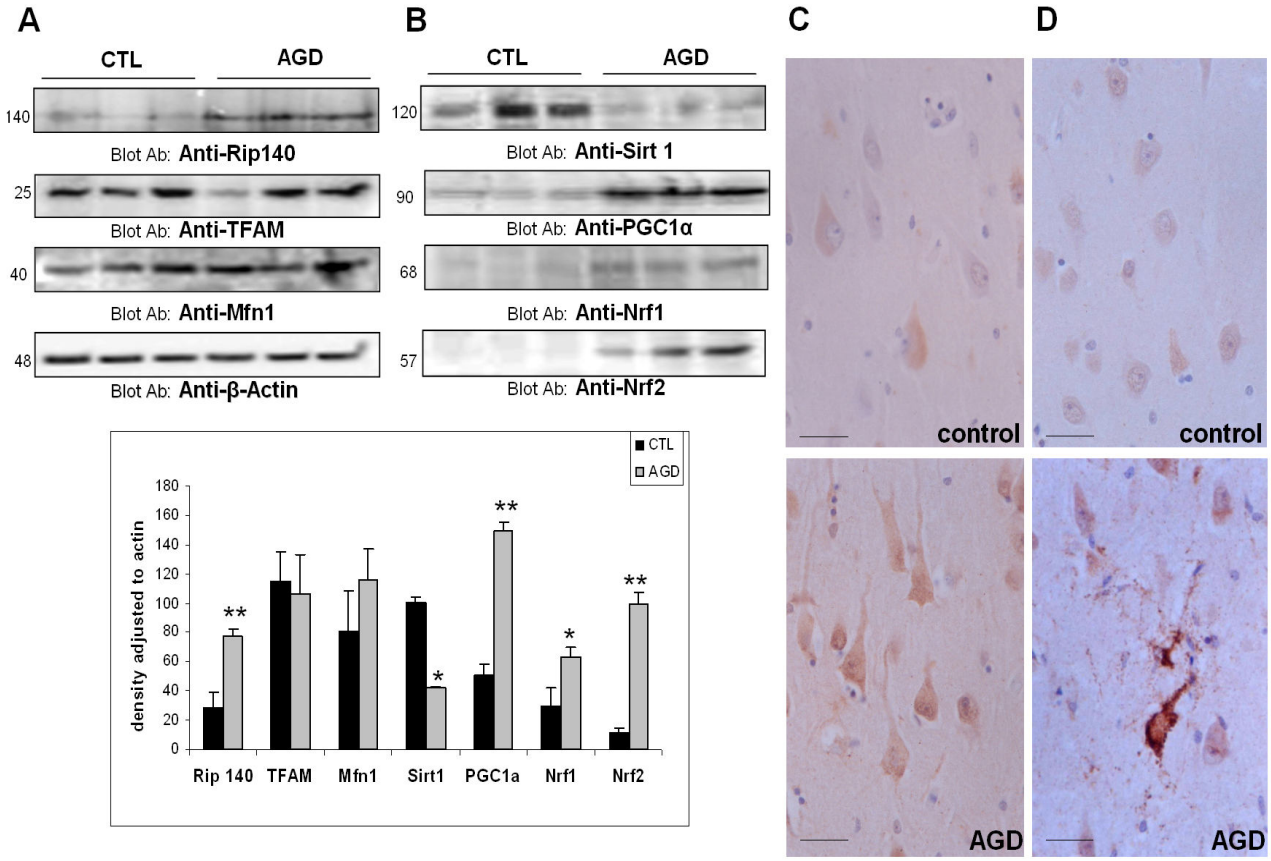

A
CTL
AGD
140
Blot Ab: Anti-Rip140
25
Blot Ab: Anti-TFAM
40
Blot Ab: Anti-Mfn1
48
Blot Ab: Anti-β-Actin
B
CTL
AGD
120
Blot Ab: Anti-Sirt 1
90
Blot Ab: Anti-PGC1α
68
Blot Ab: Anti-Nrf1
57
Blot Ab: Anti-Nrf2
CTL
AGD
density adjusted to actin
180
160
140
120
100
80
60
40
20
0
**
**
*
*
**
Rip 140
TFAM
Mfn1
Sirt1
PGC1a
Nrf1
Nrf2
C
D
control
control
AGD
AGD

Figure 4. Schematic representation of the biochemical processes occurring in the hippocampus of AGD patients

Disruption of the Sirt1-PGC1α-Rip140 physiological interplay leads to the mitochondrial biogenesis impairment and mitochondrial dysfunction triggering sustained oxidative stress that could not be overcome despite the increased levels of the antioxidant enzymes SOD1 (cytosolic) and SOD2 (mitochondrial). Oxidative stress leads to increased levels of oxidatively modified proteins prone to aggregate that may perturb the normal function of the proteasome by physical block of its entry. The perturbed proteasome that participate in UPR on its part leads to ER stress and activation of ER stress transducers IRE, ATF6; phosphorylation of eIF-2a and overexpression of ER chaperones CGR78/BiP, Grp94 and KDEL.

Figure 4

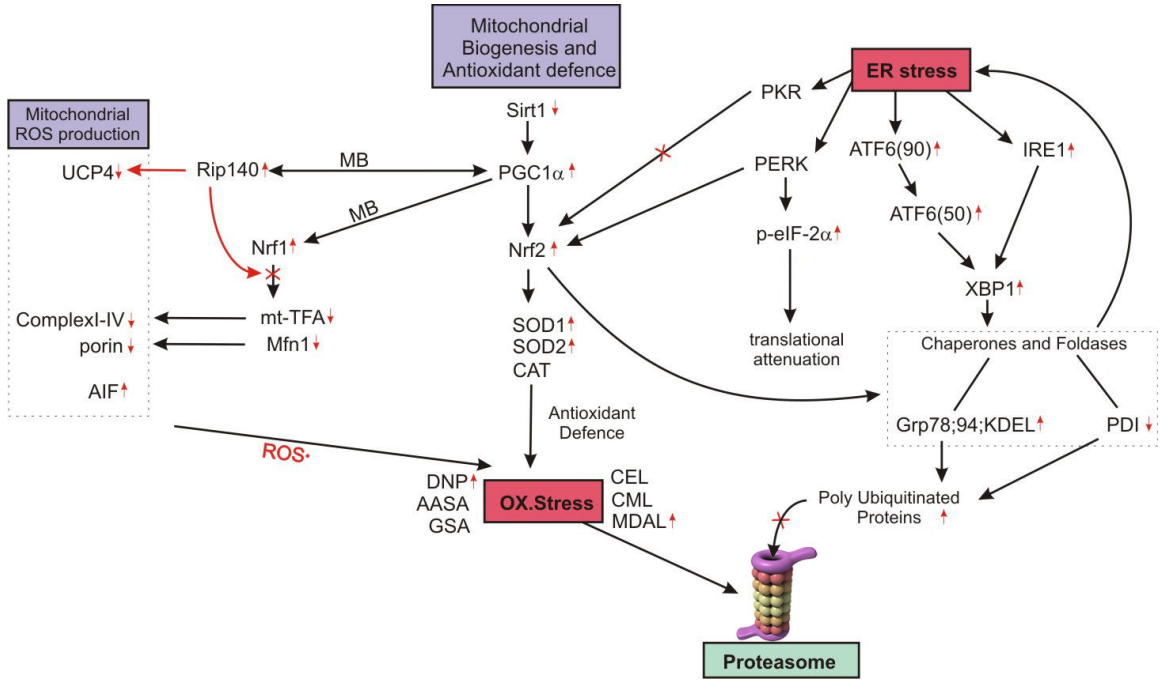

Mitochondrial Biogenesis and Antioxidant defence
Sirt1
PGC1α
Mitochondrial ROS production
UCP4
Rip140
MB
MB
Nrf1
Nrf2
ComplexI-IV
porin
AIF
mt-TFA
Mfn1
SOD1
SOD2
CAT
Antioxidant Defence
ROS·
DNP
AASA
GSA
OX.Stress
CEL
CML
MDAL
ER stress
PKR
PERK
p-eIF-2α
translational attenuation
ATF6(90)
ATF6(50)
IRE1
XBP1
Chaperones and Foldases
Grp78;94;KDEL
PDI
Poly Ubiquitinated Proteins
Proteasome

Figure S1. Proteasomal dysfunction as ER stress contributor in AGD Representative western blot with anti-Ubiquitin reveals high molecular weight bands corresponding to polyubiquitinated proteins, suggesting decreased ubiquitin degradation in hippocampus samples from AGD patients. Right numbers of the blot indicate apparent molecular weight. Immunoblotting of actin is also shown as a loading control. The lower panel indicates bars represent blot quantifications by densitometry, adjusted to actin content. Differences were analyzed respect to control group by Student's t test being *p<0,05.

Figure S1

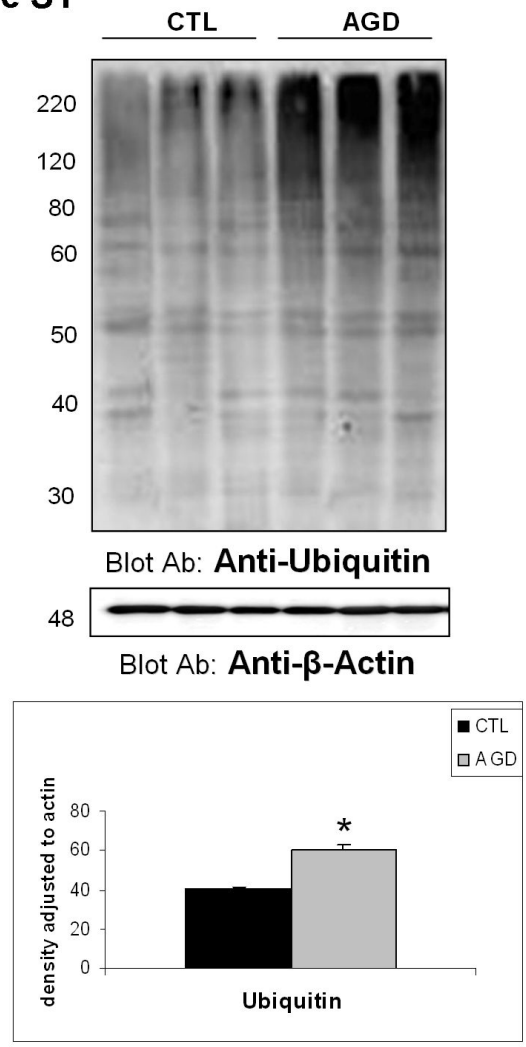

CTL
AGD
220
120
80
60
50
40
30
Blot Ab: Anti-Ubiquitin
48
Blot Ab: Anti-β-Actin
CTL
AGD
density adjusted to actin
80
60
40
20
0
*
Ubiquitin

Figure S2. Increased expression of AGE receptor (RAGE) in AGD Representative immunohistochemistry of the sensitive to the generation of reactive oxygen species RAGE reveals increased labelling in neurons of the hippocampus CA1 (**A**) and entorhinal cortex (**B**) from AGD patients. Bar = 25 microns

Figure S2

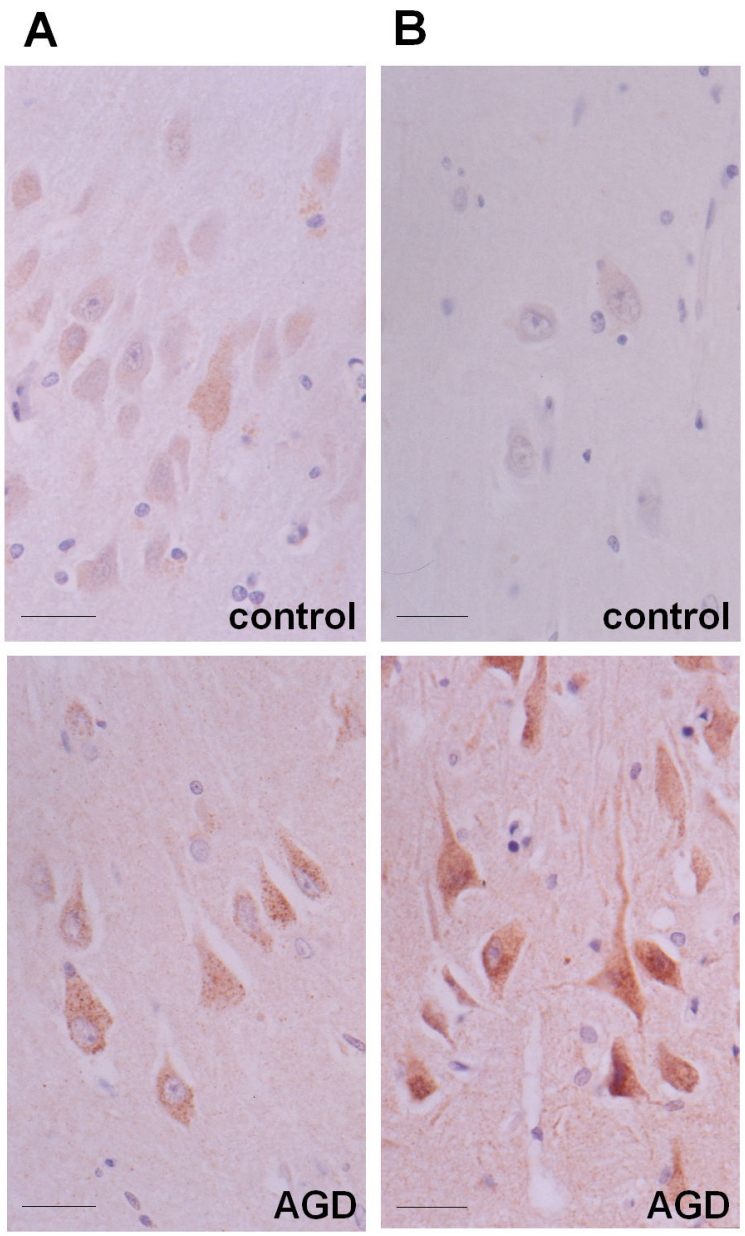


Figure S3. Characterization of protein oxidative damage targets in AGD hippocampus

2D gel electrophoresis and western blotting of membranes stained with Anti-CML. Densitometric analyses, after adjustment to protein content quantified by silver stain, demonstrated differentially stained spots (marked by letters a to s). Right numbers of the blot indicate apparent molecular weight. Bi-dimensional gels processed in parallel and stained with silver were also used to obtain the spots for mass spectrometry analysis. Dissection of the spots and their analysis revealed several isoforms of fructose bisphosphate aldolase (access No P04075; P09972) and phosphatidylethanolamine-binding protein (access No P30086).

Figure S3

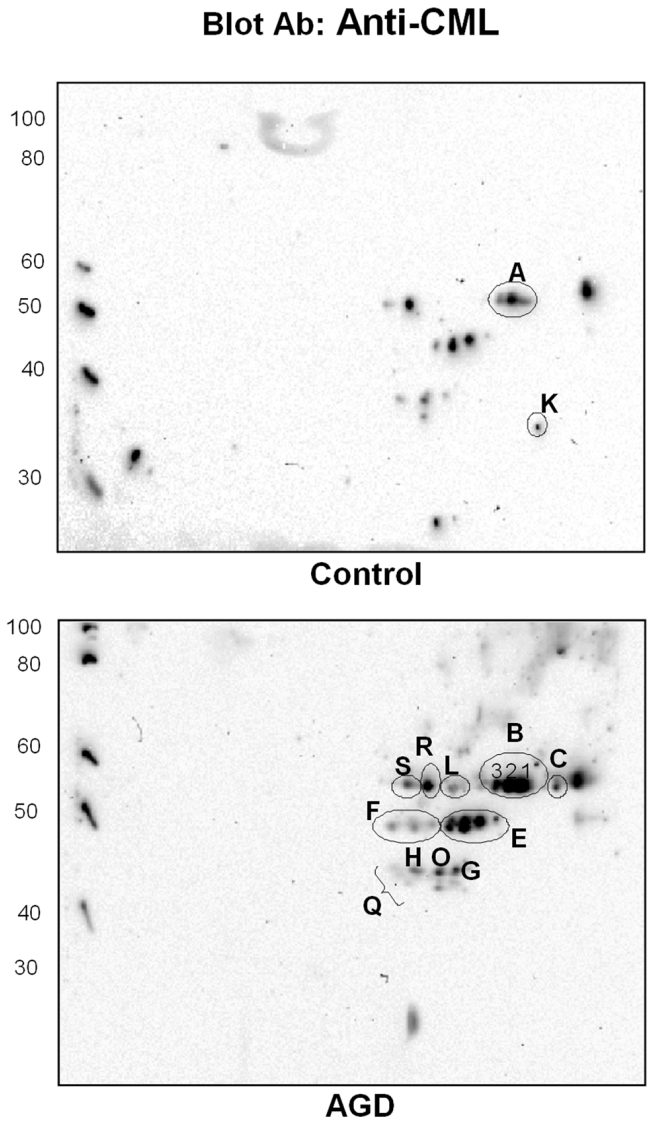

Blot Ab: Anti-CML
100
80
60
50
40
30
A
K
Control
100
80
60
50
40
30
R
B
S
L
321
C
F
E
H
O
G
Q
AGD

Figure S4. Lack of porin and cytochrom C oxidaze in AGD grains

A-C: tau-P (A, green) and mitochondrial porin (B, red); no porin localization in grains (C, merge); D-F: tau-P (D, green) and cytochrome C oxidase subunit 4 (E, red); no Cox localization in grains. G-I: parallel sections immunostained without the primary antibodies are used as negative controls. Nuclei are stained with TO-PRO. Bar = 40 microns

Figure S4

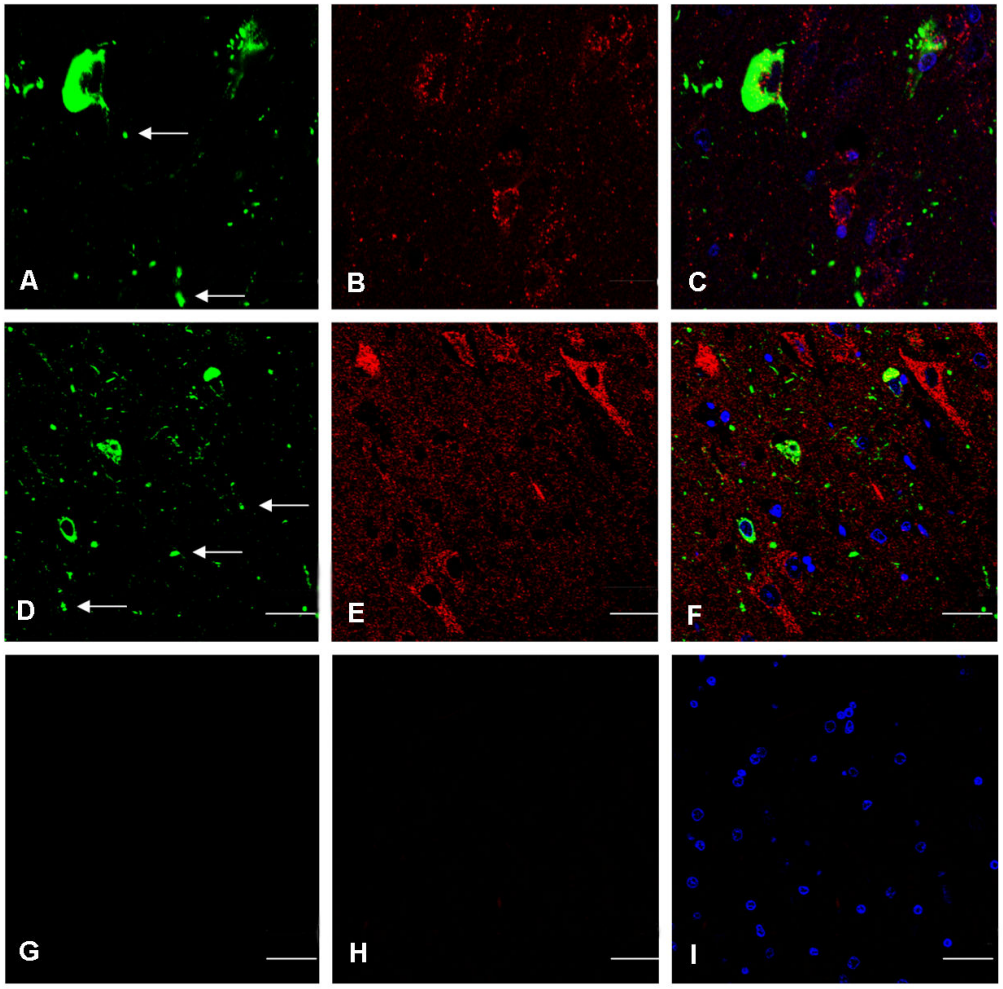


Table S1. Summary of the main characteristics in the present series

M: male; F: female; AGD: argyrophilic grain disease; AA: amyloid angiopathy; Crib: status cribosus; AD: Alzheimer disease, I-II: stages entorhinal and transentorhinal of neurofibrillary pathology, 0, A, B: stages of amyloid plaque pathology; post-mortem delay in hours.

Table S1. Clinical and pathological characteristics of analyzed samples

	Age	Gender	AGD	Other findings	Post-mortem delay
1	82	M	AGD 2	AA	13
2	74	M	AGD 3	Crib	4
3	88	F	AGD 3	Crib	9
4	79	F	AGD 2	AD II-0, Crib	6
5	95	M	AGD 2	AD I-0, Crib	10
6	66	M	AGD 2	AD 0-A	5
7	65	F	AGD 2	AD I-0	2
8	74	F	AGD 3	AD II-0	9
9	76	F	0	Crib	11
10	78	M	0	AD II-0	9
11	76	F	0	AD I-B	2
12	82	F	0	AD I-B	10
13	82	M	0	AD I-0	8
14	68	F	0	Crib, AD I-0	4
15	74	F	0	Crib	6

Table S2. Antibodies and conditions used in the present work

Antigen	Supplier	Use(dilution)
1. Phosporylated (S51) eIF-2α	Cell Signaling	WB (1:1 000)
2. eIF-2α	Cell Signalling	WB (1:1 000)
3. IRE 1	ProSci	WB (1:1 000)
4. XBP1	ProSci	WB (1:1 000)
5. ATF6	ProSci	WB (1:1 000)
6. Grp78/BiP	Stressgen	WB (1:1 000)
7. Grp94	Santa Cruz	WB (1:500)
8. PDI	Abcam	WB (1:250)
9. Ubiquitin	Sigma	WB (1:1 000)
10. Mitochondrial complex I (NDUFS3 subunit)	Invitrogen	WB (1:2 000)
11. Mitochondrial complex II (SDHA subunit)	Invitrogen	WB (1:1 000)
12. Mitochondrial complex III (core II subunit)UQCRC2	Invitrogen	WB (1:2 000)
13. Mitochondrial complex IV (MTCO1 subunit)	Invitrogen	WB (1:1 000)
14. Nrf1	Santa Cruz	WB (1:200)

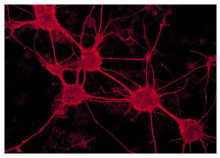
15. Nrf2	Santa Cruz	WB (1:750)
16. PGC1α	Santa Cruz	WB (1:200)
17. AIF (apoptosis inducing factor)	Sigma	WB (1:5 000)
18. Mfn1	Santa Cruz	WB (1:200)
19. TFAM	Santa Cruz	WB (1:200)
20. Sirt1	Santa Cruz	WB (1:200)
21. UCP4	Santa Cruz	WB (1:200)
22. β-Actin	Sigma	WB (1:5 000)
23. Porin	Invitrogen	WB (1:5 000)
24. CEL	TransGenicInc.	WB (1:1 000)
25. CML	Academy Bio-Medical Company	WB (1:1 000)
26. MDAL	Academy Bio-Medical Company	WB (1:1 000)
27. KDEL	Abcam	WB(1:250)
28. DNP	Sigma	WB (1:2 500)
29. anti-mouse	Amersham	WB (1:30 000)
30. anti-rabbit	Pierce	WB (1:100 000)
31. anti-goat	Vector	WB (1:15 000)

Table S3. Fatty acid profile and derived indexes in hippocampus from AGD patients

Values shown are mean ±S.E. Fatty acid indexes were calculated as follow: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids from n-3 and n-6 series (PUFAn-3 and PUFAn-6); average chain length (ACL) = [(Σ%Total14 x 14) + (Σ%Total16 x 16) + (Σ%Total18 x 18) + (Σ%Total20 x 20) + (Σ%Total22 x 22)]/100]; double bond index (DBI) = [(1 x Σmol% monoenoic) + (2 x Σmol% dienoic) + (3 x Σmol% trienoic) + (4 x Σmol% tetraenoic) + (5 x Σmol% pentaenoic) + (6 x Σmol% hexaenoic)], and peroxidizability index (PI) = [(0.025 x Σmol% monoenoic) + (1 x Σmol% dienoic) + (2 x Σmol% trienoic) + (4 x Σmol% tetraenoic) + (6 x Σmol% pentaenoic) + (8 x Σmol% hexaenoic)].

Table S3.

	Hipocamp Ctl	**Hipocamp AGD**	**P<**
n	4	6	
14:0	0.41±0.10	0.58±0.02	0.085
16:0	19.56±0.18	18.83±0.23	0.056
16:1n-7	0.94±0.15	0.70±0.04	0.114
18:0	21.87±0.08	22.08±0.26	0.553
18:1n-9	26.62±0.14	27.11±0.25	0.180
18:2n-6	0.60±0.01	0.48±0.10	0.058
18:3n-3	0.18±0.01	0.20±0.01	0.300
20:0	1.51±0.05	2.16±0.23	0.060
20:1	0.35±0.004	0.32±0.02	0.239
20:2n-6	0.19±0.08	0.30±0.08	0.080
20:3n-6	0.95±0.18	0.73±0.05	0.205
20:4n-6	7.55±0.10	7.46±0.26	0.806
22:4n-6	6.31±0.14	5.52±0.20	0.054
22:5n-6	0.79±0.06	0.73±0.05	0.471
22:5n-3	0.35±0.09	0.57±0.07	0.068
22:6n-3	11.06±0.23	11.36±0.18	0.346
24:0	0.39±0.05	0.47±0.005	0.117
24:1n-9	0.29±0.07	0.31±0.008	0.690



DISCUSSION

DISCUSSION

Whatever the source, ROS can be produced in all cellular compartments. The exposure of biologic systems to various conditions of oxidative stress leads to age-dependent increases in the cellular levels of oxidatively modified proteins, lipids, and nucleic acids, and subsequently predisposes to the development of age-related disorders that cause impaired cognitive function and metabolic integrity. Accumulating evidence suggests that protein folding and production of ROS are closely linked events. Because oxidative protein folding occurs in the ER and perturbations in protein folding can cause deleterious consequences, alterations in redox status or generation of ROS could directly and/or indirectly affect ER homeostasis and protein folding. The relation between oxidative stress and oxidative protein folding in ER represents a major and not well explored area for research.

The goal of this thesis was to investigate the interplay between oxidative and ER stress and the underling signalling pathways, as a potential mechanism involved in the pathogenesis of three neurodegenerative disorders that affect different locations (spinal cord, frontal cortex and hippocamus), different neuron populations (cortical neurons and motoneurons) and are characterized by disparate clinical symptoms. The results presented in this thesis reveal that oxidative stress and ER stress could be related through the proteasomal dysfunction and may play an important role in the pathogenesis of ALS, PiD and AGD. However, despite these three human NDDs show remarkable heterogeneity of their symptoms, which may be a result of activation of different pathways in different locations, they share common features, such as deposition of aggregated proteins, mitochondrial and proteasome disturbances, increased oxidative damage, ER stress and activated UPR.

1. Protein oxidative modifications in human NDDs

Among the markers of oxidative damage that were evaluated in these diseases the concentrations of three markers showed a significant increase in the three studied diseases MDAL (an adduct between the major oxidative breakdown product of PUFA and Lys, lipoxidation marker), NKTLs (a unique lipoxidation marker of oxidative injury in the brain) and DNP (a marker for reactive carbonyl groups that ensue from different pathways, being those derived from lipid oxidation a very important source). Thus, western-blot and highly selective GC/MS analyses suggest that protein oxidative modifications in ALS, PiD and AGD result predominantly from lipoxidation. These findings agree with the quantitative analysis by GC/MS that revealed increased lipoxidative damage (increased MDAL content) in FC from AD [Pamplona 2005], in

neocortex, amygdala and substantia nigra in early stages of PD and DLB [Dalfo 2005], and in CJD [Pamplona 2008]. Densitometric measurements also revealed increased DNP levels, respectively increased protein carbonyl levels, in FC of AD samples and in SN of PD [Pamplona 2005; Chong 2005; Basso 2004]. It was also demonstrated that protein carbonyls show an increased amount in cells and tissues during pathological events, associated with elevated oxidative damage and also associated with aging and senescence processes [Jung 2007]. The MDAL levels were significantly increased not only in the pathologically affected zone but also in the pathologically preserved BC of ALS and OC of PiD, whereas the NKTLs levels were unchanged in the BC of ALS but also raised in the OC of PiD. DNP showed a clear tendency that the reactive carbonyl species increase only in the affected zone of the diseases. In addition, markers of direct protein oxidation AASA (which results from lysine oxidation) and GSA (which derives from the metal-catalysed oxidation of proline and arginine) analysed by GC/MS were found significantly increased in ALS and in PiD in both (affected and preserved) studied areas, suggesting increased free radical modification. Whereas, in HC of AGD there were not changes in the concentrations of the markers of direct protein oxidation presumed that the observed modifications in this disease evidenced by western-blot of reactive carbonyl species could originate from other sources, such as lipid peroxidation as supported by increased MDAL levels. Increased GSA and AASA were likewise reported in CJD-affected human brains [Pamplona 2008] and in AD samples [Pamplona 2005]. Furthermore, the markers of glycoxidation CEL and CML presented the most different dynamics in the three diseases. Their levels were significantly increased in the both areas BC and SC in ALS, similarly to AD FC [Pamplona 2005] and CJD [Pamplona 2008], also increased in apparently preserved OC in PiD, but significantly decreased in the disease-affected FC, which is compatible with defects in glycolytic potential in this location previously reported in other NDDs [Hoyer 1991; Hollán 1998; Powers 2007]. Probably the pathogenesis of AGD shares the same glycolytic disturbance in view of the fact that both glycoxidative markers CEL and CML, analysed by western-blot as well as by GC/MS, were found with unchanged levels, when compared to control samples. As not all oxygen consumed by the brain is used for glucose oxidation, oxidation of substrates other than glucose (endogenous amino acids and free fatty acids) is assumed to minimize the energy loss from glucose, evidenced by the other oxidative modification markers in PiD (AASA, GSA, MDAL, NKTLS) and in AGD (MDAL, NKTLs). Defects or modification of glycolytic enzyme that has also the capacity to protect against oxidative stress was reported to impair the protection against oxidative stress with consecutive worsening of the neurodegenerative process. Because glycolytic metabolism is predominantly

astrocytic, the selective reduction in the levels of CEL and CML markers raises the possibility that astrocyte dysfunction [Martinez 2008] may be involved in the pathogenesis of PiD and AGD as it was reported for HD [Powers 2007].

Oxidative stress	**ALS**		**PiD**		**AGD**
	BC	**SC**	**OC**	**FC**	**HC**
CEL	↑ *	↑ **	↑ *	↓*	—
CML	↑ *	↑ **	↑*	↓*	—
MDAL	↑ *	↑ **	↑ *	↑ **	↑ *
NKTLs	—	↑ *	↑ **	↑ **	↑ *
DNP	—	↑ **	—	↑ *	↑ *
AASA	↑ *	↑ **	↑ **	↑ **	—
GSA	↑ **	↑ **	↑ *	↑ *	—

↑-significant increase; ↓-significant decrease; — without significant changes

BC-brain cortex; SC-spinal cord; OC-occipital cortex; FC-frontal cortex; HC-hippocampus

Undoubtedly, increased levels of the end products of direct, glyco- and lipoxidation of intracellular proteins suggest increased ROS levels and consequently increased oxidative stress in the ALS, PiD and AGD patients, supporting the notion that oxidative stress is involved in its pathophysiology. The fact that various markers for oxidative modification were increased not only in the disease-affected area but also in apparently pathologically preserved areas of the brain cortex, suggest that the oxidative stress is an early event in the pathogenesis of the NDDs. For this reason it is important to know which proteins are more prone to modifications in these disorders.

2. Identification of oxidatively modified proteins after 2D electrophoresis in NDDs

Although many scientific advances have been made in understanding the pathobiology of NDDs including AD, PD, and ALS, few studies have yielded disease specific biomarkers. Proteomic approaches using two dimensional gel electrophoresis (2D-GE) and MALDI-TOF-TOF MS have been utilized to discover biomarkers for these disorders. Differences in a number of individual proteins such as Haemoglobin α&β

chain, Thioredoxin mitochondrial precursor, Myelin basic protein, Myelin protein zero, Cholineacetyltransferase, Tubulin α&β chain, Collagen α 1&2 (I), Glyceraldehyde 3-phosphate dehydrogenase and human GFAP have been identified in SC of ALS patients (group 1). Ubiquitin conjugating enzyme E2 variant 1, Glucosamin-fructose-6-phosphate aminotransferase, Thioredoxin mitochondrial precursor, and Bcl-2 interacting killer have been identified in microdissected SC-motoneurons from ALS patients (group 2) [Ekegren 2006]. In both examined groups between the detected targets coincide Thioredoxin mitochondrial precursor (TRMP) a protein with anti-apoptotic function that plays an important role in the regulation of mitochondrial membrane potential, and glial fibrillary acidic protein (GFAP) an intermediate filament protein which exact function remains poorly understood. The most abundant protein in both groups has been the GFAP. GFAP is closely related to its family member vimentin that also have been described to participate in the pathogenesis of ALS. Both proteins are also involved in the structure and function of the cell's cytoskeleton. Hence, from the two proteins identified in SC and SC-motoneurons of ALS patients, TRMP involvement supports a mitochondrial impairment hypothesis of ALS and is in agreement with the mitochondrial dysfunction that we detected in SC samples of sALS patients, while as the alteration of GFAP supports the cytoskeleton impairment hypothesis of ALS attended with disruption in axoplasmic transport that may be related with impaired myelination. The impaired myelination, on other hand can be associated again with GFAP since it is believed to be involved in the long term maintenance of normal CNS myelination, or both proteins GFAP and TRMP may interact in the described mitochondrial abnormalities in proximal axons in SC of sALS patients.

All the identified proteins have been proposed as ALS biomarkers [Ekegren 2006] but none has achieved the level of specificity required for proper early diagnosis of the disease. A recent study using 2D-GE compared tissue and CSF from healthy living and post mortem subjects to uncover potential biomarkers of neurodegeneration. Among the identified proteins GFAP was denoted as marker of numerous NDDs [Ranganathan 2007]. Analysis with mass spectrometry after 2D-GE and immunoblotting with anti-DNP antibody revealed three important proteins as targets of oxidation in SC of a G93A-SOD1 transgenic mouse: Cu/Zn-superoxide dismutase, Ubiquitin carboxyl-terminal hydrolase isozyme L1 and αB-crystallin [Poon 2005] but none of them coincided with the target proteins found in human SC of ALS patients.

In our results there was no coincidence between the identified oxidatively modified proteins in PiD and AGD. However, two of the identified proteins in PiD FC, Glyceraldehyde 3-phosphate dehydrogenase and GFAP, were identified also in ALS patients. Dissection of the DNP-modified proteins spots of FC in PiD identified through

MALDI-TOF-TOF and their analysis revealed as targets of oxidation: Carbonyl reductase NADPH1 (an antioxidant defence enzyme), Cathepsin D precursor (an intracellular protease); Vesicle - fusing ATPase (implicated in neurotransmition); GFAP (glial marker of brain aging and glial activation which increased in astrocytes [Nichols 1999]); heat shock protein 70kDa (Hsp70) (Grp78/BiP is the main representative protein of this family); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (is an enzyme that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules); and Peroxiredoxin 6 (antioxidant enzyme).

Spot	Protein	Calculated PI	kDa nominal mass	No. of peptides match	Assecc No	ratio PiD/ctl
c-d	**Carbonyl reductase NADPH1**	8.55	30375.1	6	P16152	7.84
e-f	**Cathepsin D precursor**	6.10	44552.5	9	P07339	3.12
k	**Vesicle - fusing ATPase**	6.38	82654	17	P46459	7.15
b	**GFAP**	5.42	49880.5	21	P14136	3.17
a	**heat shock protein 70kDa**	5.48	70052.6	9	P08107	8.42
g	**Glyceraldehyde 3-phosphate dehydrogenase**	8.57	36053.4	13	P04406	4.5
r	**Peroxiredoxin 6**	6	25035.1	12	P30041	2.38

Table 1d: *DNP-modified proteins in frontal cortex of Pick's disease*

GAPDH is multifunctional enzyme, which acts as reversible metabolic switch under oxidative stress. When cells are exposed to oxidants, some of their antioxidant systems need excessive amounts of the antioxidant cofactor NADPH essential for the recycling of gluthathione. Oxidant-treatments in cell cultures cause an inactivation of GAPDH. This inactivation re-routes temporally the metabolic flux from glycolysis to the Pentose Phosphate Pathway, allowing the cell to generate more NADPH [Ralser

2007]. Thus, the oxidatively modified GAPDH may disrupt the glycolysis process contributing to the defects in glycolytic potential and energy loss. This is in conformity with the significantly decreased levels of glycoxidative markers CEL and CML in the affected from the disease FC. On the other hand the modified GAPDH could not realize the metabolic switch to generate more NADPH allowing the cells without effective antioxidant defence under the conditions of oxidative stress and thus may participate to the development of PiD pathogenesis. To this process it may contribute also another antioxidant enzyme modified by the oxidative stress in FC of PiD, the Carbonyl reductase NADPH1. Carbonyl reductase has a physiological role for neuroprotection, by detoxifying reactive aldehydes derived from lipid peroxidation thereby confering protection against oxidative stress-induced brain degeneration [Maser 2006; Botella 2004]. This enzyme participates in arachidonic acid (ARA) metabolism as well. This fact could be related with the significant changes in the ARA levels, induced by the oxidative stress, in the fatty acid profile of FC cells' homogenate when compared with ARA levels in OC of PiD. Similar functions possess another antioxidant enzyme - the peroxiredoxin 6 - that was identified as a target of oxidative modification in FC of PiD. This protein is a member of the thiol-specific antioxidant protein family. This protein is a bifunctional enzyme with two distinct active sites. It is involved in redox regulation of the cell that may be related to the ER stress that we registered in PiD; but also it can reduce H_2O_2 and short chain organic, fatty acid, and phospholipid hydroperoxides which is in concordance with the significant changes observed in the fatty acids compositions and derived indexes. It may play also a role in the regulation of phospholipid turnover as well as in protection against oxidative injury. The potential modification of GAPDH, Carbonyl reductase NADPH1 and Peroxiredoxin 6 activity by oxidation could contribute to a vicious cycle generating further oxidative damage.

Envisaging that glycolytic metabolism is predominantly astrocytic, evidence in corroboration of the above assumed speculation that astrocytes' dysfunction may be involved in the pathogenesis of PiD is the oxidatively modification of GFAP. GFAP is expressed in the CNS mainly in astrocytes. The predominant change that occurs in glia during aging and some NDD is glial activation, which can progress to reactive gliosis in response to neurodegeneration. Such gliosis is a pathological feature of PiD. During aging, astrocytes hypertrophy and exhibit signs of metabolic activation. GFAP is reported to increase dramatically in response to acute infection or neurodegeneration [Johnston-Wilson 2000]. As well, GFAP was reported previously as a major target of glycoxidative and lipoxidative damage in PiD [Muntané 2006]. Its alteration could be associated with the oxidative stress observed in the FC of PiD as

recent work shows that astrocytes are targets of oxidative damage in NDDs [Martínez 2008]. GFAP is proposed to play a role in astrocyte-neuron interactions although its exact function remains poorly understood. The altered GFAP expression observed in FC of PiD might represent an additional mechanism involved in neuron-astrocyte dysfunction and changes in glial-neuronal communication in the course of the neurodegenerative process.

OS promotes a variety of protein modifications, some of which are incompatible with proper protein function [Stadtman 1998]. Oxidative modification of the proteins makes them prone to proteolytic degradation. Cathepsin D precursor, another modified protein, is an intracellular protease, which dysfunction would lead to awkward degradation and protein accumulation in the cells. It is known that its deficit leads to high rate of neuronal death with accrual of undigested lipofuscin [Shacka 2007]. Its pathogenical role was previously reported in several diseases, including AD. Dysfunction of the ubiquitin-proteasomal degradation system has been implicated in the process of neuronal degeneration. Thus, oxidatively modified Cathepsin D precursor probably contributes to the pathogenesis of PiD by disturbing proteolysis. Dysfunction of proteolysis is related with disturbance of ERAD, which acts to relieve the stress within the ER [Marciniak 2006]. When the ERAD of misfolded proteins is affected, it is the UPR that is down-regulated.

Grp78/BiP is the master regulator of UPR during the ER stress and the main chaperon of Hsp 70 family, which takes part in many aspects of ER quality control. In the most common tauopathy, up-regulation of molecular chaperones - particularly Hsp70 – could suppress formation of neurofibrillary tangles by directing tau into a productive folding pathway and thereby preventing tau aggregation [Petrucelli 2004]. Thus, the modification of Grp78/BiP in FC of PiD could be associated with the abnormal response to the stress conditions observed in the disease, due to the chaperones depletion, that could contribute to the severe neuronal loss in PiD. Most molecular chaperones can passively prevent protein aggregation by binding misfolding intermediates. Hsp70 binds to small hydrophobic stretches in proteins, in cooperation with a cochaperone of the Hsp40 family. This binding to the substrate requires ATP hydrolysis [Gao 2008] to forcefully convert stable harmful protein aggregates into harmless natively refoldable, or protease-degradable, polypeptides. Molecular chaperones and chaperone-related proteases thus control the balance between natively folded functional proteins and aggregation-prone misfolded proteins, which may form during the lifetime and lead to cell death. Abundant data now point at the molecular chaperones and the proteases as major clearance mechanisms to remove

toxic protein aggregates from cells, delaying the onset and the outcome of protein-misfolding diseases.

The two substrates of the vesicle-fusing ATPase enzyme are ATP and H_2O. This enzyme belongs to the family of hydrolases, specifically those acting on acid anhydrides to facilitate cellular and subcellular transport. On one hand damage of vesicle-fusing ATPase together with the modification of Hsp70 may contribute to the unsuccessful response to the stress conditions observed in FC of PiD. On the other hand the modifications of vesicle-fusing ATPase and the GAPDH that appears to be involved also in the vesicle transport, may contribute to defects in synaptic function, which could contribute to clinical manifestations of PiD.

The modification of above discussed proteins suggest that the reduction of oxidative damage precursors (carbonyl reductase), the antioxidant enzymes dysfunction (GAPDH, peroxiredoxin 6), the clearance systems impairment (cathepsin D and Hsp) as well as the disturbance in synaptic function (vesicle-fusing ATPase and GAPDH) together can contribute to increased oxidative damage and unmastered ER stress in PiD.

Spot	Protein	Calculated PI	kDa nominal mass	No. of peptides match	Assecc No	ratio AGD/ctl
b	**Fructose bisphosphate aldolase A**	8.30	39420.2	12	P04075	7.84
l,r,s	**Fructose bisphosphate aldolase C**	6.41	39456.1	16	P09972	5.12
k	**Phosphatydil-ethanolamine binding protein**	7.01	21056.9	11	P30086	2.15

Table 2d: *CML-modified proteins in hippocampus of Argyrophilic grain disease*

Analysis of CML-modified proteins spots in HC of AGD identified through MALDI-TOF-TOF revealed as targets of oxidation two isoforms of Fructose bisphosphate aldolase A & C, and a Phosphatydil-ethanolamine binding protein. Fructose-bisphosphate aldolase catalyse the enzymatic cleavage of β-D-Fructose-1,6-bisphosphate leading to the formation of glyceraldehyde 3-phosphate. The non

enzymatic and/or enzymatic elimination of phosphate from glyceraldehyde 3-phosphate is considered as the major source of methylglyoxal formation in physiological systems [Hamada 1996]. Methylglyoxal is the main precursor of CEL, formed as a side-product of several metabolic pathways, being the glycolysis the most important among them. The proteomic approach revealed that fructose-bisphosphate aldolase is modified in AGD samples. When the enzyme is modified lose its function and leads to abnormal glycolysis described in other NDDs (AD, PD). A consequence of such modification could be impeded formation of methylglyoxal that could be associated with the lower levels of CEL observed in AGD cases in comparison with the control case both by WB and GC/MS approach. The other modified protein PEBP, an individual signalling protein in the brain is developmentally regulated in hippocampus [Weitzdörfer 2008]. PEBP was described in AD as an inhibitor of the chymotrypsin-like activity of proteasome [Chen 2006]. We found this protein more modified in control samples than in HC of AGD. Diminished AGD-PEBP may be a compensatory cellular response to the proteasomal dysfunction evidenced in AGD by high molecular weight polyubiquitinated proteins.

3. Fatty acid profile in NDDs favour lipoxidation. Correlations between fatty acid composition and protein oxidative damages

While several different classes of molecules may be affected by oxidative stress, lipid peroxidation is thought to be a prominent and especially deleterious form of oxidative damage in brain due to this organ's relative enrichment in polyunsaturated fatty acids. Lipids are not an inert environment of membrane proteins. Modifications in the levels of certain lipids are predisposing factors to further cellular damage. Changes in brain lipid levels due to their increased or decreased synthesis or metabolism may result in homeostatic dysregulation. Dysregulation of lipid metabolism and/or the formation of lipid peroxidation products may lead to or accelerate neurodegeneration and therefore be important in the pathogenesis of NDD. Homeostasis of membrane lipids in neurons and myelin is essential to prevent the loss of synaptic plasticity and cell death [Simons 1998]. Phospholipids constitute the backbone of neural membranes. Omega-3 (alpha-linolenic acid, ALA; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA), and omega-6 FA (lineoleic acid, LA; arachidonic acid, ARA), are their important components, and are themselves important for normal brain functioning throughout the lifespan. Deficits in DHA abundance are associated with cognitive decline during aging and in NDDs. Lipid peroxidation appear to directly coincide with progressive neuronal degeneration in AD patients [Montine1999]. Deficits in DHA or its peroxidation appear to contribute to inflammatory signalling,

apoptosis, and neuronal dysfunction in AD. It have been reported that exposure of living mesencephalic neuronal cells from normal and α-synuclein-transgenic mice brains, and normal, PD, and DLB human brains to PUFAs increase α-synuclein oligomer levels, whereas saturated FAs decrease them. PUFAs directly promote oligomerization of recombinant α-synuclein. Whereas transgenic mice accumulated soluble oligomers with age, PD and DLB brains have elevated amounts of the soluble, lipid-dependent oligomers. It have been concluded that α-synuclein interacts with PUFAs *in vivo* to promote the formation of highly soluble oligomers that precede the insoluble α-synuclein aggregates associated with neurodegeneration.

Our results show significant changes in fatty acid (FA) composition in the three studied diseases. Significant differences in global indexes were associated with ALS (BC and SC), and with PiD (FC and OC), but the correlation between the affected zone and the preserved one was reverse. Changes in PI were also observed only in ALS and PiD. In SC of ALS and in FC of PiD PI was significantly decreased suggesting a functional collapse and/or a lower content of neurons, while as in BC of ALS and OC in PiD PI was significantly increased associated with preserved neurnal function.

Lipid peroxidation-associated processes seem to be the more sensible cellular oxidative phenomenon, based on MDAL values in ALS, PiD and AGD. ALS also presents organ-specific changes in desaturation indexes (18:1/18:0) and n6/n3 ratios. Omega-3 FA, and/or the ratio of omega-3/omega-6 FA, is thought to increase the expression of many genes in the brain involved in synaptic plasticity, signal transduction, cytoskeleton and membrane formation, regulation of lipid synthesis, and greater fluidity of synaptic membranes [Clarke 2001]. Nonetheless, long-chain unsaturated fatty acids (UFA) contribute to the formation of cytotoxic aggregates in proteinopathies, thereby stressing the importance of FA changes in this pathogenesis (Kim et al., 2005). Changes in DHA levels in PiD can be attributed to a potential defensive response in OC, that is not present in FC. In contrast, ARA levels are increased in FC but not in OC. This may reflect in an increased inflammatory status in this stage. A highly significant correlation between CEL and DHA levels suggest that this marker, in this tissue, is more related to metabolic potential (e.g. glycolysis) of cells than to damage to proteins, as DHA modulation is usually viewed as a protective mechanism for neuronal cells. DHA levels associated with ALS in SC differ from those present in BC. While PI is still increased in BC, basically due to increases in DHA content, as a potential defensive response of nervous tissue (Akbar et al., 2005), PI and DHA content were decreased in SC. Decreased DHA availability in ALS SC and in PiD FC neurons could be due to increased lipoxidative consumption in this location. Rather than being a general phenomena, the selectivity for changes in n-3 strongly

suggest specific mechanisms of the disease depending on these fatty acids, maybe affecting biosynthetic pathways and/or membrane remodelling systems. A loss of omega-3 and omega-6 FA in neuronal cell membranes is associated with a reduction in fluidity, worse functioning of the membrane components, and apoptosis [Choe, Jackson, & Yu, 1995]. Omega-3 and omega-6 FA are important to the structural stability of neuronal plasma membranes. The loss of neuronal membrane phospholipids and FA has been hypothesized to be an early metabolic event in the loss of synapses resulting in neurodegeneration [Mielke 2006].

Analysis of FA composition revealed significant differences in HC associated with AGD in some individual FA but not in global indexes. The saturated hexadecanoic acid (16:0), monounsaturated octadecenoic acid (18:1n-9) and 22:4n-6 (PUFA) fatty acids exhibited significantly reduced proportions in their % content in AGD samples compared with the control samples ($p<0.05$ for all of cases). There were no changes in DHA and ARA levels in HC of AGD, probably because in this disease the antioxidant response was activated and may be thereby compensates partially the lipid peroxidative damages, whereas in PiD such defence was missing. With reference to the FA-derived indexes no differences were observed either in DBI or in PI as well.

4. Proteasome dysfunction as a potential mechanism of ER stress

Some of the pathological hallmarks of NDDs e.g. tau-, TDP-43 and ubiquitin-positive inclusions are protein aggregates that may be related to oxidative stress, based on the relationship between protein oxidative damage and proteasomal activity. While moderate oxidative modification of proteins increases their susceptibility for proteasome clearance, higher rates of oxidative modification actually inhibit proteasome activity and facilitate protein aggregation [Grune 1997]. NDDs such as PD, AD, PrD and HD are pathologically characterized by the intracellular or extracellular accumulation of misfolded proteins or mutated gene products [Taylor 2002].

Since oxidative stress could contribute to inhibition of proteasome activity and our results demonstrated an increase oxidative stress in ALS, PiD and AGD, which are characterized by accumulation of protein aggregates, it was expected that the proteasomal function in these diseases could be affected. Increased amount of ubiquitinated proteins was observed in SC of ALS as well as in the affected by the disease anatomic locations in both tauopathies. That is compatible with a slight degradation and accumulation of modified and/or toxic proteins, and probably with compromised proteasomal function. It should be also considered that increased protein turnover due to massive protein degradation could physically saturate the

ubiquitin-proteasome system in spite of proteasome preserved activity. To verify this possibility we examined the proteasome activity in FC of PiD by the means of determination of two major proteolytic activities for the eukaryotic 20S proteasome: chymotrypsin-like and trypsin-like activity. The results confirmed an enhanced trypsin-like activity of the proteasome only in FC of PiD samples and steady state levels in the control OC. Recently it was described by Poppek and Davies that phosphorylated tau completely blocks tau degradation, even thought proteasome activity is preserved [Poppek 2006] as we found out in PiD. Thus, it could be supposed or indirectly concluded that despite the detected polyubiquitination in AGD patients HC, which is compatible with proteasomal dysfunction, the proteasome in AGD might also be active but saturated from undegradable hyperphosphorylated tau aggregates. In contrast, there was neither ubiquitination in the preserved OC of PiD nor in BC of ALS that are actually affected by the oxidative stress. Thus, could be suggested that the anatomic locations of the brain that were thought to be clinically preserved from the diseases might be at an early stage of the disease, when the oxidative stress already is biochemically detectable, and the proteasome and ER functions are still not touched.

Proteasome function	**ALS**		**PiD**		**AGD**
	BC	**SC**	**OC**	**FC**	**HC**
Ubiquitin	—	↑ **	—	↑ ***	↑ **
TDP-43	—	↑ **	—	—	—

↑-significant increase; ↓-significant decrease; — without significant changes

BC-brain cortex; SC-spinal cord; OC-occipital cortex; FC-frontal cortex; HC-hippocampus

A mutant ubiquitin protein (UBB^{+1}) generated by dinucleotide deletion occurring within UBB mRNA was discovered in some NDDs. UBB^{+1} is considered a specific marker of proteasomal dysfunction in tauopathies and polyglutamine diseases [Ferrer 2008]. The aberrant protein has a modified C-terminus and is unable to ubiquitinate other protein substrates [van Leeuwen 2000, 2002]. UBB^{+1} is itself ubiquitinated, and while at low levels can be degraded by the proteasome, at high levels it can inhibit the proteasomal machinery [Lindsten 2002]. UBB^{+1} was reported to be expressed in neurons with tangles as well as in AGs in AGD, whereas very low levels were noticed

in pre-tangle neurons [Ferrer 2008]. As well, previous studies have demonstrated UBB^{+1} protein accumulation in neurofibrillary tangles in AD, in the hallmark inclusions of Down syndrome, in ubiquitin containing neuropathological hallmark lesions of PGD [de Pril 2004] and several tauopathies, among them PiD, FTDP-17, PSP and AGD [Ferrer 2008].

Another protein that aggregates in various NDDs and may be implicated in the neurodegeneration is TDP-43, but the precise cellular mechanism(s) of TDP-43 degradation are unknown. It was suggested as a specific marker for two classes of disorders: TDP-43-positive and TDP-43-negative [Dickson 2007]. Our results demonstrated as a TDP-43 positive only ALS and both tauopathies as TDP-43 negative disorders. TDP-43 was accumulated only in SC but not in BC. This data suggest a role for TDP-43 in ALS pathogenesis, but do not support a role for TDP-43 in PiD and AGD, although occasional TDP-43 staining has also been noted in PiD brains [Freeman 2008]. Abnormal TDP-43 immunoreactivity has also been described in AD, DLB, Lewy body diseases [Higashi 2007], HD [Schwab 2008] and Guam parkinsonism-dementia complex. No abnormal TDP-43 inclusions were identified in PrD cases, and TDP-43 did not co-localize with ubiquitin-positive PrP plaques or with diffuse PrP aggregates [Isaacs 2008].

p62 is another protein which play a role in protein aggregation and degradation [Seibenhener 2004] and was detected in AGs and to a lesser degree in coiled bodies of AGD [Ferrer 2008]. p62 may have a role both in promoting protein aggregation and in delivering polyubiquitinated proteins to the proteasome for their degradation [Kuusisto 2001]. p62 was also present in LBDs in neurons with neurofibrillary tangles and in α-synuclein inclusions [Ferrer 2008].

Due to the important role of the proteasome in cell homeostasis, a potential consequence of proteasomal impairment may be the disruption of the ER-associated degradation. Proteasomal dysfunction is sufficient to activate the UPR that finally contributes to ER stress and aggravation of the disease [Marciniak 2006].

5. ER stress and activation of unfolded protein response in NDDs

The mechanisms that link ER stress and oxidative stress are very poorly characterized. It has become apparent that activation of the UPR on exposure to oxidative stress is an adaptive mechanism to preserve cell function and survival. Appropriate adaptation to misfolded protein accumulation in the ER lumen requires regulation at all levels of gene expression, including transcription, translation, translocation into the ER lumen, and ERAD [Malhotra and Kaufman 2007]. The physiological response to ER stress is regulated by the activation of three key ER-

localized transmembrane signal transducers: IRE1, ATF6 and PERK. In FC of PiD and in HC of AGD ATF6 (90kDa) was found cleaved (ATF6-50kDa), suggesting that this molecule are activated whereas in OC of PiD was detected only the inactive entire form of 90kDa. Activated ATF6 was also found in sALS [Atkin 2008]. Activated ATF6 translocate to the nucleus where regulate the transcription of UPR-responsive genes (such as ER chaperones and foldases involved in retrograde transport of misfolded proteins from ER to cytosol and in ER-induced protein degradation [Xu 2005] when the cleaved ATF6 activates the XBP1 promoter. Therefore, signalling through ATF6 and IRE1 merges to induce XBP1 transcription and mRNA splicing, respectively. ATF6 increases XBP1 transcription to produce more substrate for IRE1-mediated splicing that generates more active XBP1, providing a positive feedback for UPR activation [Kaufman 2002]. IRE1 levels were found elevated in AGD and FC of PiD but not in OC and XBP1 spliced form levels significantly increased in AGD and in both areas of PiD that is an indirect evidence for the activation of IRE1 as well. Increased levels of IRE1 were also detected in human sALS [Atkin 2008]. During OS conditions, XBP1 mRNA is upregulated in an Nrf2-dependent fashion [Kwak 2003]. The last key protein PERK, the major eIF-2α phosphorylation regulator during the UPR, phosphorylates and inactivates eIF-2α, thus blocks the translocational initiation of the protein synthesis to avoid their accumulation in ER lumen. eIF-2α was phosphorelated in all studied diseases that suppose translational attenuation and implication of ERstress in ALS, PiD and AGD. Phosphorylation of both PERK and eIF-2α has been detected also in neuromelanin containing dopaminergic neurons in the substantia nigra of PD patients [Hoozemans 2007]. Another eIF-2α upstream molecule is PKR. Although the UPR marker eIF-2α was phosphorilated (p-eIF-2α) in ALS SC, PiD FC and AGD HC, levels of the eIF-2α upstream molecule PKR were unchanged in PiD and AGD, suggesting that there elevated p-eIF-2α is independent of PKR. In contrast, in ALS and AD we observed augmented PKR levels, although the PERK was shown to be activated [Atkin 2008; Hoozemans 2005], presuming the role of this kinase in other pathway involved in the disease pathogenesis.

The three independent sensors of ER stress (IRE1, ATF6 and PERK) are activated by a common stimulus-Grp78/BiP, which orchestrates the UPR. Chaperones are suppressors of neurodegenerative disorders. Consistent with this notion, the presence of aggregates in cells is known to trigger the heat-shock response, which induces the expression of HSPs [Gao 2008], such as Grp78/BiP and Grp94. Furthermore, increased levels of Grp78/BiP, Grp94 and KDEL-containing proteins were detected in AGD and ALS, as also was shown in AD [Hoozemans 2005], that correspond to activated adaptive mechanisms to restore homeostasis in response to

ER stress in these disorders. The upregulation of ER stress chaperones, such as Grp78, Grp94 and Grp58/ERp57, was observed in PrPsc-infected neuroblastoma cells as well [Lindholm 2006]. In contrast, these chaperones were practically absent not only in FC but also in OC of PiD, which is compatible with abnormal ER stress response.

ER stress	**ALS**		**PiD**		**AGD**
	BC	**SC**	**OC**	**FC**	**HC**
p-eIF-2α	↑ *	↑ **	—	↑ ***	↑ **
ATF6 (90kDa)			—	↑ *	↑ **
Cleaved ATF6 (50kDa)			↑ *	↑ *	↑ ***
IRE1			—	↑ ***	↑ ***
XBP1			↑ *	↑ *	↑ **
Grp78/BiP	—	↑ *	↓*	↓***	↑ **
Grp94	↑ **	↑ **	—	↓**	↑ **
KDEL	—	↑ **			↑ ***
PDI	—	↑ **	↓*	—	↓**
PKR	—	↑ **	↑ *	—	—
Calreticulin			—	—	

↑-significant increase; ↓-significant decrease; — without significant changes

BC-brain cortex; SC-spinal cord; OC-occipital cortex; FC-frontal cortex; HC-hippocampus

Impairment of these chaperones result in aggregation of soluble substrates, and hence decreased degradation that leads to the failure of attenuating ER stress and eventual apoptosis of neuronal cells. In addition, the oxidizing environment of the ER creates a constant demand for cellular PDI to catalyze and monitor disulfide bond formation in a regulated manner [Xu 2005]. In response to ER stress, PDI is usually upregulated and protects neuronal cells. We observed an increased levels of PDI in sALS patients SC that were later reported also by other group [Atkin 2008], providing evidence that a protective mechanism for MN is present probably as an outcome of the UPR. PDI was also reported to be physically associated with mSOD1 inclusions in

SOD1-transgenic animals and in a motor neuronal-like cell line transfected with SOD1 [Atkin 2006], but not in wild-type SOD animals and cells. Since overexpression of PDI attenuates UPR and protects cells against apoptosis induced by ER stress inducers, such as tunicamycin, this could be a protective mechanism for proper folding of SOD1 and in this manner to overcome the pathogenic process. Inhibition of its enzymatic activity leads to the accumulation of polyubiquitinated proteins and activation of UPR. PDI levels were significantly decreased in AGD and in OC of PiD, but were unchanged in the pathologically affected FC of PiD that is compatible with accumulation of unfolded proteins in ER. S-nitrosylated PDI with inhibited activity was found in the brain samples of PD and AD patients [Uehara 2006].

All these results agree with the concept that cellular injury during NDDs may result from both elevated ROS generation as well as from impaired cellular repair mechanisms following oxidative injury. The elevated reactive species suggest the possible role of the altered antioxidant protection, redox changes in the ER and the consequent ER stress. ER stress and OS trigger the release of Nrf2, a downstream target of PERK, from Keap1-Cul3 cytoplasmic complexe, resulting in the accumulation of Nrf2 in the nucleus and inducing the transcription of Nrf2 target genes encoding antioxidants and detoxifying enzymes as well as chaperones [Cullinan 2004, 2006]. Nrf2 levels were significantly elevated in ALS SC and AGD as well as in AD, in contrast with PiD where they were depleted in FC and unchanged in OC. Together with the activated ATF6 (in ALS, PiD and AGD) and XBP1 (in PiD and AGD) this is in concordance with the high levels of ER-chaperones that were observed in ALS and AGD and activated antioxidant response to the OS that were detected, through the expression of the Nrf2 target genes SOD1 and SOD2. Widespread oxidative damage of proteins, and cellular responses to OS through increased SOD1 and SOD2 expression levels were reported in the cerebral cortex in CJD and PD as well [Freixes 2005; Dalfó 2005]. The low levels of the positive regulator of the antioxidant capacity Nrf2 in PiD is compatible with insufficient antioxidant defence evidenced by the decreased levels of SOD1 in FC and with the lack of ER chaperones. Increased levels of SOD1 in OC observed in the clinically preserved area of PiD patients are indicative of the activation of antioxidant response early in neurodegeneration.

6. Mitochondrial dysfunction, mitochondrial biogenesis and antioxidant defence system in NDDs

There is considerable evidence that PGC1α has a neuroprotective function [Cui 2006]. PGC1α is a master regulator of the antioxidant defence and orchestrate the mitochondrial biogenesis stimulating the expression of Nrf-1, Nrf-2 and TFAM, which in

turn activates expression of nuclear and mitochondrial genes encoding mitochondrial proteins [Wu 1999]. PGC1α and Nrf1 analyses revealed importantly raised levels in ALS and AGD as well as in AD that presume increased levels of the proteins involved in mitochondrial biogenesis. In FC of PiD PGC1α and Nrf1 showed significantly decreased levels compatible with abnormal mitochondrial biogenesis if there is any.

Co-activators & Co-repressors	**ALS**		**PiD**		**AGD**
	BC	**SC**	**OC**	**FC**	**HC**
RIP140			↑ *	↑ **	↑ **
Sirt1			—	↓**	↓*
PGC1α	—	↑ **	—	↓*	↑ ***
Nrf1	—	↑ **	—	↓**	↑ *
TFAM			—	↓*	—
Mfn1			—	↓***	—
Nrf2	—	↑ **	—	↓*	↑ **
SOD1			↑ **	↓*	↑
SOD2			↓*	↓*	↑

↑-significant increase; ↓-significant decrease; — without significant changes

BC-brain cortex; SC-spinal cord; OC-occipital cortex; FC-frontal cortex; HC-hippocampus

Recent studies revealed a critical role for PGC1α in HD. Mutant huntingtin causes disruption of mitochondrial function by inhibiting the expression of PGC1α in a mouse model of HD [Outeiro 2008]. Lack of mitochondrial fusion results in two main consequences that impact cellular survival - mitochondrial dysfunction and alterations in mitochondrial number. Interestingly, the levels of TFAM (involved in the control of replication and transcription of mtDNA) and Mfn1 (support mitochondrial fusion) upstream regulated by Nrf1 where unchanged in AGD and OC of PiD but decreased in FC of PiD. Genes involved in oxidative phosphorylation and mitochondrial biogenesis are upregulated in the absence of RIP140. PGC1α transcriptional coactivator and the RIP140 corepressor of mitochondrial biogenesis promote opposing physiological functions [Ström 2008; White 2008]. As it was expected in PiD the levels of the RIP140

were found significantly increased. Surprisingly, in AGD the levels of the RIP140 were found significantly increased as well as those of PGC1α. Therefore, the disturbed mitochondrial biogenesis and the unchanged levels of TFAM and Mfn1 could be attributed to these abnormal levels of the mitochondrial biogenesis repressor RIP140. Mutations in the mitochondrial fission and fusion machinery can cause NDDs, and the fact that the familial PD-specific genes, PINK1 and parkin, seem to play a part in mitochondrial fission underscores the role of these proteins in mitochondrial health and neuronal function [Knott 2008]. Mitochondrial transport, together with the dynamic processes of mitochondrial fission and fusion, facilitates the transmission of energy across long distances, which is particularly important in neurons given that axons can extend up to one metre in motor neurons [Knott 2008]. Although there is no information about these proteins in ALS it could be speculated, only over the decreased levels of porin compatible with the decreased mitochondrial mass, that mitochondrial biogenesis in this disease might be disrupted and may be implicated in the motor neurons degeneration.

OS and presumably cell damage increase with age due to either diminished antioxidant defences or the increase in mitochondrial dysfunction [Cassarino 1999]. The evidences of OS and disturbance of the mitochondrial biogenesis in PiD and in AGD directed to mitochondrial dysfunction. Since mitochondria are the primary producers of ROS, oxidative damage of mitochondrial proteins is likely to contribute to the mitochondrial dysfunction that is characteristic of many NDDs [Lin 2006]. Oxidative damage is an early event in human AD: Amyloid-β (Aβ) peptide inhibits complex IV and thus increases the production of damaging ROS in mitochondria [Knott 2008]. Inhibitors of complex I, such as rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), cause parkinsonism. Moreover, proteins that are mutated in familial forms of PD, associate with the mitochondrial outer membrane are involved in ROS production. 3-nitroproprionic acid (3-NP) is a mitochondrial complex II inhibitor that produces HD-like symptoms, and mutant huntingtin (mutHtt) itself seems to disrupt complex II activity.

In the examined three diseases there was no common pattern in the levels of the respiratory chain complex subunits. Different peptides of the mitochondrial complexes, in particular complex I and complex IV, revealed significantly reduced levels in AGD, whereas complex II and complex III were unchanged. In FC of PiD complex I and complex IV were significantly increased, while complex II and complex III were again unchanged. In ALS decrease complex I, III and complex IV were observed, whereas complex II was significantly increased in SC. Together these data suggest a loss of respiration capacity, free radical generation and/or disturbance in the

mitochondrial energy metabolism in ALS, PiD and AGD. Energy depletion has been suggested to play a major role in neuronal cell-death in the neurodegenerative diseases [Outeiro 2008]. Complex I is functionally related with another mitochondrial protein AIF. AIF is required for the correct assembly and/or maintenance of the respiratory chain polyprotein complex I [Vahsen 2004]. In both areas of PiD AIF levels from the mitochondrial fraction (62 kDa) were significantly increased. In contrast, in AGD where complex I was significantly diminished AIF levels (62 kDa) were significantly decreased, in concordance with the finding that AIF-deficient cells exhibit a reduced content of complex I and of its components. Only in ALS the AIF 62 kDa levels were unchanges despite the decreased levels of complex I. In view of the fact that the absence of AIF was found to compromise the composition and function of the respiratory chain, targeting mostly complex I and the speculation that AIF might act as an antioxidant enzyme [Lipton 2002], the increase of AIF could be considered as a compensatory mechanism in PiD. Another fact that should be taken into account is that except in PiD the levels of the porin were significantly decreased in ALS and AGD.

Mitochondrial ETC Mitoch.Biogenesis	**ALS**		**PiD**		**AGD**
	BC	**SC**	**OC**	**FC**	**HC**
Complex I	—	↓*	—	↑*	↓*
Complex II	—	↑**	—	—	—
Complex III	—	↓*	—	—	—
Complex IV	—	↓**	—	↑*	↓*
Porin	—	↓*	—	—	↓*
AIF 67		↓**	—	—	↓*
AIF 62		—	↑*	↑*	↓*
AIF 57	—	—	—	—	↑**
UCP4			—	↓**	↓*
Prohibitin			—	—	↓**

↑-significant increase; ↓-significant decrease; — without significant changes

BC-brain cortex; SC-spinal cord; OC-occipital cortex; FC-frontal cortex; HC-hippocampus

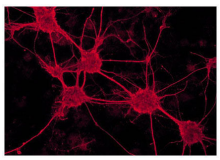
Another important and multifunctional mitochondrial protein is Prohibitin. Prohibitin was constituted as a cellular defence against oxidative stress and associated with inhibition of mitochondrial biogenesis and senescence. Its unchanged levels in PiD are in concordance with the lack of antioxidant defence and significantlly decreased levels of TFAM and Mfn1 presumably provoked by the increased levels of the mitochondrial biogenesis corepressor RIP140. In AGD Prohibitin's levels were found significantly diminished, which is also compatible with the lack of mitochondrial biogenesis on account of the abnormalities detected in the regulatory mechanisms of its activation.

The mitochondrial biogenesis activator PGC1α as well regulates the expression of another crucial mitochondrial protein – UCP4, an important regulator of the mitochondrial homeostasis and ROS formation [Dong 2006]. Increased mitochondrial membrane potential associated with increased production of ROS can be regulated by the activation of UCP [Kitahara 2004]. By shifting energy metabolism to reduce ROS production and cellular reliance on mitochondrial respiration, UCP4 can protect neurons against OS and calcium overload [Liu 2006]. Likewise the other proteins involved in mitochondrial biogenesis TFAM and Mfn1, UCP4 was seen significantly decreased in PiD and AGD. A lack of neuronal UCPs initiates mitochondrial dysfunction-decreased ATP, increased OS and calcium deregulation, all of which influence neurodegenerative pathologies [Andrews 2005]. The depletion of UCP4 in the HC of AGD and in FC of PiD patients could be associated with elevated ROS production. The high levels of PGC1α in AGD presume high levels of UCP4 as well. RIP140 antagonize the function of PGC1α thereby suppresses UCP expression and ATP uncoupling [White 2008]. In the absence of RIP140, altered expression of the PGC1α co-activator is not essential for UCP expression [Ström 2008]. Thus, it can be hypothesized that upregulation of RIP140 suppress the expression of UCP, thus probably contributing to increased production of ROS in PiD and AGD.

Furthermore, significantly decreased levels of porin were seen in ALS and AGD when compared to controls, which suggest that the mitochondria in ALS and AGD could not only be affected but also could have reduced number, probably through the repression of mitochondrial fusion by RIP140. Neuronal in vivo studies showed that Mfn-deficient cells have impaired respiratory complex activity and defects in inner membrane structure characteristic of respiratory dysfunction [Andrews 2005]. Overexpression of RIP140 is predicted to impair mitochondrial function and this could be compensated by an increase in the activity of co-regulators such as PGC1α [White 2008], since overexpression of PGC1α in cells has been shown to increase mitochondrial content [Nemoto 2005]. The upstream regulator of PGC1α - SIRT1 -

regulates the activity and acetylation status of PGC1α. SIRT1 was found depleted in PiD and in AGD. Whereas the low levels of SIRT1 and PGC1α in PiD could be explained with the increased levels of the RIP140 transcribtional repressor, the concurrent increased levels of Rip140 and PGC1α in AGD are a pathologic state that may be due to the low levels of SIRT1 regulator of the PGC1α activity. SIRT1 was found to be upregulated in mouse models for AD and ALS. In cell-based models of AD tauopathy and ALS, activation of SIRT1 promotes neuronal survival [Outeiro 2008].

Our data suggest that ER stress is a late phenomenon that arises from oxidative stress and from a mitochondrial disturbance. Although, the three NDDs that were on focus in our studies ALS, PiD and AGD showed increased oxidative stress markers, clear signs of ER stress and ER stress response systems, accompanied by mitochondrial respiratory chain disturbances, lack of mitochondrial biogenesis, proteasomal inhibition and changes in the fatty acid profile, they differ in the regulation of activation of antioxidant response, the regulation of chaperons' expression, different proteins are targets for oxidative modification and also differ in the processes that contribute to mitochondrial biogenesis failure. However, finally all similarities and distinctions lead to the neurodegeneration in the most common MND as well as in two tauopathies that also differ in between. A variety of pathological mechanisms are evidently associated with human neurodegenerative disorders, but no particular mechanism emerging as a major contributor. Thus, despite the significant progress in understanding the molecular basis of neurodegeneration, the lack of known useful molecular targets for effective therapeutic intervention impedes the development of neuroprotective strategies and drug discovery processes.



CONCLUSIONS

CONCLUSIONS

Based on our results, we can conclude:

1. There exists an increased oxidative stress, evidenced by augmentation of different markers of direct protein oxidation (AASA and GSA), glycoxidation (CEL and CML) and lipoxidation (CML and MDAL) in ALS samples compared with control samples, predominantly in spinal cord but also to a lesser extent in brain cortex.

2. Changes in fatty acid composition in ALS samples associated with increased oxidative damage, specifically the lack of the neuroprotective factor - docosahexaenoic acid - only in spinal cord, suggest a high susceptibility of the membranes in this area to be oxidized.

3. Changes in mitochondrial respiratory complexes in spinal cord of ALS, particularly the diminution of complex I and complex III may be related to increased generation of ROS and could induce alterations of energy metabolism; to loss of respiration capacity; and/or to reduction of mitochondrial mass.

4. There exists an alteration in the protein degradation system ubiquitin-proteasome, demonstrated through the presence of highly ubiquitinated proteins in spinal cord samples of ALS and accumulation of the ALS specific marker TDP-43 only in spinal cord and its absence in the control zone.

5. An endoplasmic reticulum stress, evidenced by the increased amount of ER chaperons Grp78/BiP, Grp94 and the foldase PDI as well as the phosphorylation of the eukaryotic translation initiation factor 2 that suggest a translational attenuation of protein synthesis, is present in spinal cord of ALS.

6. There exists initial biochemical alterations (OS stress) in brain cortex of patients with lumbar onset associated with lower MN symptoms of ALS without bulbar symptoms.

7. There exists an increase in direct oxidative and lipoxidative protein lesions in frontal cortex of PiD, whereas the levels of glycoxidative markers diminish that

probably could be attributed to an alteration of glycolysis in the cellular populations affected from the disease.

8. Modifications in the fatty acid composition in frontal cortex of PiD, such as increased levels of ARA, suggest a high susceptibility of the membranes to be oxidized because of the presence of more polyunsaturated fatty acids and lack of local defence because of the depletion of the neuroprotector DHA.

9. The protein oxidative modifications in PiD may be secondary to an alteration of mitochondrial energy metabolism probably derived from lack of mitochondrial biogenesis in the affected zone; to a diminution of antioxidant mediators presuming a lack of antioxidant defence; and to an alteration in the levels of protein degradation.

10. The endoplasmic reticulum stress in frontal cortex of PiD could be attributed to the oxidative stress that suffer the cell and to the improper UPR, evidenced by the depletion of ER chaperons Grp78/BiP and Grp94, and the Nrf2 regulator of the inducible expression of antioxidant response elements containing genes, in response to oxidative stress and ER stress.

11. The morphologically preserved occipital cortex of patients with PiD present biochemical alterations related to oxidative stress, such as increased markers of direct, glyco- and lipoxidation that would be compensate through a high amount of DHA-derived neuroprotector. This is compatible with a defensive response in occipital cortex, that may have been already overwhelmed in frontal cortex, with an increase in the antioxidant transcription factors and in the conserved mitochondrial biogenesis.

12. In AGD exists an increased oxidative stress that probably derive from lipoxidation, despite little significant changes have been detected in the polyunsaturated fatty acids composition.

13. The changes in mitochondrial complexes in AGD, particularly the diminution of complex I and complex IV are compatible with loss of respiratory capacity and/or alteration of energy metabolism that could be related to the absence of the uncoupling protein UCP4.

14. The reduction of mitochondrial mass in AGD associated with a potential lack of mitochondrial biogenesis in the affected area could be a consequence of the decreased levels of SIRT1, the regulator of the activity of the coactivator of the mitochondrial biogenesis PGC1α.

15. The endoplamic reticulum stress in AGD could be attributed to the oxidative stress and to the scanty antioxidant defence that is activated but insufficient to overcome the cellular stresses.

CONCLUSIONES

En relación a las patogénesis de ELA, EdP y EGA, nuestros resultados demuestran:

1. Existe un incremento del estrés oxidativo proteico, evidenciado por el aumento de los diferentes marcadores de oxidación proteica directa (AASA y GSA), de glicoxidación (CEL, CML) y de lipoxidación (CML, MDAL)) en las muestras de ELA respecto a los controles, sobretodo en médula espinal, pero también en menor medida en la corteza cerebral.

2. Los cambios en la composición de ácidos grasos en muestras de médula espinal de pacientes de ELA asociados a un incremento de la lesión oxidativa, concretamente por la pérdida del factor neuroprotector ácido docosahexaenoico, sugierieren una mayor susceptibilidad de las membranas en esta zona a ser oxidadas.

3. Los cambios en los complejos respiratorios mitocondriales en muestras de médula espinal de ELA, destacando en particular la disminución del complejo I y del complejo III, se relacionan con un incremento de ROS que puede ser causado por una alteración del metabolismo energético, una pérdida de la capacidad respiratoria y/o una reducción de la masa mitocondrial.

4. Existen alteraciones en el sistema de degradación proteica ubiquitina-proteasoma, demostradas por una mayor ubiquitinización en las muestras de médula espinal de ELA y por una acumulación del marcador específico de ELA immunoreactivo para ubiquitina, TDP-43, sólo en médula espinal, mientras que éste es ausente en la zona control de la corteza cerebral.

5. El estrés de retículo endoplasmático, evidenciado por el aumento de la cantidad de chaperonas de retículo Grp78/BiP, Grp94 y la foldasa PDI y por una mayor fosforilación del factor eIF-2α; esta última sugiere la existencia de una traducción proteica atenuada en la médula espinal de pacientes de ELA.

6. Existen alteraciones bioquímicas iniciales en la corteza cerebral de individuos únicamente con ELA medular.

7. Existe un incremento de lesión oxidativa directa y lipoxidativa proteica en la corteza frontal de pacientes EdP, mientras que la lesión glicoxidativa disminuye. Este hecho se puede atribuir probablemente a una glicolisis alterada en las poblaciones celulares afectadas por la enfermedad.

8. Modificaciones en la composición de los ácidos grasos de la corteza frontal de pacientes EdP, tales como el incremento de los niveles de ARA, sugieren una mayor susceptibilidad de las membranas celulares a ser oxidadas debido a una mayor presencia de ácidos grasos poliinsaturados y a la falta de defensa local debida a una disminución del lípido neuroprotector DHA.

9. Las modificaciones oxidativas proteicas en pacientes EdP son secundarias a la alteración del metabolismo energético mitocondrial, probablemente por una combinación de factores: falta de biogénesis mitocondrial en la zona afectada, disminución de mediadores antioxidantes causantes de la falta de defensa antioxidante y/o cambio en las tasas de degradación proteica.

10. El estrés de retículo en la corteza frontal de pacientes EdP se puede atribuir al estrés oxidativo que sufre la célula y a la respuesta inapropiada de las proteínas mal plegadas. Este fenómeno se ha evidenciado por la depleción de chaperonas de retículo Grp78/BiP y Grp94, y de Nrf2 el regulador de la expresión de los genes involucrados en la respuesta del estrés oxidativo y de retículo.

11. La corteza occipital de pacientes con EdP, morfológicamente preservada, sólo presenta alteraciones bioquímicas relacionadas con el estrés oxidativo (incremento de lesión oxidativa proteica directa, glico- y lipoxidación) pero no presenta alteración en los marcadores de estrés de retículo. Esta evidencia posiblemente esté relacionada con una mayor cantidad del lípido neuroprotector DHA, con un aumento en los factores de transcripción antioxidantes y con la conservación de la biogénesis mitocondrial en esta zona, en comparación con la corteza frontal.

12. En EGA existe un incremento del estrés oxidativo proteico que probablemente proviene de los derivados de lipoxidación. No obstante, no se han detectado grandes cambios en la composición de ácidos grasos poliinsaturados.

13. Los cambios en los complejos mitocondriales de muestras de EGA, concretamente la disminución del complejo I y del complejo IV, son compatibles con una pérdida de la capacidad respiratoria y/o una alteración del metabolismo energético, que pueden ser debidos a una falta de la proteína desacopladora UCP4.

14. La reducción de la masa mitocondrial en muestras de EGA, asociada con niveles bajos de porina, puede ser debida a una falta de biogénesis mitocondrial en la zona afectada que, a su vez, puede ser causada por una disminución de los niveles de SIRT1, el regulador de la actividad del principal activador de la biogénesis mitocondrial, el PGC1α.

15. El estrés de retículo endoplasmático en EGA se puede atribuir a la presencia de estrés oxidativo y la escasa defensa antioxidante activada, que resulta insuficiente para superar los estreses celulares.

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