# FACTORS PATOGÈNICS CONVERGENTS EN TAUPATIES

TESI DOCTORAL
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Al meu avi Josep

Als meus pares, la meva germana i l'abuelita

A la Bego

Als meus avis Gabriel i Maribel,
i al meu tiet Josep Mª

L’oblit és desmemòria, desarrel, despreocupació, formes que semblen les d’una absència o buit. Però val pensar-ho d’una altra manera: l’oblit allibera el present de tot allò que li és aliè. Brinda un present que ens repta a comprovar què som capaços de fer amb ell. I amb tot, a vegades es precisa l’oblit d’un mateix: no prendre’s massa seriosament, alleujar-se de la càrrega de ser un mateix i deixar de ser-ne esclau. Permetre la irrupció d’allò que no havíem ni sospitat.

Mariela Saez, sobre la
Farmacia del Olvido, de Rogelio Moreno

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**ABREVIATURES**

**MA: Malaltia d'Alzheimer**
**PSP: Paràlisi supranuclear progressiva**
**DCB: Degeneració cortico-basal**
**MP: Malaltia de Parkinson**
**DFTP-17: Demència frontotemporal amb parquinsonisme lligada al cromosoma 17**
**MPi: Malaltia de Pick**
**MGA: Malaltia dels grans argiròfils**
**DCL: Demència amb cossos de Lewy**
**MAP: Microtubule asociated protein (proteïna associada a microtúbuls)**
**APP: Proteïna precursora del pèptid amiloide**
**AAC: Angiopatia amiloidea cerebral**
**PSEN1 i PSEN 2: Presinilina 1 i 2**
**NGF: Nerve growth factor (factor de creixement neuronal)**
**EGF: Epidermal growth factor (factor de creixement de l'epidermis)**
**LTP: Long term potential (Potenciació a llarg termini)**
**LDL: Low density lipoprotein (lipoproteïna de baixa densitat)**
**ROS: Radical oxygen species (radicals lliures d'oxigen)**
**PET: Positron emision tomography (tomografia per emissió de positrons)**
**DFT: Demència fronto-temporal**
**CA: Cornu Ammonis**
**UCHL-1: Ubiquitin carboxi-terminal hydrolase L1 (hidrolasa carboxi-terminal de la ubiqüitina)**
**PINK-1: PTEN-induced putative kinase (cinasa putativa induïda per PTEN)**
**LRRK2: Leucine rich repeat kinase (cinasa amb repeticions riques en leucina)**
**PHF: Paired helical filaments (filaments aparellats hel·licoidalment)**
**GSK3-beta: Glycogen sintase kinase (cinasa de la glicogen sintasa)**
**Cdk5: Cyclin dependent kinase (cinasa depenent de ciclina)**
**PKA: Protein kinase A (cinasa de proteina A)**
**CaMKII: Calcium/Calmodulin-dependent protein kinase (cinasa depenent de calci/calmudulina)**
**ERK: Extracellular signal-regulated kinase (cinase regulada per senyals extracel·lulars)**
**SAPK/JNK: Stress-activated protein kinase/c-Jun NH2-terminal kinase (cinasa de proteïna activada per estrès/ cinasa de l'amino-terminal de c-Jun)**
**PP: Protein phosphatase (fosfatasa de proteïna)**
**NFT: Neurofibrillary tangles (cabdells neurofibril·lars)**
**ATF-2: Activating transcription factor (factor de transcripció d'activació)**
**AP-1: Activating protein (proteïna d'activació)**
**CREB : cAMP response element binding protein (proteïna d'unió a l'element de resposta a AMPc)**
**CBP: CREB binding protein (proteïna d'unió a CREB)**
**MAPK: Mitogen-activated protein kinase (cinasa de proteïna activada per mitògen)**
**AEC: Atàxia espino-cerebel·losa**
**TGF: Transforming growth factor (factor de cereixement transformant)**
**SOD: Superòxid dismutasa**
**HSP: Heat shock protein (proteïna de xoc tèrmic)**
**UBB: ubiqüitina**
**CEL: Carboxi-etil-lisina**
**HNE: 4-hidroxinonenal**
**MDAL: Malondialdehid-lisina**
**CML: Carboxi-metil-lisina**

**PUFA: Polyunsaturated fatty acid (àcid gras poli insaturat)**
**AGE: Advanced glycation end product (producte final de glicació avançada)**
**RAGE: Receptor d'AGEs**
**8OHG: 8-hidroxiguanosina**
**DIGE: Differential in gel electrophoresis (diferencial en electroforesis de gel)**
**NGFR: Nerve growth factor receptor (receptor del factor de creixement neuronal)**
**IL-1: Interleucina 1**
**MCI: Mild cognitive impairment (dèficit cognitiu lleu)**
**GFAP: Glial fibrillary acidic protein (proteïna fibirl·lar acídica de la glia)**
**TNF: Tumor necrosis factor (factor de necrosi tumoral)**

# Introducció

# 1- LES MALALTIES NEURODEGENERATIVES

***Demència***

**La demència senil és una síndrome que té com a principal factor de risc l'edat. Que el principal factor de risc sigui l'edat no vol dir que aquesta sigui la causa de la demència. Fins els anys 70, es considerava la demència com una conseqüència dels problemes associats a la senilitat, i els efectes d'aquesta senilitat eren prou amplis com per respondre a qualsevol problema que patís algú de més de 60 anys. La demència es defineix com la pèrdua o davallada de facultats cognitives sempre i quan aquestes afectin a diferents dominis de la cognició; si la pèrdua afecta una funció cognitiva concreta es considera un desordre específic. A més, normalment el terme demència s'aplica quan la pèrdua cognitiva afecta significativament la capacitat d'independència de l'individu. Aquesta síndrome sovint s'expressa amb pèrdua de memòria, dificultats en el llenguatge, problemes de visió de l'espai, problemes de raonament i alteracions de l'estat d'ànim i de la personalitat. La senilitat no comporta automàticament la demència, però si que comporta un cert grau de dèficit cognitiu. Aquest dèficit no està en relació amb pèrdua del volum cerebral, com sovint ocorre amb la demència, sino que es tracta de canvis com per exemple una pèrdua gradual de memòria, un augment de l'estabilitat emocional o una pèrdua d'assimetria hemisfèrica.**

**Fa dècades que sabem que la demència, tal com l'hem definit, és causada per malalties neurològiques. I en la majoria dels casos, per malalties neurodegeneratives com la malaltia d'Alzheimer.**

***Malalties neurodegeneratives***

**Les malalties neurodegeneratives són malalties molt complexes que poden involucrar diferents susceptibilitats genètiques o factors ambientals. En general presenten pèrdua neuronal, estrés oxidatiu i la presència d'unes inclusions anòmales de naturalesa proteica tant intracel·lulars (més freqüentment) com extracel·lulars (és el cas del pèptid amiloide) en el cervell. Les malalties neurodegeneratives més importants es poden classificar en funció de la proteïna majoritària dins d'aquestes inclusions: taupaties i**

**sinucleïnopaties. Les primeres presenten agregats de proteïna tau i les segones presenten agregats d'alfa-sinucleïna.**

### *1.1 Taupaties*

**Les taupaties es caracteritzen per presentar acúmuls intracel·lulars (en neurones i cèl·lules glials) de proteïna tau anormalment hiperfosforil·lada. Aquests agregats poden ser de diferents tipus i es poden trobar en regions diferents del cervell depenent de la taupatia. En el grup de les taupaties trobem: la malaltia d'Alzheimer (MA), la paràlisi supranuclear progressiva (PSP), la malaltia de Pick (MPi), la malaltia dels grans argiròfils (MGA), la degeneració cortico-basal (DCB) i un conjunt de malalties causades per diferents mutacions al gen de la tau anomenades demències frontotemporals amb parkinsonisme lligades al cromosoma 17 (FTDP-17-TAU).**

#### *1.1.1 Malaltia d'Alzheimer*

**La malaltia d'Alzheimer és la causa més freqüent de demència. La prevalència, amb dades d'Europa i Nord-Amèrica, entre la població de 65 a 69 anys és d'1 de cada 100 individus. Aquesta prevalència es dobla cada 5 anys per sobre de 69, fins arribar a ser d'entre un 20% i un 50% al voltant dels 85 anys (Hy and Keller, 2000). Clínicament comporta un seguit de dèficits cognitius que inclouen una pèrdua de memòria a curt termini, de capacitat d'orientació, dificultats del llenguatge, pèrdua d'atenció i funció visuo-espacial, així com de capacitat intel·lectual per resoldre problemes, fer judicis i l'agilitat mental en el raonament (Green et al., 1990, Welsh et al., 1991).**

**Macroscòpicament la MA presenta atròfia cerebral que afecta principalment al lòbul temporal. Això inclou estructures com l'escorça entorrinal, l'hipocamp i l'amígdala (Najlerahim and Bowen, 1988). Microscòpicament la MA presenta mort neuronal i pèrdua de sinapsis entre les neurones supervivents. La pèrdua neuronal és progressiva i afecta predominantment a l'escorça entorrinal, temporal i parietal, l'hipocamp, amígdala, nucli basal de Meynert, locus ceruleus i nuclis del rafe (Whitehouse et al., 1981, Whitehouse et al., 1982, Kremer et al., 1991). La pèrdua de sinapsis es tradueix en una reducció d'espines dendrítiques i dendrites a les neurones piramidals de l'escorça i de l'hipocamp. A més també**

**s'ha descrit una reducció de l'expressió de proteïnes sinàptiques en aquestes regions (Clinton et al., 1994).**

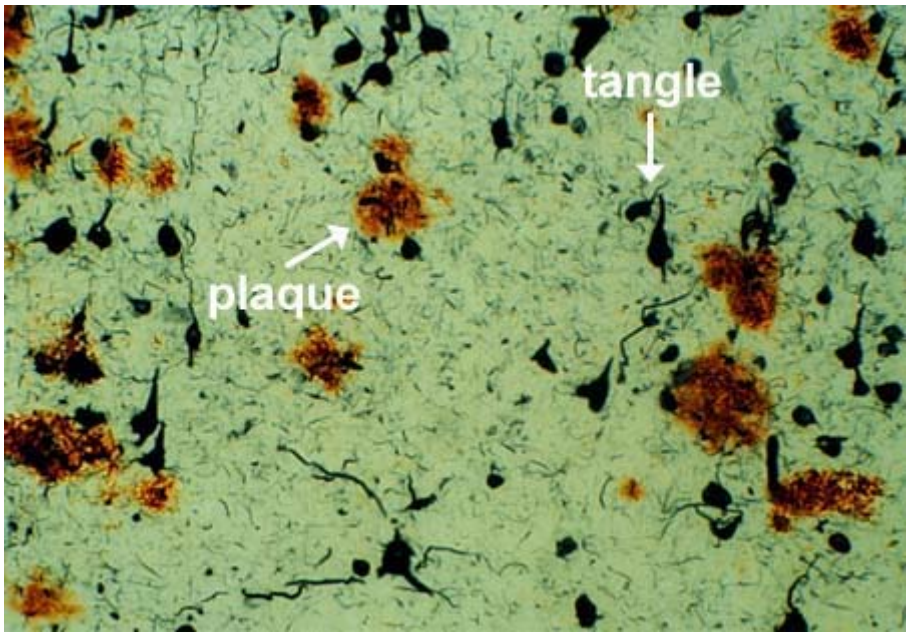
| TAUPATIA | CLÍNICA | ZONES PRINCIPALMENT AFECTADES | TIPUS D'INCLUSIONS |
|---|---|---|---|
| *Malaltia d'Alzheimer* | Dèficit cognitiu amb pèrdua de memòria, d'orientació, atenció, llenguatge, funció visual-espacial i disminució de la capacitat intel•lectual. Canvis de la pesonalitat. | Còrtex entorrinal, hipocamp, amígdala i progressió cap a la major part del còrtex. | Cabdells neurofibril•lars<br>Neurites distròfiques amb filaments de tau<br>Plaques senils |
| *Paràlisi Supranuclear Progressiva* | Parquinsonisme, inestabilitat postural, oftalmoplègia supranuclear (dificultat dels pacients per moure els ulls) i demència. | Algunes zones dels ganglis basals, el cerebel i el còrtex cerebral. | Cabdells neurofibril•lars<br>Neurites distròfiques amb filaments de tau<br>Filaments de tau a astròcits |
| *Malaltia dels Grans Argiròfils (o argirofílics)* | Canvis de personalitat, agressivitat, conducta social inapropiada, dèficit cognitiu i demència. | Àrees transentorrinal i entorrinal. Còrtex temporal. | Grans argiròfils<br>Inclusions de tau a oligodendròcits |
| *Malaltia de Pick* | Canvis de comportament i en la funció del llenguatge, que pot evolucionar fins al mutisme.<br>Problemes en distingir expressions facials.<br>Afectacions visuals. | Còrtex frontal-temporal | Cossos de Pick<br>Alguns cabdells neurofibril•lars |
| *Demència corticobasal* | Rigidesa, distònia i moviments mioclònics, membre fantasma. Demència amb canvis de personalitat, desordre de la conducta i problemes d'atenció. | Còrtex cerebral i mesencèfal. | Cossos corticobasals<br>Neurites distròfiques amb filaments de tau<br>Plaques de tau en astròcits<br>Inclusions de tau a oligodendròcits |
| *Demència frontotemporal amb parkinsonisme associada al cromosoma 17* | Canvis en el comportament i la personalitat (desinhibició, apatia, comportament compulsiu, hiper-religiositat, pèrdua d'higiene personal i altres).<br>Dèficit cognitiu.<br>Afectacions motores (parquinsonisme). | Còrtex, alguns nuclis subcorticals, substància blanca i medul•la espinal | *Depèn de la mutació que presenti el gen de la tau es poden trobar:*<br>Cabdells neurofibril•lars<br>Cossos de Pick<br>Inclusions de tau en oligodendròcits<br>Plaques de tau en astròcits |

**Taula 1.** Les pricipals taupaties i les seves característiques.

**(La demència. Omnis Cellula 13. Març 2007. Santpere G)**

**Microscòpicament, aquesta malaltia es defineix per la presència de dos tipus d'agregats proteïcs: els agregats de proteïna tau en cabdells neurofibril·lars, pre-cabdells neurofibril·lars, filaments del neuròpil i neurites distròfiques (sobretot en neurones, les cèl·lules glials es troben molt poc**

**afectades a la MA); i els agregats de pèptid amiloide extracel·lulars que s'anomenen plaques d'amiloide.**




**Plaques amiloides (plaque) i cabdells neurofibril·lars (tangle)**

***1.1.1.1 Inclusions de tau***

**La proteïna tau és una MAP o proteïna associada als microtúbuls. La seva funció fisiològica més ben caracteritzada és la d'ajudar a estabilitzar i polimeritzar els microtúbuls de tubulina. De la proteïna tau se'n parla més detalladament a l'apartat 3.**

**Els cabdells neurofibril·lars (tangles) són una estructura formada majoritàriament per tau hiperfosforil·lada que té forma de llàgrima i ocupa un gran espai dins de la cèl·lula. En aquestes estructures la proteïna tau es troba formant fibril·les. De vegades es poden observar cabdells neurofibril·lars que no es troben dins de cèl·lules. Això és degut a que la cèl·lula ha mort i el cabdell "orfe" s'anomena cabdell fantasma (o ghost tangle) (Uchihara et al., 2001).**

**Els pre-cabdells neurofibril·lars (pre-tangles) on hi ha depòsits de tau però no forma fibril·les i correspon a un estadi primerenc en l'evolució del tangle (Uchihara et al., 2001).**

**Els filaments del neuropil són acúmuls de tau en forma de fibril·les que es dipositen en feixos que es poden trobar a la dendrita apical de les neurones piramidals (Braak et al., 1986).**

**Les neurites distròfiques es formen per l'acumulació de tau hiperfosforil·lada als processos neuronals que es troben al voltant de les plaques senils. Aquests agregats formen uns engruiximents anòmals de les neurites (Dickson et al., 1999).**

***1.1.1.2 Les plaques d'amiloide***

**Les plaques d'amiloide estan formades per l'acumulació extracel·lular d'amiloide cerebral. En general les estructures amiloidees estan formades per dos components comuns a tots els amiloides que són els component AP (part proteica) i els mucopolisacàrids; i per un tercer component que és específic de cada amiloide. En aquest cas el component específic és el pèptid amiloide derivat de la proteòlis de la proteïna precursora del pèptid amiloide (APP). Aquesta proteïna es troba a la membrana, conté més de 700 aminoàcids i té una funció poc coneguda. La proteòlisi de l'APP es pot produir per l'acció de tres enzims (o conjunt de proteïnes) anomenats α, β i γ-secretases. Si tallen l' α i el γ, es produeix un pèptid no amiloidogènic; per contra, si tallen la combinació β i γ-secretases s'allibera el pèptid amiloide. Els pèptids amiloides alliberats que es troben a les plaques són l'1-40 i l'1-42, en relació al nombre d'aminoàcids que presenten.**

**Existeixen dos tipus de plaques amiloides en funció del seu grau de compactació que a la vegada depèn del grau en què el pèptid amiloide està formant fibril·les (Ikeda et al., 1989). Les plaques senils són les més compactes i tenen una mida d'unes 100 a 200 micres de diàmetre. Estan formades per una part central enriquida en pèptid 1-40 envoltada d'una capa més difosa de pèptid 1-42. Aquestes plaques són les que es troben envoltades de neurites distròfiques i de cèl·lules glials. Les plaques difoses en canvi no presenten neurites distròfiques ni cèl·lules glials al seu voltant i són molt més variables en forma i tamany (de 20 a 1000 micres de diàmetre) i també apareixen de forma més desdibuixada. No tenen un nucli central amb acumulació de pèptid 1-40 sino que estan constituïdes bàsicament de pèptid 1-42 (Wisniewski et al., 1989).**

**També es pot identificar un altre tipus de placa, anomenada placa de tipus A, que no presenta neurites distròfiques però si cèl·lules glials associades i tenen un tamany de 100 a 200 micres. S'ha proposat que la formació de les plaques es produeix passant per aquests estadis (Ikeda et al., 1989).**

### *1.1.1.3 Altres aspectes microscòpics*

**Els cossos d'Hirano**

**Són uns agregats proteics que tenen forma d'espina de peix que es troben en el citoplasma de neurones piramidals de l'hipocamp. Estan constituïts per filaments de 10nm de diametre i contenen sobretot actina i proteïnes associades a l'actina; però també s'hi pot trobar tau, neurofilaments i fragments C-terminals de l'APP.**

**Angiopatia amiloidea cerebral (AAC)**

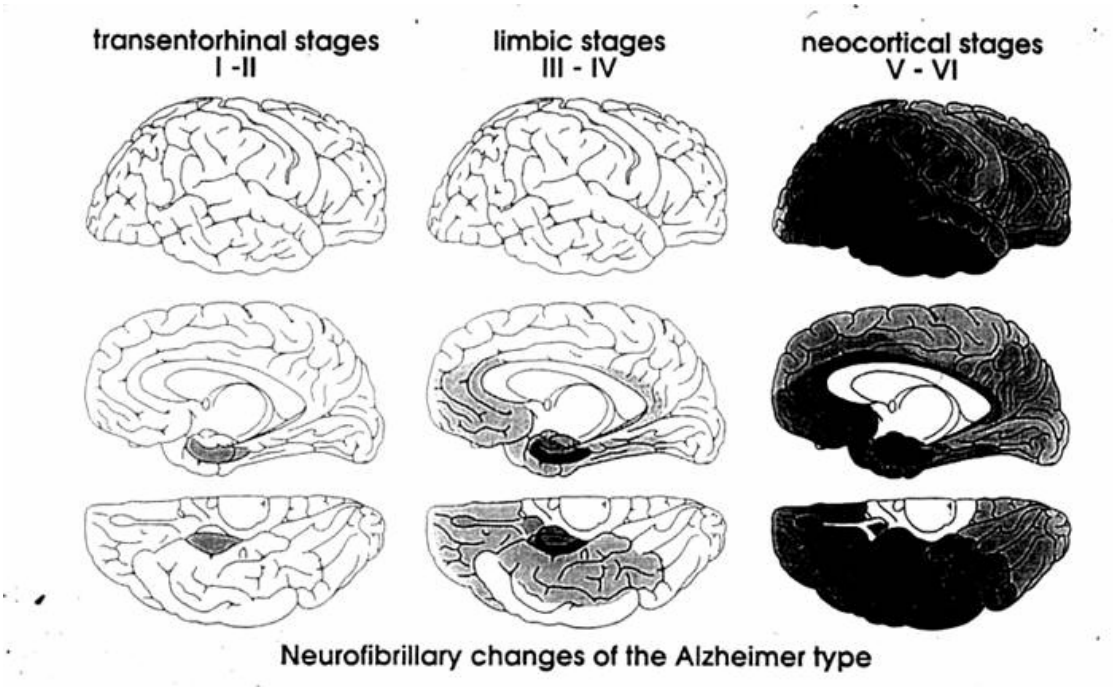
**L'angiopatia amiloidea és una malaltia que es pot trobar per si sola però la considererem en aquest subapartat de la malaltia d'Alzheimer perquè és molt freqüent trobar-la en associació amb aquesta (en un 80% dels casos de MA).**

**L'angiopatia amiloidea afecta als vasos sanguinis que irriguen tant les meninges com l'escorça cerebral i el cerebel. El pèptid amiloide (sobretot l'1-40) s'acumula a les parets dels vasos i pot extendre's pel parènquima formant plaques. Aquesta darrera forma amb plaques s'anomen angiopatia dishòrica i pot afectar la funció de la barrera hemato-encefàlica (Vinters, 1987).**

### *1.1.1.4 Estadis de la malaltia d'Alzheimer*

**Està acceptat que la malaltia d'Alzheimer progressa de forma predictible pel què fa al nombre creixent de zones per on s'extén la patologia relacionada amb els agregats de tau. La càrrega de tau és el caràcter més útil per establir un estadiatge neuropatològic que tingui una correspondència amb l'estadiatge clínic. L'amiloide no serveix en aquest sentit, perquè es distribueix de manera més caòtica essent possible trobar plaques a l'escorça quan el pacient encara no presenta cap aspecte clínic de la malaltia. El criteri utilitzat (de Braak i Braak)**

**correspon a la distribució dels agregats de tau i diferencia 6 estadis de la malaltia (Braak and Braak, 1991).**




**Estadiatge de la MA de Braak i Braak**

**Classificació de Braak i Braak:**

- **I- Escorça entorrinal**
- **II- Escorça entorrinal més afectada**
- **III- Hipocamp, zones límbiques i major afectació escorça entorrinal**
- **IV- Augmenta el nombre de lesions a les regions anteriors**
- **V- Neoescorça**
- **VI- Neoescorça més afectada**

**Els estadis I i II són pre-clínics, assimptomàtics, i la clínica es comença a veure en l'estadi III amb un discret deteriorament cognitiu. La demència apareix a partir de l'estadi V (Braak and Braak, 1991).**

### *1.1.1.5 Hipòtesis proposades per explicar la MA*

**1) La cascada amiloide**

**Segons aquesta hipòtesi, l'amiloide es situa al principi de la seqüència d'esdeveniments patològics i causa en tots els casos de MA la subsegüent aparició de degeneració neurofibril·lar, mort neuronal i al final, i a conseqüència dels processos anteriors, la demència.**

**Existeix un petit percentatge de malalts d'Alzheimer que ho són per causes hereditàries. Aquests casos d'Alzheimer familiar estan al voltant d'un 5% dels casos de MA. S'han trobat diverses i nombroses mutacions a tres gens: el gen que codifica l'APP, la presinilina-1 (PSEN1) i la presinilina-2 (PSEN2) (Goate et al., 1991, Sherrington et al., 1995). Les tres proteïnes estan involcucrades en la producció de pèptid amiloide; les PSEN col·laboren en la funció gamma-secretasa. Les mutacions a les presinilines són les més nombroses (més de 160 mutacions identificades). L'efecte d'aquestes mutacions és una alteració de la funció de tall de la presinilina que dona lloc a un augment dels nivells d'amiloide 42 respecte de l'amiloide 40, afavorint la formació de plaques (Bertram and Tanzi, 2005).**

**Les mutacions en el gen de l'APP afavoreixen l'aparició de les plaques. Un augment de la dosi gènica d'APP també pot comportar la malaltia, i s'associa amb les duplicacions gèniques o amb la Síndrome de Down. La trisomia del cromosoma 21, on està localitzat el gen de l'APP, comporta als malalts l'aparició de plaques amiloides i cabdells neurofibril·lars en els seus cervells després dels 40 o 50 anys (Selkoe, 1991).**

**Les formes familiars d'Alzheimer únicament es poden diferenciar de la forma esporàdica per l'edat d'aparició. Les formes familiars poden manifestar-se abans dels 60 anys. La resta d'aspectes clínics i neuropatològics són indistingibles. I aquest fet és l'argument clau de la hipòtesi de la cascada amiloide (Selkoe, 1991).**

**Es proposa que la placa en si mateixa no está implicada en la funció tòxica, però que és font de substàncies amb aquesta capacitat, per exemple és font de radicals lliures (McLellan et al., 2003) i d'amiloide sol·luble (Selkoe, 1991). Cada vegada es tenen més dades que apunten a la major capacitat patogènica de l'amiloide oligomèric o lliure per sobre de les plaques ja formades. Les formes solubles lliures de l'amiloide són les que exercirien la toxicitat mitjançant mecanismes encara desconeguts (Selkoe, 1991). Entre els mecanismes de toxicitat de l'amiloide soluble proposats hi ha l'estrés oxidatiu (que s'analitza en l'apartat 5) o les interaccions anòmales del pèptid amb lípids de membrana que poden afectar la seva integritat o estabilitat. Recolzant la aquesta hipòtesi, existeix el fet que la immunització passiva contra l'amiloide en ratolins transgènics és capaç de millorar el seu comportament abans de l'aparició de les primeres plaques (Kotilinek et al., 2002). El pèptid amiloide es produeix de manera normal en els individus sans i és possible detectar-lo en el liquíd cefalo-raquidi. El què es proposa és que un increment cronificat de la seva producció (com en el cas de la síndrome de Down o per determinades mutacions) o una reducció de la seva eliminació és el què acaba precipitant i formant les plaques a més d'exercir la seva funció tòxica (Selkoe, 1991, Seubert et al., 1992). La forma en què es troba en els cervells de MA és majoritàriament la de fragments N-terminals de l'amiloide 42, en concret la 3-42 en comparació a la predominant 1-42 del cervell de la gent gran sense clínica de MA (Piccini et al., 2005).**

**Les plaques senils, però, no són inoqües. A nivell morfològic s'observa que les plaques poden modificar l'entorn. Al voltant de les plaques s'hi troben les neurites distròfiques, que a més de tau hiperfosforil·lada contenen també una gran quantitat de mitocondris i de vacuoles autofàgiques (Perez-Gracia et al., 2008). Es produeix també un fenòmen d'atracció de dendrites, espines i cons de creixament que donen lloc a neurites de formes i engruximents aberrants (Knafo et al., 2009). Aquest fet pot tenir relació amb l'acúmul de factors de creixement (com el Nerve Growth Factor (NGF) o l'Epidermal Growth Factor (EGF)) a l'interior de les plaques. Aquestes malformacions en les projeccions neuronals poden perjudicar directament les projeccions cortico-corticals (D'Amore et al., 2003, Delatour et al., 2004).**

**2) Canvis en la proteïna tau**

**Una segona postura resitua la proteïna tau a l'origen de la malaltia. Principalment els arguments més forts són dos: la forta correlació entre dipòsits de tau i clínica en la MA (Gomez-Isla et al., 1997), i l'existència d'un gran nombre de mutacions al gen de la tau que comporten l'aparició del grup de malalties anomenades Demències Frontotemporals amb Parkinsonismes lligades al cromosoma 17 (DFTP17) (Lynch et al., 1994). A més, recentment s'ha vist que mutacions a la PSEN1 en pacients diagnosticats de demència fronto-temporal mostren inclusions de tau i absència d'acúmuls d'amiloide. Mutacions de PSEN1 també poden accelerar la formació de cabdells neurofibril·lars sense afectar el ritme de deposició de l'amiloide (Tanemura et al., 2006).**

**A la MA s'observa que la tau hiperfosforil·lada es transloca de l'axó, on ha de realitzar la seva funció fisiològica normal, al compartiment somatodendrític (Konzack et al., 2007). Aquesta pèrdua de funció comporta una deficiència en el transport axonal i conseqüentment, a la degeneració neuronal. Per altra banda es discuteix també sobre el poder patogènic d'un possible guany de funció tòxica o bé del propi agregat de tau, o bé de la tau lliure o oligomèrica dins de la cèl·lula. Es parla amb més detall d'aquests aspectes a l'apartat 3.**

**Altres perspectives per explicar la MA**

**1) Vulnerabilitat selectiva de diferents tipus neuronals**

**La degeneració neurofibril·lar afecta a les neurones de projecció (d'axons llargs) en primera instància i no afecta fins cap a etapes finals les neurones d'axons curts, com les piramidals de les capes II i IV de la neo-escorça (Hyman and Gomez-Isla, 1994). La manera en què progressa la distribució de la patologia, com hem vist en els criteris de Braak i Braak, sembla anar a la inversa del gradent de mielinització que es dona en el cervell (Braak et al., 2000). És a dir, les primeres zones que es veuen afectades estan molt poc mielinitzades i les darreres ho estan més. Les zones més noves evolutivament parlant són més immadures pel què fa a la seva mielinització i són les primeres zones de la neo-escorça en veure's afectades: àrees d'associació d'ordre superior i àrea pre-**

**frontal, entre d'altres. Les àrees motores o sensorials primàries, més antigues, presenten un grau de mielinització major i queden afectades molt al final de la malaltia. La idea proposada és que les neurones no mielinitzades són més sensibles i estan més desprotegides a efectes de la malaltia com l'estrés oxidatiu (Braak et al., 2000).**

**2) Increment de la càrrega de neuro-plasticitat**

**S'ha observat que els factors de risc i les mutacions causatives de la MA afecten d'alguna manera la capacitat plàstica del cervell. La primera resposta del cervell a aquesta sobrecàrrega de les necessitats plàstiques inclouria un augment de fosforil·lació de tau i un augment del recanvi de l'APP, produint al final, després d'una perturbació prolongada de la plasticitat, els dos tipus d'agregats que definieixen la malaltia (Mesulam, 2000).**

**La plasticitat neuronal inclou processos com la ramificació de dendrites, la formació d'espines, el remodelament sinàptic, les potenciacions a llarg termini (LTP), l'extensió dendrítica i axonal, la sinaptogènesi i la neurogènesi. Tots aquests processos són més presents a les estructures límbiques que inclouen l'hipocamp, l'amígdala i l'escorça entorrinal, regions on comença a aparèixer la degeneració neurofibril·lar (Mesulam, 1999).**

**Trobem doncs que L'alfa-APP soluble, el fragment gran de l'APP que es desprèn del tall de la alfa-secretasa, té propietats que promouen la neuroplasticitat (sinaptogènesi i LTPs), mentre que el pèptid amiloide inhibeix l'extensió axonal i els LTPs (Roch et al., 1994, Ishida et al., 1997). Així mateix, les mutacions a les presinilines també poden tenir un efecte sobre la plasticitat interferint en la funció normal d'aquestes o augmentant el tall de l'APP (Furukawa et al., 1998, Mesulam, 2000).**

**Entre els factors de risc que poden afectar la capacitat plàstica del cervell trobem: La deficiència d'estròg ens, donats el efectes protectors que confereixen, ja que promouen la plasticitat dendrítica en les neurones límbiques (Ferreira and Caceres, 1991). L'edat, el principal factor de risc de la MA, també està relacionada amb una pèrdua de neuroplasticitat (Mori, 1993). I finalment la presència de l'al·lel E4, de la proteïna ApoE, el més ben establert factor de risc**

**genètic de la MA (Strittmatter et al., 1993). La proteïna ApoE té una funció de transport i recaptació de colesterol també involucrada en creixement axonal i sinaptogènesi,. La presència de l'al·lel E4 redueix l'edat d'aparició de la malaltia i aquest efecte depèn de la dosi gènica (més efecte en homozigosi i menys en heterozigosi). Contràriament a altres al·lels com l'E3, l'E4 inhibeix el creixement de neurites i la plasticitat dendrítica (Nathan et al., 1994).**

**2) Cascada d'activació de proteases**

**Es centra en l'activació primerenca i progressiva de sistemes proteolítics com l'endosomal-lisosomal o el calpaïna-calpastatina. La idea es basa en la proposta que tots els factors genètics i ambiental de la MA acaben activant o alterant aquests sistemes proteolítics. S'ha observat que cathepsines i calpaïnes poden directament o indirecta promoure l'acumulació del beta-amiloide, la degeneració neurofibril·lar i la neurodegeneració (Nixon, 2000).**

**3) Desregulació de la homeostasi del colesterol. Hipòtesis metabòlica/transducció de senyal.**

**Aquesta teoria proposa que una reducció de la fluidesa de les membranes amb l'edat les podria fer més susceptibles als insults metabòlics o ambientals. Molts receptors importants es troben concentrats en els microdominis de membrana (rafts lipídics). Aquests microdominis estan molt enriquits en esfingomielina i colesterol i s'han proposat com a plataformes de transducció de senyal. Alteracions en la recaptació i/o metabolisme del colesterol podria afectar el tràfic de proteïnes de membrana causant pèrdues funcionals a la neurona i problemes de plasticitat sinàptica. La idea es sostenta bàsicament en la funció de l'APP i l'ApoE en relació al moviment de LDLs i la recaptació de colesterol (Lynch and Mobley, 2000). L'al·lel E4 de l'ApoE sembla menys apte que els altres al·lels (E3 i E2) en l'obtenció de colesterol lliure. Per altra banda, el pèptid amiloide es podria unir a l'ApoE i disminuir la recaptació de colesterol (Nathan et al., 1994, Lynch and Mobley, 2000). Aquesta teoria no dóna a la tau un paper rellevant, tot i que com es parlarà més endavant, s'ha proposat que la tau també pugui interactuar amb la membrana i tenir un paper en la transducció de senyal.**

**4) Defectes mitocondrials**

**Aquesta hipòtesis situa els mitocondris al centre d'un espiral que es retroalimenta com a causa de la neurodegeneració. Els aspectes que es veuen alterats quan es malmeten els mitocondris són el metabolisme energètic, l'estrés oxididatiu (producció de ROS) i l'homeostasi del Ca++ (Blass, 2000).**

**Altres taupaties:**

### *1.1.2 La paràlisi supranuclear progressiva (PSP)*

**La PSP és una taupatia infreqüent (amb una prevalència d'entre 3 i 6 casos per cada 100.000 habitants amb edat de tenir la malaltia). Les manifestacions clíniques inclouen desordres del moviment com bradiquinèsia, inestabilitat postural amb freqüents caigudes cap enrera, parkinsonisme i paràlisi supranuclear de la vista. Aquests desordres motors poden anar seguits, en estadis més avançats, de demència.**

**Macroscòpicament, els cervells de malalts de PSP presenten cert nivell d'atròfia en algunes regions com el tàlam i nuclis subtalàmics, i una decoloració de la substància negra, però no són canvis específics de la malaltia (Verny et al., 1996). A nivell microscòpic la PSP presenta pèrdua neuronal sobretot en nuclis del tronc de l'encèfal i diencèfal (com el globus pallidus, estriat o els nuclis subtalàmics); però també pot afectar l'escorça cerebral (Dickson, 1999). En aquestes regions, a més de pèrdua neuronal, s'observen inclusions de tau hiperfosforil·lada tant en neurones com en cèl·lules glials (Dickson, 1999). A les neurones s'hi troben cabdells neurofibril·lars, pre-cabdells o filaments del neuropil com a la MA. Els oligodendròcits poden presentar unes inclusions de tau anomenades *coiled bodies*. I als astrocits s'hi poden observar unes inclusions al citoplasma que donen lloc a diferents morfologies (Dickson, 1999).**

**Probablement per la seva raresa no es coneix gaire bé la manera en què la PSP es desenvolupa i no s'han definit estadiatges com els de Braak i Braak per la MA. La major evidència de la regió del cervell on s'origina la malaltia prové de l'estudi dels únics pacients que han desenvolupat una forma familiar de PSP, dels quals encara no se n'ha trobat el gen causant. Tal com s'observa per**

**tècniques de neuroimatge com el PET, sembla que aquesta forma autosòmica dominant de la malaltia provoca primer de tot alteracions a l'estriat (caudat/putamen) seguides d'alteracions al globus pallidus i als nuclis subtalàmics (de Yebenes et al., 1995, Piccini et al., 2001, Ros et al., 2005).**

### *1.1.3 Malaltia dels grans argiròfils (MGA)*

**La MGA és una malaltia esporàdica que està darrera d'aproximadament un 5% dels casos de demència. La malaltia a més pot donar lloc a alteracions del comportament, canvis de la personalitat, desestabilització de l'estat d'ànim, així com certa amnèsia, irritabilitat i agitació. El diagnòstic precís de la MGA ha de realitzar-se postmortem, pel fet que la clínica és força indistingible de la dels malats de MA.**

**Macroscòpicament, els cervells de malalts de MGA no presenten canvis gaire vistosos, només una lleugera atròfia fronto-temporal (Braak and Braak, 1998, Tolnay et al., 2001). Neuropatològicament la MGA es defineix per la presència d'acúmuls de tau hiperfosforil·lada a les dendrítes de les neurones que tenen forma de grans i s'anomènen grans argiròfils (perquè es poden tenyir amb tècniques de plata). A més dels grans també es poden trobar pre-cabdells neurofibril·lars en neurones, *coiled bodies* en oligodendròcits, i dipòsits de tau granulars en astròcits (menys compactes que a la PSP) (Tolnay et al., 2001).**

**Les zones que es veuen més afectades són aproximadament les mateixes que en els primers estadis de la MA: escorça entorrinal, hipocamp, amígdala i l'escorça temporal propera. Per això la clínica de la MGA pot confondre's amb la dels primers estadis de la MA.**

**La MGA es troba molt sovint en associació amb altres taupaties, com la MA, PSP o DCB; i també amb sinucleïnopaties com la MP i la DCL (Seno et al., 2000, Togo and Dickson, 2002). L'elevada co-ocurrència ha fet que alguns autors proposin que la MGA no és una malaltia independent (Martinez-Lage and Munoz, 1997). La presència de grans argiròfils al cervell sembla força freqüent en edats avançades (fins un 43%, en una de les sèries descrites) (Saito et al., 2002). Sovint la co-presència de MGA i MA s'ha vist infraestimada pel fet que amb determinades tècniques de tinció d'inclusions de tau, la major presència**

**d'estructures en la MA emmascara la presència dels grans argiròfils, molt més petits. Amb l'ajuda d'anticossos específics contra el tipus de tau que s'acumula en els grans (veure apartat de tau), o amb anticossos que detecten proteïnes associades als grans (com la p62 o la ubiquitina) s'ha vist que la freqüència de MGA en AD és major (Fujino et al., 2005, Scott and Lowe, 2007).**

**Molt recentment s'ha identificat una mutació en el gen de la tau, la S305I, que és capaç desenvolupar una neuropatologia semblant a la de MGA, amb presència de grans argiròfils (Kovacs et al., 2008). Aquest és l'únic cas que es podria comparar a una forma familiar de la malaltia, encara que es considera dins el grup de les DFTP-17.**

**La progressió de la malaltia ha estat estudiada i s'han fet algunes propostes d'estadiatge. Aquests descriuen la progressió de les zones afectades des de la part anterior de l'hipocamp cap a la posterior (Saito et al., 2004).**

### *1.1.4 La malaltia de Pick (MPi)*

**La MPi és una taupatia rara que es classifica clínicament dins del gran grup de demències fronto-temporals (DFT). La prevalència d'aquesta malaltia no es coneix amb seguretat degut al solapament clínic amb altres tipus de demència fronto-temporal (Neary et al., 1998). Aquesta malaltia comporta un deteriorament progressiu de la personalitat, del comportament, i també del llengutage. Entre els problemes comportamentals més evidents es troba la desinhibició social (Neary et al., 1998).**

**Macroscopicament, el cervell dels malalts de MPi pot presentar un elevat grau d'atròfia dels lòbuls frontals i temporals, que es pot extendre pels lòbuls parietals. Mentre que l'atròfia i mort neuronal pot afectar estructures límbiques, l'hipocamp es troba ben preservat (Dickson, 1998).**

**A nivell microscòpic s'observen els anomenats cossos de Pick, dipòsits esfèrics a l'interior de les neurones de proteïna tau hiperfosforil·lada. Aquestes inclusions s'observen majoritàriament a l'hipocamp (gir dentat, CA1) i en capes superiors de l'escorça entorrinal i la neoescorça. Un aspecte particularment**

**interessant de la malaltia és que al gir dentat no s'observa mort neuronal, mentre que gairebé totes les neurones presenten cossos de Pick a l'interior (Dickson, 1998).**

**A la MPi també es poden trobar alguns cabdells nerurofibril·lars en àrees límbiques o a la neoescorça, però en capes diferents de les que es troben a la MA. Els oligodendròcits presenten un variable nombre de *coiled bodies* a la substància blanca i els astròcits també presenten inclusions, que són diferents de les que s'observen a la PSP. Un caràcter important a assenyalar, és un elevat grau de gliosi per l'escorça i a la substància blanca de sota, així com en altres zones afectades com el nucli caudat (Komori, 1999).**

### *1.2 Alfa-sinucleïnopaties*

**Les alfa-sinucleïnopaties són malalties neurodegeneratives que comparteixen la presència d'inclusions de la proteïna alpha-sinucleïna. Aquesta proteïna és un membre de la família de les sinucleïnes i presenta 140 aminoàcids (Jakes et al., 1994). S'expressa per totes les regions del cervell i es troba particularment enriquida en terminals sinàptics. La seva funció dins la cèl·lula no es coneix, però se la ralaciona amb el transport de vesícules sinàptiques i amb la funció de xaperona (Clayton and George, 1999).**

**La malaltia de Parkinson, la demència amb cossos de Lewy i l'atròfia multisistèmica són alpha-sinucleïnopaties. Les dues primeres fa temps que es proposen per diversos autors com estadis diferents d'una mateixa malaltia (Jellinger, 2008). De manera que la malaltia de Parkinson, que afecta sobretot el mesencèfal, en progressar i extendre's per l'escorça cerebral esdevindria la demència amb cossos de Lewy (Braak et al., 2002, Braak et al., 2003). Els agregats de sinucleïna que presenten les neurones tant la MP com la DCL són iguals, mentre que de l'atròfia multisistèmica presenta un altre tipus d'agregats al citoplasma de cèl·lules oligodendroglials (Papp et al., 1989).**

### *1.2.1 La malaltia de Parkinson (MP)*

**La MP és la malaltia neurodegenerativa més prevalent entre la gent gran després de la MA. Clínicament es pot diagnosticar de manera força específica però pel fet que hi ha altres malalties que poden donar parkinsonisme el diagnòstic definitiu s'ha de donar postmortem. La clínica de la malaltia cursa amb tremolors, inestabilitat de la postura, acinèsia i rigidesa (Forno, 1996).**

**A nivell macroscòpic s'observa poc més que una depigmentació de la substantia nigra i del locus ceruleus. Microscòpicament, hi ha una elevada mortalitat de neurones dopaminèrgiques a la substantia nigra pars compacta, i es defineix la malaltia com a Parkinson quan la mortalitat en aquesta zona arriba al 60%. A més de la pèrdua neuronal en aquestes regions es troba una elveda gliosi i les inclusions d'alfa-sinucleïna al citoplasma d'algunes neurones que s'anomenen cossos de Lewy; i també a les neurites, que reben el nom de neurites de Lewy (Forno, 1996, Goedert, 2001, Shults, 2006).**

**Diferents mutacions a un nombre considerable de gens poden donar lloc a formes familiars de MP. Trobem mutacions a la pròpia alfa-sinucleïna, però també al gens de UCHL-1 (ubiquitin carboxi-terminal hydrolase L1), Parkin, DJ-1, PINK-1 (PTEN-induced putative kinase), ATP13A2 (p-type ATPase), HTRA2 (HtrA serine peptidase 2) i LRRK2. També s'han identificat mutacions en altres loci (PARK3, 10, 11, i 12) tot i que el gen concret encara no es coneix (Tan and Skipper, 2007).**

### *1.2.2 La demència amb cossos de Lewy (DCL)*

**És una malaltia que comporta una demència progressiva (la forma més comuna de demència després de la MA) a més dels fenòmens neurològics associats a la MP. Les manifestacions clíniques més comunes impliquen els dèficits d'atenció, alucinacións visuals i parquinsonisme (Weisman and McKeith, 2007). Macroscòpicament, la malaltia no presenta atròfia cerebral significativa, però si pot presentar de forma variable una pal·lidesa de la substància negra i del locus ceruleus (Weisman and McKeith, 2007) Els aspetes microscòpics són els mateixos que a la MP però extesos per l'escorça cerebral: cossos de Lewy i**

**neurites de Lewy. Sovint es troba associada a la MA donant lloc a la DCL forma comuna (un 80% dels casos). Quan no hi ha carácters de tipus MA s'anomena DCL forma pura (el 20% restant dels casos) (Weisman and McKeith, 2007).**

**Mutacions en el gen de la sinucleïna poden donar com a resultat la DCL (Bonifati, 2008). És un argument més per considerar aquestes dues malalties (DCL i MP) com a parts d'un mateix espectre.**

## 2- L'ESTUDI DE PROTEÏNES, I MODIFICACIONS POST-TRADUCCIONALS ASSOCIADES, EN TEIXIT CEREBRAL HUMÀ CONGELAT

**Els estudis bioquímics que es fan sobre malalties neurodegeneratives tenen major valor si es fan sobre el substrat real, el cervell humà, que sobre models experimentals. Però existeixen una sèrie de factors que fan que treballar amb mostres humanes pugui ser complicat pel fet que la mostra pot no estar òptimament conservada. És prioritari establir les condicions de preservació òptimes de les mostres per tal d'evitar la degradació o la modificació de les proteïnes, dels lípids i dels àcids nucleics. Treballar amb mostres subòptimament preservades pot portar a resultats alterats i a conclusions errònies. També és necessari establir quins efectes reals tenen els diferents factors que poden convertir una mostra en subòptima sobre els diferents elements d'aquesta.**

**Els factors més rellevants que poden influir en la preservació del teixit cerebral humà, tant abans de la mort com després, són:**

**<u>Premortem</u>. L'estat metabòlic, drogues i substàncies tòxiques, infeccions i hipòxies (un estat d'hipòxia prolongat o l'acidosi metabòlica poden alterar el pH del teixit). La duració prolongada del periode agònic incrementa l'efecte d'aquests factors.**

**<u>Postmortem</u>. Els dos factors principals que intervenen després de la mort són la rapidesa en què s'extreu el cervell, es processa i es congela, per una banda, i la**

**temperatura a què ha estat el cervell fins la congelació, per l'altra (Buesa et al., 2004).**

**D'especial interès per la feina que aquí es presenta és el que fa referència a la preservació de les proteïnes i de les seves modificacions post-traduccionals, en especial la fosforil·lació. Els estudis que s'han fet en aquest sentit fins ara permeten arribar a una conclusió: les proteïnes són sensibles al postmortem, però ho són de manera diferent. Algunes són més sensibles a la temperatura, altres ho són més al temps entre la mort i la congelació, i altres resisteixen els dos factors i són estables.**

**La majoria d'estudis s'han realitzat en proteïnes concretes i de manera secundària, i molts d'aquests s'han portat a terme sobre teixit animal, de rata o ratolí (Schwab et al., 1994, Li et al., 1996, Siew et al., 2004). Són necessaris estudis metodològics generals en cervell humà que puguin servir de referència per la manipulació d'aquestes mostres per tal de què s'optimitzin al màxim per la recerca i que siguin aplicables als diferents bancs de teixits.**

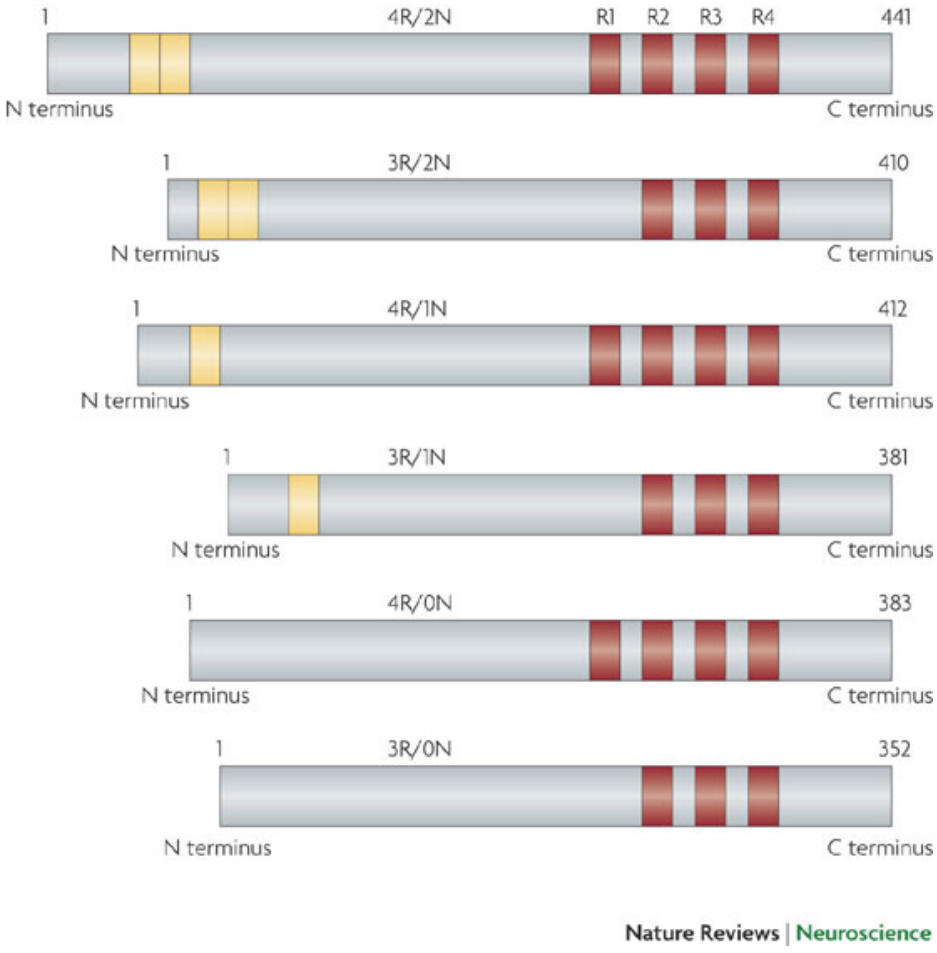
## 3- LA PROTEÏNA TAU

**Als anys 80 es va identificar la proteïna tau com el principal component dels filaments aparellats hel·licoidalment (paired helical filaments, PHF), que formen els cabdells neurofibril·lars i les neurites distròfiques en la malaltia d'Alzheimer (Weingarten et al. 1975), així com les inclusions pròpies de les altres taupaties.**

**La tau és una de les MAP o proteïnes associades a microtúbuls (Microtubule associated protein). La seva funció és la d'ajudar a estabilitzar i polimeritzar el microtúbuls (Weingarten et al., 1975), per la qual cosa està dotada de tres o quatre dominis repetits d'unió a la tubulina.**

**En el cervell adult s'expressen sis isoformes de tau generades a partir de l'empalmament alternatiu d'un gen situat al cromosoma 17q21. Les sis isoformes**

**es diferencien per la presència o absència dels exons 2, 3 i 10. La nomenclatura pels exons N-terminals és 0N, 1N i 2N en funció del nombre d'exons presents. L'exó 10 és funcionalment important perquè conté un dels quatre dominis d'unió a la tubulina. Les tres isoformes amb l'exó 10 s'anomenen 4R i les altres tres, sense l'exó 10, 3R. En etapes fetals, només s'expressa la isoforma més petita (0N3R) (Lee et al., 1988).**




**Les sis isoformes de tau que s'expressen al cervell adult (Ballatore et al., 2007)**

**A més dels dominis d'unió a la tubulina, situats a la part mitja de l'extrem C-terminal, la tau presenta una regió N-terminal anomenda domini de projecció. S'ha proposat que aquest domini pot interactuar amb la membrana i conferir a la tau altres funcions més enllà de la que se li atribueix actualment (Lee et al., 2004).**

**La unió de la tau al microtúbul està controlada per un balanç de fosforil·lació i desfosforil·lació de múltiples epítops (Serines i Treonines) en els dominis d'unió. Quan els dominis estan fosforil·lats la tau es desenganxa del microtúbul (Drechsel et al., 1992).**

### *3.1 Modificacions post-traduccionals*

#### *3.1.1 Hiperfosforil·lació*

##### *3.1.1.1 Diferents isoformes per diferents taupaties*

**En les taupaties, la tau es troba hiperfosforil·lada i forma agregats anòmals en forma de filaments. Les isoformes que s'hiperfosforilen i s'agreguen no sempre són les mateixes en totes les taupaties. En la MA ho fan totes sis, en la PSP, DCB i MGA ho fan només les 4R i en la MPi, ho fan només les 3R. En els cas de les DFTP-17, depèn de la mutació de tau que presentin; per exemple, les mutacions que dificulten l'empalmament alternatiu de l'exó 10 presenten només les isoformes 4R (Lee et al., 2001). Aquestes diferències queden ben reflectides quan s'estudia la tau hiperfosforil·lada per Western Blot: la MA presenta un patró de tres bandes a 68, 64 i 62 KDa, les taupaties amb 4R presenten només les de 68 i 64 KDa, i les 3R presenten les de 64 i 62 KDa. Sovint es pot observar també una banda a 72KDa corresponent a la isoforma més gran (Lee et al., 2001).**

##### *3.1.1.2 Cinases involucrades en la hiperfosforil·lació*

**La hiperfosforil·lació de tau afecta a més de 30 serines i treonines, i s'han identificat moltes cinases amb la capacitat de fosforil·lar la tau. Les més importants són la GSK3-beta, la Cdk5, la PKA, la CaMKII, la ERK1/2, p38 i SAPK/JNK (Ferrer et al., 2005). A més de serines i treonines, la tau és substrat de tirosina-cinases com la Fyn (Lee et al., 2004) o c-Abl (Derkinderen et al., 2005). A la taula apareixen els epítops de tau específics d'algunes d'aquestes i altres cinases.**

| Residue[a] | Foetal rat tau[b] | Adult rat tau[b] | PHF-tau[c] | Protein kinase[d] |
|---|---|---|---|---|
| Thr 175 | | | * | MAPK |
| Thr 181 | + | + | + | MAPK |
| Ser 184/185 | | | * | |
| Ser 198 | + | | + | |
| Ser 199 | + | | + | MAPK, GSK-3 |
| Ser 202 | + | + | + | MAPK, CDK5 |
| Ser 208 | | | + | |
| Ser 210 | | | + | |
| Thr 212 | | | + | MAPK, GSK-3 |
| Ser 214 | | | + | PKA, PKC |
| Thr 217 | + | | + | GSK-3 |
| Thr 231 | + | + | + | GSK-3, CDK5 |
| Ser 235 | + | | + | MAPK, GSK-3, CDK5 |
| Ser 237 | | | * | PK |
| Ser 238 | | | * | |
| Ser 262 | | + | + | GSK-3, PKA, PKC, p110K, PK |
| Ser 356 | | | * | GSK-3, PKA, p110K |
| Ser 396 | + | | + | MAPK, GSK-3 |
| Ser 400 | + | | + | GSK-3 |
| Thr 403 | | | + | |
| Ser 404 | + | + | + | MAPK, GSK-3, CDK5 |
| Ser 409 | + | | + | PKA |
| Ser 412 | | | + | |
| Ser 413 | + | | + | GSK-3 |
| Ser 422 | | | + | MAPK |

### *3.1.1.3 Fosfatases que desfosforil·len la tau*

**La desfosforil·lació de tau va a càrrec de la família de les PP (protein phosphatase). *In vitro*, s'ha observat que algunes de les PP que s'expressen en el cervell poden desfosforil·lar la tau: PP1, PP2A, PP2B i PP5 (Liu et al., 2005). *In vivo*, PP1, PP2A i PP5 són les més importants. La PP2A sembla ser la més activa desfosforil·lant tau i en la MA presenta una disminució del seu mRNA (Vogelsberg-Ragaglia et al., 2001) i, junt amb la PP1 i PP5, una disminució de la seva activitat (Sontag et al., 2004b, Liu et al., 2005). La metil·lació de la subunitat catalítica de la PP2A sembla que juga un paper important alhora de reclutar les altres dues subunitats reguladores de l'holoenzim funcional. En models tractats amb pèptid amiloide on s'observa un increment de fosforil·lació de tau, també s'observa una disminució de la metil·lació de PP2A (Sontag et al., 2004a, Zhou et al., 2008).**

### *3.1.2 Glicosil·lació*

**La tau també es troba anormalment glicosil·lada en la MA, i aquest canvi sembla precedir la hiperfosforil·lació. Sembla que un tipus concret de O-glicosil·lació està inversament relacionada amb la fosforil·lació de tau. Alguns autors consideren aquesta modificació un bon objectiu sobre el qual desenvolpar fàrmacs protectors de las desglicosil·lació (Robertson et al., 2004, Fischer, 2008).**

### *3.1.3 Proteòlisi*

#### *3.1.3.1 Fragments de tau i formació d'agregats*

**El truncatge de la tau està resultant ser un fenomen de gran importància en la seqüència d'esdeveniments que porta a la formació dels agregats patològics. Els PHF estan formats per un nucli resistent a proteases i una coberta sensible. S'ha vist mitjançant l'ús d'anticossos depenents de conformació que el nucli està format principalment per fragments de tau (Novak et al., 1991, Novak et al., 1993). Aquests anticossos tambñe posen de manifest la destrucció i creació d'epítops deguts a fosforil·lacions i talls. Així doncs, s'ha proposat un model per explicar la seqüència de modificacions que pateix la tau per arribar al trosset de proteïna que vertebra els PHF, en relació a la formació de cabdells neurofibril·lars (Guillozet-Bongaarts et al., 2005). El fragment final, després de tot el procés, correspon a una regió de la tau que conté només els dominis d'interacció amb la tubulina i l'extrem C-terminal tallat a l'àcid glutàmic 391 (Skrabana et al., 2004). Aquest model sobre observacions en MA, sembla adaptar-se bé també a la MPi (Mondragon-Rodriguez et al., 2008).**

#### *3.1.3.2 Efectes nocius de l'expressió dels fragments*

**Estudis *in vitro* demostren que determinats fragments poden desecandenar apoptosi (Fasulo et al., 2000), promoure la polimerització de tau (Abraha et al., 2000), així com provocar un mal ensamblatge dels microtúbuls. Estudis en ratolins transgènics que sobreexpressen alguns d'aquests fragments han aportat informació rellevant (Zilka et al., 2006). Per una banda, l'expressió del fragment tau151-391, que *in vitro* és capaç d'afavorir un mal ensamblatge dels microtúbuls, és suficient per generar degeneració neurofibril·lar del tipus MA. A més, els complexes de tau estan formats per la tau humana del transgen i la tau endògena de la rata en una proporció 1:1, és a dir, la tau truncada és capaç d'agregar la tau murina endògena (Zilka et al., 2006). I per altra banda, l'expressió de fragments truncats comporta en la rata un augment de radicals lliures i una distribució anòmala dels mitocondris (Cente et al., 2006).**

### *3.1.3.3 Proteases responsables de la fragmentació*

**Diverses proteases poden tallar la tau *in vitro*: caspases, calpaïnes, trombina, catepsina D, tripsina i quimiotripsina. El paper que juguen cada una d'elles *in vivo* encara no està ben clar, però algunes d'elles s'han trobat colocal·litzant amb les inclusions de tau en algunes taupaties. En els experiments *in vitro*, així com en el model de truncatge d'abans, s'observa una relació entre fosforil·lació i truncatge. La primera va abans que la segona i a vegades protegeix de l'acció de la protesa, com passa amb la trombina i amb la caspasa-3. La trombina pot tallar la tau a diferents dianes *in vitro,* i això es pot prevenir mitjançant la fosforil·lació de tau per GSK3-beta (Arai et al., 2005). La trombina i el seu precursor, la protrombina, estan predominantment expressades al fetge però en MA també s'expressen a les neurones i a les cèl·lules glials i s'acumulen als NFT (Arai et al., 2006).**

**Caspases i calpaïnes comparteixen molts dels seus substrats i la seva activació *in vitro* està relacionada amb fenòmens apoptòtics (Raynaud and Marcilhac, 2006). La calpaïna-1 s'ha vist activada en algunes malalties neurodegeneratives com la MA (Saito et al., 1993) i, *in vitro*, pot tallar la tau a diferents dianes (Canu et al., 1998). La calpaïna-2 s'ha trobat activada i colocal·litzant amb la tau en els NFT a la MA, la síndrome de Down, la PSP, la DCB i en alguns cossos de Pick (un 10%) a la MPi (Adamec et al., 2002). A més a més, la calpaïna està involucrada en la fosforil·lació de tau pel fet que és la responsable del tall de p35 a p25 que, unit a la Cdk5, promou la fosforil·lació de tau per part d'aquesta cinasa (Patrick et al., 1999).**

**La tau es pot tallar per múltiples caspases al residu Asp421 (caspasa-1, -3. -6 i -7) i la tau resultant s'agrega formant filaments més ràpidament que la tau sencera (Gamblin et al., 2003). La caspasa-3 es troba en els NFT en la MA. La caspasa-6 també es troba en les inclusions de tau i s'ha observat que *in vitro* és responsable del tall al residu N-terminal D13. Aquest tall, conjuntament amb el de la caspasa-3 a Asp421, sembla que es produeixen a la mateixa etapa primerenca de formació dels NFT (Horowitz et al., 2004).**

### *3.2 Poder patogènic de la tau*

**Com s'ha comentat al primer apartat, pel fet que les formes familiars de la MA estan causades per mutacions que afecten directament a la formació de pèptid amiloide i presenten també degeneració neurofibril·lar, és lògic conferir un paper a la tau que estigui subjecte als efectes de la "cascada amiloide". Els dèficits cognitius i la mort neuronal, però, presenten una més forta correlació amb els dipòsits de tau que amb les plaques d'amiloide (Ballatore et al., 2007).**

**La resta de taupaties no presenta dipòsits d'amiloide i les mutacions al gen de la tau no tenen cap efecte sobre l'acumulació d'amiloide, però si són la causa d'una subgrup de taupaties anomenades demències frontotemporal amb parkinsonisme associades a mutacions al gen de la tau (FTDP-17).**

**Aquestes dades posen de manifest la importància patogènica de la tau. Però no està clar de quina manera la tau exerceix aquest efecte nociu. La patogenicitat s'ha atribuit tant al cos d'inclusió com a la tau soluble, però tot i que el cos d'inclusió de cada taupatia ocupa un espai molt gran dins de la cèl·lula i pot dificultar funcions de transport en el seu interior, sembla més probable que la funció tòxica la desenvolupi en el seu estat soluble. De fet la cèl·lula és capaç de resistir molts anys en convivència amb el cos d'inclusió (Morsch et al., 1999); i existeix un argument molt citat que es basa en l'observació d'un ratolí transgènic, que expressa la tau mutada d'una DFTP-17, a qui se li suprimeix de sobte l'expressió d'aquesta tau patogènica (Santacruz et al., 2005). Aquesta supressió millora el dèficit de memòria i estabil.litza la supervivència neuronal però no atura la formació de cabdells neurofibril·lars, encara que l'única tau que s'expressa a partir de llavors és la del propi ratolí.**

**Els models animals, majoritàriament ratolins transgènics, són de gran ajuda per l'estudi de la funció patogènica de tau. Se n'han produït de molts tipus donant resultats molt diversos, depenent del nombre i el tipus d'isoformes de tau que sobreexpressen, si es silencia o no l'expressió de tau endògena del ratolí (que només expressa les isoformes 4R) així com del promotor que utilitzen (Duyckaerts et al., 2008, Frank et al., 2008).**

# 4- PROTEÏNES ASSOCIADES ALS DIPÒSITS DE TAU EN TAUPATIES

**La tau no està sola en les inclusions, tant siguin cabdells neurofibril·lars, cossos de Pick com grans argiròfils, etc. Mitjançant tècniques de microscopia òptica, confocal o electrònica es pot observar que els filaments de tau i, a major escala, les inclusions, estan poblades d'un notable nombre d'altres proteïnes. És de gran importància identificar quines són aquestes proteïnes, si encara són actives, si estan deixant de fer la seva funció normal pel fet de trobar-se segrestades a la inclusió, si estan col·laborant per formar o estabilitzar la inclusió o si, per contra, proven de desfer-la.**

**Un altre aspecte important en l'estudi de les proteïnes associades a les inclusions és la recerca de similituds i diferències entre taupaties i altres malalties neurodegeneratives amb agregats proteïcs. Trobar elements diferencials o comuns pel què fa a la composició proteïca de les inclusions pot donar pistes sobre els mecanismes generals o específics que donen lloc a aquestes.**

**En aquest apartat es descriuen algunes proteïnes que, per diferents motius de rellevància, s'han estudiat en relació als agregats de tau. Afegim aquí la presència en inclusions del factor de transcripció Sp1, descrita per primera vegada en un dels treballs que conforma aquesta tesi.**

### *4.1 Cinases*

**Existeixen moltes cinases que tenen la capacitat de fosforil·lar la tau. Aquestes cinases col·localitzen amb la tau a l'interior de les inclusions en totes les taupaties estudiades i algunes d'elles, com la GSK3-beta, la p38 i la SAPK/JNK, s'han aïllat i comprovat que encara són actives quan es posen en contacte amb un substrat genèric com és la proteïna bàsica de la mielina o amb la tau recombinant (Puig et al., 2004, Ferrer et al., 2005). Encara que un dels substrats d'aquestes múltiples cinases de tau és la pròpia tau, en tenen més. I com veurem, pot ocòrrer que algun d'aquests substrats (factors de transcripció, cinases i altres) puguin estar també atrapats dins de les inclusions, de manera**

**que es perpetuin o s'alimentin estats conformacionals o sublocalitzacions cel·lulars (sobretot en factors de transcripció) determinades amb conseqüències funcionals importants.**

**La regulació de l'activitat de les cinases es produeix per fosforil·lacions ens llocs específics. És important determinar en quin estat es troben aquestes cinases en les inclusions. El cas de la GSK3-beta és un cas curiós en el sentit que la forma que es troba en més abundància dins de les inclusions de tau és la forma teòricament inactiva (aquella que està fosforil·lada a la Ser9 per una altra cinasa anomenada Akt (Stambolic and Woodgett, 1994)), mentre que la forma activa (la fosforil·lada a la ser21) hi està pràcticament absent (Ferrer et al., 2005). Tot i així, com s'ha comentat, una vegada aïllada demostra que continua activa; l'explicació podria venir de l'estudi d'una xaperona de la que es parlarà més endavant a l'apartat 4.1.3.**

### *4.2 Factors de transcripció*

#### *4.2.1 Factors de transcripció induïbles: c-fos i c-jun*

**Un dels primers factors de transcripció que es va descriure en mamífers és l'AP-1 (activador de proteïna-1). Es va veure que aquest factor podia estar involucrat en una gran varietat de respostes que inclouen supervivència, proliferació, diferenciació i mort (Shaulian and Karin, 2002). Aquest ampli ventall de funcions es deu a la seva naturalesa heterogènia. L'AP-1 és un dímer que pot formar-se per homo o heterodímers de diferents proteïnes pertanyents a diferents famílies: Fos (c-Fos, Fos B, Fra-1 i Fra-2), Jun (c-Jun, Jun B, Jun D), ATF (ATF-2, LRF1/ATF3, B-ATF, JDP1 i JDP2) i la família Maf (Karin et al., 1997), i unir-se a l'ADN a la seqüència consens TGAC/GTCA. Fos i Jun són els principals components d'AP-1. Fos i jun són gens de resposta immediata que s'activen en resposta a l'estrés quan es veuen fosforil·lats per cinases d'estrés com la JNK (c-jun N-terminal kinase)/SAPK (stress-activated protein kinase) (Gupta et al., 1996) o de manera indirecta (via activació d'ATF2 i Elk-1), per la p38 (Hazzalin et al., 1996). La relació entre l'activació de c-Fos i c-Jun i la mort neuronal va quedar clara fa anys mitjançant dues observacions. La primera és que l'eliminació de l'expressió d'aquests factors *in vitro* o *in vivo* incrementa la supervivència de les neurones quan s'enfronten a diferents estímuls nocius (Martin et al., 1988, Eilers et al., 1998, Ham et al., 2000). La segona és que si**

**s'evita la fosforil·lació de c-Jun mitjançant mutacions als epítops fosforil·lables per JNK s'evita també l'apoptosi induïda per deprivació de factors tròfics i també la sensibilitat a l'excitotoxicitat induïda per àcid kaínic (Behrens et al., 1999, Le-Niculescu et al., 1999).**

**L'estudi de l'expressió d'aquests factors induïbles ha estat realitzada sobre algunes malalties neurodegeneratives com la MA i sobre alguns models animals d'aquestes malalties. Fins al moment no s'ha pogut demostrat una relació entre l'expressió anòmala d'aquests factors i els seus activadors amb un augment de mort neuronal per apoptosi en les taupaties. Probablement les conseqüències de la desregulació d'aquests factors de resposta immediata es produeixen tan ràpidament que se'n fa molt difícil la mesura en el teixit postmortem.**

**L'expressió de c-jun i c-fos ha estat particularment estudiada en la MA. En aquesta malaltia la rellevància d'AP-1 (en especial l'homodímer jun:jun) és notable pel fet que és un dels factors de transcripció que podrien regular l'expressió de la proteïna APP, i tenir per tant un efecte sobre la producció d'amiloide (King and Scott Turner, 2004). S'ha descrit que la immunoreactivitat de c-jun es veu incrementada a la MA (Anderson et al., 1996), en especial en neurones amb cabdells neurofibril·lars. L'augment d'expressió de c-jun a l'hipocamp de malalts de MA coincideix amb l'observació d'un increment de mRNA en aquesta zona. L'expressió de c-Jun en cèl·lules glials al voltant de plaques també s'ha vist incrementada. Cal mencionar que les observacions sobre el factor de transcripció c-Jun mitjançant tècniques d'immunohistoquímica poden haver-se de reinterpretar a la llum d'anticossos més específics que els utilitzats en alguns d'aquests estudis, que poden estar reconeixent epítops similars d'altres proteïnes (Ferrer, 2006).**

**El paper i localització de c-Fos en la MA també és controvertit sobretot pel què fa a la seva presència en cabdells neurofibril·lars i plaques senils. Però sembla que els seus nivells d'expressió estan augmentats en l'hipocamp de malalts de MA (Anderson et al., 1994, Marcus et al., 1998).**

### *4.2.2 CREB i ATF-2*

**CREB (cAMP response element binding protein) i ATF-2 (activating transcription factor 2) són factors de transcripció relacionats amb gran quantitat de processos cel·lulars. CREB, una vegada fosforil·lat esdevé actiu i es pot unir a la CBP (CREB binding protein) i activar la transcripció gènica; també es pot activar per altres vies que inclouen les MAPK com p38 i ERK (Yamamoto et al., 1988). CREB es troba relacionat sobretot amb fenòmens de supervivència i proliferació, així com de creixement de neurites i diferenciació neuronal en determinades poblacions de neurones. Com hem dit, aquests factors de transcripció poden estar regulats per la forma activa (fosforil·lada) de la cinasa d'estrés p38. ATF-2, a més, també pot ser fosforil·lat per JNK. Com s'ha dit, les dues cinases d'estrés (p38 i JNK) es troben a les inclusions en totes les taupaties (Ferrer et al., 2005); els nivells d'expressió i la localització d'aquests dos factors de transcripció han estat parcialment estudiats en la MA. De moment els resultats assenyalen diferències subtils, si n'hi ha, en els nivells d'expressió pel què fa a ATF-2. En el cas de CREB, els nivells totals no varien però si sembla que els nivells de la forma fosforil·lada de CREB disminueixen en la MA (Yamamoto-Sasaki et al., 1999).**

### *4.2.3 Sp1*

**La família Sp es composa d'una colla de factors de transcripció de tipus « zinc finger » (dits de zinc) que es poden unir a regions dels promotors amb caixes GC de gran quantitat de gens (Philipsen and Suske, 1999). Aquest factors poden reprimir o promoure l'expressió dels gens en funció de seu estat de fosforil·lació i dels co-activadors o co-repressors que intervinguin en el procés (Chu and Ferro, 2005).**

**El factor de transcripció Sp1 és particularment interessant perquè s'ha vist que es troba atrapat a les inclusions intranuclears de huntingtina mutada a la malaltia de Huntington. Aquesta associació té una explicació clara i es pot preveure pel fet que Sp1 posseeix uns dominis d'activació rics en glutamina. L'alteració que pateix la huntingtina en la malaltia de Huntington consisteix precisament en extensions de poli-Glutamina (DiFiglia et al., 1997). Aquestes extensions atrapen l'Sp1 enganxant-se als seus propis dominis rics en**

**glutamina i també, i pel mateix motiu, a un dels co-activadors de Sp1, (TAF)II130 (Freiman and Tjian, 2002).**

**Hi ha altres malalties neurodegeneratives que estan causades per extensions de poli-glutamina. Són el grup de les atàxies espino-cerebel·loses (AECs). En alguna AEC, causada per extensions de poli-glutamina en una proteïna anomenada ataxina (Ross, 1997) que també s'agrega formant inclusions, també s'ha trobat la presència d'Sp1 en aquestes inclusions (Shimohata et al., 2000).**

**El possible segrest d'Sp1 en inclusions en la MA i en altres taupaties és interessant per més d'un motiu. El primer és que Sp1 està directament involucrat en el control de l'expressió de l'APP depenent de TGF-beta (Docagne et al., 2004); i també de l'expressió de BACE1 i BACE2 (Christensen et al., 2004, Sun et al., 2005), que estan involucrades en la funció beta-secretasa, que com hem vist és responsable del tall de l'APP i la producció de pèptid amiloide. A més, Sp1 està també involucrat en la regulació de l'expressió de tau (Heicklen-Klein and Ginzburg, 2000). Essent un factor de transcripció amb poder regulador de les principals proteïnes de la neuropatologia de la MA i altres taupaties, queda pendent l'estudi de l'expressió d'Sp1 en aquestes malalties.**

**Un segon motiu per estudiar l'Sp1 en les malalties neurodegeneratives és el fet que la seva expressió pugui estar en relació a una resposta compensatòria davant de l'estrés oxidatiu. Això és el que sembla indicar el fet que es detecta un fort increment d'expressió d'aquest factor en resposta a l'estrés oxidatiu sobre neurones corticals embrionàries. L'estrés oxidatiu incrementat sembla que és un fenòmen comú en moltes malalties i en especial en malalties neurodegeneratives. El següent apartat està exclusivament dedicat a parlar d'aquest aspecte. El rol d'Sp1 en relació a l'estrés oxidatiu queda reforçat per la seva capacitat d'induir l'expressió de la superoxid dismutasa 2 (SOD-2) (Xu et al., 2002). Aquest enzim s'encarrega en els mitocondris de protegir contra els excessos de les espècies radicals d'oxigen (ROS) (Melov, 2002).**

### *4.3 14-3-3*

**Als agregats proteics s'hi troben xaperones com algunes de la família de les "heat shock proteins" (HSP) o la família de les 14-3-3. La 14-3-3 és una família de proteïnes composta de 7 isoformes diferents (beta, epsilon, eta, gamma, tau, sigma, zeta) que representen aproximadament un 1% del total de proteïnes solubles del cervell. Exerceixen la seva funció unint-se a proteïnes que presentin un motiu fosfo-serina determinat. Entre les múltiples funcions que duen a terme hi ha la de xaperona, però poden veure's involucrades en funcions reguladores de diferents vies de senyalització (d'apoptosi, control del cicle celular i transport vesicular, entre d'altres) (Ferl, 1996, Darling et al., 2005, Muslin and Lau, 2005, Aitken, 2006, Hermeking and Benzinger, 2006). Tota cèl·lula eucariota expressa una isoforma o altra de 14-3-3 i s'han identificat més de 100 proteïnes que hi poden interaccionar (Dougherty and Morrison, 2004). En el cervell humà adult estan molt expressades i dins les neurones d'un cervell normal es poden detectar en el citoplasma i a les sinapsis (Fu et al., 2000).**

**Alguns estudis han indicat que algunes proteïnes 14-3-3, i en especial la isoforma zeta, es troben localitzades en els cabdells neurofibril·lars en la MA (Layfield et al., 1996, Umahara et al., 2004b). A més de trobar-se en les inclusions, altres estudis assenyalen que algunes isoformes (zeta, gamma i epsilon) es troben sobre-expressades en MA i dues d'elles també en la síndrome de Down (Fountoulakis et al., 1999, Soulie et al., 2004).**

**La localització d'isoformes de 14-3-3 en les inclusions de tau té sentit per dos motius. El primer és que la tau pot interactuar almenys amb dues isoformes de 14-3-3 (zeta i beta) en extractes de cervell; però no amb dues altres (gamma i epsilon) (Hashiguchi et al., 2000). El segon motiu és que aquestes proteïnes tenen entre les seves funcions la de regular algunes cinases com la GSK3-beta i la CAMK (Hashiguchi et al., 2000), les dues amb l'habilitat de fosforil·lar tau i presents en les inclusions. S'ha descrit que dímers de la isoforma zeta poden unir-se al mateix temps amb la tau i la GSK3-beta de manera que es promou la fosforil·lació de la primera per part de la segona (Agarwal-Mawal et al., 2003). I a més, la 14-3-3 és capaç, mitjançant la seva unió a la GSK3-beta fosforil·lada a la serina 9, la forma inactiva, de mantenir aquesta cinasa activa (Yuan et al., 2004). Aquesta és una possible explicació, com s'avançava a l'apartat 4.1.1, de perquè**

**la forma inactiva de la GSK3-beta sembla mantenir la seva activitat tan a jutjar pels epítops de tau que es troben fosforil·lats en les inclusions, com pels assajos d'activitat cinasa.**

***4.4 UBB+1 i p62***

**A més de les xaperones, fins a cert punt és esperable que en els agregats proteics tant de tau com d'altres proteïnes que s'observen a les malalties neurodegeneratives s'hi involucrin proteïnes relacionades amb la degradació proteosomal. Així doncs, la totalitat d'inclusions proteïques que s'han identificat en malalties neurodegeneratives es poden detectar amb anticossos que reconeguin la ubiqüitina. Un cert tipus de complex proteosomal (26S) és capaç d'eliminar proteïnes mal plegades un cop aquestes s'associen a la ubiqüitina. L'objectiu d'aquest marcatge és la degradació d'aquestes proteïnes mal plegades, i el motiu de què la inclusió no desaparegui una vegada les seves proteïnes són poli-ubiqüitinitzades s no es coneix amb exactitut però poden tenir-hi a veure dues observacions. La primera és que el sistema de degradació proteosomal pot estar alterat i la seva activitat, disminuïda. I això s'ha pogut observar en MA i altres taupaties, així com en sinucleïnopaties (Layfield et al., 2003).**

**La segona observació és la presència en les inclusions d'una forma mutada de la ubiqüitina, la UBB+1 (van Leeuwen et al., 1998). Aquesta, no es tracta d'una mutació hereditària sinó somàtica, que es produeix a nivell d'ARNm i dona lloc a una ubiqütina modificada en el seu extrem C-terminal. Aquesta forma mutada no serveix per ubiqüitinitzar proteïnes però en canvi pot ser ubiqüitinitzada per la ubiqüitina normal i degradada al proteasoma. El problema apareix quan els nivells de UBB+1 són alts; llavors el proteasoma queda saturat i inutil·litzat (Lindsten et al., 2002). La UBB+1 es troba en les inclusions de tau en MA i en altres taupaties, la qual cosa suggereix un motiu adicional de mal funcionament del proteasoma en aquestes cèl·lules.**

**La proteïna p62 o Sequestosome 1 s'ha vist involucrada en processos d'agregació proteica i en la funció de llançadora de proteïnes poli-ubiqüitinitzades cap al proteasoma (Nakaso et al., 2004, Seibenhener et al., 2004). A la seva regió C-terminal, la p62 conté un domini d'unió a la ubiqüitina i**

**s'ha vist que és capaç de facilitar la degradació proteosomal de la proteïna tau. La regulació de la seva expressió corre a càrrec majoritàriament de factors de transcripció com l'Sp1, AP-1 i NFKbeta; i determinats insults, com són la inhibició dels proteasoma o l'augment de radicals lliures, poden induir-ne l'expressió. Una sobreexpressió de p62 comporta en cèl·lules la formació de grans inclusions. Aquestes dues últimes observacions suggereixen un paper per la p62 com a promotora de la formació d'inclusions. La intepretació d'aquest fenomen està en la linia de considerar les inclusions un mecanisme protector per empaquetar proteïnes anòmales o mal plegades. La p62 es troba present en inclusions de tau en diferents taupaties i també en inclusions d'alfa-sinucleïna en sinucleïnopaties (Zatloukal et al., 2002, Scott and Lowe, 2007); així com en agregats d'altres malalties com són les atàxies espinocerebelars (observacions pròpies).**

### *4.5 LRRK2*

**La LRRK2 (Leucine Rich Repeat Kinase-2) és una proteïna enorme (de 2527 aminoàcids) el gen de la qual fa pocs anys es va trobar mutat en diverses famílies amb Parkinson familiar. Des de llavors s'han identificat al voltant de 30 mutacions en el seu gen (Mata et al., 2005). Aquestes mutacions en conjunt però en especial una d'elles (G2019S, la més freqüent) poden explicar fins un 13% dels casos totals de Parkinson familiar, i fins un 2% dels esporàdics (Di Fonzo et al., 2005, Farrer et al., 2005, Mata et al., 2005, Clark et al., 2006, Ishihara et al., 2006).**

**La funció d'aquesta proteïna no es coneix del cert però s'ha descrit que: 1) S'uneix a moltes proteïnes com xaperones (HSP90) (Hurtado-Lorenzo and Anand, 2008), proteïnes del citoesquelet (Jaleel et al., 2007, Gandhi et al., 2008) i de transport de vesícules entre d'altres (Shin et al., 2008). 2) Té capacitat d'hidrolitzar l'ATP amb el seu domini GTPasa (Guo et al., 2007). 3) Té activitat cinasa (Guo et al., 2007); amb el domini cinasa s'ha vist que és capaç de fosforil·lar el residu regulador de proteïnes d'unió a l'actina i la membrana com són l'ezrina, la radixina i la moesina (Jaleel et al., 2007). 4) Regular l'endocitosi regulada per clatrina mitjançant la interacció amb la pròpia clatrina, i amb la Rab5a (Shin et al., 2008).**

**S'hipotetitza que aquesta proteïna pot tenir d'alguna manera relació amb les taupaties, bàsicament per dos motius. El primer és que algunes mutacions en el gen de la LRRK2 poden donar patologies pleiomòrfiques, és a dir que la mateixa mutació pot donar lloc a un Parkinson típic, o pot donar lloc a mort neuronal sense inclusions, o amb inclusions només reactives per ubiquitina i d'altres fenotips (Mata et al., 2006). És d'especial interès que un dels fenotips que pot donar és l'acúmul de tau en forma de cabdells neurofibril·lars distribuïts de manera que recorda una PSP (Zimprich et al., 2004, Rajput et al., 2006). Per tant, la mateixa mutació pot causar agregats de sinucleïna, de tau, d'ubiquitina o cap inclusió (Zimprich et al., 2004). S'ha proposat que els efectes patogènics d'aquesta proteïna mutada es troben al capdamunt de les vies de neurodegeneració que porten a la formació d'inclusions, tant de tau com d'alpha-sinucleïna (Mata et al., 2006).**

**El segon motiu és que alguns autors han assenyalat que la LRRK2 es troba present tant en agregats d'alpha-sinucleïna (cossos de Lewy) (Miklossy et al., 2006, Higashi et al., 2007, Melrose et al., 2007, Perry et al., 2008) com en inclusions de tau en diferents taupaties (Miklossy et al., 2006, Miklossy et al., 2007), tot i que aquest fet és motiu de controvèrsia. Mentre que alguns anticossos detecten LRRK2 a les inclusions, altres no ho fan (Giasson et al., 2006). Una possible explicació és que depèn de l'epítop. En general sembla que quan l'anticos està fabricat per reconèixer extrems de la proteïna (en especial el C-terminal) és capaç de tenyir les inclusions tant de tau com de sinucleïna. Per contra, quan l'epítop contra el qual ha estat dirigit es troba a regions internes de LRRK2 no es detecta la proteïna en cap inclusió. És per aquest motiu que alguns autors han proposat que el què realment es troba dins de les inclusions són fragments de LRRK2, i que la forma sencera o no hi és, o té els epítops emmascarats (Higashi et al., 2007).**

**La qüestió no està encara gens clara perquè un altre aspecte a tenir en compte és l'especificitat d'aquests anticossos, de la qual cosa en pot servir de reflexe el patró de bandes que detecten per Western blot. Algunes conclusions s'han tret d'estudis on s'utilitzen només determinats anticossos que generen molts dubtes respecte la seva especificitat quan s'analitzen per Western blot, pel fet que reconeixen moltes altres bandes apart de la corresponent a LRRK2, i en alguns casos aquesta no apareix (Miklossy et al., 2006, Melrose et al., 2007,**

**Alegre-Abarrategui et al., 2008). Abans de continuar especulant sobre el paper de LRRK2 en les inclusions tant en taupaties com en sinucleïnopaties, cal revisar de manera conjunta els resultats obtinguts fins ara i els anticossos utilitzats per obtenir-los.**

## 5- ESTRÉS OXIDATIU EN TAUPATIES

**L'estrés oxidatiu és un fenòmen que generalment es defineix com un excés de radicals lliures d'oxigen (ROS) que sobrepassa, o com a mínim obliga a contra-actuar, els sistemes antioxidants de defensa de l'organisme. Existeix un gran nombre d'evidències que vinculen l'estrés oxidatiu amb moltes malalties neurodegeneratives.**

**Els radicals lliures d'oxigen són produïts pel fet de dur a terme un metabolisme aeròbic. Aquest es produeix als mitocondris, que esdevenen així el principal lloc de producció de radicals lliures, com el superòxid (02-). Els radicals lliures es caracteritzen per ser formes molt reactives, propietat que s'explica pel fet de posseir un o més electrons desaparellats. També entren en aquest grup les molècules òxid nítric (NO), el radical hidroxil (OH-) i el peròxid d'hidrogen (H2O2). L'organisme compta amb una sèrie d'enzims que permeten l'eliminació d'aquests ROS. La superòxid dismutassa (SOD), que és capaç de transformar el superòxid en peròxid d'hidrogen (H2O2). També col·laboren a l'eliminació de ROS la catalasa, la glutatió reductasa i la glutatió peroxidasa. Es proposa que la glia, en concret els astròcits, tenen un paper molt important en la protecció de la neurones de l'acció dels ROS. A més, els astròcits posseeixen una major concentració de glutatió reduït així com d'enzims del metabolisme del glutatió.**

**Quan els sistemes anti estrés oxidatiu fallen i l'estrés supera les defenses, sembla que les cèl·lules moren per apoptosi en poques hores (Perry et al., 1998a, Perry et al., 1998b). Per tant, i tenint en compte que les cèl·lules dels malalts de diferents malalties neurodegeneratives no moren de cop, el què sembla és que en aquest tipus de malalties les defenses en realitat no estan sobrepassades,**

**sinó, com es demostra en molts casos, estan augmentades per compensar l'amenaça (Perry, 2003)..**

**Els ROS poden actuar sobre proteïnes, lípids i àcids nucleics i alterar el funcionament d'aquestes molècules. Els estudis que aquí es presenten es centren en la identificació de proteïnes modificades per l'efecte de l'estrés oxidatiu. Els ROS poden actuar directament sobre les proteïnes, o bé indirectament modificant àcids grassos i glúcids, i creant adductes reactius amb la capacitat d'unir-se a les proteïnes.**

**Els adductes que provenen de la glicoxidació o bé de la lipoxidació es poden reconèixer amb anticossos específics que permeten la identificació de les proteïnes modificades. El carboxi-etil-lisina (CEL) prové de la glicoxidació, el 4-hidroxinonenal (HNE) i el malondialdehid-lisina (MDAL) de la lipoxidació dels àcids grassos poli-insaturats (PUFAs) i el carboxi-metil-lisina (CML) prové tant d'un com de l'altre procés. Un altre marcador és l'increment d'AGEs (advanced glycation end products), així com del seu receptor, RAGE, que és un membre de la superfamília de les immunoglobulines a través dels quals els AGEs realitzen els seus efectes sobre les cèl·lules com pot ser l'activació de cinases (Schmidt et al., 2000). Els AGEs són grups carbonils producte de la glicooxidació que reaccionen amb les lisines de les proteïnes (Dalle-Donne et al., 2006). Altres adductes en proteïnes poden venir del peroxinitrit que prové de radicals lliures d'oxigen i nitrogen; aquests poden unir-se a les tirosines de les proteïnes donant lloc a les nitrotirosines, que també es poden usar de marcador amb anticossos específics.**

**S'ha descrit que el dany oxidatiu en proteïnes, lípids i àcids nuclèics es veu incrementat amb l'edat (Stadtman, 2006), i alguns d'aquests danys també es detecten en models murins de senilitat accelerada (SAMP8) (Nabeshi et al., 2006). Però aquest augment del dany oxdatiu és major en malalts de diferents malalties neurodegeneratives (Perry, 2003).**

**En el cas de la MA s'han trobat nivells més elevats de diferents marcadors d'estrés: AGEs (Smith et al., 1994), nitració (Smith et al., 1997b) , lipoxidació i glicooxidació (Smith et al., 1996, Sayre et al., 1997). L'origen d'aquesta major concentració de dany oxidatiu es relaciona amb diverses possibles fonts: el**

**pèptid amiloide soluble, el pèptid amiloide fibril·lar, els cabdells neurofibril·lars, problemes mitocondrials i l'edat. Els metalls que es concentren a les plaques amiloides, com el coure, el ferro o l'alumini (Good et al., 1992, Smith et al., 1997a), són també fonts d'estrés oxidatiu. Per últim, la microglia també contribueix amb l'alliberació de NO i O2- que poden combinar-se i formar el peroxinitrit. S'ha proposat també, a partir de certes observacions, que entre els efectes dels radicals lliures hi ha un increment de la producció d'amiloide i de tau fosforil·lada. Per exemple, un estudi indica que l'estrés oxidatiu induït per H2O2 augmenta l'activitat del promotor de BACE1 i, per tant, l'activitat Beta-secretasa i la producció de pèptid amiloide (Tong et al., 2005). Així mateix, el tractament en cultius primaris corticals de rata amb Fe i H2O2 augmenta notablement la fosforil·lació de tau (Lovell et al., 2004). La relació causa efecte entre el péptid amiloide i l'increment d'estrès oxidatiu s'ha pogut observar en diferents models. En cèl·lules, per exemple, l'adició de vitamina E, un conegut antioxidant, fa disminuir la toxicitat provocada pel pèptid amiloide (Yatin et al., 2000, Munoz et al., 2005).**

**Mentre que determinats autors proposen, en base a diferents treballs *in vitro* o en models animals, que l'amiloide o la tau són una font capital de producció de radicals lliures, altres paren atenció a una paradoxa evident quan s'estudia aquesta associació d'una manera diferent. Observar les modificacions de les proteïnes per dany oxidatiu pot ser enganyós pel fet que una vegada modificades poden formar agregats i enlentir el seu recanvi. Això pot portar a situar en el present d'una cèl·lula un dany oxidatiu que va ocórrer en el passat, mentre que en la realitat present la cèl·lula no es troba estressada (Perry, 2003). L'estrés oxidatiu immediat es mesura millor observant les modificacions oxidatives dels àcids nuclèics mitjançant la detecció de la 8-hidroxiguanosina (8OHG), resultant de l'atac de grups hidroxils a les guanidines. El què s'ha vist és que neurones amb cabdells neurofibril·lars, encara que mostraven un augment de marcadors de lipo o glicoxidació, tenien uns nivells molt baixos de 8OHG. També és paradoxal que els malalts de MA amb més dipòsits d'amiloide presenten els nivells més baixos de 8OHG (Hensley et al., 1994). Aquestes observacions han portat a alguns autors a proposar un paper per les inclusions de tau i d'amiloide com a un mecanisme, de la cèl·lula, antioxidant compensador. Tanmateix, cal mencionar el fet que estudis sobre l'activitat del proteasoma 20S, particularment avesat en la degradació de les proteïnes oxidades, han mostrat**

**que les proteïnes malmeses pel dany oxidatiu són ràpidament degradades i proteïnes noves es comencen a sintetitzar, de manera que el recanvi es produeix més ràpidament (Davies, 2001). Però si no es degraden al ritme necessari, aquestes proteïnes modificades poden formar agregats degut a unións covalents i un augment de la seva hidrofobicitat (Grant et al., 1993), la qual cosa si és capaç de retardar el seu recanvi.**

**Diversos marcadors d'estrés oxidatiu s'han trobat augmentats en altres taupaties menys estudiades com la PSP (Albers et al., 1999, Albers et al., 2000), MPi (Montine et al., 1997, Zarkovic, 2003) o DFTP-17 (Martinez et al., 2008a), però no hi ha tanta feina feta com a la MA; això és especialment cert pel què fa a la MGA, on no s'han realitzat encara molts d'aquests anàlisis. Molt més ben descrit està l'increment de marcadors d'estrés oxidatiu en sinucleïnopaties, com el PD o la DCL (Dexter et al., 1989, Castellani et al., 1996, Floor and Wetzel, 1998, Jenner, 2003).**

**Les modificacions causades per l'estrès oxidatiu sobre els aminoàcids de les proteïnes sovint pot comportar una pèrdua de funció o d'activitat enzimàtica (Uchida et al., 1997). És per això que un dels principals interessos consisteix en identificar quines són exactament les proteïnes diana del dany oxidatiu en les malalties neurodegeneratives. És d'especial interès conèixer si existeixen proteïnes diana en zones del cervell encara no afectades per la malaltia quan aquesta es troba en una fase més primerenca. Conèixer les proteïnes que comencen a fer fallida previament a la clínica i/o a l'aparició d'inclusions podria tenir un valor diagnòstic o terapèutic.**

# Objectius

***Objectiu general:***

- **Estudiar els components proteïcs i les seves possibles modificacions post-traduccionals en les inclusions anòmales de diferents taupaties i sinucleïnopaties.**

***Objectius concrets:***

**1.- Establir la sensibilitat de les mostres de teixit cerebral humà en relació a la posible degradacio de les proteïnes i de les seves modificacions post-traduccionals (en especial la fosforil·lació) com a conseqüència del inevitable retard en el processament postmortem del sistema nerviós humà. Establir les condicions bàsiques per treballar amb condicions òptimes de no degradació de proteïnes.**

**2.- Estudiar els patrons de possibles fragments de tau en diferents taupaties així com l'expressió de diferents proteases de tau.**

**3.- Estudiar l'expressió de diferents factors de transcripció relacionats amb estrés (c-fos, c-jun, ATF2, CREB) i amb la regulació de proteïnes clau (Sp1) en taupaties i sinucleïnopaties i la seva associació amb les inclusions proteiques.**

**4.- Estudiar mecanismes patogènics en la poc coneguda taupatia malaltia de grans argiròfils (MGA).**

**5.- Revisar i tractar d'establir la presència o absència de la proteïna LRRK2 en els agregats de tau i de sinucleïna en taupaties i sinucleïnopaties, respectivament.**

**6.- Identificar proteïnes modificades per l'estrés oxidatiu en fraccions enriquides en filaments de tau hiperfosforil·lada en la malaltia d'Alzheimer.**

**7.- Caracteritzar neuropatològica i bioquímicament els estadiatges primerencs i pre-clínics de la taupatia paràlisi supranuclear progressiva. Estudiar els patrons de tau hiperfosforil·lada i possibles fragments en aquesta malaltia així com dianes d'estrès oxidatiu.**

# Resultats

# 1

## Brain protein preservation largely depends on the postmortem storage temperature: implications for study of proteins in human neurologic diseases and management of brain banks: a BrainNet Europe Study

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ORIGINAL ARTICLE

# Brain Protein Preservation Largely Depends on the Postmortem Storage Temperature: Implications for Study of Proteins in Human Neurologic Diseases and Management of Brain Banks: A BrainNet Europe Study

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**Abstract**

The present study was designed to reveal protein modifications in control cases related with postmortem delay and temperature of storage in 3 paradigms in which the same postmortem tissue sample (frontal cortex) was frozen a short time after death or stored at 1°C, 4°C, or room temperature and then frozen at −80°C at different intervals. No evidence of protein degradation as revealed with monodimensional gel electrophoresis and Western blotting was observed in samples artificially stored at 1°C and then frozen at different intervals up to 50 hours after death. However, the levels of several proteins were modified in samples stored at 4°C and this effect was more marked in samples stored at room temperature. Two-dimensional gel electrophoresis and mass spectrometry further corroborated these observations and permitted the identification of other proteins vulnerable or resistant to postmortem delay. Finally, gel electrophoresis and Western blotting of sarkosyl-insoluble fractions in Alzheimer disease showed reduced intensity of phospho-tau-specific bands with postmortem delay with the effects being more dramatic when the brain samples were stored at room temperature for long periods. These results emphasize the necessity of reducing the body temperature after death to minimize protein degradation.

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**Key Words:** Brain banks, Human brain tissue, Postmortem delay, Protein preservation.

## INTRODUCTION

Although several experimental approaches and animal models have been used to mimic situations occurring in human degenerative diseases of the nervous system, the direct study of human brain tissue is crucial to increasing our understanding of real human neurodegenerative disorders. Optimal material quality is, however, a determining condition to avoid pitfalls related to tissue obtained at postmortem under suboptimal conditions. Many factors can affect the preservation of brain tissue before and after death. Premortem, the most relevant factors are the metabolic state, the use of toxic substances and drugs, infections, seizures, and hypoxia. Moreover, their detrimental effects can be augmented by a prolonged agonal state. At postmortem, delay among brain extraction, storage and tissue processing, as well as environmental temperature, are the most critical factors (1). Because the 2 last factors are the easiest to control, we have focused our study on postmortem delay and temperature to determine their relevance in the degree of preservation and degradation of brain proteins. This is an important issue because protein expression levels, as revealed by gel electrophoresis and Western blotting, are currently used in studies of human diseases of the nervous system.

One-time studies with human brain tissue have shown the importance of assessing the effects of postmortem delay on the preservation of target proteins. According to these observations, it has been shown that some proteins are very resistant to postmortem delay, whereas others are not. For example, Li et al studied the effects of postmortem delay (between 5 and 21 hours) in the expression levels of G-proteins in human prefrontal cortex (2). Under the same conditions, Gα-i1, Gα-i2, Gα-S, and Gβ were stable, whereas Gα-q and Gα-o were vulnerable to postmortem delay. Similarly, Siew et al, focusing on the expression of pre- and postsynaptic proteins in the rat cerebral cortex at

different intervals postmortem, showed that synaptophysin and PSD-95 remained stable throughout the period studied, whereas the expression levels of syntaxin decreased at 48 hours when the brain was stored at room temperature and at 72 hours when the brain was maintained at 4°C (3). Similar results were obtained when examining the effects of postmortem delay and temperature on selected synaptic proteins by immunohistochemistry (4). Delayed tissue processing after death results in variable modifications in the expression levels of the microtubule-associated proteins tau, MAP2, MAP1B, and MAP5 (5, 6). Nucleosides in the brain are also subject to postmortem degradation (7).

Effects of postmortem delay are also important when studying posttranslational modifications in certain human degenerative diseases as hyperphosphorylated tau band profiles in sarkosyl-insoluble fractions in Alzheimer disease (AD) and other tauopathies (8, 9). Modifications in the expression levels of phosphorylated proteins may be related to the presence of still active proteases and phosphatases in the postmortem brain (10, 11).

The present BrainNet Europe study concerning preservation trials was designed to reveal protein modifications in human postmortem brains in several paradigms. For this purpose, the same tissue sample was frozen shortly after death or stored at 1°C, 4°C, or at room temperature for varying time periods and then frozen at −80°C until use. The samples were analyzed by gel electrophoresis and Western blotting with a battery of antibodies including synapsis-related proteins, kinases, proteins of the cytoskeleton, trophic factor receptors, membrane protein, protein related with oxidative stress, protein associated with apoptotic pathway, and members of the ubiquitin proteasome system. The selection of these proteins was made at random to represent a varied range of proteins that can be possible targets for study in human diseases of the nervous system. Other samples were analyzed by bidimensional (2D) gel electrophoresis, in-gel digestion, and mass spectrometry. This permitted the identification of other proteins vulnerable to postmortem delay. Finally, the effects of postmortem delay on posttranslational modifications related to tau hyperphosphorylation in tauopathies were examined by gel electrophoresis and Western blotting of sarkosyl-insoluble fractions in cases with Alzheimer disease. Because it has been reported that another source of variation in the postmortem vulnerability of certain proteins is as a result of their regional location in the brain (3), the present study focused on human brain frontal cortex to eliminate possible regional bias.

## MATERIALS AND METHODS

### Human Brain Tissue

Protein preservation was examined in several paradigms that try to mimic postmortem delay degradation in the human brain. Review of clinical records and general autopsy reports revealed that all patients showed minimal evidence of prolonged agonal state. No evidence of neurologic disease, drug intake, or metabolic disease was noticed. The patients studied included 3 men and one woman. The age at death was 68, 66, 65, and 63 years, and the cause of death was heart failure in 3 cases and pneumonia in the fourth. The brains were obtained for research after written consent from the relatives following the guidelines of the local ethics committee of the IDIBELL-Hospital Universitari de Bellvitge. In all cases, half of the brain was stored in 4% paraformaldehyde in phosphate buffer for 3 weeks and then processed for the current neuropathologic study. The other half of the brain, except the frontal lobe, was immediately cut in coronal sections, frozen, and stored at −80°C. Part of the left frontal lobe, including Brodmann areas 8, 9, 45, and 46, was used for the present study. The basal pH of the brain tissue was 6.8, 7.2, and 6.9 in the 4 cases.

Finally, the frontal cortex (areas 8) of 4 cases with AD stage VI/C according to Braak and Braak was used in the study of phospho-tau degradation in relation to postmortem delay (12). AD cases were 2 men and 2 women aged 72, 68, 78, and 81 years. The neuropathologic diagnosis was carried out using the same regions and methods as described previously.

### Paradigms Mimicking Delayed Postmortem Delay; Modifications of Storage Time and Ambient Temperature

In case 1, samples were obtained and frozen 2 hours after death or stored for 3, 6, 21, and 48 hours (i.e. 5, 8, 23, and 50 hours of postmortem delay) at 4°C and then frozen at −80°C. In case 2, samples were obtained and frozen 2 hours and 15 minutes after death or stored for 2 hours 45 minutes, 5 hours 45 minutes, 13 hours 45 minutes, 22 hours 45 minutes, and 48 hours (i.e. 5, 8, 16, 23, and 50 hours of postmortem delay) at 1°C and then frozen at −80°C. In case 3, samples were obtained and frozen 2 hours after death or stored for 3, 6, 21, and 48 hours (i.e. 5, 8, 23, and 50 hours of postmortem delay) at room temperature (23°C) and then frozen at −80°C. In case 4, samples were obtained and frozen 2 hours after death or stored for 3, 6, 22, and 46 hours (i.e. 5, 8, 24, and 48 hours of postmortem delay) at room temperature (22°C) and then frozen at −80°C.

Regarding AD cases, the brains were obtained 2 hours after death in 2 cases (cases AD1 and AD2) and the samples of the left frontal cortex were rapidly frozen at −80°C or stored at room temperature for different time periods and then frozen at 5, 8, 26, and 50 hours after death. Another case (case AD3) was obtained at 6 hours after death and then frozen or stored at room temperature for different time periods and then frozen at 8, 12, 18, 24, and 48 hours after death. The fourth case (case AD4) was obtained 8 hours after death and then frozen. Later, part of the sample was thawed and pieces were maintained at room temperature until 12, 18, 24, and 48 hours after death and then frozen until use. In every case, the blocks were wrapped in normal kitchen aluminum foil and maintained at room temperature in a plastic box with appropriate humidity conditions until frozen.

### Biochemical Studies

Frontal cortex homogenates at the different time points in the first 3 paradigms were subjected to monodimensional gel electrophoresis and Western blotting. Twenty-three

antibodies recognizing specific proteins were analyzed. These antibodies included proteins involved in the cytoskeleton and synapses, membrane proteins, trophic factors and their receptors, proteins involved in cell survival and cell death, and transcription factors (Table 1). Every sample was processed per triplicate on different days.

In addition, the same tissue samples were examined in several human brain banks: Milan, Vienna, Munich, Edinburgh, and Bologna. Western blotting was restricted to 4 proteins (AKT-P, CaM kinase II, β-actin, and α-tubulin) in these laboratories. The antibodies were provided by the laboratory of reference so that the same aliquots were used by the different partners.

Bidimensional gel electrophoresis of the first, second, and fourth case was carried out in Barcelona. Finally, monodimensional gel electrophoresis and Western blotting of sarkosyl-insoluble fractions was carried out in the 4 cases with AD in Barcelona.

## Monodimensional Gel Electrophoresis and Western Blotting

Brain samples (0.2 g) were homogenized separately in a glass homogenizer in 1 mL of homogenizer Buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.5% sodium deoxycholate, and 0.5% NP40) and complete protease inhibitor cocktail (Roche Molecular Systems, Madrid, Spain). After a brief centrifugation at 15,000 rpm (4°C for 5 minutes), the pellet was discarded and the concentration of the resulting supernatant was determined by the bicinchoninic acid (BCA) method with bovine serum albumin as a standard.

For Western blot studies, 30 μg was mixed with reducing sample buffer and processed for 10% SDS-PAGE electrophoresis and then transferred to nitrocellulose membranes (400 mA, 90 minutes). Immediately afterward, the membranes were incubated with 5% skimmed milk in TBS-T buffer (100 mM Tris-buffered saline, 140 mM NaCl, and 0.1% Tween 20, pH 7.4) for 30 minutes at room temperature and then incubated with the primary antibody in TBS-T containing 3% BSA (Sigma, Madrid, Spain) at 4°C overnight. The characteristics of the primary antibodies are given in Table 1.

Subsequently, the membranes were incubated for 45 minutes at room temperature with the corresponding secondary antibody labeled with horseradish peroxidase (Dako, Madrid, Spain) at a dilution of 1:1000 and washed

**TABLE 1.** Characteristics of the Antibodies Used

| Antibody | Origin | Supplier | Location | Dilution |
|---|---|---|---|---|
| Synaptic proteins | | | | |
| Rabphilin | Mouse | Transduction | BD Biosciences, Madrid | 1/350 |
| Syntaxin | Rabbit | Calbiochem | Bionova | 1/500 |
| Rab3a | Rabbit | Santa Cruz | Quimigranel, Barcelona | 1/200 |
| α-synuclein | Rabbit | Chemicon | Pacisa Giralt, Madrid | |
| Kinases | | | | |
| Mek 1 | Mouse | Transduction | BD Biosciences | 1/500 |
| P38-P | Rabbit | Calbiochem | Bionova, Madrid | 1/100 |
| SAPK-JNK-P | Rabbit | Cell Signaling | Servicios Hospitalarios, Barcelona | 1/50 |
| Erk 42-44 | Mouse | Transduction | BD Biosciences | 1/500 |
| AKT-P | Rabbit | Cell Signaling | Servicios Hospitalarios | 1/500 |
| Camk II α | Mouse | Zymed | Servicios Hospitalarios | 1/200 |
| Cdk 5 | Rabbit | Calbiochem | Bionova | 1/500 |
| Fyn k | Mouse | Transduction | BD Biosciences | 1/500 |
| GSK-P Ser 9 | Rabbit | Oncogene | Bionova | 1/500 |
| Cytoskeletal proteins | | | | |
| α-tubulin | Mouse | Sigma | Sigma, Madrid | 1/4,000 |
| β-tubulin | Mouse | Sigma | Sigma | 1/4,000 |
| β-actin | Mouse | Sigma | Sigma | 1/5,000 |
| Trophic factor receptors | | | | |
| EGF-R | Mouse | Chemicon | Pacisa Giralt, Madrid | 1/200 |
| TrkB | Rabbit | Santa Cruz | Quimigranel | 1/200 |
| Membrane protein | | | | |
| PLC β1 | Rabbit | Santa Cruz | Quimigranel | 1/200 |
| Oxidative stress | | | | |
| iNOS | Rabbit | Santa Cruz | Quimigranel | 1/1,000 |
| Apoptosis | | | | |
| Bcl-2 | Mouse | Novocastra | Servicios Hospitalarios | 1/50 |
| Proteasome | | | | |
| Proteasome 11 | Rabbit | Affinity | Bionova | 1/500 |
| Proteasome 20 | Mouse | Biomol Intl. | Quimigranel | 1/500 |

with TBS-T for 30 minutes. Protein bands were visualized with the chemiluminescence ECL method (Amersham, Barcelona, Spain).

The protocols used for gel electrophoresis and Western blotting differed slightly from one center to another. Methodological differences can be found on the BrainNet web site (http://www.brainnet-europe.org/activities/frameset_activities.htm).

## Densitometry and Processing of Data

Protein expression levels were determined by densitometry of the specific bands using Total Lab v2.01 software (Pharmacia, Orsay, France). The results were normalized for β-actin. The numeric data obtained per triplicate for every protein at a given time period were expressed as a percentage of decrease compared with the corresponding basal (2-hour) values.

## Two-Dimensional Gel Electrophoresis

Samples of the frontal cortex (0.1 g) were homogenized in homogenizer buffer (50 mM Tris pH 7.4 containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and a cocktail of protease and phosphatase inhibitors) and centrifuged at 15,000 rpm for 5 minutes. The pellet was discarded and the concentration of protein from the resulting supernatant was determined by BCA method. Equal amounts of protein were mixed with a buffer containing, at final concentrations, 40 mM Tris pH 7.5, 7 M urea, 2 M thiourea 0.2% Biolite (v/v), 4% CHAPS (Bio-Rad, Barcelona, Spain), 2 mM Tributylphosphine solution, and bromophenol blue in a total volume of 350 μL.

In the first dimension electrophoresis, 350 μL of sample solution was applied on an immobilized 17-cm pH 3–10 nonlinear gradient ReadyStrip IPG strip (Bio-Rad, Barcelona, Spain) at both the basic and acidic ends of the strip. The strips were actively rehydrated for 16 hours at 50 V and the proteins were focused at 300 V for 2 hours after which time the voltage was gradually increased over 4 hours to 1,000 V. Focusing was continued at 1,000 V for 2 hours and gradually increased to 8,000 V over 8 hours; it then continued at 8,000 V for 10 hours. For the second dimension separation, IPG strips were equilibrated for 15 minutes in 50 mM Tris-HCl (pH 6.8) containing 6 M urea, 2% (wt/v) SDS, 30% (v/v) glycerol, and 2% dithiotreitol and then reequilibrated for 15 minutes in the same buffer containing 2.5% iodoacetamide. The strips were placed on 10% polyacrylamide gels and electrophoresed at 50 V overnight. For gel staining, an MS-modified silver staining method (Amersham) was used as described by the manufacturer.

## In-Gel Digestion

Proteins were in-gel-digested with trypsin (Sequencing grade modified; Promega, Barcelona, Spain) in the automatic Investigator ProGest robot of Genomic Solutions. Briefly, excised gels spots were washed sequentially with ammonium bicarbonate buffer and acetonitrile. Proteins were reduced and alkylated for 30 minutes with 10 mM DTT solution and 100 mM solution of iodine acetamide, respectively. After sequential washings with buffer and acetonitrile, proteins were digested overnight at 37°C with trypsin 0.27 nM. Tryptic peptides were extracted from the gel matrix with 10% formic acid and acetonitrile. The extracts were pooled and dried in a vacuum centrifuge.

## Acquisition of MS and MS/MS Spectra

Proteins manually excised from the 2D gels were digested and analyzed by CapLC-nano-ESI-MS-MS mass spectrometry. The tryptic digested peptide samples were analyzed using on-line liquid chromatography (CapLC; Micromass-Waters, Beverly, MA) coupled to tandem mass spectrometry (Q-TOF Global; Micromass-Waters). Samples were resuspended in 12 μL of 10% formic acid solution, and 4 μL was injected for chromatographic separation into a reverse-phase capillary $C_{18}$ column (75 μm of internal diameter and 15 cm in length, PepMap column; LC Packings, Sunnyvale, CA). The eluted peptides were ionized through coated nano-ES needles (PicoTip; New Objective, Woburn, MA). A capillary voltage of 1,800 to 2,200 V was applied together with a cone voltage of 80 V. The collision in the collision-induced dissociation was 25 to 35 eV, and argon was used as the collision gas. Data were generated in PKL file format and submitted for database searching in MASCOT server (Matrix Science, Boston, MA) using the NCBI database with the following parameters: trypsin enzyme, one missed cleavage, carbamidomethyl C as fixed modification and oxized (M) as variable modification, and mass tolerance of 150 to 250 ppm. Probability-based MOWSE score was used to determine the level of confidence in the identification of specific isoforms from the mass spectra. This probability equals $10^{(-\text{MOWSE score}/10)}$. MOWSE scores greater than 48 were considered to offer high confidence of identification.

## Gel Electrophoresis and Western Blotting of Sarkosyl-Insoluble Fractions in Alzheimer Disease

Frozen samples of approximately 2 g from the frontal cortex (area 8) were gently homogenized in a glass tissue grinder in 10 vol (w/v) with cold suspension buffer (10 mM TRIS-HCl, pH 7.4, 0.8 M NaCl, 1 mM EGTA, 10% sucrose). The homogenates were first centrifuged at 20,000 rpm and the supernatant (S1) was retained. The pellet was rehomogenized in 5 vol of homogenization buffer and recentrifuged. The 2 supernatants (S1 and S2) were then mixed and incubated with 0.1% N-lauroylsarcosinate (sarkosyl) for 1 hour at room temperature while being shaken. Samples were then centrifuged at 100,000 rpm in a Ti70 Beckman rotor. Sarkosyl-insoluble pellets (P3) were resuspended (0.2 mL/g starting material) in 50 mM TRIS-HCl (pH 7.4). Protein concentrations were determined with the BCA method. Equal amounts of protein (75 μg) were loaded on to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis and then electrophoretically transferred to nitrocellulose membranes (Hybond-C Extra; Amersham) at 400 mA/gel at 4°C. The membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS) and were then incubated with rabbit polyclonal phosphospecific antibodies to tauSer422 (Calbiochem, La Jolla, CA). After washing with TTBS, blots were incubated with anti-rabbit IgG conjugated with horseradish peroxidase 1:1000 (Dako)

for 45 minutes at room temperature. Immunoreactive bands were visualized by chemiluminescence using the ECL method (Amersham).

## RESULTS

### Monodimensional Gel Electrophoresis and Western Blotting

#### Samples Stored at 4°C and Then Frozen

Monodimensional gel electrophoresis and Western blotting in case 1 (samples obtained and frozen at 2 hours (basal values) or stored at 4°C and then frozen at 5, 8, 16, 23, and 50 hours after death showed variable patterns of protein preservation with time (Fig. 1). Some proteins were vulnerable, particularly after 23 hours, whereas others remained practically unaffected at 50 hours. The most resistant proteins were β-actin, p38-P, proteasome 20, and proteasome 11. These proteins were considered suitable for the control of protein loading, and β-actin in particular was used for densitometric studies. Therefore, densitometric values for a given protein were normalized for the corresponding values of β-actin. Examples of the diagrams and densitometric values are shown in Figure 2.

For comparative purposes, proteins were categorized according to the percentage of reduction of the densitometric intensity normalized for β-actin through time of postmortem. Data are summarized as follows: 1) proteins with no reduction at 23 and 50 hours when compared with basal values: p38-P, proteasome 11, β-actin, and proteasome 20S; 2) proteins with a percentage of reduction between 30% and 50% of basal values at 50 hours: SAPK/JNK-P, syntaxin, α-tubulin, and Fyn K; 3) proteins with a percentage of reduction between 60% and 90% from control values at 50 hours: Mek 1, rabphilin, α-synuclein, P-MAPK-ERK44/ERK42, rab3a, AKT-P, β-tubulin, CamK II, Cdk5, EGF-R, TrkB, Bcl-2, GSK Ser9, iNOS, and PLC β1; and 4) percentage of reduction between 40% and 60% at 23 hours: Mek 1, P-MAPK/ERK 44, AKT-P, CamK II, Cdk5, TrkB, Bcl-2, and iNOS.

Interestingly, the expression levels of all the proteins examined were preserved at 5 and 8 hours when compared

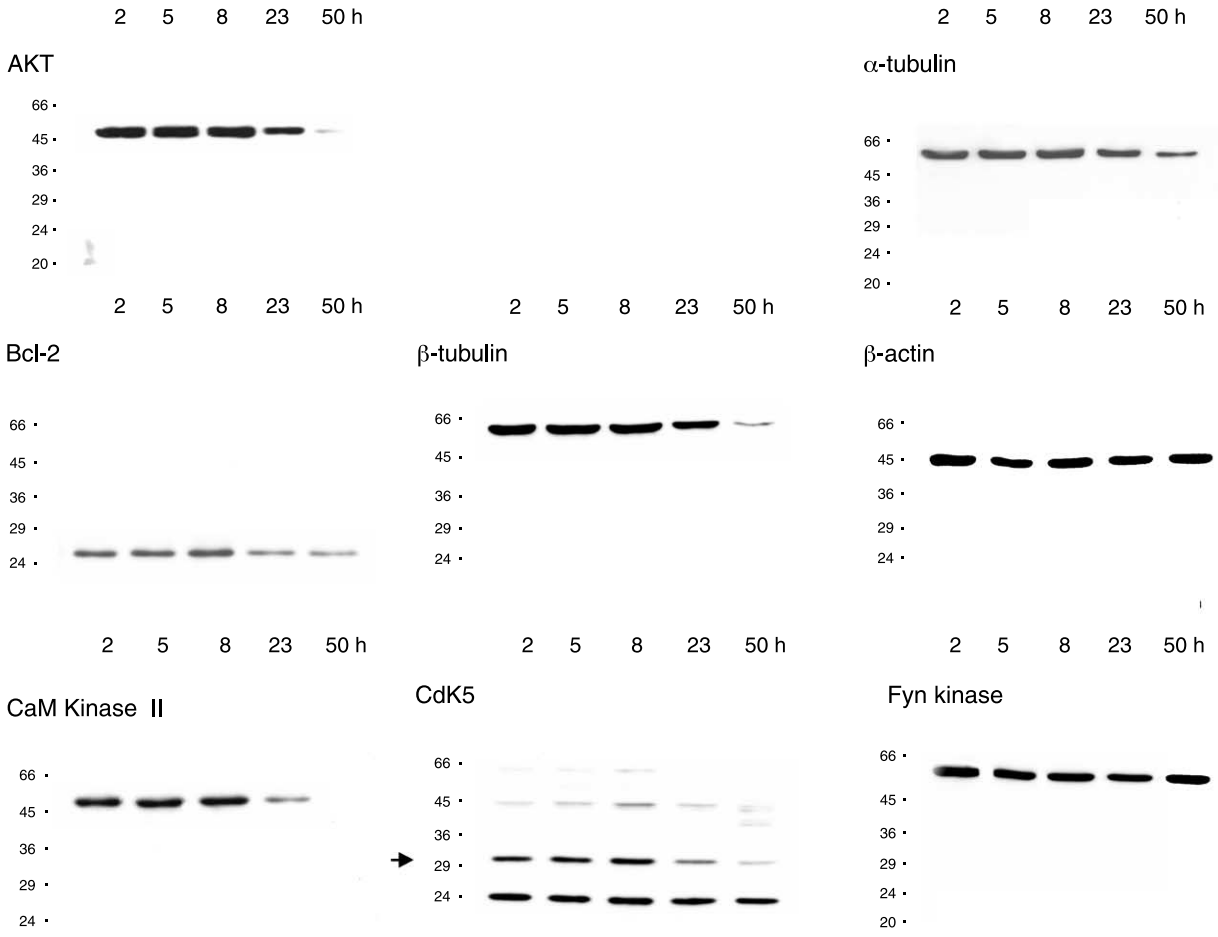
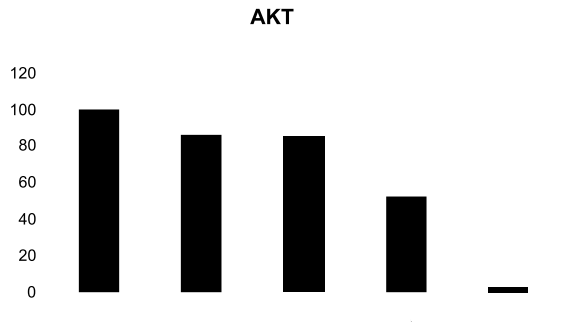
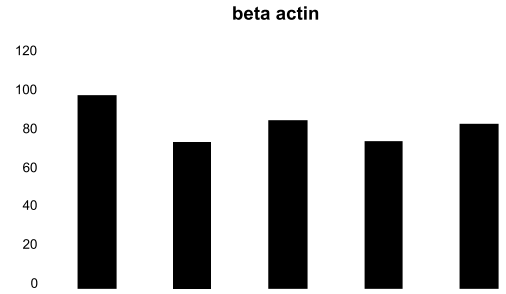



**FIGURE 1.** Western blots to several proteins in frontal cortex homogenates obtained and frozen 2 hours after death or stored for 3, 6, 21, and 48 hours (i.e. 5, 8, 23, and 50 hours of postmortem delay) at 4°C and then frozen at −80°C (case 1). Note that the decrease in the intensity of the bands with time postmortem is variable from one protein to another. Degradation of the majority of proteins occurs at 50 hours.




| Time | Bcl2 values | actin | actin/Bcl/2 | percentage |
|---|---|---|---|---|
| 2,00 | 1.270.085,00 | 1.803.917,00 | 0,70407064 | 100 |
| 5,00 | 1.375.515,00 | 2.138.985,00 | 0,64306903 | 91,3358671 |
| 8,00 | 1.611.141,00 | 2.142.628,00 | 0,75194621 | 106,799824 |
| 23,00 | 814.354,00 | 1.867.727,00 | 0,4360134 | 61,9275076 |
| 50,00 | 674.346,00 | 2.139.399,00 | 0,31520348 | 44,7687287 |


**FIGURE 2.** Examples of protein expression levels as determined by densitometry using Total Lab v2.01 software (Pharmacia, Orsay, France). The results are normalized for β-actin. The numeric data obtained for every protein at a given time period were finally expressed as a percentage of decrease compared with the corresponding basal values. Note variable vulnerability to postmortem delay in samples stored at 4°C and then frozen. The levels of AKT-P are markedly decreased with time, whereas the expression levels of β-actin remain stable. Graphics and numeric data of Bcl-2 have been used as an example.

with basal values. Day-to-day variations were ruled out by carrying out the experiments per triplicate in different days. The same results were obtained in the 3 different days, thus indicating high reproducibility.

### Samples Stored at 1°C and Then Frozen

No modifications in staining were observed in the frontal samples of case 2 obtained and frozen 2 hours and 15 minutes after death (basal values) or stored for 2 hours 45 minutes, 5 hours 45 minutes, 22 hours 45 minutes, and 48 hours (i.e. 5, 8, 23, and 50 hours of postmortem delay) at 1°C, and then frozen at −80°C. Like in the previous paradigm, densitometric studies for every protein were normalized for β-actin.

### Samples Stored at Room Temperature (23°C) and Then Frozen

#### *Monodimensional Gel Electrophoresis and Western Blotting in Cases 3 and 4*

Samples were obtained and frozen at 2 hours (basal values) or stored at 23°C and then frozen at 5, 8, 23, and 50 hours after death showed more severe patterns of protein degradation with time. All proteins examined except proteasome components were reduced at 50 hours. Data are summarized as follows: 1) proteins with no reduction at 23 hours when compared with basal values: p38-P, proteasome 11, β-actin, and proteasome 20S; 2) proteins with a percentage of reduction between 30% and 50% of basal values at 23 hours: SAPK/JNK-P, syntaxin, α-tubulin, Fyn K, and α-synuclein; 3) proteins with a percentage of reduction between 60% and 90% from control values at 23 hours: Mek 1, rabphilin, α-synuclein, P-MAPK-ERK44/ERK42, rab3a, AKT-P, β-tubulin, CamK II, Cdk5, EGF-R, TrkB, Bcl-2, GSK Ser9, iNOS, and PLCβ1; and 4) percentage of reduction between 40% and 60% at 8 hours: Mek 1, P-MAPK/ERK 44, AKT-P, TrkB, and PLCβ1.

### Interlaboratory Variations

Some experiments were carried out in parallel in other laboratories using similar protocols although with individual variations. Four proteins were validated in these studies: β-actin, AKT-P, Cam kinase II, and α-tubulin. Similar results were obtained for the various paradigms in the different laboratories.

## Two-Dimensional Gel Electrophoresis, Silver Staining Detection, In-Gel Digestion, and Mass Spectrometry Analysis

Two-dimensional gels of frontal homogenates of case 2 stored at 1°C for different time periods up to 50 hours postmortem and then frozen at −80°C were stained with silver. Under these specific conditions, no differences were seen in the number and quality of the silver spots at 2 hours 15 minutes, 5, 8, 23, and 50 hours (data not shown).

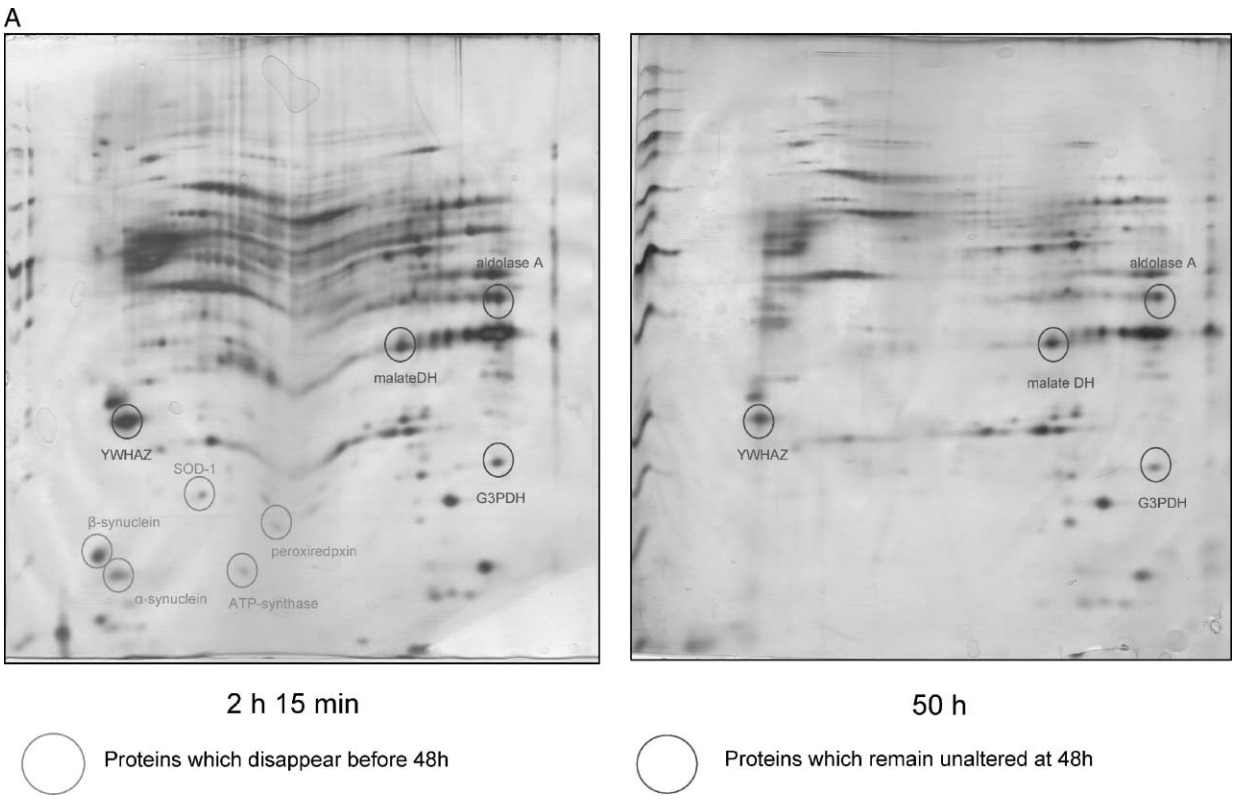



**FIGURE 3.** **(A)** Two-dimensional gels from the same case (case 1) to show spots, the intensity of which is reduced (red circles) or preserved (blue circles) in samples with 50 hours of artificial postmortem delay at 4°C (right panel) when compared with the same case stored for 2 hours and 15 minutes at room temperature and then frozen at −80°C. **(B)** Higher magnification to show reduced intensity of α- and β-synuclein compared with preserved aldolase A expression through time in the same gel. **(C)** Relative reduction of protein levels of α-synuclein and β-synuclein in relation to aldolase A, which appears better preserved with time.

**TABLE 2.** Identification of Proteins Vulnerable to Postmortem Delay in Samples Stored at 4°C for 50 Hours and Then Frozen at −80°C (case 1)

| Spot | Calculated pI | KDa Nominal mass | Protein | Score Coverage | Number of Peptides Matched | G1 Accession Number |
|---|---|---|---|---|---|---|
| 1 | 4.59 | 14.5 | α-synuclein | 69 sequence: 11% | 1 | gi\|1230575 |
| 2 | 4.41 | 14.3 | β-synuclein | 78 sequence: 16% | 2 | gi\|48255903 |
| 3 | 5.54 | 15.9 | Chain E, superoxide dismutase | 55 sequence: 15% | 2 | gi\|349912 |
| 4 | 6.60 | 15.8 | ATP synthase | 73 sequence: 18% | 2 | gi\|51479152 |
| 5 | 5.66 | 22.0 | peroxiredoxin 2 | 158 sequence: 22% | 4 | gi\|77744389 |

Score coverage: MOWSE scores greater than 48 are considered to offer high confidence of identification. Number of peptides indicates the number of peptides used to identify the protein.

In contrast, 2-dimensional gels of frontal homogenates of case 1 stored at 4°C for different time periods up to 50 hours and then frozen at −80°C disclosed marked differences in the silver staining of several spots. Interestingly, several spots progressively decreased in intensity until disappearance at the time point of 50 hours. Yet, other silver spots did not show differences in the intensity of staining (Fig. 3A, B).

Several spots at 2 hours 15 minutes and 50 hours were in-gel-digested and analyzed by mass spectrometry. The proteins resistant to postmortem delay were YWHAZ, G3PDH, malate dehydrogenase, and aldolase A. Several proteins were vulnerable to postmortem delay, including α-synuclein, β-synuclein, peroxiredoxin, ATP synthase, and superoxide dismutase 1 (Fig. 3C; Table 2). A semiquantitative study of selected proteins that are degraded with time is presented in Table 3, in which the percentage of reduction is expressed as a percentage of reduced density values in relation with a control protein (aldolase A), which is more stable during the period of the study.

Two-dimensional gels of frontal homogenates in case 4 in which samples were stored at room temperature (22°C) and then frozen disclosed several differences when comparing gels of 2 hours and 48 hours. Several proteins were identified as vulnerable to postmortem delay, including α-synuclein, β-synuclein, vacuolar proton ATPase, fructose-biphosphate aldolase C, amphiphysin, and α-enolase (Fig. 4; Table 4). Other proteins, dihydropyrimidinase-like 2, manganese superoxide dismutase, G3PDH, 14-3-3, HSP90, and HSPgp96, were resistant. A semiquantitative study of selected proteins that are degraded with time is presented in Table 5, in which the percentage of reduction is expressed as a percentage of reduced density values in relation with a control protein (protein 14-3-3), which is more stable in these experimental circumstances during the period of the study.

## Posttranslational Modifications in Alzheimer Disease: Phospho-tau Pattern in Sarkosyl-Insoluble Fractions

Blots of AD samples at 2 hours stored at 4°C revealed 3 bands of 68, 64, and 60 kDa, several bands between 60 kDa and 24 kDa, and a band of approximately 22 kDa for up to 26 hours in one case, but the phospho-tau bands were markedly decreased at the same time period in another case. Phospho-tau degradation, as revealed by reduction or near disappearance of the bands, was observed at 50 hours postmortem (Fig. 5A, B). More marked and rapid degradation was seen in samples obtained at 6 hours and stored at room temperature for different time periods when compared with the same tissue frozen at 6 hours after death (Fig. 5C). Interestingly, the lower bands were barely visible in this condition. Finally, poor

**TABLE 3.** Percentage of Reduction Through Time of Selected Proteins in Samples Stored at 4°C for 2, 5, 8, 23, and 50 Hours and then Frozen at −80°C (case 1)

| | 2 Hours | 5 Hours | 8 Hours | 23 Hours | 50 Hours | Percent of Reduction (between 2 and 50 hours) |
|---|---|---|---|---|---|---|
| Aldolase A | 100 | 88.6 | 84.7 | 79.3 | 74.2 | 26 |
| α-synuclein | 81.1 | 27.5 | 1.5 | 0.4 | 0.3 | 99.6 |
| α-synuclein | 93.4 | 40.4 | 20.2 | 1.6 | 3.1 | 96.7 |
| SOD1 | 32.1 | 27.9 | 40.5 | 21.2 | 4.8 | 85 |
| ATP-synthase | 12.5 | 8.9 | 3.2 | 1.5 | 3.4 | 72.8 |
| Peroxiredoxin | 21.3 | 6.6 | 11.4 | 11.9 | 4.7 | 77.9 |
| 14-3-3 | 110 | 91.6 | 107 | 102 | 76.6 | 30.4 |

Aldolase A (optical density arbitrarily considered as 100%) is chosen because its preservation was good up to 50 hours postmortem. Numbers in the table express the percentage of optical density when compared with the optical density of aldolase at every time point. Densitometric measurements were obtained by drawing a monodimensional transect across the spot and measuring the optical density along the transect.

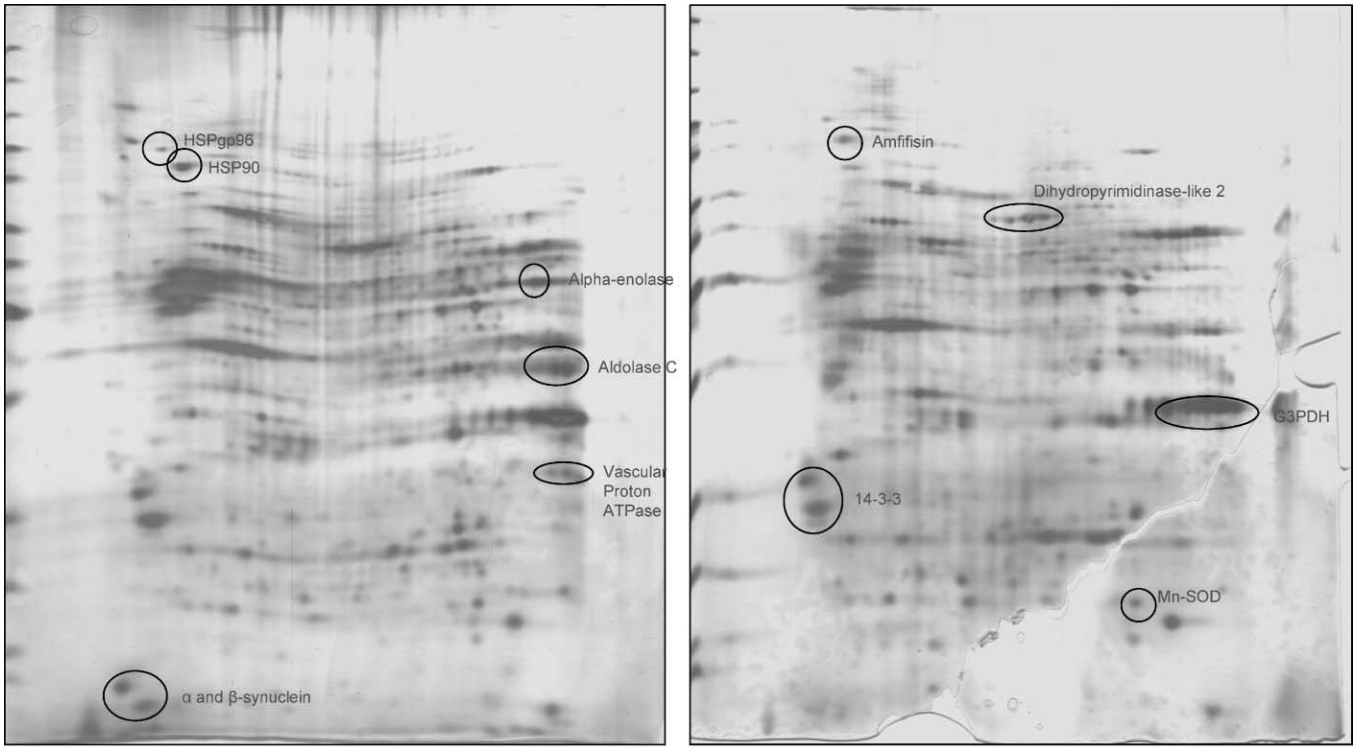



**FIGURE 4.** Two-dimensional gels of frontal cortex homogenates, stained with silver, of case 4 frozen at 2 hours after death or stored at room temperature (22°C) until 48 hours and then frozen. Labeled spots in the left panel correspond to proteins no longer present in samples stored at room temperature for 48 hours. Spots circled in the right panel correspond to proteins that are still present in samples at 48 hours. Vulnerable proteins are α-synuclein, β-synuclein, vacuolar proton ATPase, aldolase C, amphiphysin, and α-enolase. Resistant proteins are dihydropyrimidinase-like 2, manganese superoxide dismutase, G3PDH, 14-3-3, HSP90, and HSPgp96.

results were found in samples obtained at 8 hours after death (with the corpse maintained at room temperature) and then frozen. Moreover, no signal was seen in thawed samples further maintained at room temperature for variable periods and then frozen until use (Fig. 5D).

## DISCUSSION

This study was designed to learn the effects of delay and temperature during the postmortem period in human brain tissue. Other factors such as premortem metabolic status and agonal state were minimized in the present study. The pH of the tissues was neutral at the first time postmortem; thus, further indicating no major metabolic disturbances related with prolonged hypoxia and acidosis before death.

By using monodimensional gel electrophoresis and Western blotting to selected proteins, the present results support the concept that postmortem delay can affect protein levels by selective decay of vulnerable proteins.

**TABLE 4.** Identification of Proteins Vulnerable to Postmortem Delay in Samples Stored at Room Temperature (22°C) for 50 Hours and Then Frozen at −80°C (case 4)

| Spot | Calculated pI | KDa Nominal mass | Protein | Score Coverage | Number of Peptides Matched | G1 Accession Number |
|---|---|---|---|---|---|---|
| 1 | 4.67 | 14.45 | α-synuclein | 628 sequence: 54% | 15 | gi\|80475099 |
| 2 | 4.41 | 14.3 | α-synuclein | 78 sequence: 16% | 2 | gi\|48255903 |
| 3 | 8.45 | 26.3 | Vacuolar proton ATPase | 85 sequence: 34% | 8 | gi\|313014 |
| 4 | 6.41 | 39.8 | Fructose-biphosphate aldolase C | 82 sequence: 21% | 6 | gi\|78070601 |
| 5 | 4,58 | 76,4 | Amphiphysin | 208 sequence: 23% | 14 | gi\|4502081 |
| 6 | 7.01 | 47.5 | α-enolase | 292 sequence: 39% | 16 | gi\|14530765 |

Score coverage: MOWSE scores greater than 48 are considered to offer high confidence of identification. Number of peptides indicates the number of peptides used to identify the protein.

**TABLE 5.** Percentage of Reduction Through Time of Selected Proteins in Samples Stored at Room Temperature (22°C) for 2, 5, 8, 23, and 50 Hours and Then Frozen at −80°C (case 4)

| | 2 Hours | 5 Hours | 8 Hours | 23 Hours | 50 Hours | Percent of Reduction (between 2 and 50 hours) |
|---|---|---|---|---|---|---|
| 14-3-3 | 100 | 75.6 | 75.3 | 70.4 | 67.1 | 32.9 |
| α-synuclein | 91.1 | 47.2 | 42.8 | 56.9 | 0.5 | 99.5 |
| β-synuclein | 45.3 | 53.7 | 36.8 | 35.9 | 1.1 | 97.6 |
| Vacuolar proton ATPase | 84 | 85.2 | 83.2 | 73.5 | 1.4 | 93.3 |
| α-enolase | 74.4 | 37.2 | 43.1 | 8.8 | 1.2 | 98.4 |
| Amphiphysin | 13.7 | 12.8 | 5.4 | 5.3 | 0.3 | 97.8 |
| Aldolase C | 108 | 79.4 | 34.8 | 36.4 | 1 | 99 |

Protein 14-3-3 (optical density arbitrarily considered as 100%) is chosen because its preservation was good up to 50 hours postmortem. Numbers in the table express the percentage of optical density when compared with the optical density of 14-3-3 at every time point. Densitometric measurements were obtained by drawing a monodimensional transect across the spot and measuring the optical density along the transect.

Moreover, reduced temperature during the postmortem period is vital for protein preservation. Although the number of assays was limited, similar observations were obtained in the several laboratories participating in this study, thus indicating that the present observations are applicable to different settings.

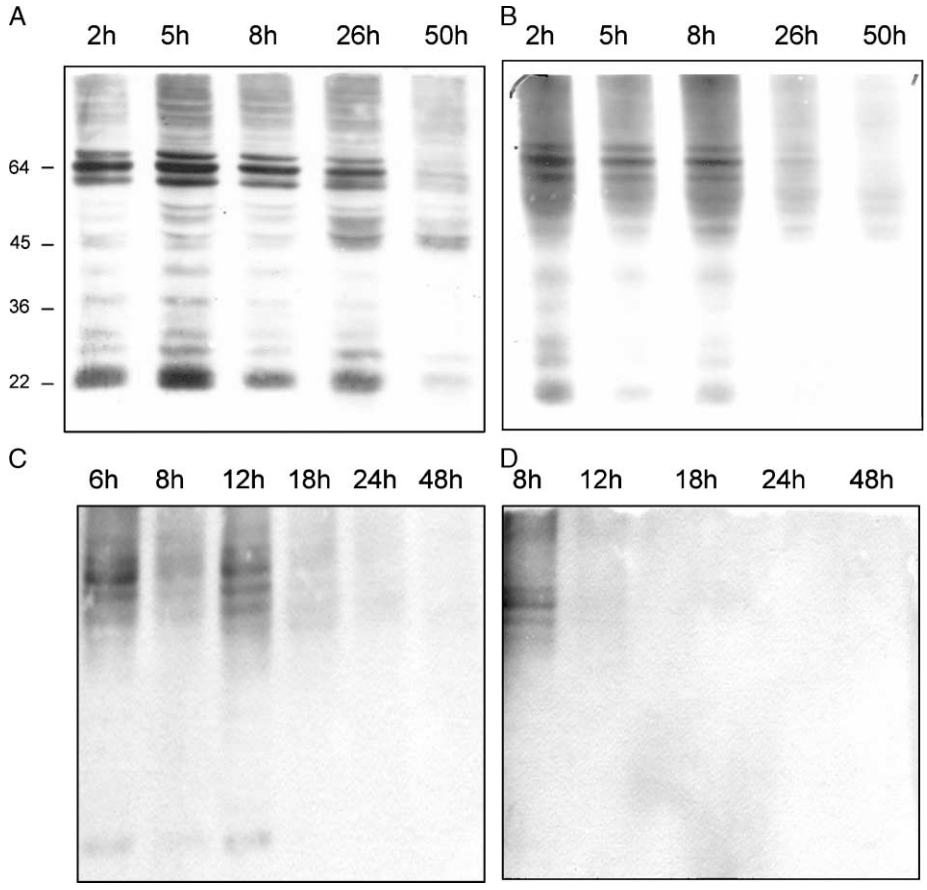



**FIGURE 5.** Effects of artificial postmortem delay in Alzheimer disease as revealed by gel electrophoresis and Western blotting of sarkosyl-insoluble fractions immunostained with specific anti-phospho-tau Ser 422 antibodies. **(A)** Bands of 68, 64, and 60 kDa, several bands between 60 kDa and 24 kDa, and a band of approximately 22 kDa are seen in samples obtained 2 hours after death and then frozen or stored at 4°C at 5, 8, and 26 hours and then frozen. Immunoreactivity decays at 50 hours in one case (case AD1). **(B)** The same apparent conditions result in reduced phospho-tau expression at 26 hours in another (case AD2). **(C)** Reduced immunoreactivity is found in samples obtained 6 hours after death and maintained at room temperature (23°C) and then frozen at progressive time periods. No signal is seen at 18, 24, and 48 hours of artificial postmortem delay (case AD3). **(D)** Very poor results are obtained in postmortem samples obtained at 8 hours and then frozen and later thawed and refrozen at 12, 18, 24, and 48 hours after death (case AD4).

The variable vulnerability of proteins to postmortem delay in the human brain is in line with histochemical and immunohistochemical observations in mice. Hilbig et al studied the effects of postmortem delay and storage temperature on the detection of antigens (13). They found that some proteins were more sensitive to storage temperature, other proteins were more sensitive to postmortem delay, and yet other proteins were always stable under their experimental conditions. The present findings also confirm previous observations on certain proteins such as the relative vulnerability of PLCβ1 (14) and the variable vulnerability of synaptic proteins (4). Synaptophysins are resistant proteins, whereas synucleins are not. It is worth noting that the present study is focused on normal brains and the vulnerability of synucleins refers to "normal" synuclein. It is likely that pathologic proteins forming aggregates such as α-synuclein in Lewy bodies in Lewy body diseases and glial cytoplasmic inclusions in multiple system atrophy are more resistant to degradation, a feature that permits their identification by immunohistochemistry. Reduced protein levels with postmortem may have implications in forensic pathology. Imidazoline receptor-binding protein immunoreactivity is reduced with postmortem delay, a feature that has implications in the postmortem study of depressed suicide victims (15). The effects of postmortem temperature here observed in the human brain are also in accordance with previous one-time studies showing that hypothermia reduces the postmortem degradation of MAP2, whereas normothermia makes MAP2 progressively undetectable with increasing postmortem interval in the rat (16).

Protein degradation in relation to increasing postmortem delay and storage temperature has been further analyzed by 2D gel electrophoresis, in-gel digestion, and mass spectometry in the human brain. Spots corresponding to α-synuclein and β-synuclein gradually disappear with increasing postmortem intervals. Chain E, superoxide dismutase, ATP synthase, and peroxiredoxin 2 are no longer observed at 48 hours. Interestingly, aldolase A and malate dehydrogenase remain unaltered at 50 hours. Storage of the sample at room temperature further disclosed proteins, in addition to synucleins, which were vulnerable to postmortem delay. Vacuolar proton ATPase, fructose-biphosphate aldolase C, amphiphysin, and α-enolase were in this group. Why some proteins are more resistant than others is not clear. It can be suggested that housekeeping proteins are less vulnerable to postmortem delay, whereas proteins related to receptors and certain cytosolic proteins are more prone to degradation. However, this suggestion has to be validated with a more extensive sampling and identification of more proteins.

Postmortem-dependent variable reduction in the levels of proteins has implications not only in the identification of quantitative changes related with pathology in neurologic diseases, but may also have an impact on the study of the function of proteins in the postmortem brain. Preserved levels and activity of cathepsin D are stable for long periods at room temperature in human and mouse postmortem brain (17). Similarly, the expression levels of proteasome subunits are resistant to postmortem delay, and this resistance is accompanied by preserved proteasomal activity in human and mouse brains (18). However, the study of ATP synthase activity in human postmortem samples stored at room temperature for 24 hours can be assumed to be unreliable as deduced from the observed decreased expression levels of the protein at this time point.

Postmortem delay in tissue processing is accompanied by decreased intensity of phospho-tau bands (9). Also, postmortem delay in tissue processing affects the phospho-tau band pattern in AD. This deleterious effect is markedly related with the temperature. Preservation of the samples at room temperature for more than 12 hours makes the biochemical study less reliable. This observation may not be limited to AD, but rather is probably applicable also to the study of other tauopathies. As a marginal note, thawed tissue stored at room temperature and then refrozen is definitely no longer usable for biochemical studies.

Together, these results confirm that the control of brain (body) temperature and the postmortem delay are crucial in the study of proteins in human postmortem samples. The present observations based on mono- and bidimensional gel electrophoresis, Western blotting, and mass spectrometry are useful in determining, on a large scale, the degradation of a brain sample. Therefore, it is probably prudent to carry out preliminary studies mimicking increased postmortem delay under conditions similar to those applied to the sample in question when analyzing the expression of proteins with unknown postmortem preservation (19, 20). Brain banks, as providers of tissue samples for research, as well as neuroscientists, must be concerned about the possibilities and limitations of the human postmortem material available for study.

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*The paper reflects only the authors' views and the community is not liable for any use that may be made of it. The authors thank T. Yohannan for editorial assistance.*

## REFERENCES

1. Buesa C, Maes T, Subirada F, Barrachina M, Ferrer I. DNA chip technology in brain banks: Confronting a degrading world. J Neuropathol Exp Neurol 2004;63:1003–14
2. Li X, Greenwood AF, Powers R, Jope RS. Effects of postmortem interval, age, and Alzheimer's disease on G-proteins in human brain. Neurobiol Aging 1996;17:115–22
3. Siew LK, Love S, Dawbarn D, Wilcock GK, Allen SJ. Measurement of pre- and post-synaptic proteins in cerebral cortex: Effects of postmortem delay. J Neurosci Methods 2004;139:153–59
4. Liu X, Brun A. Synaptophysin immunoreactivity is stable 36 hours postmortem. Dementia 1995;6:211–17
5. Schwab C, Bondada V, Sparks DL, Cahan LD, Geddes JW. Postmortem changes in the levels and localization of microtubule-associated proteins (tau, MAP2 and MAP1B) in the rat and human hippocampus. Hippocampus 1994;4:210–25
6. Irving EA, McCulloch J, Dewar D. The effect of postmortem delay on the distribution of microtubule-associated proteins tau, MAP2, and MAP5 in the rat. Mol Chem Neuropathol 1997;30:253–71
7. Kovács Z, Kékesi KT, Bobest M, et al. Post mortem degradation of nucleosides in the brain: Comparison of human and rat brains for estimation of in vivo concentrations of nucleosides. J Neurosci Meth 2005;148:88–93
8. Puig B, Rey MJ, Ferrer I. Individual and regional variations of phospho-tau species in progressive supranuclear palsy. Acta Neuropathol 2005;110:261–68

9. Santpere G, Puig B, Ferrer I. Low molecular weight species of tau in Alzheimer's disease are dependent on tau phosphorylation sites but not on delayed postmortem delay in tissue processing. Neurosci Lett 2006; 399:106–10
10. Sorimachi Y, Harada K, Yoshida K. Involvement of calpain in postmortem proteolysis in the rat brain. Forensic Sci Int 1996;81:165–74
11. Buée-Scherrer V, Condamines O, Mourton-Gilles C, et al. AD2, a phosphorylation-dependent monoclonal antibody directed against tau proteins found in Alzheimer's disease. Mol Brain Res 1996;39:79–88
12. Braak H, Braak E. Temporal sequence of Alzheimer's disease related pathology. In: Peters A, Morrison JH, eds. *Cerebral Cortex, vol. 14, Neurodegenerative and Age-Related Changes in Structure and Function of Cerebral Cortex.* Kluwer Academic/Plenum Publishers; 1999: 475–512
13. Hilbig H, Bidmon HJ, Oppermann OT, Remmerbach T. Influence of postmortem delay and storage temperature on the immunohistochemical detection of antigens in the CNS of mice. Exp Toxicol Pathol 2004;56: 159–71
14. Garro MA, Lopez de Jesus M, Ruiz de Azua I, Callado LF, Meana JJ, Salles J. Differential postmortem delay effect on agonist-mediated phospholipase Cβ activity in human cortical crude and synaptosomal brain membranes. Neurochem Res 2004;29:1461–65
15. Ma JK, Zhu HE, Piletz JE. Effect of postmortem delay on imidazoline receptor-binding proteins in human and mouse brain. Ann N Y Acad Sci 2003;1009:341–46
16. Kitamura O, Gotohda T, Ishigami A, Tokunaga I, Kubo S, Nakasono I. Effect of hypothermia on postmortem alterations in MAP2 immunostaining in the human hippocampus. Leg Med (Tokyo) 2005;7:340–44
17. Compaine A, Schein JD, Mohan PS, Nixon RA. Limited proteolytic processing of the mature form of cathepsin D in human and mouse brain: Postmortem stability of enzyme structure and activity. Neurochem Int 1995;27:385–96
18. Diaz-Hernandez M, Hernadez F, Martin-Aparicio E, et al. Neuronal induction of the immunoproteasome in Huntington's disease. J Neurosci 2003;23:11653–61
19. Ferrer I, Marín C, Rey MJ, et al. BDNF and full-length and truncated TrkB expression in Alzheimer disease. Implications in therapeutic strategies. J Neuropathol Exp Neurol 1999;58:729–39
20. Ferrer I, Blanco R, Carmona M, et al. Phosphorylated MAP kinase (ERK1, ERK2) expression is associated with early tau deposition in neurones and glial cells, but not with increased nuclear DNA vulnerability and cell death, in Alzheimer disease, Pick's disease, progressive supranuclear palsy and corticobasal degeneration. Brain Pathol 2001;11:144–58

# 2

## Low molecular weight species of tau in Alzheimer's disease are dependent on tau phosphorylation sites but not on delayed post-mortem delay in tissue processing

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# Low molecular weight species of tau in Alzheimer's disease are dependent on tau phosphorylation sites but not on delayed post-mortem delay in tissue processing

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**Abstract**

Gel electrophoresis and Western blotting of sarkosyl-insoluble fractions enriched in hyper-phosphorylated tau in Alzheimer disease (AD) have been used to analyze the pattern of phospho-tau by using different antibodies directed to the amino-terminal, core and carboxyl terminus of tau, and by using samples with increased artificial post-mortem delay in order to gain understanding on the characteristics of the band pattern and its vulnerability to post-mortem degradation. In addition to the typical profile of three major bands of 68, 64 and 60 kDa, several bands of lower molecular weight have been distinguished in frontal cortex homogenates in four AD cases stage V of Braak and Braak in optimal samples with 2 h of post-mortem delay. Lower bands, ranging from 60 to 22 kDa, are best seen with antibodies directed to the core of tau protein and, particularly, to the carboxy-terminus, thus suggesting the presence of truncated or cleaved forms of tau containing the C-terminal region. This pattern is not the result of post-mortem degradation, as artificial post-mortem delay of the same sample does not reveal the appearance of new bands with time. On the contrary, tau degradation, manifested as a reduction in the number and intensity of the bands, may occur between 8 and 26 h post-mortem and is universal in samples with post-mortem delays of 50 h.


*Keywords:* Alzheimer disease; Tau; Truncated tau; Post-mortem delay; Brain bank

One of the pathological hallmarks of Alzheimer's disease (AD) is the intracellular accumulation and aggregation of hyper-phosphorylated microtubule-associated protein tau in the form of paired helical filaments (PHF) and straight filaments localized in neurofibrillary tangles, neuropil threads and dystrophic neurites surrounding β-amyloid plaques. Accumulation of hyper-phosphorylated tau is also a common feature in other tauopathies including progressive supranuclear palsy (PSP), corticobasal degeneration, argyrophilic grains disease, Pick's disease and frontotemporal dementia and parkinsonism linked to mutations in the tau gene [15]. Tau is found in the brain in six isoforms generated by alternative splicing from a single gene which is located in chromosome 17q21, with N-terminal inserts of 0 (0N), 29 (1N) or 58 (2N) amino acids, in combination with three (3R) or four (4R) microtubule-binding repeat regions at the C-terminal region [1,9–11]. In AD, gel electrophoresis and Western blotting of fractions enriched in PHFs have classically disclosed a pattern of three bands of 68, 64 and 60 kDa, often accompanied by a weak fourth band of about 72 kDa in advanced cases, as revealed with several phospho-specific anti-tau antibodies. These bands derive from the six full-length tau isoforms in their insoluble form detected after dephosphorylation. In addition to these bands, other bands of lower molecular weight have been recognized with a variety of anti-tau antibodies [9,14,16–18]. Although the nature of these bands appears to be related to truncated tau, it is not clear whether they correspond to amino-terminal or C-terminal fragments (or both), and whether these bands are influenced by fragmentation of tau during the process of post-mortem delay.

The present study is geared toward increasing understanding of the banding profile of tau from sarkosyl-insoluble fractions in AD considering two variables: antibodies directed against different regions of tau, and effects of post-mortem interval between death and tissue processing.

Studies were carried out in the frontal cortex of four AD cases stage V of Braak and Braak, artificially subjected to increasing

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post-mortem delay. Clinically, the patients had suffered from severe dementia of Alzheimer type following the diagnostic criteria of the NINCDS-ADRDA, and all of them had a clinical dementia rating scale stage 3. Brain tissues were obtained as the result of a generous donation for research to the Institute of Neuropathology following strict criteria of full disclosure and approval by the Ethics Committee of the IDIBELL-Hospital Universitari de Bellvitge. The post-mortem neuropathological examination was carried out on multiple and representative formalin-fixed, paraffin-embedded sections which were stained with haematoxylin and eosin, Klüver-Barrera, and immunohistochemistry to phospho-tau (antibody AT8), β-amyloid ($A\beta_{1-40}$ and $A\beta_{1-42}$), α-synuclein, ubiquitin, phosphorylated neurofilament epitopes, glial fibrillary acidic protein, αB-crystallin and *Licoperium esculentum* lectin for microglia. AD was the only relevant brain pathology which was staged following the instrumental criteria of Braak and Braak [3]. Samples were excluded based on evidence of fever, seizures, infection, metabolic disturbances and drugs after revision of the clinical records.

Brain samples were obtained 2 h after death and rapidly frozen at −80 °C until use, or stored at 4 °C and then frozen at 3, 6, 24 and 48 h of additional artificial post-mortem interval (in fact 5, 8, 26 and 50 h of post-mortem). The pH of the brain samples was between 6.8 and 7.1. Frozen samples of about 2 g from the frontal cortex (area 8) were gently homogenized in a glass tissue grinder in 10 volumes (w/v) with cold suspension buffer (10 mM Tris–HCl, pH 7.4, 0,8 M NaCl, 1 mM EGTA, 10% sucrose). The homogenates were first centrifuged at 20,000 × *g* and the supernatant (S1) was retained. The pellet was re-homogenized in 5 volumes of homogenization buffer and re-centrifuged. The two supernatants (S1 + S2) were then mixed and incubated with 0.1% *N*-lauroylsarkosynate (sarkosyl) for 1 h at room temperature while being shaken. Samples were then centrifuged at 100,000 × *g* in a Ti70 Beckman rotor. Sarkosyl-insoluble pellets (P3) were re-suspended (0.2 ml/g starting material) in 50 mM Tris–HCl (pH 7.4). Protein concentrations were determined with the BCA method. Equal amounts of protein (75 μg) were loaded onto 10% sodium dodecylsulfate polyacrilamide gel electrophoresis and then electrophoretically transferred to nitrocellulose membranes (Hybond-C Extra, Amersham, Freiburg, Germany) at 400 mA/gel at 4 °C. The membranes were blocked for 1 h at room temperature with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS) and were then incubated with the primary antibodies. After washing with TTBS, blots were incubated with the secondary antibody (anti-mouse/anti-rabbit IgG conjugated with horseradish peroxidase 1:1000, DAKO, Denmark) for 45 min at room temperature. Immunorreactive bands were visualized by chemiluminescence using the ECL method (Amersham). Western blot analysis was carried out using several anti-tau antibodies. The monoclonal antibody tau-13 (MBL, Nagoya, Japan) is raised against the amino-terminal domain of tau (2–18 residues from the longest tau isoform), and was used at a dilution of 1:1000. The goat polyclonal antibody N-terminus (Chemicon International, Hampshire, UK), used at a dilution 1:2000, is directed against amino acids 1–16 of the human tau protein. Rabbit polyclonal phospho-specific antibodies to tau Thr212 and tau Ser214, both used at a dilution of 1:500, were used to map the core of phospho-tau. Rabbit polyclonal phospho-specific antibodies to tau tauSer396 and tauSer422, both used at

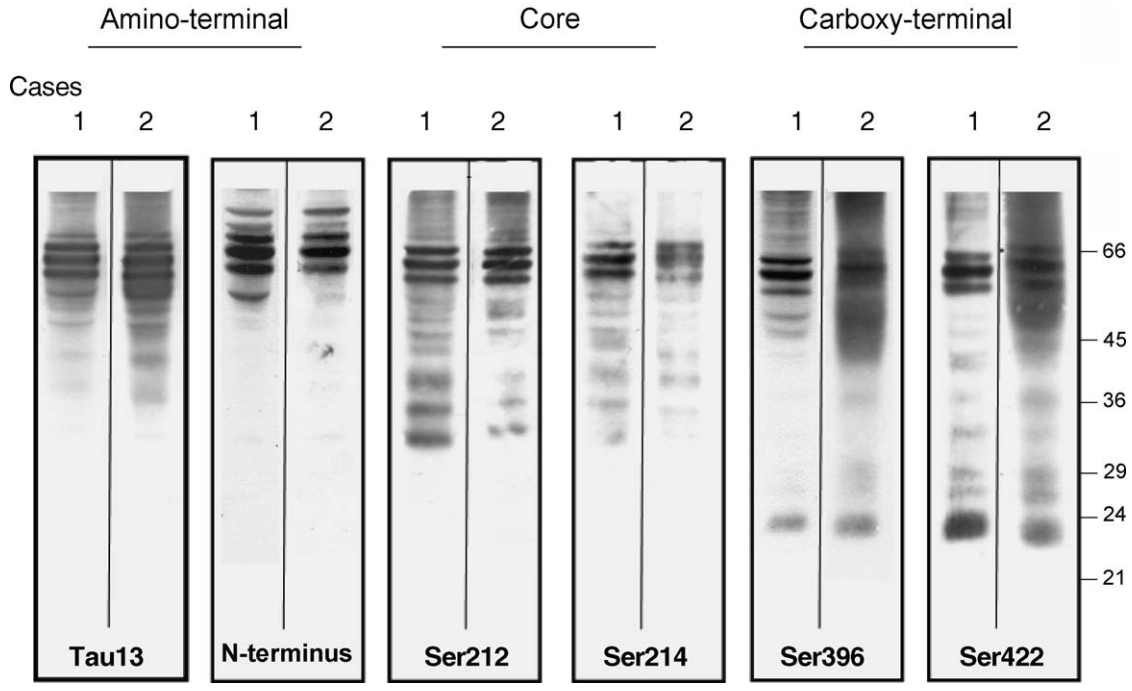


Fig. 1. Western blots of frontal cortex (area 8) homogenates from two cases (1 and 2) of AD, Braak and Braak stage V, at 2 h of post-mortem delay analysed with antibodies to the amino-terminal, core and carboxy-terminal regions of tau. The typical three-band pattern of 68, 64 and 60 kDa is found independently of the antibody used. However, the number of bands increases when using phospho-tau antibodies directed to the core region. The carboxy-terminal band of 22 kDa is only observed with phospho-specific anti-tauSer396 and Ser422 antibodies.

a dilution of 1:1000, were used to detect de C-terminal domain of phospho-tau protein. All phospho-specific anti-tau antibodies were purchased from Calbiochem (La Jolla, CA, USA).

Two different antibodies for N-terminal, core and C-terminal regions of tau protein were used in order to detect duplicates of possible differences in the tau profile banding, depending on target regions of tau. The examination of samples with optimal preservation (2 h) revealed that all the antibodies detected the same three-bands at 68, 64 and 60 kDa (Fig. 1). In addition, the antibodies tau-13 and N-terminus, which are directed to the amino-terminus, detected a few weak bands between 60 and 36 kDa. However, antibodies to phospho-tauThr212 and phospho-tauSer214, directed to the core of phospho-tau protein, detected several bands between 60 and 29 kDa. The lower molecular weight species of about 37, 32 and 29 kDa were moderately immunoreactive. Finally, antibodies to phospho-tauSer396 and phospho-tauSer422, both directed against the carboxyl terminus, detected several bands between 60 and 24 kDa. A well-defined band of about 22 kDa was only recognized with these two anti-phospho-tau antibodies directed to the C-terminal domain of phospho-tau (Fig. 1).

To test whether these patterns were modified with post-mortem delay (a common scenario when using human post-mortem material), the effects of post-mortem delay were examined by processing, in parallel, samples with post-mortem delays of 2, 5, 8, 26 and 50 h, using antibodies directed to the amino-terminal (tau-13), core (phospho-tauThr212) and carboxy-terminus (phospho-tauSer422).

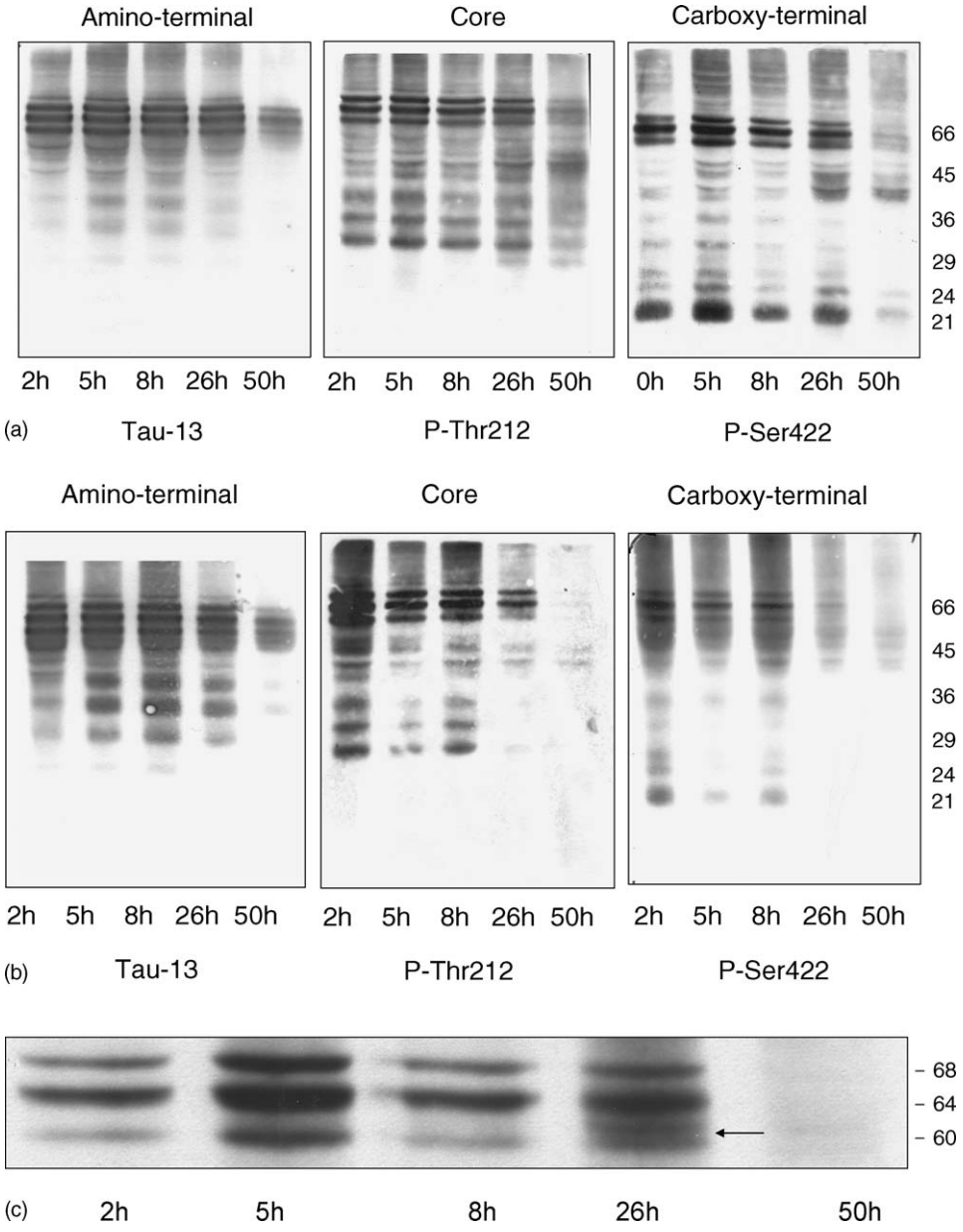


Fig. 2. Effect of artificial post-mortem delay in tau banding pattern in two AD cases. Samples of 2, 5, 8, 26 and 50 h of post-mortem delay are processed in parallel with the antibody tau-13 and phospho-specific antibodies raised against phospho-tauThr212 and phospho-tauSer422. (a) This case shows a degraded tau profile at 48 h. (b) This case shows marked tau degradation at 26 and 50 h. (c) High magnification of the first case to show a doublet of 60–62 kDa at 26 h.

Tau degradation, as revealed by the reduction or near disappearance of the bands, occurred in every case with 50 h of post-mortem (Fig. 2a). However, degradation was already observed at 26 h of post-mortem delay in some cases (Fig. 2b). Modifications over time in the banding pattern were more marked when using phospho-specific anti-tau antibodies than with pan-tau antibodies, suggesting that phospho-tau is subjected to post-mortem degradation of endogenous phosphatases.

The typical three bands of 68, 64 and 60 kDa were well defined in all non-degraded samples in every case, but a double-band of 60–62 kDa was observed at 26 h in one case (Fig. 2a and c). This band was detected with all anti-phospho-tau antibodies, but not with the antibody tau-13, thus suggesting that this doublet is the result of partial tau dephosphorylation with increasing post-mortem time.

The present findings show that tau profile bands are dependent on the phospho-specific anti-tau antibody. Antibodies directed against C-terminal epitopes detect more bands than those directed to the core or the N-terminus. The strong immunoreactive band of 22 kDa is detected only with phospho-tau antibodies raised against the carboxy terminal of phospho-tau.

Recent studies have shown enzymatic cleavage of tau in AD by several proteases such as caspase 3, caspase 6, calpain-1 and thrombin [2,7,8,12]. As a result of these observations, it has been proposed that abnormal phospho-tau proteolysis may alter tau turnover, thereby triggering intracellular accumulation [13]. Other studies point to the neurotoxicity of certain tau cleavage products, particularly those related with the C-terminal region [4–6]. Moreover, PHFs are composed of a protease-sensitive coat and a protease resistant core [21]. The core is enriched in C-terminal fragments of tau, and the minimum protease-resistant fragment of tau is found around the glutamic acid at position 391 [18,20]. Together, these findings are consistent with the idea that the band profile of phospho-tau in AD is more complex and functionally relevant than previously supposed.

The present observations in AD are similar to those recently described in PSP [19] in which complex band patterns are partly related with the phospho-specific anti-tau antibody used, thus indicating variable products of truncated or cleaved tau as common features in tauopathies.

Finally, the present observations show that the complex band pattern is not dependent on post-mortem degradation, as no differences were seen between 2 and 8 h of post-mortem delay. In fact, reduced numbers of bands of low molecular weight rather than newly appearing bands are seen with sub-optimal preservation. These findings may have practical implications in the study of phospho-tau profiles in other tauopathies.

Nonetheless, longer post-mortem delays may affect phospho-tau examination, as degrading phospho-tau is currently observed at 50 h and may also be present in samples with artificial post-mortem delay of 26 h. It is, however, worth stressing that the present paradigm refers to samples obtained at 2 h of post-mortem in cases in whom the body was maintained at room temperature from the time of death until the autopsy. Then, selected brain samples were stored at 4 °C and frozen at progressive intervals. This scenario is not frequent in the common practice in which the body is usually kept at room temperature for longer periods. Therefore, one might predict a more rapid protein degradation in these cases.

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## References

[1] A. Andreadis, W.M. Brown, K.S. Kosik, Structure and novel exons of the human tau gene, Biochemistry 31 (1992) 10626–10633.
[2] T. Arai, J. Guo, P. McGeer, Proteolysis of non-phosphorylated and phosphorylated tau by thrombin, J. Biol. Chem. 280 (2005) 5145–5153.
[3] H. Braak, E. Braak, Temporal sequence of Alzheimer's disease related pathology, in: A. Peters, J.H. Morrison (Eds.), Cerebral Cortex, vol. 14: Neurodegenerative and Age-related Changes in Structure and Function of Cerebral Cortex, Kluwer Academic/Plenum Publishers, New York, Boston, Dordrecht, 1999, pp. 475–512.
[4] N. Canu, L. Dus, C. Barbato, M.T. Ciott, C. Brancolini, A.M. Rinaldi, M. Novak, A. Cattaneo, A. Bradbury, P. Calissano, Tau cleavage and dephosphorylation in cerebellar granule neurons undergoing apoptosis, J. Neurosci. 18 (1998) 7061–7074.
[5] C.W. Chung, Y.H. Song, I.K. Kim, W.J. Yoon, B.R. Ryu, D.G. Jo, H.N. Woo, Y.K. Kwon, H.H. Kim, B.J. Gwag, I.H. Mook-Jung, Y.K. Jung, Pro-apoptotic effects of tau cleavage product generated by caspase-3, Neurobiol. Dis. 8 (2001) 162–172.
[6] L. Fasulo, G. Ugolini, M. Visintin, A. Bradbury, C. Brancolini, V. Verzillo, M. Novak, A. Cattaneo, The neuronal microtubule-associated protein tau is a substrate for caspase-3 and an effector of apoptosis, J. Neurochem. 75 (2000) 624–633.
[7] T.C. Gamblin, F. Chen, A. Abraha, R. Miller, Y. Fu, F. Garcia-Sierra, S. Lagalwar, R.W. Berry, L.I. Binder, V.L. Cryns, Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 10032–10037.
[8] F. García-Sierra, N. Ghoshal, B. Quinn, R. Berry, L.I. Binder, Conformational changes and truncation of tau protein during tangle evolution in Alzheimer's disease, J. Alzheimer's Dis. 5 (2003) 65–77.
[9] M. Goedert, M.G. Spillantini, N.J. Cairns, R.A. Crowther, Tau-proteins of Alzheimer paired helical filaments—abnormal phosphorylation of all six brain isoforms, Neuron 8 (1992) 159–168.
[10] M. Goedert, M.G. Spillantini, R. Jakes, F.A. Crowther, E. Vanmechelen, A. Probst, J. Gostz, K. Burki, P. Cohen, Molecular dissection of the paired helical filament, Neurobiol. Aging 16 (1995) 325–334.
[11] M. Goedert, M.G. Spillantini, R. Jakes, D. Rutherford, R.A. Crowther, Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimers disease, Neuron 3 (1989) 519–526.
[12] P.M. Horowitz, K.R. Patterson, A.L. Guillozet-Bongaarts, M.R. Reynolds, C.A. Carroll, S.T. Weintraub, D.A. Bennett, V.L. Cryns, R.W. Berry, L.I. Binder, Early N-terminal changes and caspase-6 cleavage of tau in Alzheimer's disease, J. Neurosci. 24 (2004) 7890–7895.
[13] K. Ikeda, H. Akiyama, T. Arai, H. Kondo, C. Haga, S. Iritani, K. Tsuchiya, Alz-50/Gallyas-positive lysosome-like intraneuronal granules in Alzheimer's disease and control brains, Neurosci. Lett. 258 (1998) 113–116.
[14] G.V. Johnson, P. Seubert, T.M. Cox, R. Motter, J.P. Brown, D. Galasko, The protein in human cerebrospinal fluid in Alzheimer's disease consists of proteolytically derived fragments, J. Neurochem. 68 (1997) 430–433.
[15] V.M. Lee, M. Goedert, J.Q. Trojanowski, Neurodegenerative tauopathies, Annu. Rev. Neurosci. 24 (2001) 1121–1159.
[16] M. Mercken, F. Grynspan, R.A. Nixon, Differential sensitivity to proteolysis by brain calpain of adult human tau, fetal human tau and PHF-tau, FEBS Lett. 368 (1995) 10–14.

[17] M. Novak, R. Jakes, P.C. Edwards, C. Milstein, C.M. Wischik, Difference between the tau protein of Alzheimer paired helical filament core and normal tau revealed by epitope analysis of monoclonal antibodies 423 and 7.51, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 5837–5841.
[18] M. Novak, J. Kabat, C.M. Wischik, Molecular characterisation of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament, EMBO J. 12 (1993) 365–370.
[19] B. Puig, M.J. Rey, I. Ferrer, Individual and regional variations of phospho-tau species in progressive supranuclear palsy, Acta Neuropathol. 110 (2005) 261–268.
[20] R. Skrabana, P. Kontsek, A. Mederlyova, K. Iqbal, M. Novak, Folding of Alzheimer's core PHF subunit revealed by monoclonal antibody 423, FEBS Lett. 568 (2004) 178–182.
[21] C.M. Wischik, M. Novak, H.C. Thogersen, P.C. Edwards, M.J. Runswick, R. Jakes, J.E. Walker, C. Milstein, M. Roth, A. Klug, Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease, Proc. Natl. Acad. Sci. U.S.A. 85 (1998) 4506–4510.

# 3

## Expression of transcription factors c-Fos, c-Jun, CREB-1 and ATF-2, and caspase-3 in relation with abnormal tau deposits in Pick's disease.

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ORIGINAL PAPER

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# Expression of transcription factors c-Fos, c-Jun, CREB-1 and ATF-2, and caspase-3 in relation with abnormal tau deposits in Pick's disease

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**Abstract** Hyper-phosphorylated tau deposition in Pick bodies and neuron loss are major hallmarks of Pick's disease (PiD). However, there is no regional correlation between neuron loss and Pick bodies, as illustrated in dentate gyrus, where Pick bodies are present in almost every neuron, whereas cell death, if present, is not a major event. In order to better understand the possible role of selected transcription factors and members of the caspase family in cell death and cell survival, immunohistochemistry to c-Fos, c-Jun, CREB-1, ATF-2; c-Fos$^P$, c-Jun$^P$ and CREB-1$^P$; and procaspase-8, procaspase-3 and active (cleaved) caspase-3 immunohistochemistry was carried out in the frontal cortex and hippocampus. Increased expression of c-Fos, c-Jun, CREB-1 and ATF-2 was observed in PiD cases. Increased c-Fos$^P$, c-Jun$^P$ and CREB-1$^P$ was also found in the nuclei of neurons in diseased brains. Interestingly, c-Fos but not c-Fos$^P$ co-localized in many Pick bodies, as observed by double labelling-immunofluorescence and confocal microscopy. Pro-caspase-8 and pro-caspase-3 were increased in PiD. Moreover, granular active caspase-3 was observed in the nuclei as was aggregated active caspase-3 in the cytoplasm of neurons in PiD. Finally, double-labelling immunofluorescence and confocal microscopy disclosed co-localization of cytoplasmic active caspase-3 only in neurons with Pick bodies. Together, these findings show an increased expression of selected transcription factors and active (phosphorylated) forms in PiD, c-Fos sequestration in Pick bodies, and increased active caspase-3 expression in relation with Pick bodies. Since all these findings were observed equally in neurons of both vulnerable regions (frontal cortex) and resistant regions (dentate gyrus), it may be suggested that transcription factors are only barely related with cell death. Active caspase-3 is associated with tau deposition in Pick bodies, but it is not a marker of cell death in the dentate gyrus in PiD. The present findings are in line with the previous studies showing tau products cleaved by caspase-3, as recognized by specific tau-cleaved antibodies, in Alzheimer's disease and other tauopathies.

**Keywords** Pick's disease · tau · c-Fos · c-Jun · CREB · ATF-2 · caspase-3

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## Introduction

Pick's disease (PiD) is a fronto-temporal dementia characterized by marked neuron loss, mainly in the upper cortical layers, and the appearance of typical phospho-tau-immunoreactive intraneuronal inclusions named Pick bodies principally in the dentate gyrus, CA1 region of the hippocampus and upper layers of the entorhinal cortex and neocortex, together with phospho-tau-immunoreactive inclusions in astrocytes and oligodendroglia [3, 6, 12, 29]. Intraneuronal inclusions do not match neuron loss; specifically, Pick bodies are encountered in the majority of, if not all, granule cells of the dentate gyrus, although these neurons are apparently preserved in terms of cell survival. The reasons for this selective vulnerability are not known although specific signals of cell death and cell survival are probably involved in this process.

Inducible transcription factors of the Jun and Fos families, and constitutive transcription factors activating transcription factor-2 (ATF-2) and calcium/cAMP response element binding protein (CREB), have been implicated in a large number of cellular events including

regulation of cell survival, as derived from in vitro and in vivo paradigms [9, 20, 22, 23, 25, 37, 39].

Transcription factor activator protein 1 (AP1) composed of jun:jun dimers seems to play a pivotal role in the regulation of amyloid precursor protein (APP) production in Alzheimer's disease (AD) [28, 38]. Increased c-jun immunoreactivity has been associated with apoptosis in AD [2]. c-Fos immunoreactivity is increased in certain areas of the hippocampus in patients with AD [1, 30, 31, 41]. Yet practically nothing is known about transcription factors and PiD.

The present study is focused on the expression of c-Fos, c-Jun, CREB-1 and ATF-2 in the hippocampus (particularly in the dentate gyrus) and frontal cortex in PiD cases in order to gain insights concerning the association of these factors with cell survival. In addition, expression of pro-caspase-3 and 8, and cleaved (active) caspase-3 (17 kDa), which are crucial in apoptotic pathways [13], has been examined in the same regions to increase the understanding of the possible role of caspases in relation with tau deposits in PiD.

## Materials and methods

### Subjects

Samples of the hippocampus and frontal cortex (area 8 Brodmann) were obtained from four men with PiD and five control cases (two men and three women with no neurological disease), with ages ranging from 56 to 68 years, and from 55 to 73 years, respectively. The delay between death and tissue processing did not exceed 14 h in control or diseased brains. Human brain tissue in this study was provided by the Institute of Neuropathology and University of Barcelona/Hospital Clinic Brain Banks following the guidelines of the local ethics committees. At autopsy, half of the brain was fixed in 10% formalin for no less than 3 weeks, whereas the other half was cut in coronal sections 1 mm thick, frozen on dry ice and stored at −80°C until use. Control and diseased cases were processed in parallel. Clinical and neuropathological findings in PiD were in accordance with well-established criteria [6]. Optimal preservation of these samples for functional studies has been reported elsewhere [35]. Neurofibrillary tangles, senile plaques, and synuclein deposits were not observed in PiD cases. Control cases had no neurological or metabolic disease, and the neuropathological study was normal. The clinical and neuropathological findings of control and PiD cases are detailed elsewhere [10].

### Gel electrophoresis and Western blotting

Western blots were carried out with total homogenates of the hippocampus from PiD and control brains processed in parallel. Samples about 1.5 g were dissected and homogenized in a glass homogenizer containing 1.5 ml of PBS and a protease inhibitor cocktail (Boehringer Mannheim). The homogenates were then sonicated and centrifuged at $2{,}650 \times g$ at 4°C for 10 min and at $100{,}000 \times g$ at 4°C for 1 h. Supernatants of total homogenates were collected and protein concentrations were determined using the BCA method (Pierce) with bovine serum albumin as a standard. Equal amounts of protein (40–50 μg) were loaded onto 7.5–10% sodium dodecylsulfate polyacrylamide gels, and then proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-C Extra, Amersham) at 200 mA/gel and 4°C. The membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 1 h at room temperature and incubated with one of the primary antibodies, as follows: mouse anti-human PHF-tau (clone AT8, Innogenetics) at a dilution of 1:80; rabbit polyclonal anti-c-Fos (Santa Cruz Biotechnology) at a dilution of 1:200; anti c-fos (Oncogene science, Calbiochem) at a dilution 1:250; phospho-specific monoclonal anti-c-Fos$^{P}$ (Ser$^{374}$) (Calbiochem) at a dilution of 1:100; rabbit polyclonal anti-c-Jun (AB1, Oncogene) diluted 1:100; phospho-specific polyclonal rabbit anti-c-Jun$^{P}$ (Ser$^{63}$) (Cell Signalling) diluted 1:100; rabbit anti-CREB-1 (Santa Cruz Biotechnology) at 1:500; phospho-specific rabbit anti-CREB-1$^{P}$ (Ser$^{133}$) (Cell Signalling) at 1:100; rabbit anti-ATF-2 (Santa Cruz Biotechnology) at 1:200; and phospho-specific rabbit anti-ATF-2$^{P}$ (Thr69/71) (Cell Signalling) at 1:100. After washing with TTBS, blots were incubated with the corresponding secondary antibody conjugated with horseradish peroxidase (Dako) at a dilution of 1:1000 for 45 min at room temperature. Immunoreactive bands were visualized by chemiluminescence using the ECL method (Amersham). The same membranes were incubated after stripping with anti-$\beta$-actin (Sigma) at a dilution of 1:1000 for the control of protein loading.

### Immunohistochemistry

Sections 40 μm thick were processed free-floating with the labelled streptavidin–biotin method (Dako LSAB+ kit, Dako) following the instructions of the supplier. Briefly, after blocking endogenous peroxidases, sections were blocked in 10% normal horse serum for 2 h and then incubated overnight at room temperature with the same primary antibodies used for western blotting. In addition, rabbit anti-pro-caspase-3 at a dilution of 1:200, mouse anti-pro-caspase-8 at 1:200, and rabbit anti-cleaved caspase-3 (Asp$^{175}$) at 1:100 (all of them from Cell Signalling) were also employed. After washing, the sections were then incubated with LSAB and streptavidin–peroxidase for 15 min each at room temperature. The peroxidase reaction was visualized as a dark blue precipitate with $NH_4NiSO_4$ (0.05 M) in phosphate buffer (0.1 M), 0.05% diaminobenzidine, $NH_4Cl$ and 0.01% hydrogen peroxide. Some sections were stained without the primary antibody to rule out non-specific immunoreactivity.

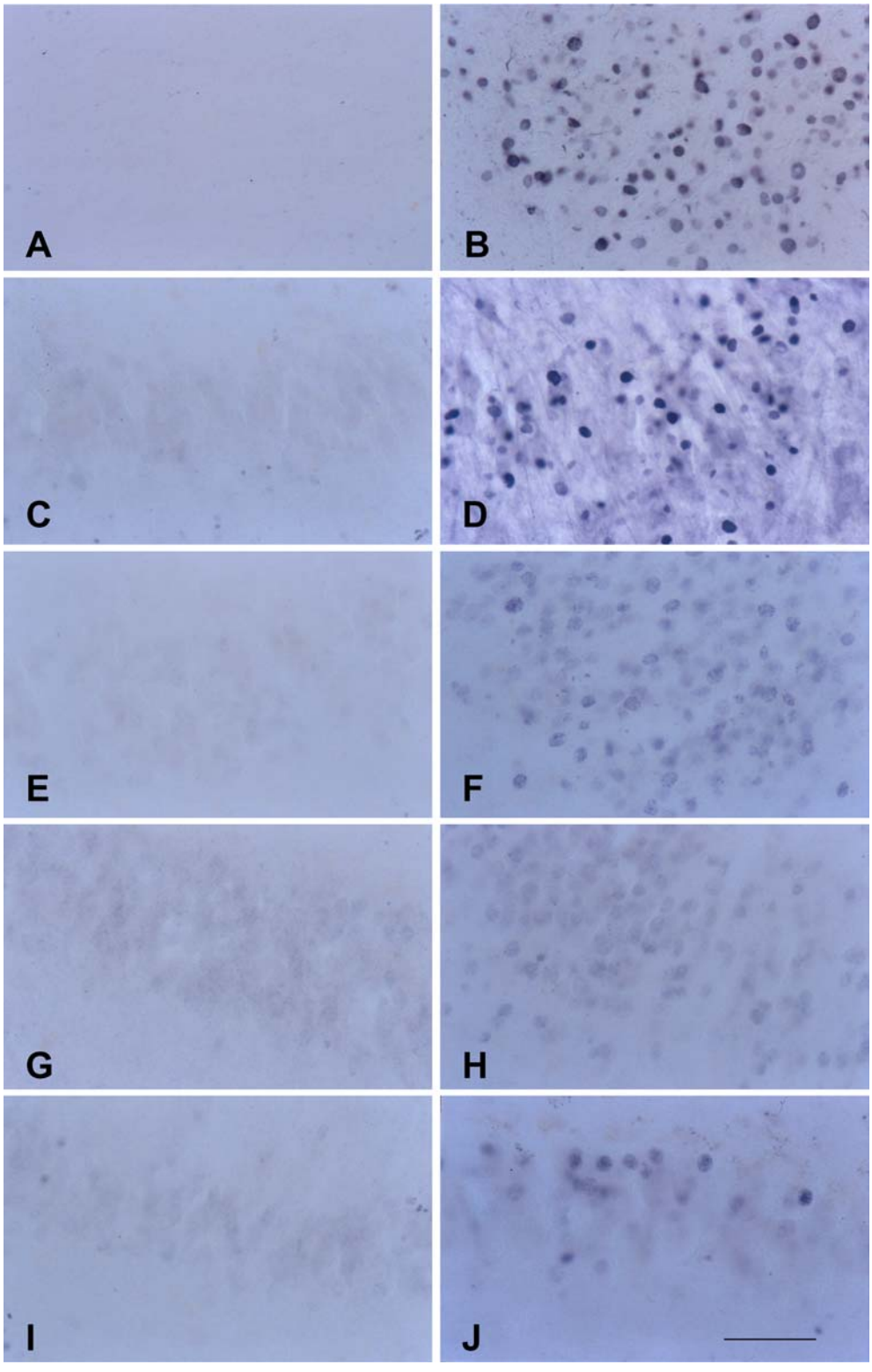


**Fig. 1** Immunohistochemistry to phospho-tau, as revealed with the AT8 antibody (**a, b**), c-Fos (**c, d**), c-Fos$^{P}$ (**e, f**), c-Jun (**g-h**) and c-Jun$^{P}$ (**i-j**) in dentate gyrus of control (**a, c, e, g, i**) and PiD brains (**b, d, f, h, j**). Increased expression of TFs is observed in PiD cases. Strong c-Fos immunoreactivity seems to localize in globular structures reminiscent of Pick bodies. Moreover, phosphorylated c-Fos and c-Jun occur in the nuclei of dentate gyrus neurons in PiD cases. Cryostat sections, *bar* 25 μm

Double-labelling immunofluorescence and confocal microscopy

Sections 40 μm thick were rinsed in PBS, mounted on glass slides, and stained with a saturated solution of Sudan black B (Merck) for 15 min to block the autofluorescence of lipofucsin granules present in nerve cell bodies. After washing with 70% ethanol and distilled water, sections were washed in PBS, pH 7.5 and co-incubated at 4°C overnight with the monoclonal antibody to phospho-tau clone AT8 (1:50, Innogenetics), and the rabbit polyclonal anti-c-Fos

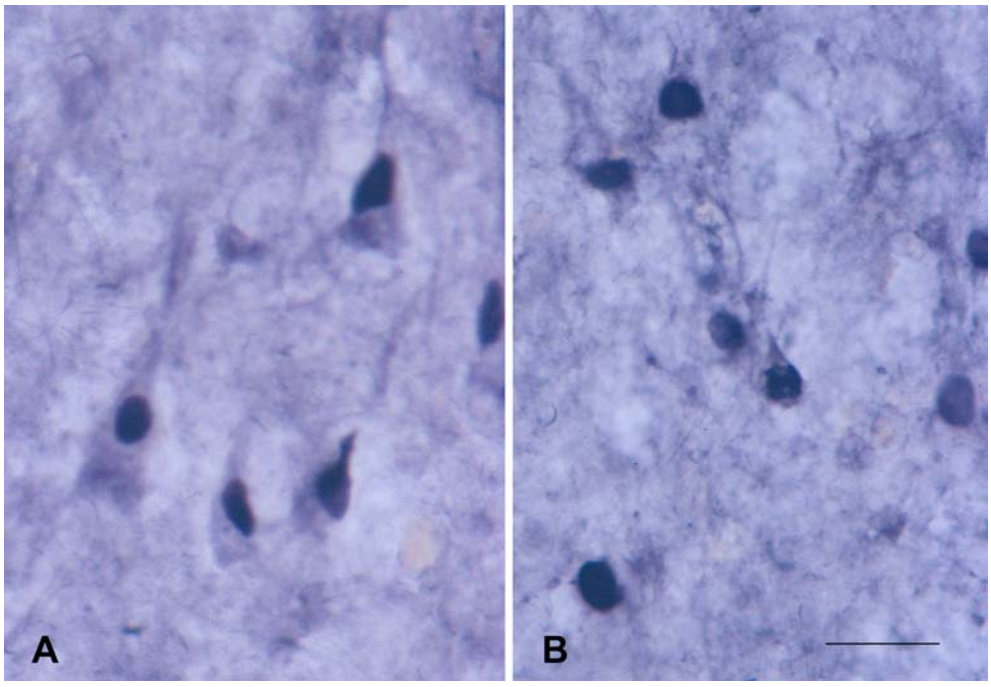


**Fig. 2** Immunohistochemistry to c-Fos in the frontal cortex (**a**) and CA1 area of the hippocampus (**b**). c-Fos immunoreactivity is observed in globular structures reminiscent of Pick bodies. Cryostat sections, *bar* 25 μm

(Santa Cruz Biotechnology) at a dilution of 1:200, or the rabbit anti-cleaved caspase-3 ($Asp^{175}$) antibody (Cell Signalling) at 1:100. After washing with PBS, the sections were incubated with a cocktail of secondary antibodies (in dark conditions) for 45 min at room temperature. Secondary antibodies were Alexa456 anti-mouse (red) and Alexa588 anti-rabbit (green) (1:400, Molecular Probes). Finally, the sections were washed with distilled PBS, mounted in immuno-Fluore Mounting medium (ICN Biomedicals), sealed and dried overnight at 4°C. All images were analysed with a Leica TCS-SL confocal microscope. Sections incubated only with the cocktail of secondary antibodies were used as controls. TO-PRO-3 (Invitrogen Life Technologies, Barcelona, Spain) was used to detect, in blue, the cell nuclei.

## Results

### Western blot analysis

Antibodies used in the present study recognized immunoreactive bands at the appropriate molecular weights in total hippocampal homogenates of control and diseased brains processed in parallel. In addition, several non-specific bands of lower and higher molecular weights were observed in both control and diseased hippocampus. None of these bands replicated the typical tau doublet of PiD [4, 7, 11, 42]. Most particularly, antibody to c-Fos recognized a single molecular band at the appropriate molecular weight. However, the phospho-specific rabbit anti-ATF-$2^{P}$ (Thr69/71) antibody recognized two bands with identical molecular weight to those recognized with anti-phospho-tau antibodies (data not shown).

### c-Fos and c-Jun immunohistochemistry

Anti-phospho-tau antibodies decorated Pick bodies in the expected regions. Pick bodies were stained with anti-phospho-tau antibodies, whereas no immunostaining was found in controls (Fig. 1a, b).

In normal brain, c-Fos weakly and diffusely stained the cytoplasm of a few neurons (Fig. 1c). Weak c-Fos immunoreactivity in PiD was found in the cytoplasm of neurons of the dentate gyrus and CA1 area of the hippocampus and dentate gyrus, but also as strong c-Fos immunoreactive deposits in the cytoplasm mimicking Pick bodies in the dentate gyrus (Fig. 1d). Similar findings were observed in the frontal cortex (Fig. 2a) and CA1 are of the hippocampus (Fig. 2b). Antibodies to c-Fos also stained scattered astrocytes. Antibodies to c-$Fos^{P}$ rarely stained cells in control brains (Fig. 1e), but they revealed a nuclear staining in the frontal cortex, CA1 area of the hippocampus and dentate gyrus in PiD cases (Fig. 1f).

In control brains, weak c-Jun immunoreactivity was rarely seen in the cytoplasm of neurons and astrocytes (Fig. 1g). Moderate c-Jun immunoreactivity was seen in the nuclei of neurons in PiD cases (Fig. 1h). Weak c-Jun-$^{P}$ immunoreactivity was localized in a few scattered neocortical and CA1 neurons in controls, but was extremely rare in dentate gyrus (Fig. 1i). In contrast, moderate c-Jun-$^{P}$ immunoreactivity occurred in the nucleus of many neurons in PiD cases (Fig. 1j).

### Double labelling immunofluorescence and confocal microscopy to c-Fos and phospho-tau

Double-labelling immunoflourescence and confocal microscopy disclosed co-localization of c-Fos and

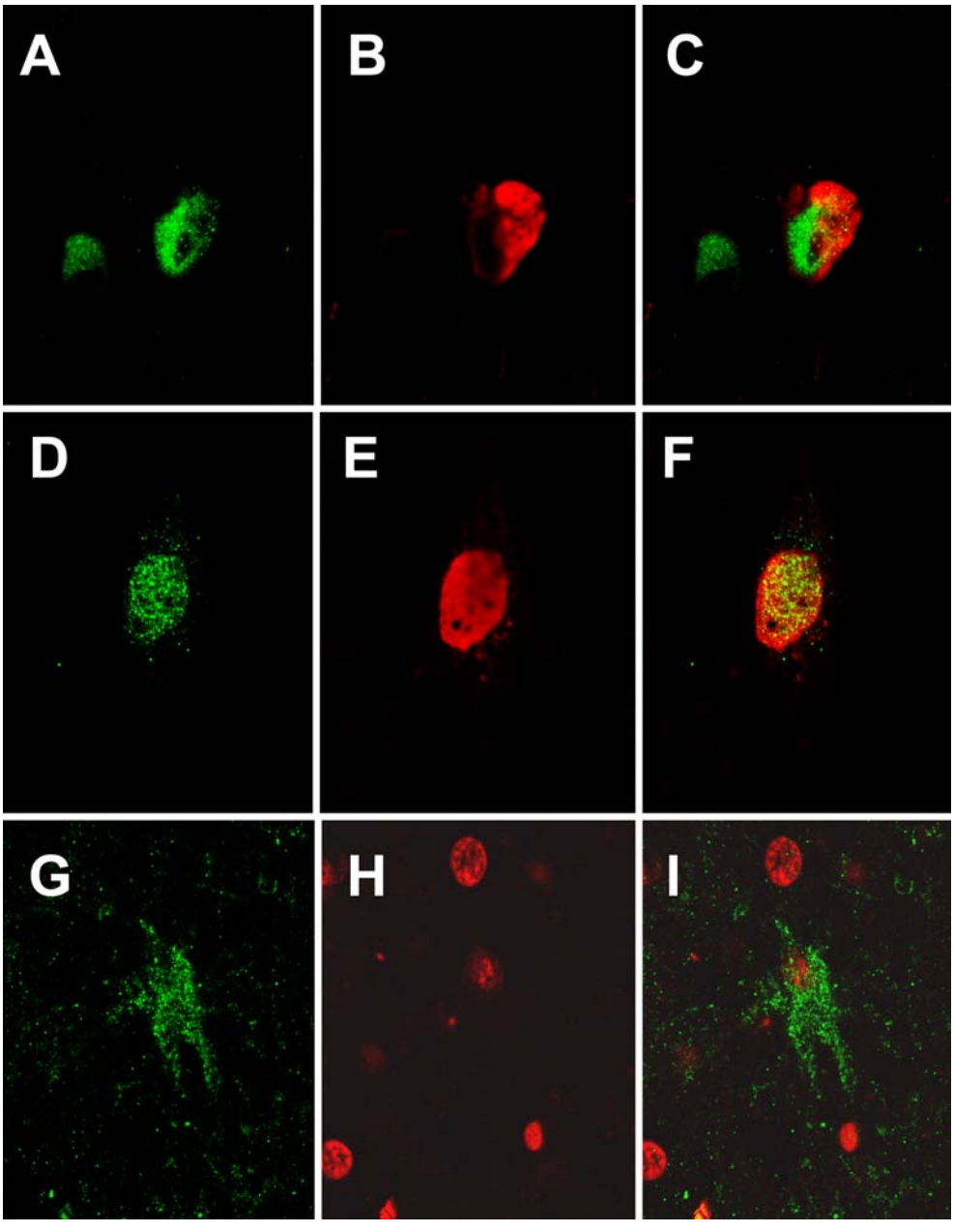


**Fig. 3** c-Fos (*green*) and phospho-tau (*red*) immunofluorescence in Pick bodies of the dentate gyrus (**a–c**) and CA1 area (**d–f**). c-Fos co-localizes phospho-tau in the majority of Pick bodies. In addition, scattered astrocytes are immunostained with anti-c-Fos and AT8 antibodies (**g–i**)

phospho tau (as revealed with the AT8 antibody) in the majority but not all neurons of the dentate gyrus and CA1 area of the hippocampus containing Pick bodies in PiD brains. In addition, a few astrocytes were also stained with anti-c-Fos and anti-phospho-tau antibodies (Fig. 3).

## CREB-1 and ATF-2 immunohistochemistry

In control brains, weak CREB-1 immunoreactivity was localized in neurons (Fig. 4a). However, moderate nuclear CREB-1 immunoreactivity was observed in PiD neurons of the dentate gyrus (Fig. 4b), CA1 and frontal cortex. CREB-$1^P$ immunoreactivity in controls was rarely observed (Fig. 4c). Yet extensive and strong CREB-$1^P$ immunoreactivity occurred in PiD, localized in the nuclei of nerve cells (Fig. 4d).

In control brains, weak ATF-2 immunoreactivity localized in the cytoplasm occurred in neurons of the frontal cortex and neurons of the CA1 area of the hippocampus, but the cytoplasm of neurons of the dentate gyrus was negative at the concentrations of the antibody used for the other regions (Fig. 4e). Increased ATF-2 immunoreactivity was found in the dentate gyrus in PiD cases; the immunoreaction decorated the nuclei of granule cells (Fig. 4f).

Pick bodies were stained with phospho-specific rabbit anti-ATF-$2^P$ (Thr69/71) antibodies (data not shown).

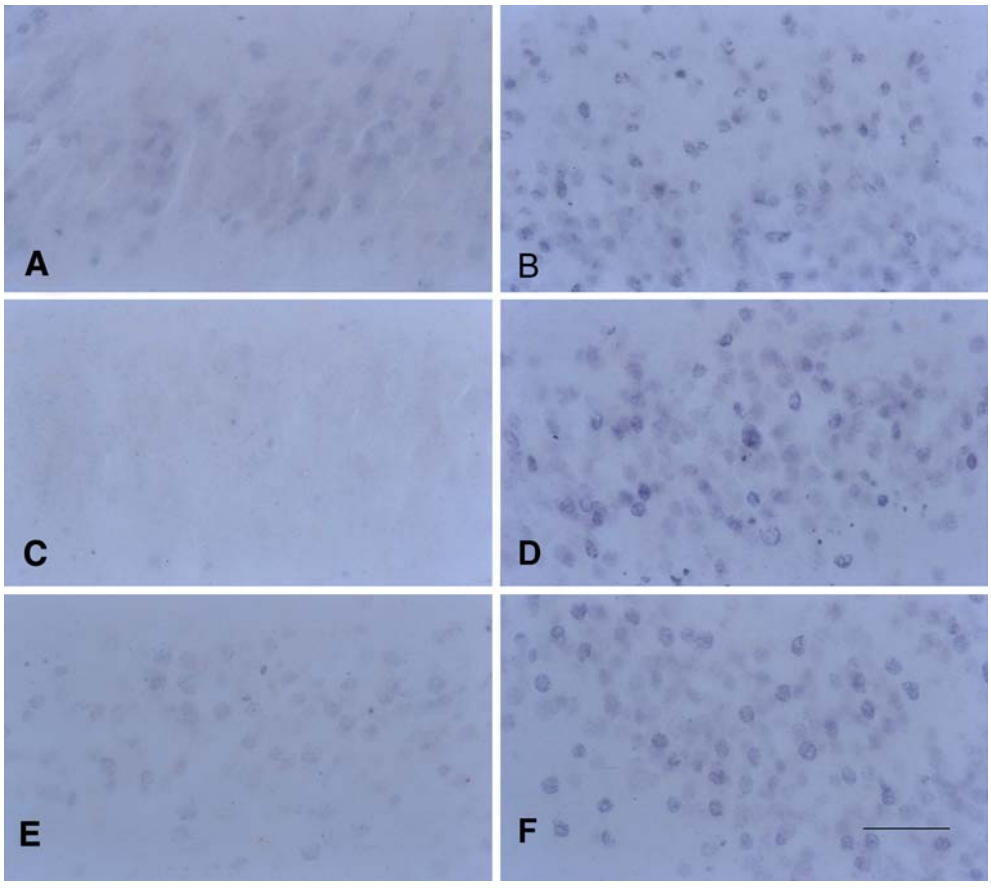


**Fig. 4** Immunohistochemistry to CREB-1 (**a, b**), CREB-1$^{P}$ (**c, d**) and ATF-2 (**e, f**) in control (**a, c, e**) and PiD (**b, d, f**) dentate gyrus brains. Increased CREB-1, CREB-1 $^{P}$ and ATF-2, with predominant nuclear staining, is observed in PiD cases. Cryostat sections, *bar* 25 μm

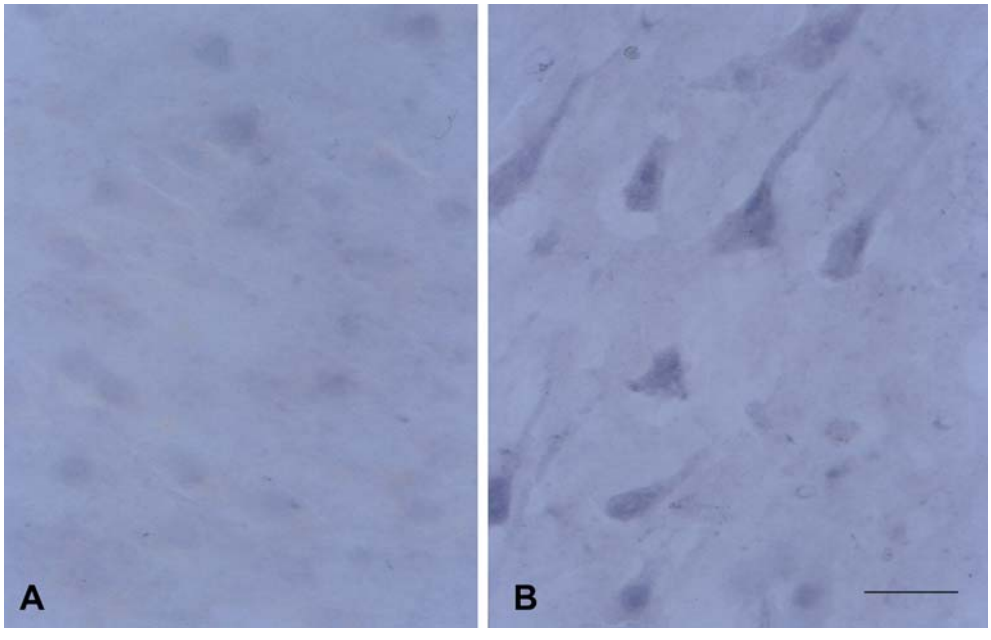


**Fig. 5** Pro-caspase-8 immunohistochemistry in dentate gyrus of control (**a**) and PiD (**b**) brains. Pro-caspase-8 immunoreactivity is increased in diseased brains when compared with controls. Cryostat sections, *bar* 25 μm

Pro-caspase-8, pro-caspase-3, and active caspase-3 immunohistochemistry

Pro-caspase-8 immunochemistry was practically negative in the frontal cortex, CA1 area and dentate gyrus in control brains (Fig. 5a). In contrast, moderate to strong pro-caspase-8 immunoreactivity was found in the cytoplasm of nerve cells in PiD cases (Fig. 5b). Similar findings were observed with pro-caspase-3 antibodies (data not shown).

Active (cleaved) caspase-3 immunohistochemistry was negative in control brains. However, strong active

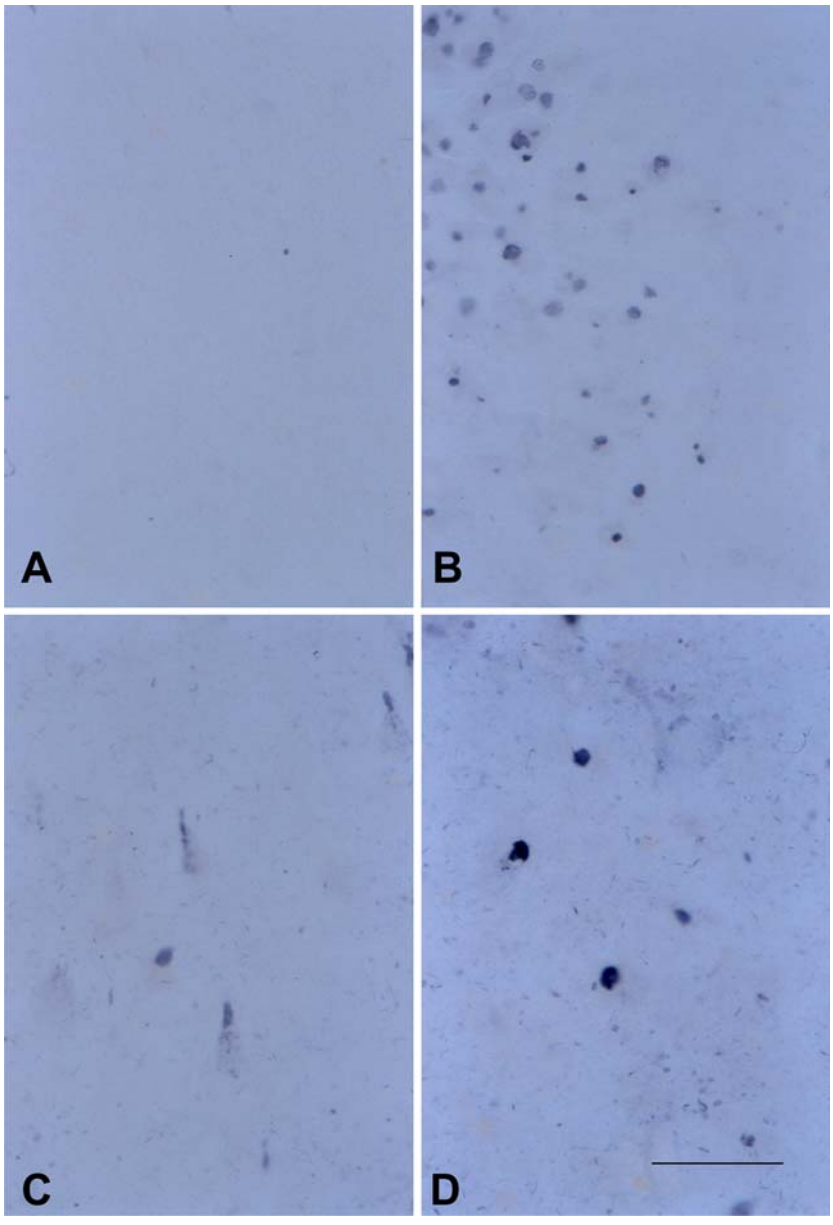


**Fig. 6** Active caspase-3 immunohistochemistry in the dentate gyrus (**a, b**), CA1 (**c**) and frontal cortex (**d**) in controls (**a**) and PiD cases (**b–d**). Strong caspase-3-positive aggregates occur in the cytoplasm of neurons in PiD. Cryostat sections, *bar* 40 μm

caspase-3 immunoreactivity was observed in the cytoplasm of neurons of the frontal cortex, hippocampus and dentate gyrus in PiD cases (Fig. 6). The number of labelled cells varied from one region to another. In the dentate gyrus, the number of positive cells ranged from 25% to 50% of the total number of neurons.

### Double labelling immunofluorescence and confocal microscopy to active caspase-3 and phospho-tau

Double-labelling immunofluorescence revealed co-localization of caspase-3 and phospho-tau in Pick bodies of the dentate gyrus (Fig. 7). Not all Pick bodies were decorated with anti-phospho-active caspase-3 antibodies, but expression of active caspase-3 was restricted to Pick bodies. Interestingly, caspase-3 and AT-8 staining did not overlap. Rather, the staining was complementary in most examples.

## Discussion

The present study has shown increased expression of transcription factors c-Jun, c-Fos, CREB-1 and ATF-2 in neurons of the dentate gyrus, CA1 area of the hippocampus, and frontal cortex in PiD cases, when compared with age-matched controls with no neurological or metabolic disease and with no neuropathological abnormalities in the post-mortem study. Moreover, phosphorylated (active) c-Jun, c-Fos and CREB-1 ($c\text{-}Jun^{P}$, $c\text{-}Fos^{P}$ and $CREB\text{-}1^{P}$) are markedly increased in the nuclei of neurons in all these regions, and particularly in granule neurons of the dentate gyrus.

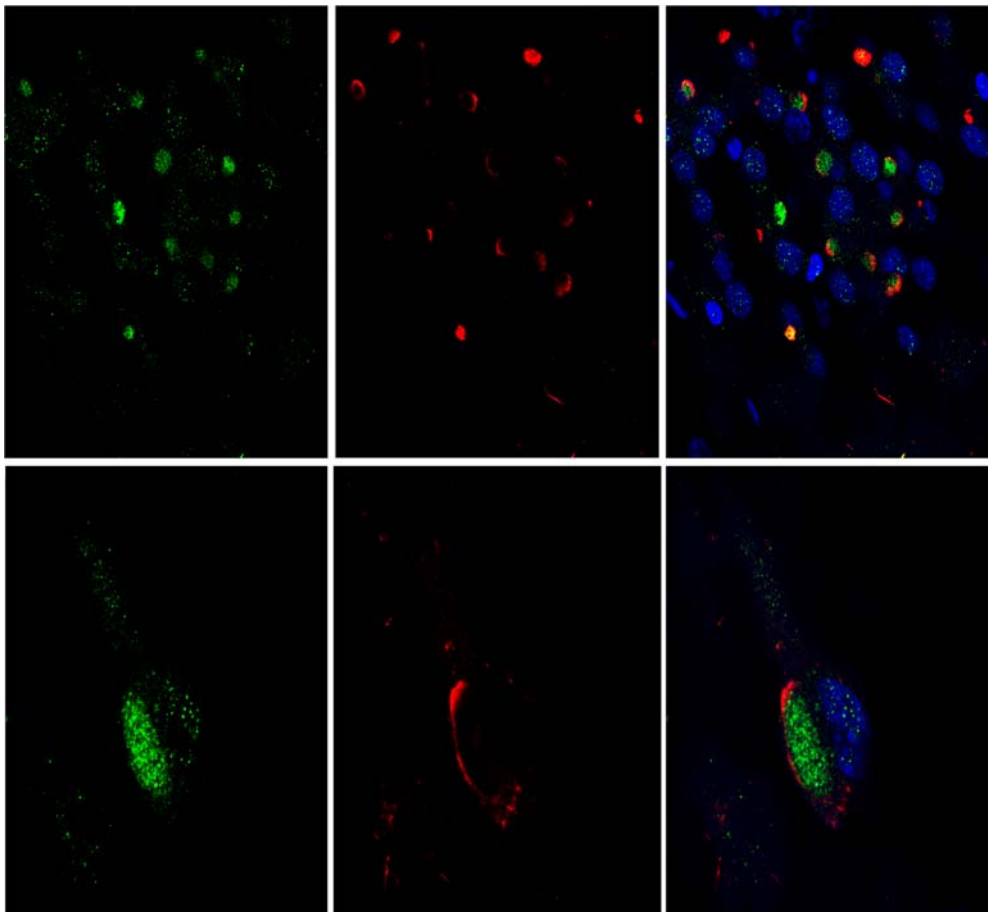

**Fig. 7** Active caspase-3 (*green*) and phospho-tau (*red*) immunofluorescence in Pick bodies of the dentate gyrus (*upper panel*) and CA1 area (*inner panel*). The nuclei are visualized with TO-PRO-3 (*blue*). Note granular caspase-3 immunfluorescence in the nuclei of granule cell neurons and aggregated caspase-3 inmunoreactivity in association with Pick bodies

The phospho-specific rabbit anti-ATF-$2^P$ (Thr69/71) (Cell Signalling) decorated Pick bodies and showed a two-band pattern in cortical homogenates of PiD cases which was the same as the pattern of phospho-tau. For these reasons, cross-reaction of ATF-$2^P$ and phospho-tau was considered, and this antibody was no longer used in the study.

Based on these findings, it is clear that the activation of these transcription factors occurs in vulnerable neurons in PiD. Whether the presence of activated transcription factors is associated with cell death or with cell survival is not clear, because they are present in neurons in which neurons are sensitive, in terms of cell death, to PiD (i.e. frontal cortex), as well as in neurons typically resistant to the disease (i.e. granule cells of the dentate gyrus). Previous studies in a paradigm of kainic acid excitotoxicity, which selectively produces cell damage in the hippocampus accompanied by preservation of granule cells, have shown increased expression of c-$Jun^P$ and CREB-$1^P$ in granule cells of the dentate gyrus, thus suggesting association with cell survival [14]. Furthermore, phosphorylated CREB is associated with cell survival in resistant regions of the hippocampus after hypoxic insults [39]. c-Jun and c-Fos are also induced in neurons of the CA1 area and dentate gyrus that show delayed onset of pyknosis or complete resistance to hypoxic–ischaemic injury [19]. Following focal cerebral ischaemia, CREB-$1^P$ is reduced very early on in the core of the infarction, whereas phosphorylated transcription factors are expressed in the nuclei of neurons in the penumbra area [16].

Transcription factors are regulated by phosphorylation, and several mitogen-activated protein kinases (MAPKs), as well as stress kinase c-Jun-N-terminal kinases (SAP/JNKs) and p38-kinases, which regulate phosphorylation of transcription factors, have also been implicated in the control of cell death and cell survival in various scenarios [21, 27, 33, 34].

Interestingly, MAPKs, SAPK/JNK, and p38 also have the capacity to phosphorylate tau in vitro and in vivo, and active (phosphorylated) MAPKs, SAP/JNK and p-38 are expressed in AD, PiD, and other tauopathies in association with hyperphosphorylated tau deposits in neurons and glial cells [17]. Yet there is no relation between the expression of active kinases and cell death in these diseases [5, 14].

In spite of the ambiguity concerning the role of these factors in the regulation of cell death and cell survival, increased expression of c-Jun, c-Fos, CREB-1 and ATF-2, and, more importantly, of the active forms translocated to the nuclei, is probably associated with gene regulation related with the deposition of hyper-phosphorylated tau. Whatever the function of this increased expression may be, these results suggest active gene

transcription in PiD granule cells and other neurons sensitive to PiD. Similar conclusions have been suggested in AD [40].

A very interesting point is the localization of c-Fos (but not phosphorylated c-Fos) in many Pick bodies in the dentate gyrus and CA1 area of the hippocampus. Since Western blots disclosed no ambiguity on the unique band at the corresponding molecular weight recognized by the anti-c-Fos antibody, and c-Fos co-localized phospho-tau, as revealed with the AT8 antibody, in many but not all Pick bodies, a cross-reaction of c-Fos and AT8 seems very unlike. It is feasible, however, that c-Fos is sequestered by protein aggregates in Pick bodies, as it happens in protein aggregates in many other neurodegenerative diseases. The reasons and the effects of this situation are largely unknown.

Another important aspect is the increased expression of pro-caspase-8 and pro-caspase-3 in PiD neurons of the frontal cortex, CA1 area of the hippocampus and dentate gyrus. More importantly, active (cleaved) caspase-3 is strongly expressed in the cytoplasm of neurons, including granule cells of the dentate gyrus in PiD brains. Furthermore, active caspase-3 is closely related with Pick bodies, as revealed by double labelling immunofluorescence and confocal microscopy.

In addition to the well-known involvement of caspases in apoptotic pathways, several studies have demonstrated the participation of caspases in several settings, including cellular proliferation, cell cycle regulation, and differentiation [8, 24, 26]. Furthermore, caspase-3 is probably involved in the cleavage of tau in neurofibrillary tangles in AD and other tauopathies [18, 36]. Therefore, it may be postulated that caspase-3 activation in PiD is associated, rather, with tau cleavage in Pick bodies. In this line, accumulation of $Asp^{421}$-caspase-cleaved tau (as recognized with the $\Delta$Tau antibody) in Pick bodies in PiD, as well as in other tau deposits in distinct tauopathies [32], has been reported. Together, these findings support the idea that caspase-3 activation is involved in tau cleavage in Pick bodies.

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## References


1. Anderson AJ, Cummings BJ, Cotman CW (1994) Increased immunoreactivity for Jun- and Fos-related proteins in Alzheimer's disease: association with pathology. Exp Neurol 125:286–295
2. Anderson AJ, Su JH, Cotman CW (1996) DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area, and effect of postmortem delay. J Neurosci 16:1710–1719
3. Arai T, Ikeda K, Akiyama H, Shikamoto Y, Tsuchiya K, Yagishita S, Beach T, Rogers J, Schwab C, McGeer PL (2001) Distinct isoform of tau aggregated in neurons and glial cells in brains of patients with Pick's disease, corticobasal degeneration and progressive supranuclear palsy. Acta Neuropathol (Berl) 101:167–173
4. Arai T, Ikeda K, Akiyama H, Tsuchiya K, Iritani S, Ishiguro K, Yagishita S, Oda T, Odawara T, Iseki E (2003) Different immunoreactivities of the microtubule-binding region of tau and its molecular basis in brains from patients with Alzheimer's disease, Pick's disease, progressive supranuclear palsy and corticobasal degeneration. Acta Neuropathol (Berl) 105:89–98
5. Atzori C, Ghetti B, Piva R, Srinivasan AN, Zolo P, Delisle MB, Mirra SS, Migheli A (2001) Activation of the JNK/p38 pathway occurs in diseases characterized by tau protein pathology and is related to tau phosphorylation but not to apoptosis. J Neuropathol Exp Neurol 60:1190–1197
6. Bergeron C, Morris HR, Rossor M. 2003 Pick's disease. In: Dickson D (ed) Neurodegeneration: The molecular pathology of dementia and movement disorders. ISN Neuropath Press, Basel, pp 124–131
7. Buée L, Bussière T, Buée-Scherrer V, Delacourte A, Hof PR (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res Rev 33:95–130
8. Chun HJ, Zheng L, Ahmad M, Wang J, Speirs CK, Siegel RM, Dale JK, Puck J, Davis J, Hall CG, Skoda-Smith S, Atkinson TP, Straus SE, Lenardo MJ (2002) Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. Nature 419:395–399
9. Colby GP, Coon AL, Connolly ES Jr, Ambron RT (2005) Activation of c-Jun and ATF-2 in primate motor cranial nerve nuclei is not associated with apoptosis following axotomy. Exp Neurol 194:57–65
10. Dalfó E, Albasanz JL, Rodríguez A, Martín M, Ferrer I (2005) Abnormal group I metabotropic glutamate receptor expression and signalling in the frontal cortex in Pick disease. J Neuropathol Exp Neurol 64:638–647
11. Delacourte A, Robitaille Y, Sergeant N, Buee L, Hof PR, Wattez A, Laroche-Cholette A, Mathieu J, Chagnon P, Gauvreau D. (1996) Specific pathological tau protein variants characterize Pick's disease. J Neuropathol Exp Neurol 55:159–168
12. Dickson D (1998) Pick's disease: a modern approach. Brain Pathol 8:339–354
13. Earnshaw WC, Martins LM, Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68:383–424
14. Ferrer I, Blanco R, Carmona M, Puig B (2001) Phosphorylated mitogen-activated protein kinase (MAPK/ERK-P), protein kinase of 38 kDa (p38-P), stress-activated protein kinase (SAPK/JNK-P), and calcium/calmodulin-dependent kinase II (CaM kinase II) are differentially expressed in tau deposits in neurons and glial cells in tauopathies. J Neural Transm 108:1397–1415
15. Ferrer I, Blanco R, Carmona M, Puig B, Dominguez I, Vinals F (2002) Active, phosphorylation-dependent MAP kinases, MAPK/ERK, SAPK/JNK and p38, and specific transcription factor substrates are differentially expressed following systemic administration of kainic acid to the adult rat. Acta Neuropathol (Berl) 103:391–407
16. Ferrer I, Friguls B, Dalfo E, Planas AM (2003) Early modifications in the expression of mitogen-activated protein kinase (MAPK/ERK), stress-activated kinases SAPK/JNK and p38, and their phosphorylated substrates following focal cerebral ischemia. Acta Neuropathol (Berl) 105:425–437
17. Ferrer I, Gomez-Isla T, Puig B, Freixes M, Ribe E, Dalfo E, Avila J (2005) Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. Curr Alzheimer Res 2:3–18
18. Gambin TC, Chen F, Zambrano A,m Abraha A, Lagalwar S, Guillozet AL, Lu M, Fu Y, García Sierra F, LaPointe N, Miller R, Berry RW, Binder LI, Cryns VL (2003) Caspase cleavage of tau: linking amyloid and neurofinbrillary tangles in Alzheimer's disease. Proc Natl Acad Sci USA 100:10032–10037
19. Gilby KL, Armstrong JN, Currie RW, Robertson HA (1997) The effects of hypoxia-ischemia on expression of c-Fos, c-Jun and Hsp70 in the young rat hippocampus. Brain Res Mol Brain Res 48:87–96

20. Glover CP, Heywood DJ, Bienemann AS, Deuschle U, Kew JN, Uney JB (2004) Adenoviral expression of CREB protects neurons from apoptotic and excitotoxic stress. Neuroreport 15:1171–1175
21. Herdegen T, Claret FX, Kallunki T, Martin-Villalba A, Winter C, Hunter T, Karin M (1998) Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. J Neurosci 18:5124–5135
22. Herdegen T, Leah JD (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Brain Res Rev 28:370–490
23. Herdegen T, Waetzig V (2001) AP-1 proteins in the adult brain: facts and fiction about effectors of neuroprotection and neurodegeneration. Oncogene 20:2424–2437
24. Ishizaki Y, Jacobson MD, Raff MC (1998) A role for caspases in lens fiber differentiation. J Cell Biol 140:153–158
25. Kapinya K, Penzel R, Sommer C, Kiessling M (2000) Temporary changes of the AP-1 transcription factor binding activity in the gerbil hippocampus after transient global ischemia, and ischemic tolerance induction. Brain Res 872:282–293
26. Kennedy NJ, Kataoka T, Tschopp J, Budd RC (1999) Caspase activation is required for T cell proliferation. J Exp Med 190:1891–1896
27. Kikuchi M, Tenneti L, Lipton SA (2000) Role of p38 mitogen-activated protein kinase in axotomy-induced apoptosis of rat retinal ganglion cells. J Neurosci 20:5037–5044
28. King GD, Scott Turner R (2004) Adaptor protein interactions: modulators of amyloid precursor protein metabolism and Alzheimer's disease risk? Exp Neurol 185:208–219
29. Komori T (1999) Tau-positive glial inclusions in progressive supranuclear palsy, corticobasal degeneration and Pick's disease. Brain Pathol 9:663–679
30. MacGibbon GA, Lawlor PA, Walton M, Sirimanne E, Faull RL, Synek B, Mee E, Connor B, Dragunow M (1997) Expression of Fos, Jun, and Krox family proteins in Alzheimer's disease. Exp Neurol 147:316–332
31. Marcus DL, Strafaci JA, Miller DC, Masia S, Thomas CG, Rosman J, Hussain S, Freedman ML (1998) Quantitative neuronal c-fos and c-jun expression in Alzheimer's disease. Neurobiol Aging 19:393–400
32. Newman J, Rissman RA, Sarsoza F, Kim RC, Dick M, Bennett DA, Cotman CW, Rohn TT, Head E (2005) Caspase-cleaved tau accumulation in neurodegenerative diseases associated with tau and α-synuclein pathology. Acta Neuropathol (Berl) 110:135–144
33. Okamoto S, Krainc D, Sherman K, Lipton SA (2000) Antiapoptotic role of the p38 mitogen-activated protein kinase-myocyte enhancer factor 2 transcription factor pathway during neuronal differentiation. Proc Natl Acad Sci USA 97:7561–7566
34. Oo TF, Henchcliffe C, James D, Burke RE (1999) Expression of c-fos, c-jun, and c-jun N-terminal kinase (JNK) in a developmental model of induced apoptotic death in neurons of the substantia nigra. J Neurochem 72:557–564
35. Puig B, Viñals F, Ferrer I, (2004) Active stress kinase p38 enhances and perpetuates abnormal tau phosphorylation and deposition in Pick's disease. Acta Neuropathol (Berl) 107: 185–189
36. Rissman RA, Poon WW, Blurton-Jones M, Oddo S, Torp R, Vitek MP, LaFerla FM, Rohn TT, Cotman CW (2004) Caspase-cleavage of tau is an early event in Alzheimer disease tangle pathology. J Clin Invest 114:212–130
37. Schenkel J (2004) Activation of the c-Jun transcription factor following neurodegeneration in vivo. Neurosci Lett 361:36–39
38. Taru H, Iijima K, Hase M, Kirino Y, Yagi Y, Suzuki T (2002) Interaction of Alzheimer's beta-amyloid precursor family proteins with scaffold proteins of the JNK signaling cascade. J Biol Chem 277:20070–20078
39. Walton MR, Dragunow I (2000) Is CREB a key to neuronal survival? Trends Neurosci 23:48–53
40. Yamada T, Yoshiyama Y, Kawaguchi N (1997) Expression of activating transcription factor-2 (ATF-2), one of the cyclic AMP response element (CRE) binding proteins, in Alzheimer disease and non-neurological brain tissues. Brain Res 749:329–334
41. Zhang P, Hirsch EC, Damier P, Duyckaerts C, Javoy-Agid F (1992) c-fos protein-like immunoreactivity: distribution in the human brain and over-expression in the hippocampus of patients with Alzheimer's disease. Neuroscience 46:9–21
42. Zhukareva V, Mann D, Pickering-Brown S, Uryu K, Shuck T, Shah K, Grossman M, Miller BL, Hulette CM, Feinstein SC, Trojanowski JQ, Lee VM (2002) Sporadic Pick's disease: a tauopathy characterized by a spectrum of pathological tau isoforms in gray and white matter. Ann Neurol 51:730–739

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# Abnormal Sp1 transcription factor expression in Alzheimer disease and tauopathies

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# Abnormal Sp1 transcription factor expression in Alzheimer disease and tauopathies

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**Abstract**

Sp1 transcription factor expression was examined by immunohistochemistry, immunofluorescence and confocal microscopy in Alzheimer disease (AD), Pick disease (PiD), progressive supranuclear palsy (PSP), Parkinson disease (PD) and Dementia with Lewy bodies (DLB). Sp1 partly co-localizes with hyper-phosphorylated tau deposits in neurofibrillary tangles, dystrophic neurites of senile plaques and neuropil threads in AD, and in neurons, astrocytes and oligodendrocytes bearing hyper-phosphorylated tau in PiD and PSP. Sp1 is not found in α-synuclein inclusions in PD and DLB. These modifications are not associated with changes in the total expression levels of Sp1, as revealed with gel electrophoresis and Western blotting of brain homogenates. Furthermore, no co-immunoprecipitation of Sp1 and phospho-tau was observed in AD and PiD cases. Since Sp1 binding sites are present in the promoters of several genes involved in amyloid and tau, and Sp1 is regulated by oxidative stress, the present findings suggest that Sp1 deposition in hyper-phosphorylated tau deposits may have functional consequences in the pathology of AD and other tauopathies.


*Keywords:* Alzheimer disease; Pick disease; Progressive supranuclear palsy; Parkinson disease; Sp1

Sp1 is a zinc finger transcription factor which binds to GC boxes of the promoters of several genes expressed in a wide variety of tissues [6,18]. The phosphorylation of Sp1 and the adaptor proteins which bind to Sp1 both regulate the positive and negative effects of Sp1 on gene expression [5,15]. Sp1 participates in many physiological processes including angiogenesis and cell cycle regulation [2,3].

Sp1 has also been related to Alzheimer Disease (AD). Major hallmarks of AD are the abnormal deposition of β-amyloid, which results from the combined cleavage of the β-amyloid precursor protein (APP) by β- and γ-secretases, in the form of β-amyloid or senile plaques, and the accumulation of hyper-phosphorylated tau comprising neurofibrillary tangles, dystrophic neurites surrounding amyloid plaques, and neuropil threads [10]. In vitro studies have shown that Sp1 is involved in the positive regulation of the TGF-beta-dependent expression of APP [9]. Sp1 also regulates the expression of BACE1, the major β-secretase involved in APP cleavage, and BACE2, a homologue of BACE1 [4,22]. Finally, Sp1 regulates the expression of tau [13].

The present study examines the expression of Sp1 in AD and in the tauopathies Pick disease (PiD) and progressive supranuclear palsy (PSP), which are characterized by abnormal hyper-phosphorylated tau deposition in neurons, astrocytes and oligodendrocytes [1,12,19]. For comparative purposes, Sp1 expression was also examined in another group of diseases characterized by abnormal α-synuclein deposition in the form of Lewy bodies and neurites, Parkinson disease and Dementia with Lewy bodies [14,16].

The brains of AD ($n=7$, Braak stages V-VIC), PiD ($n=3$), PSP ($n=5$), PD ($n=4$), DLB ($n=6$) and age-matched controls ($n=6$) were obtained as a generous donation following the guidelines of the local ethical committee. The postmortem delay was between 3 and 10 h in control and diseased brains. One hemisphere was cut in coronal sections, immediately frozen and stored at −80 °C until use. The other hemisphere was fixed in 4% buffered formalin for no less than 3 weeks. Samples of selected regions of the brain ($n=22$) were embedded in paraffin and the sections were examined for neuropathological study using current histological and immunohistochemical methods.

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Finally, samples of the frontal cortex (area 8), entorhinal cortex and hippocampal complex, and mesencephalon containing substantia nigra, were fixed in 4% paraformaldehyde in phosphate buffer for 24–48 h, cryoprotected and stored at −80 °C until use. Control and diseased brains were processed in the same way. Pathological cases fulfilled the neuropathological criteria for the corresponding disease. Control cases did not have evidence of neurological or metabolic disease during life, and did not show morphological abnormalities in the neuropathological examination.

Paraformaldehyde-fixed cryostat sections, 15 μm thick, were processed for Sp1 immunohistochemistry following the streptavidin LSAB method (Dako). After incubation with methanol and normal serum, the sections were incubated free-floating with rabbit polyclonal anti-Sp1 specific antibody raised to residues 750–785 (Serotec) or with anti-Sp1 antibody raised against full length Sp1 (Upstate) used at dilutions of 1:500 at 4 °C overnight. Following incubation with the primary antibody, the sections were incubated with LASB for 1 h at room temperature. The peroxidase reaction was visualized with diaminobenzidine, $NH_4NiSO_4$ 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.

Other sections in AD cases were stained with a saturated solution of Sudan black B (Merck) for 30 min to block the autofluorescence of lipofuscin granules present in nerve cell bodies, rinsed in 70% ethanol and washed in distilled water. The sections were incubated, at 4 °C overnight, with rabbit polyclonal anti-Sp1 specific antibody (Serotec) used at a dilution of 1:500 and monoclonal AT8 antibody (Innogenetics) used at a dilution of 1:50 in a vehicle solution composed of Tris buffer, pH 7.2, containing 15 mmol/L $NaN_3$ (Dako). After washing in PBS, the sections were incubated in the dark with the cocktail of secondary antibodies and diluted in the same vehicle solution as the primary antibodies for 45 min at room temperature. Secondary antibodies were Alexa488 anti-mouse (red) and Alexa546 anti-rabbit (green) (both from Molecular Probes), used at a dilution of 1:400. After washing in PBS, the sections were mounted in immuno-Fluore Mounting medium (ICN Biomedicals), sealed and dried overnight. Sections were examined in a Leica TCS-SL confocal microscope.

Weak Sp1 immunoreactivity in control brains was restricted to the nuclei of scattered neurons throughout the brain. Immunohistochemistry in AD cases showed Sp1 immunoreactivity in neurons with neurofibrillary tangles, as well as in dystrophic neurites of senile plaques and in neuropil threads (Fig. 1A and B). Astrocytes and oligodendrocytes were not labeled with anti-Sp1 antibodies in AD. No Sp1 immunoreactivity was observed in the vicinity of diffuse plaques as revealed in consecutive sections proccessed for β-amyloid immunohistochemistry using polyclonal antibodies to $\beta_{1–40}$ and $\beta_{1–42}$ (Dr. M. Sarasa, Zaragoza) used at dilutions of 1:500 and 1:2000, respectively.

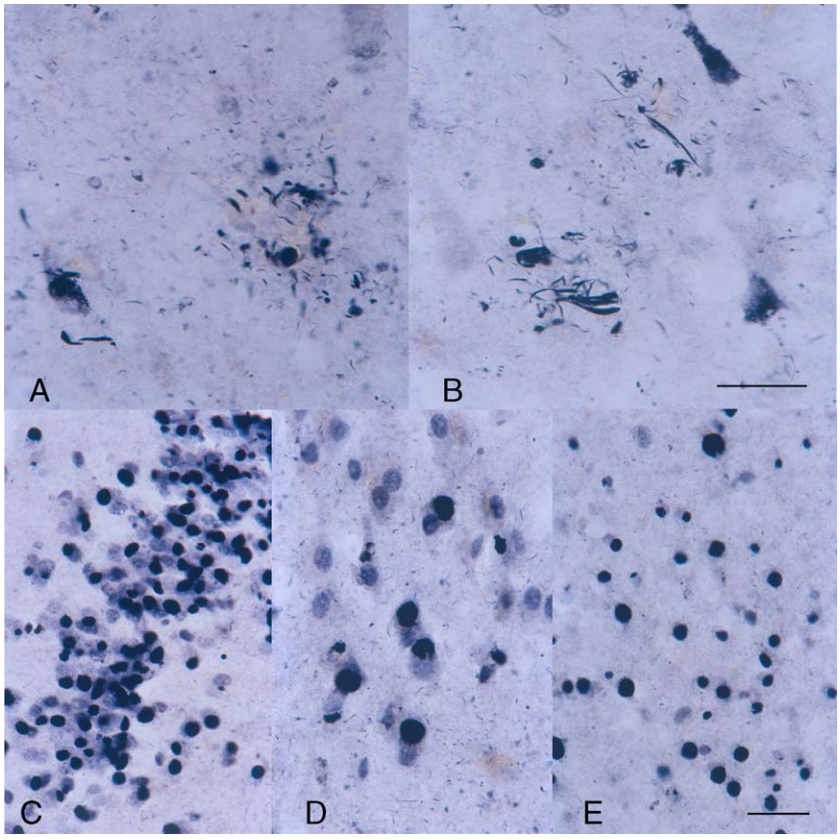


Fig. 1. Sp1 immunoreactivity in Alzheimer disease (A, B), and Pick disease (C–D). Sp1 decorates neurofibrillary tangles, dystrophic neurites and neuropil threads (A, B), and Pick bodies in the dentate gyrus (C), CA1 area of the hippocampus (D) and frontal cortex (E). Cryostat sections processed free-floating without haematoxylin counterstaining. Bar = 25 μm.

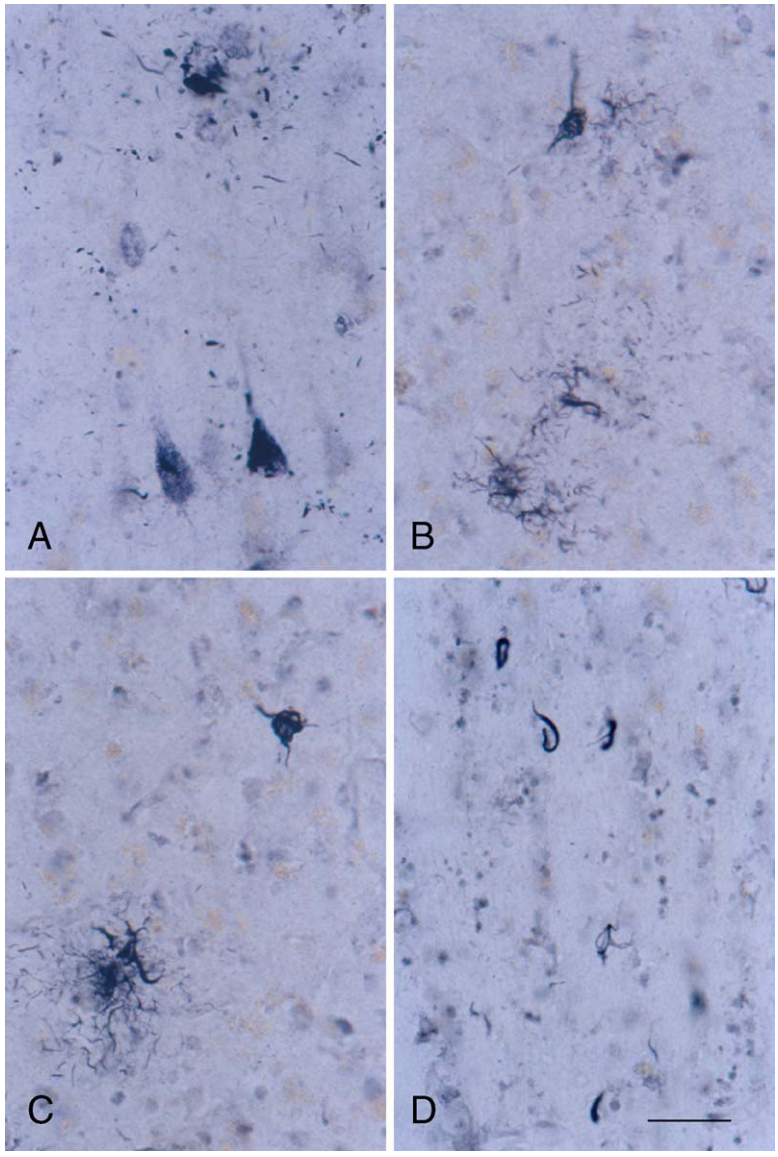


Fig. 2. Sp1 immunoreactivity in progressive supranuclear palsy. Sp1 decorates many neurons (A–C), astrocytes (B, C) and coiled bodies (D). Cryostat sections processed free-floating without haematoxylin counterstaining. Bar = 25 μm.

Sp1 immunoreactivity also decorated cytoplasmic structures resembling Pick bodies in the dentate gyrus, CA1 area of the hippocampus, entorhinal cortex and frontal cortex in PiD. Sp1 immunoreactivity was also observed in scattered neurons in the cerebral cortex, diencephalic nuclei, selected nuclei of the brain stem, and astrocytes and coiled bodies in PSP (Fig. 2 ).

In contrast, Lewy bodies and neurites in PD and DLB were not stained with anti-Sp1 antibodies.

Double-labeling immunofluorescence using AT8 and anti-Sp1 antibodies, and confocal microscopy, disclosed partial co-localization of Sp1 and hyper-phosphorylated tau in neurofibrillary tangles and dystrophic neurites of senile plaques. Similarly, Sp1 co-localized hyper-phosphorylated tau in many Pick bodies in PiD (Fig. 3 ).

Gel electrophoresis and Western blotting was carried in the frontal cortex in AD cases and controls. No differences in the expression levels of Sp1 were found between control and diseased brains. In order to understand whether abnormal tau in AD and tauopathies interacts with Sp1, immunoprecipitation studies were performed following the same methods described elsewhere [7]. No co-immunoprecipitation of Sp1 and phospho-tau was found in AD and PiD cases (Fig. 4 ).

The present observations suggest a sequestration of Sp1 by tau aggregates in AD and other tauopathies. Since no co-immunoprecipitation of Sp1 and phospho-tau was demonstrated, we cannot rule out this possibility. Sp1 might also be associated with intermediate molecules in NFTs. Whether Sp1 trapping by hyper-phosphorylated tau deposits may imply abnormal Sp1 function on target genes is a matter for speculation. Sp1 regulates the expression of BACE1, BACE2, APP and tau [4,9,13,22]. Sp1 is also dramatically increased in response to oxidative stress in embryonic cortical neurons [20], and it induces the expression of manganese superoxide dismutase (SOD2), which protects the mitochondrion from ROS-induced

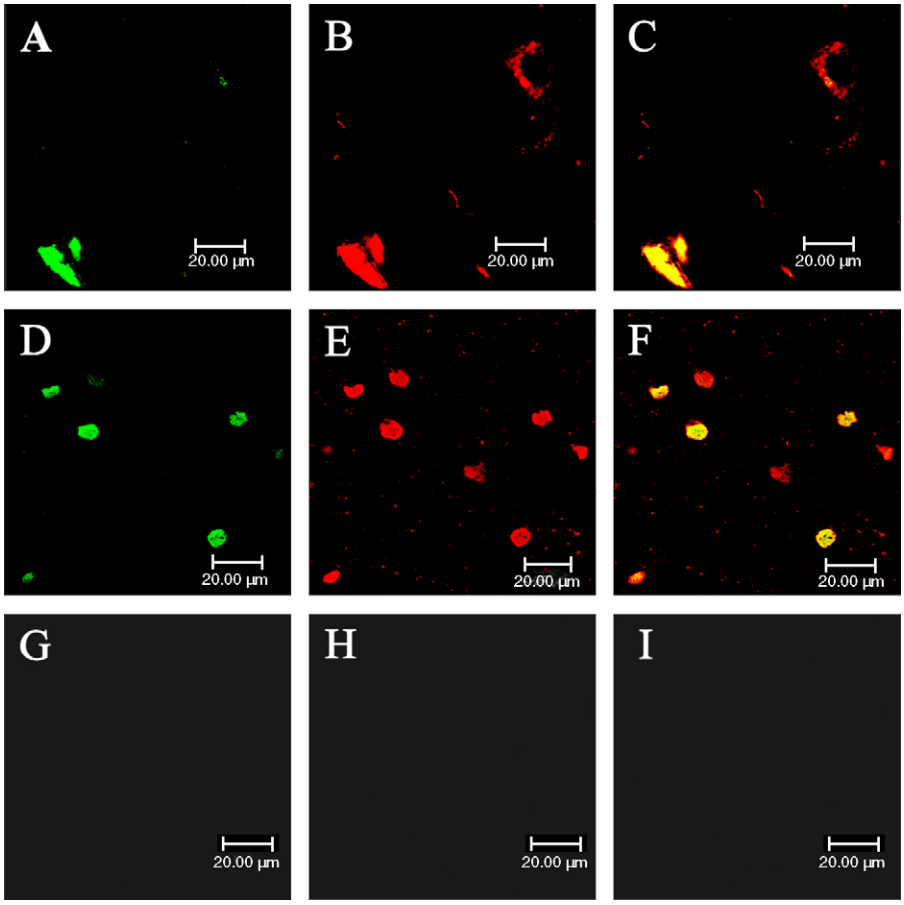


Fig. 3. Sp1 (green) partly co-localizes phospho-tau (red) in neurons with neurofibrillary tangles in AD (A–C), and in Pick bodies in Pick disease (D–F). Merge: yellow. G–I: negative controls incubated only with the secondary antibodies.

damage [23]. Therefore, possible targets of abnormal Sp1 distribution could be genes involved in amyloid and tau processing, and in mechanisms involved in the response to oxidative stress.

No relationship of Sp1 expression and cell death was seen in sections processed for Sp1 and active caspase 3 immunohistochemistry (Cell Signaling) (data not shown).

Huntington disease (HD) is a neurodegenerative disease, mainly involving the striatum and cerebral cortex, that is caused by polyglutamine expansions in the huntingtin gene, the product of which accumulates in the cytoplasm and aggregates as intranuclear inclusion bodies [8]. Expansion of glutamine amino acids in mutant huntingtin binds to the glutamine-rich activation domains of Sp1 [11]. Intranuclear aggregates of huntingtin sequestrate Sp1 and one of its co-activators (TAF)II130, inhibiting its binding to gene promoters [17,21]. Studies are needed to elucidate whether Sp1 binding to specific gene promoters is also reduced in AD and tauopathies.

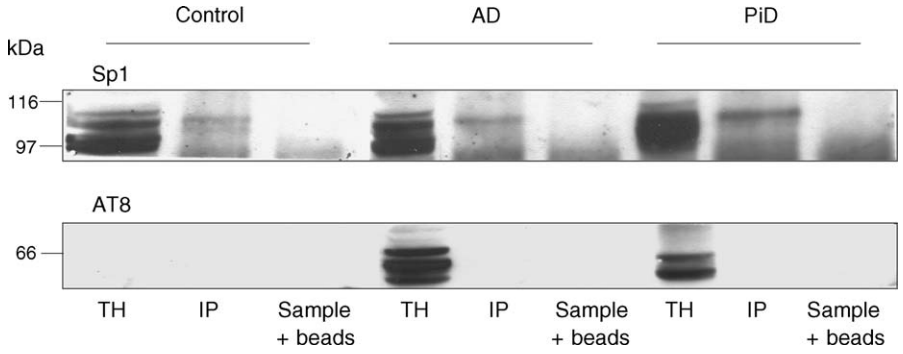


Fig. 4. Sp1 immunoprecipitation studies in control, Alzheimer disease (AD) and Pick disease (PiD) with anti-Sp1 (Upstate) blotted with anti-Sp1 (Serotec) show positive bands in the lanes corresponding to total homogenates (TH) and in the lanes of immunoprecipitates (IP), but not in control lanes with samples and beads but without the antibody. The same membranes blotted for AT8 show typical phospho-tau profiles in TH fractions in AD (three bands) and PiD (two bands), but not in IP fractions.

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## References

[1] C. Bergeron, H.R. Morris, M. Rossor, Pick's disease, in: D. Dickson (Ed.), Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders, ISN Neuropath Press, Basel, 2003, pp. 124–131.
[2] A.R. Black, J.D. Black, J. Azizkhan-Clifford, Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer, J. Cell. Physiol. 188 (2001) 143–160.
[3] A.R. Black, D. Jensen, S.Y. Lin, J.C. Azizkhan, Growth/cell cycle regulation of Sp1 phosphorylation, J. Biol. Chem. 274 (1999) 1207–1215.
[4] M.A. Christensen, W. Zhou, H. Qing, A. Lehman, S. Philipsen, W. Song, Transcriptional regulation of BACE1, the beta-amyloid precursor protein beta-secretase, by Sp1, Mol. Cell. Biol. 24 (2004) 865–874.
[5] S. Chu, T.J. Ferro, Sp1: regulation of gene expression by phosphorylation, Gene 348 (2005) 1–11.
[6] T. Cook, B. Gebelein, R. Urrutia, Sp1 and its likes: biochemical and functional predictions for a growing family of zinc finger transcription factors, Ann. N.Y. Acad. Sci. 880 (1999) 94–102.
[7] E. Dalfó, I. Ferrer, α-synuclein binding to rab3a in multiple system atrophy, Neurosci. Lett. 380 (2005) 170–175.
[8] M. DiFiglia, E. Sapp, K.O. Chase, S.W. Davies, G.P. Bates, J.P. Vonsattel, N. Aronin, Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain, Science 277 (1997) 1990–1993.
[9] F. Docagne, C. Gabriel, N. Lebeurrier, S. Lesne, Y. Hommet, L. Plawinski, E.T. Mackenzie, D. Vivien, Sp1 and Smad transcription factors co-operate to mediate TGF-beta-dependent activation of amyloid-beta precursor protein gene transcription, Biochem. J. 383 (2004) 393–399.
[10] C. Duyckaerts, D.W. Dickson, Neuropathology of Alzheimer's disease, in: D. Dickson (Ed.), Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders, ISN Neuropath Press, Basel, 2003, pp. 47–65.
[11] R.N. Freiman, R. Tjian, Neurodegeneration. A glutamine-rich trail leads to transcription factors, Science 296 (2002) 2149–2150.
[12] J.-J. Hauw, Y. Agid, Progressive supranuclear palsy (PSP) or Steele-Richardson-Olszewski disease, in: D. Dickson (Ed.), Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders, ISN Neuropath Press, Basel, 2003, pp. 103–114.
[13] A. Heicklen-Klein, I. Ginzburg, Tau promoter confers neuronal specificity and binds Sp1 and AP-2, J. Neurochem. 75 (2000) 1408–1418.
[14] P.G. Ince, I.G. McKeith, Dementia with Lewy bodies, in: D. Dickson (Ed.), Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders, ISN Neuropath Press, Basel, 2003, pp. 188–199.
[15] S.P. Jackson, J.J. MacDonald, S. Lees-Miller, R. Tjian, GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase, Cell 63 (1990) 155–165.
[16] K.A. Jellinger, Y. Mizuno, Parkinson's disease, in: D. Dickson (Ed.), Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders, ISN Neuropath Press, Basel, 2003, pp. 159–187.
[17] S.H. Li, A.L. Cheng, H. Zhou, S. Lam, M. Rao, H. Li, X.J. Li, Interaction of Huntington disease protein with transcriptional activator Sp1, Mol. Cell. Biol. 22 (2002) 1277–1287.
[18] S. Philipsen, G. Suske, A tale of three fingers: the family of mammalian Sp/XKLF transcription factors, Nucleic Acids Res. 27 (1999) 2991–3000.
[19] L. Rampello, V. Buttà, R. Raffaele, I. Vecchio, G. Battagli, G. Cormaci, A. Alvano, Progressive supranuclear palsy: a systematic review, Neurobiol. Dis. 20 (2005) 179–186.
[20] H. Ryu, J. Lee, K. Zaman, J. Kubilis, R.J. Ferrante, B.D. Ross, R. Neve, R.R. Ratan, Sp1 and Sp3 are oxidative stress-inducible, anti-death transcription factors in cortical neurons, J. Neurosci. 23 (2003) 3597–3606.
[21] D. Saluja, M.F. Vassallo, N. Tanese, Distinct subdomains of human TAFII130 are required for interactions with glutamine-rich transcriptional activators, Mol. Cell. Biol. 18 (1998) 5734–5743.
[22] X. Sun, Y. Wang, H. Qing, M.A. Christensen, Y. Liu, W. Zhou, Y. Tong, C. Xiao, Y. Huang, S. Zhang, X. Liu, W. Song, Distinct transcriptional regulation and function of the human BACE2 and BACE1 genes, FASEB J. 19 (2005) 739–749.
[23] Y. Xu, S. Porntadavity, D.K. St Clair, Transcriptional regulation of the human manganese superoxide dismutase gene: the role of specificity protein 1 (Sp1) and activating protein-2 (AP-2), Biochem. J. 362 (2000) 401–412.

# 5

# Argyrophilic grain disease

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REVIEW ARTICLE

# Argyrophilic grain disease

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**Argyrophilic grain disease (AGD) is a common sporadic neurodegenerative disease of old age characterized by the presence of argyrophilic grains (AGs)—dendritic-derived appendages as revealed with the Golgi method—together with pre-tangle neurons in the limbic system, which accounts for about 5% of all demented cases. AGs and pre-tangle neurons contain hyperphosphorylated 4R tau. This is associated with a typical 64 kDa and 68 kDa pattern, but also accompanied by tau truncated forms of low molecular mass, probably resulting from thrombin-mediated proteolysis. Hyperphosphorylated tau also accumulates in oligodendroglial-coiled bodies and in limbic astrocytes. Ballooning neurons in the amygdala are non-specific accompanying abnormalities. A new proposal for AG distribution considers four stages. Clinical symptoms largely depend on the extension of AGs together with the very common associated tauopathies, mainly Alzheimer's disease, progressive supranuclear palsy, corticobasal degeneration and synucleinopathies. Pathogenesis of AG and related lesions herein proposed includes oxidative stress that is followed by increased expression of oxidative response markers, and activation of stress kinases (stress activated protein kinase and p38). These kinases together with glycogen synthase kinase 3β co-localize with hyperphosphorylated tau deposits in neurons and glial cells, thus indicating a link between oxidative stress and tau phosphorylation in AGD. Hyperphosphorylated tau, in turn, co-localizes with p62/sequestosome I and ubiquitin, thus pointing to activation of protein aggregation and protein degradation pathways, respectively. Finally, AGs and tangles co-localize with mutant ubiquitin ($UBB^{+1}$) resulting from molecular misreading of mRNA, thus supporting proteasome function impairment and, therefore, impelling accumulation of hyperphosphorylated tau in AGs and tangles. The sequestration of active kinases in AGs and tangles is an additional local cause of tau hyperphosphorylation.**

**Keywords:** argyrophilic grain disease; Alzheimer's disease; tau; p62; ubiquitin; mutant ubiquitin; oxidative stress; stress kinases; GSK-3β

**Abbreviations:** AD = Alzheimer's disease; AGD = argyrophilic grain disease; AG = argyrophilic grains; CBD = corticobasal degeneration; CJD = Creutzfeldt–Jakob disease; DLB = dementia with Lewy bodies; FTD = frontotemporal dementia; PD = Parkinson's disease; PiD = Pick's disease; PSP = progressive supranuclear palsy; RAGE = AGE receptor; UPS = ubiquitin-proteasome systemb

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## Introduction

Argyrophilic grain disease (AGD) was first described as a degenerative disease characterized by argyrophilic grains (AGs) in the entorhinal cortex, hippocampus, amygdala and neighbouring temporal cortex in a subset of patients who had suffered from adult onset dementia (Braak and Braak, 1987, 1989).

Although the seminal descriptions emphasized the lack of Alzheimer changes, subsequent studies have shown frequent association of AGD with other degenerative diseases of the nervous system, including Alzheimer's disease (AD), Pick's disease (PiD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), dementia with only tangles, Creutzfeldt–Jakob disease (CJD) and

α-synucleinopathies such as Lewy body diseases [Parkinson's disease(PD) and Dementia with Lewy bodies (DLB)] and multiple system atrophy (Masliah *et al.*, 1991; Ikeda *et al.*, 1995; de Vos *et al.*, 1996; Martinez-Lage and Muñoz, 1997; Braak and Braak, 1998; Jellinger, 1998; Jellinger and Bancher, 1998; Kiwashima *et al.*, 1999; Seno *et al.*, 2000; Togo *et al.*, 2002; Ferrer *et al.*, 2003). Rare cases have been reported with combined association of AGD, Lewy body disease and motor neuron disease (Liang *et al.*, 2005; Klos *et al.*, 2005). As in AD, Lewy bodies are frequent in the amygdala in AGD (Popescu *et al.*, 2004). Finally, hippocampal sclerosis may be found in combination with AGD, as it occurs in other cases of dementia with tauopathy (Beach *et al.*, 2003; Probst *et al.*, 2007).

Together, these observations have raised cautionary comments about the reality of AGD as a distinct entity (Martinez-Lage and Munoz, 1997; Ikeda *et al.*, 2000), while the opposing view is defended by others (Tolnay *et al.*, 2003; Tolnay and Clavaguera, 2004).

AGs are not uncommon structures in aged human brains (Davis *et al.*, 1997). Their presence has been estimated at 5–9% in adult autopsy series (Braak and Braak, 1998; Tolnay and Clavaguera, 2004). In our series of 1000 consecutive autopsy cases from an adult general hospital, the percentage of cases with AGs was 4%. There is a general agreement that the incidence of AGD increases with age (Braak and Braak, 1998; Tolnay and Clavaguera, 2004). This may explain AGs in 43% of cases in a series of very aged patients (Saito *et al.*, 2002) and the occurrence of AGD in 10 of 32 centenarians (Ding *et al.*, 2006). The mean age of onset is about 75–80 years (Braak and Braak, 1998; Jellinger 1998; Tolnay *et al.*, 2001; Tolnay *et al.*, 2003). In our series, the distribution of AGD for ages was: younger than 60 years, 10%; between 61 and 70, 17%; between 71 and 80, 30%; and older than 80 years, 43%. Both genders are equally affected.

The cause of AGD is not known. The disease appears to be sporadic. Genetic studies have failed to discover a sustained link of AGD with a particular gene locus. Interestingly, a single case bearing the *MAPT* S305I mutation had AGD-like neuropathology (Kovacs *et al.*, 2007). The frequency of apolipoprotein E ε4 (ApoE ε4) allele in AGD is similar to that of the general population (Tolnay *et al.*, 1998; Togo *et al.*, 2002). Yet the frequency of ApoE ε2 is higher than that observed in AD and controls (Ghebremedin *et al.*, 1998). Polymorphisms of the low-density lipoprotein receptor-related gene protein and α2-macroglobulin gene have also been implicated in AGD (Ghebremedin *et al.*, 2002), paralleling what is known in AD. Nevertheless, association of AGD with tau H1 haplotype is confusing, with both positive and negative results (Togo *et al.*, 2002; Miserez *et al.*, 2003). Further studies are obviously needed to elucidate genetic aspects of AGD.

## Clinical symptoms

AGD may manifest with cognitive decline and dementia (Tolnay *et al.*, 2001; Saito *et al.*, 2002; Togo *et al.*, 2002;Tolnay and Clavaguera, 2004). Behavioural abnormalities, personality changes and emotional and mood imbalance have been noted in other cases (Braak and Braak, 1998; Ikeda *et al.*, 2000). Episodic memory loss has been noted in a majority of subjects in some series (Ikeda *et al.*, 2000).

Recent studies have shown that older AGD cases who were admitted to geriatric wards of mental hospitals had suffered from amnesia, irritability and agitation, followed by delusions, dysphoria and apathy (Togo *et al.*, 2005). Mild amnestic cognitive impairment is not uncommon as an initial manifestation of AGD (Jicha *et al.*, 2006; Petersen *et al.*, 2006).

A small number of patients present with progressive transcortical sensory aphasia, prominent abnormal behaviour and aggression, and moderate to severe cognitive impairment consistent with frontotemporal dementia (FTD) (Tsuchiya *et al.*, 2001; Ishihara *et al.*, 2005). These latter examples lend support to the proposal for considering AGD as one of the causes of FTD (Cairns *et al.*, 2007).

Although the variability of lesions and the common accompanying AD pathology make it difficult to assign clinical symptoms to AGs, it is necessary to emphasize the importance of clinical and pathological correlations in individual cases. It is clear that the involvement of the entorhinal cortex, hippocampus, neighbouring temporal cortex and amygdala may be the anatomical substrate for cognitive impairment and dementia in AGD. Using a logistic regression model, AGD has a significant effect on the development of dementia; demented AGD cases show lower stages of AD-related pathology than do pure AD cases, but higher stages than non-demented AGD patients (Thal *et al.*, 2005). Based on these and similar findings, it has been suggested that AGD acts as an additive pathology (Thal *et al.*, 2005; Josephs *et al.*, 2006). It is the presence of AGD plus mild-moderate AD-type pathology that results in dementia and not just the presence of AGD.

Although AGD may present as FTD, this is a very rare situation related with diffuse neocortical involvement (Hodges *et al.*, 2004). Most often, disorders of behaviour and mood imbalance and personality changes are symptoms accompanying AGD in very aged people.

There is no specific test for a clinical diagnosis of AGD. 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. Neuroimaging studies have shown atrophy of the anterior part of the temporal and frontal lobes or non-specific cerebral atrophy (Ferrer *et al.*, 2003; Ishihara *et al.*, 2005; Rippon *et al.*, 2005). Moreover, AGD and AD may occur in the same individual. Biological assays do not permit a clinical diagnosis of AGD.

Diagnosis follows neuropathological study of the post-mortem brain. Sometimes, as happened in two personal individual cases with a four-year history of personality changes, mood disorders and mild mental impairment leading to a fulminating disease with hallucinations, ataxia, dementia and sharp EEG complexes, the combination of AGD and CJD was not suspected during life.

Finally, AGD has been reported in 30% of brains from cognitive normal aged (aged 85.4 ± 5.4 years) individuals (Knopman *et al.*, 2003).

## Neuropathology of AGD

According to seminal studies, AGs localize in transentorhinal and entorhinal cortex, CA1 area of the hippocampus, presubiculum, neighbouring temporal cortex, orbitofrontal cortex, insular cortex, basolateral nuclei of the amygdala and hypothalamic lateral tuberal nucleus (Braak and Braak, 1989; Schultz *et al.*, 1998). Coiled bodies in oligodendrocytes are common additional findings (Braak and Braak, 1989) (Fig. 1).

## Electron microscopy

AGs contain straight filaments or tubules measuring 9–25 nm (Braak and Braak, 1989; Itagaki *et al.*, 1989; Ikeda *et al.*, 1995). Similar structures have been obtained in sarkosyl-insoluble fractions in AGD cases (Zhukareva *et al.*, 2002). Coiled bodies are composed of accumulations of fibrils measuring 10–13 nm in diameter (Yamada and McGeer, 1990; Ikeda *et al.*, 1995).

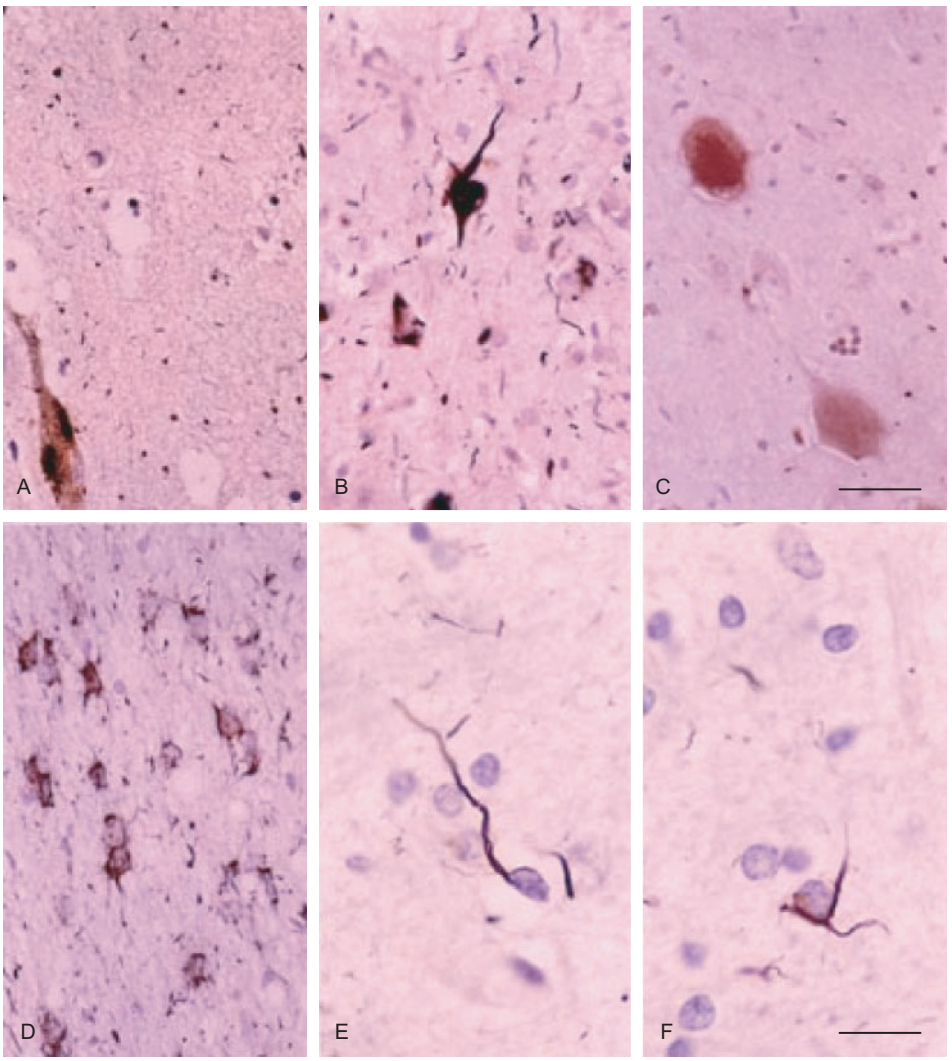



**Fig. 1** AGD, Gallyas silver staining, showing argyrophilic tangles (**A, B**), grains (**A, B**), neuropil threads (**B**) in the CA1 region (**A**) and entorhinal cortex (**B**), periventricular astrocytes (**D**), and oligodendroglial coiled bodies in the white matter of the temporal lobe (**E, F**). Ballooned neurons in the amygdala show a diffuse pale coloration (Gallyas negative). Sections lightly counterstained with haematoxylin. **A–D**, bar in C = 25 microns. **E**, **F**, bar in **F** = 10 microns.

## Immunohistochemistry

Early studies were mainly based on silver staining. However, the use of immunohistochemistry has greatly expanded the understanding of AGD pathology. The use of anti-tau antibodies has shown that phospho-tau deposition occurs in AGs, as well as in pre-tangle neurons (Gallyas-negative) and coiled bodies in oligodendrocytes. Most tau-containing astrocytes in the limbic system are Gallyas negative (Tolnay *et al.*, 1997*a*; Botez *et al.*, 1999; Spillantini *et al.*, 1999; Tolnay and Probst, 1999). Based on these crucial observations, AGD is now considered to be a tauopathy.

## Grains

The term 'argyrophilic grains' is derived from their strong staining with the Gallyas silver iodide method. However, it is worth noting that not all silver methods permit the visualization of AGs (Uchihara, 2007).

Grains are small, about 4–8 microns, spindle shaped, rod-like, button-like or round bodies in the neuropil. Single and double-labelling immunohistchemistry has suggested that AGs are preferentially localized in dendrites and dendritic branches (Ikeda *et al.*, 1995; Schultz *et al.*, 1998; Tolnay *et al.*, 1998), although association of AGs with axons has also been reported (Tolnay and Clavaguera, 2004). The origin of grains is probably in pre-tangle projection neurons of the transentorhinal and entorhinal cortex, CA1 area of the hippocampus, neurons of the dentate gyrus and hilus, presubiculum, neighbouring temporal cortex, orbitofrontal cortex, insular cortex, basolateral nuclei of the amygdala and hypothalamic lateral tuberal nucleus (Tolnay *et al.*, 1998; Tolnay and Clavaguera, 2004).

Studies with the Golgi Cox (Tolnay *et al.*, 1998) and with the rapid Golgi method in the enthorhinal cortex and hippocampus have permitted a clear visualization of AGs as small protrusions of apical, collateral and basilar dendrites of pyramidal cells (Fig. 2). These protrusions grow in normal-appearing dendrites filled with normal dendritic spines. Dendrites and dendritic spines have normal morphology distally to the protrusion. The surface of AGs is smooth, but often they are covered by a few spines or short thin processes (Fig. 2). Although rows of AGs can be seen in the CA1 area of the hippocampus, such rows have not been identified in Golgi-stained sections. These structures largely differ from varicosities, distal stumps and other dendritic abnormalities, as visualized with the Golgi method, in distinct neurological diseases (Ferrer *et al.*, 1990; Ferrer, 2000). AGs also differ from neuritic sprouts as seen in AD (Scheibel and Tomiyasu, 1978; Ferrer *et al.*, 1983; Probst *et al.*, 1983).

## Pre-tangle neurons

Pre-tangle neurons are a constant finding in AGD, and their distribution is the same as that for AGs (Tolnay *et al.*, 2003; Tolnay and Clavaguera, 2004). Pre-tangle neurons are immunostained with the same anti-tau phospho-specific antibodies that decorate AGs (Tolnay *et al.*, 1997*a*; Ferrer *et al.*, 2003). Tau-immunoreactive deposits in granule neurons of the dentate gyrus are a constant abnormality in AGD.

In agreement with previous observations, pre-tangle neurons in AGD do not apparently differ from neurons with early phospho-tau deposition in AD (Braak *et al.*, 1994; Bancher *et al.*, 1989; Tolnay *et al.*, 2003).

## Coiled bodies

Coiled bodies are similar to those observed in many other tauopathies and they lack specificity (Chin and Goldman, 1996; Ikeda *et al.*, 1998; Komori, 1999). Yet coiled bodies are a constant finding associated with AGs (Tolnay *et al.*, 2003).

## Tau-containing astrocytes

Astrocytes containing hyper-phosphorylated tau show granular immunoreactive cytoplasm rather than dense inclusions like those seen in tufted astrocytes in PSP. Rather, bush-like astrocytes show thin immunoreactive processes with anti-phospho-tau antibodies. Commonly, these delicate processes appear in clusters, thus being reminiscent of thin astrocytic plaques of CBD. In addition to bush-like astrocytes and thin astrocytic plaques in the amygdala, white matter of the temporal lobe, periventricular and subpial astrocytes, tufted astrocytes and small clusters of astrocyte processes reminiscent of astrocytic plaques are also present in some cases.

The presence of tau-containing astrocytes is variable from one case to another. Although usually confined to the limbic system, generalized astrocytosis in the subcortical white matter has been reported in some cases (Yamada *et al.*, 1992; Tsuchiya *et al.*, 2001). Whether these cases are pure AGD or different tauopathies with massive phospho-tau in astrocytes remains unresolved.

## Ballooned neurons

Ballooned neurons (Gallyas-negative) expressing αB-crystallin are commonly observed in the amygdala (Tolnay and Probst, 1998), and they have been considered as a marker of AGD (Tolnay and Probst, 1998; Togo and Dickson, 2002). Variable numbers of ballooned neurons are also present in the presubiculum and middle layers of the basal temporal cortex in AGD (Tolnay *et al.*, 2003). Yet ballooned neurons in the limbic system would be more usefully interpreted as a non-specific lesions encountered in many familial and sporadic tauopathies (CBD, PiD, PSP) and AD (Fujino *et al.*, 2004).

The cause of neuron ballooning in AGD and related settings is not known. Ballooned neurons show reduced staining of the rough endoplasmic reticulum. They accumulate phosphorylated neurofilament epitopes, and

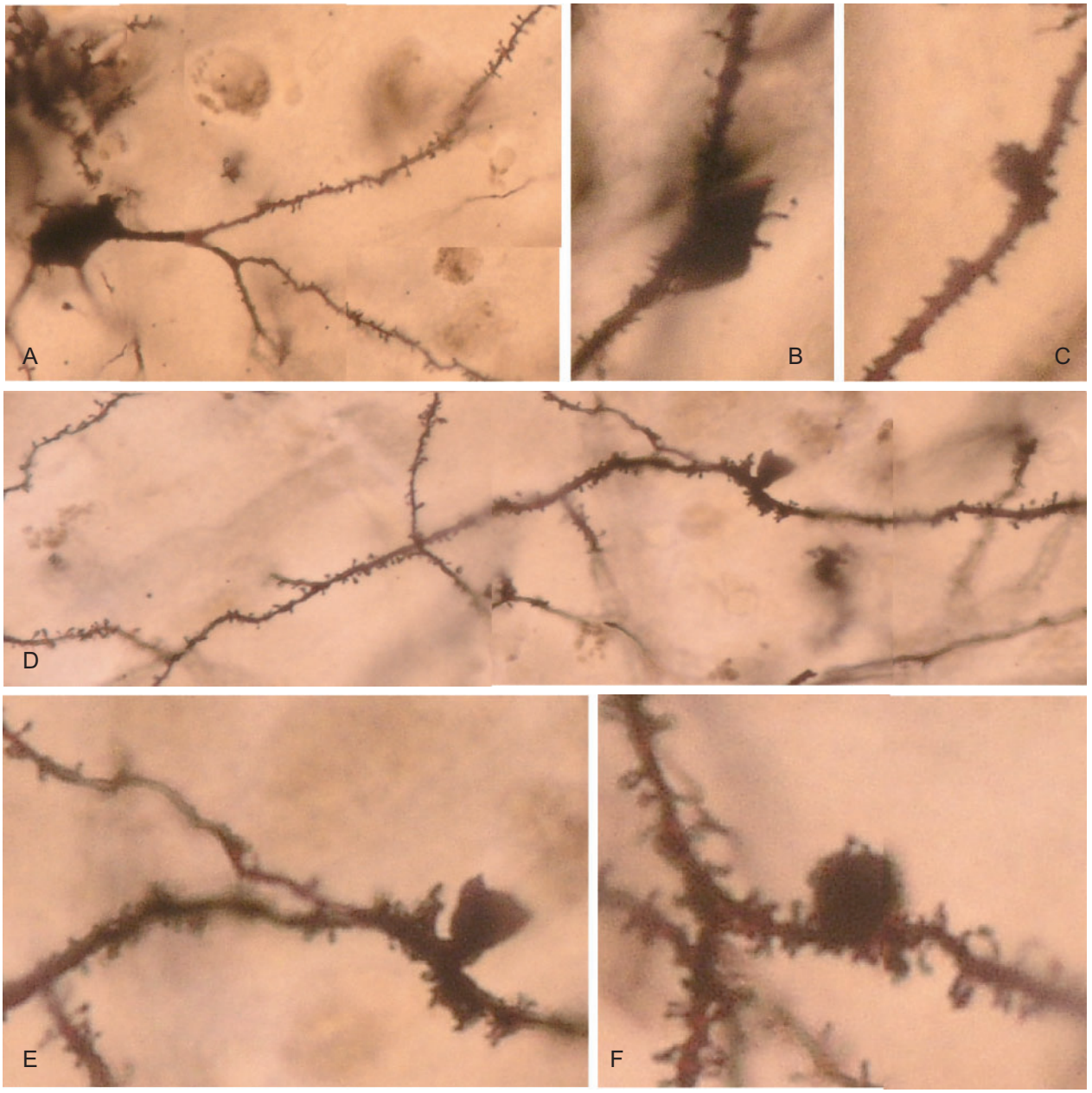



**Fig. 2** AGD, Rapid Golgi method. (**A**) Neuron of the entorhinal cortex showing the normal morphology of dendrites and dendritic spines. (**B**) Spindle-shaped and (**C**) Round protrusions in dendrites. (**D**) Basilar dendrite with inverse-cone morphology with normal-appearing proximal (right) and distal (left) processes. (**E**) High magnification showing the smooth surface of the protrusion and the normal morphology of the spines in the mother dendrite. (**F**) Round protrusion covered with a few dendritic spines in a normal-appearing dendrite filled with dendritic spines.

they are decorated at the periphery with anti-phospho-tau antibodies. Ballooned neurons may be vacuolated. These characteristics show some resemblance to denervated neurons in other regions. The role played by αB-crystallin is also obscure, although recent studies point to its implication in the modulation of cytoskeleton proteins, including filaments and actin, and microtubule assembly (Ghosh *et al.*, 2007*a*; Gosh *et al.*, 2007*b*; Ohto-Fujita *et al.*, 2007; Singh *et al.*, 2007).

## Tangles and neuropil threads

Variable numbers of tangles and neuropil threads may be present in the same regions as AGs and pre-tangle neurons. This has caused some confusion about the frontier between AGD with a few tangles and AGD with associated AD, and has led to consideration of AGD as a variant of AD (Cras and Perry, 1991). Yet AD changes (neurofibrillary tangles and neuropil threads) in AGD cases can be categorized following the guidelines of Braak and Braak

adapted to paraffin sections (Braak and Braak, 1999; Braak *et al.*, 2006).

The instrumental approach with separate consideration of AGs and AD-related changes is suitable, as this convention permits a general commitment for further studies in different settings.

In this line, Braak and collaborators have classified their own AGD cases as AGD + AD 0: 3; AGD + AD I-II: 79; AGD + AD III-IV: 41; AGD + AD V-VI: 2 (Braak and Braak, 1998). The apparently small percentage of AGs in advanced stages of AD must be interpreted cautiously, as the massive phospho-tau-immunoreactive pathology in such cases may hinder the visualization of AGs. This is particularly true when using the monoclonal AT8 antibody. Recent studies using 4R tau-specific antibodies, which highlight AGs, have shown a higher prevalence of AGs in advanced stages of AD (Fujino *et al.*, 2005).

Although often associated with AD, AGD is usually not accompanied by substantial β-amyloid deposits. In contrast to conventional AD cases, AGD brains show a very limited β-amyloid burden in the form of diffuse plaques; neuritic plaques are distinctively uncommon (Tolnay *et al.*, 1999).

## Staging of AGs

Early observations indicated that the sole involvement of the anterior part of the CA1 region of the hippocampus was found in apparently normal individuals, whereas involvement of the posterior CA1 region occurred more commonly and more severely in demented cases (Tolnay *et al.*, 1997*b*). Other studies concluded that severe involvement of the ambient gyrus (the junction between the temporal lobe and the amygdala) differentiated AG with cognitive impairment and dementia from cognitively normal AGD (Saito *et al.*, 2002). More refined analysis has established a proposal for staging AGs (Saito *et al.*, 2004) which basically reflects a similar antero-posterior gradient of putative progression of the disease. Yet rare cases have shown widespread AGs throughout the temporal lobe, limbic system, frontal cortex and brain stem (Tsuchiya *et al.*, 2001; Maurage *et al.*, 2003; Ishihara *et al.*, 2005). The term diffuse AGD has been proposed as a subgroup of AGD to differentiate these cases from the most common limbic AGD (Maurage *et al.*, 2003).

An up-dated staging based on previous descriptions (Braak and Braak, 1998; Saito *et al.*, 2004; Tolnay and Clavaguera, 2004; Ishihara *et al.*, 2005) and personal observations is shown in Table 1.

Staging of AGs does not include accompanying changes such as pre-tangle neurons, coiled bodies, bush-like astrocytes and ballooned neurons. The number and distribution of pre-tangle neurons parallels the distribution of AGs, whereas coiled bodies and tau-immunoreactive astrocytes have particular patterns in individual cases: large numbers of astrocytes may be encountered with relatively low numbers of AGs. Finally, the number of ballooned neurons in the amygdala shows important individual variation among cases with similar AG stage.

## Consideration of the neuropathological diagnosis in AGD cases

Although silver stains have been useful in the past to discover AGD, the Gallyas method and other similar silver stains are not used in current practice. This is due in part to the variations in the quality of staining from one laboratory to another. Moreover, immunohistochemistry has been demonstrated to be a powerful tool with reproducible results in different laboratories (Alafuzoff *et al.*, 2006).

Lesions in AGD are best visualized with the help of any of the several commercial anti-phospho-tau antibodies available; good results are obtained with the AT8 antibody currently used in most laboratories and institutes. The best staining of AGs is achieved with anti-4R tau antibodies (Togo *et al.*, 2002; Fujino *et al.*, 2005). Combining these two antibodies allows the visualization of AGs, neurons with pre-tangles, neurofibrillary tangles, neuropil threads, coiled bodies and different tau-immunoreactive astrocytes.

Ballooned neurons are also mildly phospho-tau-immunoreactive although the best marker of ballooned neurons is anti-αB-crystallin.

The use of additional antibodies (e.g. anti-βA amyloid, α-synuclein, ubiquitin, TDP-43, protease resistant PrP) is obviously necessary to rule out combined pathologies.

**Table 1** Argyrophilic grain (AG) staging

| Stage I | Stage II | Stage III | Stage IV |
|---|---|---|---|
| Anterior entorhinal cortex; mild involvement of the cortical and basolateral nuclei of the amygdala; mild involvement of the hypothalamic lateral tuberal nucleus | Entorhinal cortex; anterior CAI; transentorhinal cortex; cortical and basolateral nuclei of the amygdala; presubiculum; hypothalamic lateral tuberal nucleus; dentate gyrus | Entorhinal cortex; CAI; perirhinal cortex; presubiculum; amygdala; dentate gyrus; hypothalamic lateral tuberal nucleus; mild involvement of CA2 and CA3; mild involvement of the subiculum; mild involvement of other nuclei of the hypothalamus (i.e. mammillary bodies); mild involvement of the anterior temporal cortex, insular cortex, anterior cingulated gyrus, orbitofrontal cortex, nucleus accumbens, septal nuclei; rare grains in the midbrain | Moderate to severe additional involvement of the neocortex and brainstem |

Considering the high prevalence of other pathologies in association with AGs, it is probably prudent to consider a double or triple neuropathological diagnosis in the majority of AGD cases (i.e. AG + AD; AG + PSP; AG + CBD + PD). Staging of accompanying diseases may follow international agreement criteria (Braak and Braak, 1999; Braak *et al.*, 2003; McKeith *et al.*, 2005).

The majority of cases may be categorized, for example, as: AGs stage III + AD stage IIIA + PD stage 2, and probably including, as a note, the amount and distribution of tau-immunoreactive astrocytes and the presence of additional details. This complementary note may be useful as it may serve to describe early stages of tauopathies (PSP or CBD) that are commonly associated with AGs and for which there are not, at present, proposals for disease staging like those available for AD and PD.

## Biochemistry of tau in AGD

*Tau* proteins are encoded by the *tau* gene in chromosome 17. Alternative splicing of exons 2, 3 and 10 results in six isoforms, which in turn give rise to six different mRNAs. The adult *tau* isoforms are proteins of 441 amino acids (2 + 3 + 10 +), 410 amino acids (2 + 3+ 10 −), 412 amino acids (2 + 3 − 10 +), 381 amino acids (2 + 3 − 10 −) and 383 amino acids (2 − 3 − 10 +); the fetal *tau* isoform is a protein of 352 amino acids (2 − 3 − 10 −). *Tau* proteins resulting from encoding exon 10 have four repeat regions (4R tau), whereas those lacking encoding exon 10 have three repeat regions (3R tau) (Goedert *et al.*, 1989; Himmler *et al.*, 1989). The function of *tau* largely depends on post-translational modifications including phosphorylation and dephosphorylation. Phosphorylation of *tau* is the result of a balanced action between protein kinases and protein phosphatases. Several kinases have been implicated in *tau* phosphorylation: glycogen synthase kinase-3 (GSK-3), cyclin dependent kinase-5 (cdk-5), mitogen-activated protein kinase, extracellular signal-regulated kinases (MAPK/ERK1 and MAPK/ERK2, p44 and p42), stress-activated protein kinases, c-Jun N-terminal kinase (SAPK/JNK) and p-38 kinase (p38), among others. These have the capacity to phosphorylate *tau* at specific sites (Hanger *et al.*, 1992; Mandelkow *et al.*, 1992; Goedert *et al.*, 1997; Lovestone and Reynolds, 1997; Reynolds *et al.*, 1997*a*; Reynolds *et al.*, 1997*b*; Goedert *et al.*, 1998; Jenkins *et al.*, 2000; Reynolds *et al.*, 2000; Buée-Scherrer and Goedert, 2002).

## AGD is a 4R tauopathy

Early immunohistochemical studies disclosed several sites of tau phosphorylation in AGD (Tolnay *et al.*, 1997*a*; Ferrer *et al.*, 2003). Sites of tau phosphorylation in AGD do not differ from those in AD. Moreover, in contrast to results from early studies (Tolnay *et al.*, 1997*a*), tau phosphorylation at Ser262 has been found in AGs and and pre-tangle neurons (Tolnay *et al.*, 2002; Ferrer *et al.*, 2002*b*). Immunoreactivity of AGs and pre-tangle neurons to non-phosphorylation-dependent antibodies to N-terminal and C-terminal tau suggest that full tau is contained in these structures as well as in coiled bodies (Tolnay *et al.*, 1997*a*).

Gel electrophoresis of sarkosyl-insoluble fractions has been useful to recognize the band pattern of phospho-tau in AGD. In contrast to AD, characterized by bands of 68, 64 and 60 kDa often accompanied by an upper band of about 73 kDa, AGD is characterized by a double band of 68 and 64 kDa similar to that found in PSP and CBD (Togo *et al.*, 2002; Tolnay *et al.*, 2002; Ferrer *et al.*, 2003). Therefore, AGD is considered a 4R tauopathy (Togo *et al.*, 2002). The use of specific anti-4R antibodies has corroborated this biochemical observation (Togo *et al.*, 2002). Moreover, anti-4R immunohistochemistry has proved a very useful tool for the detection of AGD cases associated with AD changes (Fujino *et al.*, 2005). (Fig. 3).

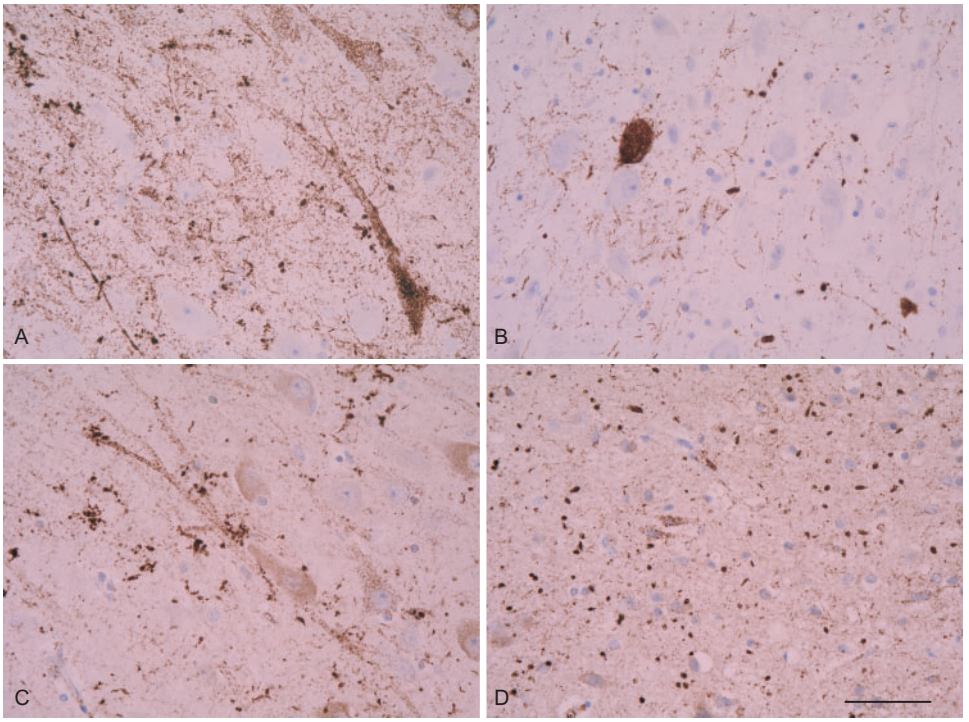
Interestingly, the presence of tangles and pre-tangles in the hippocampal CA2 area is associated with 4R tauopathy, and the most common is AGD (Ishizawa *et al.*, 2002).

In a particular series, three AGD cases showed a pattern similar to AD, but a 4R profile was found in two other cases (Zhukareva *et al.*, 2002). This example further illustrates the combination of AD and AGD changes in a number of AGD cases.

## Truncated forms of tau in AGD

In addition to characteristic bands of 68 and 64 kDa in AGD, bands of lower molecular mass have been illustrated although not described in AGD (Tolnay *et al.*, 2002). This is an important point as bands of low molecular weight are concurrently present in sarkosyl-insoluble fractions in AGD (Fig. 4). These bands are not a post-mortem artefact due to delayed tissue processing (Santpere *et al.*, 2006), but rather they evidence truncated forms of tau (Novak *et al.*, 1991, 1993; Skrabana *et al.*, 2004). The presence of truncated tau may have implications in the pathogenesis of the disease, as truncated tau promotes polymerization of tau *in vitro* (Abraha *et al.*, 2000), drives neurofibrillary degeneration (Zilka *et al.*, 2006), induces oxidative stress in a rodent model of tauopathy (Cente *et al.*, 2006), and facilitates apoptosis *in vitro* under appropriate conditions (Fasulo *et al.*, 2000).

Among several proteases capable of cleaving tau, thrombin and prothrombin are present in neurons and accumulate in neurofibrillary tangles in AD (Arai *et al.*, 2005, 2006). By using double-labelling immunohistochemistry and confocal microscopy, co-localization of 4R tau and thrombin is found in AGs (Fig. 5), thus suggesting the participation of thrombin in tau truncation in AGs. In contrast, calpain-2 and active caspase-3 (17 kDa) are only substantially expressed in tangles but not in pre-tangle neurons and AGs in AGD (Fig. 5).




AGD. A: CA1 AT8; B: laterotuberal AT8; C: CA1 4R; D: ED 4R

**Fig. 3** Strong 4R tau immunoreactivity is observed in pre-tangle neurons, tangles and grains. Paraffin sections, lightly counterstained with haematoxylin. Dilution of the 4R antibody (Upstate) 1:50. Bar = 25 microns.

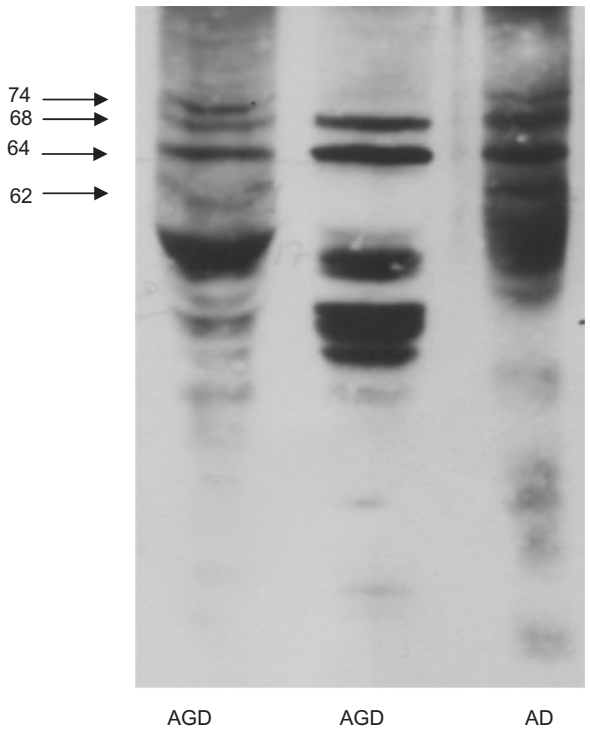



**Fig. 4** Gel electrophoresis and western blotting of sarkosyl-insoluble fractions of the hippocampus in one case with AD (right), one pure AGD (middle) and one AGD combined with AD (left) processed in parallel. Four bands of 74, 68, 64 and 60/62 kDa are characteristic of AD. Two bands of 68 and 64 kDa are seen in AGD. In addition, several bands of lower molecular mass are found in AGD.

## Tau phosphorylation and aggregation in AGD

Pre-tangle neurons contain phospho-tau, and straight filament and tubules, but do not develop paired helical filaments. Similarly, AGs are densely stained with anti-phospho-tau antibodies but they differ from neuropil threads in AD because of this particular structure. The reasons for these differences are barely known and our understanding of the factors that may distinguish pre-tangles and tangles is only fragmentary.

### Kinases involved in tau phosphorylation in AGD

A few studies have shown increased expression of active tau-kinases in AGD (Ferrer *et al.*, 2003). These include mitogen activated kinase-extracellular signal-regulated protein kinase 1 and 2 (MAPK/ERK 1 and 2), stress-activated protein kinase (SAPK-JNK), kinase p38, glycogen synthase kinase-3β (GSK-3β) and calcium/calmodulin kinase (CaMK II). Phosphorylated active kinases co-localize with phospho-tau in pre-tangles, AGs, coiled bodies and astrocytes (Ferrer *et al.*, 2003) (Fig. 6). Detailed studies focused on GSK-3β have shown early GSK-3β activation preceding and accompanying the formation of pre-tangles, tangles and other tau-immunoreactive inclusions (Leroy *et al.*, 2007).

Expression levels of the different tau-kinases in pure AGD (that is, with no associated AD pathology) are similar

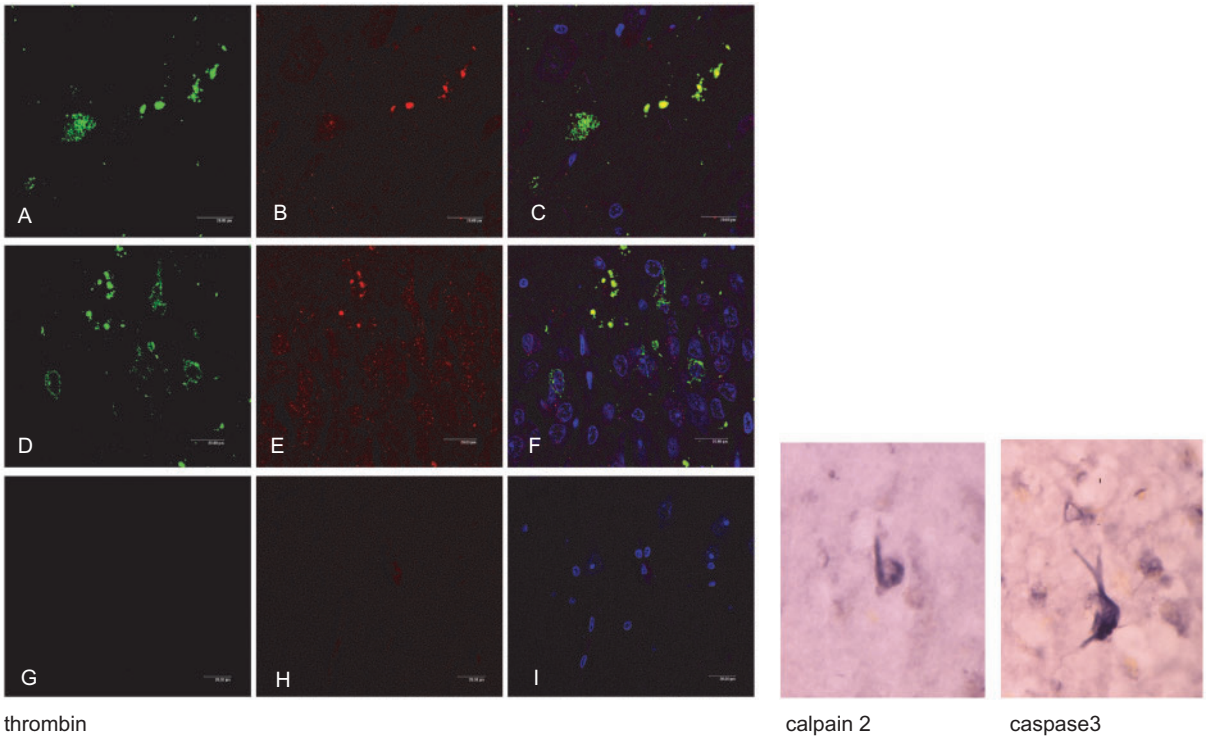



**Fig. 5** Left panel: Double-labelling immunofluorescence and confocal microscopy showing co-localization of thrombin (green: **A, D**) and 4R tau (red: **B, E**) in grains (yellow: **C, F**) in CAI area of the hippocampus (**A–C**) and dentate gyrus (**D–F**). (**G–H**): sections incubated without the primary antibodies serve as negative controls. Dilution of the thrombin antibody (American Diagnostica) 1:100 and 4R tau (Upstate) 1:50, respectively. TO-PRO counterstaining (blue) permits the visualization of nuclei. Right panel: calpain 2 (Calbiochem) 1:25 and active caspase 3 (17 kDa) (Cell Signalling) 1:25 immunohistochemistry. Immunoreactivity is restricted to neurofibrillary tangles.

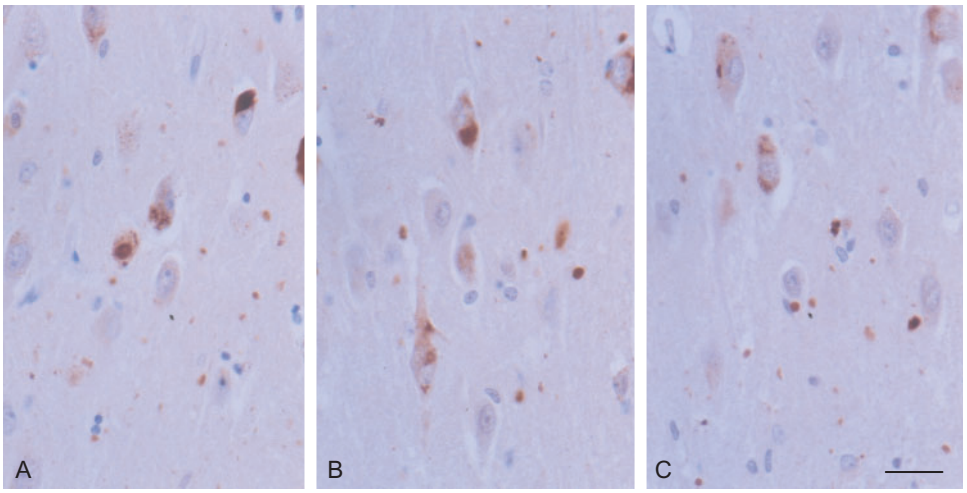



**Fig. 6** GSK-3 (**A**), SAPK/JNK-P (**B**) and p38-P (**C**) immunoreactivity in the CAI region of the hippocampus in AGD. Active tau-kinases are expressed in pre-tangle neurons, tangles and grains. Paraffin section lightly counterstained with haematoxylin. Dilution of the antibodies p38-PThr180/Tyr182 (cell Signalling) 1:200; SAPK/JNK-PThr183/Tyr185 (Cell Signalling) 1:150, GSK-3β-PSer9 (Oncogen) 1:150. Bar = 25 microns.

in total homogenates and sarkosyl-insoluble fractions, a feature which is in contrast with the sequestering of several active kinases in the sarkosyl-insoluble fraction in AD (Ferrer, 2004; Ferrer *et al.*, 2005). Therefore, one of the differential properties of tangles and pre-tangles is the sequestering of tau-kinases in tangles. Immunoprecipitation studies of tau-kinases from paired helical filament-enriched fractions in AD have shown the capacity to phosphorylate

specific substrates including recombinant tau, thus indicating that tangles have the ability to recruit tau for further phosphorylation (Ferrer *et al.*, 2002*a*; Ferrer, 2004; Ferrer *et al.*, 2005).

Components that differentiate pre-tangle neurons from tangles are tubulin (Puig *et al.*, 2005), and elevated levels of iron, ferritin and transferrin in tangles (Quintana *et al.*, 2006).

## Sequestosome 1/p62

p62 is a protein for which a role in protein aggregation and degradation has recently been attributed (Seibenhener *et al.*, 2004). p62 is able to self-aggregate and it has high affinity for multi-ubiquitin chains (Vadlamudi *et al.*, 1996; Geetha and Wooten, 2002). Based on this evidence, it has been proposed that p62 may have a role both in promoting protein aggregation and in delivering polyubiquitinated proteins to the proteasome for their degradation (Kuusisto *et al.*, 2001; Nakaso *et al.*, 2004). Previous studies have shown the presence of p62 immunoreactivity in neurons with neurofibrillary tangles and in α-synuclein inclusions within the spectrum of Lewy body diseases (Kuusisto *et al.*, 2002, 2003; Zatloukal *et al.*, 2002). p62 also decorates AGs and to a lesser degree coiled bodies (Scott and Lowe, 2007). Further analysis of p62 expression has shown diffuse moderate p62 immunoreactivity in pre-tangle neurons, sometimes with a peri-nuclear reinforcement, as well as strong localized p62 immunoreactivity in tangles and strong p62 immunoreactivity in grains (Fig. 7A and B). These findings suggest that incorporation of p62 in pre-tangle neurons and grains is probably the same as the mechanism proposed for neurofibrillary tangles in AD (Kuusisto *et al.*, 2002).

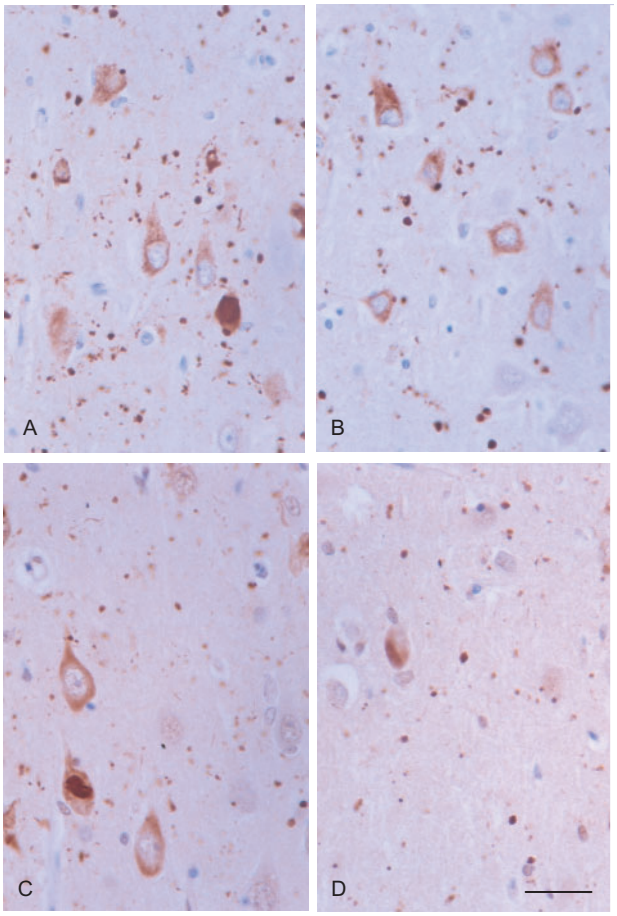



**Fig. 7** p62 immunoreactivity (**A, B**) is found in pre-tangle neurons, tangles and grains in the CA1 region of two different AGD cases. Ubiquitin immunoreactivity (**C, D**) is also observed in pre-tangle neurons, tangles and grains in the same cases. Paraffin section lightly counterstained with haematoxylin. Dilution of anti-p62 C-terminal antibody (Progen) 1:100 and anti-ubiquitin (Dako) 1:500. Bar = 25 microns.

## Ubiquitin

The ubiquitin-proteasome system (UPS) plays a crucial role in non-lysosomal protein degradation under certain physiological conditions. Misfolded proteins or un-assembled subunits of larger protein complexes and retro-translocated proteins from the endoplasmic reticulum are also subject to rapid proteasomal degradation (Herschko and Ciechanover, 1998; Glickman and Ciechanover, 2002). The ubiquitin-proteasome pathway is initiated by the conjugation of ubiquitin to the substrate leading to poly-ubiquitilation of the substrate. Most often, the 26S proteasome is composed of two caps or 19S complexes, which are responsible for recognition of ubiquitylated proteins, and the internal barrel 20S catalytic complex with three main peptidase activities: chymotrypsin-like, trypsin-like and peptidylglutamyl peptide hydrolyzing activities (Botchler *et al.*, 1999; Voges *et al.*, 1999; Herschko and Ciechanover, 1998; Glickman and Ciechanover, 2002).

The activity of the UPS is altered in many diseases characterized by aggregation of misfolded or abnormal proteins. Therefore, the proteasome has a crucial secondary role in the pathogenesis of several degenerative disorders including AD, tauopathies and synucleinopathies (Layfield *et al.*, 2003). A common feature of these disorders is the accumulation of ubiquitin bound to non-degraded proteins.

Earlier studies have established that about 50% of AGs and coiled bodies are immunostained with anti-ubiquitin antibodies (Tolnay *et al.*, 2003). However, it is our experience that ubiquitin staining of these lesions is largely sensitive to sub-optimal tissue processing due to long formalin fixation, among other factors. Ubiquitin is present in a majority of AGs and coiled bodies, as well as in many pre-tangle neurons (Fig. 7C and D). Double immunofluorescence and confocal microscopy discloses co-localization of AT8 and ubiquitin in the vast majority of AGs (Fig. 8).

## Mutant ubiquitin ($UBB^{+1}$)

$UBB^{+1}$ is generated by a non-DNA-encoded dinucleotide deletion occurring within UBB mRNA. The aberrant protein, resulting from molecular misreading, has a

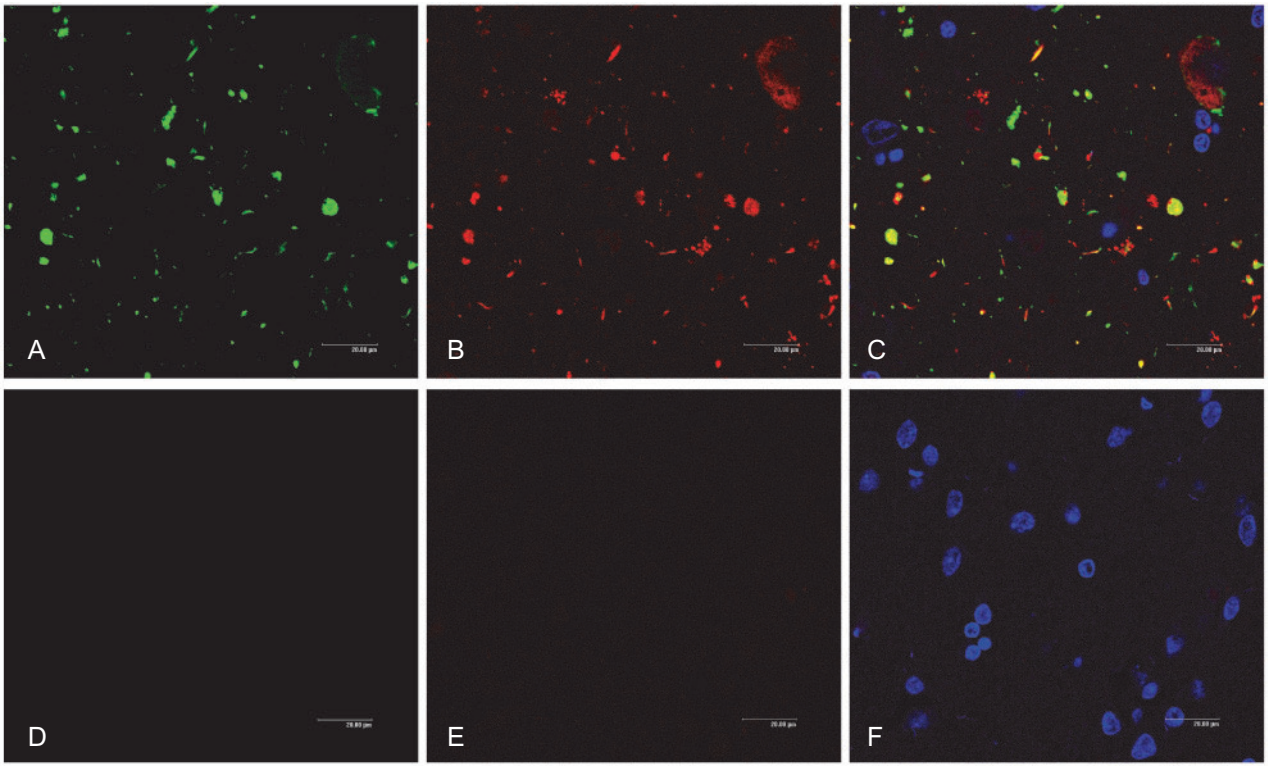



**Fig. 8** Double-labelling immunofluorescence and confocal microscopy showing co-localization of tau AT8 (green: **A**) and ubiquitin (red: **B**) in the majority of grains (yellow: **C**). **G–H**: sections incubated without the primary antibodies serve as negative controls. Dilution of AT8 (Innogenetics) 1:50 and ubiquitin (Dako) 1:500. TO-PRO counterstaining (blue) permits the visualization of nuclei.

modified C-terminus and is unable to ubiquitinate other protein substrates (van Leeuwen *et al.*, 1998). $UBB^{+1}$ is itself ubiquitinated, and while at low expression levels it can be degraded by the proteasome, at high levels it can inhibit the proteasomal machinery (Lindsten *et al.*, 2002). Previous studies have demonstrated $UBB^{+1}$ in neurofibrillary tangles in AD (Fischer *et al.*, 2003). Moreover, $UBB^{+1}$ accumulates in the hallmark inclusions of Down syndrome and all other tauopathies. Therefore, the accumulation of $UBB^{+1}$ is considered a specific marker of proteasomal dysfunction in tauopathies and polyglutamine diseases (van Leeuwen *et al.*, 1998; Fisher *et al.*, 2003; de Pril *et al.*, 2004).

Consistent with these previous observations, $UBB^{+1}$ is expressed in neurons with tangles as well as in AGs in AGD (Fisher *et al.*, 2003). However, very low levels if any are noticed in pre-tangle neurons (Fig. 9). Since the inhibition of the proteasome activity by $UBB^{+1}$ is dose-dependent (van Tijn *et al.*, 2007), it can be suggested that low levels of $UBB^{+1}$ in pre-tangle neurons would not impede proteasomal function. High levels of $UBB^{+1}$ in tangles and grains make these structures barely vulnerable to degradation via the UPS.

It has been shown that paired helical filaments (the main component of neurofibrillary tangles) may further impair the proteasomal function (Keck *et al.*, 2003). It remains to be shown whether grains also have the capacity to collapse the proteasome.

## Oxidative stress

Free radical production is a widespread phenomenon occuring under physiological aerobic metabolism in eukaryotic cells, and it is pronounced in the nervous system because of its primordial aerobic metabolism. A balance between oxidation and reduction reactions serves to maintain the physiological redox status. However, oxidative stress may cause deleterious metabolic effects when the generation of reactive oxygen species exceeds the level of antioxidant responses.

Oxidative stress plays a crucial role in the pathogenesis of AD (Moreira *et al.*, 2005; Zhu *et al.*, 2005; Nunomura *et al.*, 2006). One of the consequences of this is the lipoxidation, glycoxidation and nitration of certain proteins (Pamplona *et al.*, 2005; Butterfield *et al.*, 2006*a*; Butterfield *et al.*, 2006*b*; Sultana *et al.*, 2006*a*; Sultana *et al.*, 2006*b*; Sultana *et al.*, 2006*c*), which results in loss of function. Decline in the proteasome function in the earliest stages of AD is partially due to oxidative inactivation (Cecarini *et al.*, 2007). Another consequence is the activation of signal transduction cascades including stress kinases (Petersen *et al.*, 2007).

In spite of the important information about oxidative stress in AD, practically nothing is known about its role in AGD. Yet increased expression of advanced glycation end products (AGEs) and AGE receptor (RAGE) can be seen by immunohistochemistry in neurons of the hippocampus and

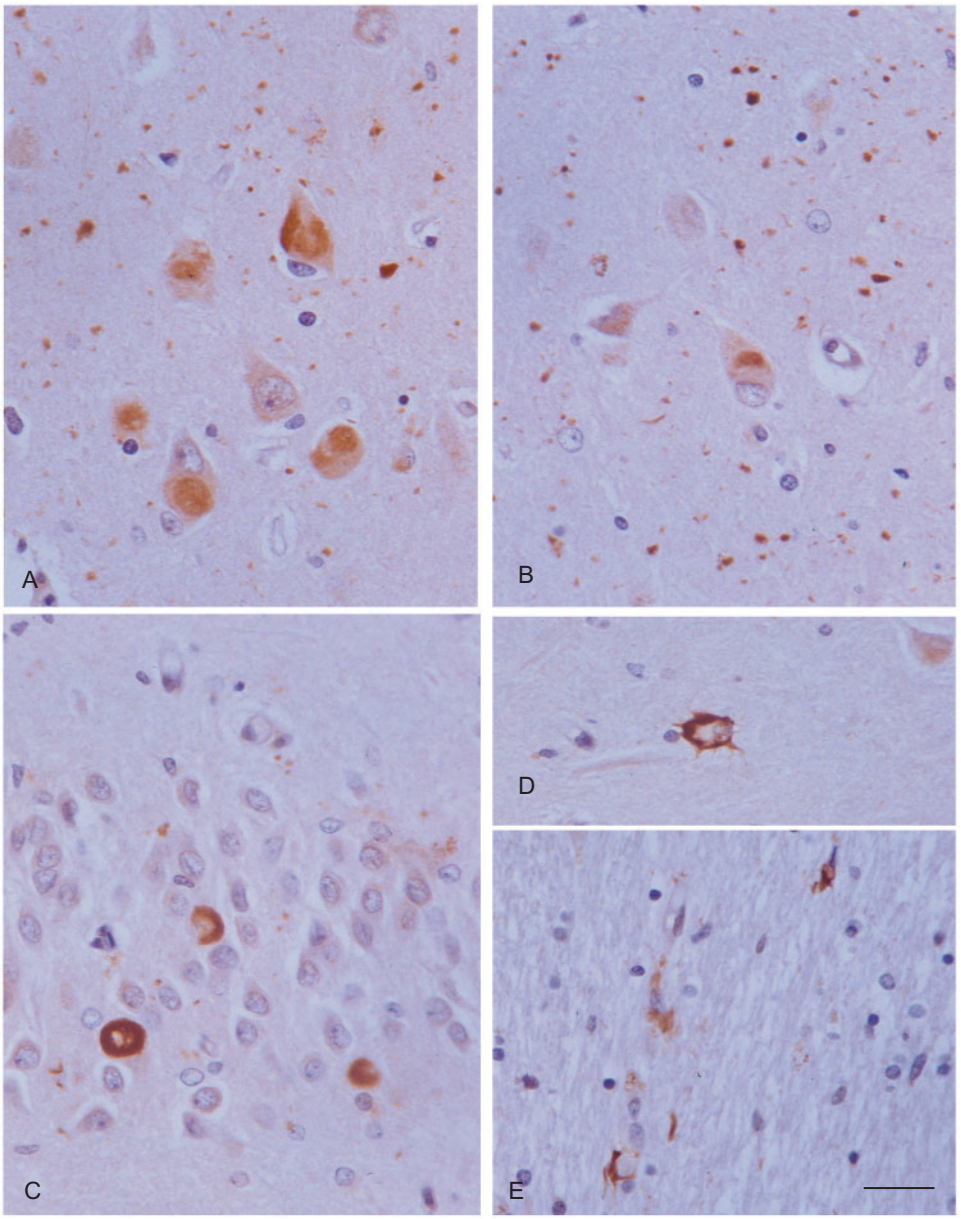



**Fig. 9** Round elongated deposits of mutant ubiquitin ($UBB^{+1}$) immunoreactivity in neurons of the CA1 region (**A**), entorhinal cortex (**B**), and granule cells of the dentate gyrus (**C**), as well as in astrocytes (**D**) and coiled bodies (**E**). $UBB^{+1}$ immunoreactivity is clearly present in grains (**A, B**). Paraffin section lightly counterstained with haematoxylin. Dilution of the rabbit polyclonal $UBB^{+1}$antibody (Dr Fred W. van Leeuwen, Ubi2+1, 140994, for details see Fisher *et al*., 2003) 1:400. **A**, **B**, bar in **B** = 25 microns; **C–E**, bar in **E** = 10 microns.

entorhinal cortex in pure forms of AGD (data not shown). AGEs are carbonyl groups generated by secondary reaction of the primary amino group of lysine residues with reactive carbonyl derivatives produced by the reaction of reducing sugars or their oxidation products with lysine residues of proteins (glycation/glycoxidation reactions) (Dalle-Donne *et al*., 2006). RAGE is a member of the immunoglobulin superfamily sensitive to the generation of reactive oxygen species that is crucial for many AGE-induced changes in cellular properties, including activation of protein kinase pathways (Lander *et al*., 1997; Schmidt *et al*., 2000). Anti-oxidant responses can also be determined by immunohistochemistry to Cu/ZN superoxide dismutase I (SOD1) and Mn superoxide dismutase II (SOD2) in the AGD brain (data not shown). Increased SOD1 and SOD2 immunoreactivity is observed in neurons in vulnerable regions in parallel with AGs and pre-tangle neurons. These findings show that increased oxidative stress and increased oxidative responses occur in the hippocampus and entorhinal cortex in pure AGD, and, therefore, that these modifications are unrelated to typical AD changes.

Evidence of oxidative stress also suggests a link between free radical production, stress kinase (basically, SAPK/JNK and p38) activation, and tau phosphorylation in

AGD. Similar scenarios have been reported in AD and other human and murine tauopathies (Zhu *et al.*, 2000, 2001, 2003; Puig *et al.*, 2004; Ferrer, 2004, Ferrer *et al.*, 2005).

## Neurotransmitters and receptors

Little is known about neurotransmitters and receptors in AGD. A single study reported normal cortical levels of choline acetyltransferase but markedly reduced levels of dopamine and its metabolites in the striatum (Yamada *et al.*, 1992). However, the description of these two cases is consistent with AGD associated with progressive subcortical gliosis.

Recently, increased adenosine receptor A (A1), but not A2A or A2B, together with increased levels of adenylyl ciclase, an effector of A1, and sensitization of this pathway, has been reported in the hippocampus but not in the frontal cortex in pure AGD (Perez-Buira *et al.*, 2007). This has been interpreted as a compensatory response geared to modulating glutamate neurotransmission and facilitating neuroprotection of this preferentially involved region in AGD.

## Concluding comments

Although very common, AGD is still a poorly understood neurological disorder. However, the recent studies discussed above allow us to delineate a pathogenic scenario for some hallmark aspects of the disease. A summary of the pathways involved and suggested activation of metabolic cascades is shown in Fig. 10. Aging is the most important determinant factor. Oxidative stress plays a crucial role in the activation of stress-activated tau-kinases, which facilitate tau-hyperphosphorylation in a subset of neurons. Abnormal tau is aggregated after binding with p62 and subsequently ubiquitinated in grains, pre-tangle neurons and tangles. Incorporation of mutant ubiquitin, $UBB^{+1}$, blocks hyperphosphorylated tau degradation in grains and tangles. These structures sequester active tau-kinases, thus further promoting local tau hyperphosphorylation in AGs and tangles. Finally, thrombin accumulated in AGs and tangles may facilitate tau truncation, which is toxic for cells and which increases oxidative stress.

In spite of these achievements, several points remain obscure, such as: (i) original causes leading to oxidative

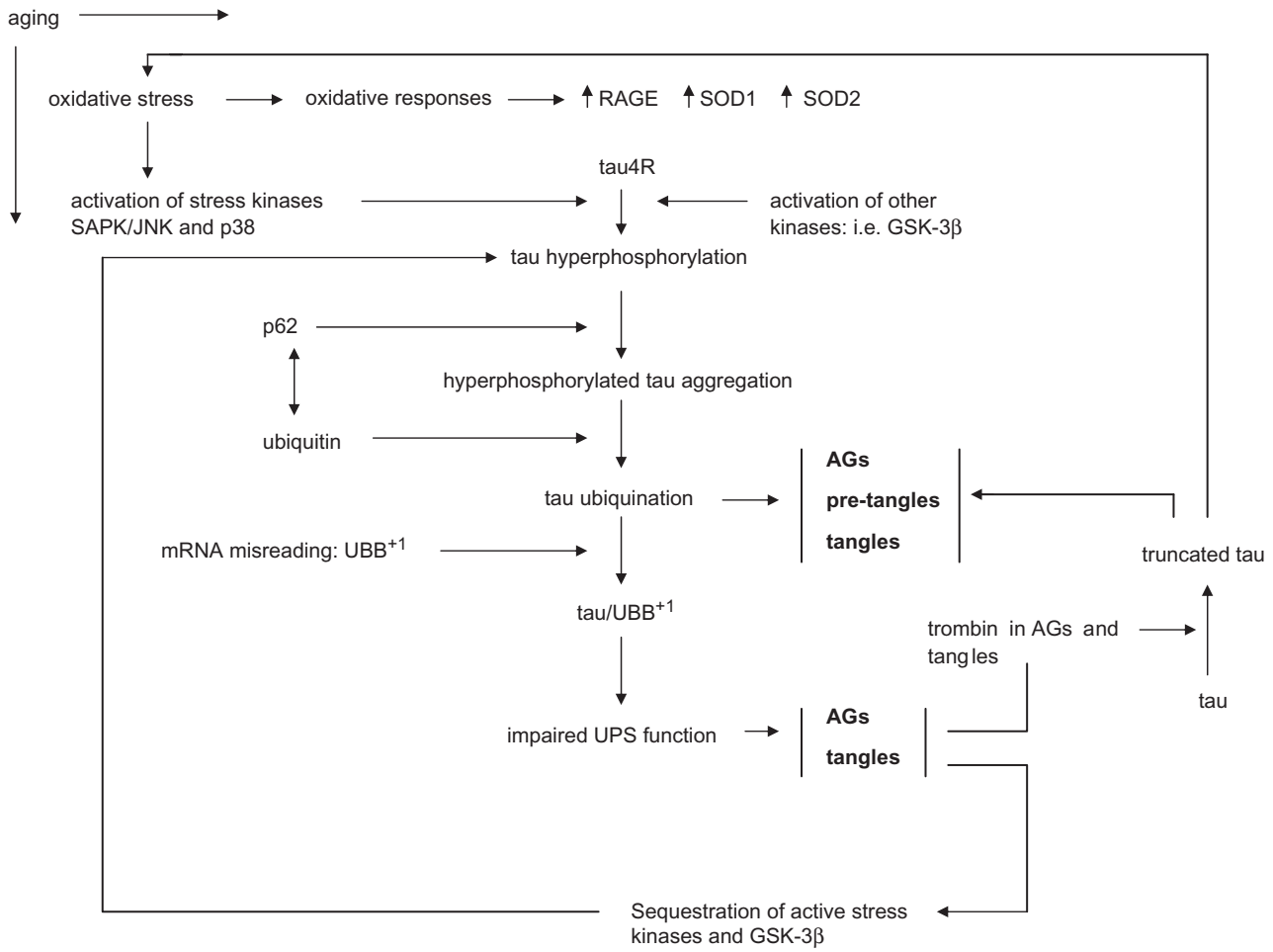



**Fig. 10** Summary of pathologic events in AGD.

stress; (ii) targets of oxidative stress; (iii) role played by phosphatases; and (iv) reasons for selective tau 4R hyperphosphorylation, among others.

AGD is restricted to human beings, as no similar lesions have been reported under natural conditions in animals. Yet deafferentation of the hippocampus after lesions of the entorhinal cortex in rats is followed by the presence of small granules containing phospho-tau in the molecular layer of the dentate gyrus (Mudher *et al.*, 2001). Whether combined deafferentation and abnormal tau metabolism localized in vulnerable points of the dendritic arbour may be causative of grain architecture is a fascinating working hypothesis.

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### References

Abraha A, Ghoshal N, Gamblin TC, Cryns V, Berry RW, Kuret J, et al. C-terminal inhibition of tau assembly in vitro and in Alzheimer's disease. J Cell Sci 2000; 113: 3737–45.

Alafuzoff I, Pikkarainen M, Al-Sarraj S, Arzberger T, Bell J, Bodi I, et al. Interlaboratory comparison of assessments of Alzheimer disease-related lesions: a study of the BrainNet Europe Consortium. J Neuropathol Exp Neurol 2006; 65: 740–57.

Arai T, Guo GP, McGeer PL. Proteolysis of non-phosphorylated and phosphorylated tau by thrombin. J Biol Chem 2005; 280: 5145–53.

Arai T, Miklossy J, Klegeris A, Guo JP, McGeer PL. Thrombin and prothrombin are expressed by neurons and glial cells and accumulate in neurofibrillary tangles in Alzheimer disease brain. J Neuropathol Exp Neurol 2006; 65: 19–25.

Bancher C, Brunner C, Lassman H, Budka H, Jellinger K, Wiche G, et al. Accumulation of abnormally phosphorylated tau precedes tau formation of neurofibrillary tangles in Alzheimer's disease. Brain Res 1989; 477: 90–9.

Beach TG, Sue L, Scott S, Layne K, Newell A, Walker D, et al. Hippocampal sclerosis dementia with tauopathy. Brain Pathol 2003; 13: 263–78.

Botchler M, Ditzel Groll M, Hartmann C, Huber R. The proteasome. Annu Rev Biophys Biomol Struct 1999; 28: 295–317.

Botez G, Probst A, Ipsen S, Tolnay M. Astrocytes expressing hyperphosphorylated tau protein without glial fibrillary tangles in argyrophilic grain disease. Acta Neuropathol 1999; 98: 251–6.

Braak H, Alafuzoff I, Arzberger T, Kretzschmar H, Del Tredici K. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. Acta Neuropathol 2006; 112: 389–404.

Braak H, Braak E. Argyrophylic grains: characteristic pathology of cerebral cortex in cases of adult-onset dementia without Alzheimer changes. Neurosci Lett 1987; 76: 124–7.

Braak H, Braak E. Cortical and subcortical argyrophylic grains characterize a disease associated with adult onset dementia. Neuropathol Appl Neurobiol 1989; 15: 13–26.

Braak H, Braak E. Argyrophilic grain disease: frequency of occurrence in different age categories and neuropathological diagnostic criteria. J Neural Transm 1998; 105: 801–19.

Braak H, Braak E. Temporal sequence of Alzheimer's disease-related pathology. In: Peters A and Morrison JH, editors. Cerebral Cortex Vol 14: Neurodegenerative and Age-related Changes in Structure and Function of Cerebral Cortex. New York, Boston, Dordrecht, London, Moscow: Kluwer Academic/Plenum Press; 1999. p. 475–512.

Braak H, Braak E, Mandelkow EM. A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. Acta Neuropathol 1994; 87: 554–67.

Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 2003; 24: 197–211.

Buée-Scherrer V, Goedert M. Phosphorylation of microtubule-associated protein *tau* by stress-activated protein kinases in intact cells. FEBS Lett 2002; 515: 151–4.

Butterfield DA, Perluigi M, Sultana R. Oxidative stress in Alzheimer's disease brain: new insights from redox proteomics. Eur J Pharmacol 2006a; 545: 39–50.

Butterfield DA, Poon HF, St Clair D, Keller JN, Pierce WN, Klein JB, et al. Redox proteomics identification of oxidatively modified hippocampal proteins in mild cognitive impairment: insights into the development of Alzheimer's disease. Neurobiol Dis 2006*b; 22: 223–32.*

Cairns NJ, Bigio EH, MacKenzie IRA, Neumann M, Lee VMY, Hatanpaa KJ, et al. Neuropathologic diagnostic and nosologic criteria for frontotemporal lobal degeneration: consensus of the consortium for frontotemporal lobar degeneration. Acta Neuropathol 2007; 114: 5–22.

Cecarini V, Ding Q, Keller JN. Oxidative inactivation of the proteasome in Alzheimer's disease. Free Radic Res 2007; 41: 673–80.

Cente M, Filipcik P, Pevalova M, Novak M. Expression of a truncated tau protein induces oxidative stress in a rodent model of tauopathy. Eur J Neurosci 2006; 24: 1085–90.

Chin SM, Goldman EJ. Glial inclusions in the CNS degenerative diseases. J Neuropathol Exp Neurol 1996; 55: 499–508.

Cras P, Perry G. Dementia with argyrophilic grains. Ann Neurol 1991; 30: 853–4.

Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction and disease progression. J Cell Mol Med 2006; 10: 389–406.

Davis DG, Ross GW, Petrovich H, White LR, Hardman JM, Nelson JS, et al. Quantitation of argyrophilic grains in hippocampal CA1 of aged Japanese-American men. J Neuropathol Exp Neurol 1997; 56: 587.

de Pril R, Fischer DF, Maat-Schieman ML, Hobo B, De Vos RA, Brunt ER, et al. Accumulation of aberrant ubiquitin induces aggregate formation and cell death in polyglutamine diseases. Hum Mol Genet 2004; 13: 1803–13.

De Vos RAI, Jansen ENH, Wesseling P, Braak H. Parkinson's disease and dementia with argyrophilic grains. Neuropathol Appl Neurobiol 1996; 22 (Suppl 1): 75.

Ding ZT, Wang Y, Jiang YP, Yoshida M, Mimuro M, Inagaki T, et al. Argyrophilic grain disease: frequency and neuropathology in centenarians. Acta Neuropathol 2006; 111: 320–8.

Fasulo L, Ugolini G, Visintin M, Bradbury A, Brancolini C, Verzillo V, et al. The neuronal microtubule-associated protein tau is a substrate for caspase-3 and an effector of apoptosis. J Neurochem 2000; 75: 624–33.

Ferrer I. El método de Golgi en neuropatología humana. In: Cruz-Sanchez F, editor. Neuropatología. Madrid: Edimsa; 2000. p. 113–6.

Ferrer I. Stress kinases involved in tau phosphorylation in Alzheimer's disease, tauopathies, and APP transgenic mice. Neurotox Res 2004; 6: 469–75.

Ferrer I, Aymami A, Rovira A, Grau Veciana JM. Growth of abnormal neurites in Alzheimer's disease. A study with the Golgi method. Acta Neuropathol 1983; 59: 167–70.

Ferrer I, Barrachina M, Puig B. Glycogen synthase kinase-3 is associated with neuronal and glial hyperphosphorylated tau deposits in Alzheimer's disease, Pick's disease, progressive supranuclear palsy and corticobasal degeneration. Acta Neuropathol 2002a; 104: 583–91.

Ferrer I, Barrachina M, Puig B. Anti-tau phosphospecific Ser262 antibody recognizes a variety of abnormal hyper-phosphorylated tau deposits in tauopathies including Pick's disease and argyrophilic grains. Acta Neuropathol 2002b; 104: 658–64.

Ferrer I, Barrachina M, Tolnay M, Rey MJ, Vidal N, Carmona M, et al. Phosphorylated protein kinases associated with neuronal and glial tau deposits in argyrophilic grain disease. Brain Pathol 2003; 13: 62–78.

Ferrer I, Gomez-Isla T, Puig B, Freixes M, Ribe E, Dalfo E, et al. Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. Curr Alzheimer Res 2005; 2: 3–18.

Ferrer I, Guionnet N, Cruz-Sanchez F, Tuñon T. Neuronal alterations in patients with dementia: a Golgi study on biopsy samples. Neurosci Lett 1990; 114: 11–6.

Ferrer I, Hernandez I, Boada M, Llorente A, Rey MJ, Cardozo A, et al. Primary progressive aphasia as the initial manifestation of corticobasal degeneration and unusual tauopathies. Acta Neuropathol 2003; 106: 419–35.

Fischer DF, De Vos RA, Van DR, De Vrij FM, Proper EA, Sonnemans MA, et al. Disease-specific accumulation of mutant ubiquitin as a marker for proteasomal dysfunction in the brain. FASEB J 2003; 17: 2014–24.

Fujino Y, Delucia MW, Davies P, Dickson DW. Ballooned neurons in the limbic lobe are associated with Alzheimer type pathology and lack diagnostic specificity. Neuropathol Appl Neurobiol 2004; 30: 676–82.

Fujino Y, Wang DS, Thomas N, Espinoza M, Davies P, Dickson DW. Increased frequency of argyrophilic grain disease in Alzheimer disease with 4R tau-specific immunohistochemistry. J Neuropathol Exp Neurol 2005; 64: 209–14.

Geetha T, Wooten MW. Structure and functional properties of the ubiquitin binding protein p62. FEBS Lett 2002; 512: 19–24.

Ghebremedin E, Schultz C, Botez G, Rub U, Sassin I, Braak E, et al. Argyrophilic grain disease is associated with apolipoprotein E epsilon 2 allele. Acta Neuropathol 1998; 96: 222–4.

Ghebremedin E, Schultz C, Thal DR, Del Tredici K, Rueb U, Braak H. Genetic association of argyrophilic grain disease with polymorphisms in alpha-2 macroglobulin and low-density lipoprotein receptor-related protein genes. Neuropathol Appl Neurobiol 2002; 28: 308–13.

Ghosh JG, Houck SA, Clark JI. Interactive domains in the molecular chaperone human αB-crystallin modulate microtubule assembly and disassembly. PLoS ONE 2007*a; 2: e498.*

Ghosh JG, Houck SA, Clark JI. Interactive sequences in the stress protein and molecular chaperone human αB-crystallin recognize and modulate the assembly of filaments. In J Biochem Cell Biol 2007b; 39: 1804–15.

Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002; 82: 373–428.

Goedert M, Hasegawa M, Jakes R, Lawler S, Cuenda A, Cohen P. Phosphorylation of microtubule-associated protein *tau* by stress-activated protein kinases. FEBS Lett 1997; 409: 57–62.

Goedert M, Spillantini MG, Davies SW. Filamentous nerve cell inclusions in neurodegenerative diseases. Curr Opin Neurobiol 1998; 8: 619–32.

Goedert M, Spillanini MG, Potier MC, Ulrich J, Crowther RA. Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein *tau* containing four tandem repeats: differential expression of tau protein mRNAs in human brain. EMBO J 1989; 8: 393–9.

Hanger DP, Hughes K, Woodgett JR, Brion JP, Anderton BH. Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of *tau*: generation of paired helical filament epitopes and neuronal localisation of the kinase. Neurosci Lett 1992; 147: 58–62.

Herschko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998; 67: 425–79.

Himmler A, Drechsel D, Kirschner MW, Martin DW. *Tau* consists of a set of proteins with repeat C-terminal microtubule-binding domains and variable N-terminal domains. Mol Cell Biol 1989; 9: 1381–8.

Hodges JR, Davies RR, Xuereb JH, Casey B, Broe M, Bak TH, et al. Clinicopathological correlates in frontotemporal dementia. Ann Neurol 2004; 56: 399–406.

Ikeda K, Akiyama H, Arai T, Matsushita M, Tsuchiya K, Miyazaki H. Clinical aspects of argyrophilic grain disease. Clin Neuropathol 2000; 19: 278–84.

Ikeda K, Akiyama H, Arai T, Nishima T. Glial tau pathology in neurodegenerative diseases: their nature and comparison with neuronal tangles. Neurobiol Dis 1998; 19: 85–91.

Ikeda K, Akiyama H, Kondo H, Haga C. A study of dementia with argyrophilic grains. Possible cytoskeletal abnormality in dendrospinal portion of neurons and oligodendroglia. Acta Neuropathol 1995; 89: 409–14.

Ishihara K, Araki S, Ihori N, Shiota J, Kawamura M, Yoshida M, et al. Argyrophilic grain disease presenting with frontotemporal dementia: a neuropsychological and pathological study of an autopsied case with presenile onset. Neuropathology 2005; 25: 165–70.

Ishizawa T, Ko LW, Cookson N, Davias P, Espinoza M, Dickson DW. Selective neurofibrillary degeneration of the hippocampal CA2 sector is associated with four-repeat tauopathies. J Neuropathol Exp Neurol 2002; 61: 1040–7.

Itagaki S, Mc Geer PL, Akiyama H, Beattie BL, Walker DG, Moore GR, et al. A case of adult-onset dementia with argyrophilic grains. Ann Neurol 1989; 26: 685–9.

Jellinger KA. Dementia with grains (argyrophilic grain disease). Brain Pathol 1998; 8: 377–86.

Jellinger KA, Bancher C. Senile dementia with tangles (tangle predominant form of senile dementia). Brain Pathol 1998; 8: 367–76.

Jenkins SM, Zinnerman M, Garner C, Johnson GV. Modulation of *tau* phosphorylation and intracellular localization by cellular stress. Biochem J 2000; 345: 263–70.

Jicha GA, Petersen RC, Knopman DS, Boeve BF, Smith GE, Geda YE, et al. Argyrophilic grain disease in demented subjects presenting initially with amnestic mild cognitive impairment. J Neuropathol Exp Neurol 2006; 65: 602–9.

Josephs KA, Whitwell JL, Parisi JE, Knopman DS, Boeve BF, Geda YE, et al. Argyrophilic grai: a distinct disease or an additive pathology? Neurobiol Aging 2006 Dec 22 [Epub ahead of print].

Keck S, Nitsch R, Grune T, Ullrich O. Proteasome inhibition by paired helical filament-tau in brains of patients with Alzheimer's disease. J Neurochem 2003; 85: 115–22.

Kiwashima T, Katsumi D, Iwaki T. Argyrophilic grains in late-onset Creutzfeldt-Jakob diseased brains. Pathol Internat 1999; 49: 369–73.

Klos KJ, Josephs KA, Parisi JE, Dickson DW. Alpha-synuclein immunohistochemistry in two cases of co-ocurring idiopathic Parkinson's disease and motor neuron disease. Mov Disord 2005; 20: 1515–20.

Knopman DS, Parisi JE, Salviati A, Floriach-Robert M, Boeve BF, Ivnik RJ, et al. Neuropathology of cognitively normal elderly. J Neuropathol Exp Neurol 2003; 62: 1087–95.

Komori T. Tau-positive glial inclusions in progressive supranuclear palsy, corticobasal degeneration and Pick's disease. Brain Pathol 1999; 9: 663–79.

Kovacks GK, Pittman A, Revesz T, Luk C, Lees A, et al. *MAPT* S305I mutation: implications for argyrophilic grain disease. Acta Neuropathol 2007; Dec 8 [Epub ahead of print].

Kuusisto E, Parkkinen L, Alafuzoff I. Morphogenesis of Lewy bodies: dissimilar incorporation of alpha-synuclein, ubiquitin, and p62. J Neuropathol Exp Neurol 2003; 62: 1241–53.

Kuusisto E, Salminen A, Alafuzoff I. Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies. Neuroreport 2001; 12: 2085–90.

Kuusisto E, Salminen A, Alafuzoff I. Early accumulation of p62 in neurofibrillary tangles in Alzheimer's disease: possible role in tangle formation. Neuropathol Appl Neurobiol 2002; 28: 228–37.

Lander HM, Tauras JM, Ogiste JS, Hori H, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a $p21^{ras}$-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. J Biol Chem 1997; 272: 17810–4.

Layfield R, Cavey JR, Lowe J. Role of ubiquitin-mediated proteolysis in the pathogenesis of neurodegenerative disorders. Ageing Res Rev 2003; 2: 343–56.

Leroy K, Yilmaz Z, Brion JP. Increased level of active GSK-3β in Alzheimer's disease and accumulation in argyrophilic grains in neurones at different stages of neurofibrillary degeneration. Neuropathol Appl Neurobiol 2007; 33: 43–55.

Liang TW, Forman MS, Duda JE, McCluskey L, Trojanowski JQ, Siderowf A. Multiple pathologies in a patient with a progressive neurodegenerative syndrome. J Neurol Neurosurg Psychiatry 2005; 76: 252–5.

Lindsten K, De Vrij FM, Verhoef LG, Fischer DF, van Leeuwen FW, Hol EM, et al. Mutant ubiquitin found in neurodegenerative disorders is a ubiquitin fusion degradation substrate that blocks proteasomal degradation. J Cell Biol 2002; 157: 417–27.

Lovestone S, Reynolds CH. The phosphorylation of *tau*: a critical stage in neurodevelopment and neurodegenerative processes. Neuroscience 1997; 78: 309–24.

Mandelkow EM, Drewes G, Biernat J, Gustke N, Van Lint J, Vandenheede JR, et al. Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein *tau*. FEBS Lett 1992; 314: 315–21.

Martinez-Lage M, Muñoz DG. Prevalence and disease association of argyrophilic grains of Braak. J Neuropathol Exp Neurol 1997; 56: 157–64.

Masliah E, Hansen LA, Quijata S, De Teresa R, Alford M, Kauss J, et al. Late onset dementia with argyrophilic grains and subcortical tangles or atypical progressive supranuclear palsy. Ann Neurol 1991; 29: 389–96.

Maurage CA, Sergeant N, Schraen-Maschke SS, Lebert F, Ruchoux MM, Sablonnière B, et al. Diffuse form of argyrophilic grain disease: a new variant of four-repeat tauopathy different from limbic argyrophilic grain disease. Acta Neuropathol 2003; 106: 575–83.

McKeith IG, Dickson DW, Lowe J, Emre M, O'Brien JT, Feldman H, et al. Diagnostic [Diagnostics? Diagnosis?] and management of dementia with Lewy bodies: third report of the DLB Consortium. Neurology 2005; 65: 1863–72.

Miserez AR, Clavaguera F, Monsch AU, Probst A, Tolnay M. Argyrophilic grain disease: molecular genetic difference to other four-repeat tauopathies. Acta Neuropathol 2003; 106: 363–6.

Moreira PI, Honda K, Liu Q, Santos MS, Oliveira CR, Aliev G, et al. Oxidative stress: the old enemy in Alzheimer's disease. Curr Alzheimer Res 2005; 2: 403–8.

Mudher AK, Yee B, Smith AD, Perry VH. Deafferentation of the hippocampus results in the induction of AT8 positive 'granules' in the rat. Neurosci Lett 2001; 301: 5–8.

Nakaso K, Yoshimoto Y, Nakano T, Takeshima T, Fukuhara Y, Yasui K, et al. Transcriptional activation of p62/A170/ZIP during the formation of the aggregates: possible mechanisms and the role in Lewy body formation in Parkinson's disease. Brain Res 2004; 1012: 42–51.

Novak M, Jakes R, Edwards PC, Milstein C, Wischik CM. Difference between the tau protein of Alzheimer paired helical filament core and normal tau revealed by epitope analysis of monoclonal antibodies 423 and 7.51. Proc Natl Acad Sci USA 1991; 88: 5837–41.

Novak M, Kabat J, Wischik CM. Molecular characterisation of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament. EMBO J 1993; 12: 365–70.

Nunomura A, Castellani RJ, Zhu X, Moreira PI, Perry G, Smith MA. Involvement of oxidative stress in Alzheimer disease. J Neuropathol Exp Neurol 2006; 65: 631–41.

Ohto-Fujita E, Fujita Y, Atomi Y. Analysis of the αB-crystallin domain responsible for inhibiting tubulin aggregation. Cell Stress Chaperones 2007; 12: 163–71.

Pamplona R, Dalfó E, Ayala V, Bellmunt J, Prat J, Ferrer I, et al. Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets. J Biol Chem 2005; 280: 21522–30.

Perez-Buira S, Barrachina M, Rodriguez A, Albasanz JL, Martin M, Ferrer I. Expression levels of adenosine receptors in hippocampus and frontal cortex in argyrophilic grain disease. Neurosci Lett 2007; 423: 194–9.

Petersen RB, Nunomura A, Lee HG, Casadesus G, Perry G, Smith MA, et al. Signal transduction cascades associated with oxidative stressing Alzheimer's disease. J Alzheimers Dis 2007; 11: 143–52.

Petersen RC, Parisi JE, Dickson DW, Johnson KA, Knopman DS, Boeve BF, et al. Neuropathologic features of amnestic mild cognitive impairment. Arch Neurol 2006; 63: 665–72.

Popescu A, Lippa CF, Lee VM, Trojanowski JQ. Lewy bodies in the amygdala: increase of α-synuclein aggregates in neurodegenerative diseases with tau-based inclusions. Arch Neurol 2004; 61: 1915–9.

Probst A, Basler V, Bron B, Ulrich J. Neuritic plaques in senile dementia of Alzheimer's type: a Golgi analysis in the hippocampal region. Brain Res 1983; 59: 167–70.

Probst A, Taylor KI, Tolnay M. Hippocampal sclerosis dementia: a reappraisal. Acta Neuropathol 2007; 114: 335–45.

Puig B, Ferrer I, Ludueña RF, Avila J. BetaII-tubulin and phospho-tau aggregates in Alzheimer's disease and Pick's disease. J Alzheimers Dis 2005; 7: 213–20.

Puig B, Gómez-Isla T, Ribé E, Cuadrado M, Torrejón-Escribano B, Dalfó E, et al. Expression of stress-activated kinase c-Jun N-terminal kinase (SAPK/JNK-P) and p38 (p38-P), and tau hyperphosphorylation in neurites surrounding βA plaques in APP Tg2576 mice. Neuropathol. Appl Neurobiol 2004; 30: 491–502.

Quintana C, Bellefgih S, Laval JY, Guerkin-Kern JL, Wu TD, Avila J, et al. Study of the localization of iron, ferritin, and hemosiderin in Alzheimer's disease hippocampus by analytical microscopy at the subcellular level. J Struct Biol 2006; 153: 42–54.

Reynolds CH, Betts JC, Blackstock WP, Nebreda AR, Anderton BH. Phosphorylation sites on *tau* identified by nanoelectrospray mass spectrometry: differences in vitro between the mitogen-activated protein kinases ERK2, c-Jun N-terminal kinase and p38, and glycogen synthase kinase-3β. J Neurochem 2000; 74: 1587–95.

Reynolds CH, Nebreda AR, Gibb GM, Utton MA, Anderton BH. Reactivating kinase/p38 phosphorylates *tau* protein *in vitro*. J Neurochem 1997a; 69: 191–8.

Reynolds CH, Utton MA, Gibb GM, Yates A, Anderton BH. Stress-activated protein kinase/c-Jun N-terminal kinase phosphorylates *tau* protein. J Neurochem 1997b; 68: 1736–44.

Rippon GA, Boeve BF, Parisi JE, Dickson DW, Ivnik RI, Jack CR, et al. Late-onset frontotemporal dementia associated with progressive supranuclear palsy/argyrophilic grain disease/Alzheimer's disease pathology. Neurocase 2005; 11: 204–11.

Saito Y, Nakahara K, Yamanouchi H, Murayama S. Severe involvement of ambient gyrus in dementia with grains. J Neuropathol Exp Neurol 2002; 61: 789–96.

Saito Y, Ruberu NN, Sawabe M, Arai T, Tanaka N, Kakuta Y, et al. Staging of argyrophilic grains: an age-associated tauopathy. J Neuropathol Exp Neurol 2004; 63: 911–8.

Santpere G, Puig B, Ferrer I. Low molecular weight species of tau in Alzheimer's disease are dependent on tau phosphorylation sites but not on delayed post-mortem delay in tissue processing. Neurosci Lett 2006; 399: 106–10.

Scheibel AB, Tomiyasu U. Dendritic sprouting in Alzheimer's senile dementia. Expl Neurol 1978; 60: 1–8.

Schmidt AM, Yan SD, Yan SF, Stern DM. The biology of the receptor for advanced glycation end products and its ligands. Biochim Biophys Acta 2000; 1498: 99–111.

Schultz C, Koppers D, Sassin I, Braak E, Braak H. Cytoskeletal alterations in the human tuberal hypothalamus related to argyrophilic grain disease. Acta Neuropathol 1998; 96: 596–602.

Scott IS, Lowe JS. The ubiquitin-binding protein p62 identifies argyrophylic grain pathology with greater sensitivity than conventional silver stains. Acta Neuropathol 2007; 113: 417–20.

Seibenhener ML, Babu JR, Geetha T, Wong HC, Krishna NR, Wooten MW. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol Cell Biol 2004; 24: 8055–68.

Seno H, Kobayashi S, Inagaki T, Yamamori C, Miyaoka T, Horiguchi J, et al. Parkinson's disease associated with argyrophilic grains clinically resembling progressive supranuclear palsy: an autopsy case. J Neurol Sci 2000; 178: 70–4.

Singh BN, Rao KS, Ramakrishna T, Rangaraj N, Rao ChM. Association of αB-crystallin, a small heat shock protein, with actin: role in modulating actin filament dynamics in vivo. J Mol Biol 2007; 366: 756–67.

Skrabana R, Kontsek P, Mederlyova A, Iqbal K, Novak M. Folding of Alzheimer's core PHF subunit revealed by monoclonal antibody 423. FEBS Lett 2004; 568: 178–82.

Spillantini MG, Tolnay M, Love S, Goedert M. Microtubule-associated protein tau, heparin sulphate and synuclein in several neurodegenerative diseases with dementia. Acta Neuropathol 1999; 97: 585–94.

Sultana R, Boyd-Kimball D, Poon HF, Cai J, Pierce WM, Klein JB, et al. Redox proteomics identification of oxidized proteins in Alzheimer's disease hippocampus and cerebellum: an approach to understand pathological and biochemical alterations in AD. Neurobiol Aging 2006a; 27: 1564–76.

Sultana R, Perluigi M, Butterfield DA. Protein oxidation and lipid peroxidation in brain of subjects with Alzheimer's disease: insights into mechanisms of neurodegeneration from redox proteomics. Antioxid Redox Signal 2006b; 8: 2021–37.

Sultana R, Poon HF, Cai J, Pierce WM, Merchant M, Klein JB, et al. Identification of nitrated proteins in Alzheimer's disease brain using a redox proteomics approach. Neurobiol Dis 2006c; 22: 76–87.

Thal DR, Schultz C, Botez G, Del Tedici K, Mrak RE, Griffin WST, et al. The impact or agyrophilic grain disease on the development of dementia and its relationship to concurrent Alzheimer's disease-related pathology. Neuropathol Appl Neurobiol 2005; 31: 270–9.

Togo T, Cookson N, Dickson D. Argyrophilic grain disease: neuropathology, frequency in a dementia brain bank and lack of relationship with apolipoprotein E. Brain Pathol 2002; 12: 45–52.

Togo T, Dickson DW. Ballooned neurons in progressive supranuclear palsy are usually due to concurrent argyrophilic grain disease. Acta Neuropathol 2002; 104: 53–6.

Togo T, Isojima D, Akatsu H, Suzuki K, Uchikado H, Katsuse O, et al. Clinical features of argyrophilic grain disease. A retrospective survey of cases with neuropsychiatric symptoms. Am J Geriatr Psychiatry 2005; 13: 1083–91.

Togo T, Sahara N, Yen SH, Cookson N, Ishizawa T, Hutton M, et al. Argyrophilic grain disease is a sporadic 4-repeat tauopathy. J Neuropath Exp Neurol 2002; 61: 547–56.

Tolnay M, Calhoum M, Pham HC, Egensperger R, Probst A. Low amyloid (Aβ) plaque load and relative predominance of diffuse plaques distinguish argyrophilic grain disease from Alzheimer's disease. Neuropathol Appl Neurobiol 1999; 25: 295–305.

Tolnay M, Clavaguera F. Argyrophilic grain disease: a late-onset dementia with distinctive features among tauopathies. Neuropathology 2004; 24: 269–83.

Tolnay M, Ghebremedhin E, Probst A, Braak H. Argyrophilic grain disease. In: Dickson D, editor. Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders. Basel: ISN Neuropath Press; 2003. p. 132–6.

Tolnay M, Mistl C, Ipsen S, Probst A. Argyrophilic grains of braak: occurrence in dendrites of neurons containing phosphorylated tau protein. Neuropathol Appl Neurobiol 1998; 24: 53–9.

Tolnay M, Monsch AU, Probst A. Argyrophilic grain disease. A frequent dementing disorder in aged patients. Adv Exp Med Biol 2001; 487: 39–58.

Tolnay M, Probst A. Ballooned neurons expressing αB-crystallin as a constant feature of the amygdala in argyrophilic grain disease. Neurosci Lett 1998; 246: 165–8.

Tolnay M, Probst A. Review: tau protein pathology in Alzheimer's disease and related disorders. Neuropathol Appl Neurobiol 1999; 25: 171–87.

Tolnay M, Probst A, Monsch AU, Staehlin HB, Egensperger R. Apolipoprotein E allele frequencies in argyrophilic grain disease. Acta Neuropathol 1998; 96: 225–7.

Tolnay M, Sergeant N, Ghestem A, Chalbot S, de Vos RA, Jansen Steur EN, et al. Argyrophilic grain disease and Alzheimer disease are distinguished by their different distribution of tau protein isoforms. Acta Neuropathol 2002; 104: 425–34.

Tolnay M, Spillantini MG, Goedert M, Ulrich J, Langui D, Probst A. Argyrophilic grain disease: widespread hyperphosphorylation of tau protein in limbic neurons. Acta Neuropathol 1997a; 93: 477–84.

Tolnay M, Schwietert M, Monsch AU, Staehelin HB, Langui D, Probst A. Argyrophilic grain disease: distribution of grains in patients with and without dementia. Acta Neuropathol 1997b; 94: 353–8.

Tsuchiya K, Mitani K, Arai T, Yamada S, Komiya T, Esaki Y, et al. Argyrophilic grain disease mimicking temporal Pick's disease: a clinical, radiological, and pathological study of an autopsy case with a clinical course of 15 years. Acta Neuropathol 2001; 102: 195–9.

Uchihara T. Silver diagnosis in neuropathology: principles, practice and revised interpretation. Acta Neuropathol 2007; 113: 483–99.

Vadlamudi RK, Joung I, Strominger JL, Shin J. p62, a phosphotyrosine-independent ligand of the SH2 domain of p56lck, belongs to a new class of ubiquitin-binding proteins. J Biol Chem 1996; 271: 20235–7.

van Leeuwen FW, de Kleijn DP, van den Hurk HH, Neubauer A, Sonnemans MA, Sluijs JA, et al. Frameshift mutants of beta amyloid precursor protein and ubiquitin-B in Alzheimer's and Down patients. Science 1998; 279: 242–7.

van Tijn P, de Vrij FM, Schuurman KG, Dantuma NP, Fischer DF, van Leeuwen FW, et al. Dose-dependent inhibition of the proteasome activity by a mutant ubiquitin associated with neurodegenerative disease. J Cell Sci 2007; 120: 1615–23.

Voges D, Zwickl P, Baumeister W. The 26 S proteasome: a molecular machine designed for controlled proteolysis. Annu Rev Biochem 1999; 68: 1015–68.

Yamada T, McGeer PL. Oligodendroglial microtubular masses: an abnormality observed in some human neurodegenerative diseases. Neurosci Lett 1990; 120: 163–6.

Yamada T, McGeer PL, McGeer EG. Some immunohistochemical features of argyrophilic grain dementia with normal cortical choline acetyltransferase but extensive subcortical pathology and markedly reduced dopamine. J Geriatr Psychiatry Neurol 1992; 5: 3–13.

Zatloukal K, Stumptner C, Fuchsbichler A, Heid H, Schnoelzer M, Kenner L, et al. p62 Is a common component of cytoplasmic inclusions in protein aggregation diseases. Am J Pathol 2002; 160: 255–63.

Zhu X, Lee HG, Casadesus G, Avila J, Drew K, Perry G, et al. Oxidative imbalance in Alzheimer's disease. Mol Neurobiol 2005; 31: 205–17.

Zhu X, Raina AK, Lee HG, Chao M, Nunomura A, Tabaton M, et al. Oxidative stress and neuronal adaptation in Alzheimer disease: the role of SAPK pathways. Antioxid Redox Signal 2003; 5: 571–6.

Zhu X, Raina AK, Rottkamp CA, Aliev G, Perry G, Boux H, et al. Activation and redistribution of c-Jun N-terminal kinase/stress activated protein kinase in degenerating neurons in Alzheimer's disease. J Neurochem 2001; 76: 435–41.

Zhu X, Rottkamp CA, Boux H, Takeda A, Perry G, Smith MA. Activation of p38 kinase links *tau* phosphorylation, oxidative stress, and cell cycle-related events in Alzheimer disease. J Neuropathol Exp Neurol 2000; 59: 880–8.

Zhukareva V, Shah K, Uryu K, Braak H, Del Tredici K, Sundarraj S, et al. Biochemical analysis of tau proteins in argyrophilic grain disease, Alzheimer's disease, and Pick's disease. A comparative study. Am J Pathol 2002; 161: 1135–41.

Zilka N, Filipcik P, Koson P, Fialova L, Skrabana R, Zilkova M, et al. Truncated tau from sporadic Alzheimer's disease suffices to drive neurofibrillary degeneration in vivo. FEBS Lett 2006; 580: 3582–8.

# 6

## LRRK2 in neurodegeneration. A review.

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REVIEW

# *LRRK2* and neurodegeneration

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**Abstract** Mutations in leucine-rich repeat kinase 2 gene (*PARK8*/*LRRK2*) encoding the protein Lrrk2 are causative of inherited and sporadic Parkinson's disease (PD) with phenotypic manifestations of frontotemporal lobar degeneration, corticobasal degeneration and associated motor neuron disease in some patients, and with variable penetrance. Neuropathology is characterized by loss of dopaminergic neurons in the substantia nigra pars compacta in all cases with accompanying Lewy pathology, or tau pathology or without intraneuronal inclusions, thus indicating that mutations in *LRRK2* are not always manifested as Lewy body disease (LBD) or as α-synucleinopathy. Molecular studies have not disclosed clear association between nerve cell degeneration and modifications in the kinase activity of Lrrk2, and the pathogenesis of *LRRK2* mutations remains unknown. Several morphological studies have suggested that Lrrk2 is a component of Lewy bodies and aberrant neurites in sporadic PD and Dementia with Lewy bodies, whereas other studies have indicated that Lrrk2 does not participate in Lewy body composition. Likewise, some studies have shown Lrrk2 immunoreactivity in hyper-phosphorylated tau inclusions in Alzheimer's disease (AD) and other tauopathies, whereas other studies did not find Lrrk2 in hyper-phosphorylated tau inclusions. We have used three currently used anti-Lrrk2 antibodies (NB-300-268, NB-300-267 and AP7099b) and concluded that these differences are largely dependent on the antibodies used and, particularly, on the interpretation of the origin of the multiple bands of low molecular weight species, in addition to the band corresponding to full-length Lrrk2, that recognize the majority of these antibodies. A review of the available data and our results indicate that full-length Lrrk2 is not a major component of Lewy bodies in LBDs, and of hyperphosphorylated tau inclusions in AD and tauopathies. Bands of low molecular weight are probably not the result of post-mortem artefacts as they are also present in cultured cells processed under optimal conditions. Truncated forms of Lrrk2 and additional transcripts related with *LRRK2*, in the absence of spliced forms of Lrrk2 may account for Lrrk2 immunoreactivity in distinct intraneuronal inclusions.

**Keywords** *LRRK2* · Parkinson disease · Alzheimer disease · Tauopathy · Lewy bodies

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## Parkinson disease

Parkinson disease (PD) is the second most prevalent neurodegenerative disease among the elderly population. Clinical features of PD are resting tremor, postural instability, akinesia and rigidity. Pathological findings are reduced pigmentation in the substantia nigra pars compacta due to loss of dopaminergic neurons. This is accompanied by intracytoplasmic proteinaceous inclusions in surviving neurons (Lewy bodies) and neurites [22]. Lewy bodies and aberrant neurites are composed of protein aggregates among which abnormal α-synuclein is a crucial component [88]. In addition to substantia nigra, neurons of other regions are affected, including several nuclei of the medulla oblongata, pons and midbrain, and the motor nuclei of the hypoglossal and vagal nerves, reticular formation and locus ceruleus, as well as the nucleus basalis of

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Meynert, amygdala, hippocampus, striatum and cerebral cortex [52].

The majority of PD cases (90%) are sporadic and their aetiology is unknown, but a number of mutated genes have been identified as the cause of the remaining, mostly familial, cases. Familial PD cases have been related with mutations, duplications and triplications in *SNCA* (α-synuclein)/*PARK1*, *UCHL-1* (ubiquitin carboxy-terminal hydrolase L1)/*PARK5*, parkin/*PARK2*, DJ-1/*PARK7*, PINK-1 (PTEN-induced putative kinase)/*PARK6*, ATP13A2 (p-type ATPase)/*PARK9*, HTRA2 (HtrA serine peptidase 2)/*PARK13* and *LRRK2*/*PARK8*. Other *PARK* loci have been identified: *PARK3*, *PARK10*, *PARK11* and *PARK12;* but the specific mutated gene is unknown. *LRRK2* mutations are by far the most abundant in autosomal dominant PD, and they may lead to late-onset PD in contrast to other PD-causative mutations which present more commonly as early onset PD [2, 94]. Expression of *LRRK2* mutations shows increased penetrance with age [34, 41, 43, 53]. The place of *LRRK2* in the pathogenesis of PD has been the subject of recent evaluations [6, 42, 45, 76, 96]. The present review deals with the role of Lrrk2 in neurodegeneration, including PD, and covers controversial aspects such as the presence of Lrrk2 in intraneuronal inclusions in α-synucleinopathies and tauopathies.

## Mutations in *LRRK2:* clinical features and prevalence

A linkage study performed on a family with autosomal dominant PD revealed a region in chromosome 12 containing 116 genes segregating with the disease in all affected individuals. This region was called *PARK8* [23]. In 2004, the mutated gene causative of the disease was identified as *LRRK2* by two different groups [81, 109], and the encoded protein Lrrk2 [81, 109] was named dardarin, from dardara which means tremor in Basque (several families analysed) [81].

About 30 different mutations have been identified in *LRRK2* gene. As a group, these mutations account for a maximum of 13% of familial PD and 5% of sporadic cases [9, 15, 18, 20, 24, 25, 31, 49, 53, 69, 78, 95, 105]. Most of these mutations are amino acid substitutions located in the C-terminal half of the protein [82]. It has been suggested that the most prevalent Lrrk2 mutation in Mediterranean shores is G2019S, mainly in the Arab population of North Africa, representing 40% of familial and sporadic PD [3, 57, 58], and in the Ashkenazi Jewish population representing 30% of familial PD [79, 80]. However, population studies have indicated that such high prevalence is, probably, overestimated [19]. Other reported mutations include: R1441G, Y1699C, R1441C, I1122V, I2020T, R1441H and G2385R (see OMIM 609007 for further details and references). *LRRK2* heterozygous R1628P and Gly2385Arg genotypes increase the risk of PD [95].

The majority of cases bearing Lrrk2 mutations suffer from PD [12]. However, some cases have amyotrophy and ocular supranuclear palsy. Mutations in *LRRK2* are rarely manifested as a corticobasal syndrome, primary progressive aphasia and frontotemporal lobar degeneration [6, 13].

## Varied pathology independent of mutation

Mutations in *LRRK2* can lead to PD clinically indistinguishable from idiopathic PD and neuropathologically characterized by the presence of Lewy bodies, although no inclusions occur in other cases. I1371V, A1441C, Y1699C and G2019S mutations are accompanied by Lewy bodies [30–32, 54, 86, 104, 110] (Fig. 1). However, the same mutations are not always accompanied by Lewy bodies, as in one reported case bearing the T1699C mutation [104] and in another carrying the G2019S mutation [26] (Fig. 1). tau pathology and the absence of Lewy bodies occurred in one case with the G2019S mutation [85]. The only case examined with the R1441G (Basque) mutation had loss of dopaminergic neurons in the substantia nigra, free neuromelanin in the neuropil and absence of α-synuclein-, hyperphosphorylated tau- and ubiquitin-immunoreactive inclusions. The only accompanying feature was increased αB-crystallin immunoreactivity in isolated neurons [68] (Fig. 1). Intriguingly, the R1441C mutation in one family was manifested as an α-synucleinopathy (presence of Lewy bodies) in one member, as a tauopathy with neurofibrillary tangles and Progressive Supranuclear Palsy (PSP)-like distribution in a second affected member, and as loss of neurons with no intracytoplasmic neuronal inclusions in a third [109].

Other mutations are associated with motor neuron disease features, frontotemporal lobar degeneration and nuclear ubiquitin-immunoreactive inclusions [8, 13, 29]. This varied pathology suggests that Lrrk2 is a protein which participates in different pathways and that mutated Lrrk2 causes, primarily, loss of dopaminergic neurons and, secondarily, accumulation of abnormal proteins, together with ubiquitin, such as α-synuclein and hyper-phosphorylated tau [17].

## Lrrk2 domains

*LRRK2* is composed of 51 exons and encodes Lrrk2, a protein of 2,527 amino acids. From N-terminal to C-terminal, the domains identified in Lrrk2 are named Ankyrin repeat (ANK), Leucine-Rich repeat (LRR), Ras of Complex (GTPase) (Roc), C-terminal of Ras (COR), kinase and WD40.

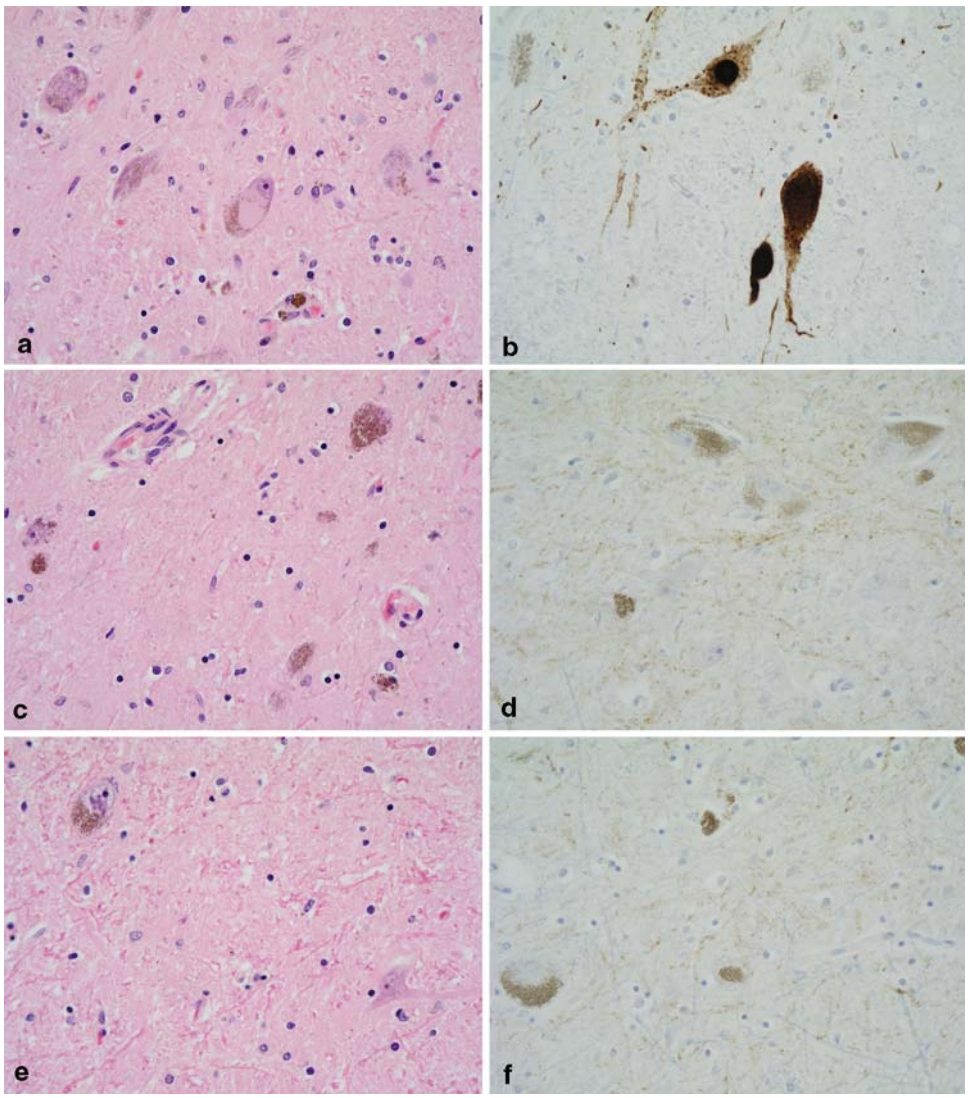


**Fig. 1** Neuropathology of the substantia nigra in Parkinson's disease bearing the G2019S mutation showing the presence of Lewy bodies (**a**, **b**). Another unrelated case with the G2019S mutation with no Lewy bodies and other inclusions (**c**, **d**, [26]). A third case carrying the R1441G Basque mutation in *LRRK2* (**e**, **f** 68) in whom neuron loss in the substantia nigra was not accompanied by Lewy bodies and neurites and hyperphosphorylated tau inclusions. **a**, **c**, **e** Haematoxylin and eosin; **b**, **d**, **f** α-synuclein immunostaining

ANK, LRR and WD40 are repeats separately found in several proteins and they confer platforms for protein interactions with different molecules such as cytoskeleton proteins, transcription factors, and signalling and cell cycle regulators. Because of this ability to bind different proteins, it has been suggested that Lrrk2 may assemble a multi-protein signalling complex [70].

The kinase domain of Lrrk2 belongs to the super-family of serine/threonine and tyrosine kinases, and is similar to the RIPK family (receptor-interacting protein kinase) which can regulate cell survival and cell death pathways in response to intracellular or extracellular stress signals [73].

The Roc and COR domains occur together as in the ROCO family of GTP-ases; the function of the COR domain is unknown, whereas the Roc domain is very homologous to members of the Rab-GTPase family [67]. This family is composed of more than 60 small GTP-ase proteins which regulate vesicle formation, actin- and tubulin-dependent vesicle movement, and membrane fusion [93].

## Functional domains

Lrrk2 is a GTP-ase which can bind and hydrolyse GTP and stimulate kinase activity, a property which suggests that the kinase domain of Lrrk2 is activated by its own Roc domain [37]. The structure of Roc domain reveals a dimeric GTPase [16]. GTP binding is essential to the protein kinase activity of Lrrk2 [50]. The ability of purified Lrrk2 to auto-phosphorylate or to phosphorylate a generic substrate such as myelin basic protein appears to be low. Yet, it has been suggested that low kinase activity in vitro could be caused by the lack of the proper co-factor, lack of appropriate stimulus or abnormal protein folding in the assay conditions [70]. It has recently been shown that at least in vitro,

moesin, ezrin and radixin were substrates of phosphorylation by mutant Lrrk2-G2019S at the residues which regulate the binding of these proteins to actin [51].

## How mutations affect Lrrk2 activity

Most of the identified Lrrk2 mutations are localized in kinase domain. Other mutations affect the GTPase (i.e. R1441C, R1441G, R1441H), the LRR (L1114L, I1122V) and Cor (Y1699C) domains. The G2019S mutation affects the kinase domain and increases kinase activity [35, 63, 100].

However, not all mutations in *LRRK2* lead to increased activity. R1441C and R1441G mutations affect the GTPase domain and they decrease GTPase activity [37, 59, 60]. Surprisingly, increased kinase activity has been reported to be associated with Lrrk2-R1441C and Lrrk2-R1441G mutants [38, 60]. However, these results were not reproduced in another study [51]. The Y1699C mutation, located in the Cor domain, seems not to be able to modify Lrrk2 kinase activity [51]. Furthermore, the T2356I mutation, affecting the WD40 domain, does not modify kinase activity. Finally, the R1941H and G2385R mutations inhibit the capacity of Lrrk2 for auto-phosphorylation, as well as for phosphorylation of MBP and moesin [51]. In contrast, increased kinase activity has been found in Lrrk2-Y1699C and Lrrk2-I2012T mutants [33, 101]. Understanding of these modifications is still fragmentary, probably due, in part, to methodological difficulties in the measurement of auto-phosphorylation [51].

Other properties of Lrrk2 may be affected depending on the site of the mutation [59]. Mutations in the residue 1441, although sited in the GTPase domain, are distant from the GTP hydrolysis region, but they are found in a region implicated in interaction with other proteins [93]. On the other hand, the G2385R mutation in WD40 domain involved in protein interaction is also able to inhibit kinase activity [44]. Finally, mutations in LRR and ankyrin domains may hamper Lrrk2-protein interactions [90].

Several small molecules may inhibit Lrrk2 activity and may have, therefore, therapeutic implications [10].

## Lrrk2 interactions

### Dimerization

Lrrk2 is predominantly found as a dimer in vivo. Dimerization occurs through the interaction of different regions of Lrrk2, including the ROC domain with several points in the LRR domain, a region close to ROC, WD40, and the N-terminal region [16, 36].

### Cytoskeleton and proteins involved in trafficking

Most of the Lrrk2-interacting proteins identified to date are cytoskeleton and trafficking proteins. Lrrk2 has been found to interact in vitro with moesin, ezrin and radixin, which regulate β-actin binding to plasma membrane [51]. The ROC domain of Lrrk2 can also interact with α/β-tubulin heterodimers in microtubules, and co-localizes with them in primary hippocampal neurons [28]. Immunoprecipitation coupled with mass-spectrometry has permitted the identification of 14 potential Lrrk2-interacting proteins, among them clathrin and vimentin [14]. Finally, Lrrk2 appears to interact with Rab5a [87], thus being involved in endocytic vesicular transport between plasma membrane and early endosomes [7]. However, further studies are needed to learn whether protein interactions observed in vitro may occur in brain tissue.

### Chaperones

Other putative interactors identified are proteins associated with phosphorylation, translation and chaperones [14]. Among these chaperones is Hsp90, which, together with its co-chaperone Cdc37, appears to play a crucial role in maintaining Lrrk2 stability [47].

### Key proteins in neurodegenerative diseases

The interaction of Lrrk2 with proteins important in neurodegenerative diseases has also been tested. No interaction was found between Lrrk2 and α-synuclein, tau or Dj-1 [95]. However, the same study revealed interaction of Lrrk2 COR domain with parkin [92]. Parkin is the name of a E3 ubiquitin ligase which is found mutated in some cases of familial PD [2].

## Expression and localization of Lrrk2

In the brain, *LRRK2* is expressed in neurons, and also in astrocytes and microglia [74]. It is also expressed in other tissues such as in liver, lung, kidney and heart [81, 109]. By using in situ hybridisation and immunohistochemistry, it has been shown that in normal brain *LRRK2* is expressed in several neuronal populations including cerebral cortex, caudate-putamen and hippocampus [27, 44, 45, 72, 89, 97, 102], as well as in dopaminergic neurons of the substantia nigra pars compacta [39, 44].

In cells, Lrrk2 is mainly found in the cytoplasm [30]. Lrrk2 is enriched in lipid raft, early endosomes, lysosomes, synaptic vesicles and plasma membrane [40, 87], as well as in the Golgi complex, endoplasmic reticulum and outer mitochondrial membrane [4, 40]. The association of Lrrk2 in

several membrane structures suggests a role for Lrrk2 in membrane trafficking [4, 40], which is further supported by the interaction of *LRRK2* with several cytoskeleton and trafficking-related proteins and with the function attributed to the RabGTPase family [93]. Moreover, with Rab5a and clathrin being possible interacting partners, a role for Lrrk2 in clathrin-mediated endocytosis was suggested and eventually demonstrated in vitro [87].

Accumulation of Lrrk2 has been observed in abnormal neurites of the substantia nigra pars compacta in one single case carrying the G2019S mutation [30]. Moreover, intracytoplasmatic Lrrk2-positive inclusions have been found in cultured cells bearing the R1441C and Y1699C mutations in Lrrk2 [35].

## Biological effects of *LRRK2* mutations

### In cells

In cell culture models, Lrrk2 can be toxic, depending on its kinase activity [35, 91]. Mutations in Lrrk2 associated with increased kinase activity increase apoptotic cell death in dopaminergic cell lines and primary neurons [62]. Decreased kinase activity, resulting from alteration of key residues in ROCO and kinase domains, reduces neuronal toxicity [91]. These observations led to the gain-of-function hypothesis to explain neurodegeneration in PD linked to mutations in *LRRK2*, particularly those associated with increased kinase activity such as G2019S.

Mutations affecting the kinase domain seem crucial also in neurite outgrowth, as expression of Lrrk2-G2019S, Lrrk2-I2010T and, to a lesser degree, Lrrk2-R1441G mutants in primary cortical neurons leads to dramatic reductions in neurite length and branching of axons and dendrites. Wild-type Lrrk2 over-expression in the same model does not alter cell morphology, whereas Lrrk2 shRNA-mediated knock-down leads to a gradual increase in neurite growth [64]. In retinoic acid-differentiated neuroblastomas, mutant Lrrk2 over-expression also causes neurite shortening in a process involving autophagy [84]. Interestingly, accumulation of Ser202 phosphorylated tau, but not of α-synuclein, was observed in spheroid-like aggregates, together with Lrrk2, within neurites in cells over-expressing Lrrk2-G2019S and Lrrk2-I2010T, and also, to a much lesser degree, in cells over-expressing wild-type *LRRK2* [64]. All these data suggest a role for Lrrk2 in the control of neurite length, ultimately regulated by its kinase activity.

Toxic effects of increased expression of Lrrk2 are probably level-dependent, as high levels of Lrrk2 over-expression produce cell death, whereas low levels of Lrrk2 over-expression are associated with impaired vesicle endocytosis [48, 87, 101]. Low Lrrk2 expression or silencing *LRRK2* in rat hippocampal neurons results in kinase-independent altered vesicle endocytosis that can be rescued by co-expression of Rab5a [87].

Finally, RNA interference of *LRRK2* in SH-SY5Y cells results in differential regulation of 187 genes, with 94 transcripts being up-regulated and 93 transcripts down-regulated compared to scrambled control siRNA transfected cells [38].

### In animal models

Lrrk2 has been studied in transgenic mice [60], pigs [55], worms [103] and flies [56, 61, 99].

Expression of Lrrk2-R1441C in mice is not accompanied by dopaminergic neuron loss [60], but degeneration of these neurons was observed following intracellular viral insertion of the Lrrk2 kinase domain fragment in rats [64].

Over-expression of wild type and human Lrrk2-G2019S in *Drosophila* results in retinal degeneration, selective loss of dopaminergic neurons, motor impairment and reduced lifespan [61]. Flies with the G2019S mutation show a significantly worse motor impairment than wild type, though this can be improved by administration of L-DOPA [61]. Previous studies in *Drosophila* expressing the CG5483 mutation, emulating human mutation R1441C, showed reduced integrity of dopaminergic neurons [56]. In contrast to these observations, no modifications of dopaminergic neurons were found in a *Drosophila* model in which the kinase domain of a Lrrk2 orthologue was removed [99]. Yet increased sensitivity to oxidative stress damage induced by hydrogen peroxide, but not with rotenone or paraquat, occurred in those transgenic flies [99]. Over-expression of wild Lrrk2 and mutated G2019S in *C. elegans* protects from toxicity mediated by rotenone, thus suggesting a role for Lrrk2 in mitochondrial physiology [103].

Observations in *C. elegans* and *Drosophila* must be considered with caution. Recent studies in cnidaria, deuterostomes (including human) and protostomes (including *Drosophila* and *C. elegans*) demonstrate a very ancient phylogenetic origin of human *LRRK2* (a bona fide orthologue of *LRRK2* is already found in cnidaria), which is lost in protostomes. Thus, *LRRK2* and *LRRK* genes in *Drosophila* and *C. elegans* are not real orthologues but rather paralogues [65, 66].

## The presence of Lrrk2 in pathological brain

### Lrrk2 immunoreactivity in α-synuclein inclusions

The presence or absence of Lrrk2 in Lewy bodies is controversial due to the distinct results obtained with different anti-Lrrk2 antibodies [5]. Antibodies AT106,

PA0362 and AP7099b, directed to functional domains of Lrrk2, fail to detect Lewy bodies [30, 44, 107]. However, antibodies NB-300-267 and NB-300-268, directed to the N-terminal and C-terminal regions of Lrrk2, respectively, do stain α-synuclein aggregates with variable intensity [1, 35, 44, 71, 74, 83, 107], and granular synuclein pathology in the brainstem of Parkinson's disease [1]. Currently, NB-300-268 stains the core and the halo of Lewy bodies, as well as aberrant neurites. In contrast, weak immunoreactivity, often decorating the halo, is usually obtained with the NB-300-267 antibody. The home-made antibodies JH5517, raised to the N-terminal part of *LRRK2* [44], ab04/11 [71] and EB06550 [1], raised to C-terminal domain of Lrrk2, also stain the halo of some Lewy bodies in PD. L955 Abgent antibody, which recognizes an epitope close to the one of NB-300-267, stains α-synuclein-immunoreactive oligodendroglial inclusions in multiple system atrophy (MSA) [46]. Based on these observations, the conclusions are divergent: (1) Lrrk2 is a component of Lewy bodies [35, 74, 83, 108], and (2) there is lack of evidence for Lrrk2 in α-synuclein pathological inclusions [11, 30].

A personal study has been carried out in order to compare results with those already available in control and diseased brains. A summary of cases examined is shown in Table 1.

We have tested the antibodies NB-300-268, NB-300-267 and AP7099b by using double-labelling immunofluorescence and confocal microscopy, and have corroborated that NB-300-268 antibody is able to stain Lewy bodies (Fig. 2), whereas Lewy bodies are barely stained with NB-300-267 and remain negative with AP7099b antibodies.

**Table 1** Summary of cases examined in the present study

| Diagnosis | Number of cases | Males/females | Mean age |
|---|---|---|---|
| Control | 8 | 4/4 | 44-82 |
| AD stage V/VIC | 10 | 5/5 | 72 |
| sPD | 6 | 5/1 | 75 |
| *LRRK2* G2019S | 2 | 1/1 | 76 |
| *LRRK2* R1441G | 1 | 1/0 | 78 |
| DLB | 6 | 4/2 | 77 |
| FTLD-tau P301L | 3 | 3/0 | 56 |
| FTLD-tau K317M | 1 | 0/1 | 48 |
| PiD | 5 | 3/2 | 67 |
| AGD | 10 | 4/6 | 78 |

Control indicates cases with no clinical neurological deficits and normal neuropathgological examination

*AD* Alzheimer's disease, *sPD* sporadic Parkinson's disease, *LRRK2* cases of PD with *LRRK2* mutations, *DLB* dementia with Lewy bodies, *FTLD-tau* frontotempopral lobar degeneration linked to mutations in the tau gene, *PiD* Pick's disease, *AGD* argyrophilic grain disease

We also tested the ab60937 antibody, which is raised against an epitope located in the C-terminal adjacent to the epitope recognized by NB-300-268. This antibody failed to stain α-synuclein inclusions.

Differences also occur between cortical-type and brainstem-type Lewy bodies [71] thus indicating that the composition of these inclusions is subject of regional differences.

## Lrrk2-immunoreactive inclusions in other neurodegenerative diseases

The antibody NB-300-268 not only recognizes α-synuclein inclusions, but is also able to stain phospho-tau, huntingtin and ubiquitin inclusions in Alzheimer disease (AD), Parkinsonism dementia complex of Guam, multiple system atrophy, Pick's disease (PiD), Huntington disease, amyotrophic lateral sclerosis and frontotemporal lobar degeneration linked to mutations in tau gene (FTLD-tau) [74, 75]. We reproduced these results in AD, PiD and FTLD-tau using the same antibody (Fig. 3).

Interestingly, not all inclusions are stained equally with the NB-300-268 antibody. By using double-labelling immunofluorescence and confocal microscopy, a variable number of phospho-tau-immunoreactive inclusions were stained with NB-300-268 antibody (Fig. 4). To learn whether Lrrk2 immunostaining was dependent on tau conformation, antibodies to Alzh 50 and tau MC1 were analysed in combination with NB-300-268. Not all Alz 50- and tau MC1-immunoreactive inclusions were stained with NB-300-268 antibodies (Fig. 5). Our studies have shown that neurofibrillary tangles and phospho-tau-immunoreactive inclusions in AD and tauopathies were weakly stained with the antibody NB-300-267 and, rarely, with the ab60937 antibody.

In PiD, NB-300-267 Lrrk2 immunoreactivity was present in the cytoplasm of neurons usually independently of the presence of phospho-tau inclusions; Lrrk2 immunoreactivity was also present in Pick bodies (Fig. 6). Diffuse cytoplasmic staining was also observed in neurons in AGD. Yet AT8-immunoreactive grains were rarely stained with anti-*LRRK2* antibodies (Fig. 6).

In contrast, the AP7099b antibody fails to detect intracellular inclusions in AD, progressive supranuclear palsy (PSP), PiD or frontotemporal lobar degeneration/motor neuron disease (FTLD-MND) [30]. Likewise, the AP7099b antibody showed a punctuate immunoreactive pattern in the cytoplasm of neurons but did not stain hyper-phosphorylated-tau inclusions in AD and tauopathies, in our hands (Fig. 7).

As with α-synucleinopathies, there is no agreement about the presence of Lrrk2 in hyper-phosphorylated tau inclusions in AD and tauopathies. Lrrk2 is thought to be a

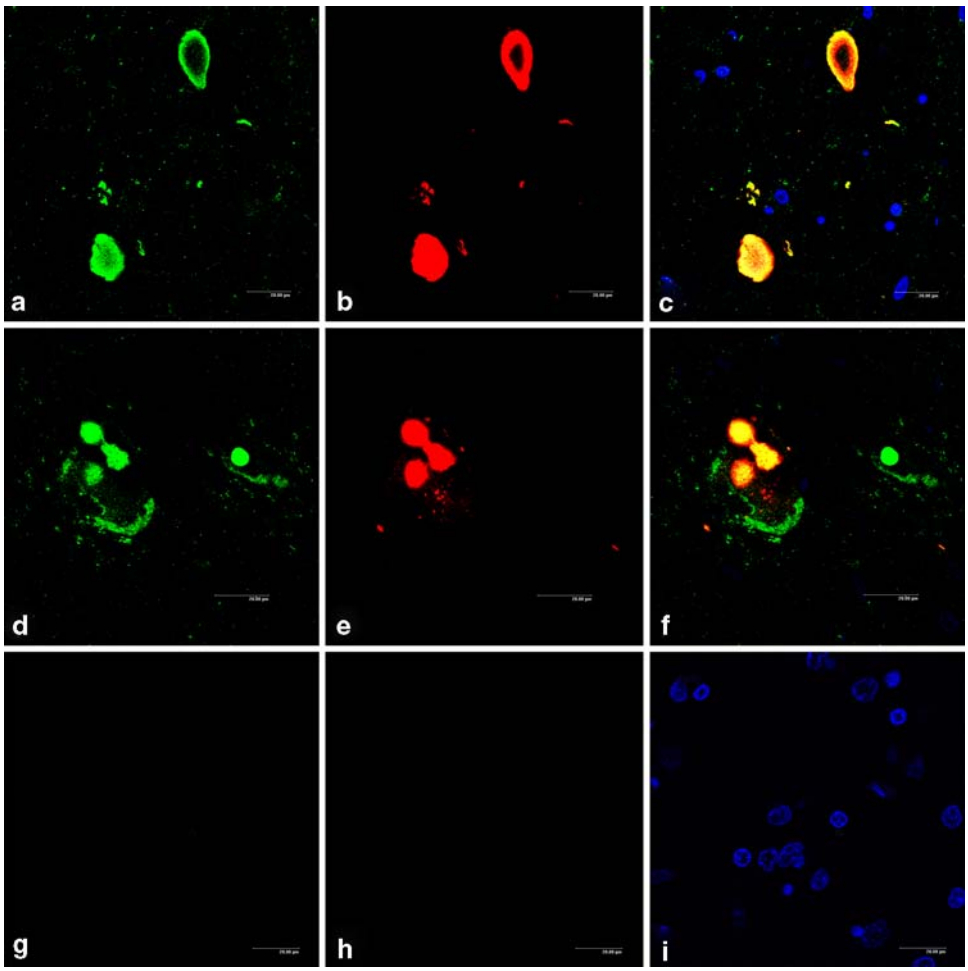


**Fig. 2** Lrrk2 immunoreactivity in the substantia nigra in Parkinson's disease (**a**–**c**) and in Dementia with Lewy bodies (**d**–**f**) using the NB-300-268 antibody directed to the C-terminus. Lrrk2 immunoreactivity (*green*) co-localizes with α-synuclein (*red*) in Lewy bodies and neurites (merge, *yellow*). **g**–**i** Sections processed without the primary antibodies are negative. Nuclei were stained with TO-PRO-3-iodide

component of tau-immunoreactive inclusions by some authors [74, 75], but not by others [83].

Several reasons may explain many of these differences, covering differences in the type of sample (paraffin vs. frozen sections), method (immunohistochemistry or immunofluorescence), pre-treatment procedures for antigen retrieval, as well as dilution of the antibody among other conditions. Table 2 summarizes protocols, antibodies and results used by different authors regarding the most commonly used Lrrk2 antibodies that may help to have a comprehensive view of observations so far reported.

## Lrrk2 antibodies in Western blots

A major point of discussion is the specificity of Lrrk2 antibodies. In principle, the primary sequences of immunogenic peptides directed to the C-terminal (TEGTQKQKEIQS CLTVWDINLPHEVQNLEKHIEVRKELAEKMRRTSVE for ab60937 and PHEVQNLEKHIEVRKELAEKMRRT SVE for NB-300-268) do not match with known sequences of other proteins. However, Western blot studies have shown that the NB-300-267 and NB-300-268 antibodies detect several bands in human brain tissue and cell homogenates, and that the band corresponding to the predicted molecular weight of Lrrk2 (about 250 kDa) is very weak when compared with the other bands [1, 71, 74]. AP7099b antibodies disclose a clear band at about 250 kDa and a few bands of lower molecular weight in Western blots [30]. A number of studies show a band of about 250 kDa with several anti-Lrrk2 antibodies-in some instances only detected when 100 g of total protein of brain homogenates was used [108]. Yet Western blots are cut at the level of 150 or 100 kDa [1, 44, 108], thus making the observation of possible bands of lower molecular weight impossible. A varying number of bands of low molecular weight are practically observed with all the anti-Lrrk2 antibodies used [11].

The possible effect of post-mortem delay in the generation of Lrrk2 fragments has been assessed in freshly dissected mouse brain. The study showed that post-mortem intervals higher than 12 h were accompanied by a decrease in full-length Lrrk2 at 250 kDa together with the appearance of lower bands of about 120 and 150 kDa [30]. Our experience is that the bands detected with the NB-300-268 and NB-300-267 antibodies are already present after very short

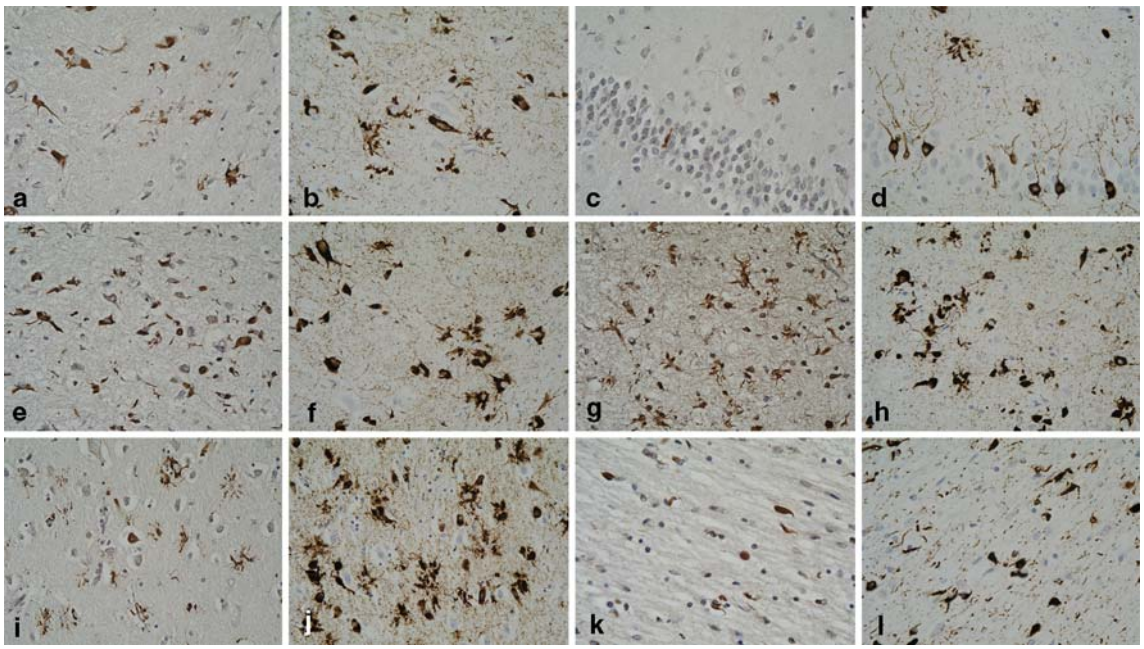


**Fig. 3** Frontotemporal lobar degeneration due to K317M mutation in *MAPT* [106]. **a**, **c**, **e**, **g**, **i**, **k** Lrrk2 immunoreactivity using the NB-300-268 antibody. **b**, **d**, **f**, **h**, **j**, **l** hyper-phosphorylated tau inclusions depicted with the antibody AT8. **a**, **b** CA1; **c**, **d** dentate gyrus; **e**, **f** entorhinal cortex; **g**, **h** temporal cortex (T3); **i**, **j** dorsomedial nucleus of the thalamus; **k**, **l** subcortical white matter, coiled bodies

post-mortem delays between death and tissue processing, and that progressive artificial post-mortem delay applied to human brain samples is accompanied by a reduction in the intensity of the bands due to protein degradation (data not shown). Strong immunoreactive bands of low molecular weight were also observed in cultured cells using NB-300-268 antibodies, giving further support to the lack of a relation between the bands of low molecular weight and delay of processing (Fig. 8). Therefore, these results point to other causes distinct from post-mortem delay-related truncation of Lrrk2 as the origin of the lower bands.

Interestingly, immunoprecipitation of Lrrk2 expressed in cells and analysed by SDS-PAGE coupled to mass spectrometry has revealed four bands in addition to the full-length one which corresponds to truncated species of Lrrk2 [14]. Whether these truncated forms exist in vivo and accumulate in Lewy bodies and hyper-phosphorylated tau inclusions in AD and tauopathies is not known.

Taking into consideration the truncation hypothesis, it can be suggested that C-terminal (and, to a lesser degree, N-terminal) truncated Lrrk2 species are detected within α-synuclein and hyper-phosphorylated tau inclusions when using NB-300-267 and NB-300-268 antibodies, while full-length Lrrk2 (apparently recognized by antibodies raised against the medial regions) is not. Alternatively, medial regions of Lrrk2 can be masked once trapped inside the inclusions, and therefore, antibodies raised against these medial regions fail to detect Lewy bodies.

Alternatively, anti-Lrrk2 antibodies do not stain Lrrk2 exclusively, but they do recognize a variegated number of other molecules as well. We have tested by Western blot the antibody NB-300-268 in sarkosyl-insoluble fractions in AD and other tauopathies to assess the possible cross-reaction of this antibody with hyper-phosphorylated tau. We found that the band of about 250 kDa was very weak in every case, a feature in striking contrast with the presence of several strong bands of lower molecular weight. No cross-reactivity with phospho-tau occurred as the molecular weight of Lrrk2-immunoreactive species did not match the molecular weight of phospho-tau in AD and other tauopathies (Fig. 9).

We also tested other anti-Lrrk2 antibodies in sarkosyl-insoluble fractions from frontal cortex in AD. The NB-300-267, NB-300-268 and ab60397 antibodies stained several bands of low molecular weight, and these bands differed depending on the antibody used in the same tissue samples processed in parallel (Fig. 10). Surprisingly, the AP7099b antibody, which was described as being highly specific for Lrrk2 [30], was not able, in our hands, to detect the band corresponding to Lrrk2 either in total homogenates or in sarkosyl-insoluble fractions from control and AD brains. However, AP7099b detected other strong bands at low molecular weight (Fig. 10). Finally, the ab60937 Abcam antibody recognized a band at about 250 kDa and an additional band at a lower molecular weight in total homogenates and sarkosyl-insoluble fractions (Fig. 10).

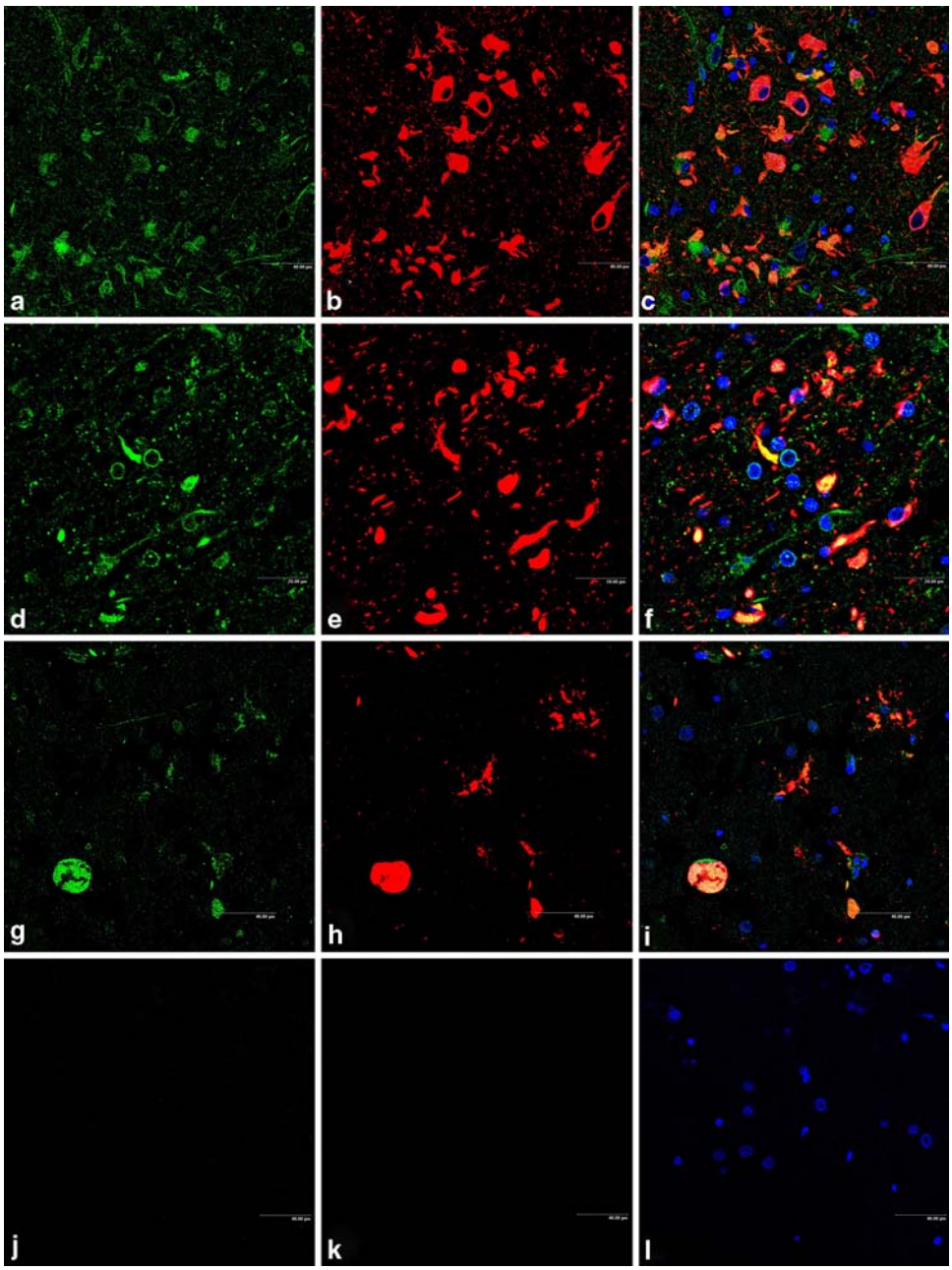


**Fig. 4** Frontotemporal lobar degeneration due to K317M mutation in *MAPT*. Partial co-localization of Lrrk2 immunoreactivity (NB-300-268 antibody, *green*) and AT8 (*red*) is found in several but not all tau inclusions. **a–c** Frontal cortex; **d–f** white matter; **g–i** brain stem; **j–l** sections processed without the primary antibodies are negative. Nuclei were stained with TO-PRO-3-iodide

Finally, the band pattern differs from one case to another even using the same antibody (Fig. 10).

Taking together immunohistochemical and Western blot studies, it may be concluded that Lewy bodies in Parkinson's disease and related α-synucleinopathies, and hyperphosphorylated tau inclusions in AD and tauopathies, are recognized by anti-Lrrk2 antibodies depicting a weak or no band at the expected molecular weight of full-length Lrrk2 but strongly immunoreactive lower bands which do not cross-react with tau. In contrast, Lewy bodies and hyperphosphorylated tau inclusions in AD and tauopathies are not recognized by antibodies which show bands at the predicted molecular weight of Lrrk2. Therefore, full-length Lrrk2 is apparently a minor component or not a component of Lewy bodies, or of hyper-phosphorylated tau inclusions in AD and tauopathies, although Lrrk2 is a component of cells. Moreover, antibodies show variegated band patterns depending on the antibody, but also the same antibody reveals different band patterns in cases with similar pathology (AD cases at the same Braak stage). Although not all neurons have tangles and neurons with phospho-tau deposits may have tangles or pre-tangles, the differences in the pattern of Lrrk2 bands with variable molecular weight in similar regions in AD cases at the same Braak stage cannot explain differences in tau composition and Lrrk2 burden, but rather on different Lrrk2 species.

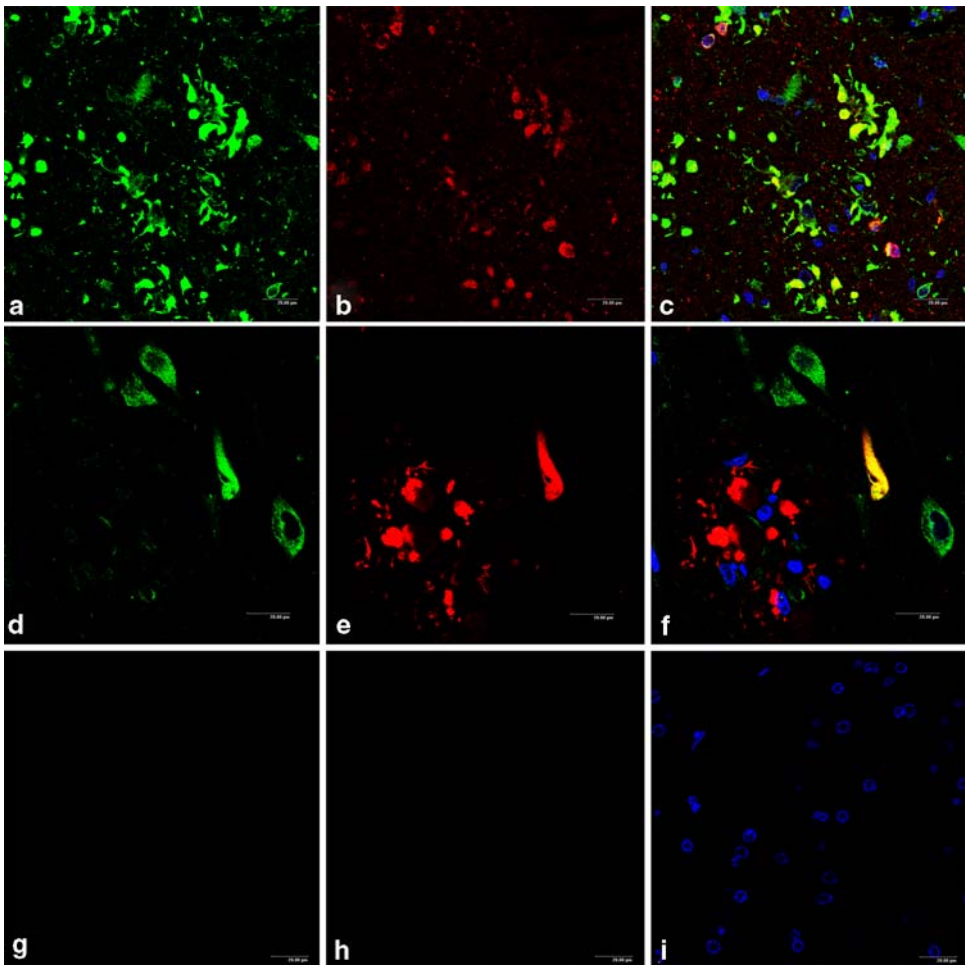


**Fig. 5** Frontotemporal lobar degeneration due to P301L mutation in *MAPT* [21] (**a**–**c**) and sporadic Alzheimer's disease (**d**–**f**). Lrrk2 immunoreactivity (NB-300-268 antibody, *green*) co-localizes partially with Alz 50 (**a**–**c**) or tau MC1 (**d**–**f**) (*red*) in neurofibrillary tangles and dystrophic neurites. **a**–**c** Frontal cortex upper layers; **d**–**f** hippocampus; **g**–**i** sections processed without the primary antibodies are negative. Nuclei stained with TO-PRO-3-iodide

## Alternative hypothesis of cross-reactivity

Although the sequences used in antigenic peptides do not match primarily with other known proteins, it cannot be ruled out that other proteins once folded may present sequences that can be bound to anti-Lrrk2 antibodies, as may be applied to all antibodies. Another possibility is that some anti-Lrrk2 antibodies recognize other possible translated Lrrk2-related transcripts.

No alternative splicing but one additional transcript with no biological significance was originally reported [100]. The possibility of transcripts of lower size than *LRRK2* was also suggested but not proved [109]. The Vertebrate Genome Annotation (VEGA) annotated transcripts in Ensembl OTTHUMT00000132847 and OTTHUMT00000132921, which are considered putative coding proteins, show maximum or high identity with RefSeq annotated proteins EAW57812.1, EAW57814.1 or EAW57815.1, respectively. EAW57812.1 (hCG1775001, isoform CRA_a) has an amino acid sequence identical to some regions of the N-terminal of *LRRK2*. EAW57814.1 (hCG39245, isoform CRA_a) and EAW57815.1 (hCG39245, isoform CRA_b) almost entirely match the C-terminal half of the *LRRK2* protein sequence. The annotation of these three proteins is referred to the sequenced human genome [98]. Finally, VEGA transcript OTTHUMT00000132920 remits to AURA17 (Augmented in rheumatoid arthritis 17) protein, which matches a large portion of the N-terminal half of Lrrk2 amino acid sequence (Fig. 11). The existence of this protein remains at transcript level [77]. Whether these transcripts are translated and trapped in α-synuclein and hyper-phosphorylated tau inclusions in α-synucleinopathies, and AD and tauopathies, respectively, is not known. If present, antibodies directed to distinct Lrrk2 epitopes might also recognize *LRRK2*-related transcripts.

## Concluding comments

*LRRK2* is composed of 51 exons and encodes the protein Lrrk2 formed by a large N-terminal tail, followed by several domains named ANK, LRR, Roc, COR, kinase and WD40, and a short C-terminus. The gene contains several regions able to encode different transcripts, and the

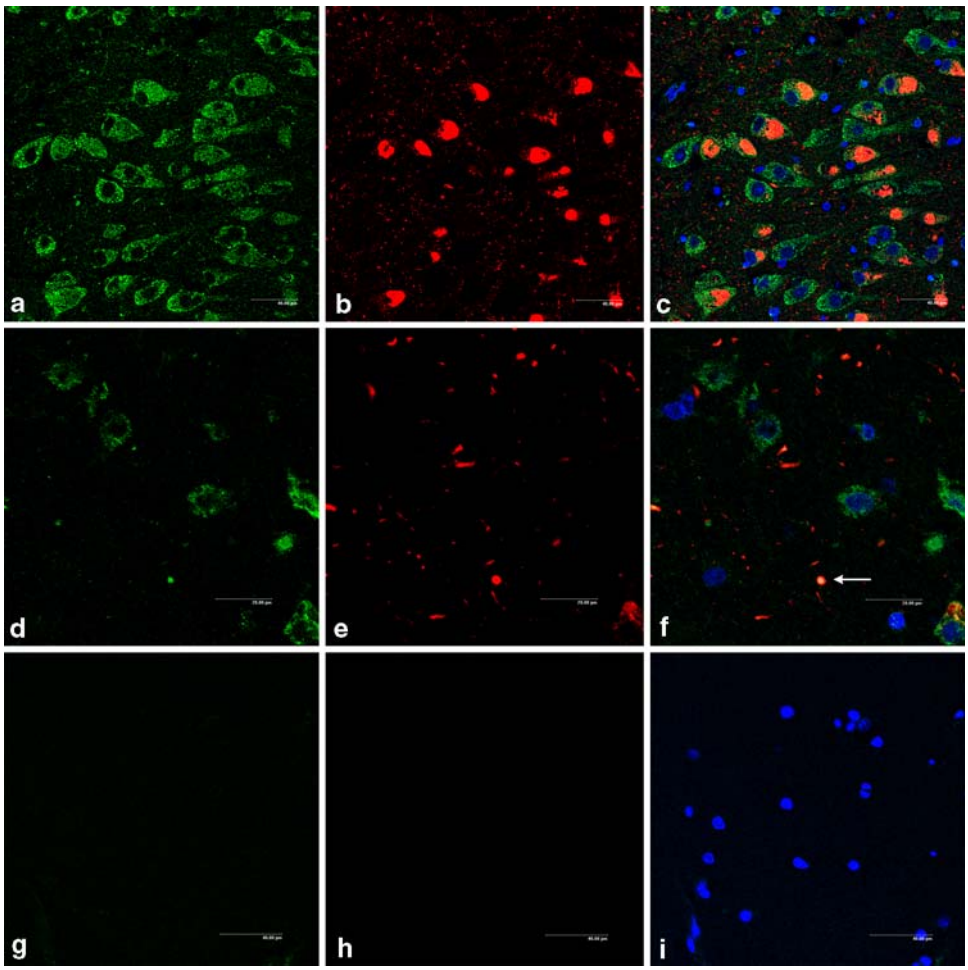


**Fig. 6** **a–c** Pick's disease. Lrrk2 immunoreactivity (NB-300-267 antibody, *green*) occurs in the cytoplasm and Pick bodies as revealed with AT8-immunoreactivity (*red*) in the CA1 region. **d–f** AGD: the same antibody stains the cytoplasm of neurons, but rarely co-localizes in grains (*arrow*). **g–i** sections processed without the primary antibodies are negative. Nuclei were stained with TO-PRO-3-iodide

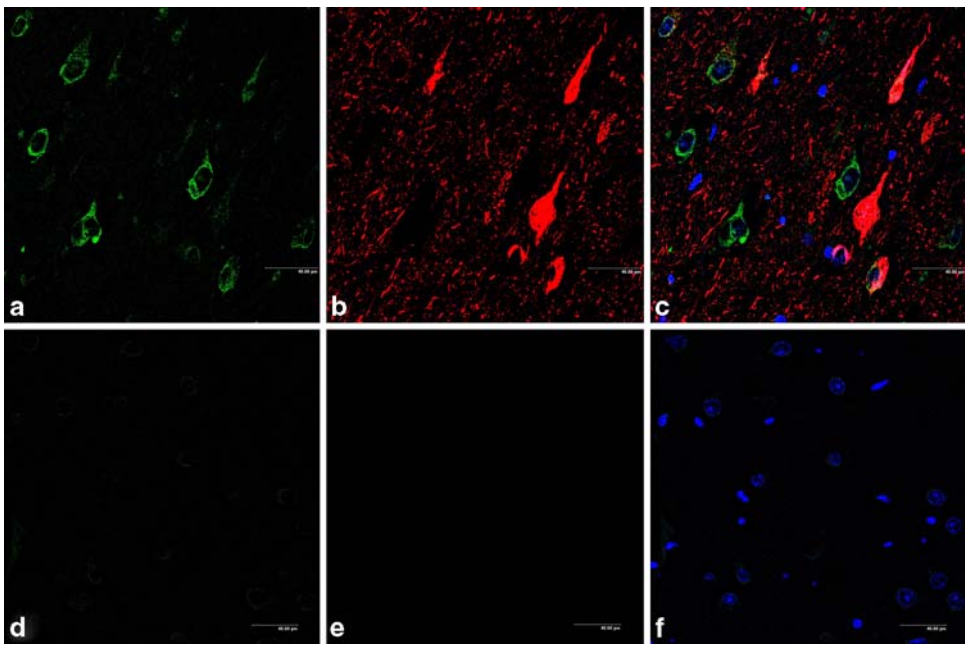


**Fig. 7** Alzheimer's disease. **a–c** Lrrk2 immunoreactivity as revealed with the AP7099b antibody does not co-localize with AT8-immunoreactive inclusions. **d–f** Sections processed without the primary antibodies are negative. Nuclei were stained with TO-PRO-3-iodide

**Table 2** Summary of antibodies (source and type), sections (paraffin: P, frozen: F, formalin: Form), epitope recognized, pretreatment, type of inclusions and corresponding references in different publications using Lrrk2 antibodies to the study of α-synuclein and phospho-tau-immunoreactive inclusions in α-synucleinopathies, Alzheimer's disease and tauopathies

| Antibody<br>NB-300-268<br>Method | Source<br>Novus Biological<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>2,500–2,527<br>Pretreatment | α-syn inclusions | tau inclusions | References |
|---|---|---|---|---|---|---|
| IHQ (DAB) | 1:500 | P 4 | 20′ CB | PD, DLB | AD, PiD, FTLD-tau, AgD | This paper |
| IF | 1:500 | P 4 | 20′ CB* | PD, DLB | AD, PiD, FTLD-tau, AgD | This paper |
| IHQ (DAB) | Not indicated | P 10 | ME and CB 3′ | GCIs MSA | – | [46] |
| IHQ (DAB) | 1:1,000 | F 30 | None/not indicated | PD, DLB, PDCG | AD, PiD, PDCG | [74] |
| IF | 1:300 | F 30 | None/not indicated | PD | AD, PiD | [74] |
| IHQ (DAB) | 1:1,000 | F 30 | None or 0.2% 5′ Triton X-100 | – | FTDP-tau (N279K) | [75] |
| IF | 1:1,000 | F 30 | None or 0.2% 5′ Triton X-100** | – | FTDP-tau (N279K) | [75] |
| IHQ (DAB) | 1:300 | Form 4–6 | ME and CB 10′ (some also with 0.1% Tryp.) | PD, DLB | – | [1] |
| IF | 1:75 | Form 4–6 | ME and CB 10′ (some also with 0.1% tryp.) | PD, DLB | – | [1] |
| IHQ (DAB) | 1:500 | P 5 | 10′ CB | Some LB, DLB | – | [71] |
| IHQ (DAB) | 1:100–1:200 | P 6 | None/not indicated | PD, DLB | – | [107] |
| Antibody<br>NB-300-267<br>Method | Source<br>Novus Biological<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>920–945<br>Pretreatment | | | |
| IHQ (DAB) | 1:500 | P 4 | 20′ CB | Barely PD, DLB | Weak AD, PiD, FTLD-tau, AgD | This paper |
| IF | 1:500 | P 4 | 20′ CB* | Barely PD, DLB | Weak AD, PiD, FTLD-tau, AgD | This paper |
| IHQ (DAB) | 1:500 | F 30 | 0.3% 60′ Triton X-100 | Faint halo some LBs PD | – | [44] |
| IF | 1:1,000 | F 30 | None/not indicated | Faint halo some LBs PD | – | [44] |
| IHQ (DAB) | Not indicated | P 10 | ME and CB 3′ | GCIs MSA | – | [46] |
| IHQ (DAB) | 1:300 | Form 4–6 | ME and CB 10′ (some also with 0.1% tryp.) | Halo some LBs PD, DLB | – | [1] |
| IHQ (DAB) | 1:200 | Wax 7 | CB 10′ | Halo some LBs PD | – | [35] |
| IHQ (DAB) | 1:900 | P 5 | 10′ CB | Some LBs DLB | – | [71] |
| IHQ (DAB) | 1:100–1:200 | P 6 | None/not indicated | PD and DLB | – | [107] |
| Antibody<br>AP7099b<br>Method | Source<br>Abgent<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>1,246-1,265<br>Pretreatment | | | |
| IHQ (DAB) | 1:100 | P 4 | 20′ CB | No, in PD, DLB | No, AD, PiD, FTLD-tau, AgD | This paper |
| IF | 1:100 | P 4 | 20′ CB* | No, PD, DLB | No, AD, PiD, FTLD-tau, AgD | This paper |
| IHQ (DAB) | Not indicated | P 6 | None, FA or ME, but not specified | No, PD, DLB, MSA | No, AD, PiD, PSP | [30] |
| IF | Not indicated | P 6 | None, FA or ME, but not specified | No, PD, DLB, MSA | No, AD, PiD, PSP | [30] |

**Table 2** continued

| | | | | | | |
|---|---|---|---|---|---|---|
| Antibody<br>AP7099b<br>Method | Source<br>Abgent<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>1,246-1,265<br>Pretreatment | | | |
| IHQ (DAB) | Not indicated | P 6 | None or FA, but not specified | No, PD + AD | No, PD + AD | [11] |
| IHQ (DAB) | 1:100–1:200 | P 6 | None/not indicated | No, PD, DLB | – | [107] |
| Antibody<br>ab60937<br>Method | Source<br>Abcam<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>2,480-2,527<br>Pretreatment | | | |
| IHQ (DAB) | 1:200 | F 40 | No | No, PD, DLB | | This paper |
| IF | 1:100 | F 40 | No* | No, PD, DLB | | This paper |
| Antibody<br>JH5517<br>Method | Source<br>Home-made<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>334–347<br>Pretreatment | | | |
| IHQ (DAB) | 1:200 | F 30 | 0.3% 60′ Triton X-100 | Faint halo some LBs PD | – | [44] |
| IF | 1:500 | F 30 | None/not indicated | Faint halo some LBs PD | – | [44] |
| Antibody<br>L955<br>Method | Source<br>Abgent<br>Dilution | Type<br>Rabbit polyclonal<br>Frozenparaffin section thickness (μm) | Epitope<br>946–962<br>Pretreatment | | | |
| IHQ (DAB) | Not indicated | P 10 | ME and CB 3′ | GCIs MSA | – | [46] |
| IF | Not indicated | P 10 | FA 3′ | GCIs MSA | – | [46] |
| Antibody<br>AP7100a<br>Method | Source<br>Abgent<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>2,008-2,027 region<br>Pretreatment | | | |
| IHQ (DAB) | 1:200 | F 30 | 0.2% 5′ Triton X-100 | – | FTDP-tau (N279K) | [75] |
| Antibody<br>EB06550<br>Technic | Source<br>Everest Biotech<br>Dilution | Type<br>Goat polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>2,015-2,026<br>Pretreatment | | | |
| IHQ (DAB) | 1:1,000 | Formalin 4–6 | ME and CB 10′<br>(some also with 0.1% tryp.) | Halo some LBs PD, DLB | – | [1] |

**Table 2** continued

| | | | | | | |
|---|---|---|---|---|---|---|
| Antibody<br>ab04/11<br>Method | Source<br>Non commercial<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>2,507–2,527<br>Pretreatment | | | |
| IHQ (DAB) | 1:3,000 | P 5 | 10′ CB | Few LBs DLB | – | [71] |
| Antibody<br>PA0362<br>Method | Source<br>Home-made<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>2,507–2,527<br>Pretreatment | | | |
| IHQ (DAB) | 1:250 | P 5 | 10′ CB | No, DLB | – | [71] |
| Antibody<br>AB9704<br>Method | Source<br>Chemicon<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section thickness (μm) | Epitope<br>Property of Chemicon<br>Pretreatment | | | |
| IHQ (DAB) | 1:3,000 | P 5 | 10′ CB | Weak in few LBs DLB | – | [71] |
| Antibody<br>AT106<br>Method | Source<br>Alexis Biochemicals<br>Dilution | Type<br>Rabbit polyclonal<br>Frozen/paraffin section/thickness (μm) | Epitope<br>1,838–2,133<br>Pretreatment | | | |
| IHQ (DAB) | 1:100–1:200 | P 6 | None/not indicated | No PD, DLB | – | [107] |

*IHQ* immunohistochemistry, *IF* immunofluorescence, *CB* citrate buffer, *ME* microwave exposure, *FA* formic acid, *+3 min or **15 min FA in double-labelling IF with α-synuclein, *Tryp* trypsine, *DAB* diamino benzidine, – not tested. *PD* Parkinson's disease, *DLB* dementia with Lewy bodies, *AD* Alzheimer's disease, *PiD* Pick's disease, *FTLD-tau* frontotemporal lobar degeneration linked to mutations in the tau gene, *AgD* argyrophilic grain disease, *GCI* glial cytoplasmic inclusions, *MSA* multiple system atrophy. The variable terminology employed to designate Lrrk2 staining has been preserved from the original sources

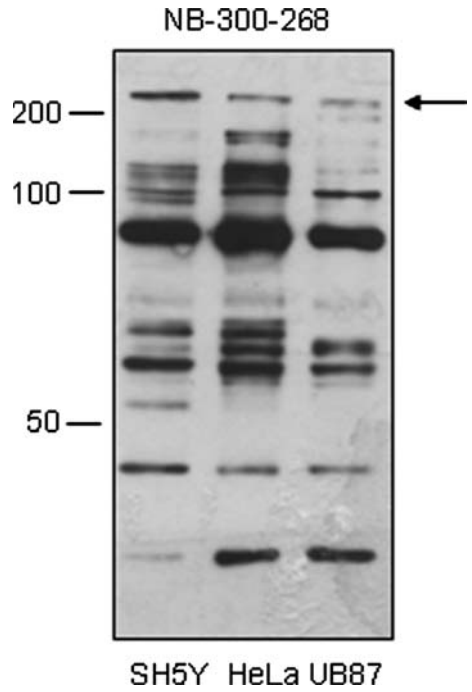


**Fig. 8** Western blot analysis of three different cell lines (SH5Y, HeLa and UB87) using NB-300-268 antibodies shows weak staining of a band consistent with the predicted full-length Lrrk2 (*arrows*). In addition, several bands of lower molecular weight are detected with this antibody

domains ANK, LRR and WD40 are separately found in several proteins. Because of its ability to bind different proteins, Lrrk2 is thought to participate in multiple signalling pathways. The most dramatic evidence of Lrrk2 involvement in degeneration of the nervous system comes from the observation that mutations in *LRRK2* cause degeneration of the human substantia nigra pars compacta clinically manifested as sporadic or familial parkinsonism with variable penetration. However, the functional consequences of the mutations are still poorly understood. Some mutations are accompanied by reduced kinase activity, whereas others are accompanied by increased kinase activity. Whether this might be due, in part, to methodological difficulties in the measurement of kinase activity is under discussion. The use of *Drosophila* and *C. elegans* as *LRRK2* mutant animal models is of limited value, as *LRRK2* in humans and rodents, and LRRK in flies and worms, are not real orthologues but rather paralogues. Moreover, studies in mice and rats expressing human *LRRK2* mutations have resulted in divergent results. Human mutations in *LRRK2* are manifested as different pathologies independently of the type of mutation; some cases are accompanied by Lewy bodies and neurites, others by hyperphosphorylated tau or ubiquitin-immunoreactive inclusions, while some of them do not have apparent inclusions in the remaining neurons of the substantia nigra. It is clear that *LRRK2* mutations may have an impact on common pathways resulting in degeneration of dopaminergic neurons, as well as on specific pathways involving α-synuclein modifications, tau hyperphosphorylation, and other proteins. Finally, whether Lrrk2 is a component of Lewy bodies and neurites in Lewy body diseases, and of hyper-phosphorylated tau inclusions in Alzheimer's disease and tauopathies, is not a subject of agreement because of the different results obtained using different anti-Lrrk2 antibodies. Other aspects are also important, as in addition to the predicted proper band at the corresponding molecular weight of Lrrk2, which is commonly weak, several strong bands of lower molecular

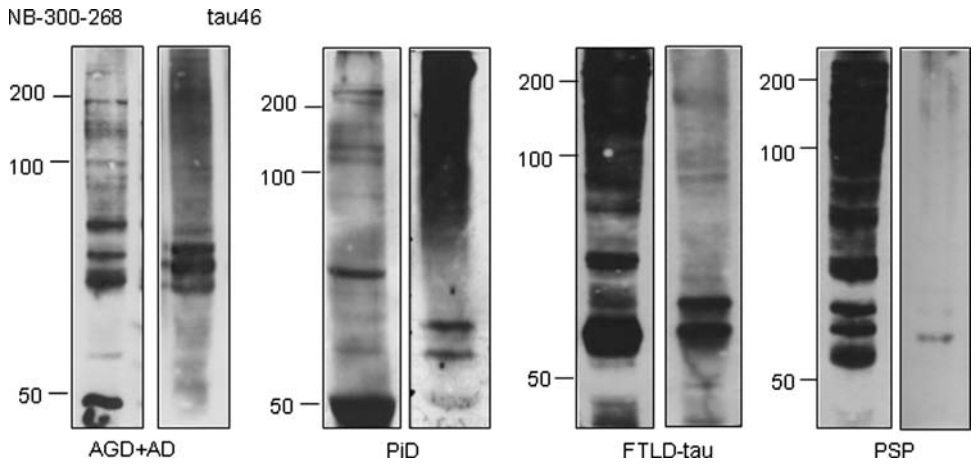


**Fig. 9** Western blot of sarkosyl-insoluble fraction from brain samples of patients with different tauopathies immunostained with NB-300-268 antibody. *AGD + AD* Argyrophilic grain disease + Alzheimer's disease stage 3 of Braak, *PiD* Pick's disease, *FTLD-tau*: frontotemporal lobar degeneration linked to mutations in the tau gene, *PSP* progressive supranuclear palsy. The band consistent with full-length Lrrk2 is hardly detected, whereas several lower bands are strongly immunoreactive. The pattern of these low bands differs among tauopathies. Re-incubation of the same membranes with an antibody against tau (tau46) reveals no match between Lrrk2 immunoreactivity and tau immunoreactivity, thus suggesting lack of cross-reactivity

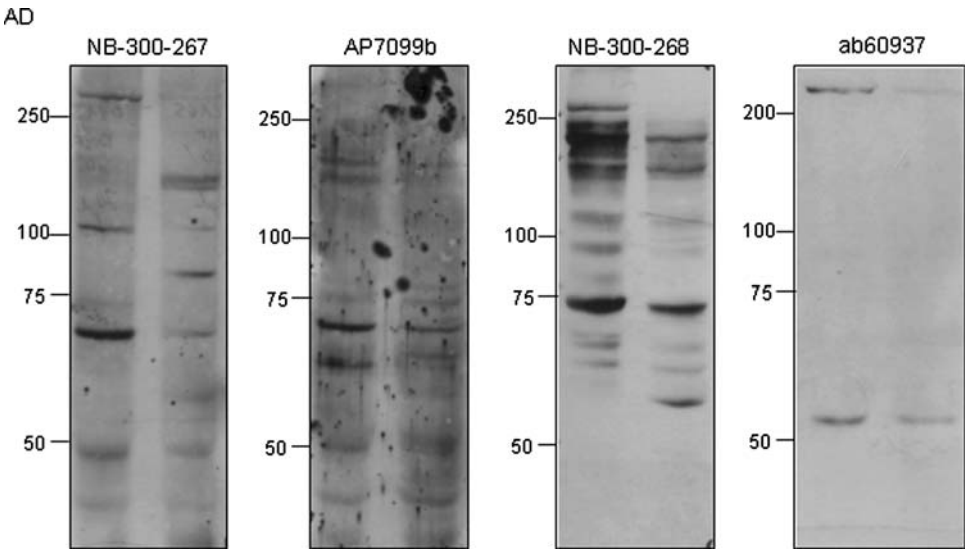


**Fig. 10** Western blots of sarkosyl-insoluble fractions in two AD cases (two columns per antibody) immunostained with NB-300-267, AP7099b, NB-300-268 and ab60937 antibodies. The four antibodies show different affinity to the predicted full-length Lrrk2, whereas all of them detect several bands at lower molecular weights. Antibodies display notably different band patterns. Moreover, the band pattern also differs, to a lesser degree, when comparing the two AD cases with the same antibody

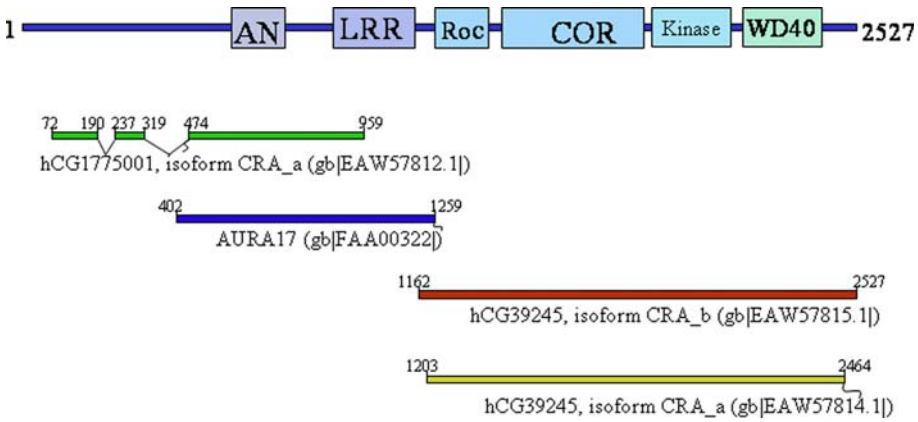


**Fig. 11** Schematic alignment of GenBank-annotated proteins related to additional putative *LRRK2*-like transcripts

weight are seen after gel electrophoresis and Western blotting with the different available antibodies. Furthermore, some but not all inclusions are stained differentially with antibodies directed to the C-terminus and N-terminus, but they are not stained at all with antibodies directed to the middle regions of Lrrk2. Available data do not permit us, at present, to state that full-length Lrrk2 is a major component of Lewy bodies in Lewy body diseases and hyper-phosphorylated tau inclusions in AD and tauopathies.

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## References

1. Alegre-Abarrategui J, Ansorge O, Esiri M, Wade-Martins R (2008) LRRK2 is a component of granular alpha-synuclein pathology in the brainstem of Parkinson's disease. Neuropathol Appl Neurobiol 34:272–283
2. Belin AC, Westerlund M (2008) Parkinson's disease: a genetic perspective. FEBS J 275:1377–1383
3. Benamer HT, de Silva R, Siddiqui KA, Grosset DG (2008) Parkinson's disease in Arabs: a systematic review. Mov Disord 23:1205–1210
4. Biskup S, Moore DJ, Celsi F, Higashi S, West AB, Andrabi SA, Kurkinen K, Yu SW, Savitt JM, Waldvogel HJ, Faull RL, Emson PC, Torp R, Ottersen OP, Dawson TM, Dawson VL (2006) Localization of LRRK2 to membranous and vesicular structures in mammalian brain. Ann Neurol 60:557–569
5. Biskup S, Moore DJ, Rea A, Lorenz-Deperieux B, Coombes CE, Dawson VL, Dawson TM, West AB (2007) Dynamic and redundant regulation of LRRK2 and LRRK1 expression. BMV Neurosci 8:102
6. Biskup S, West AB (2008) Zeroing in on LRRK2-linked pathogeneic mechanisms in Parkinson's disease. Biochem Biophys Acta. Oct 10 (Epub ahead of print) PMID 18973807
7. Carney DS, Davies BA, Horazdovsky BF (2006) Vps9 domain-containing proteins: activators of Rab5 GTPases from yeast to neurons. Trends Cell Biol 16:27–35

8. Chen-Plotkin AS, Yuan W, Anderson C, Wood EM, Hurtig HI, Clark CM, Miller BL, Lee VM, Trojanowski JQ, Grossman M, Van Deerlin VM (2008) Corticobasal syndrome and primary progressive aphasia as manifestations of LRRK2 gene mutations. Neurology 70:521–527
9. Clark LN, Wang Y, Karlins E, Saito L, Mejia-Santana H, Harris J, Louis ED, Cote L, Andrews H, Fahn S, Waters C, Ford B, Frucht S, Ottman R, Marder K (2006) Frequency of LRRK2 mutations in early- and late-onset Parkinson's disease. Neurology 67:1786–1791
10. Covy JP, Giasson BI (2008) Identification of compounds that inhibit the kinase activity of leucine-rich repeat kinase 2. Biochem Biophys Res Commun [Epub ahead of print]
11. Covy JP, Van Deerlin VM, Giasson BI (2006) Lack of evidence for LRRK2 in α-synuclein pathological inclusions. Ann Neurol 60:618–619
12. Covy JP, Yuan W, Waxman EA, Hurtig HI, Van Deerlin VM, Giasson BI (2008) Clinical and pathological characteristics of patients with leucine-rich repeat kinase-2 mutations. Mov Disord [Epub ahead of print]
13. Dächsel JC, Ross OA, Mata IF, Kachergus J, Toft M, Cannon A, Baker M, Adamson J, Hutton M, Dickson DW, Farrer MJ (2007) Lrrk2 G2019S substitution in frontotemporal lobar degeneration with ubiquitin-immunoreactive neuronal inclusions. Acta Neuropathol 113:601–606
14. Dächsel JC, Taylor JP, Mok SS, Ross OA, Hinkle KM, Bailey RM, Hines JH, Szutu J, Madden B, Petrucelli L, Farrer MJ (2007) Identification of potential protein interactors of Lrrk2. Parkinsonism Relat Disord 13:382–385
15. Deng H, Le W, Guo Y, Hunter CB, Xie W, Jankovic J (2005) Genetic and clinical identification of Parkinson's disease patients with LRRK2 G2019S mutation. Ann Neurol 57:933–934
16. Deng J, Lewis PA, Greggio E, Sluch E, Beilina A, Cookson MR (2008) Structure of the ROC domain from the Parkinson's disease-associated leucine-rich repeat kinase 2 reveals a dimeric GTPase. Proc Natl Acad Sci 105:1499–1504
17. Devine MJ, Lewis PA (2008) Emerging pathways in genetic Parkinson's disease: tangles, Lewy bodies and LRRK2. FEBS J 275:5748–5757
18. Di Fonzo A, Rohé CF, Ferreira J, Chien HF, Vacca L, Stocchi F, Guedes L, Fabrizio E, Manfredi M, Vanacore N, Goldwurm S, Breedveld G, Sampaio C, Meco G, Barbosa E, Oostra BA, Bonifati V, Italian Parkinson Genetics Network (2005) A frequent LRRK2 mutation gene mutation associated with autosomal dominant Parkinson's disease. Lancet 365:412–415
19. Elbaz A (2008) LRRK2: bridging the gap between sporadic and hereditary Parkinson's disease. Lancet Neurol 7:562–564
20. Farrer M, Stone J, Mata IF, Lincoln S, Kachergus J, Hulihan M, Strain KJ, Maraganore DM (2005) LRRK2 mutations in Parkinson disease. Neurology 65:738–740
21. Ferrer I, Hernandez I, Puig B, Rey MJ, Ezquerra M, Tolosa E, Boada M (2003) Ubiquitin-negative mini-pick-like bodies in the dentate gyrus in p30l tauopathy. J Alzheimers Dis 5:450–454
22. Forno LS (1996) Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol 55:259–272
23. Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S, Obata F (2002) A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2–q13.1. Ann Neurol 51:296–301
24. Funayama M, Hasegawa K, Ohta E, Kawashima N, Komiyama M, Kowa H, Tsuji S, Obata F (2005) An LRRK2 mutation as a cause for the parkinsonism in the original PARK8 family. Ann Neurol 57:918–921
25. Gaig C, Ezquerra M, Marti E, Muñoz E, Vallderiola F, Tolosa E (2006) LRRK2 mutations in Spanish patients with Parkinson disease: frequency, clinical features, and incomplete penetrance. Arch Neurol 63:377–382
26. Gaig C, Martí MJ, Ezquerra M, Rey MJ, Cardozo A, Tolosa E (2007) G2019S LRRK2 mutation causing Parkinson's disease without Lewy bodies. J Neurol Neurosurg Psychiatr 78:626–628
27. Galter D, Westerlund M, Carmine A, Lindqvist E, Sydow O, Olson L (2006) LRRK2 expression linked to dopamine-innervated areas. Ann Neurol 59:714–719
28. Gandhi PN, Wang X, Zhu X, Chen SG, Wilson-DElfosse AL (2008) The Roc domain of leucine-rich repeat kinase 2 is sufficient for interaction with microtubules. J Neurosci Res 86:1711–1720
29. Giasson BI, Van Deerlin VM (2008) Mutations in LRRK2 as a cause of Parkinson's disease. Neurosignals 16:99–105
30. Giasson BI, Covy JP, Bonini NM, Hurtig HI, Farrer MJ, Trojanowski JQ, Van Deerlin VM (2006) Biochemical and pathological characterization of Lrrk2. Ann Neurol 59:315–322
31. Gilks WP, Abou-Sleiman PM, Gandhi S, Jain S, Singleton A, Lees AJ, Shaw K, Bhatia KP, Bonifati V, Quinn NP, Lynch J, Healy DG, Holton JL, Revesz T, Wood NW (2005) A common LRRK2 mutation in idiopathic Parkinson's disease. Lancet 365:415–416
32. Giordana MT, D'Agostino C, Albani G, Mauro A, Di Fonzio A, Antonini A, Bonifaci V (2007) Neuropathology of Parkinson's disease associated with LRRK2 Ile1371Val mutation. Mov Disord 22:275–278
33. Gloeckner CJ, Kinkl N, Schumacher A, Braun RJ, O'Neill E, Meitinger T, Kolch W, Prokisch H, Ueffing M (2006) The Parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity. Hum Mol Genet 15:223–232
34. Goldwurm S, Zini M, Mariani L, Tesei S, Miceli R, Sironi F, Clementi M, Bonifati V, Pezzoli G (2007) Evaluation of LRRK2 G2019S penetrance: relevance for genetic counselling in Parkinson disease. Neurology 68:1141–1143
35. Greggio E, Jain S, Kingsbury A, Bandopadhyay R, Lewis P, Kaganovich A, van der Brug MP, Beilina A, Blackinton J, Thomas KJ, Ahmad R, Miller DW, Kesavapany S, Singleton A, Lees A, Harvey RJ, Harvey K, Cookson MR (2006) Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. Neurobiol Dis 23:329–341
36. Greggio E, Zambrano I, Kaganovich A, Beilina A, Taymans JM, Daniels V, Lewis P, Jain S, Ding J, Syed A, Thomas KJ, Baekelandt V, Cookson MR (2008) The Parkinson's disease associated Leucine rich repeat kinase 2 (LRRK2) is a dimer that undergoes intra-molecular autophosphorylation. J Biol Chem 283:16906–16914
37. Guo L, Gandhi PN, Wang W, Petersen RB, Wilson-Delfosse AL, Chen SG (2007) The Parkinson's disease-associated protein, leucine-rich repeat kinase 2 (LRRK2), is an authentic GTPase that stimulates kinase activity. Exp Cell Res 313:3658–3670
38. Häbig K, Walter M, Poths S, Bonin M (2008) RNA interference of LRRK2-microarray expression analysis of a Parkinson's disease key player. Neurogenetics 9:83–94
39. Han BS, Iacovitti L, Katano T, Hattori N, Seol W, Kim KS (2008) Expression of LRRK2 gene in the midbrain dopaminergic neurons of the substantia nigra. Neurosci Lett (Epub ahead of print)
40. Hatano T, Kubo S, Imai S, Maeda M, Ishikawa K, Mizuno Y, Hattori N (2007) Leucine-rich repeat kinase 2 associates with lipid rafts. Hum Mol Genet 16:678–690
41. Haugarvoll K, Rademakers R, Kachergus JM, Nuytemans K, Ross OA, Gibson JM, Tan EK, Gaig C, Tlosa E, Goldwurm S, Guidi M, Riboldazzi G, Brown L, Walter U, Benecke R, Berg D, Gasser T, Theurns J, Pals P, Cras P, DeDeyn PP, Engelborghs S, Pickut B, Uiti RJ, Foroud T, Nichols WC, Hagenah J, Klein C,

Samii A, Zabetian CP, Bonifacti V, Van Broeckhoven C, Farrer MJ, Wszolek ZK (2008) LRRK2 R1441C parkinsonism is clinically similar to sporadic Parkinson disease. Neurology 70:1456–1460
42. Haugarvoll K, Wszolek ZK (2006) PARK8 LRRK2 parkinsonism. Curr Neurol Neurosci Rep 6:287–294
43. Healy DG, Falchi M, O'Sullivan SS, Bonifati V, Durr A, Bressman S, Brice A, Aasly J, Zabetian CP, Goldwurm S, Ferreira JJ, Tolosa E, Kay DM, Klein C, Williams DR, Marras C, Lang AE, Wszolek ZK, Berciano J, Schapira AH, Lynch T, Bhatia KP, Gasser T, Lees AJ, Wood NW, International LRRK2 Consortium (2008) Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case–control study. Lancet Neurol 7:583–590
44. Higashi S, Biskup S, West AB, Trinkaus D, Dawson VL, Faull RL, Waldvogel HJ, Arai H, Dawson TM, Moore DJ, Emson PC (2007) Localization of Parkinson's disease-associated LRRK2 in normal and pathological human brain. Brain Res 1155:208–219
45. Higashi S, Moore DJ, Colebrooke RE, Biskup S, Dawson VL, Arai H, Dawson TM, Emson PC (2007) Expression and localization of Parkinson's disease-associated leucine-rich repeat kinase 2 in the mouse brain. J Neurochem 100:368–381
46. Huang Y, Song YJ, Murphy K, Holton JL, Lashley T, Revesz T, Gai WP, Halliday GM (2008) LRRK2 and parkin immunoreactivity in multiple system atrophy inclusions. Acta Neuropathol 116:639–646
47. Hurtado-Lorenzo A, Anand VS (2008) Heat shock protein 90 modulates LRRK2 stability: potential implications for Parkinson's disease treatment. J Neurosci 28:6757–6759
48. Iaccarino C, Crosio C, Vitale C, Sanna G, Carri MT, Barone P (2007) Apoptotic mechanisms in mutant LRRK2-mediated cell death. Hum Mol Genet 16:1319–1326
49. Ishihara L, EWarren L, Gibson R, Amouri R, Lesage S, Durr A, Tazir M, Wszolek ZK, Uiti RJ, Nichols WC, Griffith A, Hattori N, Leppert D, Watts R, Zabetian CP, Foroud TM, Farrer MJ, Brice A, Middleton L, Hentati F (2006) Clinical features of Parkinson disease patients with homozygous leucine-rich repeat kinase 2 G2019S mutations. Arch Neurol 63:1250–1254
50. Ito G, Okai T, Fujino G, Takeda K, Ichijo H, Katada T, Iwatsubo T (2007) GTPbinding is essential to the protein kinase activity of LRRK2, a causative gene product for familial Parkinson's disease. Biochemistry 46:1380–1388
51. Jaleel M, Nichols RJ, Deak M, Campbell DG, Gillardon F, Knebel A, Alessi DR (2007) LRRK2 phosphorylates moesin at threonine-558: characterization of how Parkinson's disease mutants affect kinase activity. Biochem J 405:307–317
52. Jellinger K, Mizuno Y (2003) Parkinson's disease. In: Dickson D (ed) Neurodegeneration: the molecular pathology of dementia and movement disorders. ISN Neuropathol Press, Basel, pp 169–187
53. Kachergus J, Mata IF, Hulihan M, Taylor JP, Lincoln S, Aaasly J, Gibson JM, Ross OA, Lynch T, Wiley J, Payami H, Nutt J, Maraganone DM, Czyzewski K, Styczynska M, Wszolek ZK, Farrer MJ, Toft M (2005) Identification of a novel LRRK2 mutation linked to autosomal dominant parkinsonism: evidence of a common founder across European population. Am J Hum Genet 76:672–680
54. Khan NL, Jain S, Lynch JM, Pavese N, Abou-Sleiman P, Holton JL, Healy DG, Gilks WP, Sweeney MG, Ganguly M, Gibbons V, Gandhi S, Vaughan J, Eunson LH, Katzenschlager R, Gayton J, Lennox G, Revesz T, Nicholl D, Bhatia KP, Quinn N, Brooks D, Lees AJ, Davis MB, Piccini P, Singleton AB, Wood NW (2005) Mutations in the gene LRRK2 encoding dardarin (PARK8) cause familial Parkinson's disease: clinical, pathological, olfactory and functional imaging and genetic data. Brain 128:2786–2796
55. Larsen K, Madsen LB (2007) Sequence conservation between porcine and human LRRK2. Mol Biol Rep (Epub ahead of print)
56. Lee SB, Kim W, Lee S, Chung (2007) Loss of LRRK2/PARK8 induces degeneration of dopaminergic neurons in *Drosophila*. Biochem Biophys Res Commun 358:534–539
57. Lesage S, Durr A, Tazir M, Lohmann E, Leutenegger AL, Janin S, Pollak P, Brice A (2006) LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. N Engl J Med 354:422–423
58. Lesage S, Leutenegger AL, Ibanez P, Janin S, Lohmann E, Durr A, Brice A (2005) LRRK2 haplotype analyses in European and North African families with Parkinson disease: a common founder for the G2019S mutation dating from the 13th century. Am J Hum Genet 77:330–332
59. Lewis PA, Greggio E, Beilina A, Jain S, Baker A, Cookson MR (2007) The R1441C mutation of LRRK2 disrupts GTP hydrolysis. Biochem Biophys Res Commun 357:668–671
60. Li X, Tan YC, Poulose S, Olanow CW, Huang XY, Yue Z (2007) Leucine-rich repeat kinase 2 (LRRK2)/PARK8 possesses GTPase activity that is altered in familial Parkinson's disease R1441C/G mutants. J Neurochem 103:238–247
61. Liu Z, Wang X, Yu Y, Li X, Wang T, Jiang H, Ren Q, Jiao Y, Sawa A, Moran T, Ross CA, Montell C, Smith WW (2008) A *Drosophila* model for LRRK2-linked parkinsonism. Proc Natl Acad Sci USA 105:2693–2698
62. Lu YW, Tan EK (2008) Molecular biology changes associated with LRRK2 mutations in Parkinson's disease. J Neurosci Res 86:1895–1901
63. Luzon-Toro B, Rubio la Torre de E, Delgado A, Perez-Tur J, Hilfiker S (2007) Mechanistic insight into the dominant mode of the Parkinson's disease-associated G2019S LRRK2 mutation. Hum Mol Genet 16:2031–2039
64. MacLeod D, Dowman J, Hammond R, Leete T, Inoue K, Abeliovich A (2006) The familial Parkinsonism gene LRRK2 regulates neurite process morphology. Neuron 52:587–593
65. Marin I (2006) The Parkinson disease gene *LRRK2*: evolutionary and structural insights. Mol Biol Evol 23:2423–2433
66. Marín I (2008) Ancient origin of the Parkinson disease gene LRRK2. J Mol Evol (Epub ahead of print)
67. Marin I, van Egmond WN, van Haastert PJ (2008) The Rocco protein family: a functional perspective. FASEB J (Epub ahead of print)
68. Martí-Massó JF, Ruiz-Martinez J, Bolaño MJ, Ruiz I, Gorostidi A, Moreno F, Ferrer I, López de Munain A (2008) Neuropathology of Parkinson disease with the R1441G mutation in LRRK2 (submitted)
69. Mata IF, Kachergus JM, Taylor JP, Lincoln S, Aasly J, Lynch T, Hulihan MM, Cobb SA, Wu RM, Lu CS, Lahoz C, Wszolek ZK, Farrer MJ (2005) Lrrk2 pathogenic substitutions in Parkinson's disease. Neurogenetics 6:171–177
70. Mata IF, Wedemeyer WJ, Farrer MJ, Taylor JP, Gallo KA (2006) LRRK2 in Parkinson's disease: protein domains and functional insights. Trends Neurosci 29:286–293
71. Melrose HL, Kent CB, Taylor JP, Dachsel JC, Hinkle KM, Lincoln SJ, Mok SS, Culvenor JG, Masters CL, Tyndall GM, Bass DI, Ahmed Z, Andorfer CA, Ross OA, Wszolek ZK, Delldonne A, Dickson DW (2007) A comparative analysis of leucine-rich repeat kinase 2 (Lrrk2) expression in mouse brain and Lewy body disease. Neuroscience 147:1047–1058
72. Melrose H, Lincoln S, Tyndall G, Dickson D, Farrer M (2006) Anatomical localization of leucine-rich repeat kinase 2 in mouse brain. Neuroscience 139:791–794
73. Meylan E, Tschopp J (2005) The RIP kinases: crucial integrators of cellular stress. Trends Biochem Sci 30:151–159
74. Miklossy J, Arai T, Guo JP, Klegeris A, Yu S, McGeer EG, McGeer PL (2006) LRRK2 expression in normal and pathologic

human brain and in human cell lines. J Neuropathol Exp Neurol 65:953–963
75. Miklossy J, Qing H, Guo JP, Yu S, Wszolek ZK, Calne D, McGeer EG, McGeer PL (2007) Lrrk2 and chronic inflammation are linked to pallido-ponto-nigral degeneration caused by the N279K tau mutation. Acta Neuropathol 114:243–254
76. Moore DJ (2008) The biology and pathobiology of LRRK2: implications for Parkinson's disease. Parkinsonism Relat Disord 14(suppl 2):S92–S98
77. Nakamura N, Shimaoka Y, Tougan T, Onda H, Okuzaki D, Zhao H, Fujimori A, Yabuta N, Nagamori I, Tanigawa A, Sato J, Oda T, Hayashida K, Suzuki R, Yukioka M, Nojima H, Ochi T (2006) Isolation and expression profiling of genes upregulated in bone marrow-derived mononuclear cells of rheumatoid arthritis patients. DNA Res 13:169–183
78. Nichols WC, Pankratz N, Hernandez D, Paisa-Ruiz C, Jain S, Halter CA, Michaels VE, Reed T, Rudolph A, Shults CW, Singleton A, Foroud T (2005) Genetic screening for a single common LRRK2 mutation in familial Parkinson's disease. Lancet 365:410–412
79. Orr-Urtreger A, Shifrin C, Rozovski U, Rosner S, Bercovich D, Gurevich T, Yagev-More H, Bar-Shira A, Giladi N (2007) The LRRK2 G2019S mutation in Ashkenazi Jews with Parkinson disease: is there a gender effect? Neurology 69:1595–1602
80. Ozelius LJ, Senthil G, Saunders-Pullman R, Ohmann E, Deligtisch A, Tagliati M, Hunt AL, Klein C, Henick B, Hailpern SM, Lipton RB, Soto-Valencia J, Risch N, Bressman SB (2006) LRRK2 G2019S as a cause of Parkinson's disease in Ashkenazi Jews. N Engl J Med 354:424–425
81. Paisan-Ruiz C, Jain S, Evans EW, Gilks WP, Simon J, van der Brug M, Lopez de Munain A, Aparicio S, Gil AM, Khan N, Johnson J, Martinez JR, Nicholl D, Carrera IM, Pena AS, de Silva R, Lees A, Martí-Massó JF, Perez-Tur J, Wood NW, Singleton AB (2004) Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron 44:595–600
82. Paisan-Ruiz C, Nath P, Washecka N, Gibbs JR, Singleton AB (2008) Comprehensive analysis of LRRK2 in publicly available Parkinson's disease cases and neurologically normal controls. Hum Mutat 29:485–490
83. Perry G, Zhu X, Babar AK, Siedlak SL, Yang Q, Ito G, Iwatsubo T, Smith MA, Chen SG (2008) Leucine-rich repeat kinase 2 colocalizes with alpha-synuclein in Parkinson's disease, but not tau-containing neurons in tauopathies. Neurodegener Dis 5:222–224
84. Plowey ED, Cherra SJ, Liu YJ, Chu CT (2008) Role of autophagy in G2019S-LRRK2-associated neurite shortening in differentiated SH-SY5Y cells. J Neurochem 105:1048–1056
85. Rajput A, Dickson DW, Robinson CA, Ross OA, Dächael JC, Lincoln SJ, Cobb SA, Rajput ML, Farrer MJ (2006) Parkinsonism, Lrrk2 G2019S, and tau neuropathology. Neurology 67:1506–1508
86. Ross OA, Toft M, Whittle AJ, Johnson JL, Papapetropoulos S, Mash DC, Litvan I, Gordon MF, Wszolek ZK, Farrer MJ, Dickson DW (2006) LRRK2 and Lewy body disease. Ann Neurol 59:388–393
87. Shin N, Jeong H, Kwon J, Heo HY, Kwon JJ, Yun HJ, Kim CH, Han BS, Tong Y, Shen J, Hatano T, Hattori N, Kim KS, Chang S, Seol W (2008) LRRK2 regulates synaptic vesicle endocytosis. Exp Cell Res 314:2055–2065
88. Shults CW (2006) Lewy bodies. Proc Natl Acad Sci USA 103:1661–1668
89. Simon-Sanchez J, Herranz-Perez V, Olucha-Bordonau F, Perez-Tur J (2006) LRRK2 is expressed in areas affected by Parkinson's disease in the adult mouse brain. Eur J NeuroSci 23:659–666
90. Skipper L, Shen H, Chua E, Bonnnard C, Kolatkar P, Tan LC, Jamora RD, Puvan K, Puong KY, Zhao Y, Pavanni R, Wong MC, Yuen Y, Farrer M, Liu JJ, Tan EK (2005) Analysis of LRRK2 functional domains in nondominant Parkinson disease. Neurology 25:1319–1321
91. Smith WW, Pei Z, Jiang H, Dawson VL, Dawson TM, Ross CA (2006) Kinase activity of mutant LRRK2 mediates neuronal toxicity. Nat Neurosci 9:1231–1233
92. Smith WW, Pei Z, Jiang H, Moore DJ, Liang West AB, Dawson VL, Dawson TM, Ross CA (2005) Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration. Proc Natl Acad Sci USA 102:18676–18681
93. Stenmark H, Olkkonen VM (2001) The Rab GTPase family. Genome Biol 2:reviews3007.1–reviews3007.7
94. Tan EK, Skipper LM (2007) Pathogenic mutations in Parkinson disease. Hum Mutat 28:641–653
95. Tan EK, Tan LC, Lim HQ, Li R, Tang M, Yih Y, Pavanni R, Prakash KM, Fook-Chong S, Zhao Y (2008) LRRK2 R1628P increases risk of Parkinson's disease: replication evidence. Hum Genet (Epub ahead of print)
96. Taylor JP, Mata IF, Farrer MJ (2006) LRRK2: a common pathway for parkinsonism, pathogenesis and prevention? Trends Mol Med 12:76–82
97. Taymans JM, Van den Haute C, Baekelandt V (2006) Distribution of PINK1 and LRRK2 in rat and mouse brain. J Neurochem 98:951–961
98. Venter JC et al (2001) The sequence of human genome. Science 291:1304–1351
99. Wang D, Tang B, Zhao G, Pan Q, Xia K, Bodmer R, Zhang Z (2008) Dispensable role of *Drosophila* ortholog of LRRK2 kinase activity in survival of dopaminergic neurons. Mol Neurodegener (Epub ahead of print)
100. West AB, Moore DJ, Biskup S, Bugayenko A, Smith WW, Ross CA, Dawson VL, Dawson TM (2005) Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. Proc Natl Acad Sci USA 102:16842–16847
101. West AB, Moore DJ, Choi C, Andrabi SA, Li X, Dikeman D, Biskup S, Zhang Z, Lim KL, Dawson VL, Dawson TM (2007) Parkinson's disease-associated mutations in LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity. Hum Mol Genet 16:223–232
102. Westerlund M, Belin AC, Anvret A, Bickford P, Olson L, Galter D (2008) Developmental regulation of leucine-rich repeat kinase 1 and 2 expression in the brain and other rodent and human organs: implications for Parkinson's disease. Neuroscience 152:429–436
103. Wolozin B, Saha S, Guillily M, Ferre A, Riley M (2008) Investigating convergent actions of genes linked to familial Parkinson's disease. Neurodegener Dis 5:182–185
104. Wszolek ZK, Pfeiffer RF, Tsuboi Y, Uitti RJ, McComb RD, Stoessl AJ, Strongosky AJ, Zimprich A, Müller-Myhsok B, Farrer MJ, Gasser T, Calne DB, Dickson DW (2004) Autosomal dominant parkinsonism associated with variable synuclein and tau pathology. Neurology 62:1619–1622
105. Zabetian CP, Hunmter CM, Yearout D, Lopez AN, Factor SA, Griffith A, Leis BC, Bird TD, Nutt JG, Higgins DS, Roberts JW, Kay DM, Edwards KL, Samii A, Payami H (2006) LRRK2 G2019S in families with Parkinson disease who originated from Europe and the Middle East: evidence of two distinct founding events beginning two millennia ago. Am J Hum Genet 79:752–758
106. Zarranz JJ, Ferrer I, Lezcano E, Forcadas MI, Eizaguirre B, Atares B, Puig B, Gomez-Esteban JG, Fernandez-Maiztegui C, Rouco I, Perez-Concha T, Fernandez M, Rodriguez O, Rodriguez-Martinez AB, Martinez de Pancorbo M, Pastor P, Perez-

Tur J (2005) A novel mutation (K317M) in the MAPT gene causes FTDP and motor neuron disease. Neurology 64:1578–1585

107. Zhu X, Babar A, Siedlak SL, Yang Q, Ito G, Iwatsubo T, Smith MA, Perry G, Chen SG (2006) LRRK2 in Parkinson's disease and dementia with Lewy bodies. Mol Neurodeg 1:1–9
108. Zhu X, Siedlak SL, Smith MA, Perry G, Chen SG (2006) LRRK2 protein is a component of Lewy bodies. Ann Neurol 60:617–618 author reply 618–619
109. Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S, Kachergus J, Hulihan M, Uitti RJ, Calne DB, Stoessl AJ, Pfeiffer RF, Patenge N, Carbajal IC, Vieregge P, Asmus F, Muller-Myhsok B, Dickson DW, Meitinger T, Strom TM, Wszolek ZK, Gasser T (2004) Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron 44:601–607
110. Zimprich A, Muller-Myhsok B, Farrer M, Leitner P, Sharma M, Hulihan M, Lockhart P, Strongosky A, Kachergus J, Calne DB, Stoessl J, Uitti RJ, Pfeiffer RF, Trenkwalder C, Homann N, Ott E, Wenzel K, Asmus F, Hardy J, Wszolek Z, Gasser T (2004) The PARK8 locus in autosomal dominant parkinsonism: confirmation of linkage and further delineation of the disease-containing interval. Am J Hum Genet 74:11–19

# 7

## Oxidative damage of 14-3-3 zeta and gamma isoforms in Alzheimer's disease and cerebral amyloid angiopathy

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# OXIDATIVE DAMAGE OF 14-3-3 ZETA AND GAMMA ISOFORMS IN ALZHEIMER'S DISEASE AND CEREBRAL AMYLOID ANGIOPATHY

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**Abstract—Previous studies have shown oxidative damage resulting from amyloid A$\beta$ exposure to cultured cells and in murine models. A target of oxidation is 14-3-3 which comprises a group of proteins involved in kinase activation and chaperone activity. The present study shows glycoxidative damage, as revealed with mono and bi-dimensional gel electrophoresis and Western blotting, followed by in-gel digestion and mass spectrometry, in the frontal cortex in Alzheimer's disease (AD) and cerebral amyloid angiopathy (CAA), a neurodegenerative disease with deposition of A$\beta$ in cerebral blood vessels and in diffuse plaques unaccompanied by intraneuronal hyper-phosphorylated tau deposition. malondialdehyde-lysine (MDA-Lys)-, but not 4-hydroxy-2-nonenal (HNE)-immunoreactive adducts, and N-carboxyethyl-lysine (CEL), but not N-carboxymethyl-lysine (CML)-products, were present in 14-3-3 involving zeta and gamma isoforms in both AD and CAA. These findings demonstrate that 14-3-3 glyco- and lipoxidation occurs in AD and CAA, probably as a direct consequence of A$\beta$ deposition.** 

**Key words: Alzheimer's disease, cerebral amyloid angiopathy, 14-3-3, A$\beta$, oxidative stress, lipoxidation.**

Neurofibrillary tangles (NFTs) and amyloid plaques are the main neuropathological markers of Alzheimer's disease (AD). NFTs are mainly composed of aggregated and hyper-phosphorylated tau protein, which forms paired helical filaments (PHFs). Tau is a microtubule-associated protein which promotes and stabilizes the microtubule network through a balance between phosphorylation and dephosphorylation states. Abnormal phosphorylation of tau reduces its affinity to microtubules, and microtubule instability is a putative cause of nerve cell degeneration in AD (Spillantini and Goedert, 1998; Buee et al., 2000; Lee et al., 2001; Avila et al., 2002; Sergeant et al., 2005). Amyloid plaques are mainly composed of A$\beta$ which is a product of the trans-membrane protein APP (amyloid precursor protein). Amyloid deposits are locally surrounded by abnormal cell processes, mainly aberrant neurites containing PHFs, and reactive microglia and astrocytes in neuritic plaques (Duyckaerts and Dickson, 2003; Masters and Beyreuther, 2003).

Recent evidence suggests that oxidative damage is an important early event, and a key factor, in the pathogenesis of AD (Butterfield et al., 2001; Cecchi et al., 2002; Chauhan and Chauhan, 2006; Pamplona et al., 2005; Nunomura et al., 2006; Butterfield et al., 2006). A$\beta_{(1-42)}$ has been shown to induce protein oxidation *in vitro* and *in vivo* (Boyd-Kimball et al., 2005; Butterfield and Lauderback, 2002; Varadarajan et al., 2000; Drake et al., 2003; Butterfield et al., 2006), whereas antioxidants such as vitamin E play a protective role against A$\beta$-mediated cytotoxicity in AD (Muñoz et al., 2005). Moreover, stress-activated protein kinases c-Jun N-terminal kinase (SAPK/JNK) and p38 pathways are activated in dystrophic neurites surrounding A$\beta$ deposits and in neurons with abnormal tau accumulation in AD and related murine models (Zhu et al., 2000, 2001; Ferrer et al., 2001, 2005; Atzori et al., 2001; Pei et al., 2001; Puig et al., 2004).

Protein oxidation occurs physiologically as a consequence of aerobic life. Oxidized proteins appear to be degraded by an ubiquitin- and ATP-independent pathway ruled by the 20S proteasome (Davies, 2001). Yet excessive oxidative stress in the brain may render the proteolytic capacity of this system insufficient, and thereby facilitate the accumulation of abnormal proteins. In addition, oxidative damage of proteins is commonly associated with their loss of function (Davies, 1987; Oliver et al., 1987; Nishikawa et al., 2003; Sultana et al., 2006). Together, these circumstances promote the accumulation of abnormal and often disabled proteins, through covalent cross-linking reactions and increased surface hydrophobicity (Garrison et al., 1962; Davies, 2001).

The present study was undertaken in an attempt to identify oxidized proteins in sarkosyl-insoluble fractions in AD. N-carboxymethyl-lysine (CML) and N-carboxyethyl-lysine (CEL) were used as markers of glycoxidation and carbonyl production. Antibodies to 4-hydroxy-2-nonenal (HNE) and malondialdehyde-lysine (MDA-Lys) were used as markers of lipoxidation. Bi-dimensional (2D) electrophoresis and Western blotting linked to mass spectrometry revealed 14-3-3 (zeta and gamma isoforms) as a major target of glycoxidative and lipoxidative damage in sarkosyl-insoluble fractions in AD brains. In order to learn whether this was specifically related to AD, the study was extended to cases with amyloid angiopathy and A$\beta$ plaques (cerebral amyloid angiopathy, CAA) without NFTs.

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*Abbreviations:* AD, Alzheimer's disease; BSA, bovine serum albumin; CAA, cerebral amyloid angiopathy; CEL, N-carboxyethyl-lysine; CML, N-carboxymethyl-lysine; HNE, 4-hydroxy-2-nonenal; MDA-Lys, malondialdehyde-lysine; NFT, neurofibrillary tangle; PHF, paired helical filament; TBS-T, 100 mM Tris-buffered saline, 140 mM NaCl and 0.1% Tween 20, pH 7.4; 2D, bi-dimensional.

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## EXPERIMENTAL PROCEDURES

### Brain tissues

Brain tissues were obtained from the Institute of Neuropathology Brain Bank following the guidelines and approval of the local ethics committee. Four patients had suffered from severe (Global Deterioration Scale) dementia of Alzheimer type. Eleven cases were neurologically normal. The postmortem delay was between 3 and 20 h. 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, samples of the frontal cortex were fixed in 4% paraformaldehyde in phosphate buffer for 24 h, cryoprotected in 30% saccharose and frozen at −80 °C. The neuropathological study was carried out on de-waxed 4-$\mu$m-thick paraffin sections of the frontal (area 8), primary motor, primary sensory, parietal, temporal superior, temporal inferior, anterior cingulated, 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 hematoxylin and eosin, Klüver Barrera, and, for immunohistochemistry to glial fibrillary acidic protein, CD68 and *Licopericum esculentum* lectin for microglia, A$\beta$-amyloid, pan-*tau*, AT8 tau, phosphorylation-specific tau Thr181, Ser202, Ser214, Ser262, Ser396 and Ser422, and $\alpha$B-crystallin, $\alpha$-synuclein and ubiquitin. Following neuropathological examination, four cases were categorized as AD stages V/VIC of Braak and Braak (1999). Eight cases with no neurological involvement suffered from AD stages I/IIIA/B and were not included in the present study. The remaining three cases did not have neuropathological abnormalities and were considered as controls. In addition, two cases with CAA were included. All these had, in addition to amyloid angiopathy, A$\beta$ diffuse plaques in the cerebral cortex consistent with stage B of Braak and Braak (1999). Hyper-phosphorylation of tau was restricted to aberrant neurites around rare neuritic plaques. A summary of the main clinical and neuropathological findings in the present series is shown in Table 1.

Brain tissue was further processed for biochemical studies and for 14-3-3 immunohistochemistry and Western blotting.

### Sarkosyl-insoluble fraction extraction

Frozen samples of about 2 g of the frontal cortex (area 8) were gently homogenized in a glass tissue grinder in 10 vol (w/v) with cold suspension buffer (10 mM Tris–HCl, pH 7.4, 0.8 M NaCl, 1 mM EGTA, 10% sucrose). The homogenates were first centrifuged at 20,000×*g* and the supernatant (S1) was retained. The pellet was re-homogenized in 5 vol of homogenization buffer and re-centrifuged. The two supernatants (S1+S2) were then mixed and incubated with 0.1% N-lauroylsarcosynate (sarkosyl) for 1 h at room temperature while being shaken. Samples were then centrifuged at 100,000×*g* in a Ti70 Beckman rotor. Sarkosyl-insoluble pellets (P3) were re-suspended (0.2 ml/g, starting material) in 50 mM Tris–HCl (pH 7.4). Protein concentrations were determined with the BCA method using bovine serum albumin (BSA) as a standard.

### Mono-dimensional gel electrophoresis and Western blotting

For mono-dimensional gel electrophoresis, 30 $\mu$g of frontal cortex was mixed with reducing sample buffer and processed for 10% SDS-PAGE electrophoresis and then transferred to nitrocellulose membranes (400 mA for 90 min). Immediately afterward, the membranes were incubated with 5% skimmed milk in TBS-T buffer (100 mM Tris-buffered saline, 140 mM NaCl and 0.1% Tween 20, pH 7.4) for 30 min at room temperature, and then incubated with the primary antibody in TBS-T containing 3% BSA (Sigma, Madrid, Spain) at 4 °C overnight. The mouse monoclonal anti-CEL and anti-CML antibodies (TransGenic, Kumamoto, Japan) were used at a dilution of 1:1000. The goat polyclonal anti-MDA-Lys (BioMedical, Houston, TX, USA), rabbit polyclonal anti-MDA-Lys (Biomedical), rabbit polyclonal anti-HNE (Calbiochem, Barcelona, Spain) and rabbit polyclonal rabbit polyclonal anti-14-3-3 (Abcam, Cambridge, UK) were used diluted 1:1000. Subsequently, the membranes were incubated for 45 min at room temperature with the corresponding secondary antibody labeled with horseradish peroxidase (Dako, Barcelona, Spain) at a dilution of 1:1000, and washed with TBS-T for 30 min. Protein bands were visualized with the chemiluminescence ECL method (Amersham, Barcelona, Spain).

### 2D Gel electrophoresis

Samples of the frontal cortex (area 8) in AD, CAA and controls were homogenized in lysis buffer (40 mM Tris, pH 7.5, containing 7 M urea (9 M when using sarkosyl-insoluble fractions), 2 M thiourea and a cocktail of protease and phosphatase inhibitors, and centrifuged at 9700 r.p.m. for 10 min. The pellet was discarded and the concentration of protein of the resulting supernatant was determined with the BCA method. Equal amounts of protein were mixed with 0.2% Byolites (v/v), 4% CHAPS (Bio-Rad, Barcelona, Spain), 2 mM tributylphosphine solution (TBP), 50 $\mu$l 8 M urea and Bromophenol Blue in a final volume of 150 $\mu$l. In the first dimension electrophoresis, 150 $\mu$l of sample solution was applied to an immobilized 7 cm pH 3–10 nonlinear gradient ReadyStrip IPG strip (Bio-Rad) at both the basic and acidic ends of the strip. The strips were actively re-hydrated for 12 h at 50 V and the proteins were focused at 300 V for 1 h, after which time the voltage was gradually increased to 3500 V within 6 h. Focusing was continued at 3500 V for 12 h and at 5000 V for 24 h. For the second dimension separation, IPG strips were equilibrated for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (wt/v)

**Table 1.** Summary of the main clinical and pathological findings in the present series

| | Age | Gender | Postmortem delay | Clinical diagnosis | Neuropathological diagnosis | Braak stage |
|---|---|---|---|---|---|---|
| 1 | 73 | F | 5.5 | Normal | No lesions | 0 |
| 2 | 58 | F | 4 | Normal | No lesions | 0 |
| 3 | 73 | F | 7 | Normal | No lesions | 0 |
| 4 | 89 | F | 4 | AD | AD | VC |
| 5 | 78 | F | 3.5 | AD | AD | VC |
| 6 | 79 | M | 7 | AD | AD | VC |
| 7 | 61 | F | 4 | AD | AD | VC |
| 8 | 69 | H | 29 | | CAA | C |
| 9 | 69 | H | 24 | | CAA | C |

SDS, 30% (v/v) glycerol and 2% dithiothreitol, and then re-equilibrated for 10 min in the same buffer containing 2.5% iodoacetamide. The strips were placed on 10% polyacrylamide gels and electrophoresed at 100 V overnight. For gel staining, Coomassie Biosave staining (Bio-Rad) was used, as described by the manufacturer.

Two 2D electrophoreses were run in parallel in every case, one for Coomassie staining and the other transferred into a nitrocellulose membrane (200 mA for 1 h 30 min). After incubation with 5% skimmed milk in TBS-T buffer for 30 min at room temperature, nitrocellulose membranes were blotted with monoclonal anti-CEL and anti-CML (TransGenic), rabbit polyclonal anti-HNE (Calbiochem), and rabbit anti-MDA-Lys (Biomedical) or rabbit polyclonal anti-14-3-3 (Abcam) antibodies used at a dilution of 1:1000. Subsequently, the membranes were processed as previously indicated for mono-dimensional gels.

### In-gel digestion

Proteins were in-gel digested with trypsin (sequencing grade modified, Promega, Barcelona, Spain) in the automatic Investigator ProGest robot of Genomic Solutions. Briefly, excised gels spots were washed sequentially with ammonium bicarbonate buffer and acetonitrile. Proteins were reduced and alkylated with 10 mM DTT solution for 30 min and 100 mM solution of iodine acetamide for 15 min, respectively. After sequential washing with buffer and acetonitrile, proteins were digested overnight at 37 °C with trypsin 0.27 nM. Tryptic peptides were extracted from the gel matrix with 10% formic acid and acetonitrile. The extracts were pooled and dried in a vacuum centrifuge.

### Acquisition of MS and MS/MS spectra

Proteins manually excised from the 2D gels were digested and analyzed by CapLCnano-ESI-MS-MS mass spectrometry. The tryptic-digested peptide samples were analyzed using on-line liquid chromatography (CapLC, Micromass-Waters, Manchester, UK) coupled with tandem mass spectrometry (Q-TOF Global, Micromass-Waters). Samples were re-suspended in 12 $\mu$l 10% formic acid solution, and 4 $\mu$l was injected for chromatographic separation into a reverse-phase capillary C18 column (75 $\mu$m internal diameter and 15 cm in length (PepMap column, LC Packings, Amsterdam, Netherlands). The eluted peptides were ionized via coated nano-ES needles (PicoTipTM, Woburn, MA, USA; New Objective). A capillary voltage of 1800–2200 V was applied together with a cone voltage of 80 V. The collision in the CID (collision-induced dissociation) was 25–35 eV, and argon was employed as the collision gas. Data were generated in PKL file format and submitted for database searching in MASCOT server (Matrix Science, Boston, MA, USA) using the NCBI database with the following parameters: trypsin enzyme, one missed cleavage, carbamidomethyl (C) as fixed modification and oxidized (M) as variable modification, and mass tolerance of 150–250 ppm.

Probability-based MOWSE score was used to determine the level of confidence in the identification of specific isoforms from the mass spectra. This probability equals 10 (−Mowse score/10). Mowse scores higher than 50 were considered to be of high confidence of identification.

### 14-3-3 Immunohistochemistry and Western blotting

Cryostat sections 7 $\mu$m thick were processed free-floating with the LSAB method. The rabbit polyclonal antibody to 14-3-3 (ab6081, Abcam) was used at a dilution of 1:200. This antibody is directed to a synthetic peptide with the sequence DKSELVQKAEQAERYD, mapping to the amino terminal domain of human 14-3-3, and reacts with alpha, beta, zeta and theta isoforms. The rabbit polyclonal to 14-3-3 (phospho S) (ab14127, Abcam) was used at a dilution of 1:200. This antibody binds peptides and proteins containing a motif composed of phospho-Ser with proline at the +2

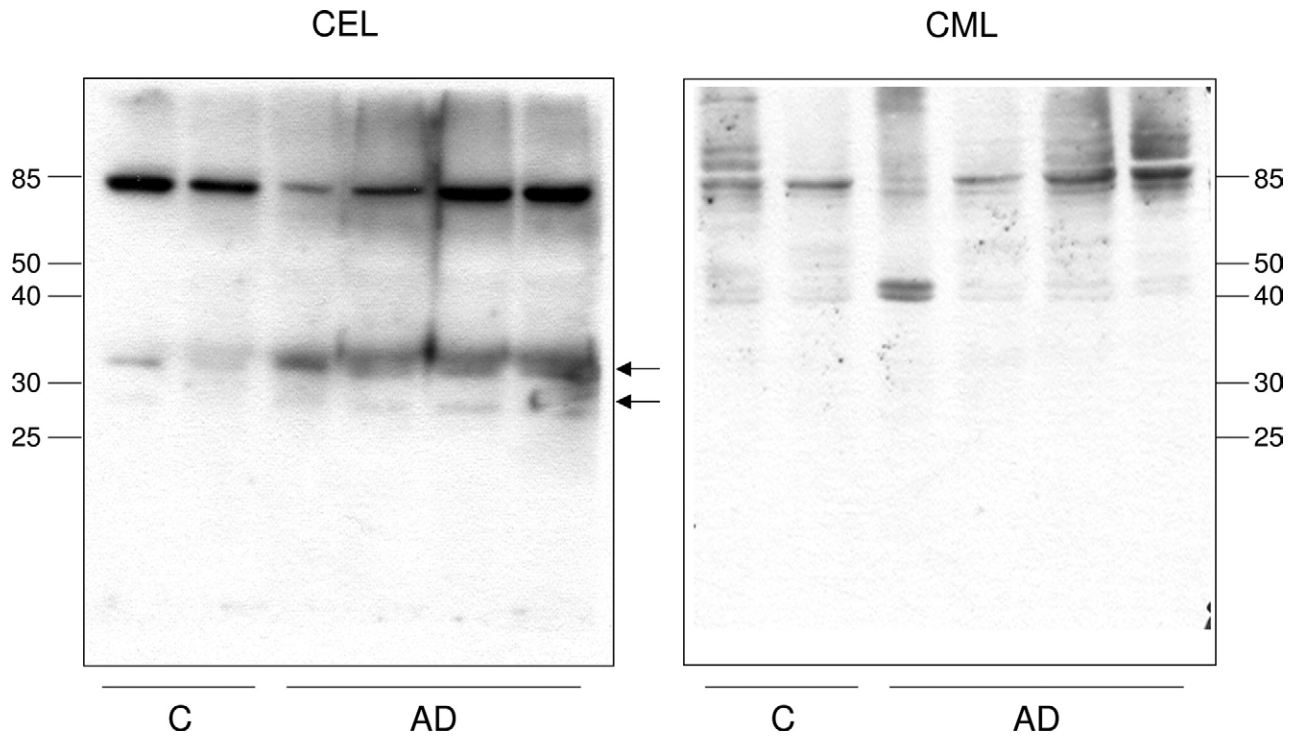


**Fig. 1.** Gel electrophoresis and Western blotting to CEL and CML in sarkosyl-insoluble fractions of the frontal cortex in two controls (C) and four cases of AD. Two strong bands of about 30 kDa are detected by CEL antibody in AD cases compared with age-matched controls. Note the presence of a band of about 85 kDa in control and AD cases. CML antibody detects several bands in C and AD. A doublet of about 40–50 kDa is present in one AD case.

position and argininine or lysine at the −3 position. Antibody binding is phospho-specific and largely independent of other surrounding amino acids. According to the supplier, the antibody recognizes a wide range of peptides containing the 14-3-3 binding motif and a large number of presumptive 14-3-3 binding proteins. Finally, the rabbit polyclonal anti-14-3-3 (18649, IBL, Gunma, Japan) was used at a dilution of 1:200. This antibody is raised against a synthetic peptide for a part of human 14-3-3 gamma, and may react to several human 14-3-3 isoforms. The sections were incubated with LSAB for 1 h at room temperature. The peroxidase reaction was visualized with $NH_4NiSO_4$ (0.05 M) in phosphate buffer (0.1 M), 0.05% diaminobenzidine, $NH_4Cl$ and 0.01% hydrogen peroxide (dark blue precipitate). Some sections were incubated without the primary antibody. No immunoreactivity was found in these samples.

To test the specificity of these antibodies, membranes of sarkosyl-insoluble fractions in control and AD cases were blotted with anti-14-3-3 antibodies, and processed as indicated for conventional mono-dimensional gels. The rabbit polyclonal antibody to phospho-tauThr181 (Calbiochem; dilution 1:250) was used as a control of phospho-tau band pattern of sarkosyl-insoluble fractions in AD.

### Double-labeling immunofluorescence and confocal microscopy

Cryostat sections, 7 m thick, of the frontal cortex were stained with a saturated solution of Sudan black B (Merck) for 30 min to block the autofluorescence of lipofuscin granules present in nerve cell bodies, rinsed in 70% ethanol and washed in distilled water. The sections were incubated at 4 °C overnight with the mouse anti-AT8 antibody (Innogenetics, Barcelona, Spain) at a dilution of 1:50 and rabbit polyclonal anti-14-3-3 (18649, IBL) used at a dilution of 1:200. After washing in PBS, the sections were incubated in the dark with the cocktail of secondary antibodies, and then diluted in the same vehicle solution as the primary antibodies for 45 min at room temperature. Secondary antibodies were Alexa488 anti-rabbit (green) and Alexa546 anti-mouse (red) (both from Molecular Probes, Invitrogen, Barcelona, Spain), and these were used at a dilution of 1:400. After washing in PBS, the sections were mounted in Immuno-Fluore Mounting medium (ICN Biomedicals, Illrich, France), sealed and dried overnight. Sections were examined with a Leica TCS-SL confocal microscope. Nuclei were visualized in blue.

## RESULTS

### Mono-dimensional gel electrophoresis and Western blotting to glycoxidized products

Two strong bands between 28 and 33 kDa were detected in sarkosyl-insoluble fractions from frontal cortex of AD cases, when compared with control samples, using anti-CEL antibodies. Although the intensity of the lower band was variable from one AD case to another, the intensity of the upper band was similar in every disease case. These antibodies also detected a band at 85 kDa, but no differences in the intensity of this band were observed between control and AD brains (Fig. 1).

Anti-CML detected the same band at 85 kDa and other bands between 40 and 120 kDa. None of them showed differences between controls and AD, except for a doublet between 40 and 50 kDa which was found in one AD case.

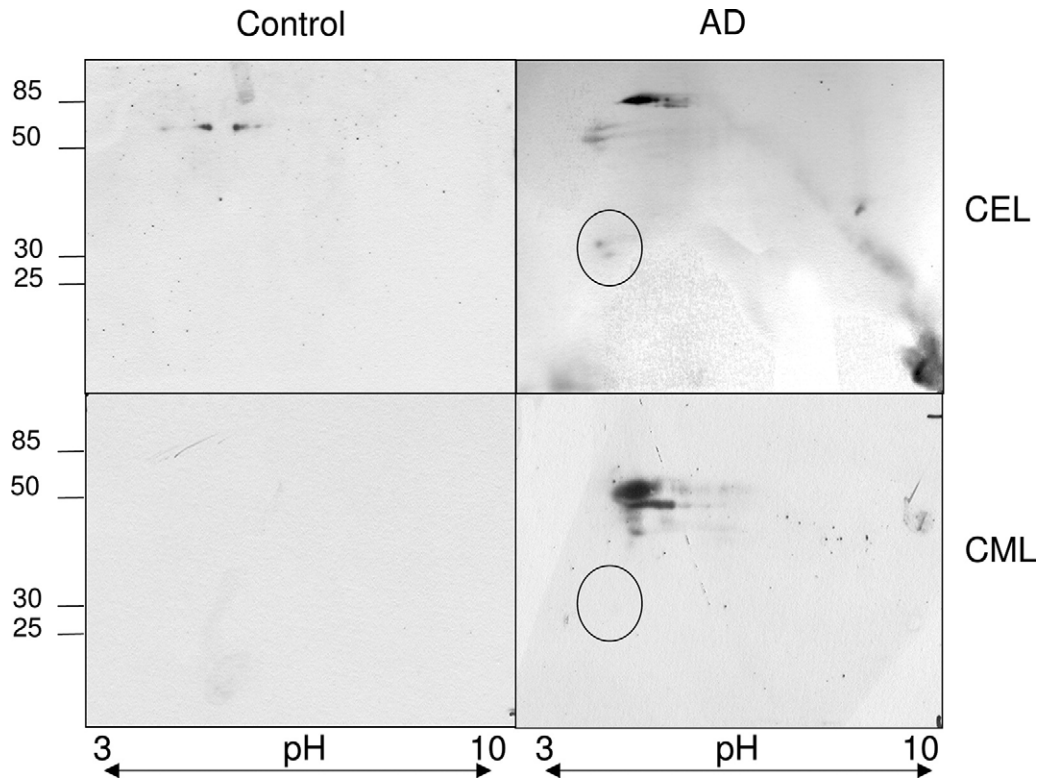


**Fig. 2.** 2D Gel electrophoresis and Western blotting to CEL and CML in sarkosyl-insoluble fractions of frontal cortex in control (C) and AD. CEL antibody reveals two spots of about 30 kDa which are not seen in control cases (circle). The CML antibody does not recognize spots of about 30 kDa (see inside circle) but a doublet of about 50 kDa (this case is the same as the first AD in Fig. 1).

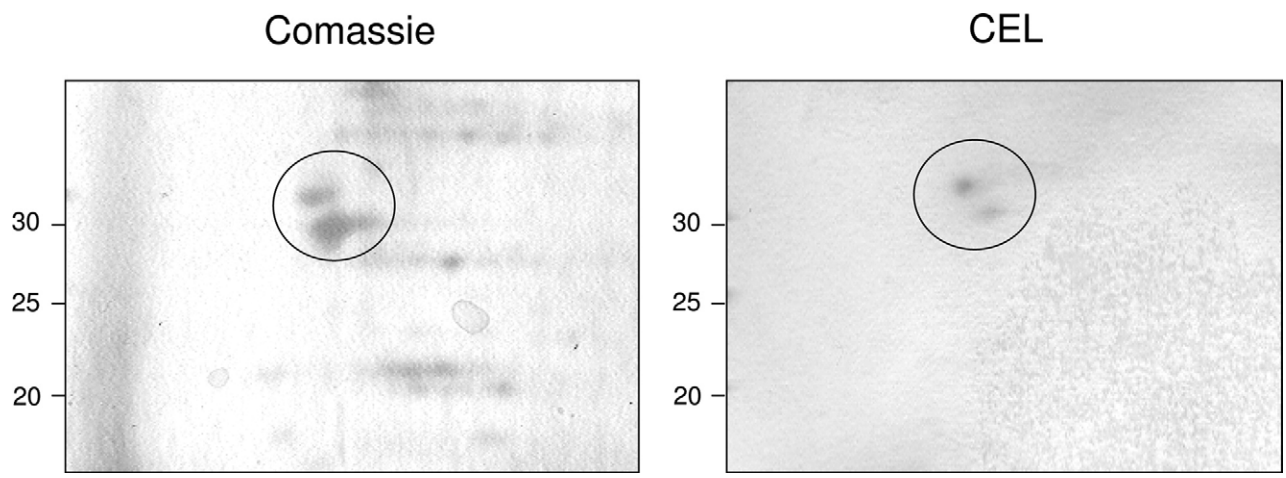


**Fig. 3.** CEL-immunoreactive oxidized spots of about 30 kDa (right panel) identified in Coomassie-stained 2D gels (left panel) processed in parallel (circles). The spots were excised from Coomassie-stained gels to perform the mass spectrometry analysis.

Yet the two bands around 30 kDa, detected with anti-CEL, were not detected with the CML antibody (Fig. 1).

### 2D Gel electrophoresis, Western blotting for oxidized proteins, and protein characterization in sarkosyl-insoluble fractions

*AD.* 2D Gels of sarkosyl-insoluble fractions of AD samples immunoblotted with anti-CEL antibody revealed oxidized spots at 85 kDa and 50 kDa, and two well-defined spots close to 30 kDa. In two controls, spots at 85 kDa were also observed. CEL-immunoreactive spots close to 30 kDa were not found in controls. 2D Gels of sarkosyl-insoluble fractions of AD samples immunoblotted with anti-CML disclosed several spots of high molecular weights but not the two spots around 30 kDa (Fig. 2). Therefore, 2D gels confirmed the presence of unique CEL-immunoreactive spots in AD brains within a molecular weight range of about 30–40 kDa. These spots were identified in Coomassie-stained 2D gels processed in parallel (Fig. 3) and excised. Mass spectrometry showed two 14-3-3 isoforms: zeta and gamma (Table 2).

The same membranes blotted for anti-14-3-3 (ab6081, Abcam) confirmed the localization of this protein in the corresponding spots (data not shown).

In order to assess lipoxidative damage of 14-3-3, additional 2D gels of sarkosyl-insoluble fractions were immunoblotted with anti-MDA-Lys and anti-HNE antibodies. Anti-MDA-Lys, but not anti-HNE, antibodies detected one spot at about 30 kDa. This spot was present only in AD and not in control samples (Fig. 4). The spot was identified in Coomassie-stained gels run in parallel, in-gel digested, and identified following MS and data searching as 14-3-3 gamma isoform (data not shown).

*CAA.* 2D Gels of sarkosyl-insoluble fractions in CAA immunoblotted with anti-MDA-Lys or with anti-CEL antibodies recognized similar spots of about 30 kDa (Fig. 5). Western blots of the same membranes with anti-14-3-3 antibody revealed the spots corresponding to 14-3-3 at the same place as the spots detected by anti-MDA-Lys and anti-CEL antibodies (Fig. 5).

### Total homogenates of AD and CAA

2D Gels of total homogenates from frontal cortex of AD were immunoblotted with anti-MDA-Lys antibodies. Anti-MDA-Lys antibody detected a pattern very similar to that of the sarkosyl-insoluble fraction in AD. Western blotting with the anti-14-3-3 antibody (ab6081, Abcam) revealed the spot corresponding to 14-3-3 at the same place as the spot detected by anti-MDA-Lys (Fig. 6). Interestingly the spot of 14-3-3 in AD cases was larger than the spot in controls thus suggesting higher amount of 14-3-3 protein in AD.

2D Gels of total homogenates from two cases of CAA showed similar spots of MDA-Lys and 14-3-3 proteins, using the corresponding specific antibodies (Fig. 6). These spots were identified in parallel Coomassie-stained 2D gels and excised. Mass spectrometry of MDA-Lys-modified proteins in CAA revealed two 14-3-3 isoforms: zeta and gamma (Table 3).

**Table 2.** Oxidized proteins excised from AD gels

| Protein | Molecular weight | pI | MOWSE score[a] | Peptides matched | ID number |
|---|---|---|---|---|---|
| 14-3-3 Protein gamma | 28.3 | 4.8 | 41 (20%) | 3 | 1433G_HUMAN |
| YWHAZ protein (14-3-3 zeta) | 30.1 | 4.72 | 459 (33%) | 11 | gi 49119653 |

CEL-modified proteins in sarkosyl insoluble fractions in AD.
[a] Sequence coverage shown in parentheses.

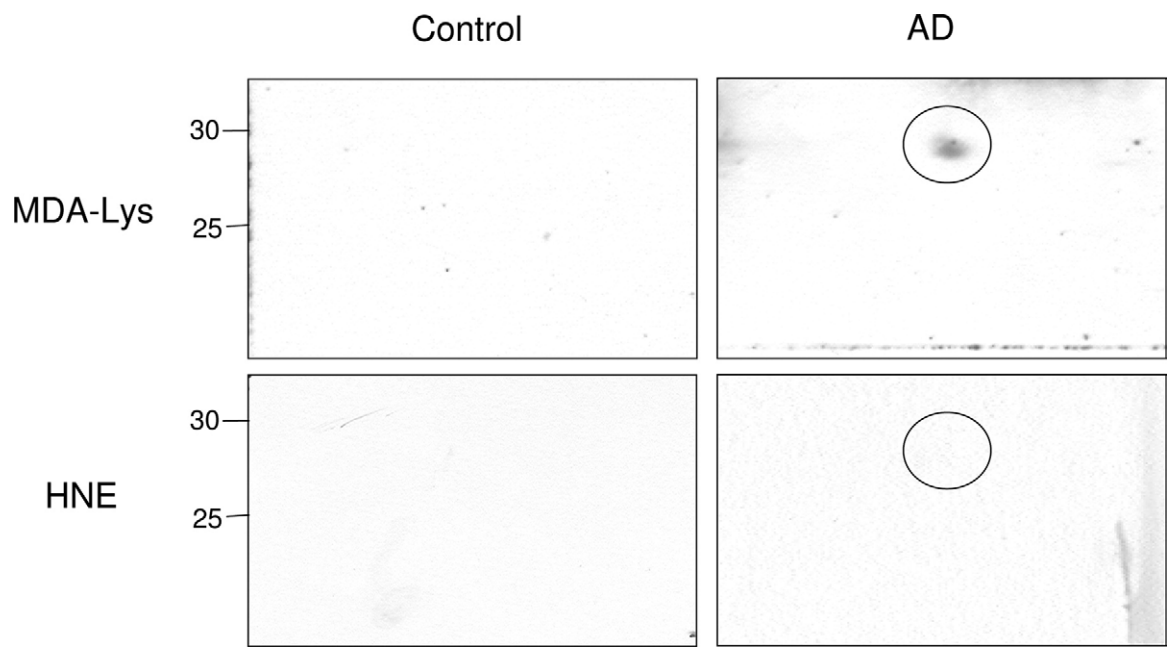


**Fig. 4.** 2D Gels and Western blotting of sarkosyl-insoluble fractions in one control and one AD case with anti-MDA-Lys and anti-HNE antibodies. MDA-Lys antibody reveals a spot of about 30 kDa. A similar spot is not recognized with anti-HNE antibodies (circles).

### 14-3-3 Immunohistochemistry, and 14-3-3 double-labeling immunofluorescence and confocal microscopy

Cryoprotected samples of the frontal cortex in control and diseased cases processed free-floating showed 14-3-3 immunoreactivity in the soma of neurons. By using antibodies ab ab6081 (Abcam) and and 18649 (IBL), immunoreactivity was present equally in neurons of control and AD cases. NFTs were not stained with these antibodies (Fig. 7). Yet immunohistochemistry with the antibody 14-3-3 (phospho S) (ab14127) revealed marked differences, as this antibody did not recognize constitutive 14-3-3 in the cytoplasm

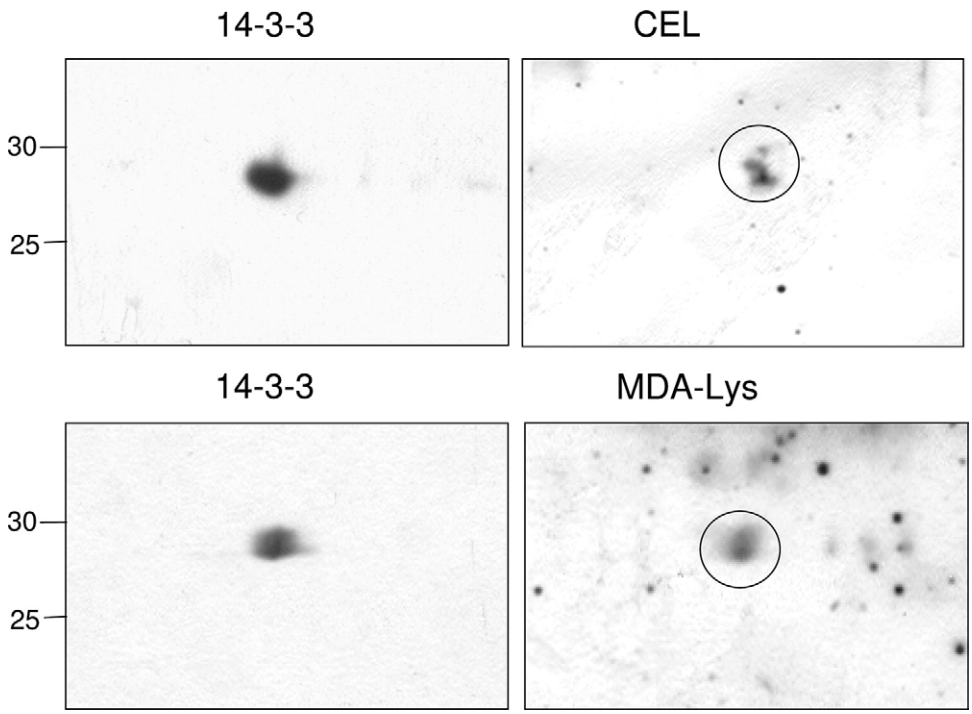


**Fig. 5.** 2D Gel electrophoresis and Western-blotting of sarkosyl-insoluble fractions in CAA. Membranes were processed in parallel with anti-CEL or anti-MDA-Lys, and with anti-14-3-3. CEL and MDA-Lys immunoreactivity matches with 14-3-3-immunoreactive spots of about 30 kDa.

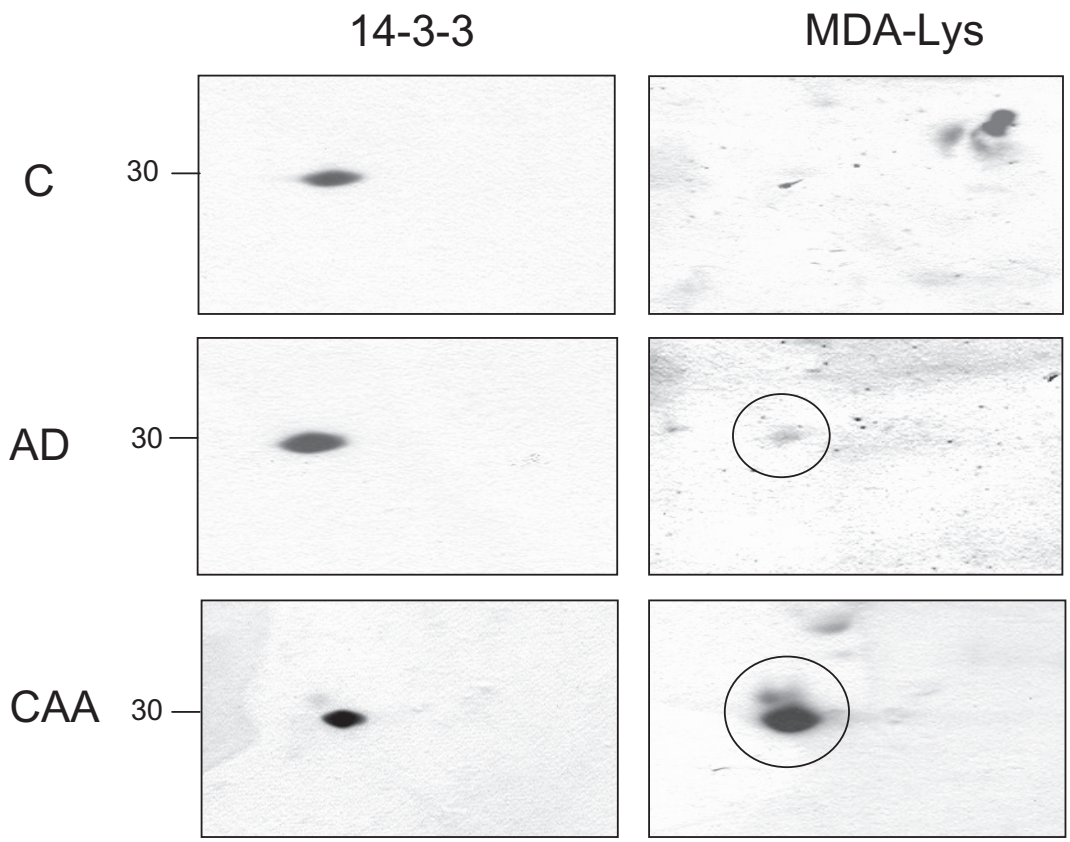


**Fig. 6.** 2D Gel electrophoresis and Western blotting of total homogenates from control, AD, and CAA with anti-14-3-3 and anti-MDA-Lys antibodies. MDA-Lys discloses spots of about 30 kDa corresponding to the spots which are also detected with the 14-3-3 antibody in AD and CAA (circles), but only barely in control cases.

of neurons but strongly immunostained NFTs, neuropil threads and dystrophic neurites of senile plaques. Consecutive sections immunostained with the AT8 antibody indicated that the majority of, if not all, NFTs were stained with the anti-14-3-3 (phospho S) antibody (data not shown).

Sections double-labeled with AT8 (anti-phospho tau antibody) and 14-3-3 disclosed that 14-3-3 protein was present in the cytoplasm of all neurons, whereas AT8 immunoreactivity was restricted to the subset of neurons with NFTs (Fig. 8). As a result, some neurons contained both 14-3-3 and phospho-tau, but the amount and the distribution of 14-3-3 in neurons with NFTs was the same as those in neurons without NFTs. Incubation without the primary antibodies revealed no immunoreactivity (Fig. 8).

### Western blotting of 14-3-3 antibodies

To further identify the characteristics of the material recognized with these antibodies, Western blots of sarkosyl-insoluble fractions were carried out in every case. Antibodies ab6081 (Abcam) and 18649 (IBL) recognized bands of about 30 kDa corresponding to 14-3-3 proteins (Fig. 9). In contrast, anti-14-3-3 (phospho S) antibody did not recognize bands of molecular weight consistent with 14-3-3 in control and diseased brains but, instead, several bands of 68, 64 and 60 kDa in AD cases only. These bands are the same as those obtained with anti-phospho-tau antibodies, and characterize the pattern of phospho-tau in AD (Fig. 9). Interestingly, similar bands were weakly immunostained with the 18649 (IBL) antibody. No bands were immunostained with the secondary antibody alone (data not shown).

## DISCUSSION

14-3-3 Is a family of acidic proteins composed of seven isoforms widely expressed in all tissues. This family of proteins is involved in kinase activation and chaperone

**Table 3.** Oxidized spots excised from CAA gels

| Protein | Molecular weight | pI | MOWSE score[a] | Peptides matched | ID number |
|---|---|---|---|---|---|
| 14-3-3 Protein gamma | 28.3 | 4.8 | 64 (39%) | 3 | 1433G_HUMAN |
| YWHAZ protein (14-3-3 zeta) | 30.1 | 4.72 | 529 (39%) | 12 | gi 49119653 |

MAD-Lys-modified proteins in total frontal cortex homogenates in CAA.
[a] Sequence coverage shown in parentheses.

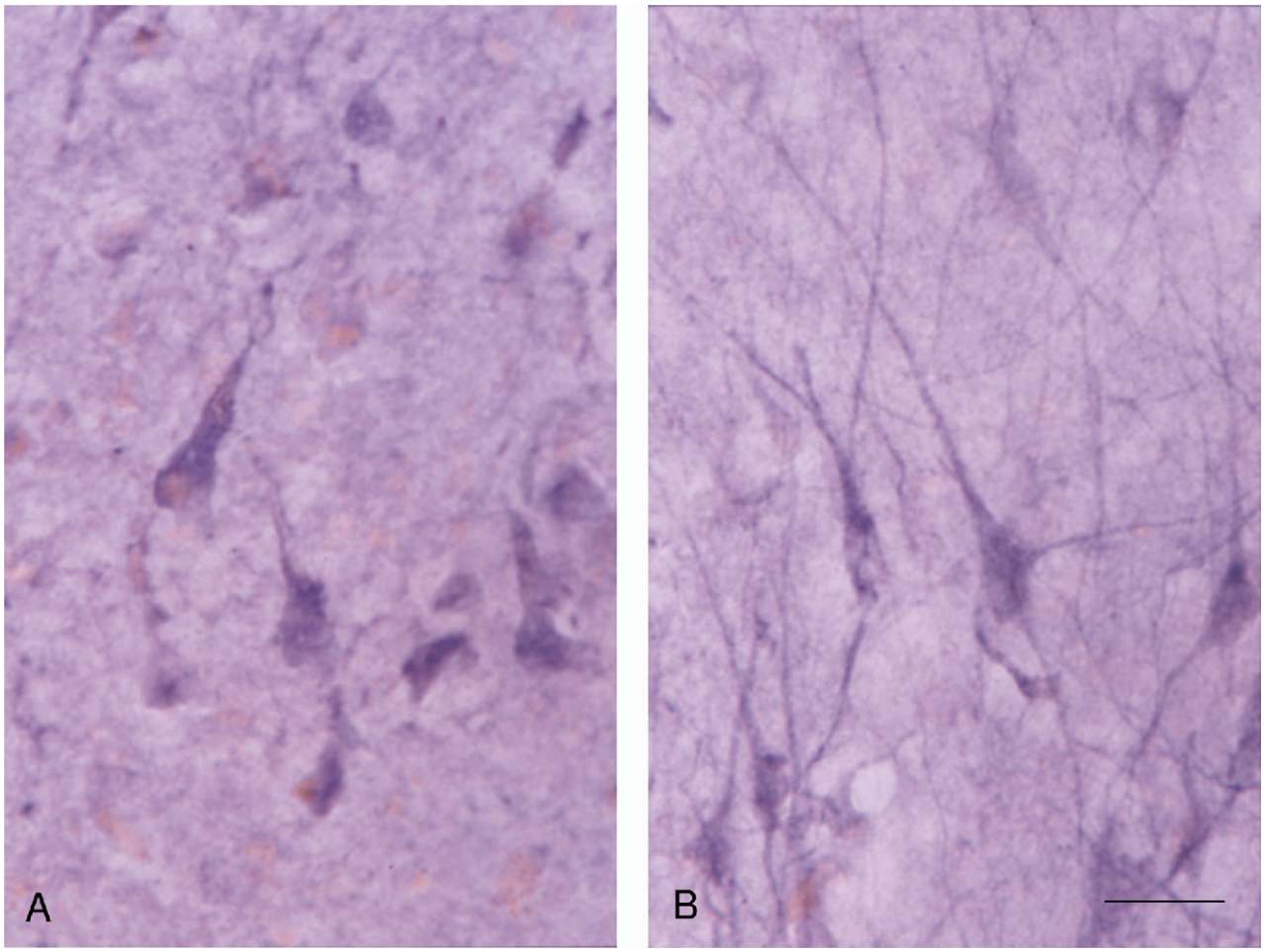


**Fig. 7.** Immunohistochemistry to 14-3-3 by using ab6081 (Abcam) (A) and 18649 (IBL) (B) in Alzheimer disease (B–D) cases. Antibodies recognize cytosolic 14-3-3 but not NFTs. Cryostat sections processed free-floating with no hematoxylin counterstaining. Scale bar=25 $\mu$m.

activity, among other functions related with signaling pathways, in addition to key roles in cell cycle regulation and development (Ferl, 1996; Muslin and Lau, 2005; Mhawech, 2005; Darling et al., 2005; Hermeking and Benzinger, 2006; Aitken, 2006).

14-3-3 Proteins are highly expressed in the adult brain where they play important functions including protein interactions, protein folding and effects on protein kinases (Takahashi, 2003; Berg et al., 2003). In neurons, 14-3-3 is mainly found in cytoplasm and synapses (Fu and Subramanian, 2000). The fact that 14-3-3 proteins are phosphoserine/phosphothreonine-binding proteins implies important roles in cell signaling, and more than 100 binding partners have been identified (Dougherty and Morrison, 2004).

14-3-3 Zeta, gamma and epsilon are over-expressed in AD brain regions affected by tau pathology, and their levels correlate with disease progression (Fountoulakis et al., 1999; Soulié et al., 2004). 14-3-3 Gamma and epsilon isoforms are also over-expressed in the brains of cases with Down syndrome (Fountoulakis et al., 1999). Similar findings have been herein obtained in 2D gels.

Oxidative damage is increased by A$\beta$, as reported in several *in vitro* and *in vivo* studies (Butterfield and Lauderback, 2002; Varadarajan et al., 2000; Drake et al., 2003; Butterfield, 2003; Boyd-Kimball et al., 2005; Sultana et al., 2006).

In the present study, we have shown that 14-3-3, zeta and gamma isoforms are targets of oxidative damage in AD. Similar findings have been observed in every case. This is important, as monodimensional gels revealed individual differences in the intensity of the lower band of about 28 kDa, probably corresponding to oxidized levels of gamma isoform. The higher levels of MDA-Lys spots could reflect a larger content of this protein target in these pathological cases in agreement with previous observations showing 14-3-3 over-expression in AD (Fountoulakis et al., 1999). Thus, 14-3-3 oxidative damage in AD could be related to A$\beta$-induced oxidative stress. To test this hypothesis, we studied the possible oxidation of 14-3-3 in CAA. We found that 14-3-3, zeta and gamma isoforms were also MDA-Lys- and CEL-modified in CAA, thus indicating a link between A$\beta$ pathology and 14-3-3 oxidation in AD and CAA.

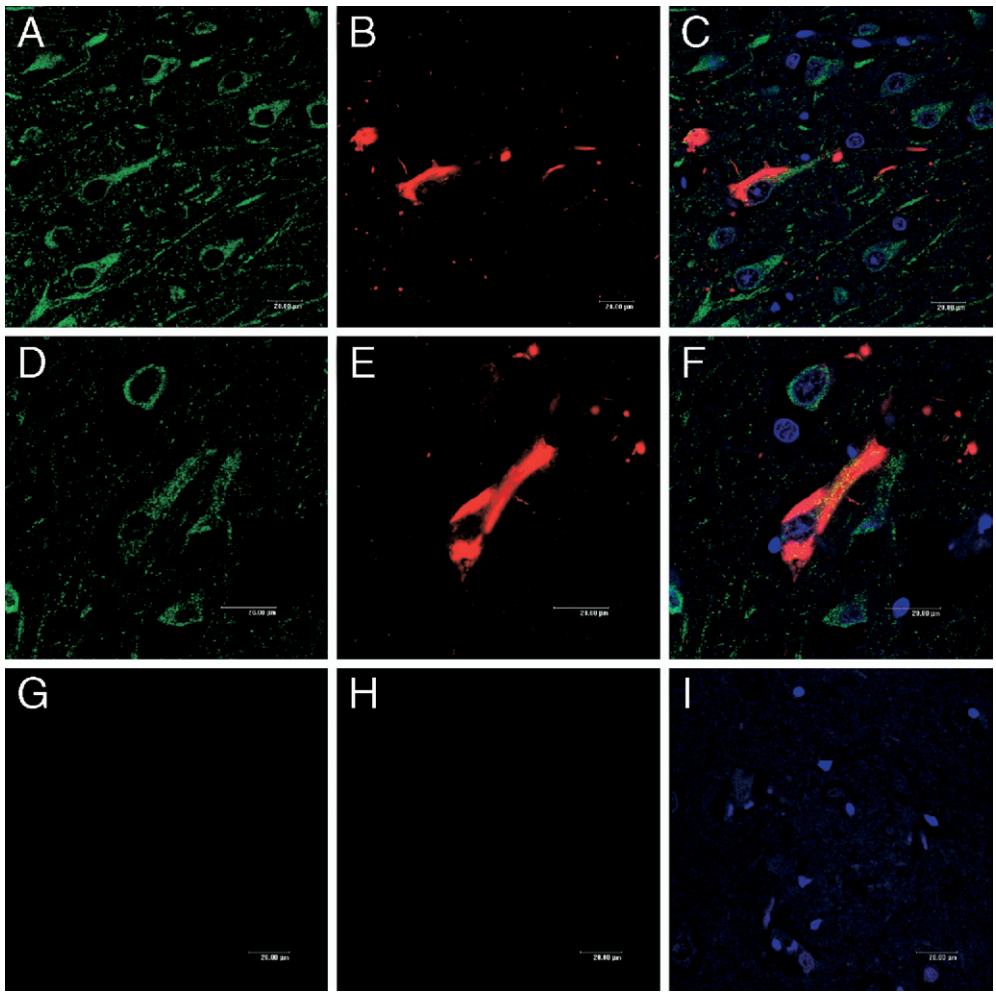


**Fig. 8.** Double-labeling immunofluorescence and confocal microscopy to 14-3-3 (green) and AT8 (red) in the frontal cortex in AD. Fine granular 14-3-3 immunoreactivity is found in the cytoplasm of all neurons and dendrites, whereas phospho-tau immunoreactivity (AT8) occurs only in neurons with NFTs. Note that 14-3-3 immunoreactivity is the same in neurons with NFTs as in neurons without NFTs. (A, D) 14-3-3; B and E: AT8; C and F: merge. (G–I) Incubation without the corresponding primary antibodies. Nuclei are visualized in blue.

Previous immunohistochemical studies have shown that 14-3-3 co-localizes with phospho-tau in NFTs in AD (Layfield et al., 1996; Umahara et al., 2004). In the first work, antibodies to sheep brain 14-3-3 were used for immunohistochemistry (Layfield et al., 1996); whereas in the second study, antibodies to human 14-3-3 common, and antibodies to 14-3-3 beta, gamma, epsilon and zeta were tested by Western blot and proved for immunohistochemistry, and double and triple fluorescence in AD brains (Umahara et al., 2004). No similar results were obtained in the present immunohistochemical and double-labeling immunofluorescence studies. Two different antibodies to 14-3-3 recognized cytosolic 14-3-3 but they did not stain NFTs. An additional 14-3-3-phospho antibody immunostained NFTs and other tau-bearing structures in AD cases. Yet this antibody identified only three bands of 68, 64 and 60 kDa which are typical of phospho-tau aggregates in AD. The later antibody was considered unsuitable for 14-3-3 immunohistochemistry because of the possible cross-reactivity of the antibody with phospho-tau proteins. Lack of 14-3-3 immunoreactivity in relation with amyloid plaques in AD, but not in PrP plaques in CJD, was also emphasized in other studies (Richard et al., 2003).

### Inconsistencies of immunohistochemical results do not undermine possible implications of oxidized 14-3-3 in AD and CAA

Some data point to the implications of 14-3-3 in tau phosphorylation.1: tau interacts with zeta and beta but not with gamma and epsilon 14-3-3 isoforms (Hashiguchi et al., 2000); 2: dimers of 14-3-3 zeta can bind tau and GSK-3$\beta$ simultaneously, enhancing tau phosphorylation (Agarwal-Mawal et al., 2003); 3: 14-3-3 is able to bind the inactive form of GSK-3$\beta$ (GSK-3$\beta$Ser9) and then preserve its activity (Yuan et al., 2004); 4: 14-3-3 zeta can stimulate the phosphorylation of tau at Ser(262)/Ser(356) through the cAMP-dependent protein kinase pathway (Hashiguchi et al., 2000); 5: a direct role for 14-3-3 zeta in tau fibril

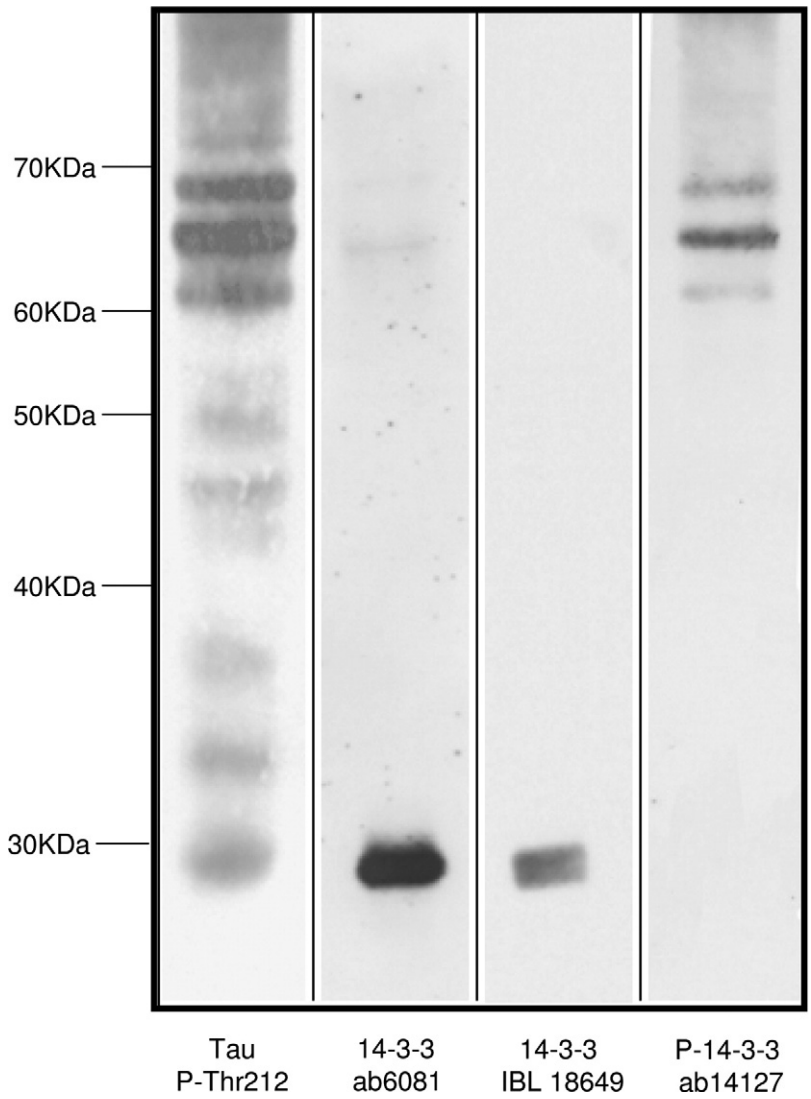


**Fig. 9.** Gel electrophoresis and Western blotting to 14-3-3 by using 18649 (IBL), ab6081 (Abcam) and 14-3-3 (phospho S) (ab14127, Abcam) antibodies in sarkosyl-insoluble fractions in AD. Anti-14-3-3 antibodies ab6081 and 18649 recognized a band of 30kDa. In contrast, 14-3-3 (phospho S) (ab14127), does not label bands at the predicted molecular weight of 14-3-3 but three bands of 68, 64 and 60 kDa corresponding to phospho-tau. Note that weak bands of high molecular weight are also seen with the IBL antibody.

formation has also been suggested (Hernández et al., 2004). Therefore, oxidized 14-3-3 may impact on tau phosphorylation in AD. Whether these possible effects are not apparently visualized in CAA is not known. Since 14-3-3 zeta is oxidized in AD and CAA, tau protein binding and tau phosphorylation may be modified in these disorders.

Additional implications of oxidized 14-3-3 in AD and CAA brains can be related to conformational changes of other proteins, abnormal scaffolding and physical occlusion of sequence-specific or structural protein features as reviewed elsewhere (Bridges and Moorhead, 2005; van Heusden, 2005; Coblitz et al., 2006). Additional studies are, however, needed to elucidate the exact impact of *in vivo* oxidized 14-3-3 in AD and CAA.

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## REFERENCES

Agarwal-Mawal A, Qureshi HY, Cafferty PW, Yuan Z, Han D, Lin R, Paudel HK (2003) 14-3-3 Connects glycogen synthase kinase-3 beta to tau within a brain microtubule-associated tau phosphorylation complex. J Biol Chem 278:12722–12728.

Aitken A (2006) 14-3-3 Proteins: a historic overview. Semin Cancer Biol 16:162–172.

Atzori C, Ghetti B, Piva R, Srinivasan AN, Zolo P, Delisle MB, Mirra SS, Migheli A (2001) Activation of the JNK/p38 pathway occurs in diseases characterized by *tau* protein pathology and is related to *tau* phosphorylation but not to apoptosis. J Neuropathol Exp Neurol 60:1190–1197.

Avila J, Lim F, Moreno F, Belmonte C, Cuello AC (2002) *Tau* function and dysfunction in neurons: its role in neurodegenerative disorders. Mol Neurobiol 25:213–231.

Berg D, Holzmann C, Riess O (2003) 14-3-3 Proteins in the nervous system. Nat Rev Neurosci 4:752–762.

Boyd-Kimball D, Sultana R, Fai Poon H, Lynn BC, Casamenti F, Pepeu G, Klein JB, Butterfield DA (2005) Proteomic identification of proteins specifically oxidized by intracerebral injection of amyloid $\beta$-peptide (1–42) into rat brain: implications for Alzheimer's disease. Neuroscience 132:313–324.

Braak H, Braak E (1999) Temporal sequence of Alzheimer's disease-related pathology. In: Cerebral cortex, Vol. 14: Neurodegenerative and age-related changes in structure and function of cerebral cortex (Peters A, Morrison JH, eds), pp 475–512. New York: Kluwer Academic/Plenum Press.

Bridges D, Moorhead GB (2005) 14-3-3 Proteins: a number of functions for a numbered protein. Sci STKE 2005: re10.

Buee L, Bussiere T, Buee-Scherrer V, Delacourte A, Hof PR (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res Rev 33:95–130.

Butterfield DA (2003) Amyloid beta-peptide (1–42)-associated free radical-induced oxidative stress and neurodegeneration in Alzheimer's disease brain: mechanisms and consequences. Curr Med Chem 10:2651–2659.

Butterfield DA, Drake J, Pocernich C, Castegna A (2001) Evidence of oxidative damage in Alzheimer's disease brain: central role for $\beta$-amyloid peptide. Trends Mol Med 7:548–554.

Butterfield DA, Lauderback CM (2002) Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. Free Rad Biol Med 32:1050–1060.

Butterfield DA, Perluigi M, Sultana R (2006) Oxidative stress in Alzheimer's disease brain: New insights from redox proteomics. Eur J Pharmacol 545:39–50.

Cecchi C, Fiorillo C, Sorbi S, Latorraca S, Nacmias B, Bagnoli S, Nassi P, Liguri G (2002) Oxidative stress and reduced antioxidant defenses in peripheral cells from familial Alzheimer's patients. Free Radic Biol Med 33:1372–1379.

Chauhan V, Chauhan A (2006) Oxidative stress in Alzheimer's disease. Pathophysiology 3:195–208.

Coblitz B, Wu M, Shikano S, Li M (2006) C-terminal binding: an expanded repertoire and function of 14-3-3 proteins. FEBS Lett 580:1531–1535.

Darling DL, Yingling J, Wynshaw-Boris A (2005) Role of 14-3-3 proteins in eukaryotic signaling and development. Curr Top Dev Biol 68:281–315.

Davies KJ (2001) Degradation of oxidized proteins by the 20S proteasome. Biochimie 83:301–310.

Davies KJ (1987) Protein damage and degradation by oxygen radicals. I. general aspects. J Biol Chem 262:9895–9901.

Dougherty MK, Morrison DK (2004) Unlocking the code 14-3-3. J Cell Sci 117:1875–1884.

Drake J, Link CD, Butterfied DA (2003) Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid beta-peptide1–42 in a transgenic Caenorhabditis elegans model. Neurobiol Aging 24:415–420.

Duyckaerts C, Dickson DW (2003) Neuropathology of Alzheimer's disease. In: Neurodegeneration: the molecular pathology of dementia and movement disorders (Dickson D, ed), pp 47–68. Basel: ISN Neuropath Press.

Ferl RJ (1996) 14-3-3 proteins and signal transduction. Annu Rev Plant Physiol Plant Mol Biol 47:49–73.

Ferrer I, Blanco R, Carmona M, Puig B (2001) Phosphorylated mitogen-activated protein kinase (MAPK/ERK-P), protein kinase of 38 kDa (p38-P), stress-activated protein kinase (SAPK/JNK-P), and calcium/calmodulin-dependent kinase II (CaM kinase II) are differentially expressed in tau deposits in neurons and glial cells in tauopathies. J Neural Transm 108:1397–1415.

Ferrer I, Gomez-Isla T, Puig B, Freixes M, Ribe E, Dalfó E, Avila J (2005) Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. Curr Alzheimer Res 2:3–18.

Fountoulakis M, Cairns N, Lubec G (1999) Increased levels of 14-3-3 gamma and epsilon proteins in brain of patients with Alzheimer's disease and Down syndrome. J Neural Transm Suppl 57:323–335.

Fu RR, Subramanian SC (2000) Masters, 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol 40: 617–647.

Garrison WM, Jayko ME, Bennet W (1962) Radiation-induced oxidation of protein in aqueous solution. Radiat Res 16:483–502.

Hashiguchi M, Sobue K, Paudel HK (2000) 14-3-3 Zeta is an effector of tau protein phosphorylation. J Biol Chem 275:25247–25254.

Hermeking H, Benzinger A (2006) 14-3-3 Proteins in cell cycle regulation. Semin Cancer Biol 16:183–192.

Hernández F, Cuadros R, Avila J (2004) Zeta 14-3-3 protein favors the formation of human tau fibrillar polymers. Neurosci Lett 357: 143–146.

Layfield R, Fergusson J, Aitken A, Lowe J, Landon M, Mayer RJ (1996) Neurofibrillary tangles of Alzheimer's disease brains contain 14-3-3 proteins. Neurosci Lett 209:57–60.

Lee VM, Goedert M, Trojanowski JQ (2001) Neurodegenerative tauopathies. Annu Rev Neurosci 24:1121–1159.

Masters CL, Beyreuther K (2003) Molecular pathogenesis of Alzheimer's disease. In: Neurodegeneration: the molecular pathology of dementia and movement disorders (Dickson D, ed), pp 69–73. Basel: ISN Neuropath Press.

Mhawech P (2005) 14-3-3 Proteins. An update. Cell Res 15:228–236.

Muñoz FJ, Sole M, Coma M (2005) The protective role of vitamin E in vascular amyloid beta-mediated damage. Subcell Biochem 38: 147–165.

Muslin AJ, Lau JM (2005) Differential functions of 14-3-3 isoforms in vertebrate development. Curr Top Dev Biol 65:211–228.

Nishikawa K, Li H, Kawamura R, Osaka H, Wang YL, Hara Y, Hirokawa T, Manago Y, Amano T, Noda M, Aoki S, Wada K (2003) Alterations of structure and hydrolase activity of parkinsonism-associated human ubiquitin carboxyl-terminal hydrolase L1 variants. Biochem Biophys Res Commun 304:176–183.

Nunomura A, Castellani RJ, Zhu X, Moreira PI, Perry G, Smith MA (2006) Involvement of oxidative stress in Alzheimer's disease. J Neuropathol Exp Neurol 65:631–641.

Oliver CN, Ahn BW, Moerman EJ, Goldstein S, Stadtman ER (1987) Age-related changes in oxidized proteins. J Biol Chem 262: 5488–5491.

Pamplona R, Dalfó E, Ayala V, Bellmunt J, Prat J, Ferrer I, Portero M (2005) Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets. J Biol Chem 280: 21522–21530.

Pei JJ, Braak E, Braak H, Grundque-Iqbal K, Winblad W, Cowburn RF (2001) Localization of active forms of c-Jun kinase (JNK) and p38 kinase in Alzheimer's disease brains at different stages of neurofibrillary degeneration. J Alzheimer's Dis 3:41–48.

Puig B, Gómez-Isla T, Ribé E, Cuadrado M, Torrejón-Escribano B, Dalfó E, Ferrer I (2004) Expression of stress-activated kinase c-Jun N-terminal kinase (SAPK/JNK-P) and p38 (p38-P), and tau hyperphosphorylation in neurites surrounding $\beta$A plaques in APP Tg2576 mice. Neuropathol Appl Neurobiol 30:491–502.

Richard M, Biacabe AG, Streichenberger N, Ironside JW, Mohr M, Kopp N, Perret-Liaudet A (2003) Immunohistochemical localization

of 14-3-3 zeta protein in amyloid plaques in human spongiform encephalopathies. Acta Neuropathol 105:296–302.
Sergeant N, Delacourte A, Buee L (2005) Tau protein as a differential biomarker of tauopathies. Biochim Biophys Acta 1739:179–197.
Soulié C, Nicole A, Delacourte A, Ceballos-Picot I (2004) Examination of stress-related genes in human temporal versus occipital cortex in the course of neurodegeneration: involvement of 14-3-3 zeta in this dynamic process. Neurosci Lett 365:1–5.
Spillantini MG, Goedert M (1998) *Tau* protein pathology in neurodegenerative diseases. Trends Neurosci 21:428–433.
Sultana R, Newman SF, Mohammad Abdul H, Cai J, Pierce WM, Klein JB, Merchant M, Butterfield DA (2006) Protective effect of D609 against amyloid-beta1-42-induced oxidative modifications of neuronal proteins: redox proteomics study. J Neurosci Res 84: 409–417.
Takahashi Y (2003) The 14-3-3 proteins: gene, gene expression, and function. Neurochem Res 28:1265–1273.
Umahara T, Uchihara T, Tsuchiya K, Nakamura A, Iwamoto T, Ikeda K, Takasaki M (2004) 14-3-3 Proteins and zeta isoform containing neurofibrillary tangles in patients with Alzheimer's disease. Acta Neuropathol 108:279–286.
van Heusden GP (2005) 14-3-3 Proteins: regulators of numerous eukaryotic proteins. IUBMB Life 57:623–629.
Varadarajan S, Yatin S, Aksenova M, Butterfield DA (2000) Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. J Struct Biol 130:184–208.
Yuan Z, Agarwal-Marwal A, Paudel HK (2004) 14-3-3 binds to and mediates phosphorylation of microtubule-associated tau protein by Ser9-phosphorylated glycogen synthase kinase 3$\beta$ in the brain. J Biol Chem 279:26105–26114.
Zhu X, Raina AK, Rottkamp CA, Aliev G, Perry G, Boux H, Smith MA (2001) Activation and redistribution of c-Jun N-terminal kinase/stress activated protein kinase in degenerating neurons in Alzheimer's disease. J Neurochem 76:435–441.
Zhu X, Rottkamp CA, Boux H, Takeda A, Perry G, Smith MA (2000) Activation of p38 kinase links tau phosphorylation, oxidative stress, and cell cycle-related events in Alzheimer disease. J Neuropathol Exp Neurol 59:880–888.

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# RETRACTED: Oxidative damage of 14-3-3 zeta and gamma isoforms in Alzheimer's disease and cerebral amyloid angiopathy

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This article has been retracted at the request of the editors and authors. Please see Elsevier Policy on Article Withdrawal (http://www.elsevier.com/locate/withdrawalpolicy).

Reason: After publication of their paper, the authors increased the number of control cases and carried out densitometric studies relating levels of malondialdehyde lysine (MDAL) immunoreactivity to levels of 14-3-3 immunoreactivity in comparative spots of membranes blotted with 14-3-3 and MDAL antibodies. Differences were not significant between Alzheimer's disease (AD) (*n*=6) and age-matched controls (*n*=8) when values of MDAL in 14-3-3 spots were corrected by values of total 14-3-3.

Therefore, the present data do not indicate significant differences between control and AD cases regarding total 14-3-3 and oxidised 14-3-3 levels in total homogenates, and the conclusion made in this article is invalidated.

**In the article it was concluded that the 14-3-3 zeta and gamma isoforms are oxidatively damaged in the aged human brain. The latter conclusion is still valid.**

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# 8

## Delineation of Early Changes in Cases with Progressive Supranuclear Palsy-Like Pathology. Astrocytes in Striatum are Primary Targets of Tau Phosphorylation and GFAP Oxidation

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RESEARCH ARTICLE

# Delineation of Early Changes in Cases with Progressive Supranuclear Palsy-Like Pathology. Astrocytes in Striatum are Primary Targets of Tau Phosphorylation and GFAP Oxidation

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argyrophilic grain disease, glial fibrillary acidic protein oxidative damage, progressive supranuclear palsy.

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## Abstract

Progressive supranuclear palsy (PSP) is a complex tauopathy usually confirmed at post-mortem in advanced stages of the disease. Early PSP-like changes that may outline the course of the disease are not known. Since PSP is not rarely associated with argyrophilic grain disease (AGD) of varible intensity, the present study was focused on AGD cases with associated PSP-like changes in an attempt to delineate early PSP-like pathology in this category of cases. Three were typical clinical and pathological PSP. Another case presented with cognitive impairment, abnormal behavior and two falls in the last three months. One case suffered from mild cognitive impairment, and two had no evidence of neurological abnormality. Neuropathological study revealed, in addition to AGD, increased intensity and extent of lesion in three groups of regions, striatum, pallidus/subthalamus and selected nuclei of the brain stem, correlating with neurological impairment. Biochemical studies disclosed oxidative damage in the striatum and amygdala. Together the present observations suggest (i) early PSP-like lesions in the striatum, followed by the globus pallidus/subthalamus and selected nuclei of the brain stem; (ii) early involvement of neurons and astrocytes, but late appearance of tufted astrocytres; and (iii) oxidative damage of glial acidic protein in the striatum.

## INTRODUCTION

Progressive supranuclear palsy (PSP) is a rare neurodegenerative disease, with an age-adjusted prevalence of 6.4 per 100 000 (43), clinically manifested by movement disorders and cognitive deficits. These include bradykinesia, postural instability with falls (usually backwards), parkinsonism, nuchal dystonia and gaze supranuclear palsy. Frontotemporal impairment and dementia can follow the appearance of movement disorders (20, 39).

Clinical diagnosis of PSP is based on both inclusion and exclusion criteria of other neurodegenerative diseases with similar clinical features as Parkinson's disease (PD), corticobasal degeneration, multiple system atrophy and Lewy body disease (28, 29). Progressive Supranuclear Palsy Rating Scale and Staging system permits an approach to the clinical staging and prognosis of the disease (19).

The main neuropathological findings in PSP are neuronal loss, astrocytic gliosis, and hyperphosphorylated-tau accumulation in neurons, astrocytes and oligodendrocytes. Cortical and subcortical structures are affected at the terminal stages of the disease. The globus pallidus, striatum, subthalamic nucleus, nucleus basalis of Meynert, colliculi, tegmentum, periaqueductal gray matter, substantia nigra, red nucleus, reticular formation of the midbrain and pons, basis pontis, and dentate nucleus are involved in the majority of cases; the cerebral cortex, locus ceruleus, oculomotor complex and inferior olive are variably affected (29, 21, 22). Tau-positive aggregates in PSP are neurofibrillary tangles (NFT) and pretangles in neurons, neuropil threads, coiled bodies in oligodendroglial cells, and heterogeneous inclusions in the cytoplasm of astrocytes that are subdivided into thorn-shaped or bush astrocytes and protoplasmic tufted astrocytes (4, 26, 46, 47). Hyperphosphorylated tau in PSP is mainly composed of 4R-tau isoforms that are resolved in two phospho-tau bands of 68 kDa and 64 kDa in Western blots of sarkosyl-insoluble fractions (11, 14).

In contrast with other degenerative diseases of the nervous system, as Alzheimer's disease (AD) or PD, practically nothing is known about early changes in PSP. This is because, in part, of the rarity of PSP in comparison with AD and PD. Yet PSP is often associated with argyrophilic grain disease (AGD) (31, 49), another tauopathy much more common than PSP. AGD was first described as a degenerative disease characterized by argyrophilic grains in the entorhinal cortex, hippocampus, amygdala and neighbouring temporal cortex in a subset of patients who had suffered from adult onset dementia (7, 8). However, AGD is frequently asymptomatic

depending on the extension of the lesions (42, 52); the presence of AGD has been estimated at 5%–9% in adult autopsy series (9, 50). The main neuropathological findings in AGD are tau hyperphosphorylation and accumulation in grains, pretangle neurons, tangles, neuropil threads, coiled bodies, and cytoplasm of astrocytes. Gel electrophoresis of sarkosyl-insoluble fractions has shown that AGD is characterized by a double band of 68 kDa and 64 kDa (16, 18, 49, 53, 54). The use of specific anti-4R tau antibodies has further categorized AGD as a 4R-tauopathy (49).

The neuropathological screening of AGD is relatively easy with the use of phospho-tau immunohistochemistry in a single section of the anterior hippocampus, and this method is routinely carried out in many laboratories because it also permits the screening of AD. Following this procedure, we have found 45 AGD cases, which represent 4% of the total autopsies, in a consecutive autopsy series in a general adult hospital. Seven cases had additional lesions of mild, moderate or severe intensity that resembled those seen in PSP. Three of them had suffered from typical clinical symptoms of PSP for 4–6 years—the neuropathological changes were also typical of terminal PSP—and these cases were diagnosed as PSP with associated AGD. Another case presented with cognitive impairment, abnormal behavior and two falls in the last 3 months. One case had suffered from mild cognitive impairment with no motor abnormalities. The remaining two cases had no evidence of neurological deficits according to the clinical report and the neurological examination.

The present study is focused on the neuropathology of the four cases with AGD and associated lesions that were evaluated as consistent with early PSP-like changes. It is worth considering that in spite of certain similarity of lesions in these cases to those encountered in advanced PSP, we will never know whether these individuals would have suffered from clinical PSP if they had survived for a long time. Therefore, the present study is geared to increase understanding about possible steps of PSP-like pathology in certain subjects rather than to establish a rigid and universal scheme of PSP stages of disease progression. In addition to the neuropathological study, gel electrophoresis and western blotting to glycoxidative and lipoxidative markers, as well as 2D gel electrophoresis and mass spectrometry, have been carried out to evaluate whether oxidative damage is an early event associated with PSP-like pathology.

# MATERIAL AND METHODS

## Cases

A summary of the cases studied is shown in Table 1.

Case 1: The patient was a 68 years old man who was admitted in the hospital because of fever, respiratory insufficiency, pancytopenia, lung infiltrates and positive cultures to pseudomona. He was diagnosed of chronic lymphocytic leukemia, bilateral pneumonia, septic shock and multiorganic failure. He died 7 days later. No clinical evidence of neurological disorder was recorded in the clinical file. An interview to the relatives after the neuropathological diagnosis was made further excluded major neurological deficits, including abnormal behavior and cognitive impairment.

Case 2: The patient was a 79 years old man with chronic respiratory insufficiency currently visited in the hospital. No evidence of neurological symptoms and signs was recorded in the clinical files. He was admitted in the hospital because of sudden respiratory failure caused by a lobar pneumonia. He died 48 h later.

Case 3: The patient was a 66 years old woman with loss of recent memory and cognitive decline during the last 2 years. The last CT examination carried out 3 months before her admittance in the hospital showed mild global cerebral atrophy. At the same time, the neurological examination did not reveal motor or sensory disturbances, gait disorders or motor ocular anomalies. The patient was admitted because of sudden respiratory failure caused by pulmonary thromboembolism and she died 4 h later.

Case 4: The patient was a 68 years old woman with progressive cognitive decline, abnormal behavior with irritability and emotional instability for the last 4 years, clinically categorized as possible Alzheimer's disease. Two falls were recorded in the last 3 months. The patient also suffered from arterial hypertension and renal failure under clinical study. The neurological examination 1 month before her admittance to the hospital revealed slight tremor and a discrete disorder of the gait; the motor ocular movements were not affected. The patient came to the hospital because of urinary infection and sepsis.

Case 5: The patient was a 75 years old man with progressive cognitive beginning at the age of 70 and characterized by loss of memory, impaired orientation and progressive aphasia, personality

| | Age | Gender | p.-m. delay | Neurological diagnosis | Neuropathological diagnosis |
|---|---|---|---|---|---|
| 1 | 68 | Man | 12 h | Normal | AGD + tauopathy |
| 2 | 79 | Man | 4 h | Normal | AGD + tauopathy |
| 3 | 66 | Woman | 6 h | MCI | AGD + tauopathy |
| 4 | 68 | Woman | 16 h | MCI + falls | AGD + PSP-like |
| 5 | 75 | Man | 18 h | PSP | PSP + AGD |
| 6 | 54 | Man | 3 h | Normal | No lesions |
| 7 | 35 | Man | 8 h | Normal | No lesions |
| 8 | 82 | Woman | 11 h | Normal | A few diffuse plaques |
| 9 | 75 | Woman | 6 h | Normal | No lesions |
| 10 | 80 | Woman | 3.5 h | Normal | A few diffuse amyloid plaques; NFTs in EC |
| 11 | 73 | Woman | 7 h | Normal | No lesions |
| 12 | 58 | Woman | 4 h | Normal | No lesions |

**Table 1.** Summary of the clinical and neuropathological diagnosis in the present series. Abbreviations: MCI = mild cognitive impairment; PSP = progressive supranuclear palsy; AGD = argyrophilic grain disease; NFTs = neurofibrillary tangles; EC = entorhinal cortex; p.-m. delay = post-mortem delay (h).

changes with apathy, frequent changes of mood, irritability, and insomnia. He also suffered from the last 3 years of frequent falls, tremor, abnormal gait, facial hypomimia and motor ocular disturbances, including slowness of saccadic movements and difficulties to look upward. The patient was admitted in the hospital because of respiratory insufficiency caused by lobar pneumonia.

Cases 6–12: No neurological abnormalities were recorded in these patients. Causes of death were respiratory infections (3), cardiac infarction (1), disseminated cancer (2) and pulmonary thromboembolism (1).

## Neuropathological methods

The time between death and tissue processing was between 2 and 14 h.

The left hemisphere was immediately cut on coronal sections, 1-cm thick, frozen on dry ice and stored at −80°C until use. For morphological examination, the brains were fixed by immersion in 10% buffered formalin for 2 or 3 weeks. The neuropathological study was carried out on dewaxed 4-μm thick paraffin sections of the frontal (area 8), primary motor, primary sensory, parietal, temporal superior, temporal inferior, anterior gyrus cinguli, anterior insular, and primary and associative visual cortices, entorhinal cortex and hippocampus, caudate, putamen and globus pallidus, medial and posterior thalamus, subthalamus, Meynert nucleus, amygdala, midbrain (two levels), pons and bulb, and cerebellar cortex and dentate nucleus. The sections were stained with haematoxylin and eosin, Klüver Barrera, and, for immunohistochemistry to glial fibrillary acidic protein (Dako, Barcelona, Spain dilution 1:250), CD68 (Dako, dilution 1:100), βA-amyloid (Boehringer, Ingelheim, Germany, dilution 1:50), tau AT8 (Innogenetics, Gent, Belgium, dilution 1:500), tau 4R and tau 3R (Upstate, Gent, Belgium, dilution 1:200 and 1:2 000, respectively), phosphorylation-specific *tau* Thr181, Ser202, Ser214, Ser262, Ser396 and Ser422 (all of them Calbiochem, LaJoya, CA, USA, dilution 1:100, except Thr181 1:250), and αB-crystallin (Abcam, Cambridge, UK, dilution 1:100), α-synuclein (Chemicon, Millipore, MA, USA, dilution 1:500) and ubiquitin (Dako, dilution 1:200). AD stages were established according to the amyloid deposition burden and neurofibrillary pathology following the nomenclature of Braak and Braak (10). Stages of amyloid deposition refer to initial deposits in the basal neocortex (stage A), deposits extended to the association areas of the neocortex (stage B), and heavy deposition throughout the entire cortex (stage C). Stages of neurofibrillary pathology correspond to transentorhinal (I–II), limbic (III–IV) and neocortical (V and VI). AGD stages were established following the nomenclature of Saito *et al* (42), slightly modified (18). Stage I is characterized by mild involvement of the anterior entorhinal cortex, cortical and basolateral nuclei of the amygdala, and hypothalamic lateral tuberal nucleus. Stage II is defined by moderate involvement of the entorhinal cortex, anterior CA1, transentorhinal cortex, cortical and basolateral nuclei of the amygdala, presubiculum, hypothalamic lateral tuberal nucleus and dentate gyrus. Stage III involves the entorhinal cortex, CA1, perirhinal cortex, presubiculum, amygdala, dentate gyrus, hypothalamic lateral tuberal nucleus. In addition, there is mild involvement of CA2 and CA3, subiculum, other nuclei of the hypothalamus (ie, mammillary bodies), anterior temporal cortex, insular cortex, anterior gyrus cinguli, orbitofrontal cortex, nucleus accumbens and septal nuclei.

## Sarkosyl-insoluble fraction extraction

Frozen samples of about 2 g of the amygdala and striatum were gently homogenized in a glass tissue grinder in 10 vol (w/v) with cold suspension buffer (10 mM Trishydroxymethylaminomethane (TRIS-HCl), pH 7.4, 0.8 M NaCl, 1 mM Ethylene-glycol tetraacetic acid (EGTA), 10% sucrose). The homogenates were first centrifuged at 20 000 g, and the supernatant (S1) was retained. The pellet was rehomogenized in 5 vol of homogenization buffer and recentrifuged. The two supernatants (S1 + S2) were then mixed and incubated with 0.1% N-lauroylsarcosynate (sarkosyl) for 1 h at room temperature while being shaken. Samples were then centrifuged at 100 000 g in a Ti70 Beckman rotor. Sarkosyl-insoluble pellets (P3) were resuspended (0.2 mL/g, starting material) in 50 mM TRIS-HCl (pH 7.4). Protein concentrations were determined with the Bradford method using bovine serum albumin (BSA) as a standard.

## Brain homogenates

Brain samples (0.1 g) of the striatum and amygdala of cases 2, 3 and 5, and controls 6, 7 and 8 were homogenized in 1 mL of lysis buffer (40 mM Tris, pH 7.5, 7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl)dimethylammonia]-1-propanesulphonate (CHAPS) (BioRad, Barcelona, Spain) and complete protease inhibitor cocktail (Roche Molecular Systems, Barcelona, Spain), and centrifuged at 15 000 rpm for 10 minutes at 4°C. The pellets were discarded and protein concentrations of the supernatants were determined by Bradford method with bovine serum albumin (BSA) (Sigma, Barcelona, Spain) as a standard.

## Western blot

For monodimensional gel electrophoresis, 20 μg of each sample from total homogenates, 250 ug from sarkosyl-insoluble fraction of case 2 and 150 ug from the same fraction of case 3 and case 5 were loaded for 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and then transferred to nitrocellulose membranes (400 mA for 90 minutes). Mini-protean system (BioRad) was used for brain homogenates, and maxi-protean system (16 × 20 cm) was used for sarkosyl-insoluble fractions. Immediately afterwards, the membranes were incubated with 5% skimmed milk in tris buffered saline Tween 20 (TBS-T) buffer (100 mM Tris-buffered saline, 140 mM NaCl and 0.1% Tween 20, pH 7.4) for 30 minutes at room temperature, and then incubated with the primary antibody in TBS-T containing 3% BSA (Sigma, Madrid, Spain) at 4°C overnight. The mouse monoclonal anti-carboxy-ethyl-lysine (CEL) and anti-carboxy-methyl-lysine (CML) (TransGenic, Kumamoto, Japan), the mouse-monoclonal anti-advanced glycation end products (AGE) (TransGenic), and the rabbit polyclonal anti-malondyaldehyde-lysine (MDA-L) (Biomedical, Houston, TX, USA) antibodies were used diluted 1:1000. The rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) (Dako) was used at a dilution of 1:4000. The monoclonal antibody to β-actin (Sigma) was used at a dilution of 1:30 000 as a control of

protein loading. In sarkosyl-insoluble fractions, rabbit polyclonal antiphospho-tau(Ser422) (Calbiochem) was used at a dilution of 1:1000. Subsequently, the membranes were incubated for 45 minutes at room temperature with the corresponding secondary antibody labeled with horseradish peroxidase (Dako) at a dilution of 1:1000, and washed with TBS-T for 30 minutes. Protein bands were visualized with the chemiluminescence ECL method (Amersham, Barcelona, Spain).

Control and diseased cases were processed in parallel.

### 2D gel electrophoresis

A 150-µg protein was mixed with 2% Byolites (v/v), 2 mM tributylphosphine solution and bromophenol blue in a final volume of 150 µL. In the first-dimension electrophoresis, 150 µL of sample solution was applied to an immobilized 7-cm pH 3–10 nonlinear gradient ReadyStrip immobilized pH gradient (IPG) strip (Bio-Rad) at both the basic and acidic ends of the strip. The strips were actively rehydrated for 12 h at 50 V, and the proteins were focused at 300 V for 1 h, after which time the voltage was gradually increased to 3500 V within 6 h. Focusing was continued at 3500 V for 12 h and at 5000 V for 24 h. For the second dimension separation, IPG strips were equilibrated for 10 minutes in 50 mM Tris-HCl (pH 6.8) containing 6 M urea, 1% (wt/v) SDS, 30% (v/v) glycerol and 2% dithiotreitol, and then reequilibrated for 10 minutes in the same buffer containing 2.5% iodacetamide. The strips were placed on 10% polyacrylamide gels and electrophoresed at 0.02 A. For gel staining, Comassie Biosave staining (Biorad) was used as described by the manufacturer.

Two 2D gels for every case were run in parallel, one for Comassie staining and the other transferred to a nitrocellulose membrane (200 mA for 1 h 30 minutes). Diseased cases were processed in parallel with control cases. After incubation with 5% skimmed milk in TBS-T buffer for 30 minutes at room temperature, nitrocellulose membranes were blotted with mouse monoclonal anti-AGE (TransGenic). Membranes were stripped by two incubations of 20 minutes at 64°C with stripping buffer (0,1 mM B-mecap, 2% SDS, 62.5 mm Tris HCL pH 6.8) and incubated with 5% skimmed milk in TBS-T buffer for 30 minutes at room temperature. Membranes were then incubated with rabbit polyclonal anti-GFAP (Dako) used at a dilution of 1:4000. Subsequently, the membranes were processed as previously indicated for monodimensional gels. Several combinations of disease cases with at least three control cases were performed in order to prove reproducibility.

### In-gel digestion

Proteins were in-gel digested with trypsin (sequencing grade modified, Promega, Barcelona, Spain) in the automatic Investigator™ ProGest robot of Genomic Solutions, Michigan, USA. Briefly, excised gel spots were washed sequentially with ammonium bicarbonate buffer and acetonitrile. Proteins were reduced and alkylated with 10 mM DTT solution for 30 minutes and 100 mM solution of iodine acetamide for 15 minutes, respectively. After sequential washing with buffer and acetonitrile, proteins were digested overnight at 37°C with trypsin 0.27 nM. Tryptic peptides were extracted from the gel matrix with 10% formic acid and acetonitrile. The extracts were pooled and dried in a vacuum centrifuge.

### Acquisition of Mass spectrometry (MS) and MS/MS spectra

Proteins manually excised from the 2D gels were digested and analyzed by CapLCnano-ESI-MS-MS mass spectrometry. The tryptic digested peptide samples were analyzed using on-line liquid chromatography (CapLC, Micromass-Waters, Manchester, UK) coupled with tandem mass spectrometry (Q-TOF Global, Micromass-Waters, Manchester, UK). Samples were resuspended in 12-µL 10% formic acid solution, and 4 µL was injected for chromatographic separation into a reverse-phase capillary C18 column [75-µm internal diameter and 15 cm in length (PepMap™ column, LC Packings, Amsterdam, The Netherlands)]. The eluted peptides were ionized via coated nano-ES needles (PicoTip™, New Objective, Woburn, MA, USA). A capillary voltage of 1800–2200 V was applied together with a cone voltage of 80 V. The collision in the collision-induced dissociation was 25 to 35 eV, and argon was employed as the collision gas. Data were generated in PKL file format and submitted for database searching in Mascot server (Matrix Science, Boston, MA, USA) using the NCBI database with the following parameters: trypsin enzyme, one missed cleavage, carbamidomethyl (C) as fixed modification and oxidized (M) as variable modification, and mass tolerance of 150–250 ppm.

Probability-based Mowse score was used to determine the level of confidence in the identification of specific isoforms from the mass spectra. This probability equals 10 (-Mowse score/10). Mowse scores higher than 50 were considered to be of high confidence of identification.

## RESULTS

### Neuropathology

Representative lesions are shown in Figure 1. Characteristic lesions in individual cases 1, 2, 3 and 5 are shown in Figure 1S–4S (supplementary data).

AGD pathology was characterized by the presence of grains and pretangles in the entorhinal cortex, CA1 region of the hippocampus and amygdala in every case, although the intensity of lesions was variable depending on the presence of associated lesions. The amygdala was slightly damaged in cases 1 and 2, but it was severely affected in cases 3, 4 and 5. Grains and pretangles, together with deposits in dentate gyrus neurons, were also seen in the temporal cortex and subiculum in cases 3, 4 and 5. Astrocytic inclusions and coiled bodies were noticed in every case, although with variable intensity.

On the basis of neuropathological data, staging of AGD was established as follows: case 1: early stage 2, case 2: stage 2, and cases 3, 4 and 5: stage 3.

Neurofibrillary tangles in the same regions were categorized as stage I in cases 1, 2 and 4; and stage II in case 5. A few diffuse plaques were seen in the temporal and orbital cortices in case 3 (stage A of Braak and Braak).

PSP-like pathology was examined in three different groups of regions: caudate/putamen. globus pallidus/sbthalamic nucleus, and brain stem nuclei (substantia nigra, locus ceruleus, colliculi, peri-aqueductal gray matter and ventral pons).

Lesions in the caudate/putamen were characterized by astrocytic gliosis, as revealed with GFAP-immunostaining, and gel electro-

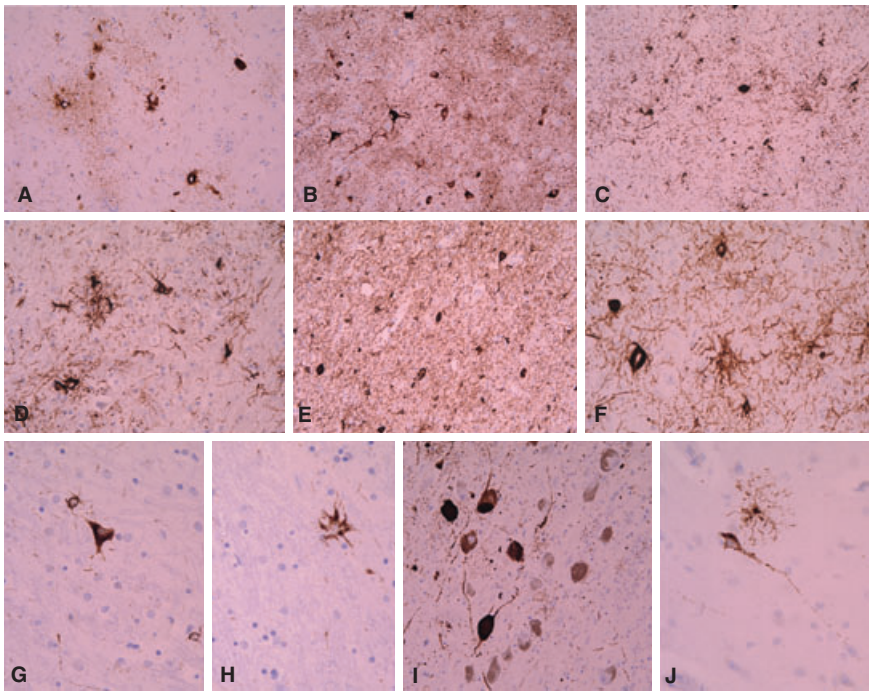


**Figure 1.** *Phospho-tau pathology in cases with AGD and PSP-like pathology.* **A.** caudate, case 2. **B.** amygdala, case 3. **C.** globus pallidus, case 5. **D.** CA1, case 3. **E.** subthalamus, case 5. **F.** superior colliculus, case 5. **G.** ventral pons, case 3. **H.** midbrain, case 3. **I.** locus coeruleus, case 3. **J.** gyrus cinguli, case 3. Paraffin section slightly counterstained with haematoxylin. Abbreviations: AGD = argyrophilic grain disease; PSP = progressive supranuclear palsy.

phoresis and GFAP immunoblotting (Figure 2), phospho-tau-immunoreactive astrocytes, and phospho-tau-immunoreactive neurons. These lesions were present in every case, although with variable intensity. The intensity of lesions was mild in case 1 and 2, moderate in cases 3 and 4, and severe in case 5.

Lesions in the globus pallidus and subthalamic nuclei were scanty in cases 1, 2 and 3, moderate in case 4 and severe in case 5. Neuronal pathology rather than astrocytic pathology predominated in these regions.

Lesions in the brain stem were characterized by neurofibrillary tangles and phospho-tau-immunoreactive inclusions in astrocytes. Discrete lesions were restricted to the ventral pons and ceruleus in cases 1 and 2. The intensity of lesions increased in these nuclei and the distribution of lesions extended to the substantia nigra, colliculus, red nucleus and peri-aqueductal gray matter in case 3 and particularly in case 4. Severe phospho-tau deposition in neurons and astrocytes was found in case 5.

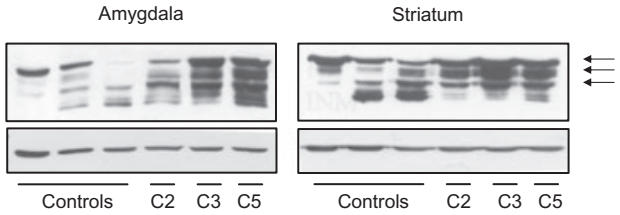


**Figure 2.** *Monodimensional gel electrophoresis and western blotting to GFAP in the amygdala and striatum in cases 2, 3 and 5, and age-matched controls.* Several bands of high molecular weight and strong density (arrows) are seen in diseased cases when compared with controls. Abbreviation: GFAP = glial fibrillary acidic protein.

Interestingly, in spite of the substantial numbers of phospho-tau-immunoreactive astrocytes, tufted astrocytes were absent in case 1, exceptional or very rare in cases 2 and 3, occasional in case 4 and frequent in case 5.

Coiled bodies in these regions were absent in cases 1 and 2, they were mild in case 3, moderate in case 4 and severe in case 5. These lesions were stained with anti-4R-tau antibodies. 3R-tau immunoreactivity was restricted to small numbers of tangles in the entorhinal cortex, thus suggesting combined stage I–II AD (data not shown).

Mild involvement of the Meynert nucleus occurred in cases 1 and 2, moderate in cases 3 and 4, and severe in case 5. The frontal cortex was involved in cases 3, 4 and 5, the parietal cortex was additionally involved in case 4, and the occipital cortex in case 5.

A summary of neuropathological observations is shown in Table 2, whereas individual changes in cases 1–5 are shown in the corresponding Tables 1S–5S (supplementary data).

No lesions were seen in cases 6–12. More explicitly, tau, α-synuclein and ubiquitin inclusions, and β-amyloid plaques were absent, excepting a few diffuse plaques in the orbital and temporal cortex in two cases and a few neurofibrillary tangles in the entorhinal cortex in one.

## Tau banding pattern in sarkosyl-insoluble fraction

Western blots of sarkosyl-insoluble fractions incubated with antiphospho-tau(Ser422) antibody revealed a double band of 68 kDa and 64 kDa in the striatum and amygdala in case 3 and in case 5 (established PSP). In addition to these two bands, other bands of about 50 kDa and lower were detected in the striatum

**Table 2.** Summary of main neuropathological findings related with tau pathology. Involvement of three combined regions, presence of tufted astrocytes and AGD changes are expressed semiquantitative ly. +: mild; ++: moderate; +++: severe; +/– indicates lack of involvement of one of the mentioned regions. Abbreviation: AGD = argyrophilic grain disease.

| Cases/region affected | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Caudate/putamen | + | + | + | + | ++ |
| Globus pallidus/subthalamus | +/– | +/– | ++/+ | + | ++ |
| Substantia nigra/coeruleus/colliculi/ventral pons | +/– | +/– | + | ++/+ | +++/++ |
| Tufted astrocytes | no | exceptional | very rare | occasional | very common |
| AGD stage | 2 | 2 | 3 | 3 | 3 |

and amygdala (Figure 3). Similar bands representing full-length 4R tau and truncated forms of tau have also been described in PSP and AGD (18, 38). The pattern in case 2 was lightly different because the two upper bands appeared to be composed of doublets. The reasons are not clearly understood, but the signal in this case was low when compared with case 3 and canonical PSP. The protein needed was higher in case 2 (250 μg) than in case 3 (50 μg).

## Oxidative stress markers (MDA-L, AGE, CML and CEL)

Gel electrophoresis was carried out in total homogenates of the amygdala and striatum in three controls (cases 6, 7 and 8), and diseased cases 2, 3 and 5. Membranes were immunoblotted with anti-MDAL, AGE, CEL and CML antibodies. Control and diseased cases were run in parallel.

Several bands were obtained in the amygdala in control and diseased cases with the different antibodies. Yet a band of about 50 kDa was detected with the four antibodies in cases 3 and 5. This band was not detected, or it was very faint in the three controls and in case 2. However, anti-AGE antibodies recognized a band of about 70 kDa only in case 2 (Figure 4, upper panel).

Several bands were also present in the striatum in control and diseased cases. Yet an AGE band of about 50 kDa was increased only in diseased brains, but not in controls (Figure 4, lower panel). A band of higher molecular weight (about 70 kDa) was also present in case 2. The intensity of MDA-L, CEL and CML bands was similar in disease cases and controls, thus indicating a preferential damage related with advanced glycation end products.

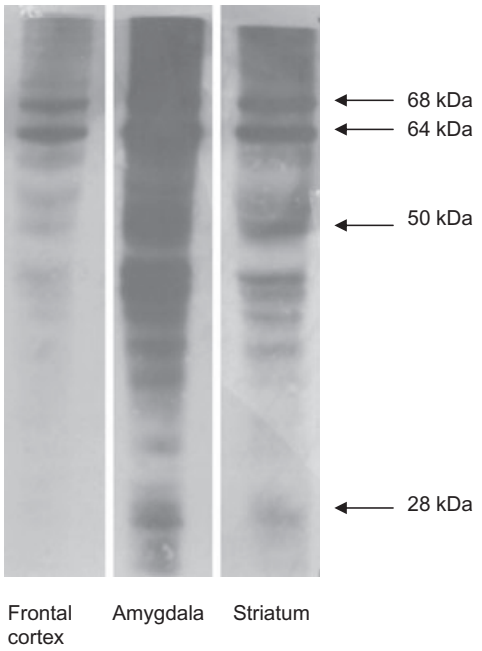


**Figure 3.** *Western blots of sarkosyl-insoluble fractions in the amygdala and striatum in case 3 stained with the antiphospho-tau(Ser422) antibody.* Bands of 68 kDa and 64 kDa together with bands of about 50 kDa and lower are present in the amygdala and striatum. These bands are similar to hose currently found in PSP and AGD cases. Abbreviations: PSP = progressive supranuclear palsy; AGD = argyrophilic grain disease.

## 2D gel electrophoresis, western blotting and mass spectrometry

### Amygdala

2D gels stained with Comassie revealed three spots close to 50 kDa in case 3 when compared with controls. Parallel membranes blotted for AGE disclosed AGE immunoreactivity in these spots. The three spots were excised from the gels and identified by mass spectrometry as glial fibrillary acidic protein. Mowse scores higher than 50 (96, 204 and 273) were considered of high confidence of identification (Table 3).

Validation was carried out in 2D gels of one control and case 3 run in parallel and processed for Western blot with anti-GFAP antibody. Increased expression of GFAP was found in case 3 as three spots of high density and about 50 kDa of molecular weight.

No differences were seen between controls and case 2 (data not shown).

### Striatum

2D gels stained with Comassie revealed five spots (three from case 2, two from case 3) close to 50 kDa in cases 2 and 3 when compared with controls. The five spots were excised and identified by mass spectrometry as glial fibrillary acidic protein (GFAP) (Table 3). All the spots excised were consistent with GFAP. No other proteins or negative results were obtained in

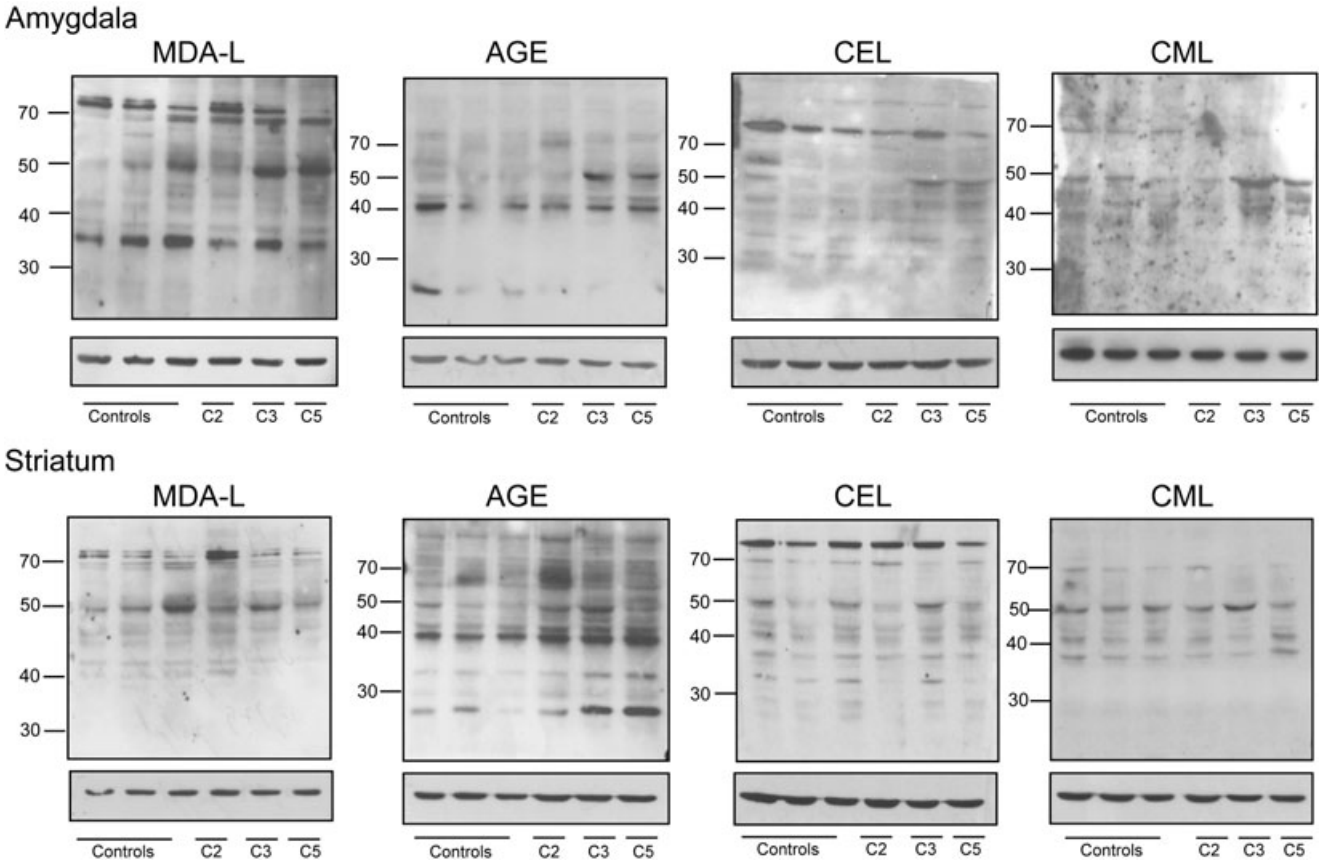


**Figure 4.** *Monodimensional gel electrophoresis and western blotting to MDA-L, AGE, CEL and CML in the amygdala and striatum in three controls, and in cases 2, 3 and 5.* Increased density of one band of about 50 kDa is found in the amygdala in cases 3 and 5, and in the striatum in cases 2, 3 and 5 with in membranes processed with anti-AGE antibodies. Lower intensity of the band in the striatum in case 5 when compared with case 2 and 3 can be related with the longer post-mortem delay in this case. Abbreviations: MDA-L = malondyaldehyde-lysine; AGE = advanced glycation end products; CEL = carboxy-ethyl-lysine; CML = carboxy-methyl-lysine.

the present study. Mowse scores varied from 222 to 377, and they were considered to be of high confidence of identification (above 50).

Parallel membranes of cases 2 and 3, and corresponding controls showed the five spots immunoreactive for AGE and for GFAP (Figure 5).

## DISCUSSION

The present study was carried out in an attempt to delineate neuropathological modifications consistent with early changes in PSP. As this is a small number of cases, and they have been selected from a series of AGD with associate PSP-like pathology, we can not

**Table 3.** Excised spots from 2D gels in the amygdala in case 3 and in the striatum in cases 2 and 3. Mowse scores higher than 50 are considered of high confidence of identification. Abbreviation: GFAP = glial fibrillary acidic protein.

| Protein | Molecular weight | pI | Mowse score | Number of peptides matched | ID number |
|---|---|---|---|---|---|
| Amygdala case 3 | | | | | |
| GFAP | 49.7 kDa | 5.42 | 204 | 12 | gil38566198 |
| GFAP | 47.6 kDa | 5.4 | 96 | 5 | gil119571954 |
| GFAP | 49.7 kDa | 5.42 | 273 | 16 | gil38566198 |
| Striatum case 2 | | | | | |
| GFAP | 49.7 kDa | 5.42 | 286 | 22 | gil38566198 |
| GFAP | 49.7 kDa | 5.42 | 226 | 21 | gil38566198 |
| GFAP | 49.7 kDa | 5.42 | 222 | 17 | gil38566198 |
| Striatum case 3 | | | | | |
| GFAP | 49.7 kDa | 5.42 | 377 | 22 | gil38566198 |
| GFAP | 49.7 kDa | 5.42 | 279 | 21 | gil38566198 |

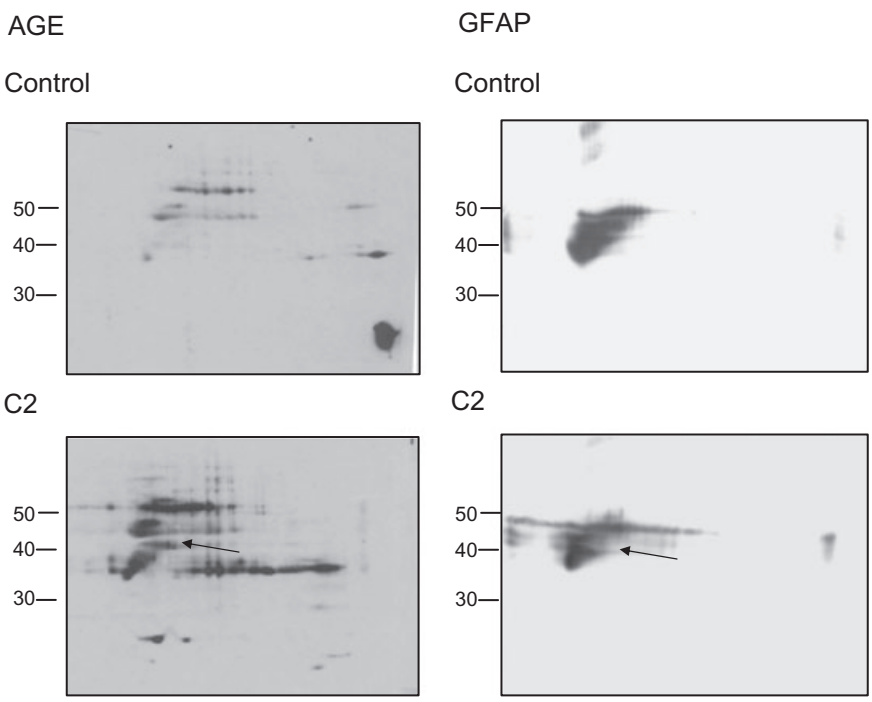


**Figure 5.** *2D gel electrophoresis and western blotting to AGE shows the presence of differential spots in case 2 when compared with the corresponding control run in parallel (arrows).* Parallel membranes blotted for GFAP show that AGE-immunoreactive spots are also GFAP positive. Abbreviations: AGE = advanced glycation end products; GFAP = glial fibrillary acidic protein.

exclude certain bias in their selection and therefore that results of the present series may be not universal for PSP. This remark is even more relevant considering the heterogeneity in terms of clinical manifestations and neuropathological findings in established PSP (6, 12, 24, 36, 44, 55). As stated at the beginning, the present observations do not attempt to establish a rigid and universal scheme of PSP progression, but rather to increase our understanding about early changes related with PSP-like pathology.

Categorization of AGD pathology was carried out following established criteria (18, 42). Cases 1 and 2 were considered as stage 2, and cases 3, 4 and 5 as stage 3. Previous studies have shown that early stages in AGD are usually asymptomatic, and this is also observed in cases 1 and 2. AGD stage 3 is often associated with a variety of clinical symptoms, including cognitive decline, dementia, behavioral abnormalities, personality changes, and emotional and mood imbalance (8, 23, 41, 48–51). Cognitive and behavioral abnormalities were also present in cases 3 and 4, and these changes were ascribed to AGD. Cognitive impairment in case 5 was considered as a combined result of AGD and PSP.

For instrumental purposes, three main groups of regions were considered in the study of PSP-like pathology: (i) caudate/putamen; (ii) globus pallidus/subthalamus; and (iii) substantia nigra/ceruleus/colliculi/ventral pons.

The most remarkable lesions in cases 1 and 2 were astrocytic gliosis and hyperphospho-tau accumulation in caudate and putamen. Changes increased in intensity in case 3, where involvement of the globus pallidus and subthalamus, together with mild involvement of brain stem structures, was also observed. The intensity of lesions, ranging from mild to moderate, augmented in all three groups of regions in case 4. All these regions were severely affected in case 5 (prototypical PSP).

No motor deficits were present in cases 1, 2 and 3. The neurological examination carried out at the time of admission was not relevant in cases 1 and 2. Cognitive impairment in case 3 was considered consistent with AGD. Mild motor deficits were present in case 4. Two falls in the last 3 months was the only additional complain in this patient. However, the neurological examination disclosed, in addition to memory loss and cognitive impairment, slow saccadic eye movements and slight tremor in the upper extremities, as well as certain loss of stability. Motor symptoms and signs we considered the consequence of PSP-like pathology. Finally, neurological deficits and neuropathological findings in case 5 were typical of PSP.

Together, these observations point to early involvement in the caudate/putamen in cases with PSP-like pathology, followed by globus pallidus/subthalamus and selected nuclei of the brain stem. It may be postulated that clinical symptoms appear as the intensity of lesions increase in these regions from a determinate threshold in a particular individual. Yet it is noteworthy that mild or moderate lesions in the caudate/putamen, even associated with mild tau pathology in the globus pallidus/subthalamus and selected nuclei of the brain stem, are not accompanied by significant clinical deficits, at least those discriminated in the current clinical practice.

Interestingly, midbrain hypometabolism has been identified as an early diagnostic sign in PSP (32), and dopaminergic dysfunction has been characterized in subclinical familial PSP (45). More pertinent in the present context is the observation of early striatal abnormalities in nonaffected individuals in a large kindred with autosomal dominant PSP linked to 1q31.1, as revealed by 18F-dopa and 18-fluordeoxyglucose positron emission tomography (15, 37, 40). Together, neuropathological and imaging data point to the caudate/putamen as the initial vulnerable region in PSP, followed by brain stem and globus pallidus/subthalamus.

Accumulation of phospho-tau in neurons and astrocytes occurred in cases consistent with early stages of PSP-like pathology, whereas coiled bodies in oligodendrocytes were rarely seen.

Moreover, abnormal astrocytes rarely had the morphology of tufted astrocytes. Early astrocytic lesions were rather characterized by fine tau-immunoreactive networks and tau-immunoreactive astrocytes. Tufted astrocytes were absent in case 1, exceptional or very rare in cases 2 and 3, and occasional in case 4. On the one hand, these findings suggest that tufted astrocytes and coiled bodies appear later in the course of the disease. On the other, these observations also indicate that astrocytes are early targets of abnormal tau phosphorylation in the caudate and putamen in cases with early PSP-like pathology. These observations are in line with previous studies suggesting that tau accumulation (and pathology) in astrocytes is a degenerative rather than a reactive process in PSP (47).

Previous studies have shown increased lipid peroxidation, as revealed by increased tissue levels of MDAL and HNE in the subthalamic nucleus, midbrain and superior frontal cortex in PSP brains (1, 2, 30, 34). Moreover, the activity of antioxidant systems such as superoxide dismutase and reduced glutathione is increased in multiple brain regions of PSP with reactive gliosis (13). Finally, stress-related proteins, as stress-activated protein kinase (SAPK/JNK) and p38SAPK, are activated and expressed in neurons and glial cells in PSP (3, 17). In line with these findings, evidence of oxidative stress damage was also observed in the amygdala (selected as a vulnerable region in AGD) and striatum (selected as a susceptible region in cases with PSP-like pathology). Single gel electrophoresis and western blotting showed increased lipoxidative and glycoxidative damage in diseased cases when compared with controls. Changes were observed in the amygdala and striatum thus showing that oxidative damage affects regions vulnerable to AGD and PSP-vulnerable regions as well.

2D gel electrophoresis, western blotting, in gel digestion and mass spectrometry revealed GFAP as a major target of oxidative damage in the striatum in conventional PSP and in the two cases with PSP-like pathology. GFAP was also oxidized in the amygdala in AGD associated with conventional PSP and in one case (case 3) with PSP-like pathology. GFAP has been found modified by oxidation in AD (27, 35) and Pick's disease (33). GFAP oxidation has also been reported in conditions not associated with tau pathology as in aceruloplasminemia (25) and diabetic retina (5). Whether GFAP oxidation is the result of phospho-tau deposition or an unrelated event in astrocytes associated with PSP-like pathology is not solved.

Oxidative damage to GFAP is associated with increased expression of GFAP, as revealed with western blotting and with increased astrogliosis as shown by immunohistochemistry. The present findings further support the concept of early involvement of astrocytes (increased numbers, increased GFAP levels and increased astroglial tau phosphorylation), and of GFAP as a target of oxidative damage in cases with PSP-like pathology.

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## REFERENCES

1. Albers DS, Augood SJ, Martin DM, Standaert DG, Vonsattel JP, Beal MF (1999) Evidence for oxidative stress in the subthalamic nucleus in progressive supranuclear palsy. *J Neurochem* **73**:881–884.
2. Albers DS, Augood SJ, Park LC, Browne SE, Martin DM, Adamson J *et al* (2000) Frontal lobe dysfunction in progressive supranuclear palsy: evidence for oxidative stress and mitochondrial impairment. *J Neurochem* **74**:878–881.
3. Atzori C, Ghetti B, Piva R, Srinivasan AN, Zolo P, Delisle MB *et al* (2001) Activation of the JNK/p38 pathway occurs in diseases characterized by tau protein pathology and is related to tau phosphorylation but not to apoptosis. *J Neuropathol Exp Neurol* **60**:1190–1197.
4. Arima K, Nakamura M, Sunohara N, Ogawa M, Anno M, Izumiyama Y *et al* (1997) Ultrastructural characterization of the tau-immunoreactive tubules in the oligodendroglial perikarya and their inner loop processes in progressive supranuclear palsy. *Acta Neuropathol* **93**:558–566.
5. Baydas G, Tuzcu M, Yasar A, Baydas B (2004) Early changes in glial reactivity and lipoid peroxidation in diabetic rat retina: effects of melatonin. *Acta Diabetol* **41**:123–128.
6. Birdi S, Rajput AH, Fenton M, Donat JR, Rozdilsky B, Robinson C *et al* (2002) Progressive supranuclear palsy diagnosis and confounding features: report on 16 autopsied cases. *Mov Disord* **17**:1255–1264.
7. Braak H, Braak EArgyrophylic grains: characteristic pathology of cerebral cortex in cases of adult-onset dementia without Alzheimer changes. *Neurosci Lett* **76**:124–127.
8. Braak H, Braak E (1989) Cortical and subcortical argyrophylic grains characterize a disease associated with adult onset dementia. *Neuropathol Appl Neurobiol* **15**:13–26.
9. Braak H, Braak E (1998) Argyrophilic grain disease: frequency of occurrence in different age categories and neuropathological diagnostic criteria. *J Neural Transm* **105**:801–819.
10. Braak H, Braak E (1999) Temporal sequence of Alzheimer's disease-related pathology. In: *Cerebral Cortex Vol 14: Neurodegenerative and Age-Related Changes in Structure and Function of Cerebral Cortex*. A Peters, JH Morrison (eds), pp. 475–512. Kluwer Academic/Plenum Press: New York, Boston, Dordrecht, London, Moscow.
11. Buee L, Bussiere T, Buee-Scherrer V, Delacourte A, Hof PR (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res Brain Res Rev* **33**:95–130.
12. Burn DJ, Lees AJ (2002) Progressive supranuclear palsy: where are we now? *Lancet Neurol* **1**:359–369.
13. Cantuti-Castelvetri I, Keller-McGandy CE, Albers DS, Beal MF, Vonsattel JP, Standaert DG, Augood SJ (2002) Expression and activity of antioxidants in the brain in progressive supranuclear palsy. *Brain Res* **930**:170–181.
14. Delacourte A, Buee L (2000) Tau pathology: a marker of neurodegenerative disorders. *Curr Opin Neurol* **13**:371–376.
15. de Yebenes JG, Sarasa JL, Daniel SE, Lees AJ (1995) Familial progressive supranuclear palsy. Description of a pedigree and review of the literature. *Brain* **118**:1095–1103.
16. Ferrer I, Barrachina M, Tolnay M, Rey MJ, Vidal N, Carmona M *et al* (2003) Phosphorylated protein kinases associated with neuronal and glial tau deposits in argyrophilic grain disease. *Brain Pathol* **13**:62–78.
17. Ferrer I, Blanco R, Carmona M, Puig B (2001) Phosphorylated mitogen-activated protein kinase (MAPK/ERK-P), protein kinase of 38 kDa (p38-P), stress-activated protein kinase (SAPK/JNK-P), and calcium/calmodulin-dependent kinase II (CaM kinase II) are

differentially expressed in tau deposits in neurons and glial cells in tauopathies. *J Neural Transm* **108**:1397–1415.
18. Ferrer I, Santpere G, van Leeuwen FW (2007) Argyrophilic grain disease. *Brain* On line, February 2008 (publication ahead).
19. Golbe LI, Ohman-Strickland PA (2007) A clinical rating scale for progressive supranuclear palsy. *Brain* **130**:1552–1565.
20. Grafman J, Litvan I, Stark M (1995) Neuropsychological features of progressive supranuclear palsy. *Brain Cogn* **28**:311–320.
21. Hauw JJ, Agid Y (2003) Progressive supranuclear palsy (PSP) or Steele-Richardson-Oslzewski disease. In: *Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders*. DW Dickson (ed), pp. 103–114. ISN Neuropath Press: Basel.
22. Hauw JJ, Verny M, Ruberg M, Duyckaerts C (1988) The neuropathology of progressive supranuclear palsy (PSP) or Steele-Richardson-Olszewski disease. In: *The Neuropathology of Dementing Disorders*. W Markesberry (ed), pp. 193–218. Edward Arnold: London.
23. Ikeda K, Akiyama H, Arai T, Matsushita M, Tsuchiya K, Miyazaki H (2000) Clinical aspects of argyrophilic grain disease. *Clin Neuropathol* **19**:278–284.
24. Josephs KA, Petersen RC, Knopman DS, Boeve BF, Whitwell JL, Duffy JR *et al* (2006) Clinicopathologic analysis of frontotemporal and corticobasal degeneration and PSP. *Neurology* **66**:41–48.
25. Kaneko K, Nakamura A, Yoshida K, Kametani F, Higuchi K, Ikeda S (2002) Glial fibrillary acidic protein is greatly modified by oxidative stress in aceruloplasminemia brain. *Free Radic Res* **36**:303–306.
26. Komori T (1999) Tau-positive glial inclusions in progressive supranuclear palsy, corticobasal degeneration and Pick's disease. *Brain Pathol* **9**:663–679.
27. Korolainen MA, Auriola S, Nyman TA, Alafuzoff I, Pirtilla T (2005) Proteomic analysis of glial fibrillary acidic protein in Alzheimer's disease and aging brain. *Neurobiol Dis* **20**:858–870.
28. Litvan I, Agid Y, Calne D, Campbell G, Dubois B, Duvoisin RC *et al* (1996a) Clinical research criteria for the diagnosis of progressive supranuclear palsy (Steele-Richardson-Olszewski syndrome): report of the NINDS-SPSP international workshop. *Neurology* **47**:1–9.
29. Litvan I, Agid Y, Jankovic J, Goetz C, Brandel JP, Lai EC *et al* (1996b) Accuracy of clinical criteria for the diagnosis of progressive supranuclear palsy (Steele-Richardson-Olszewski syndrome). *Neurology* **46**:922–930.
30. Martinez A, Dalfó E, Muntane G, Ferrer I (2007) Glycolitic enzymes are targets of oxidation in aged human frontal cortex and oxidative damage of these proteins is increased in progressive supranuclear palsy. *J Neural Transm* August 21 (e-published ahead of print).
31. Martinez-Lage M, Muñoz DG (1997) Prevalence and disease association of argyrophilic grains of Braak. *J Neuropathol Exp Neurol* **56**:157–164.
32. Mishina M, Ishii K, Mitani K, Ohyama M, Yamazaki M, Ishiwata K *et al* (2004) Midbrain hypometabolism as early diagnostic sign for progressive supranuclear palsy. *Acta Neurol Scand* **110**:128–135.
33. Muntane G, Dalfo E, Martinez A, Rey MJ, Avila J, Perez M *et al* (2006) Glial fibrillary acidic protein is a major target of glycoxidative and lipoxidative damage in Pick's disease. *J Neurochem* **99**:177–185.
34. Odetti P, Garibaldi S, Norese R, Angelini G, Marinelli L, Valentini S *et al* (2000) Lipoperoxidation is selectively involved in progressive supranuclear palsy. *J Neuropathol Exp Neurol* **59**:393–397.
35. Pamplona R, Dalfó E, Ayala V, Bellmunt J, Prat J, Ferrer I *et al* (2005) Proteins in human cortex are modified by oxidation, glycoxidation, and lipoxidation. *J Biol Chem* **280**:21522–21530.
36. Papapetropulos S, Gonzalez J, Mash DC (2005) Natural history of progressive supranuclear plasy: a clinicopathological study from a population of brain donors. *Eur Neurol* **54**:1–9.
37. Piccini P, de Yebenez J, Lees AJ, Ceravolo R, Turjanski N, Pramstaller P *et al* (2001) Familial progressive supranuclear palsy: detection of subclinical cases using 18F-dopa and 18fluorodeoxyglucose positron emission tomography. *Arch Neurol* **58**:1846–1851.
38. Puig B, Rey MJ, Ferrer I (2005) Individual and regional variations of phospho-tau species in progressive supranuclear palsy. *Acta Neuropathol* **110**:261–268.
39. Rampello L, Butta V, Raffaele R, Vecchio I, Battaglia G, Cormaci G *et al* (2005) Progressive supranuclear palsy: a systematic review. *Neurobiol Dis* **20**:179–186.
40. Ros R, Gomez Garre P, Hirano M, Tai YF, Ampuero I, Vidal L *et al* (2005) Genetic linkage of autosomal dominant progressive supranuclear palsy to 1q31.1. *Ann Neurol* **57**:634–641.
41. Saito Y, Nakahara K, Yamanouchi H, Murayama S (2002) Severe involvement of ambient gyrus in dementia with grains. *J Neuropathol Exp Neurol* **61**:789–796.
42. Saito Y, Ruberu NN, Sawabe M, Arai T, Tanaka N, Kakuta Y *et al* (2004) Staging of argyrophilic grains: an age-associated tauopathy. *J Neuropathol Exp Neurol* **63**:911–918.
43. Schrag A, Ben-Shlomo Y, Quinn NP (1999) Prevalence of progressive supranuclear palsy and multiple system atrophy: a cross sectional study. *Lancet* **354**:1771–1775.
44. Steele JC, Richardson JC, Olszewski J (1964) Progressive Supranuclear Palsy. A heterogeneous degeneration involving the brain stem, basal ganglia and cerebellum with vertical gaze and pseudobulbar palsy, nuchal dystonia and dementia. *Arch Neurol* **10**:333–359.
45. Tai YF, Ahsan RL, de Yebenes JG, Pavese N, Brooks DJ, Piccini P (2007) Characterization of dopaminergic dysfunction in familial progressive supranuclear palsy: an 18F-dopa PET study. *J Neural Transm* **114**:337–340.
46. Takahashi M, Weidenheim KM, Dickson DW, Ksiezak-Reding H (2002) Morphological and biochemical correlations of abnormal tau filaments in progressive supranuclear palsy. *J Neuropathol Exp Neurol* **61**:33–45.
47. Togo T, Dickson DW (2002) Tau accumulation in astrocytes in progressive supranuclear palsy is a degenerative rather than a reactive process. *Acta Neuropathol* **104**:398–402.
48. Togo T, Isojima D, Akatsu H, Suzuki K, Uchikado H, Katsuse O *et al* (2005) Clinical features of argyrophilic grain disease. A retrospective survey of cases with neuropsychiatric symptoms. *Am J Geriatr Psychiatry* **13**:1083–1091.
49. Togo T, Sahara N, Yen SH, Cookson N, Ishizawa T, Hutton M *et al* (2002) Argyrophilic grain disease is a sporadic 4-repeat tauopathy. *J Neuropath Exp Neurol* **61**:547–556.
50. Tolnay M, Clavaguera F (2004) Argyrophilic grain disease: a late-onset dementia with distinctive features among tauopathies. *Neuropathology* **24**:269–283.
51. Tolnay M, Monsch AU, Probst A (2001) Argyrophilic grain disease. A frequent dementing disorder in aged patients. *Adv Exp Med Biol* **487**:39–58.
52. Tolnay M, Schwietert M, Monsch AU, Staehelin HB, Langui D, Probst A (1997b) Argyrophilic grain disease: distribution of grains in patients with and without dementia. *Acta Neuropathol* **94**:353–358.
53. Tolnay M, Sergeant N, Ghestem A, Chalbot S, de Vos RA, Jansen Steur EN *et al* (2002) Argyrophilic grain disease and Alzheimer disease are distinguished by their different distribution of tau protein isoforms. *Acta Neuropathol* **104**:425–434.
54. Tolnay M, Spillantini MG, Goedert M, Ulrich J, Langui D, Probst A (1997a) Argyrophilic grain disease: widespread hyperphosphorylation of tau protein in limbic neurons. *Acta Neuropathol* **93**:477–484.
55. Williams DR, de Silva R, Paviour DC, Pittman A, Watt HC, Kilford L *et al* (2005) Characteristics of two distinct clinical phenotypes in pathologically proven progressuive supranuclear palsy: richardson's syndrome and PSP-parkinsonism. *Brain* **128**:1247–1258.

## SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

**Figure 1S.** Case 1: GFAP (**A**) and AT8 (**B–D**) immunoreactivity in the caudate (**A**,**B**), globus pallidus (**C**) and subthalamic nucleus (**D**). Moderate gliosis is observed in the caudate together with phosphor-tau accumulation in neurons and glial cells. Only rare neurons are immunoreactive with the AT8 antibody in the globus pallidus and subthalamus. Paraffin section slightly counterstained with haematoxylin. **A**, bar = 50 μm; **B–D**, bar in **D** = 50 μm.

**Figure 2S.** Case 2: AT8 immunoreactivity in the caudate (**A**), amygdala (**B**), CA1 region of the hippocampus (**C**) and dentate gyrus (**D**). Tau immunoreactivity is observed in astrocytes and in neurons in every region. In addition, grains are clearly visible in CA1. Paraffin section slightly counterstained with haematoxylin. Bar = 25 μm.

**Figure 3S.** Case 3: AT8 immunoreactivity in the putamen (**A**), caudate (**B**), amygdala (**C**), CA1 region of the hippocampus (**D**), dentate gyrus (**E**), nucleus basalis of Meynert (**F**), ventral pons (**G**), midbrain (**H**), ceruleus (**I**) and gyrus cinguli (**J**). Tau immunoreactivity is observed in astrocytes and in neurons in every region. Note grains in the CA1 region and amygdale. Rare tufted astrocytes are seen in the caudate (**A**) and midbrain (**H**). Paraffin section slightly counterstained with haematoxylin. Bar = 25 μm.

**Figure 4S.** Case 5: AT8 immunoreactivity in the putamen (**A**), amygdala (**B**), globus pallidus (**C**), dorsomedial nucleus of the thalamus (**D**), subthalamus (**E**), superior colliculus (**F**), ventral pons (**G**), frontal cortex (**H**) and CA1 region of the hippocampus. Paraffin section slightly counterstained with haematoxylin. **A–G**, **I**, bar in **I** = 25 μm; **C**, **E**, **H**, bar in **H** = 50 μm.

**Table 1S.** Summary of neuropathological findings in case 1. AGD classification: early stage 2. AD classification: ADI; no β-amyloid deposition

**Table 2S.** Summary of neuropathological findings in case 2. AGD classification: stage 2. AD classification: ADI; no β-amyloid deposition

**Table 3S.** Summary of neuropathological findings in case 3. AGD classification: stage 3. AD classification: ADIIA

**Table 4S.** Summary of neuropathological findings in case 4. AGD classification: stage 3. AD classification: ADI; no β-amyloid deposition

**Table 5S.** Summary of neuropathological findings in case 5. AGD classification: stage 3. No β-amyloid deposition

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# Discussió

***1-Sensibilitat del teixit cerebral humà als factors postmortem***

***1.1- Degradació de proteïnes***

**La utilització de teixit cerebral postmortem humà va lligada de manera inevitable a condicions de treball sub-òptimes. Això es deu principalment a l'intèrval de temps entre la necròpsia i la congel·lació del cervell, així com a la temperatura a la que es troba el cervell durant aquest intèrval.**

**Els efectes d'aquests factors ja fa més de 30 anys que es van plantejar i començar a estudiar (Kosik 1982), però la majoria dels estudis realitzats (resistència al postmortem de la seva estructura, activitat o modificacions post-traduccionals), fins la moment de la realització dels nostres experiments s'han dut a terme sobre proteïnes concretes i moltes vegades en models animals (Fountoulakis, 2001).**

**Per aquesta raó, el nostre disseny experimental va consistir en:**

1. **Sotmetre teixit cerebral (de l'escorça frontal) que ja de per si teni un postmortem molt curt (al voltant de les 2 hores) a diferents condicions de temperatura (1ºC, 4ºC i temperatura ambient) i durant diferents intervals de temps (2, 5, 8, 23 i 50 hores).**
2. **A partir d'aquí, per una banda es va sel·leccionar una sèrie de proteïnes (proteïnes sinàptiques, cinases, receptors de factors de creixament, del citosesquelet, de membrana; així com proteïnes relacionades amb l'estrès oxidatiu, amb vies apoptòtiques o amb el sistema proteosomal) per tal d'estudiar la seva sensibilitat al postmortem. I, per altra banda, es va dur a terme un anàlisi més general mitjançant gels bidimensionals per tal d'analitzar el comportament de les proteïnes del cervell a "grosso modo".**

**Les dues aproximacions ens van portar a concloure la mateixa idea ja descrita anteriorment per altres autors (Hilbig et al., (Li et al., 1996), (Schwab et al., 1994), (Irving et al., 1997): a una determinada temperatura cada proteïna resisteix a la seva manera particular l'intèrval postmortem. Algunes són molt sensibles i es degraden ràpidament com l'alfa-sinucleïna, mentre que d'altres resisteixen fins al máxim de temps estudiat, com la 14-3-3. A nivell pràctic, això**

**implica que quan es treballa amb aquest tipus de material, és recomanable analitzar prèviament resistència de la proteïna d'interès al postmortem per descartar la possibilitat que es tracti d'una proteïna sensible a les poques hores. Depenent dels resultats l'investigador es pot permetre uns temps postmortem de les seves mostres o bé uns altres.**

**La temperatura també és un factor rellevant en la preservació de les proteïnes. Les condicions de menor temperatura, en el nostre cas 1ºC, va acomanyada d'una menor degradació, on proteïnes especialment vulnerables, com la sinucleïna, es mantenen constants durant els intèrvals analitzats. Tenint en compte que la preservació del cos es produirà en el millor dels casos a 4ºC i mai o quasi a 1ºC, és esperable que la nostra mostra haurà estat subjecte a la dregradació.**

**Posteriorment als nostres estudis sobre la degradació de les proteïnes n'han sorgit d'altres en la mateixa linia. Amb una aproximació similar, mitjançant gels bidimensionals però utilitzant mètodes amb més possibilitats de quantificació (DIGEs), es van identificar altres proteïnes sensibles al postmortem artificial (Crecelius et al 2008). A més de parar atenció a les proteïnes que disminuien o que es mantenien, també es van llistar proteïnes que augmentaven la seva presència. El perquè hi ha augment de determinades proteïnes amb el temps postmortem és un enigma; però es podria deure a la creació de formes truncades, a un augment de síntesi o bé a una disminució de l'eliminació normal d'aquestes proteïnes.**

**A més de la degradació proteïca, altres estudis estant tractant la degradació de l'ADN o de l'ARN en les mostres postmortem preservades de diferent manera (incloses en parafina o congelades). A més, també s'ha estudiat la sensibilitat al temps postmortem de les principals modificacions epigenètiques de l'ADN: la metil·lació de CpGs, i l'acetil·lació d'histones.**

***1.2- Fosforil·lació i truncatge de la proteïna tau***

**En un segon estudi ens vam marcar l'objectiu d'estudiar els efectes del temps postmortem sobre una proteïna concreta de gran importància en la malaltia d'Alzheimer, la proteïna tau. En aquest cas, estàvem més interessats en**

**dos aspectes post-traduccionals de suma importància tant en MA com en altres taupaties, descrits en aquesta proteïna: la fosforil·lació i el truncatge. La fosforil·lació, però, està molt més estudiada que el truncatge.**

**Les fraccions enriquides en filaments de tau, de les mostres d'escorça frontal de malalts d'Alzheimer, sotmeses al postmortem artificial van mostrar que el triplet de tau hiperfosforil·lada pateix una modificació apreciable amb l'aparició d'una nova banda, de pes molt semblant, en algun moment entre les 6 i les 24 hores de postmortem. Aquesta banda podria ser resultat de l'acció de fosfatases endògenes, i posa de manifest que la fosforil·lació de les proteïnes és un fenòmen sensible a l'intèrval entre la mort i la congelació. De totes maneres, la hiperfosforil·lació de tau en MA resulta ser més resistent que la fosforil·lació de la tau normal (la qual s'ha descrit que es desfoforil·la en qüestió de minuts després de la mort (Sorimachi et al. 1996)).**

**Els anticossos fabricats per reconèixer proteïna tau sovint detecten altres bandes de menor pes molecular. La naturalesa d'aquestes bandes no es coneix però s'hipotetitza que es podria tractar de fragments de tau. El model experimental anterior ens indica que les bandes de baix pes molecular, siguin o no fragments de proteïna tau, no són producte de la degradació postmortem pel fet que el patró de bandes baixes es troba present des del primer punt analitzat amb el menor temps postmortem possible.**

***1.3- Estrés oxidatiu i localització cel·lular***

**Altres estudis publicats s'han centrart en la preservació d'altres tipus de modificacions proteïques, presents a les malalties neurodegeneratives, com són les modificacions per estrès oxidatiu. La detecció d'adductes de lipo i glico-oxidació en les proteïnes com MDAL, HNE, CEL o CML s'ha observat que es manté constant al llarg del postmortem artificial fins a les 8 hores. Al voltant de les 20 hores, algunes bandes augmenten i d'altres disminueixen. Això sembla indicar que durant el postmortem algunes proteïnes continuen essent oxidades mentre d'altres són degradades (Ferrer et al., 2008).**

**Per últim, les condicions de postmortem poden afectar la localització cel·lular de les proteïnes. En particular s'ha observat que factors de transcripció**

**que es troben en el nucli quan es fa una biòpsia de teixit (com c-fos, jun i factors de transcripció de la família Sp) , es troben en el citoplasma quan s'observen em el teixit postmortem (MacGibbon et al., 1997), (Boutillier et al., 2007).**

**Aquests tipus d'estudis tenen especial importància en bancs de teixit, on es dipositen i preserven mostres de cervell que han de servir per la recerca. És important que els protocols s'estandaritzin entre els diferents hospitals i bancs. La qüestió radica en identificar les limitacions que presenta la recerca amb aquest tipus de mostra i com, en alguns casos, es poden millorar les pràctiques per fer recular el màxim possible aquestes limitacions. És també rellevant la delimitació de l'efecte del postmortem en les diferents tècniques de preservació (congel·lació, parafina, etc) per tal de fer una correcta comparació dels experiments. Pot passar que un increment d'expressió observat en immunohistoquímica no s'observi per Western Blot pel fet que determinada proteïna es preserva de manera diferent en una mostra parafinada o congel·lada.**

**En algun grau o altre, la degradació de proteïnes sempre serà present en la recerca en malalties neurodegeneratives, perquè el factors pre-, peri- i postmortem es poden control·lar només fins a cert punt. Mentre es fan aquest tipus d'estudi per tal acotar aquest problema, altres investigadors busquen possibilitats d'estudi alternatives. Una d'elles és, per exemple, estudiar la microglia, un factor important en la neuroinflamació present en la MA, a partir de les seves cèl·lules precursores de la sang, els monòcits de cada individu. Allunyant-se una mica del cervell, sortegen el problema de la degradació (Hamacher et al., 2007).**

***2- Tau en MGA, PSP i MA***

***2.1- Bandes de baix pes molecular***

**Un dels aspectes que vam voler analitzar en el nostre estudi és el patró per Western blot de la proteïna tau en les fraccions sarkosyl-insolubles (ens referim a la mateixa fracció que l'anomenem fracció enriquida en PHFs). Una de les preguntes que volíem resoldre, una vegada observat el fet que les bandes baixes de tau no semblen producte de la degradació post-mortem, era si aquest patró era igual entre totes les taupaties o, per contra, era específic de cada**

**malaltia. En aquests experiments es va inlcoure la MGA, que és una taupatia que acapara relativament poca atenció, encara que explica al voltant del 5% dels casos de demència i apareix molt freqüentment en combinació amb altres malalties neurodegeneratives.**

**Són molt pocs els estudis sobre el patró de bandes baixes de tau. En un d'ells, els autors proposaven que la taupatia 4R PSP es podia diferenciar de la també taupatia 4R DCB per la presència d'una banda forta a 37KDa en la PSP, i un doblet a 42KDa en la DCB (Arai et al., 2001, Arai et al., 2004). Un estudi posterior realitzat en el nostre grup va mostrar que tant la banda de 37KDa com el doblet de 42KDa estaven presents també en la PSP (Puig et al., 2005). En MGA es mostren però no es descriuen (Tolnay et al., 2002).**

**L'estudi del patró de bandes baixes de la MGA mostra que el patró resultant és molt similar al d'altres taupaties 4R com la PSP, la DCB i algunes DFTP-17. En canvi, el patró difereix del que es troba en la MA, on tant isoformes 3R com 4R es troben fosforil·lades. En el cas del malalt que presentem que patia al mateix temps MA i MGA, el patró és molt similar al de la MA.**

**En el conjunt dels treballs que presentem aquí en tres malalties diferents (MA, PSP i MGA) on s'han estudiat els patrons complets de tau, i d'altres reportats en la literatura, sembla que el patró de bandes baixes depèn en gran mesura de les isoformes que es veuen hiperfosforil·lades. Altres estudis realitzats pel nostre grup, però que no es presenten en aquesta tesi sobre DFTP-17 de tipus 4R donen el mateix patró que la resta de taupaties 4R, cosa que reforça la hipotesi que el patró de bandes baixes depèn de les isoformes hiperfosfoforil·lades.**

**A partir dels resultats obtinguts en el treball en MA, on estudiávem el possible efecte del postmortem en la presència de bandes baixes, també podem afirmar que la regió de la proteïna cap on está dirigit cada anticòs també és un aspecte rellevant en el tipus de patró de bandes baixes que apareix. Com més cap al domini C-terminal està dirigit l'anticòs, major nombre de bandes baixes es reconeixen i també de menor pes molecular. Aquest resultat suggereix que aquestes bandes més baixes corresponen a fragments C-terminals de la proteïna.**

***2.2 Estudi de l'expressió de proteases de tau***

**Com dèiem a la introducció, la trombina és una proteasa que s'expressa al cervell i és capaç de tallar la proteïna tau. En els nostres experiments hem confirmat que s'acumula en els cabdells neurifibril·lars en diferents taupaties (incloent la MA i i el cossos de Pick a la MPi) i vam voler comprovar si també ho fèia en els grans argiròfils, i en les estructures pre-cabdell, característics de la MGA. També vam estudiar l'associació d'aquestes estructures amb altres proteases amb l'habilitat de tallar la tau, com la caspasa-3 o la calpaïna-2.**

**El resultat va ser que, mentre la trombina si que es trobava present en els grans argiròfils i en els pre-cabdells, la calpaïna-2 i la caspasa-3 no. Aquestes últimes es trobaven present en el cabdells neurofibril·lars madurs, però no en els pre-cabdells ni als grans argiròfils. Per tant, si alguna proteasa pot ser candidata a tallar la tau *in vivo* en estadis primerencs de la formació de les inclusions i també en els grans, una d'elles pot ser la trombina.**

**Per altra banda, igual que ocorre en els cabdells ja formats, a la MPi hem trobat la caspasa-3 present en els cossos de Pick, la qual cosa suggereix la possibilitat de que aquesta proteasa estigui digerint la tau en aquestes inclusions.**

***3- Factors de transcripció en inclusions de tau***

**Els factors de transcripció són proteïnes que poden regular l'expressió de determinats gens unint-se a seqüències específiques dels seus promotors. Sovint, l'activació d'aquests factors de transcripció succeeix al citoplasma i posteriorment hi ha una translocació al nucli on finalment realitzen la seva funció. Algunes inclusions proteïques en diferents malalties neurodegeneratives atrapen factors de transcripció en el lloc de la inclusió, la qual cosa podem pensar que tindrà un efecte en la regulació dels gens que controla el factor de transcripció atrapat.**

### *3.1 Sp1*

**Un exemple de l'efecte del segrest de factors de transcripció sobre l'expressió gènica el trobem en un experiment en cultiu cel·lular on s'estudia l'expressió de la proteïna huntingtina mutada, causant de les inclusions intranuclears de la malaltia de Huntington (DiFiglia et al., 1997). En aquest experiment *in vitro* s'observa que la huntingtina mutada efectivament segresta l'Sp1, i aquest ja no es pot unir al promotor del gen de NGFR (Nerve Growth Factor Receptor) suprimint així la seva expressió (Li et al., 2002).**

**Els nostres resultats mostren que, mitjançant immunohistoquímica i immunoflorescència, Sp1 es troba també a les inclusions intracitoplasmàtiques de tau a la MA, PSP i MPi, però no en les inclusions de sinucleïna de les sinucleïnopaties estudiades. Els efectes del segrest d'Sp1 observat en les cèl·lules que contenen inclusions encara són desconeguts. Però una possibilitat que es pot contemplar és el descontrol de l'expressió de proteïnes regulades per Sp1 entre les que poden tenir especial rellevància l'APP (Docagne et al., 2004), BACE 1 i 2 (Sun et al., 2005), tau (Heicklen-Klein and Ginzburg, 2000), caspasa-3 (Liu et al., 2002), IL-1beta (involucrades totes elles en la MA) o proteïnes relacionades amb la defensa contra l'estrès oxidatiu com la SOD-2 (Xu et al., 2002). De totes manetes, existeixen moltes altres proteïnes amb caixes Sp1 en els seus promotors, de manera que és difícil de saber la magnitut del dany que pot produir l'absència d'Sp1 al nucli quan se'l necessita. De fet, degut a la diversitat d'observacions sobre el seu efecte en diferents models, no hi ha consens en si es podria considerar l'Sp1 una diana terapèutica amb l'objectiu d'estimular-lo o d'inhibir-lo.**

**En el nostre estudi també vam tractar d'obervar variacions en l'expressió d'Sp1 en els cervells de pacients de MA comparats amb els controls. Segons la nostra experiència, els nivells d'Sp1 eren similars en tots els casos. Posteriorment s'ha publicat que els nivells d'expressió a nivell de ARN missatger d'Sp1 són superiors en els cervells de malalts de MA comparats amb els controls; així com els dels cervells de ratolins transgènics per PSEN1 i/o APP comparats amb els Wt; en els ratolins la variació es descriu també a nivell de proteïna (Citron et al., 2008). El motiu d'aquesta discrepància no està clar i**

**caldran més experiments en aquest sentit per establir si els nivells d'Sp1 varien o no en la MA.**

**Finalment, un estudi recent es fixa en l'expressió en cervell de malalts de MA d'altres membres de la família Sp, com són Sp3 i Sp4. Els autors observen un forta presència d'aquests dos factors en els dipòsits de tau, igual que Sp1, i a més, un fort augment de la seva expressió (Boutillier et al., 2007). Els membres de la família Sp reconeixen el mateix motiu d'ADN i competeixen per la regulació dels gens diana. Tenint en compte això i que els factors de transcripció Sp regulen cadascú a la seva manera l'expressió gènica, és molt rellevant el ratio d'expressió d'uns respecte els altres (Li et al., 2004); però fa igualment molt difícil de predir l'efecte gobal sobre l'expressió gènica.**

***3.2 c-Fos, c-Jun, ATF2 i CREB***

**Quan s'estudia mitjançant tècniques d'immunohistiquímica la presència de proteïnes en inclusions de tau hiperfosforil·lada, especialment quan l'estudi es fa amb anticossos contra epítops fosforil·lats, és molt important descartar en la mesura possible la reacció creuada de l'anticos amb els múltiples epítops fosforil·lats de la pròpia tau. El què fem en aquests casos és provar els anticossos per Western blot sobre fraccions enriquides en filaments de tau de diferents taupaties i observar si l'anticos reconeix el patró de bandes de tau, a més de la banda pròpia de la proteïna. En el cas de l'anticos contra ATF2 fosforil·lat això és exactament el què vam observar, de manera que la presència d'ATF2 fosforil·lat a les inclusions de la MPi és probablement un artefacte, sobretot si tenim en compte que l'anticos contra ATF2 no fosforil·lat no tenyeix les inclusions.**

**En un estudi molt similar al de l'apartat anterior, a la MPi hem observat un augment d'expressió tant de c-Fos, com de c-Jun, ATF2 i CREB. Aquesta sobreexpressió s'observa tant a l'escorça frontal com a l'hipocamp. Es ben conegut que la fosforil·lació juga un paper fonamental en l'activació d'aquests factors, i per això es van util·litzar anticossos contra les seves formes fosforil·lades i actives. El resultat va ser que els nuclis d'aquestes regions estudiades mostraven un augment de les formes fosforil·lades de c-Fos, c-Jun i**

**CREB. A més, c-Fos i ATF2 fosforil·lat es van trobar a l'interior dels cossos de Pick en colocalització amb la tau hiperfosforil·lada.**

**A priori es relaciona c-Jun amb mort neuronal per apoptosi, via l'alliberació del citocrom C o a través del control de l'expressió del gen del Fas-L (Kasibhatla et al., 1998, Ham et al., 2000). Els nostres resultats però, també mostren que aquest increment respecte els casos control es presenten tant en zones amb mort neuronal (l'escorça frontal), com en poblacions neuronals que tot i presentar patologia de tau (cossos de Pick) resisteixen i sobreviuen (les cèl·lules granulars del gir dentat de l'hipocamp). Es fa doncs complicat establir una relació directa entre l'activació i increment d'aquests factors i les respostes de supervivència o mort neuronal.**

**Estudis previs realitzats en el nostre laboratori havien mostrat que c-Jun i CREB s'expressaven de manera diferencial en models en rata d'excitoxicitat per àcid kaínic (Ferrer et al. 2002). A l'escorça entorrinal, on les cèl·lules moren per l'acció del kaínic, hi havia una forta expressió de c-Jun fosforil·lat però no de CREB. Però de manera similar als resultats obtinguts ara en la MPi, c-Jun també es trobava augmentat en les cèl·lules del gir dentat, que si sobrevivien a la matança provocada per l'àcid kaínic (Ferrer et al., 2002). Els presents resultats en la MPi confirmen que la fosforil·lació de c-Jun no ha d'estar relacionada per força amb la mort neuronal. En el model de rata, CREB fosforil·lat es veia disminuit a l'escorça entorrinal i no variava els seus nivells en el gir dentat. En cervells de malalts de la malaltia de Creutzfeldt-Jakob, CREB també es troba disminuït, però a nivell d'expressió total, no només de CREB fosforil·lat (Rodriguez and Ferrer, 2007). A partir d'aquests treballs sembla que la disminució de CREB fosforil·lat es produeix en zones afectades amb mort neuronal i que en les resistents, o no varia o augmenta la seva expressió. En aquestes regions resistents de l'hipocamp CREB fosforil·lat augmenta els seus nivells en resposta a la hipòxia (Walton and Dragunow, 2000). Es podria pensar que el fet de trobar CREB fosforil·lat augmentat en MPi també a l'escorça frontal pot tenir relació amb el nivell d'afectació. En estadis tardans de malalties com el Creutzfeldt-Jakob de l'estudi citat, la disminució de CREB fosforil·lat podria explicar-se com a un esgotament de la resposta anti-apoptòtica o anti-excitotòxica protagonitzada per aquest factor de transcripció.**

***4- Aspectes patològics de la MGA***

**La revisió de la taupatia MGA ha inclòs experiments originals que han aportat noves dades sobre l'estrès oxidatiu, els patrons de bandes de tau, i la presència de p62, UBB+1, cinases i proteases a les inclusions; així com una proposta d'estadiatge de la malaltia i un paradigma integrador dels aspectes patogènics centrat en l'estrès oxidatiu, el segrestosoma (p62/UBB) i el truncatge de tau.**

**En resum, l'edat avançada, l'estrès oxidatiu i l'activació de cinases de tau com la GSK3-beta comporten un augment de la hiperfosforil·lació de tau. Aquesta s'associa a la p62 i a la UBB formant les inclusions patològiques en forma de cabdells, pre-cabdells i grans argiròfils. Paral·lelament, l'activació de proteases com la trombina tallen la tau tot fomant fragments que participen també en la formació dels agregats i en augmentar l'estrès oxidatiu. A més, la presència de UBB+1 saturaria la capacitat del proteasoma de degradar la tau fosforil·lada i pre-agregada, participant també en la formació de les inclusions.**

**Cal dir que aquesta paradigma podria ser aplicable a altres taupaties donat el fet que l'edat, l'estrés oxidatiu i l'activació de cinases i proteases és un fenomen no exclusiu de la MGA. Per què en cada taupatia es fosforil·len unes isoformes específiques, es formen inclusions específiques, seguint uns estadiatges i unes regions diferents, és encara un misteri.**

***5- LRRK2 en taupaties***

**Entre els possibles components de les inclusions de tau n'hi ha un que és especialment interessant, l'Lrrk2. Aquesta proteïna acapara l'atenció desde que es va descobrir, pel fet que pot estar darrera de mecanismes comuns de neurodegeneració tant de sinucleïnopaties com de taupaties (Mata et al., 2006), tal i com s'ha comentat a la introducció.**

**El nostre objectiu va ser el de revisar tots els treballs on s'hagués estudiat la presència de Lrrk2 en les inclusions tant de sinucleïna com de tau, atès que existia un controvèrsia molt forta sobre aquest tema propiciada per l'obtenció de resultats diferents en funció dels anticossos util·litzats.**

**En la revisió presentem una sèrie d'experiments originals que inclouen la util·lització de quatre anticossos comercials dirigits contra diferents epítops de la proteïna. Els anticossos es van util·litzar tant per anàlisi immunohistoquímics de diferents sinucleïnopaties i taupaties, com per Western blot en extractes de cultius cel·lulars (de tres línies diferents), o en homogenats totals i fraccions sarkosil-insolubles de diferents taupaties.**

**Els resultats obtinguts són consistents amb els obtinguts per la resta d'autors. *I.e*: els anticossos NB-300-268 i NB-300-267, dirigits contra epítops C-terminal i N-terminal respectivament, van ser els únics capaços de marcar les inclusions tant de sinucleïnopaties com de taupaties. En el grup de taupaties vam afegir la MGA, que no havia estat estudiada, i es va veure que aquests anticossos també marcaven els grans argiròfils. Per contra, l'anticos AP7099b, dirigit contra epítops més interns de la proteïna i que havia estat descrit com un dels més específics (Giasson et al., 2006), no va ser capaç de marcar cap tipus d'inclusió.**

**Una de les hipòtesi que suggereixen alguns autors és que les inclusions estan enriquides en formes truncades de Lrrk2, o bé que els epítops interns queden emmascarats (Higashi et al., 2007). En el nostre cas però, vam util·litzar un anticos que reconeix un epítop C-terminal molt proper al del l'anticos NB-300-268, que tampoc va ser capaç de marcar cap inclusió.**

**Vist que tots aquests anticossos en major o menor grau detecten bandes adicionals a la de Lrrk2 sencera en Western blot, cal plantejar-se la possibilitat que els anticossos que tenyeixen les inclusions siguin inespecífics. Aquesta revisió té la intenció de servir de punt de partida pels propers anàlisi immunohistoquímics que es realitzin, coneixent quins han estat els resultats obtinguts fins ara, amb quines tècniques, amb quins protocols i sobretot, amb quins anticossos.**

***6- Estrés oxidatiu***

**Tal i com comentàvem a la introducció, sembla prou consistent l'afirmació de què l'estrès oxidatiu es troba incrementat en diferents malalties neurodegeneratives, i aquest increment s'observa amb diferents marcadors (de**

**glicooxidació, de lipoxodacio, nitrotirosines o modificacions dels àcids nucleics). En els nostres estudis ens hem centrat en el dany oxidatiu que pateixen les proteïnes amb un objectiu: identificar quines proteïnes estan més oxidades en la patologia respecte les mostres que provenen de persones control. L'oxidació d'una proteïna pot afectar la seva activitat, de manera que identificar les proteïnes diana ens pot donar pistes per identificar les vies susceptibles o perjudicades a la malaltia.**

***6.1- Proteïnes oxidades en inclusions de tau a la MA***

**Un dels possibles efectes de l'adhesió d'adductes d'oxidació a les proteïnes és el de fer-les susceptibles a l'agregació (Garrison et al., 1962, Davies, 2001). En la MA s'acumul·la tau hiperfosforil·lada a l'interior d'algunes neurones formant els cabdells neurofibril·lars. La nostra idea va ser intentar identificar proteïnes oxidades que puguessin estar atrapades en aquests cabdells de tau.**

**Per estudiar això vam realitzar el mateix protocol per tal d'enriquir en filaments de tau en fraccions sarkosil-insolubles, principal component dels cabdells neurofibril·lars, i provar diferents marcadors d'estrés (CML, CEL, HNE i MDAL) en gels monodimensionals. D'aquesta manera vam identificar dues bandes comparativament més oxidades en malalts d'Alzheimer, que mitjançant gels bidimensionals vam relacionar amb dues isoformes de la família de proteïnes 14-3-3 (la gamma i la zeta). Aquestes apareixien modificades tant per CEL com per MDAL (glico i lipooxidació, respectivament), però no per altres modificacions com CML o HNE.**

**Un cop realitzat aquest estudi, vam creure interessant saber si aquestes formes de 14-3-3 estaven situades ens els cabdells quan s'analitzava mitjançant immunohistoquímica. Aquest aspecte ja havia estat estudiat per altres autors, com es comentava a la introducció, que havien trobat diferents isoformes i amb diferents anticossos, una col·localització amb la tau en les inclusions en la MA (Layfield et al., 1996, Umahara et al., 2004b); així com en els cossos de Pick a la MPi (Umahara et al., 2004a). Per contra, el nostre propi anàlisi immunohistoquímic convencional o amb immunofluorescència realitzat amb dos anticossos contra diferents epítops de 14-3-3 va mostrar només marcatge al citosol de les neurones, i no en els cabdells neurofibril·lars. Aquests dos**

**anticossos no presentaven gens ni mica de reactivitat creuada amb la tau quan es van provar en Western blot. No podem assegurar el mateix dels anticossos emprats pels dos equips anteriorment citats. La presència o no d'aquestes isoformes de 14-3-3 en les inclusions de tau, i l'origen de les discrepàncies al respecte, requerirá aprofundir més en aquest estudi.**

**La possibilitat que la 14-3-3 (la forma zeta en especial) estigui present pot basar-se en diverses observacions, principalment: que les isoformes zeta i beta de 14-3-3 poden interactuar amb la tau en extracte de cervell (Hashiguchi et al., 2000); que la 14-3-3 pot mantenir activa la forma inactiva d'una de les principals cinases de tau, la GSK3-beta (Yuan et al., 2004); que és capaç també de interaccionar simultàniament amb la GSK3-beta i la tau (Agarwal-Mawal et al., 2003); i que és capaç de promoure la fosforil·lació de tau estimul·lant la proteïna cinasa depenent d'AMPc (Hashiguchi et al., 2000). A més, també s'ha vist que la 14-3-3 zeta és capaç, *in vitro*, de facilitar l'agregació de la tau (Hernandez et al., 2004).**

**De totes maneres, tot i que les cinases amb les que la 14-3-3 pot interactuar es troben en els cabdells de tau i que tots aquests processos descrits fins ara recolzen la presència de 14-3-3 en el seu interior, res no impedeix suggerir que aquests mateixos processos es podrien estar produint fora dels cabdell o fins i tot abans de la seva formació.**

**Tornant a l'objectiu del nostre treball, la 14-3-3, estigui o no en els cabdells neurofibril·lars, apareix oxidada en les fraccions sarkosyl-insolubles. Per veure si aquesta oxidació afectava només a la 14-3-3 present en aquesta fracció o a tota la citosòlica, vam repetir l'experiment però utilitzant homogenats totals. Els nivells d'oxidació de 14-3-3 en aquesta fracció total eren indetectables amb CEL i pràcticament indistingibles dels controls, amb MDAL. Així doncs, tant si és perquè un cop oxidada s'associa als filaments de tau, o perquè forma altres agregats o conformacions més insolubles, la 14-3-3 (zeta i gamma) es troba més oxidada en la fracció sarkosyl-insoluble en la MA.**

**Poc abans de l'inici del nostre estudi, s'havia identificat la proteïna 14-3-3 zeta com una de les quatre proteïnes significativament oxidades en neurones després del tractament *in vitro* amb pèptid amiloide (Sultana et al., 2006). La 14-**

**3-3 zeta també apareix oxidada en sinaptosomes aïllats i tractats amb pèptid amiloide (Boyd-Kimball et al., 2005a), així com a l'hipocamp d'un model de rata al qual es realitza una injecció intracerebral del mateix pèptid (Boyd-Kimball et al., 2005b). Finalment, la 14-3-3 gamma s'ha trobat nitrada en estadis intermitjos de MA (III i IV de Braak i Braak) (Sultana et al., 2007). Realment, l'oxidació d'aquestes isoformes de 14-3-3 sembla estretament lligada a la presència d'amiloide, per això vam estudiar l'oxidació d'aquestes proteïnes en una malaltia que presenta acumul·lació d'amiloide però no de tau: l'angiopatia amiloidea (AAC). En el cas d'aquesta malaltia la 14-3-3 es va trobar oxidada per CEL només a les fraccions sarkosil-insolubles, mentre que amb MDAL també es trobava oxidada en les fraccions totals. Pel què fa a la glicooxidació per CEL, tan en la MA com en AAC, la 14-3-3 només queda marcada en les fraccions sarkosil insolubles, indicant que aquesta proteïna pateix un canvi de solubilitat que no ha de tenir res a veure amb els acúmuls de tau, els quals estan totalment absents en els casos de AAC.**

**En el transcurs de posteriors estudis sobre marcadors d'estrès oxidatiu, el nostre equip ha trobat moltes vegades un gran variabilitat entre els nivells d'oxidació dels casos control, que no només s'explica per les diferències d'edat. La tan subtil diferència entre els controls i el casos de MA en els nivells de MDAL dels homogenats totals ens van fer replantejar la possibilitat d'augmentar molt més el nombre de casos per establir més correctament aquest aspecte particular del treball. L'experiment va mostrar nivells similars d'expressió tant de MDAL com de la pròpia 14-3-3, la qual cosa va invalidar la nostra conclusió sobre els majors nivells d'oxidació per MDAL als homogenats totals. Es va advertir a la revista i l'article es va retirar. El fet que els nivells de 14-3-3 zeta siguin similar als controls a la MA contrasta amb certs treballs publicats que presenten una sobreexpressió de 14-3-3 zeta a nivell de transcripció (Soulie et al., 2004). També s'ha vist que les isoformes gamma i epsilon (Fountoulakis et al., 1999) estan més presents en regions afectades per tau en cervells de MA.**

### *6.2- Estrés en estadis primerencs de malalties neurodegeneratives*

**Les malalties neurodegeneratives en estadis finals són incurables i el máxim interès es centra en identificar els canvis patològics que es produeixen**

**com més al principi millor de la malaltia, amb l'objectiu de millorar el diagnòstic precoç i de trobar dianes terapèutiques que l'aturin només començar.**

**Un dels aspectes que acapara l'atenció és la presència o no de formes d'estrés oxidatiu en etapes molt primarenques de la malaltia, o en regions del cervell encara no afectades pels aspectes neuropatològics d'aquesta. Sobretot, el què a nosaltres ens interessa és la identificació de les proteïnes diana d'aquest estrès, que poden donar pistes de quines proteïnes perden la seva funció normal en les primeres etapes de cada malaltia.**

**Un exemple d'aquest tipus d'estudi és el que s'ha portat a terme en els darrers anys en l'espectre de sinucleïnopaties que anomenem malalties amb cossos de Lewy (MP i DCL). En escorça cerebral de cervells amb MP o amb MP incidental (preclínica), on no hi ha presència de cossos de Lewy, s'han trobat ja augmentats marcadors de glicooxidació i lipooxidació, així com un increment de l'expressió del receptor d'AGEs (el RAGE) (Dalfo et al., 2005). Entre les proteïnes possiblement oxidades que s'han identificat fins al moment a l'escorça cerebral de cervells amb MP, hi ha: la pròpia alpha-sinucleïna (Dalfo and Ferrer, 2008), gamma-sinucleïna i SOD2 (Dalfo et al., 2005), SOD1 (Choi et al., 2005) i UCHL-1 (Choi et al., 2004). Recentment, s'ha trobat que un sèrie d'enzims del metabolisme energètic (aldolasa A, enolasa 1 i gliceraldehid deshidrogenasa) es troben marcats per l'adducte de lipooxidació HNE també en l'escorça frontal de MP incidental i MP (Gomez and Ferrer, 2008).**

**En el cas de la MA, alguns estudis s'han dedicat a analitzar els nivells d'oxidació en els cervells amb estadis intermitjos de la malaltia (III i IV de Braak i Braak), on la clínica es comença a manifestar d'una forma lleu amb discretes alteracions cognitives; estadi clínic que s'anomena MCI (de l'anglès, Mild Cognitive Impairment). Aquest estrès s'observa en forma d'un increment dels nivells d'HNE i d'un considerable llistat de proteïnes oxidades (Keller et al., 2005, Butterfield et al., 2006, Williams et al., 2006, Sultana et al., 2007). Molt recentment ha sortit el primer estudi sobre dianes proteïques d'estrès oxidatiu en estadis encara més primerencs de la malaltia (I i II de Braak i Braak). Aquest treball identifica la proteïna ATP-sintasa com a diana de lipooxidació a l'escorça entorrinal, així com una disminució de la seva activitat, amb possible conseqüències per la producció mitocondrial d'energia en aquesta primera etapa de la MA (Terni et al, 2009, en premsa).**

### *6.2.1- Estadis primerencs de PSP*

**Aquí s'han presentat resultats referents a l'estrès oxidatiu en estadis primerencs de PSP, aspecte aquest que fins ara no havia estat mai analitzat. Un dels motius, és que la progressió de la PSP es coneix molt poc i no n'existeix un patró anotat com en el cas de la MA. El què se sap de l'inici de la PSP, com s'ha comentat a la introducció, prové de l'estudi d'una forma familiar autosòmica dominant de la malaltia on, mitjançant la tomografia per emissió de positrons (PET), s'ha assenyalat el caudat/putamen com el lloc que primer queda afectat, seguit del tronc i del globus pallidus/subtàlam (de Yebenes et al., 1995, Piccini et al., 2001, Ros et al., 2005).**

**Els nostres casos estudiats provenen d'una sèrie amb diversos estadis de MGA on s'hi va veure PSP associada. Les observacions no són, per tant, sobre casos purs de PSP i no podem afirmar que la MGA no representi un cert biaix. De tota manera, estem davant de casos inicials de PSP sense clínica rellevant.**

**L'estudi neuropatològic d'aquest casos es va realitzar sobre tres regions: caudat/putamen, globus pallidus/subtàlam i substància negra/ceruleus/colliculi/pons ventral. En els casos més primerencs, el caudat/putamen és el que es veia més afectat d'astrogliosi i acumulament de tau hiperfosforil·lada. En un cas més afectat, els canvis en aquesta regió eren més acusats però també es va observar l'alteració del globus pallidus/subtàlam, i també, però més lleugerament, de la tercera regió estudiada. El cas estudiat amb finalitat comparativa de PSP típica, amb tota la clínica present, mostrava afectació severa de les tres regions. Aquests resultats coincideixen en assenyalar el caudat/putamen com la primera regió afectada en la PSP, seguit del globus pallidus/subtàlam i de determinats nuclis basals. Els resultats també indiquen que el caudat/putamen pot estar afectat gliosi i de cert grau d'acumulament de tau sense presentar problemes clínics detectables en les proves habituals. A partir de quan les lesions comporten dèficits clinics no está ben establert i podria variar entre diferents individus.**

**Com s'ha comentat a la introducció, alguns astròcits en la PSP presenten tau hiperfosforil·lada formant inclusions de morfologia variable. També els oligodendròcits presenten acúmuls anomenats *coiled bodies*. Tant els *coiled***

***bodies*** **com un certa morfologia dels astròcits (tufted astrocytes) pràcticament es troben absents en els casos més primerencs de PSP. Aquestes lesions apareixen, per tant, més tard en el transcurs de la malaltia. Per altra banda, astròcits immunoreactius per tau hiperfosforil·lada si que es poden observar en el caudat/putamen en aquests casos més primerencs, la qual cosa suggereix que la fosforil·lació i acumulament de tau a la glia és un fenòmen associat a la degeneració i no als processos reactius secundaris de la glia.**

### *6.2.2- Gliosi i oxidació de la GFAP*

**En condicions normals els astròcits es dediquen al suport tròfic de les neurones, a regular els nivells extracel·lulars de glutamat, proveïr de factors tròfics i mantenir l'homeòstasi iònica; i també, com hem comentat a la introducció, ajuden a protegir de l'estrès oxidatiu. Quan parlem de gliosi, ens referim als canvis que experimenten els astròcits i la microglia. L'activació de la glia, o gliosi reactiva, és una fenomen comú en el cervell en cas de múltiples malalties o lesions. L'activació de la microglia produeix la resposta inflamatòria en el cervell. Les cèl·lules activades migren cap a la zona lesionada, proliferen i expressen factors proinflamatoris així com proteïnes del complex major d'histocompatibilitat; actuen com a cèl·lules presentadores d'antigen i es poden transformar al fenotip de cèl·lula fagocitària. L'alliberació de citoquines per la microglia com IL-1beta, TNF o TGF-beta tenen, no en exclusiva, la capacitat d'activar un astròcit. Quan parlem únicament de l'activació d'astròcits, ens referim a l'astrogliosi.**

**S'ha vinculat una activació desmesurada i sostinguda de la glia amb la neurodegeneració. Fins i tot, alguns autors li confereixen un paper capital en algunes malalties neurodegeneratives, a les que anomenen "gliodegeneratives" (Croisier and Graeber, 2006). questa activació d'astròcits està present en moltes malalties neurodegeneratives, inclosa la PSP.**

**Els astròcits reactius canvien la seva morfologia, la qual cosa está relacionada amb l'expressió de diferents gens. Alguns dels gens que augmenten la seva expressió en aquestes condicions tenen a veure amb els filaments intermedis, i són la GFAP (glial fibrillary acidic protein), la vimentina i la nestina**

**(Mucke and Eddleston, 1993). Els ratolins deficients en GFAP sembla que poden viure bé mentre no se'ls pertorbi el sistema nerviós (McCall et al., 1996), sota determinats estressors experimentals de tipus mecànic, aquests Knock Out resisteixen molt menys que els salvatges. Els filaments intermedis de GFAP tenen molta importància en mantenir mecànicament o estructuralment el sistema nerviós quan aquest rep estressors mecànics severs (Lundkvist et al., 2004, Pekny and Pekna, 2004). La funció d'aquests filaments intermedis s'està veient que va més enllà de la seva funció purament estructural i que determina estats funcionals de l'astròcit contra diversos estímuls perjudicials de tipus agut, mentre que poden perjudicar la regeneració dels sistema nerviós central si es perpetua la seva activació (Lepekhin et al., 2001, Pekny and Pekna, 2004). Altres funcions en les quals participa la GFAP són la de regular el volum dels astròcits (Ding et al., 1998) o lligar els receptors de glutamat a la membrana plasmàtica (Sullivan et al., 2007).**

**Els nostres resultats apunten a una tendència de la GFAP a veure's oxidada en el cervell ja en estadis primerencs de PSP. Mitjançant gels bidimensional i Western blot contra diferents marcadors d'estrès oxidatiu (CML, MDAL, AGE i CEL) es pot veure un increment d'una sèrie d'espots al voltant de 50 KDa, que la identificació per espectrometria de masses va revel·lar que es tractava de GFAP. Els Western monodimensional contra GFAP en diferents casos de PSP i els dos casos de PSP incidental comparats amb els controls mostra en les diferents àrees estudiades un augment dels nivells de GFAP en consonància amb els elevats nivells d'astrogliosi associats a la malaltia i observats en l'estudi neuropatològic. Els majors nivells d'oxidació que observem mitjançant Western blot sobre gels monodimensionals al pes de GFAP podrien correspondre no a un augment real d'aquests nivells sinó a un augment proporcional a l'augment d'expressió de GFAP. El Western contra CML, on s'observa la banda corresponent al pes de la GFAP en tots el casos, va permetren's un abordatge quantitatiu d'aquesta qüestió. Els nivells de CML a la banda de GFAP normalitzats amb actina dividits pels nivells de GFAP normalitzats amb actina ens va donar el ratio d'oxidació per CML de la GFAP en cada cas. Si l'oxidació fos únicament deguda als nivells d'expressió de GFAP el ratio hauria de ser el mateix entre els casos. Va resultar que el ratio era major en els cas de PSP incidental més afectat i més gran encara en el de PSP establerta, indicant una major oxidació de la GFAP independentment de la quantitat**

**d'aquesta. Aquest abordatge no es va poder realitzar amb els altres marcadors on no tot els casos mostraven la banda i per tant feien impossible la quantificació. Hem de tenir en compte, per tant, que l'augment observat amb certs marcadors pot está únicament relacionat amb un augment d'expressió de GFAP.**

**L'oxidació de la proteïna GFAP s'ha pogut observar en altres malalties neurodegeneratives amb gliosi com la MA (Korolainen et al., 2005, Pamplona et al., 2005), la MPi (Muntane et al., 2006) o la DFT-Tau, DFT amb inclusions d'ubiqüitina i les DFT-amb malalties de motoneurones (Martinez et al., 2008b); i també en altres malalties com l'aceruloplasminemia (Kaneko et al., 2002) o la malaltia de Huntington (Sorolla et al., 2008).**

**La GFAP presenta cinc isoformes conegudes (alpha, beta, gamma, delta i epsilon) (Nielsen et al., 2002). La isoforma més abundant i que més s'expressa quan comença l'astrogliosi és l'alpha. Fa cinc anys es va interrompre la creença de què la GFAP era una proteïna exclusivament glial. Es va veure que en regions dels cervell afectades per la MA, com l'escorça entorrinal o l'hipocamp, les neurones expressaven GFAP. A més, els mateixos autors van descubrir unes formes noves d'empalmament alternatiu dels transcrits de GFAP, dues de les quals eren "out-of-frame" i s'expressaven prácticament només en les neurones de les zones afectades per la MA, es va anomenar GFAP+1 (com la UBB+1, deguda també a errors de lectura en la fabricació del tràns crit) (Hol et al., 2003). Desconeixem quina de les cinc isoformes, o de les formes "out-of-frame", és la que nosaltres trobem incrementada i oxidada en els casos de PSP i PSP incidental estudiats. Creiem que el motiu és que les diferents formes (incluïdes les dues "out-of-frame"), que hem pogut comprovar al GeneBank o Ensembl, es diferencien en el seu extrem C-terminal. L'extrem C-terminal és particularment ric en lisines, de manera que la digestió amb tripsina, pas previ a l'espectrometria de masses, dona com a resultat pèptids massa petits per poder-se identificar correctament. La seqüència coberta pels pèptids identificats sempre s'ha situat en la part mitja-N-terminal de la proteïna.**

**No es coneix l'efecte que les modificacions oxidatives de la GFAP poden tenir en la seva funció, recanvi o localització. A més de l'oxidació se n'han identificat altres que podrien estar relacionats amb estats patològics com són la**

**O-glicosilació, la N-glicosilació o la fosforil·lació. En la MA, s'ha vist que tant la fosforil·lació com la N-glicosilació de la GFAP estan augmentades (Korolainen et al., 2005). Algunes d'aquestes modificacions, en especial la fosforil·lació, regulen la polimerització de la GFAP en la formació de filaments intermedis (Takemura et al., 2002). Però pràcticament res es coneix sobre aquests aspectes en la PSP.**

# Conclusions

**Conclusions:**

**1.1) Les proteïnes són sensibles a l'intèrval postmortem i a la temperatura d'emmagatzematge en diferent grau i de manera particular. Algunes d'elles, com l'alfa-sinucleïna, són molt sensibles als dos factors i requereixen la util·lització de teixit conservat en condicions de mínima temperatura i intèrval postmortem.**

**1.2) La fosforil·lació de la proteïna tau és sensible als factors postmortem. En l'estudi de modificacions post-traduccionals sobre teixit nerviós congel·lat ha de tenir-se molt en compte aquesta limitació.**

**2.1) Les bandes de baix pes molecular que mostren gran quantitat d'anticossos de tau no té relació amb la degradació postmortem, si en canvi, presenta certa associació amb la localització intramolecular dels epítops contra els quals han estat dirigits.**

**2.2) El patró de bandes de baix pes molecular reconegudes pels anticossos contra epítops C-terminals de la proteïna tau (o anticossos policlonals contra diversos epítops) presenta semblances entre les malalties que presenten només isoformes 4R hiperfosforil·lades i diferències amb les que presenten isoformes hiperfosforil·lades tant 4R com 3R.**

**2.3) La proteasa trombina es pot observar associada als grans argiròfils i als pre-cabdells neurofibril·lars en la MGA la qual cosa suggereix un paper per aquesta proteasa en el truncatge de tau en aquestes inclsions. La calpaïna-2 i la caspasa-3 només estan presents als cabdells madurs en la MGA.**

**2.4) La proteasa caspasa-3 es troba present als cossos de Pick a la MPi, la qual cosa suggereix un paper per aquesta proteasa en el truncatge de tau en aquestes inclusions.**

**3.1) El factor de transcripció Sp1 es troba anormalment localitzat a l'interior de les inclusions de tau presents en diverses taupaties (MA, MPi i PSP), però no a les inclusions d'alfa-sinucleïna en sinucleïnopaties (MP i DCL).**

**3.2) Els factors de transcripció c-Fos, c-Jun, ATF2 i CREB es troben sobreexpressats en hipocamp i escorça frontal de la MPi així com en col·localització amb la tau als cossos de Pick. Ni hi ha una relació clara entre l'activació d'aquests factors i les respostes de supervivència i mort neuronal en aquesta malaltia.**

**4) La proteïna p62 i la ubiqüitina mutada estan presents en els grans argiròfils en la MGA. La qual cosa suggereix la formació del segrestosoma i també dificultats en la degradació proteosomal en la MGA.**

**5) La presència de LRRK2 a les inclusions d'alfa-sinucleïna i de tau depèn en gran mesura de l'anticòs util·litzat. Cal determinar l'especificitat dels anticossos comunament util·litzats abans de continuar extreient conclusions sobre el paper de LRRK2 basades en els estudis immunohistoquímics.**

**6) La proteïna 14-3-3 (gamma i zeta) es troba glico-oxidada (CEL) i lipo-oxidada (MDAL) en les fraccions sarkosil-insolubles en la MA i la AAC.**

**7.1) La proteïna GFAP es troba oxidada en diverses regions en estadis primerencs i pre-clínics de la malaltia PSP.**

**7.2) Els estadis primerencs de la PSP comencen a nivell neuropatològic amb la hiperfosforil·lació de tau en astròcits en el caudat/putamen, des d'on s'extén cap al globus pallidus/subtàlam i d'allà a certs nuclis basals.**

# Materials i mètodes

## 1- HOMOGENEÏTZACIÓ DEL TEIXIT

**Tampó d´homogenat:**

**Per la major part d'experiments, els homogenats totals de teixit s'han realitzat amb tampó RIPA, que permet analitzar proteïnes citosòliques i de membrana i de l'interior d'orgànuls, pel fet de portar detergents. La composició de RIPA util·litzat és:**

**Tris-HCl 10mM, NaCl 100mM, EDTA 10mM, 0,5% deoxicolat de sodi 0,5% NP40.**

**Ajustar el PH a 7´4.**

**Els inhibidors emprats sempre han estat per defecte:**

**1mM de PMSF (inhibidor d'algunes serina-proteases)**
**1 pastilla/10ml d'un còctel complet d'inhibidor de proteases (Roche)**
**1uM d'ortovanadat de sodi (Na3VO4) (inhibidor de fosfatases de tirosina i alcalines)**

**I en cas de voler detectar epítops fosforil·lats augmentem el nombre d'inhibidors de fosfatases:**

**25mM de fluorur sòdic (NaF) (inhibidor de fosfatases de serines i treonines)**
**20mM de beta-glicerofosfat (inhibidor de fosfatases de serines i treonines)**

**Procediment:**

- **S'homogenitza el teixit en aproximadament 10 volums de tampó. Emprem tan homogenitzadors de vidre, com de plàstic com homogenitzadors per vibració.**

- **Centrifugar l´homogenat a unes 12.000rpm durant 5 min per tal de precipitar les restes cel·lulars no solubilitzades.**

- **El sobrenedant es guarda a –80ºC o se'n mesura directament la concentració de proteïna mitjançant Bradford o BCA.**

**BRADFORD o BCA**

**En una placa de 96 pouets es pipetegen quantitats creixents d'albúmina sèrica bovina (BSA). I les mostres es posen per duplicat en quantitat depenent del grau de turbidesa que s'observi, però sempre la mateixa quantitat per totes les mostres. S'afegeixen 200ul del reactius Bradford, o BCA (una vegada mesclats els dos components en una relació 1:50), es deixa uns minuts que es produeixi la reacció i es llegeix la placa a l'espectrofotòmetre a la longitud d'ona a que emet cada reactiu.**

## 2-ELECTROFORESI

**La fabricació dels gels es realitza segons la recepta següent:**

**Gels d´acrilamida: (Bio-rad, 30%, 29:1)**

**RESOLVING (separació)**

| | 8% | 10% | 12% | 15% |
|---|---|---|---|---|
| Acrilamida | 2´66ml | 3.33ml | 4ml | 5ml |
| Tampó (R,S) | 2´5ml | 2´5ml | 2´5ml | 2´5ml |
| $H_2O$ | 4´69ml | 4´02ml | 3´35ml | 2´35ml |
| Temed | 10ul | 10ul | 10ul | 10ul |
| AP | 100ul | 100ul | 100ul | 100ul |

**STACKING (concentració)**

| | 4% |
|---|---|
| Acrilamida | 1´3ml |
| Tampó (R,S) | 2´5ml |
| $H_2O$ | 6´1ml |
| Temed | 10ul |
| AP | 100ul |

**<u>SOLUCIONS</u>:**

**Tampó R: 1´5M Tris HCl PH= 8´8 (18´7 grs Tris/ 100ml $H_2O$ destil)**
**0´1% SDS**

**Tampó S: 0´5M TrisHCl PH= 6´8 (6´05 grs/ 100ml $H_2O$ destil)**

**Amoni Persulfat: 0´1 mgrs/ml $H_2O$ destil**
**TAMPÓ D´ELECTRODES (10x)**

**30´28 grs/l Tris (0´25M)**
**144´13 grs/l glicina (1´92M)**
**10 grs/l SDS (0´1%)**

**Les mostres es barregen amb el tampó de mostra següent:**

**TAMPÓ DE MOSTRA (SAMPLE BUFFER) 2X**

**4% SDS**
**10% 2-mercaptoetanol**
**20% glicerol**
**0.004% blau de bromofenol**
**0.125 M Tris HCl**
**Ajustar PH=6´8**

**Les concentracions del tampó de mostra més emprades han estat 2x i 4x.**
**El beta-mercaptoetanol o el DTT, els agents reductors que trenquen els ponts disulfur que mantenen la forma nativa a les proteïnes, s'afegeixen al tampó abans de afegir-ho a la mostra, d'estar pre-mesclats amb el tampó de mostra es degradarien i perdrien l'efecte.**

**Un cop la mostra s´ha barrejat amb el tampó de mostra, es desnaturalitza la proteïna per calor en posar-ho a 95ºC uns 5´. La mostra bullida es pot guardar al congelador de –20ºC.**

**Un cop carregades les mostres al gel, conectar la font:**

**O bé marquem un voltatge constant de 60-70 volts mentre el front es troba a l'stacking, i després el pugem a 150 omés quan ja es troba al resolving; o bé, i això és completament equivalent, marquem l'amparatge constant a 20mA per gel durant tot el procés i el voltatge ja s'adequa sol als canvis de resistència del gel.**

## 3- TRANSFERÈNCIA

**2 papers whatman + membrana de nylon + gel + 2 papers whatman**

**Tot ha d´estar empapat de tampó de transferència. Treure les bombolletes amb un tub.**

**TAMPÓ DE TRANSFERÈNCIA GEL GRAN (SANDWICH):**

**250 mM Tris (Tris base) ⟶ 30´3 grs**

**1´92 mM Glicina ⟶ 144 grs**

**Enrasar a 1 l. No cal mesurar el pH.**

**Abans d´utilitzar, afegir 10% de metanol**

**Un cop feta la transferència, de forma rutinària posem la membrana en solució de Ponceau. Aquesta és capaç de tenyir de forma reversible les proteïnes de manera que ens permet assegurar dues coses: 1) que la transferència a sortit bé, i 2) que no hi ha hagut error de quantificació o de càrrega molt aparents.**

**I**

**4- IMMUNOBLOT**

**TAMPÓ TBST (10x)**

**12´11 grs (100 mM) Tris**
**81´81 grs (1´4 M) NaCl**

**Ajustar el PH= 7´4 amb HCl i llavors afegir 1% de Tween-20**
**Enrasar a 1 l amb $H_2O$d**

**<u>Procediment:</u>**

**- Bloqueig de les unions inespecífiques . 30´amb TBSt-llet (TBST+ 5% llet)**

**- Anticòs primari dissolt en TBST-llet. Overnight a 4ºC (o 1 hora a temperatura ambient si l'anticòs ho permet)**

**- 3 X 5 TBST-llet**

**- Anticòs secundari dissolt en TBST-llet a 1:1000. 45´**

**- 3 X 5´TBST-llet**

**- 3 X 5´TBST**

**- Revelar amb ECL**

**Revelador → 1:5 amb $H_2O$ aixeta**

**Fixador → 1:5 amb $H_2O$ aixeta**

**5- DESLLIGAMENT DE MEMBRANES**

**TAMPÓ DE DESLLIGAMENT:**

**3´78 grs de Tris en 500 ml $H_2O$ destil. i ajustar PH= 6´8 amb HCl**
**10 grs SDS (2%)**
**3´48 ml beta-mercaptoetanol**

**<u>Procediment</u>:**

- **Netejar la membrana amb TBST**
- **Incubar 20´la membrana amb el tampó de deslligament a 66ºC aprox**
- **Canviar el tampó i tornar a incubar 20´més**
- **3 X 5´TBST**
- **Tornar a fer el bloqueig de les unions inespecífiques i continuar a partir d´aquí.**

## 5- GELS BIDIMENSIONALS

**Per tal de realitzar els gels bidimensionals quasi sempre partim de mostra lisada en un tampó amb urea, tiourea i CHAPS. En algunes ocasions, la mostra es trobava en algun altre tampó. Si la mostra està molt concentrada, es pot disoldre en el tampó d'urea i ja està, però si no, val la pena precipitar-la amb TCA, acetona o metanol, i resupendre-la en el tampó d'urea, a risc de perdre part de les proteïnes.**

**El tampó de lisi emprat més regularment és:**

**40 mM Tris, pH 7,5. 7M Urea (9M si es tracta de fraccions sarkosil-insolubles), 2M tiourea, 4% CHAPS (un detergent zwitteriònic molt apte per solubil·litzar proteines sense desnaturalitzar-les), i els inhibidors de proteases i fosfatases.**

**Per la primera dimensió per tal de separar les proteïnes en funció del seu punt isoelèctric agafem:**

**150ug de la mostra en el tampó s'urea i afegim un 2% d'amfolits en el rang de pH que necessitem (p.e. 3-10). Els amfolits també són formes zwiteriòniques que serviran per establir un gradient estable de pH sobre la tira d'acrilamida de la primera dimensió. Afegim 2% de TBP com a agent reductor. No s'util·litza DTT perquè no és tan eficient en aquest cas pel fet de tenir un pI que el fa migrar. En migrar, és incapaç de reduir part de la mostra. Per últim, afegim blau de bromofenol per localitzar el front en la segona dimensió.**

**La mostras es col·loca sobre les tires d'acrilamida immobil·litzades de gradient no linear. Es comença per un procés de rehidratació a 50 V durant 12 hores. L'enfoc de les proteïnes en el seu pH s'aconseguiex amb 300V 1h, seguit d'un augment gradual durant 6h fins a 3500V, on s'atura en aquest voltatge durant 12h més. Per últim es deixen a 5000 V 24h. En aquest últim pas es pot interrompre el procés quan es considera que els volts/hora acumulats passen dels 10000-12000 volts.**

**Després les tires s'quil·libren amb un tampó Tris 50mM, pH 6.8, Urea 6M, 1%SDS i 30% de glicerol. Primer s'afegeix al tampó un 2% de DTT i es posa a les tires 10min, i en acabat es canvia pel mateix tampó sense DTT però amb iodoacetamida que estabil·litza el procés de desnaturalització unint-se a les cisteïnes de forma covalent i evitant que es tornin a produir els ponts disulfur prèviament trencats pel DTT.**

**Finalment la tira el col·loca sobre un gel sense stacking i es continua el procés exactament igual que amb els gels bidimensionals.**

**Sovint tenyim els gels amb solució de Comassie col·loidal o plata, que ens permet la identificació dels spots i retallar-los per enviar al servei d'identificació de proteïnes.**

## 6- IMMUNOPRECIPITACIONS (IP)

**Cada immunoprecipitació és un món. El mètode correcte i l'anticòs més apte sovint es decobreix després d'un llarg camí d'assaig i error. Hem realizat IPs amb boles de proteïna G/A, amb TrueBlot per evitar el reconeixement de les immunoglobulines del primari, amb uColumns amb matrius enganxades a suports magnètics amb boles magnètiques i amb gradetes magnètiques que permeten la precipitació de les boles i optimitzen l'eixugat i recuperació d'aquestes en cada rentat, sense necessitat de centrífuga.**

**En el cas de la IP que es presenta a la tesi, la d'Sp1, es va emprar el primer dels mètodes després d'un gran nombre d'intents.**

**Breument: 0,5 mg de mostra de cada cas es barregen amb 30 ul de boles netes d'etanol durant 1h-2h a 4ºC en agitació orbital amb l'objectiu que arroseguin totes aquelles proteïnes que s'unirien de forma inespeciífica a les boles sense necessitat d'anticos primari. Després es centrifuga la mostra a 800g 3' per tal de fer baixar les boles sense trencar les interaccions i es recull el sobrenedant ja més net de inespecificitats. S'afegeix l'anticos primari contra Sp1 a la concentració indicada per la casa comercial (si és així, sinó es tracta d'assaig/error altra vegada). Es deixa o/n a la nòria a 4ºC.**

**L'endemà s'afegeixen les boles sobre la mostra amb el primari i es deixa a l'orbital 1-2h per deixar temps a formar-se el complex boles-primari-Sp1. Despres, mitjançant centrífugues de 800 g 3' es realitzen els rentats (entre 10 i 15) amb PBS per tal d'eliminar les possible unions inespecífiques que s'hagin pogut fer entre les boles i altres proteïnes de la mostra.**

**Després de l'últim rentat s'intenta deixar les boles el més seques possible i s'afegeix ràpidament el tampó de mostra (30ul o més d'1X). Es bull, es vorteja, es torna a bullir, i s'aconsegueix desfer la unió entre les boles i les Ig agafades a Sp1. Es centrifuga a máxima velocitat per baixar les boles i s'extreu el sobrenedant. Això ja es pot carregar directament en un gel.**

**Durant tot aquest procés s'ha realitzat exactament el mateix en paral·lel en un altre tub però sense anticos primari. Quan es mostri el resultat del Western blot, aquest carril servirà per descartar inespecificitats.**

## 7- PROTOCOL PER OBTENIR FRACCIONS SARKOSIL-INSOLUBLES

★ **Homogeneïtzar el teixit (congelat a –70ºC) en 10 vol de buffer**

**Buffer homogenat: 10mM Tris-HCl (PH= 7.4)**
**0.8M NaCl**
**1mM EGTA**
**10% sacarosa**
**+ tots els inhibidors de proteases i fosfatases descrits**

★ **Centrifugar a 20.000 g x 20´**

↓ ↓

**Sobrenedant 1 (guardar)** **pellet 1**
★ **resuspendre en 5 vol de buffer homogenat i recentrifugar**

**20.000g x 20´**

↓ ↓

**sobrenedant 2**
★ **ajuntar amb sobrenedant 1**

**pellet 2 (descartar)**

★ **Afegir 0,1-1% (w/v) de N- laurosylsarcosinat**

**Incubar 1h a R.T. en agitació constant**

★ **Centrifugar a 100.000g 1h**

↓ ↓

**Sobrenedant (descartar)** **pellet**

★ **Resuspendre en 50mM Tris-HCl (PH=7.4) (0.2 ml per gr de teixit del principi.**

★ **Guardar a 4ºC**

## 8- IMMUNOHISTOQUÍMICA EN PARAFINA

- **Desparafinar i posar en aigua destil·lada**
- **Rentar 2x5' PBS**
- **Bloqueig de les peroxidases endògenes (70ml de PBS + 30ml de metanol + 1ml d'aigua oxigenada) 15'**
- **Rentat 5' aigua destil·lada**
- **Rentar 3x5' PBS**
- **Posar el tractament necessari per cada primari (saponina, tampó citrat, etc.)**
- **Rentat 5' aigua destil·lada**
- **Rentar 3x5' PBS**
- **Bloqueig de les unions inespecífiques amb sèrum normal durant 2h.**
- **Incubar amb l'anticòs primari o/n a 4ºC diluït en sèrum normal.**
- **Atemperar**
- **Rentar 3x5' PBS**
- **Incubar amb el secundari biotinil·lat 10-15'**
- **Rentar 3x5' PBS**
- **Incubar amb streptavidina 10-15'**
- **Rentar 3x5' PBS**
- **Revelar. Amb DAB: 200ml de PBS + punta de pipeta Pasteur plena de DAB. Filtrar i afegir 50ul d'aigua oxigenada.**
- **Contrastar, si es vol, amb hematoxilina diluïda**
- **Deshidratar i muntar amb medi de muntatge**

## 9- IMMUNOHISTOQUÍMICA EN FLOTACIÓ

- **Sel·leccionar els talls i posar-los en PBS**
- **Rentar 3x5' PBS**
- **Bloqueig de les peroxidases endògenes (40ml d'aigua destil·lada, 5ml de metanol, 5ml d'aigua oxigenada)**
- **Rentar 6x5' PBS**
- **Bloqueig de les unions inespecífiques amb sèrum normal 2h a temp. amb.**
- **Rentar 3x5' PBS**
- **Anticòs primari o/n**
- **Atemperar**
- **Rentar 3x5' PBS**
- **10' anticòs secundari**
- **Rentar 3x5' PBS**
- **10' streptavidina**
- **Rentar 3x5' PBS**
- **Revelar amb DAB (0,05% + 30ul d'aigua oxigenada en 10ml de PBS)**
- **Rentar 3x5' PBS**
- **Muntatge en portes de polylisina al 5%**
- **Deixar aixugar, deshidratar i muntar**

## 10- IMMUNOFLUORESCÈNCIA

**El protocol segueix els mateixos passos que als apartats anteriors (segons s'util·litzi flotació o parafina), amb modificacions:**

- **S'ha de bloquejar la possible autoflorescència de determinades substàncies amb pigments (grànuls de lipofuscina). Ho fem amb una incubació amb negre de Sudan durant 30'.**
- **Els anticossos tenen un fluorocorm associat. El còctel d'anticossos després del primari assignen a cada primari un color. Existeixen secundaris i fluorocroms amb tots les possible combinacions: anti-ratolí vermell, anti-conill verd.**
- **Una vegada realitzada la immuno, el muntatge precisa d'un medi especial en el nostre cas Immuno-Fluore Mounting medium. Els cobre-objectes es segellen i es manté la preparació en la foscor fins el moment d'observar-la al microscopi de fluorescència amb els filtres corresponents.**

## 11- Altres protocols realitzats:

**Aquí enumero sense detallar altres tècniques que s'han dut a terme en múltiples experiments que han quedat fora de la tesi de diferents línees de recerca que han quedat interrumpudes per motius diversos.**

**<u>Estudi de la proteïna CSK (C-terminal Src Kinase)</u>**

**Aquests proteïna és capaç d'inhibir l'activitat de la família de tirosina-cinases Src (Src, Yes, Fyn, Lck...) una vegada és reclutada als rafts lipídics. Algunes d'aquestes cinases poden fosforil·lar directament tau (a l'epítop Tyr18, per exemple) o bé activar cinases de tau com la GSK3-beta. Ens va interessar veure diversos aspectes d'aquesta proteïna a la MA. A més de les tècniques abans esmentades vam util·litzar també :**

**- Immunomicroscopia electrònica de CSK sobre pellets de tau-PHF per confirmar la possible interacció, suggerida per la seva localització, observada amb immunohistoquímica, en inclusions de tau.**

**- Extracció de RNA, RT PCR i Real Time amb sondes TaqMan, per veure els nivells d'expressió de la proteïna.**

**- Extracció de rafts lipídics amb gradients de sacarosa, per estudiar els nivells d'expressió de CSK en la regió on desenvolupa la seva activitat.**

**- IP de CSK (mitjançant boles magnètiques) acoblada a una assaig d'activitat cinasa amb gamma-ATP32, per veure si la seva activitat estava disminuïda en la malaltia.**

**- Transfecció amb siRNA per silenciar l'expressió de CSK i comprovar el seu efecte sobre la fosforil·lació de tau en neuroblastomes, per tal de veure si el mecanisme hipotetitzat es cumplia en un model experimental.**

**Interacció de tau-PHF o tau citosòlica amb altres proteïnes**

- **Un altre objectiu va ser el d'util·litzar membranes d'arrais d'anticossos (Hipromatrix) de diferents tamanys orientades a transducció de senyal. Es va provar en diferents malalties (MA, PSP i FTDP-17) amb tau de diferents fraccions (sarkosil-insoluble o citosòlica). L'arrai va mostrar un conjunt de proteïnes en tots els casos, algunes de les quals es van confirmar per immunohistoquímica i microscopia confocal i es va intentar validar per IP. Algunes d'elles, a més, es van provar de localitzar mitjançant immunomicroscopia electrònica sobre pellets enriquits en PHF.**

- **TAP system. Aquest sistema consisteix en clonar la proteïna (tau i tau amb diferente mutacions) en un vector que posseeix dos tags (d'unió a calmodulina i estreptavidina). La transfecció en neuroblastomes ens va permetre després realitzar els dos processos de purificació (mitjançant columnes) obtenint una fracció molt enriquida en tau. Aquesta fracció es va carregar en un gel gran i tenyir amb plata per tal de descobrir els interactors que arrossegava. Alguns d'aquests es van poder revel·lar mitjançant espectrometria de masses.**

- **Extracció d'inclusions de tau mitjançant microdissecció laser per tal de correr les inclusions en gels bidimensionals i identificar-ne el components. Aquest protocol pot resultar útil si es disposa d'una quantitat de temps enorme, motiu pel qual es va abandonar. En casos avançats de MA, si no es disposa del temps necessari, extirpar sota una lupa binocular la capa CA1 de l'hipocamp pot resultar molt més ràpid encara que molt menys precís.**

# Bibliografia

Abraha, A., Ghoshal, N., Gamblin, T. C., Cryns, V., Berry, R. W., Kuret, J. and Binder, L. I., 2000. C-terminal inhibition of tau assembly in vitro and in Alzheimer's disease. J Cell Sci. 113 Pt 21, 3737-3745.

Adamec, E., Mohan, P., Vonsattel, J. P. and Nixon, R. A., 2002. Calpain activation in neurodegenerative diseases: confocal immunofluorescence study with antibodies specifically recognizing the active form of calpain 2. Acta Neuropathol. 104, 92-104.

Agarwal-Mawal, A., Qureshi, H. Y., Cafferty, P. W., Yuan, Z., Han, D., Lin, R. and Paudel, H. K., 2003. 14-3-3 connects glycogen synthase kinase-3 beta to tau within a brain microtubule-associated tau phosphorylation complex. J Biol Chem. 278, 12722-12728.

Aitken, A., 2006. 14-3-3 proteins: a historic overview. Semin Cancer Biol. 16, 162-172.

Albers, D. S., Augood, S. J., Martin, D. M., Standaert, D. G., Vonsattel, J. P. and Beal, M. F., 1999. Evidence for oxidative stress in the subthalamic nucleus in progressive supranuclear palsy. J Neurochem. 73, 881-884.

Albers, D. S., Augood, S. J., Park, L. C., Browne, S. E., Martin, D. M., Adamson, J., Hutton, M., Standaert, D. G., Vonsattel, J. P., Gibson, G. E. and Beal, M. F., 2000. Frontal lobe dysfunction in progressive supranuclear palsy: evidence for oxidative stress and mitochondrial impairment. J Neurochem. 74, 878-881.

Alegre-Abarrategui, J., Ansorge, O., Esiri, M. and Wade-Martins, R., 2008. LRRK2 is a component of granular alpha-synuclein pathology in the brainstem of Parkinson's disease. Neuropathol Appl Neurobiol. 34, 272-283.

Anderson, A. J., Cummings, B. J. and Cotman, C. W., 1994. Increased immunoreactivity for Jun- and Fos-related proteins in Alzheimer's disease: association with pathology. Exp Neurol. 125, 286-295.

Anderson, A. J., Su, J. H. and Cotman, C. W., 1996. DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area, and effect of postmortem delay. J Neurosci. 16, 1710-1719.

Arai, T., Guo, J. P. and McGeer, P. L., 2005. Proteolysis of non-phosphorylated and phosphorylated tau by thrombin. Journal of Biological Chemistry. 280, 5145-5153.

Arai, T., Ikeda, K., Akiyama, H., Nonaka, T., Hasegawa, M., Ishiguro, K., Iritani, S., Tsuchiya, K., Iseki, E., Yagishita, S., Oda, T. and Mochizuki, A., 2004. Identification of amino-terminally cleaved tau fragments that distinguish progressive supranuclear palsy from corticobasal degeneration. Ann Neurol. 55, 72-79.

Arai, T., Ikeda, K., Akiyama, H., Shikamoto, Y., Tsuchiya, K., Yagishita, S., Beach, T., Rogers, J., Schwab, C. and McGeer, P. L., 2001. Distinct isoforms of tau aggregated in neurons and glial cells in brains of patients with Pick's disease, corticobasal degeneration and progressive supranuclear palsy. Acta Neuropathol. 101, 167-173.

Arai, T., Miklossy, J., Klegeris, A., Guo, J. P. and McGeer, P. L., 2006. Thrombin and prothrombin are expressed by neurons and glial cells and accumulate in neurofibrillary tangles in Alzheimer disease brain. J Neuropathol Exp Neurol. 65, 19-25.

Ballatore, C., Lee, V. M. and Trojanowski, J. Q., 2007. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. Nat Rev Neurosci. 8, 663-672.

Behrens, A., Sibilia, M. and Wagner, E. F., 1999. Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. Nat Genet. 21, 326-329.

Bertram, L. and Tanzi, R. E., 2005. The genetic epidemiology of neurodegenerative disease. J Clin Invest. 115, 1449-1457.

Blass, J. P., 2000. The mitochondrial spiral. An adequate cause of dementia in the Alzheimer's syndrome. Ann N Y Acad Sci. 924, 170-183.

Bonifati, V., 2008. Recent advances in the genetics of dementia with lewy bodies. Curr Neurol Neurosci Rep. 8, 187-189.

Boutillier, S., Lannes, B., Buee, L., Delacourte, A., Rouaux, C., Mohr, M., Bellocq, J. P., Sellal, F., Larmet, Y., Boutillier, A. L. and Loeffler, J. P., 2007. Sp3 and sp4 transcription factor levels are increased in brains of patients with Alzheimer's disease. Neurodegener Dis. 4, 413-423.

Boyd-Kimball, D., Castegna, A., Sultana, R., Poon, H. F., Petroze, R., Lynn, B. C., Klein, J. B. and Butterfield, D. A., 2005a. Proteomic identification of proteins oxidized by Abeta(1-42) in synaptosomes: implications for Alzheimer's disease. Brain Res. 1044, 206-215.

Boyd-Kimball, D., Sultana, R., Poon, H. F., Lynn, B. C., Casamenti, F., Pepeu, G., Klein, J. B. and Butterfield, D. A., 2005b. Proteomic identification of proteins specifically oxidized by intracerebral injection of amyloid beta-peptide (1-42) into rat brain: implications for Alzheimer's disease. Neuroscience. 132, 313-324.

Braak, H. and Braak, E., 1991. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. 82, 239-259.

Braak, H. and Braak, E., 1998. Argyrophilic grain disease: frequency of occurrence in different age categories and neuropathological diagnostic criteria. J Neural Transm. 105, 801-819.

Braak, H., Braak, E., Grundke-Iqbal, I. and Iqbal, K., 1986. Occurrence of neuropil threads in the senile human brain and in Alzheimer's disease: a third location of paired helical filaments outside of neurofibrillary tangles and neuritic plaques. Neurosci Lett. 65, 351-355.

Braak, H., Del Tredici, K., Bratzke, H., Hamm-Clement, J., Sandmann-Keil, D. and Rub, U., 2002. Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). J Neurol. 249 Suppl 3, III/1-5.

Braak, H., Del Tredici, K., Rub, U., de Vos, R. A., Jansen Steur, E. N. and Braak, E., 2003. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging. 24, 197-211.

Braak, H., Del Tredici, K., Schultz, C. and Braak, E., 2000. Vulnerability of select neuronal types to Alzheimer's disease. Ann N Y Acad Sci. 924, 53-61.

Buesa, C., Maes, T., Subirada, F., Barrachina, M. and Ferrer, I., 2004. DNA chip technology in brain banks: confronting a degrading world. J Neuropathol Exp Neurol. 63, 1003-1014.

Butterfield, D. A., Reed, T., Perluigi, M., De Marco, C., Coccia, R., Cini, C. and Sultana, R., 2006. Elevated protein-bound levels of the lipid peroxidation product, 4-hydroxy-2-nonenal, in brain from persons with mild cognitive impairment. Neurosci Lett. 397, 170-173.

Canu, N., Dus, L., Barbato, C., Ciotti, M. T., Brancolini, C., Rinaldi, A. W., Novak, M., Cattaneo, A., Bradbury, A. and Calissano, P., 1998. Tau cleavage and dephosphorylation in cerebellar granule neurons undergoing apoptosis. Journal of Neuroscience. 18, 7061-7074.

Castellani, R., Smith, M. A., Richey, P. L. and Perry, G., 1996. Glycoxidation and oxidative stress in Parkinson disease and diffuse Lewy body disease. Brain Res. 737, 195-200.

Cente, M., Filipcik, P., Pevalova, M. and Novak, M., 2006. Expression of a truncated tau protein induces oxidative stress in a rodent model of tauopathy. European Journal of Neuroscience. 24, 1085-1090.

Choi, J., Levey, A. I., Weintraub, S. T., Rees, H. D., Gearing, M., Chin, L. S. and Li, L., 2004. Oxidative modifications and down-regulation of ubiquitin carboxyl-terminal hydrolase L1 associated with idiopathic Parkinson's and Alzheimer's diseases. J Biol Chem. 279, 13256-13264.

Choi, J., Rees, H. D., Weintraub, S. T., Levey, A. I., Chin, L. S. and Li, L., 2005. Oxidative modifications and aggregation of Cu,Zn-superoxide dismutase associated with Alzheimer and Parkinson diseases. J Biol Chem. 280, 11648-11655.

Christensen, M. A., Zhou, W., Qing, H., Lehman, A., Philipsen, S. and Song, W., 2004. Transcriptional regulation of BACE1, the beta-amyloid precursor protein beta-secretase, by Sp1. Mol Cell Biol. 24, 865-874.

Chu, S. and Ferro, T. J., 2005. Sp1: regulation of gene expression by phosphorylation. Gene. 348, 1-11.

Citron, B. A., Dennis, J. S., Zeitlin, R. S. and Echeverria, V., 2008. Transcription factor Sp1 dysregulation in Alzheimer's disease. J Neurosci Res. 86, 2499-2504.

Clark, L. N., Wang, Y., Karlins, E., Saito, L., Mejia-Santana, H., Harris, J., Louis, E. D., Cote, L. J., Andrews, H., Fahn, S., Waters, C., Ford, B., Frucht, S., Ottman, R. and Marder, K., 2006. Frequency of LRRK2 mutations in early- and late-onset Parkinson disease. Neurology. 67, 1786-1791.

Clayton, D. F. and George, J. M., 1999. Synucleins in synaptic plasticity and neurodegenerative disorders. J Neurosci Res. 58, 120-129.

Clinton, J., Blackman, S. E., Royston, M. C. and Roberts, G. W., 1994. Differential synaptic loss in the cortex in Alzheimer's disease: a study using archival material. Neuroreport. 5, 497-500.

Croisier, E. and Graeber, M. B., 2006. Glial degeneration and reactive gliosis in alpha-synucleinopathies: the emerging concept of primary gliodegeneration. Acta Neuropathol. 112, 517-530.

Dalfo, E. and Ferrer, I., 2008. Early alpha-synuclein lipoxidation in neocortex in Lewy body diseases. Neurobiol Aging. 29, 408-417.

Dalfo, E., Portero-Otin, M., Ayala, V., Martinez, A., Pamplona, R. and Ferrer, I., 2005. Evidence of oxidative stress in the neocortex in incidental Lewy body disease. J Neuropathol Exp Neurol. 64, 816-830.

Dalle-Donne, I., Aldini, G., Carini, M., Colombo, R., Rossi, R. and Milzani, A., 2006. Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med. 10, 389-406.

D'Amore, J. D., Kajdasz, S. T., McLellan, M. E., Bacskai, B. J., Stern, E. A. and Hyman, B. T., 2003. In vivo multiphoton imaging of a transgenic mouse model of Alzheimer disease reveals marked thioflavine-S-associated alterations in neurite trajectories. J Neuropathol Exp Neurol. 62, 137-145.

Darling, D. L., Yingling, J. and Wynshaw-Boris, A., 2005. Role of 14-3-3 proteins in eukaryotic signaling and development. Curr Top Dev Biol. 68, 281-315.

Davies, K. J., 2001. Degradation of oxidized proteins by the 20S proteasome. Biochimie. 83, 301-310.

de Yebenes, J. G., Sarasa, J. L., Daniel, S. E. and Lees, A. J., 1995. Familial progressive supranuclear palsy. Description of a pedigree and review of the literature. Brain. 118 ( Pt 5), 1095-1103.

Delatour, B., Blanchard, V., Pradier, L. and Duyckaerts, C., 2004. Alzheimer pathology disorganizes cortico-cortical circuitry: direct evidence from a transgenic animal model. Neurobiol Dis. 16, 41-47.

Derkinderen, P., Scales, T. M., Hanger, D. P., Leung, K. Y., Byers, H. L., Ward, M. A., Lenz, C., Price, C., Bird, I. N., Perera, T., Kellie, S., Williamson, R., Noble, W., Van Etten, R. A., Leroy, K., Brion, J. P., Reynolds, C. H. and Anderton, B. H., 2005. Tyrosine 394 is phosphorylated in Alzheimer's paired helical filament tau and in fetal tau with c-Abl as the candidate tyrosine kinase. J Neurosci. 25, 6584-6593.

Dexter, D. T., Carter, C. J., Wells, F. R., Javoy-Agid, F., Agid, Y., Lees, A., Jenner, P. and Marsden, C. D., 1989. Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. J Neurochem. 52, 381-389.

Di Fonzo, A., Rohe, C. F., Ferreira, J., Chien, H. F., Vacca, L., Stocchi, F., Guedes, L., Fabrizio, E., Manfredi, M., Vanacore, N., Goldwurm, S., Breedveld, G., Sampaio, C., Meco, G., Barbosa, E., Oostra, B. A. and Bonifati, V., 2005. A frequent LRRK2 gene mutation associated with autosomal dominant Parkinson's disease. Lancet. 365, 412-415.

Dickson, D. W., 1998. Pick's disease: a modern approach. Brain Pathol. 8, 339-354.

Dickson, D. W., 1999. Neuropathologic differentiation of progressive supranuclear palsy and corticobasal degeneration. J Neurol. 246 Suppl 2, II6-15.

Dickson, T. C., King, C. E., McCormack, G. H. and Vickers, J. C., 1999. Neurochemical diversity of dystrophic neurites in the early and late stages of Alzheimer's disease. Exp Neurol. 156, 100-110.

DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. W., Bates, G. P., Vonsattel, J. P. and Aronin, N., 1997. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science. 277, 1990-1993.

Ding, M., Eliasson, C., Betsholtz, C., Hamberger, A. and Pekny, M., 1998. Altered taurine release following hypotonic stress in astrocytes from mice deficient for GFAP and vimentin. Brain Res Mol Brain Res. 62, 77-81.

Docagne, F., Gabriel, C., Lebeurrier, N., Lesne, S., Hommet, Y., Plawinski, L., Mackenzie, E. T. and Vivien, D., 2004. Sp1 and Smad transcription factors co-operate to mediate TGF-beta-dependent activation of amyloid-beta precursor protein gene transcription. Biochem J. 383, 393-399.

Dougherty, M. K. and Morrison, D. K., 2004. Unlocking the code of 14-3-3. J Cell Sci. 117, 1875-1884.

Drechsel, D. N., Hyman, A. A., Cobb, M. H. and Kirschner, M. W., 1992. Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. Mol Biol Cell. 3, 1141-1154.

Duyckaerts, C., Potier, M. C. and Delatour, B., 2008. Alzheimer disease models and human neuropathology: similarities and differences. Acta Neuropathol. 115, 5-38.

Eilers, A., Whitfield, J., Babij, C., Rubin, L. L. and Ham, J., 1998. Role of the Jun kinase pathway in the regulation of c-Jun expression and apoptosis in sympathetic neurons. J Neurosci. 18, 1713-1724.

Farrer, M., Stone, J., Mata, I. F., Lincoln, S., Kachergus, J., Hulihan, M., Strain, K. J. and Maraganore, D. M., 2005. LRRK2 mutations in Parkinson disease. Neurology. 65, 738-740.

Fasulo, L., Ugolini, G., Visintin, M., Bradbury, A., Brancolini, C., Verzillo, V., Novak, M. and Cattaneo, A., 2000. The neuronal microtubule-associated protein tau is a substrate for caspase-3 and an effector of apoptosis. Journal of Neurochemistry. 75, 624-633.

Ferl, R. J., 1996. 14-3-3 Proteins and Signal Transduction. Annu Rev Plant Physiol Plant Mol Biol. 47, 49-73.

Ferreira, A. and Caceres, A., 1991. Estrogen-enhanced neurite growth: evidence for a selective induction of Tau and stable microtubules. J Neurosci. 11, 392-400.

Ferrer, I., Blanco, R., Carmona, M., Puig, B., Dominguez, I. and Vinals, F., 2002. Active, phosphorylation-dependent MAP kinases, MAPK/ERK, SAPK/JNK and p38, and specific transcription factor substrates are differentially expressed following systemic administration of kainic acid to the adult rat. Acta Neuropathol. 103, 391-407.

Ferrer, I., Gomez-Isla, T., Puig, B., Freixes, M., Ribe, E., Dalfo, E. and Avila, J., 2005. Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. Curr Alzheimer Res. 2, 3-18.

Ferrer, I., Martinez, A., Boluda, S., Parchi, P. and Barrachina, M., 2008. Brain banks: benefits, limitations and cautions concerning the use of post-mortem brain tissue for molecular studies. Cell Tissue Bank. 9, 181-194.

Ferrer, I. S., G. Puig, B., 2006. Immediate early genes, inducible transcription factors and stress kinases in Alzheimer's disease. In: R. Pinaud, L. A. T. (Ed.), Immediate early genes in sensory processing, cognitive performance and neurological disorders. Springer Science and Business Media, pp. 243-260.

Fischer, P. M., 2008. Turning down tau phosphorylation. Nat Chem Biol. 4, 448-449.

Floor, E. and Wetzel, M. G., 1998. Increased protein oxidation in human substantia nigra pars compacta in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. J Neurochem. 70, 268-275.

Forno, L. S., 1996. Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol. 55, 259-272.

Fountoulakis, M., Cairns, N. and Lubec, G., 1999. Increased levels of 14-3-3 gamma and epsilon proteins in brain of patients with Alzheimer's disease and Down syndrome. J Neural Transm Suppl. 57, 323-335.

Frank, S., Clavaguera, F. and Tolnay, M., 2008. Tauopathy models and human neuropathology: similarities and differences. Acta Neuropathol. 115, 39-53.

Freiman, R. N. and Tjian, R., 2002. Neurodegeneration. A glutamine-rich trail leads to transcription factors. Science. 296, 2149-2150.

Fu, H., Subramanian, R. R. and Masters, S. C., 2000. 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol. 40, 617-647.

Fujino, Y., Wang, D. S., Thomas, N., Espinoza, M., Davies, P. and Dickson, D. W., 2005. Increased frequency of argyrophilic grain disease in Alzheimer disease with 4R tau-specific immunohistochemistry. J Neuropathol Exp Neurol. 64, 209-214.

Furukawa, K., Guo, Q., Schellenberg, G. D. and Mattson, M. P., 1998. Presenilin-1 mutation alters NGF-induced neurite outgrowth, calcium homeostasis, and transcription factor (AP-1) activation in PC12 cells. J Neurosci Res. 52, 618-624.

Gamblin, T. C., Chen, F., Zambrano, A., Abraha, A., Lagalwar, S., Guillozet, A. L., Lu, M. L., Fu, Y. F., Garcia-Sierra, F., LaPointe, N., Miller, R., Berry, R. W.,

Bincler, L. I. and Cryns, V. L., 2003. Caspase cleavage of tau: Linking amyloid and neurofibrillary tangles in Alzheimer's disease. Proceedings of the National Academy of Sciences of the United States of America. 100, 10032-10037.

Gandhi, P. N., Wang, X., Zhu, X., Chen, S. G. and Wilson-Delfosse, A. L., 2008. The Roc domain of leucine-rich repeat kinase 2 is sufficient for interaction with microtubules. J Neurosci Res. 86, 1711-1720.

Garrison, W. M., Jayko, M. E. and Bennett, W., 1962. Radiation-induced oxidation of protein in aqueous solution. Radiat Res. 16, 483-502.

Giasson, B. I., Covy, J. P., Bonini, N. M., Hurtig, H. I., Farrer, M. J., Trojanowski, J. Q. and Van Deerlin, V. M., 2006. Biochemical and pathological characterization of Lrrk2. Ann Neurol. 59, 315-322.

Goate, A., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L. and et al., 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature. 349, 704-706.

Goedert, M., 2001. Parkinson's disease and other alpha-synucleinopathies. Clin Chem Lab Med. 39, 308-312.

Gomez, A. and Ferrer, I., 2008. Increased oxidation of certain glycolysis and energy metabolism enzymes in the frontal cortex in Lewy body diseases. J Neurosci Res.

Gomez-Isla, T., Hollister, R., West, H., Mui, S., Growdon, J. H., Petersen, R. C., Parisi, J. E. and Hyman, B. T., 1997. Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. Ann Neurol. 41, 17-24.

Good, P. F., Perl, D. P., Bierer, L. M. and Schmeidler, J., 1992. Selective accumulation of aluminum and iron in the neurofibrillary tangles of Alzheimer's disease: a laser microprobe (LAMMA) study. Ann Neurol. 31, 286-292.

Grant, A. J., Jessup, W. and Dean, R. T., 1993. Inefficient degradation of oxidized regions of protein molecules. Free Radic Res Commun. 18, 259-267.

Green, J., Morris, J. C., Sandson, J., McKeel, D. W., Jr. and Miller, J. W., 1990. Progressive aphasia: a precursor of global dementia? Neurology. 40, 423-429.

Guillozet-Bongaarts, A. L., Garcia-Sierra, F., Reynolds, M. R., Horowitz, P. M., Fu, Y. F., Wang, T. Y., Cahill, M. E., Bigio, E. H., Berry, R. W. and Binder, L. I., 2005. Tau truncation during neurofibrillary tangle evolution in Alzheimer's disease. Neurobiology of Aging. 26, 1015-1022.

Guo, L., Gandhi, P. N., Wang, W., Petersen, R. B., Wilson-Delfosse, A. L. and Chen, S. G., 2007. The Parkinson's disease-associated protein, leucine-rich repeat kinase 2 (LRRK2), is an authentic GTPase that stimulates kinase activity. Exp Cell Res. 313, 3658-3670.

Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B. and Davis, R. J., 1996. Selective interaction of JNK protein kinase isoforms with transcription factors. Embo J. 15, 2760-2770.

Ham, J., Eilers, A., Whitfield, J., Neame, S. J. and Shah, B., 2000. c-Jun and the transcriptional control of neuronal apoptosis. Biochem Pharmacol. 60, 1015-1021.

Hamacher, M., Meyer, H. E. and Marcus, K., 2007. New access to Alzheimer's and other neurodegenerative diseases. Expert Rev Proteomics. 4, 591-594.

Hashiguchi, M., Sobue, K. and Paudel, H. K., 2000. 14-3-3zeta is an effector of tau protein phosphorylation. J Biol Chem. 275, 25247-25254.

Hazzalin, C. A., Cano, E., Cuenda, A., Barratt, M. J., Cohen, P. and Mahadevan, L. C., 1996. p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are insufficient. Curr Biol. 6, 1028-1031.

Heicklen-Klein, A. and Ginzburg, I., 2000. Tau promoter confers neuronal specificity and binds Sp1 and AP-2. J Neurochem. 75, 1408-1418.

Hensley, K., Carney, J. M., Mattson, M. P., Aksenova, M., Harris, M., Wu, J. F., Floyd, R. A. and Butterfield, D. A., 1994. A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. Proc Natl Acad Sci U S A. 91, 3270-3274.

Hermeking, H. and Benzinger, A., 2006. 14-3-3 proteins in cell cycle regulation. Semin Cancer Biol. 16, 183-192.

Hernandez, F., Cuadros, R. and Avila, J., 2004. Zeta 14-3-3 protein favours the formation of human tau fibrillar polymers. Neurosci Lett. 357, 143-146.

Higashi, S., Biskup, S., West, A. B., Trinkaus, D., Dawson, V. L., Faull, R. L., Waldvogel, H. J., Arai, H., Dawson, T. M., Moore, D. J. and Emson, P. C., 2007. Localization of Parkinson's disease-associated LRRK2 in normal and pathological human brain. Brain Res. 1155, 208-219.

Hol, E. M., Roelofs, R. F., Moraal, E., Sonnemans, M. A., Sluijs, J. A., Proper, E. A., de Graan, P. N., Fischer, D. F. and van Leeuwen, F. W., 2003. Neuronal expression of GFAP in patients with Alzheimer pathology and identification of novel GFAP splice forms. Mol Psychiatry. 8, 786-796.

Horowitz, P. M., Patterson, K. R., Guillozet-Bongaarts, A. L., Reynolds, M. R., Carroll, C. A., Weintraub, S. T., Bennett, D. A., Cryns, V. L., Berry, R. W. and Binder, L. I., 2004. Early N-terminal changes and caspase-6 cleavage of tau in Alzheimer's disease. Journal of Neuroscience. 24, 7895-7902.

Hurtado-Lorenzo, A. and Anand, V. S., 2008. Heat shock protein 90 modulates LRRK2 stability: potential implications for Parkinson's disease treatment. J Neurosci. 28, 6757-6759.

Hy, L. X. and Keller, D. M., 2000. Prevalence of AD among whites: a summary by levels of severity. Neurology. 55, 198-204.

Hyman, B. T. and Gomez-Isla, T., 1994. Alzheimer's disease is a laminar, regional, and neural system specific disease, not a global brain disease. Neurobiol Aging. 15, 353-354; discussion 379-380.

Ikeda, S., Allsop, D. and Glenner, G. G., 1989. Morphology and distribution of plaque and related deposits in the brains of Alzheimer's disease and control cases. An immunohistochemical study using amyloid beta-protein antibody. Lab Invest. 60, 113-122.

Irving, E. A., McCulloch, J. and Dewar, D., 1997. The effect of postmortem delay on the distribution of microtubule-associated proteins tau, MAP2, and MAP5 in the rat. Mol Chem Neuropathol. 30, 253-271.

Ishida, A., Furukawa, K., Keller, J. N. and Mattson, M. P., 1997. Secreted form of beta-amyloid precursor protein shifts the frequency dependency for induction of LTD, and enhances LTP in hippocampal slices. Neuroreport. 8, 2133-2137.

Ishihara, L., Warren, L., Gibson, R., Amouri, R., Lesage, S., Durr, A., Tazir, M., Wszolek, Z. K., Uitti, R. J., Nichols, W. C., Griffith, A., Hattori, N., Leppert, D., Watts, R., Zabetian, C. P., Foroud, T. M., Farrer, M. J., Brice, A., Middleton, L. and Hentati, F., 2006. Clinical features of Parkinson disease patients with homozygous leucine-rich repeat kinase 2 G2019S mutations. Arch Neurol. 63, 1250-1254.

Jakes, R., Spillantini, M. G. and Goedert, M., 1994. Identification of two distinct synucleins from human brain. FEBS Lett. 345, 27-32.

Jaleel, M., Nichols, R. J., Deak, M., Campbell, D. G., Gillardon, F., Knebel, A. and Alessi, D. R., 2007. LRRK2 phosphorylates moesin at threonine-558: characterization of how Parkinson's disease mutants affect kinase activity. Biochem J. 405, 307-317.

Jellinger, K. A., 2008. A critical reappraisal of current staging of Lewy-related pathology in human brain. Acta Neuropathol. 116, 1-16.

Jenner, P., 2003. Oxidative stress in Parkinson's disease. Ann Neurol. 53 Suppl 3, S26-36; discussion S36-28.

Kaneko, K., Nakamura, A., Yoshida, K., Kametani, F., Higuchi, K. and Ikeda, S., 2002. Glial fibrillary acidic protein is greatly modified by oxidative stress in aceruloplasminemia brain. Free Radic Res. 36, 303-306.

Karin, M., Liu, Z. and Zandi, E., 1997. AP-1 function and regulation. Curr Opin Cell Biol. 9, 240-246.

Kasibhatla, S., Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A. and Green, D. R., 1998. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol Cell. 1, 543-551.

Keller, J. N., Schmitt, F. A., Scheff, S. W., Ding, Q., Chen, Q., Butterfield, D. A. and Markesbery, W. R., 2005. Evidence of increased oxidative damage in subjects with mild cognitive impairment. Neurology. 64, 1152-1156.

King, G. D. and Scott Turner, R., 2004. Adaptor protein interactions: modulators of amyloid precursor protein metabolism and Alzheimer's disease risk? Exp Neurol. 185, 208-219.

Knafo, S., Alonso-Nanclares, L., Gonzalez-Soriano, J., Merino-Serrais, P., Fernaud-Espinosa, I., Ferrer, I. and DeFelipe, J., 2009. Widespread changes in dendritic spines in a model of Alzheimer's disease. Cereb Cortex. 19, 586-592.

Komori, T., 1999. Tau-positive glial inclusions in progressive supranuclear palsy, corticobasal degeneration and Pick's disease. Brain Pathol. 9, 663-679.

Konzack, S., Thies, E., Marx, A., Mandelkow, E. M. and Mandelkow, E., 2007. Swimming against the tide: mobility of the microtubule-associated protein tau in neurons. J Neurosci. 27, 9916-9927.

Korolainen, M. A., Auriola, S., Nyman, T. A., Alafuzoff, I. and Pirttila, T., 2005. Proteomic analysis of glial fibrillary acidic protein in Alzheimer's disease and aging brain. Neurobiol Dis. 20, 858-870.

Kotilinek, L. A., Bacskai, B., Westerman, M., Kawarabayashi, T., Younkin, L., Hyman, B. T., Younkin, S. and Ashe, K. H., 2002. Reversible memory loss in a mouse transgenic model of Alzheimer's disease. J Neurosci. 22, 6331-6335.

Kovacs, G. G., Pittman, A., Revesz, T., Luk, C., Lees, A., Kiss, E., Tariska, P., Laszlo, L., Molnar, K., Molnar, M. J., Tolnay, M. and de Silva, R., 2008. MAPT S305I mutation: implications for argyrophilic grain disease. Acta Neuropathol. 116, 103-118.

Kremer, B., Swaab, D., Bots, G., Fisser, B., Ravid, R. and Roos, R., 1991. The hypothalamic lateral tuberal nucleus in Alzheimer's disease. Ann Neurol. 29, 279-284.

Layfield, R., Cavey, J. R. and Lowe, J., 2003. Role of ubiquitin-mediated proteolysis in the pathogenesis of neurodegenerative disorders. Ageing Res Rev. 2, 343-356.

Layfield, R., Fergusson, J., Aitken, A., Lowe, J., Landon, M. and Mayer, R. J., 1996. Neurofibrillary tangles of Alzheimer's disease brains contain 14-3-3 proteins. Neurosci Lett. 209, 57-60.

Lee, G., Cowan, N. and Kirschner, M., 1988. The primary structure and heterogeneity of tau protein from mouse brain. Science. 239, 285-288.

Lee, G., Thangavel, R., Sharma, V. M., Litersky, J. M., Bhaskar, K., Fang, S. M., Do, L. H., Andreadis, A., Van Hoesen, G. and Ksiezak-Reding, H., 2004. Phosphorylation of tau by fyn: implications for Alzheimer's disease. J Neurosci. 24, 2304-2312.

Lee, V. M., Goedert, M. and Trojanowski, J. Q., 2001. Neurodegenerative tauopathies. Annu Rev Neurosci. 24, 1121-1159.

Le-Niculescu, H., Bonfoco, E., Kasuya, Y., Claret, F. X., Green, D. R. and Karin, M., 1999. Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. Mol Cell Biol. 19, 751-763.

Lepekhin, E. A., Eliasson, C., Berthold, C. H., Berezin, V., Bock, E. and Pekny, M., 2001. Intermediate filaments regulate astrocyte motility. J Neurochem. 79, 617-625.

Li, L., He, S., Sun, J. M. and Davie, J. R., 2004. Gene regulation by Sp1 and Sp3. Biochem Cell Biol. 82, 460-471.

Li, S. H., Cheng, A. L., Zhou, H., Lam, S., Rao, M., Li, H. and Li, X. J., 2002. Interaction of Huntington disease protein with transcriptional activator Sp1. Mol Cell Biol. 22, 1277-1287.

Li, X., Greenwood, A. F., Powers, R. and Jope, R. S., 1996. Effects of postmortem interval, age, and Alzheimer's disease on G-proteins in human brain. Neurobiol Aging. 17, 115-122.

Lindsten, K., de Vrij, F. M., Verhoef, L. G., Fischer, D. F., van Leeuwen, F. W., Hol, E. M., Masucci, M. G. and Dantuma, N. P., 2002. Mutant ubiquitin found in neurodegenerative disorders is a ubiquitin fusion degradation substrate that blocks proteasomal degradation. J Cell Biol. 157, 417-427.

Liu, F., Iqbal, K., Grundke-Iqbal, I., Rossie, S. and Gong, C. X., 2005. Dephosphorylation of tau by protein phosphatase 5: impairment in Alzheimer's disease. J Biol Chem. 280, 1790-1796.

Liu, W., Wang, G. and Yakovlev, A. G., 2002. Identification and functional analysis of the rat caspase-3 gene promoter. J Biol Chem. 277, 8273-8278.

Lovell, M. A., Xiong, S., Xie, C., Davies, P. and Markesbery, W. R., 2004. Induction of hyperphosphorylated tau in primary rat cortical neuron cultures mediated by oxidative stress and glycogen synthase kinase-3. J Alzheimers Dis. 6, 659-671; discussion 673-681.

Lundkvist, A., Reichenbach, A., Betsholtz, C., Carmeliet, P., Wolburg, H. and Pekny, M., 2004. Under stress, the absence of intermediate filaments from Muller cells in the retina has structural and functional consequences. J Cell Sci. 117, 3481-3488.

Lynch, C. and Mobley, W., 2000. Comprehensive theory of Alzheimer's disease. The effects of cholesterol on membrane receptor trafficking. Ann N Y Acad Sci. 924, 104-111.

Lynch, T., Sano, M., Marder, K. S., Bell, K. L., Foster, N. L., Defendini, R. F., Sima, A. A., Keohane, C., Nygaard, T. G., Fahn, S. and et al., 1994. Clinical characteristics of a family with chromosome 17-linked disinhibition-dementia-parkinsonism-amyotrophy complex. Neurology. 44, 1878-1884.

MacGibbon, G. A., Lawlor, P. A., Walton, M., Sirimanne, E., Faull, R. L., Synek, B., Mee, E., Connor, B. and Dragunow, M., 1997. Expression of Fos, Jun, and Krox family proteins in Alzheimer's disease. Exp Neurol. 147, 316-332.

Marcus, D. L., Strafaci, J. A., Miller, D. C., Masia, S., Thomas, C. G., Rosman, J., Hussain, S. and Freedman, M. L., 1998. Quantitative neuronal c-fos and c-jun expression in Alzheimer's disease. Neurobiol Aging. 19, 393-400.

Martin, D. P., Schmidt, R. E., DiStefano, P. S., Lowry, O. H., Carter, J. G. and Johnson, E. M., Jr., 1988. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J Cell Biol. 106, 829-844.

Martinez, A., Carmona, M., Portero-Otin, M., Naudi, A., Pamplona, R. and Ferrer, I., 2008a. Type-Dependent Oxidative Damage in Frontotemporal Lobar Degeneration: Cortical Astrocytes Are Targets of Oxidative Damage. J Neuropathol Exp Neurol.

Martinez, A., Carmona, M., Portero-Otin, M., Naudi, A., Pamplona, R. and Ferrer, I., 2008b. Type-dependent oxidative damage in frontotemporal lobar degeneration: cortical astrocytes are targets of oxidative damage. J Neuropathol Exp Neurol. 67, 1122-1136.

Martinez-Lage, P. and Munoz, D. G., 1997. Prevalence and disease associations of argyrophilic grains of Braak. J Neuropathol Exp Neurol. 56, 157-164.

Mata, I. F., Kachergus, J. M., Taylor, J. P., Lincoln, S., Aasly, J., Lynch, T., Hulihan, M. M., Cobb, S. A., Wu, R. M., Lu, C. S., Lahoz, C., Wszolek, Z. K. and Farrer, M. J., 2005. Lrrk2 pathogenic substitutions in Parkinson's disease. Neurogenetics. 6, 171-177.

Mata, I. F., Wedemeyer, W. J., Farrer, M. J., Taylor, J. P. and Gallo, K. A., 2006. LRRK2 in Parkinson's disease: protein domains and functional insights. Trends Neurosci. 29, 286-293.

McCall, M. A., Gregg, R. G., Behringer, R. R., Brenner, M., Delaney, C. L., Galbreath, E. J., Zhang, C. L., Pearce, R. A., Chiu, S. Y. and Messing, A., 1996. Targeted deletion in astrocyte intermediate filament (Gfap) alters neuronal physiology. Proc Natl Acad Sci U S A. 93, 6361-6366.

McLellan, M. E., Kajdasz, S. T., Hyman, B. T. and Bacskai, B. J., 2003. In vivo imaging of reactive oxygen species specifically associated with thioflavine S-positive amyloid plaques by multiphoton microscopy. J Neurosci. 23, 2212-2217.

Melov, S., 2002. Therapeutics against mitochondrial oxidative stress in animal models of aging. Ann N Y Acad Sci. 959, 330-340.

Melrose, H. L., Kent, C. B., Taylor, J. P., Dachsel, J. C., Hinkle, K. M., Lincoln, S. J., Mok, S. S., Culvenor, J. G., Masters, C. L., Tyndall, G. M., Bass, D. I., Ahmed, Z., Andorfer, C. A., Ross, O. A., Wszolek, Z. K., Delldonne, A., Dickson, D. W. and Farrer, M. J., 2007. A comparative analysis of leucine-rich repeat kinase 2 (Lrrk2) expression in mouse brain and Lewy body disease. Neuroscience. 147, 1047-1058.

Mesulam, M. M., 1999. Neuroplasticity failure in Alzheimer's disease: bridging the gap between plaques and tangles. Neuron. 24, 521-529.

Mesulam, M. M., 2000. A plasticity-based theory of the pathogenesis of Alzheimer's disease. Ann N Y Acad Sci. 924, 42-52.

Miklossy, J., Arai, T., Guo, J. P., Klegeris, A., Yu, S., McGeer, E. G. and McGeer, P. L., 2006. LRRK2 expression in normal and pathologic human brain and in human cell lines. J Neuropathol Exp Neurol. 65, 953-963.

Miklossy, J., Qing, H., Guo, J. P., Yu, S., Wszolek, Z. K., Calne, D., McGeer, E. G. and McGeer, P. L., 2007. Lrrk2 and chronic inflammation are linked to pallido-ponto-nigral degeneration caused by the N279K tau mutation. Acta Neuropathol. 114, 243-254.

Mondragon-Rodriguez, S., Mena, R., Binder, L. I., Smith, M. A., Perry, G. and Garcia-Sierra, F., 2008. Conformational changes and cleavage of tau in Pick bodies parallel the early processing of tau found in Alzheimer pathology. Neuropathol Appl Neurobiol. 34, 62-75.

Montine, K. S., Kim, P. J., Olson, S. J., Markesbery, W. R. and Montine, T. J., 1997. 4-hydroxy-2-nonenal pyrrole adducts in human neurodegenerative disease. J Neuropathol Exp Neurol. 56, 866-871.

Mori, N., 1993. Toward understanding of the molecular basis of loss of neuronal plasticity in ageing. Age Ageing. 22, S5-18.

Morsch, R., Simon, W. and Coleman, P. D., 1999. Neurons may live for decades with neurofibrillary tangles. J Neuropathol Exp Neurol. 58, 188-197.

Mucke, L. and Eddleston, M., 1993. Astrocytes in infectious and immune-mediated diseases of the central nervous system. Faseb J. 7, 1226-1232.

Munoz, F. J., Sole, M. and Coma, M., 2005. The protective role of vitamin E in vascular amyloid beta-mediated damage. Subcell Biochem. 38, 147-165.

Muntane, G., Dalfo, E., Martinez, A., Rey, M. J., Avila, J., Perez, M., Portero, M., Pamplona, R., Ayala, V. and Ferrer, I., 2006. Glial fibrillary acidic protein is a major target of glycoxidative and lipoxidative damage in Pick's disease. J Neurochem. 99, 177-185.

Muslin, A. J. and Lau, J. M., 2005. Differential functions of 14-3-3 isoforms in vertebrate development. Curr Top Dev Biol. 65, 211-228.

Nabeshi, H., Oikawa, S., Inoue, S., Nishino, K. and Kawanishi, S., 2006. Proteomic analysis for protein carbonyl as an indicator of oxidative damage in senescence-accelerated mice. Free Radic Res. 40, 1173-1181.

Najlerahim, A. and Bowen, D. M., 1988. Regional weight loss of the cerebral cortex and some subcortical nuclei in senile dementia of the Alzheimer type. Acta Neuropathol. 75, 509-512.

Nakaso, K., Yoshimoto, Y., Nakano, T., Takeshima, T., Fukuhara, Y., Yasui, K., Araga, S., Yanagawa, T., Ishii, T. and Nakashima, K., 2004. Transcriptional activation of p62/A170/ZIP during the formation of the aggregates: possible mechanisms and the role in Lewy body formation in Parkinson's disease. Brain Res. 1012, 42-51.

Nathan, B. P., Bellosta, S., Sanan, D. A., Weisgraber, K. H., Mahley, R. W. and Pitas, R. E., 1994. Differential effects of apolipoproteins E3 and E4 on neuronal growth in vitro. Science. 264, 850-852.

Neary, D., Snowden, J. S., Gustafson, L., Passant, U., Stuss, D., Black, S., Freedman, M., Kertesz, A., Robert, P. H., Albert, M., Boone, K., Miller, B. L., Cummings, J. and Benson, D. F., 1998. Frontotemporal lobar degeneration: a consensus on clinical diagnostic criteria. Neurology. 51, 1546-1554.

Nielsen, A. L., Holm, I. E., Johansen, M., Bonven, B., Jorgensen, P. and Jorgensen, A. L., 2002. A new splice variant of glial fibrillary acidic protein, GFAP epsilon, interacts with the presenilin proteins. J Biol Chem. 277, 29983-29991.

Nixon, R. A., 2000. A "protease activation cascade" in the pathogenesis of Alzheimer's disease. Ann N Y Acad Sci. 924, 117-131.

Novak, M., Jakes, R., Edwards, P. C., Milstein, C. and Wischik, C. M., 1991. Difference between the Tau-Protein of Alzheimer Paired Helical Filament Core

and Normal Tau Revealed by Epitope Analysis of Monoclonal Antibodies-423 and Antibodies-7.51. Proceedings of the National Academy of Sciences of the United States of America. 88, 5837-5841.

Novak, M., Kabat, J. and Wischik, C. M., 1993. Molecular Characterization of the Minimal Protease Resistant Tau-Unit of the Alzheimers-Disease Paired Helical Filament. Embo Journal. 12, 365-370.

Pamplona, R., Dalfo, E., Ayala, V., Bellmunt, M. J., Prat, J., Ferrer, I. and Portero-Otin, M., 2005. Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets. J Biol Chem. 280, 21522-21530.

Papp, M. I., Kahn, J. E. and Lantos, P. L., 1989. Glial cytoplasmic inclusions in the CNS of patients with multiple system atrophy (striatonigral degeneration, olivopontocerebellar atrophy and Shy-Drager syndrome). J Neurol Sci. 94, 79-100.

Patrick, G. N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P. and Tsai, L. H., 1999. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature. 402, 615-622.

Pekny, M. and Pekna, M., 2004. Astrocyte intermediate filaments in CNS pathologies and regeneration. J Pathol. 204, 428-437.

Perez-Gracia, E., Torrejon-Escribano, B. and Ferrer, I., 2008. Dystrophic neurites of senile plaques in Alzheimer's disease are deficient in cytochrome c oxidase. Acta Neuropathol. 116, 261-268.

Perry, G., 2003. The role of oxidative mechanisms in neurodegenerative diseases. In: Dickson, D. (Ed.), Neurodegeneration: The molecular pathology of dementia and movement disorders. ISN Neuropath Press, pp. 8-10.

Perry, G., Nunomura, A., Lucassen, P., Lassmann, H. and Smith, M. A., 1998a. Apoptosis and Alzheimer's disease. Science. 282, 1268-1269.

Perry, G., Nunomura, A. and Smith, M. A., 1998b. A suicide note from Alzheimer disease neurons? Nat Med. 4, 897-898.

Perry, G., Zhu, X., Babar, A. K., Siedlak, S. L., Yang, Q., Ito, G., Iwatsubo, T., Smith, M. A. and Chen, S. G., 2008. Leucine-rich repeat kinase 2 colocalizes with alpha-synuclein in Parkinson's disease, but not tau-containing deposits in tauopathies. Neurodegener Dis. 5, 222-224.

Philipsen, S. and Suske, G., 1999. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. Nucleic Acids Res. 27, 2991-3000.

Piccini, A., Russo, C., Gliozzi, A., Relini, A., Vitali, A., Borghi, R., Giliberto, L., Armirotti, A., D'Arrigo, C., Bachi, A., Cattaneo, A., Canale, C., Torrassa, S., Saido, T. C., Markesbery, W., Gambetti, P. and Tabaton, M., 2005. beta-amyloid is different in normal aging and in Alzheimer disease. J Biol Chem. 280, 34186-34192.

Piccini, P., de Yebenez, J., Lees, A. J., Ceravolo, R., Turjanski, N., Pramstaller, P. and Brooks, D. J., 2001. Familial progressive supranuclear palsy: detection of subclinical cases using 18F-dopa and 18fluorodeoxyglucose positron emission tomography. Arch Neurol. 58, 1846-1851.

Puig, B., Gomez-Isla, T., Ribe, E., Cuadrado, M., Torrejon-Escribano, B., Dalfo, E. and Ferrer, I., 2004. Expression of stress-activated kinases c-Jun N-terminal kinase (SAPK/JNK-P) and p38 kinase (p38-P), and tau hyperphosphorylation in neurites surrounding betaA plaques in APP Tg2576 mice. Neuropathol Appl Neurobiol. 30, 491-502.

Puig, B., Rey, M. and Ferrer, I., 2005. Individual and regional variations of phospho-tau species in progressive supranuclear palsy. Acta Neuropathologica. 110, 261-268.

Rajput, A., Dickson, D. W., Robinson, C. A., Ross, O. A., Dachsel, J. C., Lincoln, S. J., Cobb, S. A., Rajput, M. L. and Farrer, M. J., 2006. Parkinsonism, Lrrk2 G2019S, and tau neuropathology. Neurology. 67, 1506-1508.

Raynaud, F. and Marcilhac, A., 2006. Implication of calpain in neuronal apoptosis. A possible regulation of Alzheimer's disease. Febs J. 273, 3437-3443.

Robertson, L. A., Moya, K. L. and Breen, K. C., 2004. The potential role of tau protein O-glycosylation in Alzheimer's disease. J Alzheimers Dis. 6, 489-495.

Roch, J. M., Masliah, E., Roch-Levecq, A. C., Sundsmo, M. P., Otero, D. A., Veinbergs, I. and Saitoh, T., 1994. Increase of synaptic density and memory retention by a peptide representing the trophic domain of the amyloid beta/A4 protein precursor. Proc Natl Acad Sci U S A. 91, 7450-7454.

Rodriguez, A. and Ferrer, I., 2007. Expression of transcription factors CREB and c-Fos in the brains of terminal Creutzfeldt-Jakob disease cases. Neurosci Lett. 421, 10-15.

Ros, R., Gomez Garre, P., Hirano, M., Tai, Y. F., Ampuero, I., Vidal, L., Rojo, A., Fontan, A., Vazquez, A., Fanjul, S., Hernandez, J., Cantarero, S., Hoenicka, J., Jones, A., Ahsan, R. L., Pavese, N., Piccini, P., Brooks, D. J., Perez-Tur, J., Nyggard, T. and de Yebenes, J. G., 2005. Genetic linkage of autosomal dominant progressive supranuclear palsy to 1q31.1. Ann Neurol. 57, 634-641.

Ross, C. A., 1997. Intranuclear neuronal inclusions: a common pathogenic mechanism for glutamine-repeat neurodegenerative diseases? Neuron. 19, 1147-1150.

Saito, K., Elce, J. S., Hamos, J. E. and Nixon, R. A., 1993. Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. Proc Natl Acad Sci U S A. 90, 2628-2632.

Saito, Y., Nakahara, K., Yamanouchi, H. and Murayama, S., 2002. Severe involvement of ambient gyrus in dementia with grains. J Neuropathol Exp Neurol. 61, 789-796.

Saito, Y., Ruberu, N. N., Sawabe, M., Arai, T., Tanaka, N., Kakuta, Y., Yamanouchi, H. and Murayama, S., 2004. Staging of argyrophilic grains: an age-associated tauopathy. J Neuropathol Exp Neurol. 63, 911-918.

Santacruz, K., Lewis, J., Spires, T., Paulson, J., Kotilinek, L., Ingelsson, M., Guimaraes, A., DeTure, M., Ramsden, M., McGowan, E., Forster, C., Yue, M., Orne, J., Janus, C., Mariash, A., Kuskowski, M., Hyman, B., Hutton, M. and Ashe, K. H., 2005. Tau suppression in a neurodegenerative mouse model improves memory function. Science. 309, 476-481.

Sayre, L. M., Zelasko, D. A., Harris, P. L., Perry, G., Salomon, R. G. and Smith, M. A., 1997. 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. J Neurochem. 68, 2092-2097.

Schmidt, A. M., Yan, S. D., Yan, S. F. and Stern, D. M., 2000. The biology of the receptor for advanced glycation end products and its ligands. Biochim Biophys Acta. 1498, 99-111.

Schwab, C., Bondada, V., Sparks, D. L., Cahan, L. D. and Geddes, J. W., 1994. Postmortem changes in the levels and localization of microtubule-associated proteins (tau, MAP2 and MAP1B) in the rat and human hippocampus. Hippocampus. 4, 210-225.

Scott, I. S. and Lowe, J. S., 2007. The ubiquitin-binding protein p62 identifies argyrophilic grain pathology with greater sensitivity than conventional silver stains. Acta Neuropathol. 113, 417-420.

Seibenhener, M. L., Babu, J. R., Geetha, T., Wong, H. C., Krishna, N. R. and Wooten, M. W., 2004. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol Cell Biol. 24, 8055-8068.

Selkoe, D. J., 1991. The molecular pathology of Alzheimer's disease. Neuron. 6, 487-498.

Seno, H., Kobayashi, S., Inagaki, T., Yamamori, C., Miyaoka, T., Horiguchi, J., Wada, M. and Harada, T., 2000. Parkinson's disease associated with argyrophilic grains clinically resembling progressive supranuclear palsy: an autopsy case. J Neurol Sci. 178, 70-74.

Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C. and et al., 1992. Isolation and quantification of soluble Alzheimer's beta-peptide from biological fluids. Nature. 359, 325-327.

Shaulian, E. and Karin, M., 2002. AP-1 as a regulator of cell life and death. Nat Cell Biol. 4, E131-136.

Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K. and et al., 1995. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature. 375, 754-760.

Shimohata, T., Nakajima, T., Yamada, M., Uchida, C., Onodera, O., Naruse, S., Kimura, T., Koide, R., Nozaki, K., Sano, Y., Ishiguro, H., Sakoe, K., Ooshima, T., Sato, A., Ikeuchi, T., Oyake, M., Sato, T., Aoyagi, Y., Hozumi, I., Nagatsu, T., Takiyama, Y., Nishizawa, M., Goto, J., Kanazawa, I., Davidson, I., Tanese, N., Takahashi, H. and Tsuji, S., 2000. Expanded polyglutamine stretches interact with TAFII130, interfering with CREB-dependent transcription. Nat Genet. 26, 29-36.

Shin, N., Jeong, H., Kwon, J., Heo, H. Y., Kwon, J. J., Yun, H. J., Kim, C. H., Han, B. S., Tong, Y., Shen, J., Hatano, T., Hattori, N., Kim, K. S., Chang, S. and Seol, W., 2008. LRRK2 regulates synaptic vesicle endocytosis. Exp Cell Res.

Shults, C. W., 2006. Lewy bodies. Proc Natl Acad Sci U S A. 103, 1661-1668.

Siew, L. K., Love, S., Dawbarn, D., Wilcock, G. K. and Allen, S. J., 2004. Measurement of pre- and post-synaptic proteins in cerebral cortex: effects of post-mortem delay. J Neurosci Methods. 139, 153-159.

Skrabana, R., Kontsek, P., Mederlyova, A., Iqbal, K. and Novak, M., 2004. Folding of Alzheimer's core PHF subunit revealed by monoclonal antibody 423. Febs Letters. 568, 178-182.

Smith, M. A., Harris, P. L., Sayre, L. M. and Perry, G., 1997a. Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. Proc Natl Acad Sci U S A. 94, 9866-9868.

Smith, M. A., Perry, G., Richey, P. L., Sayre, L. M., Anderson, V. E., Beal, M. F. and Kowall, N., 1996. Oxidative damage in Alzheimer's. Nature. 382, 120-121.

Smith, M. A., Richey Harris, P. L., Sayre, L. M., Beckman, J. S. and Perry, G., 1997b. Widespread peroxynitrite-mediated damage in Alzheimer's disease. J Neurosci. 17, 2653-2657.

Smith, M. A., Taneda, S., Richey, P. L., Miyata, S., Yan, S. D., Stern, D., Sayre, L. M., Monnier, V. M. and Perry, G., 1994. Advanced Maillard reaction end products

are associated with Alzheimer disease pathology. Proc Natl Acad Sci U S A. 91, 5710-5714.

Sontag, E., Hladik, C., Montgomery, L., Luangpirom, A., Mudrak, I., Ogris, E. and White, C. L., 3rd, 2004a. Downregulation of protein phosphatase 2A carboxyl methylation and methyltransferase may contribute to Alzheimer disease pathogenesis. J Neuropathol Exp Neurol. 63, 1080-1091.

Sontag, E., Luangpirom, A., Hladik, C., Mudrak, I., Ogris, E., Speciale, S. and White, C. L., 3rd, 2004b. Altered expression levels of the protein phosphatase 2A ABalphaC enzyme are associated with Alzheimer disease pathology. J Neuropathol Exp Neurol. 63, 287-301.

Sorolla, M. A., Reverter-Branchat, G., Tamarit, J., Ferrer, I., Ros, J. and Cabiscol, E., 2008. Proteomic and oxidative stress analysis in human brain samples of Huntington disease. Free Radic Biol Med. 45, 667-678.

Soulie, C., Nicole, A., Delacourte, A. and Ceballos-Picot, I., 2004. Examination of stress-related genes in human temporal versus occipital cortex in the course of neurodegeneration: involvement of 14-3-3 zeta in this dynamic process. Neurosci Lett. 365, 1-5.

Stadtman, E. R., 2006. Protein oxidation and aging. Free Radic Res. 40, 1250-1258.

Stambolic, V. and Woodgett, J. R., 1994. Mitogen inactivation of glycogen synthase kinase-3 beta in intact cells via serine 9 phosphorylation. Biochem J. 303 ( Pt 3), 701-704.

Strittmatter, W. J., Saunders, A. M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G. S. and Roses, A. D., 1993. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. Proc Natl Acad Sci U S A. 90, 1977-1981.

Sullivan, S. M., Lee, A., Bjorkman, S. T., Miller, S. M., Sullivan, R. K., Poronnik, P., Colditz, P. B. and Pow, D. V., 2007. Cytoskeletal anchoring of GLAST determines susceptibility to brain damage: an identified role for GFAP. J Biol Chem. 282, 29414-29423.

Sultana, R., Newman, S. F., Abdul, H. M., Cai, J., Pierce, W. M., Klein, J. B., Merchant, M. and Butterfield, D. A., 2006. Protective effect of D609 against amyloid-beta1-42-induced oxidative modification of neuronal proteins: redox proteomics study. J Neurosci Res. 84, 409-417.

Sultana, R., Reed, T., Perluigi, M., Coccia, R., Pierce, W. M. and Butterfield, D. A., 2007. Proteomic identification of nitrated brain proteins in amnestic mild cognitive impairment: a regional study. J Cell Mol Med. 11, 839-851.

Sun, X., Wang, Y., Qing, H., Christensen, M. A., Liu, Y., Zhou, W., Tong, Y., Xiao, C., Huang, Y., Zhang, S., Liu, X. and Song, W., 2005. Distinct transcriptional regulation and function of the human BACE2 and BACE1 genes. Faseb J. 19, 739-749.

Takemura, M., Nishiyama, H. and Itohara, S., 2002. Distribution of phosphorylated glial fibrillary acidic protein in the mouse central nervous system. Genes Cells. 7, 295-307.

Tan, E. K. and Skipper, L. M., 2007. Pathogenic mutations in Parkinson disease. Hum Mutat. 28, 641-653.

Tanemura, K., Chui, D. H., Fukuda, T., Murayama, M., Park, J. M., Akagi, T., Tatebayashi, Y., Miyasaka, T., Kimura, T., Hashikawa, T., Nakano, Y., Kudo, T., Takeda, M. and Takashima, A., 2006. Formation of tau inclusions in knock-in mice with familial Alzheimer disease (FAD) mutation of presenilin 1 (PS1). J Biol Chem. 281, 5037-5041.

Togo, T. and Dickson, D. W., 2002. Ballooned neurons in progressive supranuclear palsy are usually due to concurrent argyrophilic grain disease. Acta Neuropathol. 104, 53-56.

Tolnay, M., Monsch, A. U. and Probst, A., 2001. Argyrophilic grain disease. A frequent dementing disorder in aged patients. Adv Exp Med Biol. 487, 39-58.

Tolnay, M., Sergeant, N., Ghestem, A., Chalbot, S., De Vos, R. A., Jansen Steur, E. N., Probst, A. and Delacourte, A., 2002. Argyrophilic grain disease and Alzheimer's disease are distinguished by their different distribution of tau protein isoforms. Acta Neuropathol. 104, 425-434.

Tong, Y., Zhou, W., Fung, V., Christensen, M. A., Qing, H., Sun, X. and Song, W., 2005. Oxidative stress potentiates BACE1 gene expression and Abeta generation. J Neural Transm. 112, 455-469.

Uchida, K., Hasui, Y. and Osawa, T., 1997. Covalent attachment of 4-hydroxy-2-nonenal to erythrocyte proteins. J Biochem. 122, 1246-1251.

Uchihara, T., Nakamura, A., Yamazaki, M. and Mori, O., 2001. Evolution from pretangle neurons to neurofibrillary tangles monitored by thiazin red combined with Gallyas method and double immunofluorescence. Acta Neuropathol. 101, 535-539.

Umahara, T., Uchihara, T., Tsuchiya, K., Nakamura, A., Ikeda, K., Iwamoto, T. and Takasaki, M., 2004a. Immunolocalization of 14-3-3 isoforms in brains with Pick body disease. Neurosci Lett. 371, 215-219.

Umahara, T., Uchihara, T., Tsuchiya, K., Nakamura, A., Iwamoto, T., Ikeda, K. and Takasaki, M., 2004b. 14-3-3 proteins and zeta isoform containing neurofibrillary tangles in patients with Alzheimer's disease. Acta Neuropathol. 108, 279-286.

van Leeuwen, F. W., de Kleijn, D. P., van den Hurk, H. H., Neubauer, A., Sonnemans, M. A., Sluijs, J. A., Koycu, S., Ramdjielal, R. D., Salehi, A., Martens, G. J., Grosveld, F. G., Peter, J., Burbach, H. and Hol, E. M., 1998. Frameshift mutants of beta amyloid precursor protein and ubiquitin-B in Alzheimer's and Down patients. Science. 279, 242-247.

Verny, M., Duyckaerts, C., Agid, Y. and Hauw, J. J., 1996. The significance of cortical pathology in progressive supranuclear palsy. Clinico-pathological data in 10 cases. Brain. 119 ( Pt 4), 1123-1136.

Vinters, H. V., 1987. Cerebral amyloid angiopathy. A critical review. Stroke. 18, 311-324.

Vogelsberg-Ragaglia, V., Schuck, T., Trojanowski, J. Q. and Lee, V. M., 2001. PP2A mRNA expression is quantitatively decreased in Alzheimer's disease hippocampus. Exp Neurol. 168, 402-412.

Walton, M. R. and Dragunow, I., 2000. Is CREB a key to neuronal survival? Trends Neurosci. 23, 48-53.

Weingarten, M. D., Lockwood, A. H., Hwo, S. Y. and Kirschner, M. W., 1975. A protein factor essential for microtubule assembly. Proc Natl Acad Sci U S A. 72, 1858-1862.

Weisman, D. and McKeith, I., 2007. Dementia with Lewy bodies. Semin Neurol. 27, 42-47.

Welsh, K., Butters, N., Hughes, J., Mohs, R. and Heyman, A., 1991. Detection of abnormal memory decline in mild cases of Alzheimer's disease using CERAD neuropsychological measures. Arch Neurol. 48, 278-281.

Whitehouse, P. J., Price, D. L., Clark, A. W., Coyle, J. T. and DeLong, M. R., 1981. Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. Ann Neurol. 10, 122-126.

Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T. and Delon, M. R., 1982. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science. 215, 1237-1239.

Williams, T. I., Lynn, B. C., Markesbery, W. R. and Lovell, M. A., 2006. Increased levels of 4-hydroxynonenal and acrolein, neurotoxic markers of lipid peroxidation, in the brain in Mild Cognitive Impairment and early Alzheimer's disease. Neurobiol Aging. 27, 1094-1099.

Wisniewski, H. M., Bancher, C., Barcikowska, M., Wen, G. Y. and Currie, J., 1989. Spectrum of morphological appearance of amyloid deposits in Alzheimer's disease. Acta Neuropathol. 78, 337-347.

Xu, Y., Porntadavity, S. and St Clair, D. K., 2002. Transcriptional regulation of the human manganese superoxide dismutase gene: the role of specificity protein 1 (Sp1) and activating protein-2 (AP-2). Biochem J. 362, 401-412.

Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H., 3rd and Montminy, M. R., 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature. 334, 494-498.

Yamamoto-Sasaki, M., Ozawa, H., Saito, T., Rosler, M. and Riederer, P., 1999. Impaired phosphorylation of cyclic AMP response element binding protein in the hippocampus of dementia of the Alzheimer type. Brain Res. 824, 300-303.

Yatin, S. M., Varadarajan, S. and Butterfield, D. A., 2000. Vitamin E Prevents Alzheimer's Amyloid beta-Peptide (1-42)-Induced Neuronal Protein Oxidation and Reactive Oxygen Species Production. J Alzheimers Dis. 2, 123-131.

Yuan, Z., Agarwal-Mawal, A. and Paudel, H. K., 2004. 14-3-3 binds to and mediates phosphorylation of microtubule-associated tau protein by Ser9-phosphorylated glycogen synthase kinase 3beta in the brain. J Biol Chem. 279, 26105-26114.

Zarkovic, K., 2003. 4-hydroxynonenal and neurodegenerative diseases. Mol Aspects Med. 24, 293-303.

Zatloukal, K., Stumptner, C., Fuchsbichler, A., Heid, H., Schnoelzer, M., Kenner, L., Kleinert, R., Prinz, M., Aguzzi, A. and Denk, H., 2002. p62 Is a common component of cytoplasmic inclusions in protein aggregation diseases. Am J Pathol. 160, 255-263.

Zhou, X. W., Gustafsson, J. A., Tanila, H., Bjorkdahl, C., Liu, R., Winblad, B. and Pei, J. J., 2008. Tau hyperphosphorylation correlates with reduced methylation of protein phosphatase 2A. Neurobiol Dis. 31, 386-394.

Zilka, N., Filipcik, P., Koson, P., Fialova, L., Skrabana, R., Zilkova, M., Rolkova, G., Kontsekova, E. and Novak, M., 2006. Truncated tau from sporadic Alzheimer's disease suffices to drive neurofibrillary degeneration in vivo. Febs Letters. 580, 3582-3588.

Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., Kachergus, J., Hulihan, M., Uitti, R. J., Calne, D. B., Stoessl, A. J., Pfeiffer, R. F., Patenge, N., Carbajal, I. C., Vieregge, P., Asmus, F., Muller-Myhsok, B., Dickson, D. W., Meitinger, T., Strom, T. M., Wszolek, Z. K. and Gasser, T., 2004. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron. 44, 601-607.