Modificacions post-traduccionals de l'α-sinucleïna en les malalties neurodegeneratives

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Als papes

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I. Índex

II. Abreviatures

AD: Malaltia d'Alzheimer
ADN: Àcid desoxiribonucleic
AGD: Malaltia de grans argiròfils
APP: Proteïna precursora de l'amiloide
CBD: Degeneració corticobasal
Cdk-5: Cyclin-dependent kinase 5
CK: Casein-kinase
CMA: Autofàgia mitjançada per xaperones
DA: Dopamina
DFT: Demència frontotemporal
DLB: Demència amb cossos de Lewy
ERK ½: Extracellular signal-regulated kinases 1 and 2
FTDP-17: Demències fontotemporal amb parkinsonisme lligada al cromosoma 17
GCI: Inclusions citoplasmàtiques glials
GSK3-β: Glycogen synthase kinase 3
kDa: Kilodaltons
KO: Knock-out
LAMP2A: Lysosome-associated membrane protein type 2A
LB: Cossos de Lewy
LBD: Malalties amb cossos de Lewy
LN: Neurites de Lewy
LRRK2: Leucine-rich repeat kinase 2
MBD: Domini d'unió a microtúbuls
MMP: Matrix metalloproteinase
MPTP: 1-metil-4-fenil-1,2,3,6-tetrahidropiridina
mRNA: Missatger d'àcid ribonucleic
MSA: Atròfia multisistèmica
NAC: Non-amyloid component
NFT: Neurofibrillary tangles (Cabdells neurofibril·lars)
NO: Òxid nítric
PiD: Malaltia de Pick
PLD2: Fosfolipasa D2
PD: Malaltia de Parkinson
PDD: Malaltia de Parkinson amb demència
PDGF: Platelet-derived growth factor
PHF: Paired helical filaments (filaments aparellats helicoidalment)
PrP: Proteïna priònica
PUFA: Àcids grassos insaturats
PINK1: PTEN induced putative kinase 1
PKA: Proteïna kinase A
PSP: Paràlisi supranuclear progressiva
ROS: Espècies reactives d'oxigen
SDS: Dodecilsulfat sòdic
SNC: Sistema nerviós central
SNCA: gen de l'α-sinucleïna
TH: Tirosina hidroxilasa
UCHL-1: Ubiquitin carboxy-terminal hydrolase L1
UPS: Sistema ubiqüitina-proteasoma

III. Introducció

El cervell en ple rendiment és una meravella. És un òrgan que ens ajuda a percebre, a comprendre i a interaccionar amb el nostre interior i amb el nostre entorn. Entre les seves funcions s'hi poden comptar: el control de les funcions homeostàtiques de l'organisme, el processament de la informació sensorial i la coordinació de la resposta adient, funcions mentals superiors com la cognició i la presa de decisions i la coordinació dels moviments voluntaris. Això ho aconsegueix mitjançant diferents estructures especialitzades en processos determinats, però que a la vegada mantenen una estreta i evident relació les unes amb les altres.

El cervell humà té un pes aproximat de 1350 grams i està composat principalment per neurones i cèl·lules glials. Es calcula que en un cervell humà hi pot haver 100 mil milions de neurones i unes 10 vegades més de cèl·lules glials, totes elles estretament interconnectades. Quan ens trobem amb la mort d'aquestes neurones, s'observa un mal funcionament de les estructures de les que formen part, la qual cosa comporta defectes en la funcionalitat del cervell. És el que ocorre, per exemple, en les malalties neurodegeneratives, quan alguns tipus neuronals concrets moren abandonant la seva funció habitual i afectant a la cognició, a la memòria o al control dels moviments.

1. Les malalties neurodegeneratives

Sota aquest nom s'agrupen un conjunt de malalties que afecten al sistema nerviós central (SNC) i que cursen durant llargs períodes de temps. Sovint, el seu inici és silenciós i tenen un curs progressiu sense remissions. Quan la malaltia evidencia el seu fenotip, el procés de mort neuronal s'ha iniciat en el cervell amb anterioritat i resulta molt difícil recuperar el dany ocasionat.

Els fenotips que manifesten les malalties neurodegeneratives són molt diversos. Es poden presentar com un trastorn de l'equilibri, del moviment, de la parla, de la respiració o de la funció cardíaca.. En la gran majoria de les malalties neurodegeneratives encara que el principal factor de risc és l'edat, se'n desconeix la causa exacta, són les que es coneixen amb el nom d'esporàdiques. D'altres són hereditàries i afecten a tots els individus portadors de la mutació, són les que s'anomenen formes familiars. D'altres, en canvi, són degudes a un quadre clínic concret com un virus, l'alcoholisme o l'exposició a toxines entre d'altres.

La classificació de les malalties neurodegeneratives no és una tasca gens fàcil ja que molts cops una mateixa malaltia pot presentar una clínica i una neuropatologia variables solapant-se amb les altres.

En els últims anys s'han produït grans avenços en els camps de la biologia molecular i la genètica i s'ha observat que aquestes malalties són conseqüència de l'agregació d'algunes proteïnes vulnerables localitzades en el citoplasma o el nucli de la neurona o en les seves proximitats, formant acumulacions proteiques. Fet que comportaria la disminució o la pèrdua total de la seva funció. Se les engloba amb el nom de proteïnopaties [1].

L'acumulació d'aquestes proteïnes pot ser deguda a diversos factors que sovint es troben interelacionats formant un cercle viciós. Entre d'altres: mutacions, una expressió inapropiada, un plegament anormal de la proteïna, modificacions post-traduccionals, estrès oxidatiu, exposició a factors tòxics externs o anormalitats en el procés proteolític [1, 2].

El fet de conèixer millor les alteracions proteiques que es donen en aquests processos ens és útil per classificar les malalties neurodegeneratives en base a l'alteració genètica o molecular que les caracteritza [1]. Depenent de la proteïna que forma part dels agregats les classifiquem en:

	Proteïna tòxica	**Malalties**
Taupaties	Tau	Malaltia d'Alzheimer (AD), Malaltia de Pick (PiD), Demència Frontotemporal amb parkinsonisme lligada al cr.17 (FTDP-17)…
α-sinucleïnopaties	α-sinucleïna	Malaltia de Parkinson (PD), Demència amb Cossos de Lewy (DLB), Atrofia multisistèmica (MSA), etc.
Amiloidopaties	APP(pèptid amiloide)	Malaltia d'Alzheimer (AD), Síndrome de Down (DS), etc.
TDP-43 proteïnopaties	TDP-43, dinactina	Demències frontotemporals (FTLD), Síndrome de Perry.
Trastorns per repetició de triplets CAG	Huntingtina, Choreïna, Ataxina, Atrofina-1	Malaltia de Huntington (HD), Ataxies espinocerebelars (SCA), etc.
Altres trastorns neuromotors	Sod1, Alsina, Atlastina,…	Esclerosi lateral amiotròfica (ALS), Malaltia de Fazio-Londe.
Neurofilamentopaties	α-internexin	Malaltia d'inclusions neuronals de filaments intermedis (NIFID)
Neuroserpinopaties	Neuroserpina	Encefalopatia amb cossos d'inclusió de neuroserpina
Neuroferritinopatia hereditèria	Pèptid 175AA	Neuroferritinopatia hereditària
Malalties priòniques	Proteïna priònica PrP	Malaltia de Creutzfeldt-Jakob (CJD), Insomni Fatal Familiar (FFI)
Altres trastorns heredodegeneratius	Torsina, Laforina,…	Malaltia de Hallervorden-Spatz pura, Distònia genètica, Adrenoleucodistrofia, Malaltia d'Alexander, etc.

Taula 1. En la taula es troben classificades les malalties neurodegeneratives tenint en compte la proteïna tòxica que les provoca i alguns exemples de les malalties més representatives de cada grup.

De totes aquestes malalties, pararem especial atenció en les taupaties i en les α-sinucleïnopaties, ja que són els grups més comuns i els que han estat subjecte d'estudi al llarg d'aquest treball.

2. Les taupaties

Les taupaties són un conjunt de malalties que comparteixen un fet patològic comú: la presència d'agregats de proteïna tau anormalment fosforilada a l'interior de les cèl·lules neuronals. Dins d'aquest grup trobem: la malaltia d'Alzheimer (AD), la malaltia de Pick (PiD), la paràlisi supranuclear progressiva (PSP), la malaltia dels grans argiròfils (AGD), la degeneració cortico-basal (CBD) 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). En aquest treball es tractaran les dues primeres.

2.1 La tau

La tau és una proteïna abundant en el sistema nerviós central (SNC), que es troba enriquida en els axons de les neurones. La seva localització és bàsicament citosòlica tot i que també es pot trobar associada a la membrana cel·lular [3].

És una proteïna d'associació als microtúbuls (MAP) que té la funció de promoure l'assemblatge i l'estabilització dels microtúbuls, fent-la molt important en la neurogènesi i en el transport axonal. Els dominis d'unió a microtúbuls (MBD) que es troben situats es l'extrem C-terminal de la proteïna permeten aquesta unió. A més, aquests MBD es troben flanquejats per dues regions riques en residus serina i treonina (més de 30), que poden fosforilar-se gràcies a l'acció de diferents cinases i regular així la seva unió als microtúbuls. Existeix una llarga llista de cinases que fosforilen tau, entre les que hi ha la GSK3-β, la Cdk-5, la PKA, la ERK1/2 o la p38 [4], i entre les encarregades de defosforilar-la trobem les fosfatases de la família de les Protein Phophatase (PP) [5].

2.2 Regulació transcripcional

El gen de la tau, està situat al cromosoma 17 i té 16 exons. Degut a l'empalmament alternatiu en el cervell adult s'expressen 6 isoformes diferents (Figura 1) i aquestes es diferencien per la presència o absència del producte dels exons 2, 3 i 10. Com que l'exó 10 codifica per una regió d'unió als microtúbuls, les isoformes que el contenen s'anomenen 4R (4 dominis d'unió), mentre que les que no el tenen s'anomenen 3R [6]. Les proporcions relatives de les isoformes no són sempre constants i van canviant

durant el desenvolupament. És interessant observar que les isoformes tenen també un poder diagnòstic, ja que depenent del tipus de taupatia a la què ens referim, podem trobar unes isoformes enriquides en els agregats o unes altres. Així, en la PiD es troba que els filaments de tau estan enriquits en les formes 3R, mentre que en l'AD podem trobar totes les formes [7].

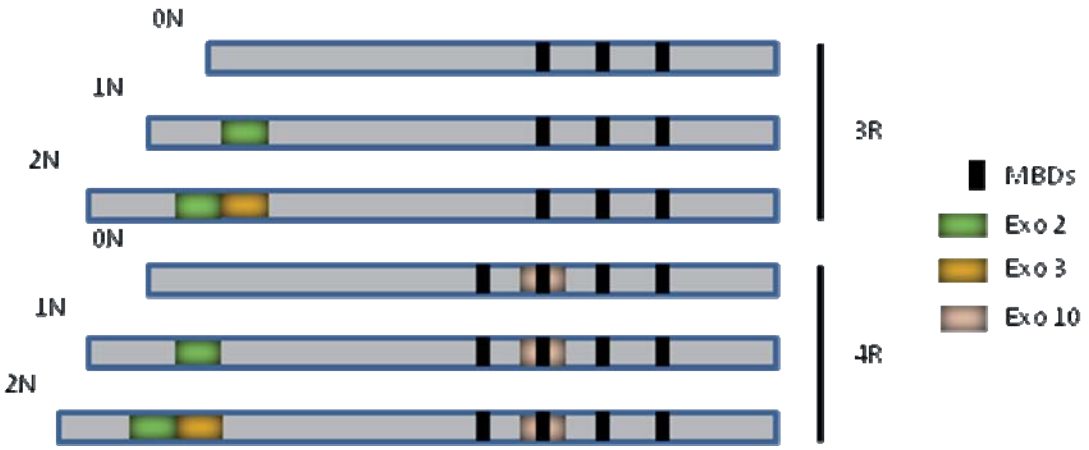

Figura 1.

Les 6 isoformes de tau. Les isoformes que contenen l'exó 10 presenten 4 dominis d'unió als microtúbuls i s'anomenen 4R. Depenent de la presència dels dos exons N-terminals, es poden generar formes 0N, 1N o 2N (modificat de Spillantini i col. [8]).

2.3 Agregats de tau

En certes condicions patològiques el balanç entre fosforilació i defosforilació s'altera i la tau s'hiperfosforila irreversiblement [9], la qual cosa fa que la tau es desuneixi dels microtúbuls. La contínua fosforilació de tau comporta la seva fibril·lació i compactació formant:

a) Cabdells neurofibril·lars (*Neurofibrillary tangles*, NFT): Són unes inclusions citoplasmàtiques en forma de llàgrima compostes bàsicament per la proteïna tau hiperfosforilada [10]. En els NFT, la proteïna tau s'organitza formant filaments que s'aparellen helicoïdalment, donant nom als PHF (filaments aparellats helicoïdalment o *paired helical filaments*).

Alguns cops es poden trobar cabdells que no tenen estructura filamentosa, es creu que són un estadi anterior a la creació dels NFT i se'ls anomena pre-cabdells (pre-tangles) [11].

b) Neurites distròfiques: Es troben en els processos neuronals al voltant de les plaques d'amiloide compactes i estan compostos també per tau hiperfosforilada [12].

c) Filaments del neuropil: Són unes estructures que es troben principalment en les dendrites distals i s'ha suggerit que podrien estar relacionats amb el deteriorament cognitiu de l'AD. Estan formades per tau hiperfosforilada formant feixos o filaments aparellats [13].

2.4 Malaltia d'Alzheimer (AD)

L'AD és la causa més freqüent de demència en la població. Com en totes les malalties neurodegeneratives, l'edat és el factor de risc més gran. La prevalença de la malaltia (amb dades dels Estats Units) és d'1'6% en la població general, però per al grup superior als 75 anys, és d'un 19%, arribant quasi al 50% en els majors de 85 anys [14]. Clínicament, la malaltia es caracteritza per un dèficit cognitiu a diferents nivells que inclou pèrdues en el llenguatge, en la memòria, en les habilitats perceptives, en l'atenció, en les habilitats constructives, en l'orientació, en la resolució de problemes i en les habilitats funcionals.

Macroscòpicament, l'AD presenta una atròfia cerebral en el lòbul temporal degut a una pèrdua neuronal progressiva que afecta predominantment a l'escorça entorrinal, temporal i parietal, el hipocamp, l'amígdala, el nucli basal de Meynert, el locus coeruleus i els nuclis del rafe.

Neuropatològicament, es caracteritza per la presència d'acúmuls de la proteïna tau sobretot en neurones, però també en cèl·lules glials. També s'observa la presència d'uns agregats extracel·lulars anomenats plaques d'amiloide. El patró de distribució de la càrrega de tau és predictible i s'utilitza per a dividir la progressió de la malaltia en 6 estadis ben diferenciats [15], alguns d'ells representats a la figura 2:

Estadis I-II: Corresponen a estadis pre-clínics de la malaltia i es troba afectada l'escorça entorrinal.

Estadis III-IV: En aquests estadis es troben alteracions en el hipocamp i en el sistema límbic, també es comença a fer patent un cert deteriorament cognitiu.

Estadis V-VI: Apareixen els primers símptomes de demència associats a l'afectació del neocòrtex.

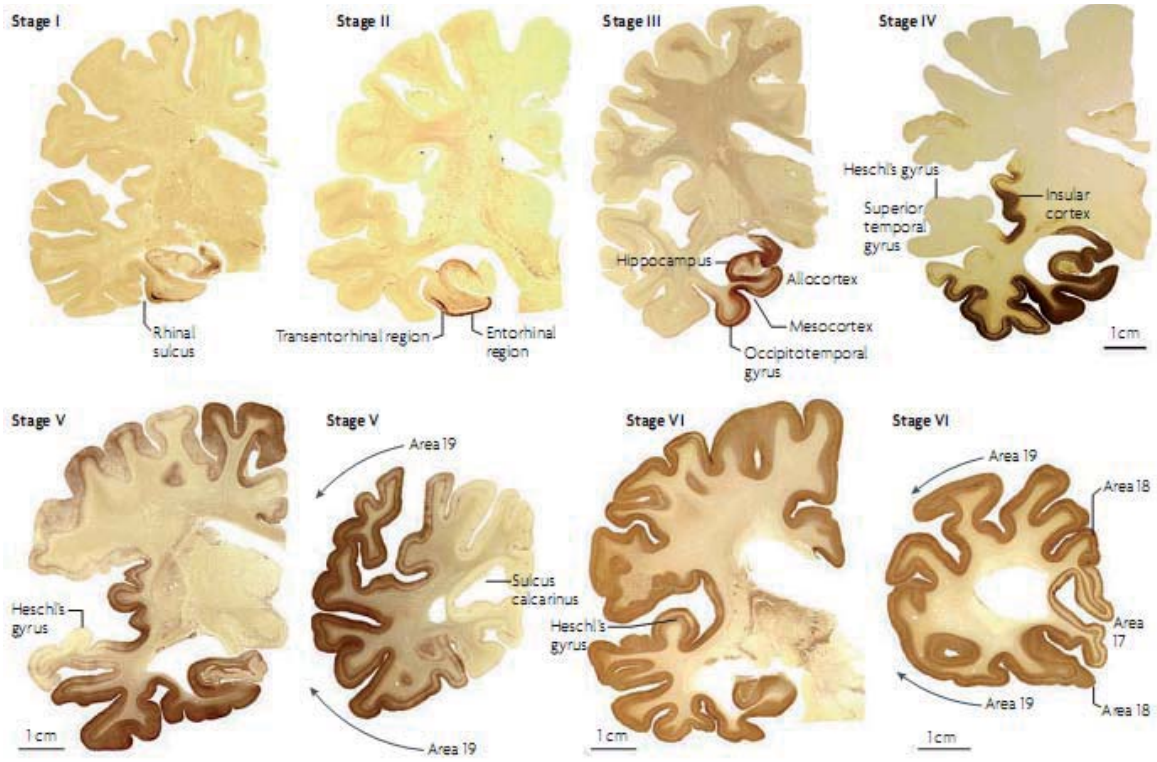

Figura 2.

A mesura que avança la malaltia, el patró d'afectació amb dipòsits de tau es va estenent per tot el cervell. En la imatge, utilitzant un anticòs contra tau fosforilat, es pot observar com en l'estadi III tan sols queda marcada una petita zona en el hipocamp. Mentre que en l'estadi VI el marcatge ja ha progressat cap a totes les regions del cervell. Extret de [16]

S'han descrit 34 mutacions en el gen de la tau que desencadenen malalties, però de totes elles cap produeix AD familiar. En canvi, sí que es relaciona amb mutacions en el gen tant de la presenilina 1 com la 2, que incrementen la deposició del pèptid β-amiloide. El fenotip d'aquests malalts és indistingible dels malalts d'AD esporàdics, el que reflexa la importància de l'amiloide en l'acumulació de tau.

2.4.1 Plaques d'amiloide

També anomenades plaques senils, les va descriure per primer cop Alois Alzheimer el 1907. Són dipòsits anormals fibril·lars resistents a detergents i a desnaturalitzants, on el major constituent n'és la pèptid β-amiloide [17].

La proteòlisi seqüencial de la proteïna precursora de l'amiloide (APP) duta a terme per una γ-secretasa, formada per les presenilina 1 i 2 (PS1 i PS2), genera majoritàriament dos pèptids que estan formats pels residus 1-40 o l'1-42, que són el que es coneix com pèptid amiloide. Existeixen dos tipus de plaques en funció del seu grau

de compactació: les anomenades plaques senils que tenen un nucli compacte format pel pèptid 1-40 i una capa externa formada pel pèptid 1-42 més difosa. A més, reuneixen al seu voltant neurites distròfiques i cèl·lules glials, cosa que no ocorre en les plaques difoses, que estan formades majoritàriament pel pèptid 1-42 i són de mides variables [18].

2.5 Malaltia de Pick (PiD)

La malaltia de Pick (PiD) és un taupatia poc freqüent que actualment es classifica dins del grup de les demències fronto-temporals (DFT). La prevalença de la malaltia sembla que podria estar al voltant de l'1 per 100 000 entre persones de 60 anys. La PiD es considera una malaltia esporàdica, però mutacions en el gen de la tau poden donar un fenotip de DFT indistingible de la forma esporàdica [19].

Clínicament es caracteritza per canvis en el comportament, desinhibició social, falta de cura personal i afàsia que pot finalitzar en mutisme. Patològicament presenta un grau elevat d'atròfia als lòbuls frontals i temporals, amb espongiosi superficial. En la PiD també es pot trobar mort neuronal i alguns dipòsits de tau hiperfosforilada en àrees límbiques o al neocòrtex, però en capes diferents de les que es troben en la AD. També es possible trobar una gliosi important tant a l'escorça com en el nucli caudat [20, 21]. El diagnòstic final es realitza a nivell microscòpic on s'observen els anomenats cossos de Pick: dipòsits esfèrics de tau hiperfosforilada al citosol de les neurones. Aquestes inclusions s'observen majoritàriament al hipocamp (gir dentat) i en capes superiors de l'escorça entorrinal i el neocòrtex [22].

3. Les α-sinucleïnopaties

Les α-sinucleïnopaties comprenen un grup divers de malalties neurodegeneratives que comparteixen una lesió comú: agregats de la proteïna α-sinucleïna en algunes poblacions de neurones i/o glia. Són representatives d'aquest grup la malaltia de Parkinson (PD), la demència amb cossos de Lewy (DLB) i l'atròfia multisistèmica (MSA).

3.1 L'α-sinucleïna

L'α-sinucleïna és una proteïna que fins al dia d'avui roman força desconeguda. Rep el seu nom de la descripció original que es va efectuar en el *Torpedo californica,* on es definia com a una proteïna que aparentment es trobava en les sinapsis i en el nucli cel·lular [23]. Sabem que és una proteïna bàsicament neuronal tot i que també pot trobar-se en cèl·lules glials i que correspon a un 1% de tota la càrrega proteica citosòlica [24]. Es troba localitzada i enriquida en els axons presinàptics en estreta relació amb les vesícules sinàptiques. Pertany a la família de les sinucleïnes, una família de gens altament conservats en vertebrats, aquestes proteïnes conserven algunes semblances amb les apolipoproteïnes i es troben de forma abundant en teixit neuronal. Es produeixen per mitjà de tres gens independents i el seu producte són: l'α-sinucleïna, la β-sinucleïna i la γ-sinucleïna [25]. L'α-sinucleïna està molt lligada a la PD i a l'AD, ha estat un focus molt important en el present estudi i en parlarem detingudament en l'apartat 4.

La β-sinucleïna, és una proteïna de 134 aminoàcids que va ser inicialment identificada en el cervell, tot i que s'ha identificat posteriorment en altres teixits [26]. Aquesta proteïna és quasi igual a l'α-sinucleïna, tant en estructura com en distribució (manté una homologia d'un 78% amb l'α-sinucleïna humana), però no conté una regió d'11 aminoàcids del domini central *non-amyloid component* (NAC), la qual cosa podria explicar, tal i com han suggerit alguns autors, que la seva capacitat per agregar-se sigui molt més reduïda que la de l'α-sinucleïna [24, 27]. La seva funció també és desconeguda, però es creu que podria fer un paper redundant a l'α-sinucleïna en l'alliberament dels neurotransmissors i en la plasticitat sinàptica [28]. A més, podria estar inhibint l'agregació de l'α-sinucleïna [29].

Finalment, la γ-sinucleïna s'ha relacionat amb el progrés del càncer de pit i se la va anomenar, en primera instància, *gen 1 específic de càncer de mama* (BCSG1) [25]. És encara una mica més petita, té 127 aminoàcids i conserva un 60% d'homologia amb l'α-

sinucleïna, diferenciant-se sobretot en la part C-terminal dels altres dos membres de la família. Tampoc conté la regió d'11 aminoàcids entre el fragment NAC. Contràriament a l'α- i a la β-sinucleïna, es troba distribuïda àmpliament pel citoplasma neuronal en regions com la medul·la espinal o els ganglis sensorials. Tot i ser una proteïna majoritàriament neuronal, també s'ha descrit en l'epidermis, l'ovari, els testicles i el cor i s'ha trobat alterada en el càncer de pit [24].

3.2 Agregats d'α-sinucleïna

Els agregats d'α-sinucleïna, en els que es pot trobar la proteïna mal plegada i que ha sofert modificacions post-traduccionals, formen la major part del que s'anomenen cossos de Lewy (LB), neurites distròfiques de Lewy (LN) i inclusions citoplasmàtiques glials (GCI) [30].

a) Cossos de Lewy (LB)

A l'any 1913 el patòleg alemany Friederich Lewy (1885-1950) va descriure la presència d'una estructura arrodonida que es tenyia de rosa en el citoplasma de les neurones de pacients morts amb Parkinson. Aquestes inclusions intraneuronals s'observaven sempre en el soma neuronal de les cèl·lules que romanien vives (Figura 3A). Va anomenar aquestes estructures com els Cossos de Lewy (*Lewy Bodies,* LB), i fins que no han millorat les tècniques d'histoquímica s'han definit com uns cossos intraneuronals, esfèrics i eosinofílics amb un nucli central fibril·lar dens envoltat per una aurèola que mesuren de 8 a 30 μm de diàmetre i que es tenyeixen de rosa en les preparacions de hematoxilina-eosina. La presència de LB no està restringida tan sols a la substància negra, poden trobar-se també en l'àrea tegmental ventral, el locus coeruleus, el nucli del rafe, el nucli dorsal motor del nervi vague, la formació reticular o el bulb olfactori anterior (on apareixen les primeres inclusions) i en altres zones amb menys freqüència [31].

Els LB tot i ser molt importants en el diagnòstic post-mortem de les α-sinucleïnopaties, no en són un tret característic, ja que es poden trobar en d'altres malalties (com la malaltia d'Alzheimer, la malaltia de Pick o la síndrome de Down). De fet, en un gran nombre de persones sense una malaltia diagnosticada clínicament, s'han trobat inclusions en el nucli dorsal motor, en la substància negra i/o en altres regions [32]. S'ha arribat al conveni que aquests casos podrien ser la representació dels estadis

pre-clínics de les α-sinucleïnopaties, on la patologia encara no es prou important per desencadenar el fenotip.

El fet que l'α-sinucleïna sigui la proteïna clau en la composició d'aquestes inclusions en diferents malalties ha provocat que s'agrupin totes elles sota el nom d'α-sinucleïnopaties [33]. De totes formes no és la única proteïna present, ja que s'han trobat al voltant de 300 proteïnes en agregats aïllats de pacients amb LB corticals, entre les que es troben proteïnes del citoesquelet, relacionades amb metabolisme o amb la degradació proteica o de matriu extracel·lular [34], i la llista continua creixent. El fet de conèixer les proteïnes que vertebren els LB ens podria ajudar a entendre els mecanismes que duen a la formació d'aquestes inclusions neuronals.

Tòxic o protector?

El fet de trobar l'α-sinucleïna formant part dels LB, i com que les mutacions en aquesta proteïna conduïen a la PD, es va pensar que els agregats eren tòxics i que podien dur a la mort neuronal. No obstant, amb el temps, es va observar que podia existir la PD sense aquests agregats, com per exemple en les PD familiars amb mutacions en el gen de la parkina [35, 36]. Ja que els LB contenen també ubiqüitina, proteïnes ubiqüitinades, i components del sistema ubiqüitina-proteasoma (UPS), s'ha suggerit que la formació de l'agregat podria tenir un origen protector, en el intent d'eliminar el que podria estar fent mal a la neurona [37].

Conjuntament amb aquestes observacions, diversos autors han suggerit que la presència de LB en el cervell i el seu nombre no correlacionarien amb la gravetat de la malaltia, fet que eliminaria també la hipòtesi tòxica dels agregats [38, 39]. A més, a l'analitzar el cervell d'un pacient postmortem es veu que les neurones que contenen LB estan més sanes que les neurones veïnes, si tenim en compte criteris morfològics i bioquímics [40]. Per altra banda, està prenent cada cop més força la hipòtesi que són les fibres immadures (o oligòmers) les que serien tòxiques [41-43] i que els agregats podrien funcionar com un espai on s'hi guardaria tot allò que fos tòxic per la neurona, protegint-la.

Totes aquestes observacions serveixen per a totes les proteïnopaties que comparteixen com a conseqüència final l'agregació d'una proteïna tòxica en uns agregats neuronals, ja sigui en el nucli, en el citosol o en l'espai extracel·lular.

b) Neurites de Lewy (LN)

Conjuntament amb els LB, en el cervell dels malalts també s'hi troben les neurites de Lewy (LN). Són uns dipòsits d'α-sinucleïna que es troben en els processos axonals i tenen formes allargades com de serpentina (Figura 3B). Sovint les LN es poden trobar en absència de LB, la qual cosa suggereix que podrien precedir-los en el temps.

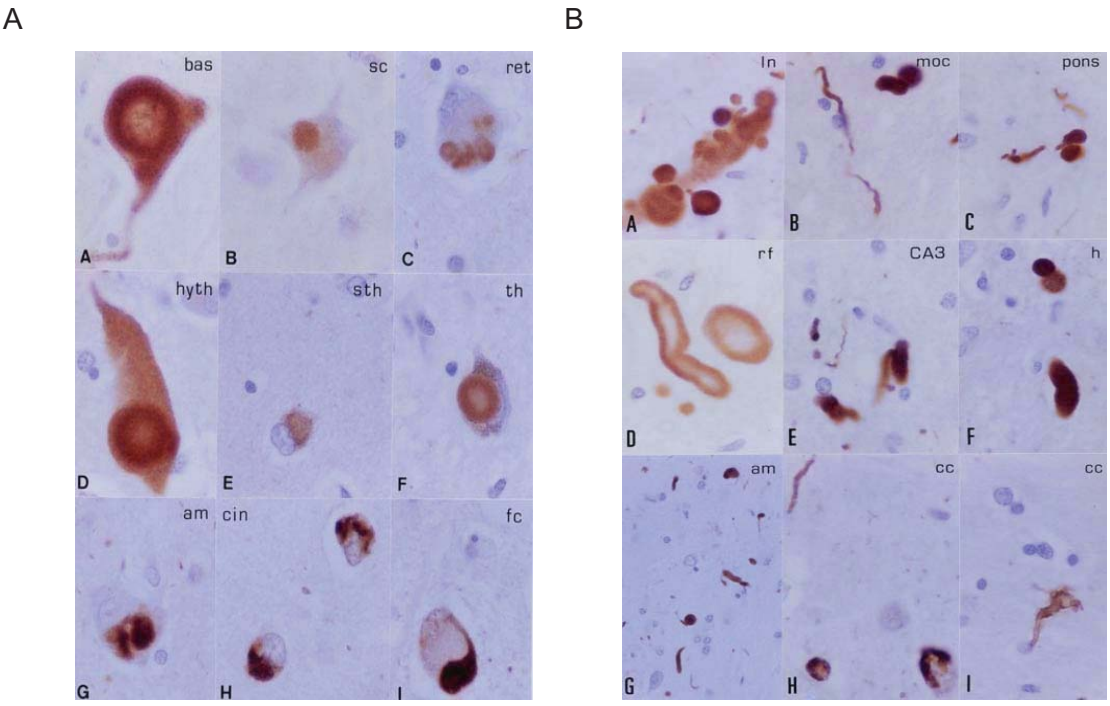

Figura 3.

Tall histològic en camp clar on es poden observar les estructures clàssiques en diferents àrees del cervell de pacients de LBD a l'utilitzar un anticòs contra la proteïna α-sinucleïna. Al panell de l'esquerra s'observen els Cossos de Lewy (LB) i a la dreta les neurites de Lewy (LN). (Foto: INP)

bas: Nucli basal de Meynert, *sc*: coeruleus, *ret*: formació reticular, *hyth*: hipotàlem, *sth*: subtàlem, *th*: tàlem, *am:* amígdala, *cin*: gir cinglat, *fc*: escorça frontal, *ln*: substància negra, *pons*: protuberància, *rf*: formació reticular, *h*: hipotàlem.

c) Les inclusions citoplasmàtiques glials (GCI)

Les inclusions citoplasmàtiques a la glia (GCI) són una de les característiques patològiques distintives de l'atròfia multisistèmica (MSA). Són uns agregats proteics que es troben en la oligodendròglia i que estan formats majoritàriament per α-sinucleïna nitrada, fosforilada i modificada per ubiqüitina [44-46], tot i que també s'hi pot trobar tubulina, αB-cristal·lina o tau sense fosforilar [47, 48].

3.3 Malalties amb cossos de Lewy (LBD)

Amb aquest nom s'agrupen dues malalties que durant anys s'han considerat com a dues entitats diferents: la malaltia de Parkinson (PD) i la demència amb cossos de Lewy (DLB). Molts cops, tant el diagnòstic clínic com el neuropatològic d'aquestes dues malalties se solapa i es fa difícil discernir entre una entitat i una altra, així que s'ha proposat que poden formar part d'un espectre patològic comú que anomenem les malalties amb cossos de Lewy (LBD). Això queda palès, per exemple, en els casos avançats de Parkinson, quan els malalts desenvolupen demència, ho anomenem malaltia de Parkinson amb demència (PDD). Aquests casos són neuropatològicament indistingibles de la DLB. Aquesta diferenciació és una mica artificial i es tendeix a utilitzar-la tan sols en el diagnòstic clínic dels malalts. Avui per avui, s'aplica una regla per distingir-les anomenada "1-year-rule". Si el pacient desenvolupa demència durant el primer any de la malaltia, es classifica com a DLB. Si, en canvi, el pacient triga uns anys a manifestar-la, mentre arrossega un diagnòstic parkinsonià motor, se la classifica com a PDD.

Per a dur a terme el diagnòstic neuropatològic d'aquestes malalties es considera que tant la PD com la DLB formen part d'aquest espectre comú [30] i es realitza seguint un criteri consensuat d'avaluació semi quantitativa de la presència de cossos de Lewy en zones corticals i subcorticals. Aquesta classificació ha estat proposada per Braak [32] i contempla la progressió dels LB des de la medul·la i el bulb olfactori fins al mesencèfal i a l'escorça (Figura 4). Es classifica en els següents estadis:

***Estadi 1*)** Es troba afectació a la medul·la oblongata: lesions en el nucli motor dorsal IX/X i/o la zona reticular intermèdia.

***Estadi 2*)** Trobem la patologia de l'estadi I més l'afectació del nucli del rafe, el nucli reticular gigantocel·lular i el complex coeruleus/sub-coeruleus.

***Estadi 3*)** Es veu afectat el mesencèfal, en particular la substància negra pars compacta.

***Estadi 4*)** S'observa la patologia de l'estadi 3 acompanyada d'afectació del prosencèfal, de la zona transentorrinal i del plexe CA2.

***Estadi 5*)** Es troben lesions en àrees d'associació del neocòrtex i de l'escorça prefrontal, a part de les lesions característiques de l'estadi 4.

***Estadi 6*)** És l'estadi més avançat i es troba afectació en àrees sensorials primàries del neocòrtex i àrees premotores a més de les lesions corresponents a l'estadi 5.

En els tres primers estadis la patologia no s'associa a cap alteració clínica motora. Són els estadis que se'n diuen asimptomàtics, tot i que es poden trobar alteracions olfactives, digestives o trastorns de la son. Al trobar-se afectada gran quantitat de la substància negra, en l'estadi 4, es manifesten els primers símptomes de parkinsonisme. Finalment, quan es lesionen les estructures corticals s'associa a un deteriorament cognitiu i a una DLB clínica. Tot i que aquesta classificació conserva una bona correlació entre la neuropatologia i la clínica, s'ha criticat en un estudi clínic-patològic retrospectiu per falta de relació entre l'estadiatge de Braak i el dèficit cognitiu, ja que una bona part dels malalts clínics de PD no segueixen aquest patró [38].

També cal tenir en compte la presència de molts casos atípics que no s'ajusten als criteris d'aquestes classificacions. Per exemple, en els casos anomenats amígdala predominants, la patologia es concentra en l'amígdala mentre l'afectació en el tronc de l'encèfal és molt menor [49]. També s'han descrit altres casos amb predominança cortical de patologia Lewy [50, 51]. Tots aquests casos atípics corresponen al 5-10% de les LBD esporàdiques [52].

Malgrat no es pot utilitzar per a classificar tots els casos que presenten patologia de Lewy, aquesta és l'aproximació més acceptada, i els casos atípics es classifiquen com a α-sinucleïnopaties diferents [53]. Aquesta és la nomenclatura que hem utilitzat en el present treball, deixant de banda els casos atípics.

A B

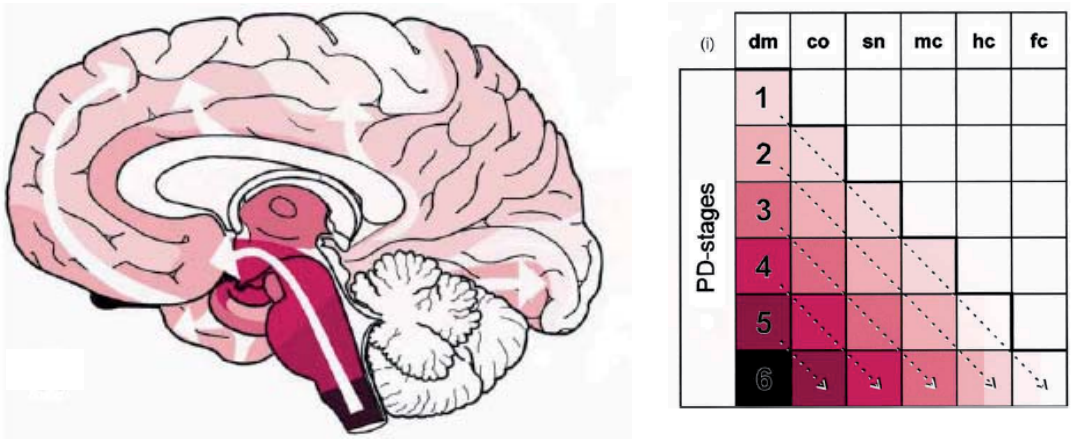

Figura 4.

a) Esquema de la progressió de les zones afectades en la malaltia de Parkinson. S'inicia afectant la medul·la, avança cap al mesencèfal, i acaba afectant el neocòrtex. Els colors foscos marquen les primeres estructures en afectar-se, i el color més clar assenyala les que s'afecten en estadis més tardans.

b) Correlació d'estructures afectades per la patologia de Lewy en concordança amb l'estadiatge proposat per Braak.

dm: nucli dorsal motor; *co*: locus coeruleus; *sn*: substantia nigra; *mc*: mesocortex; *hc*: àrees associació sensorial; *fc*: arres associació sensorial primàries.

Imatges extretes de Braak i col. [32].

3.3.1 Malaltia de Parkinson (PD)

La malaltia de Parkinson fou descrita l'any 1817 pel metge anglès James Parkinson (1755-1824) en l'assaig "An Essay on the Shaking Palsy" [54]. Allà, Parkinson assenyala que els malalts tenien "tremolors involuntaris, amb disminució de la potència muscular en la mobilitat passiva i activa, amb tendència a encorbar el tronc endavant i a passar de caminar a córrer; mentre els sentits i el intel·lecte no pateixen cap dany" [54]. Parkinson creia que la malaltia es devia a una alteració en el funcionament de la medul·la espinal i descartava una vinculació de l'encèfal ja que no trobava modificació del intel·lecte. No va ser fins a finals del segle XIX que Jean Martin Charcot (1825-1893) va descriure la típica rigidesa de la malaltia i la rebatejà amb el nom de malaltia de Parkinson. Tot i que alguns autors afirmin que aquesta malaltia no existia abans del segle XIX, ja hi ha una àmplia evidència en textos antics on es fa referència a la malaltia. Per exemple, el tractat clàssic de medicina ayurveda (1500-1000 aC) fa referència a una malaltia anomenada *kampavata* (kampa: tremolor) que produeix escàs moviment, excés de saliva, somnolència i una mirada reptiliana. Curiosament, per al seu tractament

recomanaven la utilització d'una planta de la família de les fabàcies: *Mucuna pruriens* que conté aproximadament un 3% de levodopa [55].

La malaltia de Parkinson és el segon trastorn neurodegeneratiu més important, després de la malaltia d'Alzheimer. A nivell clínic es presenta com un trastorn motor amb rigidesa muscular, acinèsia, tremolor en repòs i inestabilitat postural [56]. La mitjana d'edat d'aparició de la malaltia és entre els 60 i els 80 anys i afecta entre l'1 i el 2% de la població major de 60 anys [57] i augmenta a mida que ens fixem en grups de població d'edat més elevada. De totes formes, tot i que existeixen formes familiars de la malaltia, aquestes expliquen menys del 5% de tots els casos de PD i acostumen a tenir una edat d'aparició inferior als 45 anys [58]. Es desconeixen les causes que expliquen el 95% de casos de Parkinson esporàdic, però s'assumeix que es tracta d'una malaltia multifactorial, on l'edat és el factor més important a sobre el qual actuen uns factors de susceptibilitat genètica i l'ambient.

Macroscòpicament, les diferències entre un cervell afectat de Parkinson i el d'un individu sa es troben a l'observar la substància negra, on s'evidencia que l'habitual pigmentació es torna pàl·lida com a resultat de la pèrdua específica de neurones pigmentades en aquesta zona (Figura 5).

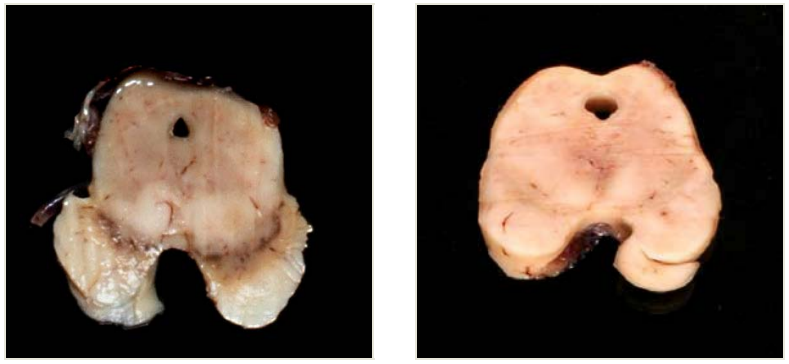

Figura 5.
Comparació entre una substancia negra d'un pacient de Parkinson (dreta) i d'un individu no afectat (esquerra). Es pot observar una disminució important en la pigmentació.
(Foto: INP)

Els estudis neuropatològics evidencien una pèrdua selectiva de les neurones dopaminèrgiques de la substància negra, així com una denervació dopaminèrgica a l'estriat. Quan la pèrdua neuronal en la substancia negra pars compacta representa més d'un 60% del total, és el moment en que es fan palesos els primers símptomes de la malaltia. A nivell microscòpic també es troben els LB en el citoplasma de les neurones i les LN en els axons. La pèrdua neuronal i la presència dels LB en diferents regions del mesencèfal és sovint acompanyada per la presència de macròfags i de gliosi [56, 59].

Acompanyant els LB en la substancia negra i el locus coeruleus podem trobar unes altres estructures conegudes amb el nom de pale bodies (*cossos pàl·lids*). Aquests cossos han estat proposats com a precursors dels LB i tenen un perfil immunocitoquímic molt semblant [60].

3.3.2 Demència amb cossos de Lewy (DLB)

A partir de 1960, alguns autors van començar a descriure casos de demència que tenien LB en l'escorça cerebral. Aquests casos però, eren anecdòtics fins que al voltant dels anys 80 van començar a millorar les tècniques de detecció dels LB. Actualment, aquesta malaltia és la segona causa més freqüent de demència, explicant-ne més d'un 20% dels casos. Normalment, està associada clínicament a un conjunt de trets neuropsiquiàtrics com el coneixement fluctuant i al·lucinacions visuals, associats a parkinsonisme [61].

La demència amb cossos de Lewy (DLB) es caracteritza patològicament per tenir unes lesions idèntiques a la malaltia de Parkinson (LB i LN positius per α-sinucleïna), però molt més esteses per l'escorça cerebral i el nucli diencefàlic [52].

En la gran majoria dels pacients també es troba una patologia típica de malaltia d'Alzheimer en forma de plaques d'amiloide difoses, és el que s'anomena la forma comú de DLB i es dóna en un 90% dels casos. Per altra banda, en la resta dels casos els canvis patològics del tipus Alzheimer són molt escassos o nuls, llavors parlem de la forma pura de DLB [50].

3.4 Genètica en les LBD

Encara no estan clars quins són els mecanismes que condueixen a les DLB esporàdiques, però gràcies a que amb els anys s'han anat descrivint algunes mutacions que produeixen malalties hereditàries, algunes de les rutes afectades es poden començar a entreveure.

S'han descrit 11 locus relacionats amb les LBD familiars [62]. El primer gen que es va identificar relacionat amb la PD va ser el PARK1 que es va identificar com una mutació (A53T) en el braç llarg del cromosoma 4 en el gen que codificava per l'α-sinucleïna. Posteriorment es van trobar dues mutacions més en el mateix gen que produïen formes familiars de la PD, són la mutació A30P en una família alemanya [63] i la E46K en una

família del País Basc [64]. Mentre que el fenotip dels portadors de les mutacions A53T i E46K és bastant agressiu i una edat d'inici primerenca, la substitució A30P produiria un fenotip moderat i un inici de la malaltia més tardà [63].

Més tard es va descriure el locus PARK4. Originàriament no es va relacionar amb l'α-sinucleïna, però més endavant es va veure que era degut a replicacions del gen. L'edat d'aparició de la malaltia és bastant primerenca produint-se als 30 anys en el cas de triplicacions del gen o als 60 en el cas de duplicacions [65]. Aquestes mutacions donen suport al paper central de l'α-sinucleïna i a la seva dosi en les DLB. De mica en mica s'han anat trobant d'altres locus, tots ells descrits en la Taula 2. El cas del PARK11 i el PARK13 encara tenen un paper controvertit, ja que les famílies són massa curtes o perquè no s'ha trobat encara una relació directa entre la mutació i la malaltia.

De tots els locus descrits, n'hi ha alguns que es troben en gens que codifiquen per proteïnes relacionades amb el proteasoma (UPS). Normalment, les proteïnes mal plegades o danyades es degraden per mitjà d'aquest mecanisme. El locus PARK2, codifica per la parkina, una E3 ubiquitin ligasa que marca la proteïna danyada i la transfereix al proteasoma. El locus PARK5 codifica per l'UCHL1 que recicla les molècules d'ubiqüitina utilitzades. Sembla doncs, que el sistema UPS està molt relacionat amb la malaltia i un defecte en algun dels enzims necessaris portaria a la falta d'eliminació de proteïnes no-funcionals i a la mort neuronal.

Però també hi ha altres mutacions descrites (DJ1, PINK1 i OMI/HTRA2), que desencadenen una PD amb patró d'herència recessiva, aquestes es troben en locus que codifiquen proteïnes relacionades amb el mitocondri, suggerint una relació, entre el mitocondri i la PD.

Locus	Proteïna	Mecanisme implicat	Herència	Inici malaltia	Neuro-patologia	Referència
PARK1	α-sinucleïna	Funció sinàptica	Dominant	30-60 anys	Igual a la PD esporàdica	[66]
PARK2	Parkina	Sistema ubiqüitina-Proteasoma	Recessiva	30 anys	Sense LB	[67]
PARK3	sepiapterina reductasa??	Síntesi Dopamina	Dominant	60 anys	Degen. SN i LBs	[68]
PARK4	α-sinucleïna	Funció sinàptica	Dominant	30 anys (triplic.)	Igual a la PD esporàdica	[65, 69]
PARK5	UCHL-1	Sistema ubiqüitina-Proteasoma	Dominant	?	?	[70]
PARK6	PINK1	Mitocondri	Recessiva	30-50 anys	?	[71]
PARK7	DJ-1	Antioxidant	Recessiva	20-40 anys	?	[72]
PARK8	LRRK2	Cinasa	Dominant	40-80 anys	LB i LN i/o Tau i/o Amilode	[73]
PARK9	ATP13A2	Bomba ions	Recessiva	>20 anys	?	[74]
PARK11	GIGYF2 ?	Senyalització insulina	Dominant	?	?	[75]
PARK13	Omi/HTRA2	Proteasa	Esporàdic?	?	?	[76]

Taula 2.

En aquesta taula es representen tots els gens associats a la PD, la proteïna que codifiquen i el possible mecanisme involucrat, així com l'edat d'aparició de la malaltia en els malalts. En alguns casos es desconeixen les dades (marcat amb ?).

3.4.1 Paper de les mutacions de l'α-sinucleïna

Degut a la transmissió autosòmica dominant de la malaltia, l'efecte de les mutacions s'associa a un guany de funció a la proteïna [62]. Encara no es coneix del cert quina seria aquesta nova funció que prendria la proteïna mutada, però les hipòtesis més destacades plantegen que podrien afavorir la conformació en làmina β plegada de la proteïna per sobre de la forma nativa desplegada; la qual cosa portaria a un increment en la tendència a oligomeritzar-se [77-79].

De les substitucions descrites sembla que la mutació A30P podria ser la menys agressiva de les tres [80]. A més, fenotípicament es la que produeix un fenotip més suau i amb una edat d'aparició de la malaltia més tardana.

3.5 Atròfia Multisistèmica (MSA)

L'any 1969 Graham i Oppenheimer van introduir el terme d'atròfia multisistèmica (MSA) per parlar d'una malaltia que combinava la degeneració estriatal, l'atàxia olivopontocerebelosa i la síndrome de Shy-Drager. La MSA és una malaltia neurodegenerativa poc comú que cursa de forma progressiva i els afectats presenten un dany que s'estén pel sistema nerviós autònom i afecta la funció cardíaca, la pressió arterial, l'aparell digestiu i la sudoració. Els trets neuropatològics més importants comprenen la pèrdua neuronal en els ganglis basals, cerebel, protuberància, nucli de l'oliva inferior i la medul·la espinal; acompanyat de gliosi [81].

La característica central i obligatòria per a realitzar un diagnòstic definitiu, és la presència patològica d'unes inclusions en les cèl·lules oligodendroglials anomenades inclusions citoplasmàtiques glials (GCI). Tot i que es coneix des de 1989 la seva presència en els malalts de MSA, no va ser fins més tard que es va veure que les inclusions estaven compostes per α-sinucleïna agregada [82]. També s'ha trobat α-sinucleïna acumulada en el citoplasma i en el nucli neuronals, així com en les neurites [83]. L'any 2005 es va proposar un estadiatge de la malaltia tenint en compte la densitat de GCI en el cervell i la pèrdua neuronal associada [84].

4. L'α-sinucleïna

4.1. Regulació gènica

L'expressió d'α-sinucleïna està controlada pel gen SNCA que va ser mapat en el cromosoma 4q21.3-q22. Prèviament ja s'havia descrit el seu homòleg en el peix *Torpedo californica* [85]. Aquest gen consta de 7 exons, dels quals 6 són codificants. El trànscrit sencer del gen, produeix una proteïna de 140 aminoàcids, però s'ha descrit que es poden generar en el cervell humà almenys dues isoformes més per *splicing* (empalmament) alternatiu conegudes com a Syn112 [86] i Syn126 [87].

La isoforma syn112 es produeix per *splicing* alternatiu de l'exó 5, generant una proteïna hipotètica de 112 aminoàcids i un pes aproximat de 11,4 kDa [88]. L'exó 5 es localitza en la meitat C-terminal de la proteïna i conté els residus 103-130. La pèrdua de l'exó 5 generaria una proteïna que li mancaria el principal lloc de fosforilació de la proteïna en posició 129. Estudiant els nivells de mRNA (àcid ribonucleic missatger) en cervells post-mortem s'ha assenyalat un increment de la isoforma 112 en malalts amb LBD i no en AD o individus control, el que suggereix un paper de la syn112 en la formació d'agregats [88].

Per altra banda, la isoforma syn126 es produeix per *splicing* alternatiu de l'exó 3 (una mica més petit), produint una proteïna hipotètica de 126 aminoàcids i uns 13 kDa [88]. L'exó 3 es localitza a la part N-terminal de la proteïna, comprèn els residus 41-54 i el seu clivellament alteraria les hèlix α que conformen el domini d'interacció amb membranes, transformant possiblement la seva unió a membranes [89]. És destacable també que en l'exó 3 es situen dues de les mutacions que duen a la PD familiar: les substitucions E46K i A53T. Aquestes mutacions augmenten la capacitat d'agregació de l'α-sinucleïna, la qual cosa fa pensar que aquesta isoforma podria alterar la propensió de la proteïna resultant a agregar. Els nivells del mRNA de la syn126 s'han trobat reduïts en cervells post-mortem de pacients amb LBD o AD [90], la qual cosa estaria a favor de suggerir un paper preventiu de la isoforma en la formació d'agregats. Finalment, s'ha descrit una nova isoforma en el cervell que seria molt més petita i consistiria en la pèrdua dels dos exons, tant el 3 com el 5, generant una proteïna hipotètica de 98 aminoàcids [91].

Tot i que s'han descrit a nivell de mRNA encara es desconeix si aquestes isoformes acaben produint una proteïna que sigui estable i funcional *in vivo*.

4.2 Estructura de la proteïna

Estructuralment l'α-sinucleïna es pot dividir en tres grans dominis (Figura 6):

- **Domini N-terminal** (aminoàcids 1-60): comprèn la major part de les 7 repeticions imperfectes d'11 aminoàcids que inclouen l'hexàmer KTKEGV (que es troba entre els residus 1-87). Aquest domini ric en repeticions pren una estructura d'hèlix α i li permet unir-se de forma reversible amb membranes de fosfolípids. A més, en aquesta regió es troben les tres posicions que mutades donen lloc a malalties hereditàries de Parkinson (A30P, E46K i A53T).

- **Domini central** (aminoàcids 61-95): també conegut com a domini NAC (de l'anglès *non-amyloid component*). Aquest domini va ser descrit ja fa més de 10 anys, fins i tot abans que l'α-sinucleïna, com el component no-amiloideu en les plaques senils dels pacients d'AD [92]. Aquest domini altament hidrofòbic és indispensable per a l'agregació de la proteïna, fins i tot el fragment sintètic tot sol és capaç de formar fibres [93]. L'α-sinucleïna difereix de les altres dues sinucleïnes en una regió de 12 aminoàcids dins d'aquest domini (71-82).

- **Domini C-terminal** (aminoàcids 96 al 140): és molt acídic ja que conté 10 residus glutamat i 5 aspartats. Aquest domini confereix a la proteïna una elevada estabilitat i solubilitat a altes temperatures, també és essencial per l'activitat xaperona i finalment jugaria un paper important augmentant l'estabilitat de la proteïna protegint-la de l'agregació. La regió C-terminal, degut a la seva naturalesa acídica, també s'uneix específicament a compostos catiònics com el Ca^{2+} i el Cu^{2+} [94-96].

Figura 6.

Representació esquemàtica dels 3 dominis de l'α-sinucleïna. Els símbols (-) representen l'elevada quantitat de càrregues negatives en el domini C-terminal degut als residus glutamat i aspartat. Els quadrets negres simbolitzen els dominis imperfectes de repetició de l'hexàmer KTKEGV en el domini N-terminal, on també es marquen amb asteriscs les mutacions puntuals (*) que comporten un malaltia de Parkinson familiar.

4.3 Configuracions de l'α-sinucleïna

L'α-sinucleïna en forma soluble es troba de forma totalment desplegada i sense estructura, però es una proteïna molt dinàmica que pot adoptar diverses conformacions depenent de l'entorn en el qual es troba. Es pot trobar en forma d'hèlix α en associació a membranes, o com a làmina β en certes condicions de nucleació. Aquestes estructures es troben en equilibri en la cèl·lula, que es pot veure alterat depenent de les condicions [97]. Quan es troba en condicions patològiques, com una disminució en el pH, un augment de temperatura, presència de metalls, un increment de radicals lliures, o la presència de l'α-sinucleïna mutada, l'α-sinucleïna incrementa la seva relació a favor de la conformació en làmina β, formant-se petits oligòmers que són relativament solubles. Aquest oligòmers es van associant entre ells per formar les fibril·les insolubles que finalment es dipositarien formant el LB [98]. Els oligòmers, al seu torn, es poden trobar amb morfologies molt diferents. Els primers que es formen tenen conformació esfèrica, que en les condicions adequades esdevenen estructures anulars (semblants a un donut). Un cop les fibres insolubles formen agregats, aquests es poden presentar en forma compacta semblant a les fibres d'amiloide o amb configuració totalment amòrfica [99].

En la figura 7 hi ha representada l'excepcional habilitat de l'α-sinucleïna per prendre diferents configuracions.

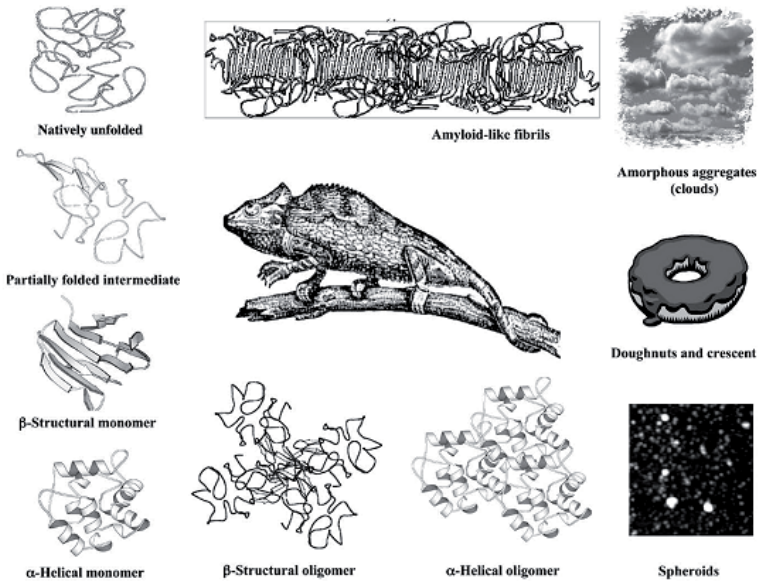

Figura 7.

L'α-sinucleïna pot adoptar un ampli ventall de conformacions en condicions fisiològiques *in vitro*. L'adopció d'una conformació o una altra depèn en gran mesura de les condicions de l'entorn de la proteïna. Aquesta enorme capacitat plàstica ha dut a alguns investigadors a comparar-la amb un camaleó [99].

4.4 Expressió en cervell

Des del descobriment de les PD familiars degudes a duplicacions i triplicacions del gen de l'α-sinucleïna i de la observació que en ratolins un increment en el nombre d'agregats es correlaciona amb els nivells d'expressió del transgen salvatge (WT) de l'α-sinucleïna, s'ha obert una possibilitat a l'estudi dels nivells d'expressió de la proteïna com a causa del procés neurodegeneratiu. En aquesta línia s'han dut a terme varis estudis analitzant les possibles variacions de la seva expressió en l'evolució de les LBD esporàdiques. Per estudiar aquestes alteracions, s'han utilitzat dos abordatges diferents: esbrinar els nivells d'expressió de la proteïna directament o utilitzar el mRNA per inferir-los.

Fins a la data tan sols s'han realitzat estudis a nivell proteic en rata, on les àrees més proclius a degenerar en PD, com la substància negra o el nucli estriat, tenen els nivells més baixos d'expressió, mentre que les àrees que normalment no degeneren en PD, com l'escorça o el cerebel expressen els nivells més alts de proteïna [100]. A més, es redueixen aquests nivells de forma depenent de l'edat tant en estriat com en cerebel [101].

L'estudi dels nivells del mRNA d'α-sinucleïna en el cervell són conflictius. En individus control els graus d'expressió més baixos es registren en els ganglis basals i la substància negra i els més alts en l'escorça, el hipocamp i el cerebel [102]. Tanmateix, al comparar-los amb cervells afectats s'observen resultats contradictoris. Alguns autors han detectat que els nivells de mRNA es troben reduïts en la substància negra de pacients de PD enfront a individus control [103, 104], mentre que més recentment s'han descrit augmentats quatre vegades en la mateixa zona i estables en l'escorça [105], o també augmentats en escorça temporal [102]. Altres estudis suggereixen un manteniment del nivell d'expressió entre malalts de PD esporàdic i de MSA comparat amb controls [106, 107]. Mentre que no s'han trobat variacions en els nivells del mRNA entre afectats i controls en el gir cinglat (estructura que també es troba afectada en la PD i que s'utilitza en el seu diagnòstic) [108].

Totes aquestes evidències suggereixen que a falta d'estudis més il·luminadors sobre aquest tema, hi ha d'haver algun altre factor a part de la sobreexpressió que estigui involucrat en l'acumulació de l'α-sinucleïna en agregats.

4.5 Funcions de l'α-sinucleïna

Tot i que encara no estigui clara, la funció de l'α-sinucleïna ha de ser realment transcendental ja que es troba molt conservada al llarg de l'evolució. Segurament, tot i no ser una funció estructural, ha de tenir una funció de regulació subtil i necessària en situacions d'estrès i/o lesió, que es pot predir gràcies a la seva semblança estructural amb d'altres proteïnes ja conegudes o gràcies als estudis d'interacció realitzats.

Algunes de les funcions proposades per a l'α-sinucleïna són:

· Regulació de l'alliberament de vesícules sinàptiques:

Des del descobriment de l'α-sinucleïna se l'ha relacionada amb les vesícules sinàptiques degut a la seva localització en els terminals axònics en estreta connexió amb les membranes. En condicions normals, fins a un 15% de la proteïna podria estar unida a les vesícules sinàptiques [109, 110].

No es coneix exactament quin podria ser el mecanisme pel qual actuaria l'α-sinucleïna per mobilitzar el neurotransmissor des del fons de reserva cap a les vesícules presinàptiques, així com en l'alliberament, però donen suport a aquest fet els treballs amb animals KO per l'α-sinucleïna que tot i no exhibir un fenotip clar, presenten unes cinètiques alterades en l'alliberament dels neurotransmissors en el hipocamp i l'estriat [111, 112]. Altres models que expressen la proteïna mutada A30P també s'han descrit incapaços de mobilitzar la dopamina des del fons de reserva [113]. Majoritàriament, el seu efecte queda palès en l'alliberament de dopamina (DA), però també s'ha vist que la proteïna juga un paper en l'alliberament en sinapsis glutamatèrgiques [114].

Una possible solució al mecanisme l'ofereix el fet que l'α-sinucleïna actua com a inhibidora de la fosfolipasa D2 (PLD2). La PLD2 és un enzim que es troba en la membrana plasmàtica i funciona com a recaptador d'altres molècules que, a la vegada, són necessàries per empaquetar les noves vesícules sinàptiques a partir de les membranes donadores [115]. Així, modulant l'activitat de la PLD2, l'α-sinucleïna podria regular la formació de les vesícules sinàptiques (Figura 8). En casos de mutacions, com per exemple, en les mutacions A30P i A53T, s'ha descrit que perden l'habilitat d'unir-se a membranes, perdent la regulació a través de la PLD2 i reduint la formació de vesícules sinàptiques [109].

L'α-sinucleïna també interactua amb les proteïnes Rab3a i Rabphilina , que es troben associades a les vesícules sinàptiques i juguen un paper en el transport de la vesícula cap a la zona activa i en l'acoblament a la membrana. Aquesta interacció es deixa de produir en les LBD, limitant la funció conjunta d'aquestes proteïnes en l'exocitosi i disminuint l'alliberament i el buidatge de la vesícula sinàptica [116].

Per altra banda, degut a que entre un 15 i un 50% de la proteïna es troba unida a membranes, també podria jugar-hi un paper interactuant de forma directa. Durant un procés patogènic, els oligòmers d'α-sinucleïna podrien prendre forma anul·lar i formar un porus a la membrana que permeabilitzaria el compartiment i en permetria la sortida de la DA [117, 118]. La DA en el citosol s'oxida i modifica l'α-sinucleïna, que incrementa la seva capacitat d'agregar-se i s'inicia un procés que acaba desembocant en l'acumulació de LB [109, 119, 120].

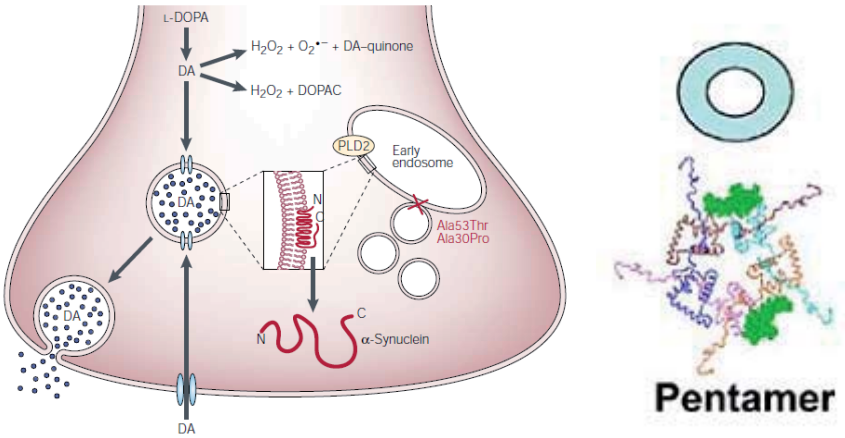

Figura 8.

L'α-sinucleïna es troba unida a la membrana de les vesícules en condicions normals i interactua amb la PLD2. Les formes mutades no poden unir-se a la membrana (sobretot la A30P), però formen estructures anulars que permeabilitzen les vesícules sinàptiques ocasionant la sortida aberrant de dopamina al citoplasma. Extret de [27] i [109].

· Sinaptogènesi:

Ja fa més de 10 anys que es va descriure un augment d'expressió en un gen anomenat *synelfin* durant períodes d'aprenentatge del cant en uns ocells d'Austràlia (*Taeniopygia guttata)*. Aquest gen, relacionat amb la plasticitat sinàptica, resultà ser un homòleg de l'α-sinucleïna [121]. L'expressió augmentada que es dóna durant el desenvolupament del cervell, lligada a altres marcadors clàssics de sinaptogènesi, també reforça el seu paper en la formació de les sinapsis i la diferenciació neuronal [122]

· Proteïna d'unió a àcids grassos (FABP):

L'α-sinucleïna s'ha vist que és important en l'absorció i la circulació dels àcids grassos gràcies a un domini d'homologia amb les FABP que li permet unir-se a l'àcid oleic [123].

Sorprenentment, en les LBD s'han detectat nivells elevats d'àcids grassos poli-insaturats (PUFA) en fraccions solubles del cervell. Aquest fet concorda amb l'evidència que la unió d'α-sinucleïna amb PUFA implica un increment en la formació d'oligòmers de la proteïna, mentre que l'exposició a àcids grassos saturats la disminueixen [124, 125].

A més, s'ha descrit que l'α-sinucleïna pot unir-se a membranes lipídiques de diferents naturaleses, modulant la organització dels components lipídics i també inhibint l'oxidació lipídica [110].

· Xaperona:

L'α-sinucleïna comparteix regions d'homologia amb les xaperones de la família de les 14-3-3. A més, també s'uneix a elles i interactua amb els seus lligands [126]. L'α-sinucleïna també es pot unir a la tirosina hidroxilasa (TH) [127], que és un enzim involucrat en la formació de la DA, gràcies a aquesta interacció podria modular la TH inhibint la seva activitat [127]. Curiosament, la situació contrària es dóna amb les 14-3-3 que s'uneixen a la TH augmentant la seva activitat.

4.6 Modificacions post-traduccionals

Segueixen sent una incògnita els mecanismes que porten a la neurodegeneració i la manera com una proteïna que funciona normalment pot passar a deslocalitzar-se i agregar-se de forma estable. Ja s'ha parlat de les mutacions i la relació que podrien tenir amb la pèrdua de funció de la proteïna però, aquests com a màxim explicarien un 5% dels casos. Hi ha d'haver altres factors que es repeteixin i que puguin explicar la pèrdua de funcionalitat i l'agregació anormal de l'α-sinucleïna en els casos esporàdics, aquestes són les modificacions post-traduccionals, que s'han observat formant part dels LB dels malalts:

- ***Fosforilació:***

La fosforilació de l'α-sinucleïna en la serina 129 és una modificació dominant en les α-sinucleïnopaties i s'ha trobat de forma constitutiva en els LB representant-ne un 90%. També existeix una altra serina en posició 87 que està menys estudiada, i que també es pot trobar fosforilada, però de forma menys eficient. La cinases responsables de totes dues fosforilacions són la casein-kinase 1 (CK1) i la casein-kinase 2 (CK2) [128]. Per altra banda, també s'ha descrit que aquesta fosforilació condueix a una acceleració de la oligomerització i la fibril·lació de la proteïna [45].

Al canviar el residu fosforilable 129 per una alanina en un model de PD en *Drosophila melanogaster*, es prevé la mort neuronal, però amb un increment en la formació d'agregats, suggerint que la fosforilació en la serina 129 augmenta la toxicitat de l'α-sinucleïna i que els agregats podrien protegir la neurona d'aquesta toxicitat elevada [129].

- ***Nitració:***

L'òxid nítric (NO) regula en el cervell alguns processos com la neurotransmissió, la plasticitat sinàptica o la neuromodulació. No obstant, en certes condicions es dóna una producció excessiva de NO generant espècies reactives de nitrogen que estan involucrades en varis processos patològics. Un mal funcionament en aquest mecanisme podria ser una causa de mort cel·lular en les malalties neurodegeneratives [130].

L'α-sinucleïna és una de les proteïnes que es veuen afectades per la nitració i n'augmenta la proclivitat a formar oligòmers mitjançant enllaços entre tirosines. Hi ha 4 tirosines en l'α-sinucleïna, localitzades en els residus 39, 125, 133 i 136, i la sola presència d'una nitració en un d'ells comporta la compactació en octàmers estables, en canvi, n'impedeix l'agregació [131]. Curiosament però, s'ha descrit la presència d'aquestes modificacions en els LB [44, 132, 133].

- ***Oxidació:***

L'estrès oxidatiu, es dóna en situacions en què els sistemes antioxidants intracel·lulars, es veuen desbordats i fracassen en el seu intent d'eliminar les espècies reactives d'oxigen (ROS). Les ROS tenen una potent capacitat d'afectació, i poder danyar lípids, proteïnes i l'ADN (àcid desoxiribonucleic). Aquest és un fenomen normal que ocorre durant l'envelliment i que augmenta en condicions de neurodegeneració.

Les neurones dopaminèrgiques, estan particularment exposades a l'estrès oxidatiu a causa de l'oxidació de la dopamina, i això fa que l'estrès oxidatiu sigui un dels principals contribuïdors en la patogènesi de les LBD.

L'α-sinucleïna es troba oxidada en la substància negra i en el còrtex frontal en les LBD, fins i tot en estadis primerencs de la malaltia [134], aquesta modificació la induiria a formar oligòmers, amb el conseqüent increment en el seu poder patogènic [135]. Al voltant de la patogenicitat deguda a les situacions d'estrès, són d'una importància crucial les proteïnes que confereixen un bon funcionament al mitocondri, ja que és l'encarregat de mantenir el balanç en la formació de ROS. Així, les mutacions descrites que afecten la funcionalitat mitocondrial, podrien conduir a un augment de l'estrès cel·lular ocasionant un mal funcionament de l'α-sinucleïna i desencadenant-ne l'agregació.

- ***Ubiqüitinació:***

La ubiqüitina és una proteïna petita que s'afegeix a les proteïnes mal plegades per a la seva degradació en el proteasoma. Els agregats insolubles d'α-sinucleïna presents en les LBD s'ha demostrat que es troben ubiqüitinats [46, 136], però en menys mesura que la proteïna soluble [137].

- ***Truncament:***

El truncament de l'α-sinucleïna també s'ha descrit com a un mecanisme influent en la patogènesi de les LBD. Degut a la importància creixent que té i a que és un punt important del present treball se li dedicarà el següent apartat.

4.7 Truncament de l'α-sinucleïna

Malgrat que la gran majoria dels estudis es focalitzen en l'agregació de la proteïna sencera, ja fa temps que es coneix que la proteïna tau es troba truncada en la malaltia d'Alzheimer i que podria ser el primer pas en la formació dels PHF. A més, aquests fragments formen part del nucli dels NFTs [138]. Aquest mateix fenomen ha esdevingut important també en l'estudi de les malalties que presenten agregats d'α-sinucleïna i, de mica en mica es va revelant quina podria ser la seva localització, el mecanisme pel qual es formen i els processos implicats.

4.7.1 Proteases que tallen l'α-sinucleina

Diverses proteases poden tallar l'α-sinucleïna *in vitro*: neurosina [139], calpaïna I [140], metal·loproteïnases [141] o la catepsina D [142]. El paper que juguen *in vivo* aquestes proteases encara està per resoldre, tot i que hi ha arguments suficients per pensar que podrien tenir-hi alguna implicació.

La neurosina és una proteasa que talla per residus de lisina, i l'α-sinucleïna és una proteïna molt rica en lisines (sobretot en les seqüències repetides KTKEGV). Els estudis suggereixen que el tall que es produeix degut a l'acció de la neurosina inhibeix la polimerització de l'α-sinucleïna *in vitro*. A més, la neurosina colocalitza amb l'α-sinucleïna en neurones amb interacció directa i també es troba formant part dels cossos de Lewy [139].

La calpaïna I genera in vitro diversos fragments de l'α-sinucleïna de manera temps depenent, tant per la part amino-terminal com per la carboxi-terminal. La generació

d'aquests fragments augmenta la tendència del pèptid resultant a adoptar conformació en làmina β i la seva conseqüent agregació. A més, la calpaïna I també es localitza en els agregats d'α-sinucleïna de pacients amb PD [140]. Però sembla que l'activitat de la calpaïna I *in vivo* no és suficient per degradar la proteïna i que el truncament més important es donaria per la part N-terminal [142].

Les metal·loproteïnases de matriu són unes endopeptidases que es troben en l'espai extracel·lular, on sembla que l'α-sinucleïna podria localitzar-se en certes condicions [143]. Es suggereix que la MMP-3, en concret, generaria un tall per la part C-terminal que afavoriria la tendència de l'α-sinucleïna a polimeritzar [141].

Finalment, la catepsina D s'ha vist involucrada en el tall C-terminal de l'α-sinucleïna tant *in vitro* com amb lisosomes purificats de cervell [142].

Tot i quedar demostrat que les proteases poden tallar l'α-sinucleïna i generar fragments semblants als que s'observen en el cervell, segurament no són l'únic mecanisme implicat, perquè inhibint selectivament aquestes proteases, o el proteasoma, o el lisosoma no s'atura la formació de fragments truncats [144]. Tampoc està clar encara el poder patogènic d'aquests fragments, ja que s'obtenen resultats contradictoris en relació a si augmenten la tendència de la proteïna a agregar-se o la disminueixen.

4.7.2 Clivellament com a resultat d'una degradació incompleta

Els mecanismes encarregats de degradar la major part de les proteïnes citosòliques i mal plegades en les cèl·lules són: l'autofàgia i el que intervé l'UPS. Un mal funcionament d'aquests mecanismes condueix a l'agregació i l'acumulació de les proteïnes tòxiques i la conseqüent neurodegeneració com passa en l'envelliment [145] i en moltes de les malalties neurodegeneratives [146-148], tal i com es proposa en la següent figura.

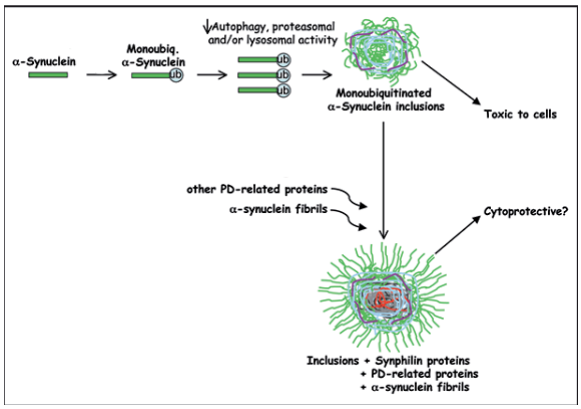

Figura 9.

Mecanisme proposat pel qual una davallada en l'activitat lisosomal i un mal funcionament del proteasoma poden portar a l'acumulació d'α-sinucleïna ubiqüitinada. Modificat de [149].

Es proposa, que la presència de formes truncades d'α-sinucleïna de baix pes molecular també podria estar associada a una degradació aberrant degut a un mal funcionament d'aquests mecanismes.

- ***Sistema ubiqüitina-proteasoma*:**

L'UPS és el responsable de la degradació selectiva de proteïnes de vida mitja curta, així com de proteïnes citosòliques, nuclears o del reticle endoplasmàtic inservibles. Perquè les proteïnes passin a través del porus del proteasoma i puguin ser degradades, cal que estiguin desplegades i marcades amb ubiqüitina.

En les regions del cervell afectades per la malaltia de Parkinson s'ha descrit una davallada del funcionament del proteasoma 20S d'un 55%. Aquest fet produiria una degradació incompleta de l'α-sinucleïna generant fragments truncats. A més, l'α-sinucleïna es troba conjugada a una, dos o tres ubiqüitines en els agregats proteics, el que fa pensar en una implicació del proteasoma en la seva degradació [136].

Mitjançant estudis *in vitro* també s'ha descrit la formació d'espècies truncades per l'extrem C-terminal per mitjà de l'activitat tripsina del proteasoma 20S que augmentarien la tendència a agregar-se de l'α-sinucleïna, en canvi, no degradaria l'α-sinucleïna unida a membranes [150]. Tot i així, encara hi ha debat en quant a la intervenció del proteasoma en la degradació de l'α-sinucleïna soluble ja que molts autors han estat incapaços de trobar-hi una relació clara [151-153].

- ***Degradació mitjançant autofàgia*:**

Els lisosomes són uns orgànuls cel·lulars que se n'encarreguen de la degradació dels components cel·lulars gràcies a diferents tipus d'autofàgia com són: l'autofàgia mitjançada per xaperones (CMA), la microautofàgia i la macroautofàgia (Figura 10).

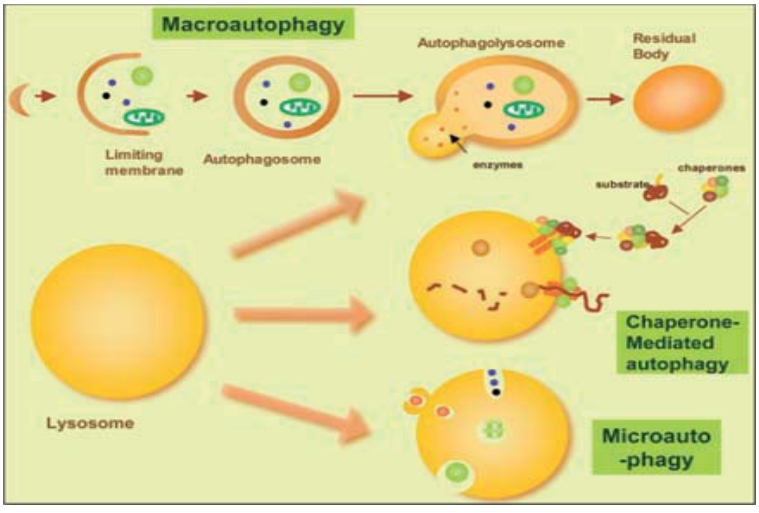

Figura 10.

Model esquemàtic dels diferents tipus d'autofàgia en els mamífers. Extret de [145].

La CMA es realitza gràcies a l'acció de xaperones a través del receptor lisosomal LAMP2A (*Lysosome-Associated Membrane Protein type 2A*), que reconeix una seqüència senyal (KFERQ-*like*) per internalitzar-la. En la microautofàgia, el lisosoma inclou petites quantitats de citosol mitjançant la invaginació directa de la membrana lisosomal. Finalment, en la macroautofàgia es segresten grans regions citoplasmàtiques en autofagosomes que transporten el seu contingut als lisosomes per a la seva degradació.

L'α-sinucleïna conté la seqüència senyal reconeguda per LAMP2A, que la fa susceptible de ser degradada a través de CMA. Aquest fet ha estat demostrat en lisosomes purificats i en models cel·lulars i s'ha proposat com el mecanisme més important en la degradació de l'α-sinucleïna en condicions normals [148, 153]. Les formes A53T i A30P mutades de la proteïna, al igual que les molècules d'α-sinucleïna modificades per DA, es podrien unir al receptor LAMP2A, però no entrarien al lisosoma, s'acumularien i bloquejarien el receptor inhibint la degradació de la resta dels substrats [148, 154]. Cal tenir en compte també, que les formes oligomèriques i agregades de la proteïna que no es poden desplegar, no es degraden per mitjà d'aquest mecanisme, sinó per macroautofàgia [155].

Els sistemes de degradació estan relacionats entre ells i un bloqueig d'un d'ells pot ser rescatat per l'augment en l'activitat d'un altre, és el que ocorre quan les cèl·lules compensen l'aturada del sistema CMA per mitjà d'un increment en el mecanisme de macroautofàgia o un bloqueig de l'UPS amb un augment de la macroautofàgia i a la inversa, per tal de mantenir els nivells de degradació proteica i garantir la supervivència [156].

4.8 L'α-sinucleïna truncada en el cervell

La detecció d'espècies de baix pes molecular amb anticossos contra l'α-sinucleïna és, avui dia, un fet innegable. Les formes truncades de l'α-sinucleïna han estat descrites en mostres postmortem de cervell humà i de ratolins que sobreexpressen la proteïna sencera. Aquestes formes truncades s'assumeix que serien patogèniques i estarien associades amb la formació dels agregats proteics, ja que s'han trobat representant un 15% de l'α-sinucleïna total en fraccions aïllades de LB [150].

La presència dels fragments d'α-sinucleïna ha estat relacionada amb l'edat [150] i amb el nombre d'agregats proteics, així, a més cossos de Lewy en el cervell més alts serien els nivells de proteïna truncada. Aquest fet donaria suport a una teoria on les formes truncades fossin les causants de la formació dels agregats d'α-sinucleïna actuant

com a origen [144]. Malgrat la bellesa de la teoria, la controvèrsia al voltant d'aquest esdeveniment ha augmentat quan també s'han identificat formes de baix pes molecular en fraccions solubles de cervell humà tant de pacients afectats com d'individus sense patologia [157, 158].

Una aproximació interessant és la realitzada per Miake i col. que estudien el nucli dels GCI presents en l'atrofia multisistèmica. Al tractar amb proteïnasa K fraccions enriquides en GCI aïllades de cervells afectats s'observa una reducció en els nivells d'α-sinucleïna sencera. En canvi, es detecta l'aparició d'una banda resistent al tractament, de pes molecular més baix, que suggereixen com el pèptid que vertebra el nucli dels filaments i que per aproximació el caracteritzen com el fragment 31-109 [159].

D'entre tots els treballs que analitzen aquestes formes truncades, la inmensa majoria assenyalen fragments truncats per l'extrem C-terminal de més o menys grandària, però mantenint el fragment central NAC [136, 144, 150, 158].

4.9 Interès del truncament en l'extrem C-terminal

L'extrem C-terminal és l'encarregat de conferir estabilitat a la proteïna. Al perdre part d'aquest domini, els fragments són més propensos i eficients en formar estructures en làmina β i organitzar-se a mida que es van escurçant per l'extrem C-terminal. Els 20 aminoàcids finals, que incorporen un gran nombre de càrregues negatives són essencials al contribuir en la funció xaperona de l'α-sinucleïna [160, 161].

S'ha proposat un model on l'extrem C-terminal oculta el fragment NAC, que té una gran tendència a agregar-se, com es mostra a la figura 11. Qualsevol canvi post-traduccional en aquest extrem, tant nitracions, com fosforilacions, com oxidacions o truncaments, alterarien la conformació de l'α-sinucleïna exposant el fragment NAC [93, 162] i multiplicant-ne la capacitat d'agregació.

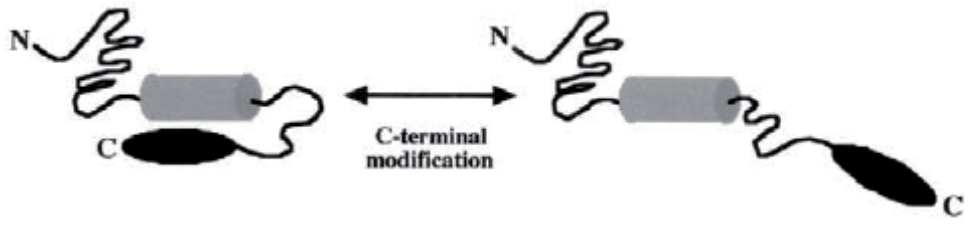

Figura 11.

Model proposat per McLean i col. [162] on l'extrem C-terminal estaria encobrint el fragment NAC. Una alteració en aquest extrem desorganitzaria aquesta conformació i exposaria el fragment NAC duent la proteïna a l'autoensamblatge.

S'han avaluat en varis models murins *in vivo* els efectes patogènics dels truncaments de l'α-sinucleïna per l'extrem C-terminal, i tots ells produeixen un augment de l'afectació en el cervell en comparació amb ratolins que expressen les formes senceres de la proteïna. Aquests models es comentaran en el següent apartat.

Per altra banda, estudis *in vitro* utilitzant fragments truncats per l'extrem N-terminal, inhibeixen la unió a membranes, però no alteren la capacitat xaperona de la proteïna ni incrementen la tendència a agregar-se [161].

5. Models animals de les LBD

L'estudi amb models animals és de gran utilitat per esbrinar els mecanismes patofisiològics de la malaltia, degut a la dificultat que té estudiar-ho en humans. A més, esdevenen essencials si volem testar teràpies potencials o identificar noves dianes terapèutiques. En molts casos però, ja que és impossible aconseguir desenvolupar una PD completa, s'ha intentat produir models que recreïn algun dels trets característics de la malaltia (resumits a la Taula 3). Així, l'estudi conjunt amb diferents models pot contribuir a obtenir una visió general de la PD.

5.1 Models tòxics

Són els primers models que es van utilitzar com a aproximació a la forma esporàdica de la malaltia de Parkinson. El interès del seu ús rau en la capacitat de imitar la pèrdua de neurones dopaminèrgiques present en la PD i la possibilitat d'avaluar l'administració de substàncies que puguin recuperar-ne els nivells. S'han utilitzat agents farmacològics com la reserpina, la rotenona, el paraquat o l'MPTP.

El MPTP (1-metil-4-fenil-1,2,3,6-tetrahidropiridina) és un compost que va ser descobert a l'estudiar la substància que s'administraven uns drogoaddictes que desenvoluparen parkinsonisme. Aquesta substància s'ha utilitzat en molts models i satisfà els requeriments d'un model parkinsonià adient, amb l'únic defecte que és un model agut [163]. El tractament amb rotenona, a més de provocar pèrdua neuronal, també produeix l'agregació d'α-sinucleïna en LB i el clàssic fenotip motor de parkinsonisme amb resposta als tractaments [164], però s'utilitza poc degut a la alta taxa de mortalitat que provoca. Tot i això, últimament s'ha observat que l'administració crònica de rotenona per via digestiva és capaç de reproduir el patró d'afectació temporal que s'observa en els malalts de PD acompanyada de disfunció motora [165].

5.2 Models genètics

Els models genètics apareixen amb el descobriment de la PD familiar degut a mutacions en diferents gens. Aquests models representen una PD genètica que permet tenir a l'abast les eines per estudiar el paper de les proteïnes afectades, els mecanismes patogènics i la utilització de noves teràpies.

A part dels models en que s'expressa l'α-sinucleïna truncada, dels que ja s'ha parlat en l'anterior apartat, n'existeixen una àmplia varietat que ens permeten abordar l'estudi de les α-sinucleïnopaties des de diferents punts de vista.

5.2.1 Ratolins nuls d'α-sinucleïna

Aquests models s'han generat eliminant el gen de l'α-sinucleïna dels ratolins. Els ratolins deficients (KO) tant en α- com en β-sinucleïna, o les dues a la vegada, no tenen cap fenotip clar ni es veu alterada cap de les funcions bàsiques del cervell ni la supervivència, tan sols s'observa una reducció del 20% en els nivells de dopamina en els dobles KO [111, 166]. A més, aquests models deplecionats són resistents a l'MPTP, evitant la inhibició del complex I, el que suggereix que la funció fisiològica de l'α-sinucleïna és important per la funcionalitat neuronal [167].

5.2.2 Transgènics amb sobreexpresió d'α-sinucleïna

S'han generat diversos models que expressen diferents formes de la proteïna (mutada o no mutada), sota el control de varis promotors que controlen la seva expressió en totes les neurones del cervell (els promotors PDGF, Prp o Thy1) o tan sols en neurones dopaminèrgiques (TH). En aquest punt ja ens podríem trobar amb el primer handicap: encara no s'ha generat cap model que reprodueixi de forma fiable l'expressió d'α-sinucleïna endògena del cervell humà.

La forma no-mutada de la proteïna sota el control del promotor PDGF ja és suficient per desenvolupar els fenòmens característics de la malaltia com les inclusions citoplasmàtiques marcades amb ubiqüitina i α-sinucleïna, una reducció de marcadors dopaminèrgics i dèficits motors. Tot aquests conjunt de trets es manifesten de forma progressiva a partir dels 2 mesos d'edat. Per altra banda, els ratolins no mostren pèrdua neuronal ni inclusions fibril·lars, sinó que són més aviat globulars [168].

Utilitzant el promotor Thy1 o el promotor priònic Prp, els ratolins transgènics amb la mutació A53T desenvolupen patologia en les motoneurones [169, 170], que no és un tret característic de la malaltia humana. El model transgènic que sobreexpressa la proteïna amb la mutació A53T sota el control del promotor Prp, ha esdevingut un dels models més acurats de la malaltia i més utilitzats. Aquest model pateix una malaltia neurodegenerativa de començament tardà, acompanyada d'una disfunció motora

progressiva [170, 171]. A més, aquests ratolins desenvolupen unes inclusions citoplasmàtiques semblants als LB humans on s'hi troba la pròpia α-sinucleïna i la ubiqüitina. Aquest model és singular ja que reprodueix molts dels trets característics de la malaltia de Parkinson humana: l'α-sinucleïna es troba en fraccions insolubles formant agregats que a vegades poden ser fibril·lars, s'observen formes truncades de la proteïna i els animals moren degut a la malaltia [170].

En els ratolins transgènics en què s'ha sobreexpressat la forma mutada A30P , no s'ha aconseguit evidenciar la formació de LB, ni l'afectació motora ni la pèrdua de marcadors dopaminèrgics, però si un increment en l'agregació de l'α-sinucleïna [172, 173]. Aquests models ens poden ajudar a estudiar la toxicitat *in vivo* i els efectes concrets dels mecanismes implicats en la neurodegeneració i ens informen de la importància del fons genètic i de la quantitat d'expressió en el desenvolupament del fenotip de la malaltia. Encara però, falta trobar un model que reprodueixi la vulnerabilitat de les neurones dopaminèrgiques pròpia de la PD.

5.2.3 Model de DLB

Per tal d'estudiar el solapament que existeix entre taupaties i α-sinucleïnopaties es poden utilitzar models que presentin les dues patologies clàssiques, tant agregats d'α-sinucleïna com plaques d'amiloide, i en permeti analitzar les vies moleculars implicades.

L'únic model que existeix fins a l'actualitat que intenta reproduir la clínica de la demència amb cossos de Lewy el van crear Masliah i col. Els dobles transgènics d'APP i α-sinucleïna humans presentaven un fenotip més agressiu que el que s'obtenia solament amb l'expressió d'α-sinucleïna, que s'evidenciava en un avançament dels dèficits motors i d'aprenentatge i en un increment del nombre d'inclusions positives per α-sinucleïna. Tanmateix, el nombre de plaques d'amiloide romania constant en comparació amb el simple transgènic d'APP [174].

MODELS EN RATOLÍ		Pèrdua neurones DA	Pèrdua neurones no-DA	Dèficits motors	Agregats LB-*like*	Referència
Tòxics	Reserpina	-	-	+	-	[164]
	MPTP	aguda	-	+	inclusions no fibril·lars	[164]
	6-OHDA	aguda	aguda	+	-	[164]
	Rotenona	gradual	-	+	+	[175]
	Paraquat	gradual	-	+	inclusions no fibril·lars	[176]
Genètics	PDGFβ-WTsyn	-	-	+	amòrfics	[168]
	PDGFβ-WTsyn + APP	-	+	+	+	[174]
	Thy1-WT/A30P/A53T syn	-	+	+	inclusions no fibril·lars	[169, 172]
	TH-A30P/A53T syn	-	-	+	inclusions no fibril·lars	[177]
	Prp- A53T syn	-	+	+	+	[171]
	Prp- A30P syn	-		+	-	[178]

Taula 3.

En aquesta taula es resumeixen les característiques generals i les limitacions d'alguns dels models de LBD més utilitzats. Els models tòxics intenten imitar la forma esporàdica de la malaltia mentre que els genètics la forma familiar.

5.2.4 Models d'α-sinucleïna truncada

En ratolins que expressen el fragment truncat per l'extrem carboxil 1-130 en neurones dopaminèrgiques es produeix una pèrdua d'aquestes que es tradueix en una ineficàcia dels terminals axònics i una caiguda dels nivells de dopamina en l'estriat. Però aquests canvis no van acompanyats de la formació de LB ni de l'activació de la glia habitual en la malaltia de Parkinson, la qual cosa fa pensar que són més aviat uns canvis que es produeixen durant el desenvolupament de l'embrió que no resultat d'un dany progressiu [179].

Expressant el fragment 1-119 s'han obtingut ratolins que presenten una pèrdua gradual dels nivells de dopamina en absència de pèrdua neuronal [180]. En canvi, els ratolins que expressen el fragment de l'α-sinucleïna 1-120 en neurones dopaminèrgiques en un fons genètic nul d'α-sinucleïna, presenten a partir dels 12 mesos, però no abans, acumulacions de la proteïna en el citoplasma de les neurones de la substància negra, i una reducció dels nivells de dopamina de manera progressiva [181].

5.2.5 Models de MSA

No s'ha detectat la presència de la proteïna en cèl·lules glials en cap dels models que sobreexpressen l'α-sinucleïna sota el control d'un promotor neuronal. Descartant així la possibilitat d'una migració de la proteïna des de la neurona cap a la oligodendroglia com s'havia formulat anteriorment per donar una explicació als GCIs [182].

Existeixen almenys dos models vàlids per intentar comprendre els mecanismes moleculars que produeixen la patogènesi en la MSA: un d'ells sobreexpressa l'α-sinucleïna sota el control del promotor proteolípid de mielina, que és específic d'oligodendroglia [183]. Aquests ratolins presenten agrupacions semblants als GCI en la oligodendroglia, on s'hi troba l'α-sinucleïna fosforilada. Per altra banda, l'altre model utilitza la mateixa construcció, però sota el control del promotor CNP (2', 3'-cyclic nucleotide 3' phosphodiesterase). Aquest transgènic reprodueix la formació de GCI de forma edat-depenent i conjuntament una degeneració axonal molt similar a la MSA humana [184].

6. Nexes entre taupaties i α-sinucleïnopaties

Normalment, cada malaltia, es defineix pels seus trets característics. Les α-sinucleïnopaties presenten LB i es consideren com a entitats diferents de les taupaties que presenten agregats de tau. Malauradament, això no succeeix sempre així, i s'observen casos on els canvis patològics característics de cada malaltia es solapen. Aquest fet es dóna en una probabilitat bastant major a l'esperada per la simple casualitat [185], indicant, que els dos mecanismes patogènics es podrien estar emfatitzant de forma sinèrgica.

A nivell clínic, aquest solapament ja es fa patent; la PD, caracteritzada per una disfunció motora extrapiramidal, pot acabar evidenciant símptomes de demència, mentre que l'AD pot acabar manifestant símptomes parkinsonians. Alguns autors han determinat els símptomes parkinsonians en malalts d'Alzheimer en un 36% dels casos, mentre que en individus control es parla d'un 5% [185]. També en la PD, els malalts tenen un risc de patir demència 6 cops més gran que la d'individus control [186]. En el camp clínic, aquest solapament queda representat per la demència amb cossos de Lewy (DLB). Aquesta entitat clínica engloba característiques d'ambdós grups i presenta canvis fluctuants en la cognició i l'atenció, al·lucinacions i parkinsonisme.

A nivell neuropatològic, es pot observar en molts casos l'existència de les dues inclusions característiques de cada un dels grups en el mateix cervell. La troballa d'inclusions esporàdiques d'α-sinucleïna en cervells controls és un fet en un 13% dels casos, però la incidència d'aquests agregats conjuntament en la AD arriba fins al 60% [187]. Pel que fa als canvis del tipus AD, en els cervells de persones control es troba un 20% d'incidència, mentre que en pacients diagnosticats amb PD es doblen aquests números, arribant al 87% en DLB [185]. Curiosament, els pacients amb la mutació A53T en el gen de l'α-sinucleïna (la família Contursi) presenten acumulacions de tau en neurites i, fins i tot, tau i α-sinucleïna arriben a colocalitzar en les inclusions d'algunes neurones [188]. El mateix ocorre en famílies amb mutacions en les presenilines 1 i 2, que presenten una AD familiar amb NFT i plaques d'amiloide. En aquests individus s'ha trobat que un 20% dels estudiats tenen inclusions d'α-sinucleïna en almenys una regió cerebral, i a més tant els NFT com els LB poden arribar a colocalitzar en la mateixa neurona [189].

Un exemple imponent és el que ens ofereixen les famílies amb mutacions al gen de la LRRK2, que clínicament es manifesten amb parkinsonisme. Les famílies portadores de les mutacions G2019S o la R1441C presenten manifestacions completament diferents en cada un dels membres afectats de la mateixa família. Alguns individus

pateixen una α-sinucleïnopatia indistingible del PD idiopàtic, mentre que altres individus es caracteritzen per una taupatia amb NFT o en el cas de la mutació G2019S, un membre de la família no té cap mena d'inclusions [190, 191].

A nivell proteic, tant la tau com l'α-sinucleïna comparteixen una llarga llista de particularitats: són neuronals, tenen una conformació nativa desplegada i poden passar de monòmer a fibres i posteriorment agregar. Totes dues presenten modificacions post-traduccionals com la oxidació, la nitració, la fosforilació i la ubiqüitinació. Un aspecte important que dóna suport a la possibilitat d'un solapament entre aquestes dues entitats separades és la interacció directa que es dóna entre les dues proteïnes. S'ha observat amb experiments *in vitro* que la tau només agrega quan s'incuba amb α-sinucleïna mentre per si sola és incapaç de fer-ho [192]. A l'inrevés, també s'ha observat que la tau potencia l'agregació de l'α-sinucleïna [193]. Finalment, models transgènics en ratolí amb mutacions en l'α-sinucleïna (A53T i A30P) o en la tau (P301L) presenten inclusions citoplasmàtiques que es tenyeixen amb anticossos contra ambdues proteïnes [173, 192].

Aquestes evidències suggereixen que malalties que en principi es consideren per separat, comparteixen uns mecanismes patogènics comuns, essent essencial el paper del vincle directe entre tau i α-sinucleïna.

IV. Objectius

Objectiu general:

- Estudiar els canvis post-traduccionals de l'α-sinucleïna comuns en la patogènia tant d'α-sinucleïnopaties com taupaties.

Objectius concrets:

1- Estudiar els canvis de solubilitat de l'α-sinucleïna, fruit de la seva fibril·lació, en mostres provinents de pacients amb malaltia de Pick (representant del grup de les taupaties).

2- Examinar la localització de l'α-sinucleïna i la tau en les regions sinàptiques en diferents estadiatges de taupaties i α-sinucleïnopaties. Explorar les possibles modificacions a les que estan subjectes ambdues proteïnes al llarg de la neurodegeneració i establir possibles rutes comuns.

3- Caracteritzar la neuropatologia del model murí que sobreexpressa l'α-sinucleïna humana mutada A53T. Establir processos comuns amb la malaltia de Parkinson per a utilitzar el model com a eina d'estudi. A més, valorar els efectes de l'administració de diferents dietes empobrides en àcids grassos n-3.

4- Establir la sensibilitat a la degradació de l'α-sinucleïna en les mostres postmortem i fixar un rang d'utilització on la degradació sigui mínima.

5- Descriure els nivells d'expressió d'α-sinucleïna en les diferents regions d'un cervell sa i les seves fluctuacions lligades a la neurodegeneració. Així com, estudiar els nivells d'expressió de la proteïna truncada depenent de l'àrea cerebral i de la patologia presentada.

6- Identificar la localització neuronal dels fragments truncats d'α-sinucleïna i estudiar el possible mecanisme involucrat en la seva formació.

7- Generar un anticòs contra la proteïna resultant de l'*splicing* alternatiu de l'α-sinucleïna (syn112) i verificar la seva expressió en el cervell humà.

V. Resultats

Resultats:

1. Dalfó E, Martinez A, **Muntané G**, Ferrer I. *Abnormal alpha-synuclein solubility, aggregation and nitration in the frontal cortex in Pick's disease*. Neurosci Lett. 2006 May 29;400(1-2):125-9.

2. **Muntané G**, Dalfó E, Martinez A, Ferrer I. *Phosphorylation of tau and alpha-synuclein in synaptic-enriched fractions of the frontal cortex in Alzheimer's disease, and in Parkinson's disease and related alpha-synucleinopathies.* Neuroscience. 2008 Apr 9;152(4):913-23.

3. **Muntané G***, Janué A*, Fernandez N, Odena MA, Oliveira E, Boluda S, Portero-Otin M, Naudí A, Boada J, Pamplona R, Ferrer I. *Modification of brain lipids but not phenotype in alpha-synucleinopathy transgenic mice by long-term dietary n-3 fatty acids.* Neurochem Int. 2009 Nov 12.

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* Coautors del treball

1- L'α-sinucleïna presenta una solubilitat, agregació i nitració anormal en l'escorça frontal de la malaltia de Pick.

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En aquest treball s'ha observat que l'α-sinucleïna presenta una solubilitat i una agregació anormal en l'escorça frontal dels casos de PiD comparant-ho amb els casos control. S'han trobat unes bandes d'un pes superior als 45 kDa en les fraccions SDS-solubles tan sols en els malalts de PiD. De totes maners, encara que posseeixin un patró d'α-sinucleïna alterat, els western blots difereixen dels que s'observen en l'escorça frontal de pacients amb LBD. A més, la utilització d'anticossos contra α-sinucleïna nitrada ha ajudat a trobar bandes de 45 i 60 kDa en les fraccions Dxc i SDS-solubles en l'escorça frontal i no en la occipital (resistent a la patologia de la malaltia) dels casos de PiD. L'α-sinucleïna també s'ha trobat nitrada en els cossos de Lewy i les neurites de Lewy dels casos amb una α-sinucleïnopatia, però de manera difosa en el citoplasma d'algunes neurones aïllades en PiD. Els resultats d'aquest treball demostren una agregació i uns canvis en la solubilitat de l'α-sinucleïna en l'escorça dels pacients amb PiD, unit a una nitració anormal però que en conjunt no comporta la formació de cossos de Lewy en aquestes regions.

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Abnormal α-synuclein solubility, aggregation and nitration in the frontal cortex in Pick's disease

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Abstract

Abnormal solubility and aggregation of α-synuclein have been observed in the frontal cortex in three cases with Pick's disease (PiD) when compared with age-matched controls. Bands of 45 kDa and higher molecular weight were detected in the SDS-soluble fractions only in PiD. Patterns in PiD differed from that observed in the cerebral cortex in Lewy body diseases which were examined in parallel. Immunoblots to α-synuclein nitrated in tyrosines revealed bands of 45 and 60 kDa in Dxc- and SDS-soluble fractions in the frontal cortex (which is vulnerable to PiD) but not in the occipital cortex (which is resistant to this degenerative disease). Moreover, nitrated α-synuclein was found in Lewy bodies and neurites in synucleinopathies but diffusely in the cytoplasm of scattered neurons in PiD. These findings demonstrate abnormal and distinct α-synuclein solubility and aggregation, and α-synuclein nitration without formation of Lewy bodies in the frontal cortex in PiD.

Keywords: Pick's disease; α-Synuclein; Parkinson's disease; Dementia with Lewy bodies

Pick's disease (PiD) is a fronto-temporal dementia disorder characterized by circumscribed cerebral atrophy and widespread occurrence of argyrophilic, globular intraneuronal inclusions named Pick bodies, of which phosphorylated tau is the major component. These inclusions are principally localized in the dentate gyrus, CA1 region of the hippocampus, amygdala, septal nuclei, and upper layers of the entorhinal cortex and isocortex, together with phospho-tau-immunoreactive thorn-shaped and ramified astrocytes, and tau-positive bodies in oligodendroglia [2,4].

α-Synuclein is a 140-amino-acid, natively unfolded, heat-stable, soluble protein that is localized in the pre-synaptic terminals of neurons of the central nervous system, where it may regulate the release of a reserved pool of synaptic vesicles. However, under pathological conditions α-synuclein can aggregate into intracellular proteinaceus inclusions such as Lewy-bodies (LBs) and Lewy neurites found in the brains of patients with Parkinson's disease (PD) and Dementia with Lewy bodies (DLB) [1,9,18]. α-Synuclein in sporadic PD and DLB cases is phosphorylated, oxidized and nitrated [5,7,8,10], and all these factors facilitate α-synuclein aggregation [13].

Lewy bodies are also found in the amygdala in Alzheimer's disease, Down syndrome, argyrophilic grain disease and PiD [17]. Although these α-synuclein inclusions and hyper-phosphorylated tau aggregates are usually located in different cell populations and regions, there is an increasing number of diseases exhibiting a combination of tau hyper-phosphorylation and α-synuclein aggregation [6,11].

C-terminal α-synuclein immunoreactivity has been observed in some Pick bodies in PiD [19]. Discrete α-synuclein immunoreactivity has also been detected in some Pick bodies in the dentate gyrus [14]. Yet no evidence of α-synuclein deposition has been found in PiD in many other studies [2,4]. The present study investigates the presence of α-synuclein insoluble aggregates in PiD.

Brain samples were obtained from the brain banks of the Institute of Neuropathology and the University of Barcelona-Clinic Hospital, following the guidelines of the local ethics committees. The brains of three patients with PiD and four age-matched controls were obtained at from 1 to 6 h after death, and were immediately prepared for morphological and biochemical studies. The cases with PiD were two men and one women aged 65, 66 and 71 years, with sporadic fronto-temporal dementia

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and severe fronto-temporal lobar atrophy on neuroimaging studies (CT and MRI). The fresh brain weights were 950, 970 and 850 g. At autopsy, half of each brain was fixed in formalin, while the other half was cut in coronal sections 1 cm thick, frozen on dry ice and stored at −80 °C until use. The neuropathological findings were characteristic of PiD [2,4]. Regarding the frontal cortex, marked neuron loss and gliosis was observed in every case. Pick bodies were present in remaining neurons in layers II and III and in scattered neurons in layer VI. Neurofibrillary tangles, amyloid plaques and α-synuclein inclusions were absent in all cases. Control cases were two men and two women (70, 62, 69 and 68 years old) with no neurological disease. The neuropathological examination was carried out in similar corresponding sections and using the same immunohistochemical methods. The seven cases (controls and PiD cases) were processed for all the immunohistochemical and biochemical studies.

In addition, and for comparative purposes, frozen samples of the frontal cortex from three patients with diffuse Lewy body disease pure form (with no accompanying lesions of Alzheimer's disease), DLBp, and three patients with advanced Parkinson's disease (PD) were used for biochemical studies. Finally, cryoprotected samples of the frontal cortex from controls, PiD and DLBp, and substantia nigra from PD were used for nitrated and non-nitrated α-synuclein immunohistochemistry.

α-Synuclein aggregates were isolated as previously described [3]. Brain samples (0.2 g) from the frontal cortex (area 8) were homogenized in a glass homogenizer in 1.5 ml of ice-cold PBS^+ (sodium phosphate buffer, pH 7.0, plus protease inhibitors), sonicated and centrifuged at 2650 × *g* at 4 °C for 10 min. The pellet was discarded and the resulting supernatant was ultracentrifuged at 100,000 × *g* at 4 °C for 1 h. The supernatant (S2) was kept as the cytosolic fraction. The resulting pellet was re-suspended in a solution of PBS, pH 7.0, containing 0.5% sodium deoxycholate, 1% Triton and 0.1% SDS, and then ultracentrifuged at 100,000 × *g* at 4 °C for 1 h. The resulting supernatant (S3) was kept as the deoxycholate-soluble fraction. The corresponding pellet was re-suspended in a solution of SDS 2% in PBS and maintained at room temperature for 2 h. Immediately afterwards, the samples were centrifuged at 100,000 × *g* at 25 °C for 1 h, and the resulting supernatant (S4) was the SDS-soluble fraction. Equal amounts of each fraction were mixed with reducing sample buffer and processed for 10% SDS-PAGE electrophoresis and Western blot analysis. The membranes were incubated with rabbit polyclonal anti-α-synuclein (Chemicon, Barcelona), mouse monoclonal anti-α-synuclein Ab-2 (Neomarkers, Bionova, Barcelona) and with mouse monoclonal anti-nitrated-α-synuclein (Zymed, Madrid) at a dilution of 1:4000, 1:1000 and 1:500, respectively. The rabbit polyclonal anti-α-synuclein antibody is raised against the 111–131 human α-synuclein residues. The Ab-2 antibody is raised against amino acids 121–125 using recombinant carboxy terminal-truncated human synuclein expressed in *E. coli*. The protein bands were visualized with the ECL method (Amersham, Barcelona).

α-Synuclein (about 20 kDa) was recovered in the PBS-soluble (cyt) in control and PiD cases, but α-synuclein-immunoreactive bands in the SDS-soluble fraction were recovered only in PiD. In addition, α-synuclein-immunoreactive bands of high molecular weight, 45 kDa and higher were recovered in the SDS-soluble fractions only in PiD with minor variations from one case to another (Fig. 1). Similar results were obtained with both antibodies to non-nitrated α-synuclein. Bands of about 66 kDa were obtained in the SDS-soluble fraction in the frontal cortex in PD, and in the cytosolic, deoxycholate (Dxc)- and SDS-soluble fractions in DLBp. Bands of lower molecular weight were also observed in the SDS fraction in DLBp but not in PD (Fig. 1).

Electrophoresed PiD samples blotted with antibodies raised against α-synuclein nitrated in tyrosines revealed a specific band of about 20 and weak bands of 36 and 45 kDa in the cytosolic fraction, and weak bands of 45 of kDa in the Dxc and SDS soluble fractions in the frontal cortex in PiD, whereas no similar pattern was seen in the occipital cortex (Fig. 2). Interestingly, basal nitrated synuclein was found in age-matched controls, thus suggesting that basal levels of nitrated synuclein may be found in the normal aged brain. Aggregates of nitrated α-synuclein were also observed in the frontal cortex in DLBp. Differences in the density of the bands between PiD and Lewy body diseases was related to lower protein loading in PD and DLBp compared with CTR and PiD (Fig. 2).

Paraformaldehyde-fixed, cryostat sections, 15 μm thick, were processed for α-synuclein immunohistochemistry following the streptavidin LSAB method (Dako). After incubation with methanol and normal serum, the sections were incubated free-floating with one of the primary antibodies at 4 °C overnight. Antibodies to α-synuclein (Chemicon) were used at a dilution of 1:2000; antibodies to anti-nitrated-α-synuclein (Zymed) were used at a dilution of 1:200. 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.

Polyclonal and monoclonal anti-α-synuclein antibodies stained Lewy bodies and neurites in PD and DLBp cases (data not shown). No α-synuclein deposits, as revealed with these polyclonal and monoclonal antibodies, were seen in the cerebral cortex in PiD. Antibodies to nitrated α-synuclein slightly stained cortical neurons in controls (Fig. 3A), thus indicating that low levels of nitrated α-synuclein do occur in normal brain aging. Strong immunoreactivity was observed in Lewy bodies and neurites in the substantia nigra (Fig. 3B) and cerebral cortex (Fig. 3C and D) in PD and DLBp, respectively. Interestingly, increased nitrated α-synuclein immunoreactivity was observed in scattered neurons in the frontal cortex (Fig. 3E–G), but not in the occipital cortex (data not shown) in PiD. Differences between antibodies to non-nitrated and nitrated α-synuclein regarding cytoplasmic staining in PiD cases may be related to differences in antigenic availability and presentation of binding sites of α-synuclein in tissue sections when compared with brain homogenates. Alternatively, α-synuclein antibodies do not particularly distinguish non-aggregated from aggregated α-synuclein, whereas nitrated synuclein antibodies recognize nitrated synuclein.

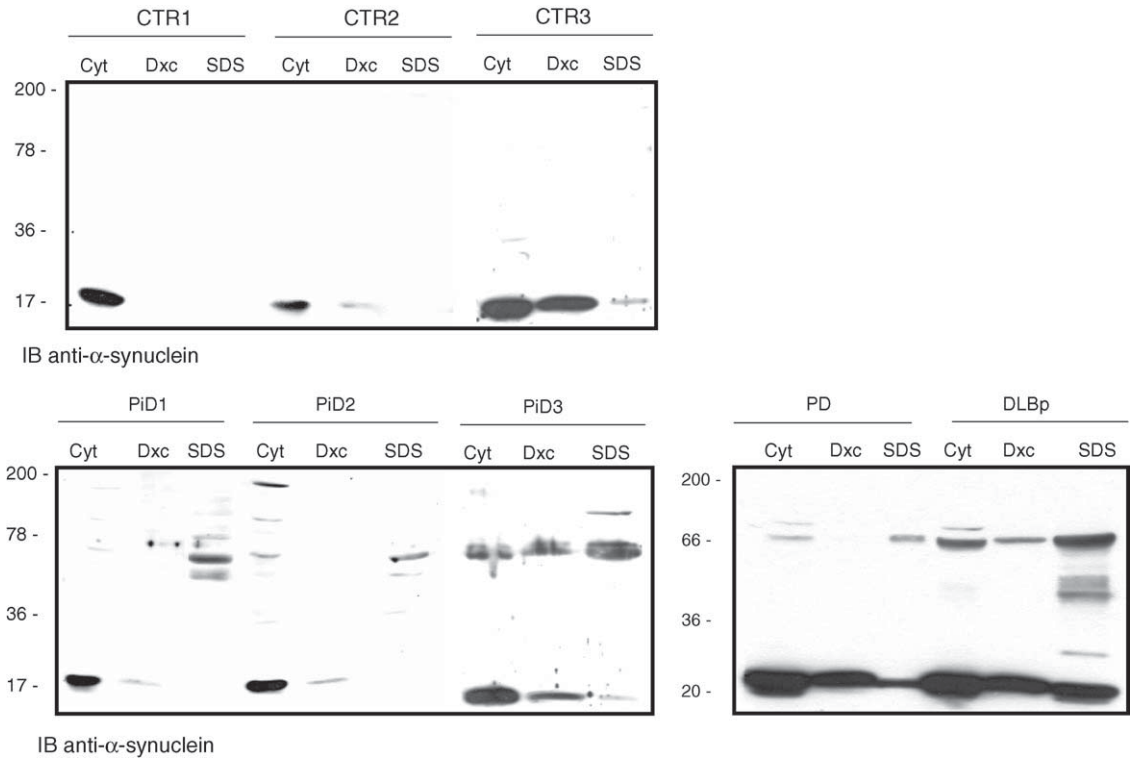

Fig. 1. Solubility and aggregation of α-synuclein examined in frontal cortex homogenates of three control (CTR1-3) and three PiD (PiD1-3) cases blotted for α-synuclein. A specific band of about 20 kDa is recovered in the PBS-soluble (Cyt) in C and PiD cases, and in the deoxycholate (Dxc) in PiD. In addition, bands of high molecular weight of 45 kDa, 66 kDa and higher are detected in the Cyt, and of 45 kDa in SDS-soluble fractions only in PiD. Solubility and aggregation of α-synuclein is also examined in frontal cortex homogenates of Parkinson's disease (PD) and diffuse Lewy body disease pure form (DLBp) blotted for α-synuclein. In addition to bands of about 20 kDa, aggregates of about 60 kDa are seen in SDS-soluble fraction in PD and in the cytosolic fraction, and in Dxc- and SDS-soluble fractions in DLBp. In addition, several bands of lower molecular weights are recovered in the SDS-soluble fraction in DLBp.

The present study shows the presence of high molecular weight α-synuclein species in frontal cortex homogenates in PiD. The present studies have also shown that although abnormal, this pattern differs from that observed in the cerebral cortex in Lewy body diseases processed in parallel. Aggregation promoting C-terminal truncation of α-synuclein is a normal cellular process [12], but abnormal α-synuclein solubility is a characteristic feature in α-synucleinopathies, and it has been associated

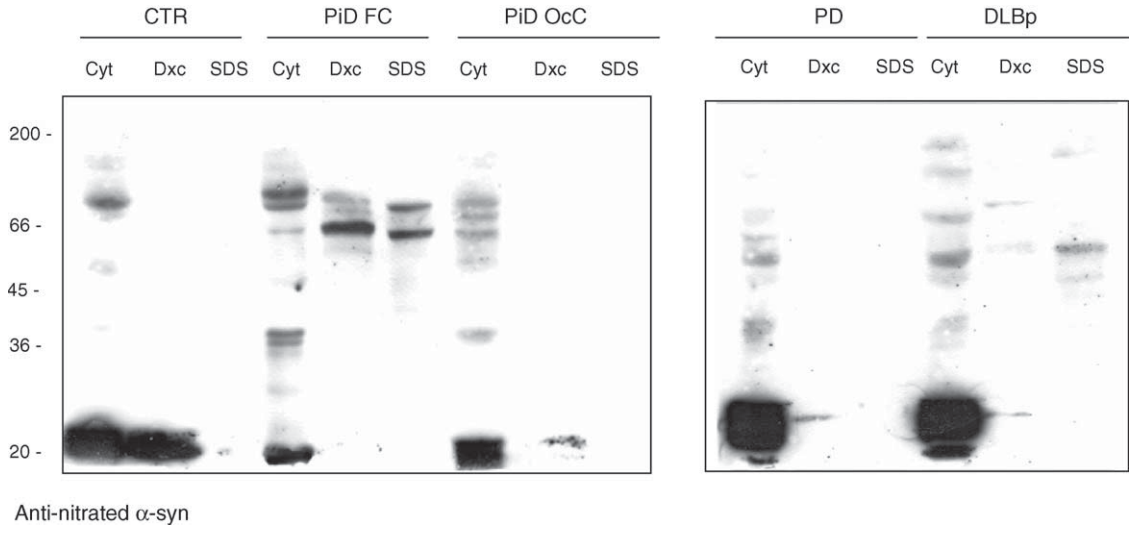

Fig. 2. Immunoblots to α-synuclein nitrated in tyrosines reveal bands of 45 kDa and about 60 kD in Dxc- and SDS-soluble fractions in the frontal cortex in Pick's disease (PiD, FC). These bands do not appear in the occipital cortex in the same case (PiD OcC). Several bands are seen in the cytosolic fraction in PiD and control (CTR) brains. Aggregates of nitrated α-synuclein are also observed in the SDS-soluble fraction in DLBp. Apparent differences in the amount of nitrated α-synuclein between PiD, and PD and DLBp are related to protein loading which was lower in PD and DLBp.

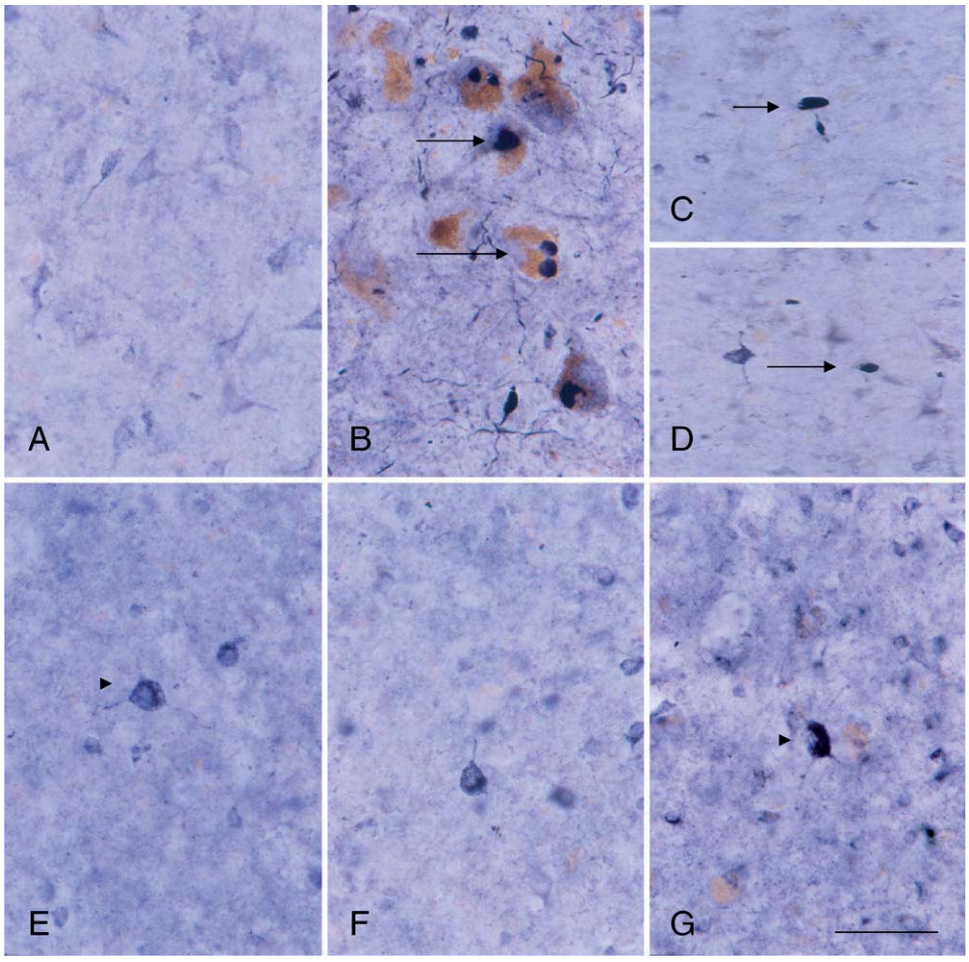

Fig. 3. Immunohistochemistry to nitrated α-synuclein in the frontal cortex of control (A), substantia nigra of Parkinson's disease (B), frontal cortex of diffuse Lewy body disease (C, D), and frontal cortex in distinct cases of Pick's disease (E–G). Weak immunostaining is seen in the control case. Strong nitrated α-synuclein immunoreactivity occurs in Lewy bodies and neurites in PD and DLBp (long arrows). Nitrated α-synuclein immunostaining is also observed in the cytoplasm of scattered neurons in the frontal cortex in PiD cases (arrowheads). Cryostat sections processed free-floating without counterstaining. Bar = 40 μm.

with α-synuclein oxidation [15,20,21] and phosphorylation [7]. This suggests that fibrillar α-synuclein does not occur in PiD, unlike PD and DLB.

Nitrated α-synuclein has also been observed in PiD aggregates. Interestingly, nitrated α-synuclein has been recovered in the frontal cortex, a region vulnerable to PiD, but not in the occipital cortex, a region rather resistant to this neurodegenerative process. These results are in agreement with the identification of nitrated α-synuclein species in synucleinopathies [5,8,15,16]. These findings indicate that α-synuclein is a target for reactive nitrogen species in vivo, but it remains unclear whether this post-translational modification is a primary event that leads to aggregation of α-synuclein, or whether it occurs upon the reaction of reactive nitrogen species with preformed fibrils. Yet, the localization of nitrated α-synuclein differs in PiD cases and Lewy body diseases. Immunohistochemistry has revealed the expected localization of nitrated α-synuclein in Lewy body and neurites in PD and DLBp. Together, these findings show that abnormal α-synuclein deposits occur in vulnerable cortical areas in PiD but also that α-synuclein aggregation and deposition differs in PiD when compared with diseases with Lewy bodies.

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2. La fosforilació de la tau i l'α-sinucleïna en les fraccions enriquides en sinapsis es produeix en l'escorça frontal de la malaltia d'Alzheimer i en les α-sinucleïnopaties.

Muntané G, Dalfó E, Martinez A, Ferrer I.Neuroscience. 2008 Apr 9;152(4):913-23.

La fosforilació de la tau i de l'α-sinucleïna són esdeveniments crucials en el desenvolupament de la malaltia d'Alzheimer (AD) i de les α-sinucleïnopaties (malaltia de Parkinson, PD i la demència amb cossos de Lewy, DLB), respectivament.

En aquest estudio s'ha examinat la presència de tau i α-sinucleïna fosforilats, mitjançant el subfraccionament de la mostra, en l'escorça frontal de pacients con diferents estadis de AD, PD, DLB forma pura i comú i en pacients control.

S'ha observat fosforilació de tau en posició Ser-396 en les fraccions enriquides amb sinapsis, en l'escorça frontal de pacients amb AD i PD en diferents estadis, així com en pacients amb DLB. També s'han observat nivells augmentats d'α-sinucleïna fosforilada i agregada en aquestes fraccions, en l'escorça frontal de pacients amb PD, DLB i estadis avançats d'AD. En les fraccions en les que s'observa un increment de l'α-sinucleïna fosforilada, es corresponen amb un descens en els nivells d'expressió de l'α-sinucleïna no modificada.

Aquests resultats indiquen una fosforilació precoç tant de l'α-sinucleïna com de la tau en regions no afectades en ambdós grups de malalties i suggereixen a les sinapsis com a dianes primerenques per a la fosforilació d'ambdues proteïnes.

A més, aquestes observacions donen suport a un solapament entre la AD i les α-sinucleïnopaties.

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PHOSPHORYLATION OF TAU AND α-SYNUCLEIN IN SYNAPTIC-ENRICHED FRACTIONS OF THE FRONTAL CORTEX IN ALZHEIMER'S DISEASE, AND IN PARKINSON'S DISEASE AND RELATED α-SYNUCLEINOPATHIES

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Abstract—Phosphorylation of tau and phosphorylation of α-synuclein are crucial abnormalities in Alzheimer's disease (AD) and α-synucleinopathies (Parkinson's disease: PD, and dementia with Lewy bodies: DLB), respectively. The presence and distribution of phospho-tau were examined by sub-fractionation, gel electrophoresis and Western blotting in the frontal cortex of cases with AD at different stages of disease progression, PD, DLB pure form and common form, and in age-matched controls. Phospho-tauSer396 has been found in synaptic-enriched fractions in AD frontal cortex at entorhinal/transentorhinal, limbic and neocortical stages, thus indicating early tau phosphorylation at the synapses in AD before the occurrence of neurofibrillary tangles in the frontal cortex. Phospho-tauSer396 is also found in synaptic-enriched fractions in the frontal cortex in PD and DLB pure and common forms, thus indicating increased tau phosphorylation at the synapses in these α-synucleinopathies. Densitometric studies show between 20% and 40% phospho-tauSer396, in relation with tau-13, in synaptic-enriched fractions of the frontal cortex in AD stages I–III, and in PD and DLB. The percentage reaches about 95% in AD stage V and DLB common form. Yet tau phosphorylation characteristic of neurofibrillary tangles, as revealed with the AT8 antibody, is found in the synaptic fractions of the frontal cortex only at advanced stages of AD. Increased phosphorylated α-synucleinSer129 levels are observed in the synaptic-enriched fractions of the frontal cortex in PD and DLB pure and common forms, and in advanced stages of AD. Since tau-hyperphosphorylation has implications in microtubule assembly, and phosphorylation of α-synuclein at Ser129 favors α-synuclein aggregation, it can be suggested that synapses are targets of abnormal tau and α-synuclein phosphorylation in both groups of diseases. Tau phosphorylation at Ser396 has also been found in synaptic-enriched fractions in 12-month-old transgenic mice bearing the A53T α-synuclein mutation.

Key words: α-synuclein, tau, Parkinson's disease, dementia with Lewy bodies, Alzheimer's disease, A53T transgenic mouse.

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Abbreviations: AD, Alzheimer's disease; BS, buffered sucrose; DLB, dementia with Lewy bodies; DLBc, common form of Dementia with Lewy bodies; DLBp, pure form of dementia with Lewy bodies; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; iPD, incidental Parkinson's disease; LB, Lewy body; LBD, Lewy body disease; NFT, neurofibrillary tangle; PD, Parkinson's disease; PHF, paired helical filament.

Alzheimer's disease (AD) is characterized by βA-amyloid deposition in senile (diffuse and neuritic) plaques, and by abnormal tau hyper-phosphorylation and accumulation in neurons. Hyper-phosphorylated tau accumulates in paired helical filaments (PHFs) and straight filaments localized in neurofibrillary tangles (NFTs), neuropil threads and in dystrophic neurites of neuritic plaques (Duyckaerts and Dickson, 2003). The microtubule-associated protein tau is involved in axonal transport stabilizing and promoting microtubule polymerization, and it participates in the transport of vesicles and organelles from axon to the synaptic terminals (Trinczek et al., 1999). Tau is found in the brain in six isoforms generated by alternative splicing from a single gene which is located in chromosome 17q21 (Goedert et al., 1989). Inserts of 0 (0N), 29 (1N) or 58 (2N) amino acids in the N-terminal region are combined with three (3R) or four (4R) microtubule-binding repeat regions at the C-terminal region (Andreadis et al., 1992; Goedert et al., 1992, 1995; Goedert, 2005). All these tau isoforms are hyper-phosphorylated in AD (Goedert et al., 1992, 1995; Mandelkow et al., 2007).

Parkinson's disease (PD) is pathologically defined by loss of neurons in the substantia nigra pars compacta, locus ceruleus, other nuclei of the brain stem, basal nucleus of Meynert and amygdala, and by the presence of Lewy bodies (LBs) and aberrant neurites (Forno, 1996; Jellinger and Mizuno, 2003). Parkinson-like pathology restricted to the medulla oblongata and pons, associated or not with mild midbrain involvement in the absence of motor symptoms, is known as pre-clinical or incidental Parkinson's disease (iPD) (Forno, 1996; Jellinger and Mizuno, 2003). Dementia with Lewy bodies (DLB) is characterized by the additional widespread distribution of LBs and neurites in the cerebral cortex (Ince et al., 1998; Jellinger and Mizuno, 2003; Ince and McKeith, 2003). iPD, PD and DLB are considered to be within the spectrum of Lewy body diseases (LBDs). α-Synuclein is localized in close proximity to synaptic vesicles and has a role in neurotransmission (Dev et al., 2003; Vekrellis et al., 2004; Beyer, 2006). Abnormal α-synuclein is the major component of protein aggregates in LBs and aberrant neurites (Baba et al., 1998; Spillantini et al., 1998; Hashimoto and Masliah, 1999; Duda et al., 2002; Iwatsubo, 2003). Mutations in the α-synuclein gene (A53T, A30P, E46K) are associated with familial PD and DLB (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004). Triplication or duplication of the α-synuclein locus is a cause of PD (Singleton et al.,

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2003; Chartier-Harlin et al., 2004; Ibañez et al., 2004; Nishioka et al., 2006). Based on these characteristics, LBDs have been categorized as α-synucleinopathies.

DLB is often accompanied by AD. This is considered the common form of dementia with Lewy bodies (DLBc), whereas the pure form of dementia with Lewy bodies (DLBp) shows minimal βA-amyloid deposits and no tau pathology (Kosaka, 1993). Interestingly, tau pathology is higher in PD cases in comparison to age-matched controls (Boller et al., 1980). Furthermore, tau has been shown to decorate LBs in sporadic PD and DLB (Arima et al., 1999; Ishizawa et al., 2003), as well as in some cases of familiar PD associated with A53T α-synuclein mutation (Duda et al., 2002; Kotzbauer et al., 2004). Finally, abnormal inclusions of phosphorylated tau are seen in transgenic mice over-expressing the A30P α-synuclein mutation (Frasier et al., 2005).

Furthermore, several studies have revealed α-synuclein-positive structures in different brain regions in sporadic and familial AD (Hamilton, 2000; Kotzbauer et al., 2001; Arai et al., 2001; Hishikawa et al., 2003; Jellinger, 2003). LB are found in the amygdala of some sporadic as well as familial cases of AD, and also in cases of Down syndrome (Trembath et al., 2003; Lippa, 2003). The distribution of LBs and neurites in some AD cases defines a new amygdala-predominant form of α-synucleinopathy (Uchikado et al., 2006).

These observations show that accumulation of abnormal tau and abnormal α-synuclein may occur simultaneously in a particular individual and that this association is more common than could be expected even considering that AD and LBDs are not rare degenerative diseases. Moreover, tau and α-synuclein proteins may interact *in vivo* and *in vitro* (Jensen et al., 1999). Furthermore, tau may enhance aggregation of α-synuclein (Giasson et al., 2003) and vice versa (Geddes, 2005), under appropriate conditions. Together observations in human and animal models, and studies *in vitro* suggest an interface between diseases with tau deposits and α-synucleinopathies (Galpern and Lang, 2006).

The present study is focused on the study of modifications of tau and α-synuclein in the frontal cortex in AD and LBDs (iPD, PD and DLB) in order to understand whether abnormalities of these proteins do exist at the synapses in both groups of diseases. Cellular sub-fractionation studies in combination with gel electrophoresis and Western blotting to non-phosphorylated and phosphorylated tau and α-synuclein have permitted the identification of abnormal phosphorylated tau and α-synuclein at the synapses in AD and synucleinopathies.

EXPERIMENTAL PROCEDURES

Human brain samples

The brains of three patients with iPD, three PD, five DLBp, two DLBc, three AD I–II, three AD III–IV, four AD V–VI, and three age-matched controls were obtained at autopsy, following informed consent of the patients or their relatives and the approval of the local ethics committee. Both genders were represented equally; age range was between 60 and 88 years (mean age 73 years), and the average time between death and tissue processing was 5.4 h. One half of the brain was immediately cut into coronal sections, 1 cm thick, frozen on dry ice and stored at −80 °C until use. For morphological examinations, the brains were fixed by immersion in 4% buffered formalin for 3 weeks.

The neuropathological study was carried out on de-waxed 4-μm-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 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 hematoxylin and eosin, Klüver Barrera, and, for immunohistochemistry to glial fibrillary acidic protein (GFAP), CD68 for microglia, βA-amyloid 1–40 and 1–42, amino terminus of tau (tau-13), phosphorylation-specific tauSer396 (phospho-tauSer396), AT8, and αB-crystallin, α-synuclein and ubiquitin.

AD stages were established depending on the neurofibrillary pathology, following the nomenclature of Braak and Braak (1999). Stages I and II correspond to entorhinal and transentorhinal involvement, stages III and IV show additional hippocampal and limbic involvement, and stages V and VI affect the neocortex. The three cases with AD transentorhinal stage were neurologically normal. Patients with AD limbic stage had suffered from discrete cognitive impairment (one case) or were normal (two cases). The remaining four cases with AD cortical stage had suffered from severe dementia (Global Deterioration Scale) of Alzheimer type.

Neuropathological characterization of DLB was according to consensus guidelines of the Consortium on DLB International Workshop (McKeith et al., 1996, 2000). To further refine α-synuclein pathology, staging of brain pathology related to sporadic PD proposed by Braak et al. (2003) was used in the present study. Basically, stages 1 and 2 affect the medulla oblongata plus the pontine tegmentum; stage 3, the midbrain; stage 4, the basal prosencephalon and mesocortex; and stages 5 and 6, the neocortex. Clinically, the three cases of PD (stages 3 and 4) had suffered from classical PD lasting from 8 to 15 years, and none of them had cognitive impairment. The seven cases with DLB (stages 5 and 6) fulfilled the clinical criteria proposed by the Consortium on DLB International Workshop (McKeith et al., 1996, 2000). No neurological symptoms were recorded in the three iPD cases (stages 2 and 3). No neurological symptoms or metabolic disorders had occurred in control cases. No abnormalities, including AD-associated changes or vascular disorders, were found in control cases.

Biochemical studies were carried out in frozen samples of the frontal cortex (area 8). Control and diseased brains were processed in parallel. The election of this area was done because of its late involvement in synucleinopathies and AD, thus permitting the comparative study of changes throughout the putative progression setting of those diseases.

Transgenic mice

A53T α-synuclein transgenic mice express both murine α-synuclein and human α-synuclein with the pathogenic A53T mutation (Martin et al., 2006). Animals were housed three or four per cage in a temperature-controlled room under a 12-h light/dark cycle with free access to food and water. Animal experiment protocols were conformed to the Animal Care and Ethics Committee of the Universitat de Barcelona and all efforts were made to minimize the number of animals used and their suffering. Protocols for the use and manipulation of the animals were approved by the Autonomous Government of Catalunya. In the present study, transgenic mice and wild type controls aged 12 months were killed under deep anesthesia and their brains immediately frozen at −80 °C for biochemical studies.

Gradient fractionation

Brain samples (0.2 g) of the frontal cortex from iPD, PD, DLBp, DLBc, AD I–II, AD III–IV, ADV–VI and control cases were homogenized separately in a glass homogenizer, in 1 mL of Buffer 1 (5 mM Tris–HCl pH 7.4 and 1 mM EGTA, 250 mM sucrose) and complete protease inhibitor cocktail (Roche Molecular Systems, Almeda, CA, USA). After a brief centrifugation at 2600×*g* at 4 °C for 5 min, the supernatant (S1) was kept and the pellet (P1) was re-suspended in 500 μL of Buffer 1. After another centrifugation at 2600×*g* at 4 °C for 5 min, the pellet (P2) was discarded and the resulting supernatant (S2) was mixed with S1 to obtain a new supernatant (S3). The protein of this resulting supernatant was determined with the BCA method, with bovine serum albumin as a standard. In parallel, nine stocks of different dilutions of sucrose, from 2 M to 0.4 M, each decreasing by 0.2 M intervals, were stored at 4 °C. Homogenized samples were added at the top of the gradient, and after ultracentrifugation at 111,132×*g* for 3 h at 4 °C, 19 fractions of 500 μL were obtained and stored at 4 °C. In addition, samples of the entorhinal cortex of AD cases and controls were processed in the same way.

A similar protocol was carried out in the processing of cerebral cortex from wild type and transgenic mice.

Mono-dimensional gel electrophoresis and Western blotting

For Western blot studies, 20 μL of each fraction 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 pH 7.4, 140 mM NaCl and 0.1% Tween 20) for 45 min at room temperature, and then incubated with one of the primary antibodies in TBS-T containing 3% BSA (Sigma, Madrid) at 4 °C overnight. The following antibodies were used: mouse monoclonal antibodies to synaptophysin (Dako, Glostrup, Denmark; 1:2000), SNAP-25 (Chemicon; 1:2000), anti-phosphorylated α-synucleinSer129 antibody (Wako; 1:3000), tau-13 (MBL, 1:1000), tau-2 (Sigma Aldrich, 1:500), PHF-tau AT8 (Innogenetics; 1:50), glycogen synthase kinase 3β (anti-phospho-GSK-3 (Tyr279/Tyr216), Upstate, 1:500) and superoxide dismutase one (SOD1, Novocastra; 1: 2000); and rabbit polyclonal antibodies to α-synuclein (Chemicon; 1:4000); cdk5 (Calbiochem, 1:500) and phospho-tauSer396 (Calbiochem; 1:500). Subsequently, the membranes were incubated with the corresponding secondary antibody labeled with horseradish peroxidase (Dako) at a dilution of 1:1000 for 45 min at room temperature, and washed with TBS-T for 30 min. Protein bands were visualized with the chemiluminescence ECL method (Amersham, Little Chalfont, Buckinghamshire, UK).

The densitometric quantification of Western blot bands was carried out with Total Laboratory v2.01 software (Pharmacia, Uppsala, Sweden). Densitometric values for phosphorylated tau in each case were normalized using tau-13.

Isolation of synaptic vesicles

Synaptic vesicles were isolated as described (Huttner et al., 1983). Frontal cortex of ADV cases was homogenized in 20 mL of ice-cold buffered sucrose (BS) (320 mM sucrose, 4 mM Hepes–NaOH, pH 7.4) plus 50 mM sodium orthovanadate, 1 mM PMSF and complete protease inhibitor cocktail (Roche Molecular Systems) using a glass homogenizer. The temperature was maintained at 4 °C from this step onwards. The crude homogenate was centrifuged for 10 min at 800×*g*, the resulting pellet (P1) was discarded and the supernatant (S1) was collected and centrifuged for 15 min at 10,000×*g*. The supernatant (S2) was removed and the pellet (P2) was washed, re-suspended in 10 volumes of BS and centrifuged for 15 min at 10,200×*g* to produce a supernatant (S2′) and a washed crude synaptosomal fraction (P2′). The pellet P2′ was lysed in 2 mL of BS and 18 mL of ice-cold water plus 50 mM sodium orthovanadate, 1 mM of PMSF and complete protease inhibitor cocktail (Roche Molecular Systems). The pH was rapidly adjusted at 7.5 mM Hepes–NaOH using a 1 M Hepes–NaOH buffer (pH 7.4). The lysate was transferred to 25 mL polycarbonate tubes and kept in ice for 30 min. Then, the lysate was centrifuged in a Beckman 70Ti rotor at 25,000×*g* for 20 min. The resulting pellet (LP1) was discarded and the lysate supernatant (LS1) was collected and centrifuged for 2 h in a Beckman 70Ti rotor at 165,000×*g* to obtain a lysate supernatant (LS2) and a crude synaptic vesicle fraction (LP2), LS1 was removed and LP2 was re-suspended in 3 mL BS.

Immunopurification of synaptic vesicles

The protein concentration of the re-suspended LP2 fraction was determined using the Bradford method using BSA as a standard. The protein concentration of samples for further processing was 1.5 μg/μL. Homogenized LP2 was blocked with 2% glycine and 2% lysine for 30 min. Meanwhile, 3 μg of the primary antibody was coupled to 100 μL of the secondary antibody labeled with magnetic beads for 1 h on ice. This procedure was followed by incubating the sample with coupled antibodies for 1 h on ice. MicroColumns (Miltenyi Biotec, Madrid, Spain) were equilibrated with 100 μL of ice-cold ethanol at 70%. After three washes of 100 μL of ice-cold filtrated PBS, 700 μL of homogenized LP2 were added to the MicroColumn. The beads containing the bound material were washed (×15) with 100 μL ice-cold filtrated PBS and collected (W). Finally, the eluted fraction (E) was collected using 100 μL of elution buffer (50 mM Tris–HCl, 50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% Bromophenol Blue, 10% glycerol, pH 6.8) at 95 °C. Homogenized LP2 incubated with non-coated secondary antibody processed in the same way was used as a negative control. Fractions analyzed by SDS-PAGE. Antibodies used for the immunopurification were mouse anti-synaptophysin (Dako, Denmark), and goat anti-mouse IgG Microbeads (Miltenyi Biotec).

Electron microscopy of synaptic-enriched fractions and immunopurified synaptic vesicles

Synaptic-enriched fractions and subcellular immunopurified LP2 fractions were separated, and the pellet was fixed in 2% glutaraldehyde in phosphate buffer for 1 h, post-fixed with 2% osmium tetroxide, and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate.

RESULTS

General neuropathological findings in human cases

A summary of the main neuropathological findings in the frontal cortex in relation with LB and Lewy neurites, and NFTs, neuropil threads and dystrophic neurites surrounding β-amyloid deposits in senile plaques is shown in Table 1.

Gradient fractionation of human cases

Synaptic-enriched fractions were characterized by the presence of synaptophysin and were recovered between fractions 7 and 11 among the 19 obtained following separation with sucrose gradient. SNAP-25 was present in the same and neighboring fractions, as it is mainly present in the pre-synaptic membrane. SOD-1 was used as a marker of the cytosol (Fig. 1). The distribution of SNAP-25 and synaptophysin was similar in control and diseased brains.

Table 1. LBs and aberrant neuritis, and NFTs, neuropil threads and dystrophic neurites around senile plaques in the frontal cortex (area 8), as revealed with anti-α-synuclein and anti-phospho-specific tau antibodies in the present series

	LBs and neurites	NFTs, neuropil threads, dystrophic neurites	Number of cases
Control	0	0	3
iPD	0	0	3
PD	0	0	3
DLBp	++	0	5
DLBc	++	++	2
AD I–II	0	0	3
AD III–IV	0	0/+	3
AD V–VI	0	+++	4

iPD, incidental Parkinson disease; AD I–VI, Alzheimer's disease staged following Braak and Braak (1999).

Morphologically, the pellet was composed of small vesicles and membrane remnants together with lower numbers of dense bodies and occasional fibrils.

Phospho-tauSer396 modifications

The distribution of total tau in selected gradient sucrose fractions was determined with anti-tau-13 antibodies. The distribution of tau-13 was similar in control and AD brains. Cytosolic and synaptic-enriched fractions were immunostained in every case (Fig. 2A).

Marked differences between control and diseased brains were observed with anti-phospho-tauSer396 antibodies. No phospho-tauSer396 was seen in the synaptic-enriched fractions in control brains. However, a marked increase in phospho-tau was found in the frontal cortex in AD cases. Such increase was noted in the cytosolic and synaptic-enriched fractions, and the intensity of the bands increased with stage progression (Fig. 2A). Phospho-tauSer396 was similarly increased in different fractions in DLBc as expected on the basis of the associated AD pathology.

Similarly, no differences in tau-2 (data not shown) and tau-13 were observed in PD and DLB cases when compared with controls (Fig. 2A). However, phospho-tauSer396 was found in the synaptic-enriched fractions of the frontal cortex in iPD, PD and DLBp but not in controls (Fig. 2A).

Densitometric studies were carried out to analyze the percentage of phospho-tauSer396 in relation with tau-13 in fractions 9–11 in every case. The percentage of phosphorylation at Ser396 in controls was about 5%, whereas the percentage of phospho-tau in synaptic-enriched fractions in PD, DLBp, AD I–II and AD III–IV varied from 20% to 40%. The percentage of phospho-tauSer396 in synaptic-enriched fractions in the frontal cortex in AD V–VI and DLBc was 90–95% of total tau.

All the experiments were carried out in duplicate or triplicate. Although the distribution of tau was similar in the different experiments, no combined quantitative determinations can be carried out because of day-to-day variations in the immunoreaction, and therefore, differences in the basal control values.

Although phospho-tauSer396 was recovered in synaptic-enriched fractions at early stages of AD and synucleinopathies, AT8 immunoreactivity was found only in AD at stages V–VI (Fig. 2B).

Gel electrophoresis and Western blotting of sub-cellular fractions from the entorhinal cortex of AD cases showed phospho-tauSer396 in AD but not in control cases in a pattern similar to that found in the frontal cortex. PhosphotaSer396 was recovered in synaptic-enriched fractions from early stages onwards (data not shown).

Tau kinase distribution

Both kinases were distributed in the different fractions. The subcellular distribution of cdk5 and phospho-GSK-3 (Tyr279/Tyr216) was similar in control and diseased brains. Both kinases were distributed equally in the different subfractions, including synaptic-enriched fractions (Fig. 3). Subtle differences in individual cases, as for example, cdk5 in DLB and advanced AD (as seen in Fig. 3) were not reproduced in other experiments.

Casein kinase II distribution

The monomeric form of casein kinase was distributed only in the two first fractions of the gradient (fractions 1 and 3) in all control and diseased brains (data not shown).

α-Synuclein modifications

In control brains, α-synuclein wasdistributed in the cytosolic and synaptic-enriched fractions. A similar pattern of distribution occurred in the frontal cortex in iPD and PD (Fig. 4A). A reduction of α-synuclein was observed in the synaptic-enriched fractions in DLBp and DLBc (Fig. 4A). Similarly, α-synuclein immunoreactivity was reduced in the synaptic-enriched fractions of the frontal cortex in AD with stage progression (Fig. 4A). Whether reduction of α-synuclein was related with real α-synuclein decrease or with post-translational modifications of α-synuclein at the synapses was examined by using an antibody to phosphorylated α-synucleinSer129. Decreased synuclein was associated with increased levels of phosphorylated α-synucleinSer129, and increased levels of phospho-α-synuclein paralleled reduced levels of α-synuclein with disease progression (Fig. 4B).

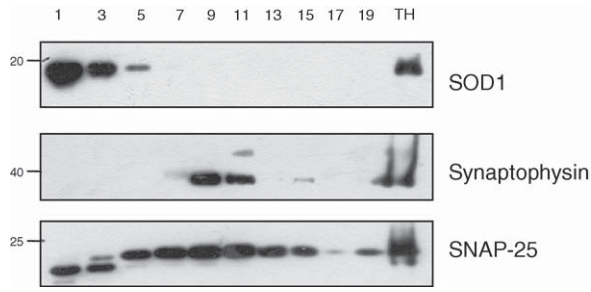

Fig. 1. Representative sub-fractionation in the frontal cortex labeled with anti-cytosolic superoxide dismutase (SOD1), synaptophysin, and SNAP-25. Antibodies to synaptophysin were used to recognize synaptic-enriched fractions. Note that synaptic-enriched fractions correspond to those numbered 7–11 in control and diseased brain samples.

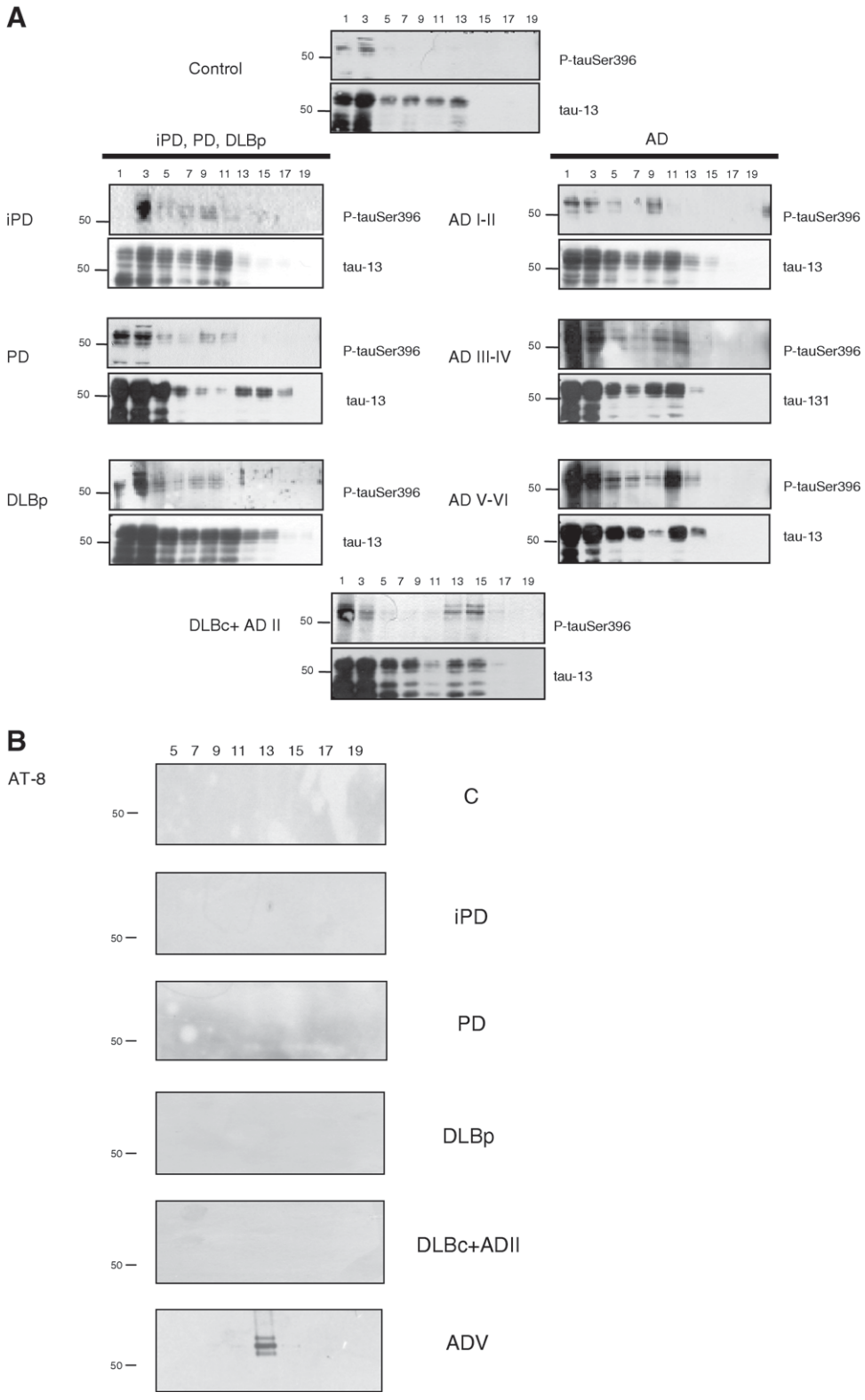

Fig. 2. (A) Representative figures of the distribution of phospho-tauSer396 (upper files) and tau-13 (lower files) in the frontal cortex in control (C), iPD, PD, DLBp, DLBc and AD stages I–II, III–IV and V–VI. Phospho-tauSer396 is not found in synaptic-enriched fractions in controls, whereas an increase of phospho-tauSer396 occurs in the synaptic-enriched fractions with stage progression in AD (and DLBc). Phospho-tauSer396 immunoreactivity is also present in synaptic-enriched fractions in iPD, PD and DLBp. This is in contrast with tau-13 which is present in synaptic-enriched fractions in control and diseased brains; 1 and 3: cytosolic fractions; 7–11: synaptic-enriched fractions. (B) Representative figures of the distribution of AT8 phospho-tau in the frontal cortex in control, iPD, PD, DLBp, DLBc+AD II and ADV, showing that AT8 immunoreactivity in the synaptic enriched fractions is restricted to advanced stages of AD.

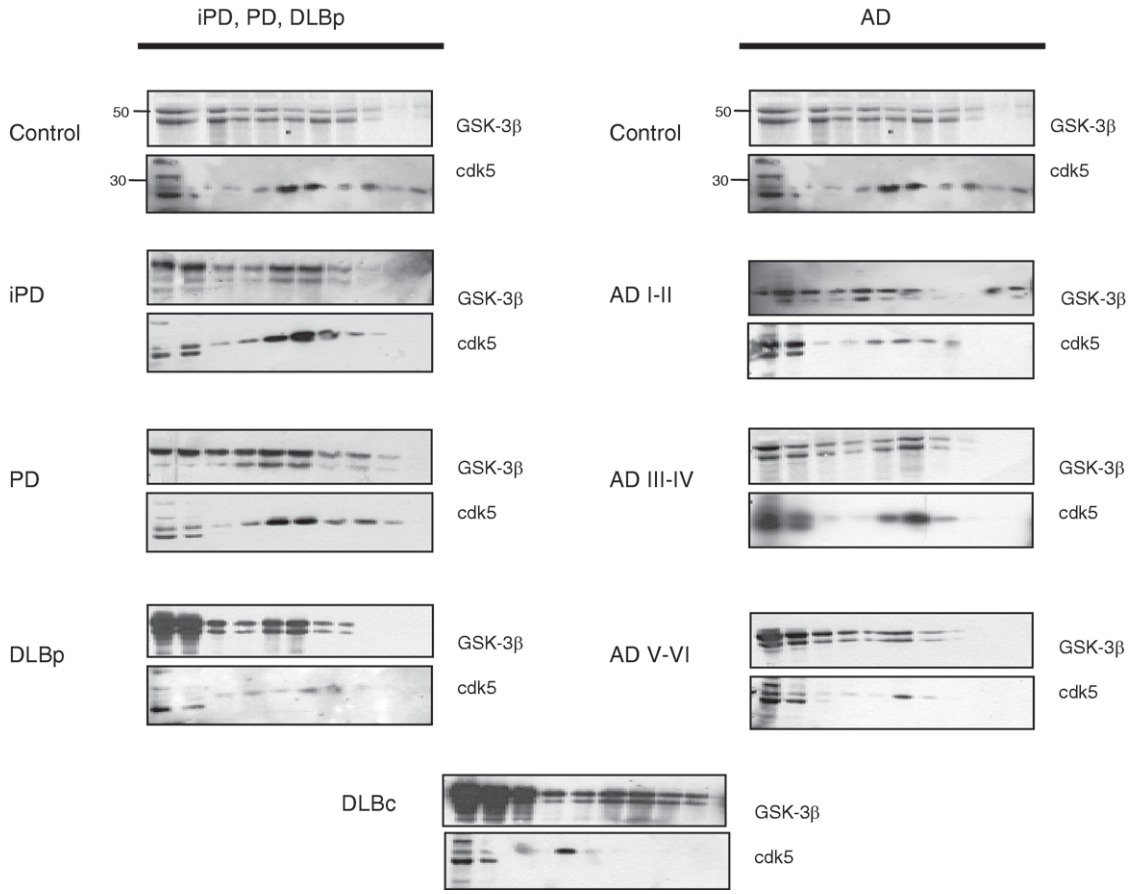

Fig. 3. Representative figures of the distribution of GSK-3 and cdk5 in the frontal cortex in control and disease. The sub-fraction distribution of both tau kinases is similar in control and diseased brains. Synaptic-enriched fractions contain both enzymes.

Phospho-tauSer396 localized in immunopurified synaptic vesicles

In order to further localize phospho-tauSer396 in fractions enriched in synaptic vesicles (LP2), this fraction from AD cases was purified following synaptophysin immunoprecipitation. Phospho-tau was recovered in LP2 fractions but only phospho-tauSer396 was found in eluate fractions obtained after synaptophysin immunoprecipitation (Fig. 5A). Electron microscopy of LP2 fraction was composed of vesicles and some other undetermined organelles (Fig. 5B).

Tau phosphorylation in A53T synuclein transgenic mice

Subfractionation studies in 12-month-old transgenic mice carrying the A53T synuclein mutation showed increased phospho-tauSer396 in synaptic-enriched fractions (Fig. 6). No differences in synuclein phosphorylation were observed in synaptic-enriched fractions in transgenic mice when compared with controls (data not shown). No further attempt was made to analyze the presence of synuclein aggregates in synaptic-enriched fractions.

DISCUSSION

By using sucrose gradient fractionation, gel electrophoresis and Western blotting, the present study has shown increased tau phosphorylation, as revealed with anti-phospho-tauSer396 antibody, in the synaptic-enriched fractions in the frontal cortex in AD when compared with age-matched controls. These changes happen at early stages of AD and occur in the absence of NFTs in the frontal cortex at entorhinal and limbic stages of the disease.

Hyper-phosphorylation of tau in synaptic-enriched fractions is not only encountered in AD, but also in the frontal cortex in LBDs. Cases with DLBc (DLB with associate AD pathology) are similar to those of advanced AD because of the combined pathology. But phospho-tauSer396 is found in synaptic-enriched fractions in the frontal cortex in PD and DLBp. These observations indicate that phospho-tauSer396 is a common sub-cellular abnormality in synaptic-enriched fractions in PD and DLB, albeit these changes are not associated with any evidence of neurofibrillary degeneration or neuropil threads in the same regions in these α-synucleinopathies. Whether tau phosphorylation occurs locally or is transported through the axon is not known, but our findings indicate that tau kinases GSK-3β and cdk5 (Anderton et al., 1999) are also present in syn-

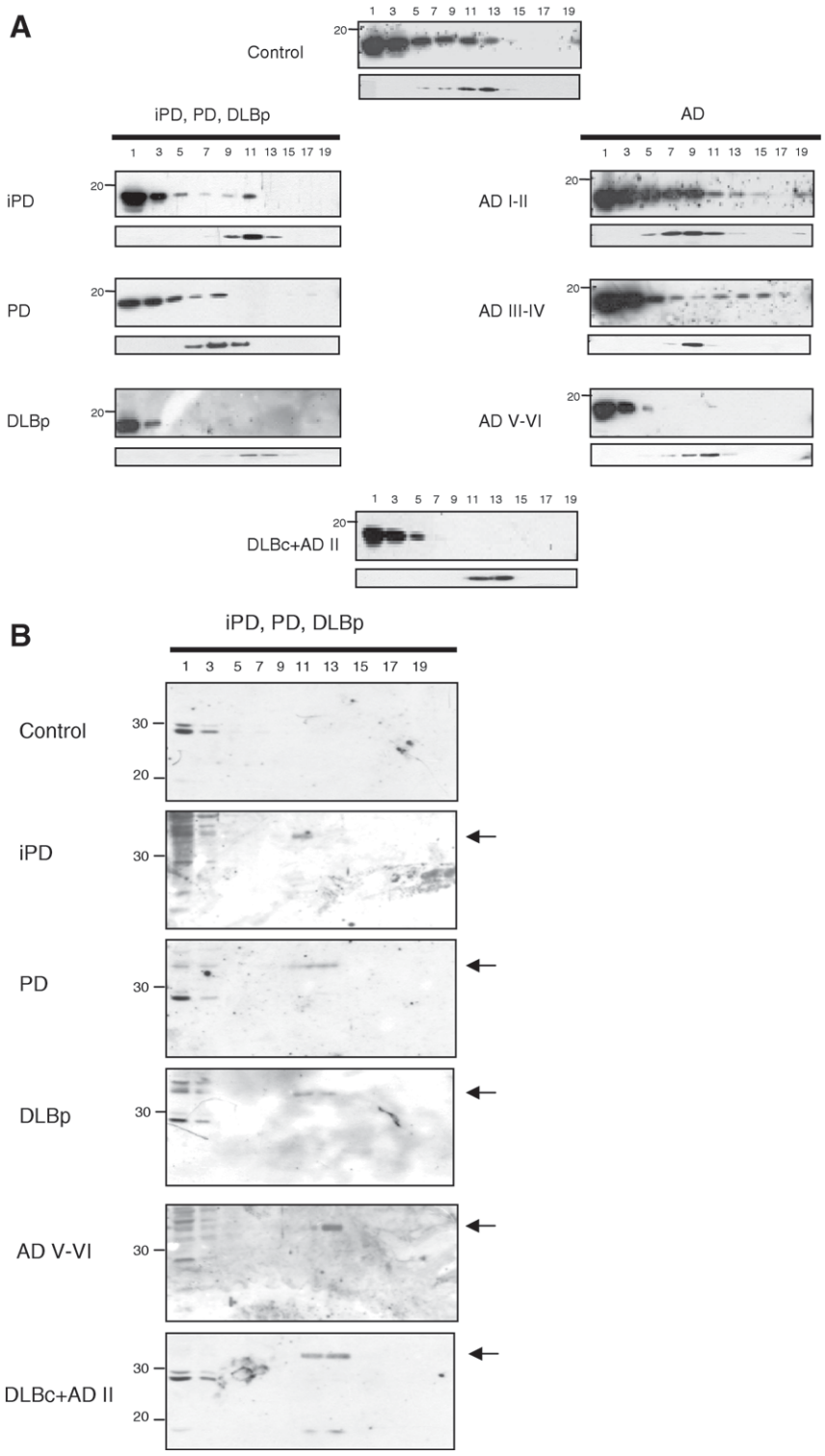

Fig. 4. (A) Representative figures of the distribution of α-synuclein in the frontal cortex in control (C), iPD, PD, DLBp, DLBc and AD stages I–II, III–IV and V–VI. Synaptophysin distribution is also indicated in the lower files to recognize synaptic-enriched fractions. Reduced α-synuclein is found in the synaptic-enriched fractions in DLBp and DLBc and in advanced stages of AD (AD V–VI). Moderate reduction occurs in iPD and PD. (B) Phospho-α-synucleinSer129 is found in synaptic-enriched fractions in DLBp and DLBc, and advanced stages of AD (arrows).

aptic-enriched fractions, thus suggesting that tau phosphorylation may occur locally upon kinase activation. Other tau kinases may participate at the synapses as well (Ferrer, 2004).

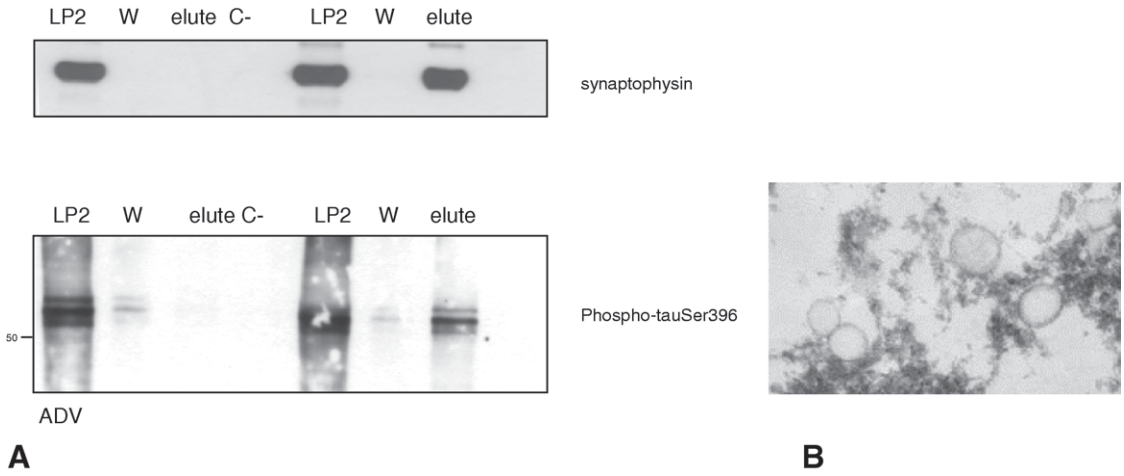

Fig. 5. (A) Fractions of AD cases enriched in synaptic vesicles (LP2) contain synaptophysin (upper panel) and phospho-tauSer396 (triplet). These proteins are not found in the supernatant (W) and in the control negative eluate purified without synaptophysin antibodies (eluate C−). Yet phospho-tauSer396 is found in the positive eluate fraction (eluate) obtained following immunoprecipitation with anti-synaptophysin antibodies. Synaptophysin immunoreactivity in this eluate is used as a positive control. (B) Electron microscopy of the eluate fraction is composed of small vesicles alone.

Fractions enriched with synaptic vesicles immunoprecipitated with anti-synaptophysin antibodies have shown the presence of phospho-tauSer396 in the eluted fractions, thus suggesting that phospho-tauSer396 is linked to synaptic vesicles. Whether phospho-tauSer396 at the synapses may have functional consequences cannot be discerned on the basis of the present and related (Ishizawa et al., 2003; Frasier et al., 2005) descriptive observations. Yet over-expression of tau results in defective synaptic transmission in *Drosophila* neuromuscular junction (Chee et al., 2005); and transgenic mice over-expressing mutant tau have deficits in synaptic plasticity (Oddo et al., 2003; Schindowski et al., 2006).

Therefore, together these observations point to the likelihood that abnormal tau phosphorylation at the synapses may impair synaptic function and plasticity in AD and synucleinopathies as it occurs in *Drosophila* and tau mutant mice.

Modifications of tau in α-synucleinopathies and AD are accompanied by distinct changes of α-synuclein in both conditions. α-Synuclein phosphorylation at Ser129 occurs in the synaptic-enriched fractions in the frontal cortex in PD and DLBp and DLBc; thus suggesting abnormal α-synuclein phosphorylation at the synapses in α-synucleinopathies. This is an important aspect as phosphorylation of α-synuclein at Ser129 is a dominant pathological modification in α-synucleinopathies (Fujiwara et al., 2002; Saito et al., 2003; Anderson et al., 2006). Furthermore, α-synuclein phosphorylation at Ser129 increases aggregation *in vivo* (Chen and Feany, 2005; Anderson et al., 2006). Oligomeric α-synuclein inhibits tubulin polymerization (Chen et al., 2007). Furthermore, synuclein modifications

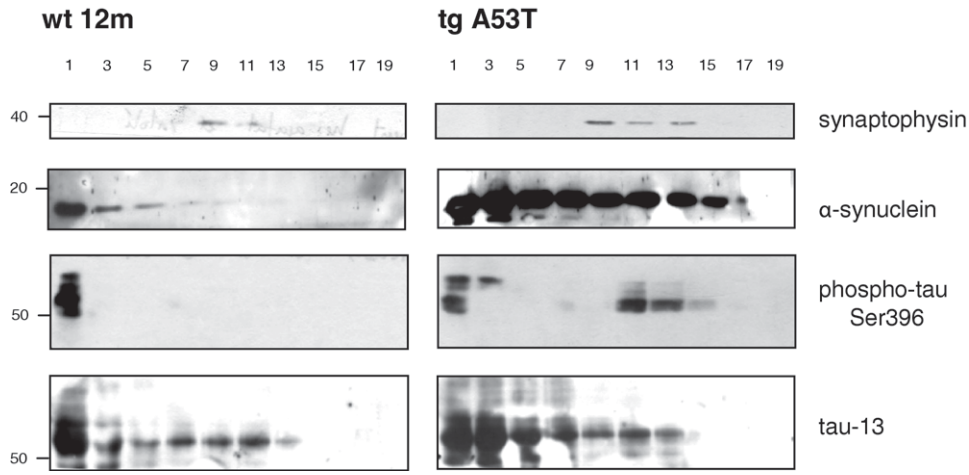

Fig. 6. Representative figures of the distribution of tau-13 and phospho-tauSer396 in cerebral fractions of control (wt) and A53T α-synuclein transgenic (tg) mice 12 months old. Synaptophysin immunoreactivity is used to recognize synaptic-enriched fractions. Phospho-tauSer396 is found in the synaptic-enriched fractions in transgenic but not in wt mice. The subfraction distribution of tau-13 is similar in control and transgenic mice. Note the impressive accumulation of α-synuclein in transgenic mice when compared with controls.

may have additional implications in synaptic trafficking and abnormal metabotropic glutamate receptor signaling (Dalfó et al., 2004a,b). Whether these changes associated with α-synuclein modifications also occur in AD need further study. Although synuclein phosphorylation is crucial for synuclein aggregation, the present findings indicate synuclein phosphorylation in the absence of Lewy pathology at the synapses. It can be argued that LB formation at the synapse is rather visible with current optical microscopy methods. Yet other factors may be necessary to the full formation of Lewy pathology *in vivo*, including aggregation of multiple proteins (Wakabayashi et al., 2007) and reduced abnormal protein elimination (Pan et al., 2008).

To rule out whether tau phosphorylation and synuclein phosphorylation may have a common mechanism linked with particular kinases, the subfractionation distribution of GSK-3, cdk5 and casein kinase II has been analyzed in normal and diseased brains. GSK-3 and cdk5 have the capacity to phosphorylate tau at different sites including Ser396 (Ferrer et al., 2005). Since the GSK-3 and cdk5 are found in synaptic-enriched fractions, it may be suggested that these kinases may act as tau kinases at the synapse. Since the distribution and expression levels of these tau kinases are similar in controls and diseased brains, the implications of these kinases in abnormal tau phosphorylation at the synapse remains obscure. α-Synuclein phosphorylation at Ser129 is mainly carried out by casein kinase 2 (Okochi et al., 2000; Ishii et al., 2007). Although GSK-3 and cdk5 have been implicated in the phosphorylation of PD-associated protein and cdk5 is constituent of LB (Takahashi et al., 2000; Tanji et al., 2003; Avraham et al., 2007), there is at present no evidence that GSK-3 and cdk5 are directly implicated in synuclein phosphorylation at Ser129.

Together these findings do not support a common phosphorylation pathway of tau and α-synuclein at the synapse. Rather, these results suggest that there are two separate pathways that are both active in AD and synucleinopathies.

Hyper-phosphorylation of tau at Ser396/404 has been observed in primary mesencephalic neurons treated with neurotoxin MPP^+ and in wild-type mice chronically treated with MPTP, used as models of PD. Interestingly, MPP^+/MPTP-inducible tau hyper-phosphorylation does not occur in transfected cells not expressing α-synuclein or in α-synuclein −/− mice, thus suggesting convergent overlapping pathways in divergent diseases such AD and PD (Duka et al., 2006). Further evidence of the association of tau pathology in synucleinopathies relies on the observation of phosphorylated tau in transgenic mice over-expressing the A30P α-synuclein mutation (Frasier et al., 2005), as well as in the present observations demonstrating phosphor-taSer396 in synaptic-enriched fractions in A53T transgenic mice.

Sub-cellular tau phosphorylation at Ser396 in the synaptic-enriched fractions of the frontal cortex converges in AD and LBDs, and it provides a rationale for the observation of tau and AD pathology in PD and DLB (Boller et al., 1980; Kosaka, 1993). Phosphorylation of α-synuclein in synaptic-enriched fractions in the neocortex is also a common feature in α-synucleinopathies and advanced stages of AD. The present observations support a link between AD and α-synucleinopathies in which both proteins tau and α-synuclein are abnormally phosphorylated in synaptic-enriched fractions.

Yet tau phosphorylation consistent with NFT formation, as revealed with the AT8 anti-tau antibody, is only found in synaptic-enriched fractions of the frontal cortex from cases with advanced cases of AD. That means that phosphorylation of tau396 at the synapses is not sufficient to produce NFTs. Similarly, α-synuclein phosphorylation at Ser129 is not sufficient to produce LB. In the same line, recent studies have shown α-synuclein lipoxidation as a major modification of synuclein in regions bearing LB and neurites in LB diseases. However, α-synuclein lipoxidation also occurs in vulnerable brain regions in the absence of LB at early stages of LBDs (Dalfó and Ferrer, 2008). Therefore, α-synuclein lipoxidation is not sufficient to produce LB.

CONCLUSION

In summary, the present findings support the concept that different mechanisms converge at the synapse causing tau and synuclein phosphorylation in AD and α-synucleinopathies. Moreover, oxidative/nitrosative stress causes nitration of α-synuclein at the synapse in AD and synucleinopathies. This scenario is not limited to the human brain as tau phosphorylation at the synapses is also observed in 12-month-old transgenic mice bearing the A53T α-synuclein mutation. Since both alterations are rate-limiting stages in the formation of abnormal tau and synuclein oligomerization and fibrillization, these changes point to abnormal tau and synuclein metabolism at the synapse which may facilitate oligomerization and fibrillization with disease progression in both AD and synucleinopathies.

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3. Modificació del perfil lipídic en el cervell però no del fenotip, en el ratolí transgènic A53T després d'una dieta pobra en n-3.

Muntané G, Janué A, Fernandez N, Odena MA, Oliveira E, Boluda S, Portero-Otin M, Naudí A, Boada J, Pamplona R, Ferrer I.

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Els ratolins que sobreexpressen l'α-sinucleïna humana mutada són un dels models més utilitzats per estudiar les malalties amb cossos de Lewy. En aquest treball, nosaltres hem caracteritzat la neuropatologia del model A53T i li hem administrat una dieta pobre en n-3 o una dieta pobre en n-3 enriquida amb DHA durant 6 mesos. Aquests animals transgènics mostren un fenotip lleu caracteritzat per un augment en l'expressió de l'α-sinucleïna humana, 4 vegades l'expressió de la pròpia del ratolí. També presenten formes truncades de l'α-sinucleïna, una agregació i una solubilitat anormals de l'α-sinucleïna en absència de cossos de Lewy, neurites de Lewy, mort neuronal aparent, astrogliosi o microgliosi.

L'administració de les dietes produeix una reducció en el contingut d'àcid linoleic i àcids grassos poliinsaturats totals en el cervell, juntament amb una reducció del dany oxidatiu proteic sense modificacions en l'expressió de l'α-sinucleïna ni en el nombre d'astròcits corticals ni cèl·lules microglials.

Aquest estudi suggereix que el model pot utilitzar-se per estudiar mecanismes concrets en la patologia de les α-sinucleïnopaties, com el truncament o l'agregació de l'α-sinucleïna, però no desenvolupa una patologia de Lewy completa. A més, la dieta és capaç de modificar la composició de lípids del cervell i la susceptibilitat al dany oxidatiu, però no interfereix en el fenotip d'aquest model genètic.

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Modification of brain lipids but not phenotype in α-synucleinopathy transgenic mice by long-term dietary *n*-3 fatty acids

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ABSTRACT

Transgenic mice expressing both wild mouse α-synuclein and the Parkinson's disease associated A53T mutated human α-synuclein were subjected to long-term diets impoverished in *n*-3 or diets impoverished in *n*-3 and supplemented with docosahexaenoic acid (DHA) for 6 months. Transgenic mice evidenced mild phenotype characterized by increased total α-synuclein expression, truncated α-synuclein forms, and abnormal solubility and aggregation, in the absence of Lewy bodies and neurites, and lack of apparent neuronal loss, astrocytosis and microgliosis. These diets produced a reduction in the content of linolenic, *n*-3 docosapentaenoic and total polyunsaturated fatty acids, leading to significantly lower double bond and peroxidizability indexes as well as to lower protein oxidative damage, with no effects in α-synuclein expression and with no modifications in the number of cortical astrocytes and microglial cells. The present results show that diets may modify brain lipid composition and susceptibility to oxidative damage that do not interfere with phenotype in models with a genetic susceptibility to develop α-synucleinopathy.

1. Introduction

Parkinson disease (PD) is a systemic illness characterized by loss of dopaminergic neurons in the substantia nigra and other brain regions, accompanied by intracytoplasmic neuronal inclusions, called Lewy bodies, and enlarged neurites and threads in vulnerable regions (Forno, 1996; Jellinger and Mizuno, 2003; Schults, 2006). The main component of Lewy bodies and aberrant neurites is abnormal α-synuclein, which is nitrated and oxidized, truncated and phosphorylated, and has abnormal solubility and aggregation (Spillantini et al., 1997; Wakabayashi et al., 1997; Baba et al., 1998; Hashimoto and Masliah, 1999; Duda et al., 2000; Giasson et al., 2000; Fujiwara et al., 2002; Iwatsubo, 2003). Three different nucleotide substitutions (A30P, E46K and A53T) in the α-synuclein gene (*SNCA*) have been described in association with autosomal dominant PD (Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004). Together, these data point to the cardinal role of α-synuclein in the pathogenesis of PD.

Several lines of evidence suggest that α-synuclein can influence brain the intracellular lipid trafficking, the regulation of lipid metabolism and the stabilization of lipid membranes (Golovko et al., 2008). In this sense, its role in brain metabolism of the polyunsaturated fatty acids (PUFAs) arachidonic (20:4*n*-6) and docosahexaenoic (DHA, 22:6*n*-3) acid (Golovko et al., 2006, 2007), the two major brain PUFAs, is especially remarkable. In addition, previous studies have shown abnormal lipid composition with increased components which raise the lipoxidation index, together with the presence of lipoxidative stress and the production of lipoxidative damage of critical proteins in PD (Dalfó et al., 2005; Dalfó and Ferrer, 2008). Moreover, the percentage of brain neutral lipids is altered in α-synuclein gene-ablated mice (Barceló-Coblijn et al., 2007). Together, these observations indicate a close relationship between α-synuclein and brain lipids, and raise the possibility of considering whether the composition of lipids in the diet may influence brain lipid composition and biochemical brain parameters linked to oxidative stress in subjects with a predisposition to suffer from PD.

Different models have been generated in an attempt to mimic unique aspects of PD, none of them fulfilling *all* the criteria of PD (van der Putten et al., 2000; Jellinger, 2003; Fernagut and Chesselet, 2004; Chesselet, 2008). However, available models are very useful to analyze specific mechanisms in PD, provided that the

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limitations and advantages of the model are adapted to the objectives to put forward. In some works, mice over-expressing the human α-synuclein A53T under the control of mouse PrP promoter recapitulate PD pathology (Lee et al., 2002; Giasson et al., 2002; Dawson et al., 2002). This PD-reminiscent phenotype has not been reproduced in other studies but novel A53T transgenic mice manifest a more benign phenotype and lack of Lewy-like inclusions (Gispert et al., 2003). The expression of human α-synuclein A53T in a null background of α-synuclein results in decreased lifespan; brain disorder is more severe in these mice when compared with animals expressing the human mutation together with wild mouse α-synuclein (Cabin et al., 2005). This observation suggests that wild mouse α-synuclein has a protective effect when expressed together with the human α-synuclein mutation.

Following this rationale, we exposed A53T α-synuclein transgenic mice to long-term diets with variable lipid composition: low in *n*-3 fatty acids (with corn oil 5.5%, safflower oil 1.5% and a ratio *n*-6/*n*-3 of 83:1) and low in *n*-3 and enriched in DHA (with 3% and 4% safflower oil in DHA powder)—to learn whether these diets might modify the fatty acid profile, the protein oxidative damage (assessing three different types of protein oxidative modifications: direct oxidation, glyco- and lipoxidation) and finally PD pathological hallmarks in this model. Special attention was paid to select an animal line with a relative benign phenotype as modifications related to external influences (i.e. diets) could not be hampered by severe phenotypic marks.

2. Materials and methods

2.1. α-Synuclein transgenic mice

The generation of transgenic mice harbouring human α-synuclein with the A53T mutation under the control of mouse PrP promoter has been described by Giasson et al. (2002). Mice were purchased from Jackson Labs (ref. B6;C3-Tg(Prnp-SNCA*A53T)83Vle/J). Animals were housed three or four per cage in a temperature-controlled room under a 12 h light/dark cycle with free access to food and water. Animal experiment protocols were approved by the Animal Care and Ethics Committee of the University of Barcelona, and all efforts were made to minimize the number of animals used and their suffering. Transgenic mice aged 6, 9, 12, 15, 18 and 24 months old were identified routinely after weaning from tail samples by PCR analysis using the primers: IMR1772: 5′-TGT AGG CTC CAA AAC CAA GG-3′, and IMR3560: 5′-TGT CAG GAT CCA CAG GCA TA-3′. Only transgenic mice expressing similar levels of human α-synuclein protein (assessed by Western blotting of brain homogenates) were selected for study.

For histological studies, mice ($n = 15$) were anaesthetized with an overdose of sodium pentobarbital and perfused through the heart with phosphate buffer followed by 4% paraformaldehyde. The brains were removed, stored for 24 h in the same fixative solution and then cryoprotected with 30% saccharose for 24 h. Slices were cut on coronal sections and frozen at −80 °C until use. Serial sections, 20 μm thick, were obtained with a cryostat and prepared for morphological and immunohistochemical studies. Other sections were embedded in paraffin and cut with a sliding microtome. Sections of were emFor biochemical studies mice ($n = 60$) were killed under deep anaesthesia using a CO_2 atmosphere and immediately decapitated. The brain was dissected and separate sample were labelled, frozen in liquid nitrogen and stored at −80 °C until use.

2.2. RT-PCR for human α-synuclein

mRNA isolation was carried out in two steps. Total RNA was isolated using TRizol Reagent (Life Technologies, Barcelona, Spain) followed by the RNeasy Midi Kit (Qiagen, Barcelona, Spain). Frozen mouse brain cortex samples were directly homogenized in 1 ml of TRizol Reagent per 100 mg tissue and left for 5 min at room temperature. Next, 200 μl of chloroform was added and mixed vigorously. After 3 min at room temperature, the samples were centrifuged (12,000 × *g*, 15 min, 4 °C). The resulting supernatants were mixed with 0.5 ml of isopropanol, left at room temperature for 10 min and then centrifuged (12,000 × *g*, 10 min, 4 °C). Afterwards, the pellets were washed with 1 ml of ethanol 70% and centrifuged at 7500 × *g* for 5 min at 4 °C. Then the pellets were dried at room temperature for 10 min and re-suspended in 100 μl of RNase-free water and incubated in a water bath at 55–60 °C for 10 min.

Purified total RNA was mixed with 350 μl of RTL buffer (containing β-mercaptoethanol) provided with the RNeasy Midi Kit and 250 μl of ethanol 96%. The resulting solution was poured into an RNeasy column and centrifuged at 8000 × *g* for 15 s. Next, 500 μl of RPE buffer (provided with the RNeasy Midi Kit) was also poured into the column and centrifuged at 8000 × *g* for 15 s. This was followed by centrifugation at 8000 × *g* for 2 min to completely elute the RPE buffer. Finally, the RNA was eluted by adding 30–40 μl of RNase-free water and centrifuging the column at 8000 × *g* for 1 min. The columns used excluded tRNA, 5S and 5.8S ribosomal RNAs.

The concentration of each sample was obtained from A_{260} measurements. RNA integrity was tested by gel electrophoresis, and confirmed by using the Agilent 2100 BioAnalyzer (Agilent).

2.2.1. cDNA synthesis

For each 20 μl reverse transcription reaction, 2 μg RNA was mixed with 2.5 μM random hexamers, 1× TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM each dATP, dTTP, dCTP and dGTP, 0.4 U/μl RNase inhibitor and 1.25 U/μl MultiScribe Reverse Transcriptase (Applied Biosystems). Reactions were carried out at 25 °C for 10 min to maximize primer–RNA template binding, followed by 120 min at 37 °C, and then by incubation for 5 s at 85 °C to de-activate reverse transcriptase. Parallel reactions for each RNA sample were run in the absence of MultiScribe Reverse Transcriptase to assess the degree of contaminating genomic DNA.

2.2.2. TaqMan probes

Mice γ-tubulin (Tub) (Mm 00506153_gH, TaqMan probe) and mouse α-synuclein (Syn) (Hs00240906_m1, TaqMan probe) (Applied Biosystems) were used in the present study.

2.2.3. TaqMan PCR

TaqMan PCR assays for Tub and Syn were performed in triplicate on cDNA samples in 96-well optical plates using an ABI Prism 7700 Sequence Detection system (Applied Biosystems). The plates were capped using optical caps (Applied Biosystems). The ABI Prism 7700 measures the fluorescent accumulation of the PCR product by continuously monitoring cycle threshold (Ct), which is an arbitrary value assigned manually to a level somewhere above the baseline but in the exponential phase of PCR where there are no rate-limiting components. The Ct value sets the point at which the sample amplification plot crosses the threshold. The Ct values correlate with the initial amount of specific template.

For each 20 μl TaqMan reaction, 9 μl cDNA (diluted 1/50, which corresponds approximately to the cDNA from 4 ng of RNA) was mixed with 1 μl 20× TaqMan® Gene Expression Assays and 10 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems). Parallel assays for each sample were carried out using primers and probes with Tub for normalization. The reactions were carried out using the following parameters: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Standard curves were prepared for Syn and Tub using serial dilutions of control mouse brain RNA. Finally, all TaqMan PCR data were captured using the Sequence Detector Software (SDS version 1.9, Applied Biosystems).

2.3. Diets

The fatty acid profile of diets used in this work is shown in Table 1. Dietary treatment reproduced the paradigm previously reported to induce pathogenically relevant changes in a murine model of neurodegeneration (Calon et al., 2004). Mice were first fed with standard laboratory chow for the first 6 months and then the

Table 1
Fatty acid composition (mol%) of diets.

	Control	↓*n*-3	↓*n*-3 + DHA
14:0	0.12 ± 0.00	0.24 ± 0.00	3.92 ± 0.06
16:0	10.46 ± 0.14	9.28 ± 0.16	10.95 ± 0.06
16:1*n*-7	0.09 ± 0.00		0.55 ± 0.02
18:0	3.82 ± 0.11	1.89 ± 0.07	2.46 ± 0.06
18:1*n*-9	23.32 ± 0.41	24.13 ± 0.14	17.72 ± 0.21
18:2*n*-6	54.24 ± 0.28	63.57 ± 0.18	51.02 ± 0.31
18:3*n*-3	7.92 ± 0.08	0.89 ± 0.01	0.27 ± 0.02
22:5*n*-3			1.71 ± 0.03
22:6*n*-3			11.40 ± 0.09
ACL	17.78 ± 0.003	17.77 ± 0.003	17.84 ± 0.006
SFA	14.41 ± 0.09	11.41 ± 0.23	17.33 ± 0.18
UFA	85.58 ± 0.09	88.59 ± 0.23	82.67 ± 0.18
MUFA	23.43 ± 0.41	24.13 ± 0.14	18.27 ± 0.23
PUFA	62.16 ± 0.32	64.46 ± 0.19	64.40 ± 0.35
PUFA*n*-6	54.24 ± 0.28	63.57 ± 0.18	51.02 ± 0.31
PUFA*n*-3	7.92 ± 0.08	0.89 ± 0.01	13.39 ± 0.10
DBI	155.67 ± 0.28	153.95 ± 0.40	198.08 ± 0.84
PI	70.67 ± 0.36	65.96 ± 0.19	153.50 ± 0.93

Values are means ± SEM from $n = 3$ samples. ↓*n*-3: diet impoverished in fatty acids from series *n*-3 with high levels of *n*-6 and *n*-9, as well as saturated fatty acids (SFA). ↓*n*-3 + DHA: similar to ↓*n*-3 but elevated levels of docohexosaenoic acid (DHA, 22:6*n*-3). For abbreviations, see Section 2.

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tested diets were given to mice during the next 6 months. At the age of 12 months the animals were killed for biochemical and morphological studies. Mice were given *ad libitum* access to water and food throughout the experiment.

Each group was composed of 12 animals (6 males and 6 females). At the end of the experiment, ten animals per group were killed under deep anaesthesia, and the brains were rapidly removed from the skull, dissected and immediately frozen at −80 °C for biochemical studies; two animals per group (one male, one female) were perfused through the heart as previosly indicated and their brains used for immunohistochemistry.

2.4. Fatty acid analysis

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

From fatty acid profile, the following fatty acid indexes were calculated: saturated fatty acids (SFAs) = Σ% of saturated fatty acids; unsaturated fatty acids (UFAs) = Σ% unsaturated fatty acids; monounsaturated fatty acids (MUFAs) = Σ% of monoenoic fatty acids; polyunsaturated *n*-3 fatty acids (PUFA*n*-3) = Σ% of polyunsaturated fatty acids *n*-3 serie; polyunsaturated *n*-6 fatty acids (PUFA*n*-6) = Σ% of polyunsaturated fatty acids *n*-6 serie; Average Chain Length (ACL) = $\Sigma[(\Sigma\%\text{Total } 14 \times 14) + \ldots + (\Sigma\%\text{Total}_n \times n)]/100$ (*n* = carbon atom number).

In addition the following indexes of lipid susceptibility to oxidative modification (Hulbert et al., 2007), were calculated: unsaturation index (UI) = [(Σmol% monoenoic × 1) + (Σmol% dienoic × 2) + (Σmol% trienoic × 3) + (Σmol% tetraenoic × 4) + (Σmol% pentaenoic × 5) + (Σmol% hexaenoic × 6)]; peroxidizability index (PI) = [(Σmol% monoenoic × 0.025) + (Σmol% dienoic × 1) + (Σmol% trienoic × 2) + (Σmol% tetraenoic × 4) + (Σmol% pentaenoic × 6) + (Σmol% hexaenoic × 8)].

2.5. Measurement of the oxidative protein damage markers GSA, AASA, CML, CEL and MDAL

Glutamic (GSA) and aminoadipic (AASA) semialdehydes, N^{ε}-carboxymethyl-lysine (CML), N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-malondialdehyde-lysine (MDAL) concentrations in total proteins from mice brain homogenates were measured by gas chromatography/mass spectrometry (GC/MS) (Dalfó et al., 2005; Pamplona et al., 2005; Pamplona et al., 2008). GSA and AASA arise from the direct reaction of lysine, arginine and proline residues with reactive oxygen species (Pamplona et al., 2005), being considered biomarkers of direct protein oxidative modification. CEL is derived from the reaction of methylglyoxal – a byproduct of glycolysis – with lysine, thus being used as surrogates for protein glycoxidation. MDAL arises from the addittion of the carbonyl compound malondialdehyde – derived from lipid peroxidation – and lysine. For this reason, its levels express protein lipoxidative modification. Finally, CML is a mixed glycoxidative–lipoxidative product, as its precursor, glyoxal is originated by both reactions.

Samples containing 0.1–0.4 mg of protein were delipidated using chloroform:methanol (2:1, v/v), and proteins were precipitated by adding 10% trichloroacetic acid (final concentration) and performing subsequent centrifugation. Protein samples were reduced overnight with 500 mM $NaBH_4$ (final concentration) in 0.2 M borate buffer, pH 9.2, containing one drop of hexanol as an anti-foam reagent. Proteins were then reprecipitated by adding 1 ml of 20% trichloroacetic acid and performing subsequent centrifugation. The following isotopically labelled internal standards were then added: [$^2H_8$]Lysine (d_8-Lys; CDN Isotopes); and [$^2H_4$]CML (d_4-CML), [$^2H_4$]CEL (d_4-CEL), [$^2H_8$]MDAL (d_8-MDAL), [$^2H_5$]glutamic semialdehyde (d_5-GSA) and [$^2H_4$]aminoadipic semialdehyde (d_4-AASA), prepared as described in Dalfó et al. (2005). The samples were hydrolyzed at 155 °C for 30 min in 1 ml of 6N HCl, and then dried in vacuo. The N,O-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared as previously described (Dalfó et al., 2005). GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30 m HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector (Hewlett-Packard Española, S.A., Barcelona, Spain). The injection port was maintained at 275 °C; the temperature program was 5 min at 110 °C, then 2 °C/min to 150 °C, then 5 °C/min to 240 °C, then 25 °C/min to 300 °C, and finally holds at 300 °C for 5 min. Quantification was performed by external standardisation using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analyses were carried out by selected ion monitoring gas chromatography/mass spectrometry. The ions used were: lysine and d_8-lysine, *m*/*z* 180 and 187, respectively; glutamic semialdehyde and d_5-glutamic semialdehyde, *m*/*z* 280 and 285, respectively; aminoadipic semialdehyde and d_4-aminoadipic semialdehyde, *m*/*z* 294 and 298, respectively; CML and d_4-CML, *m*/*z* 392 and 396, respectively; CEL and d_4-CEL, *m*/*z* 379 and 383, respectively; and MDAL and d_8-MDAL, *m*/*z* 474 and 482, respectively. The amounts of products were expressed as the ratio μmol glutamic semialdehyde, aminoadipic semialdehyde, CML, CEL or MDAL/mol lysine.

2.6. Sample fractionation

The analysis of α-synuclein distribution in different cellular fractions was performed based on modification of a previously reported protocol (Dickson et al., 1999). In brief, 0.1 g from every brain region of wild mice (20 months) and transgenic mice at different ages (6, 12 and 24 in one series; 7 and 23 months in the other) was homogenized and sonicated on ice in 0.5 mL of ice-cold PBS (sodium phosphate buffer, pH 7.0) 1 mM of phenylmethylsulfonylfluoride (PMSF) plus complete proteases inhibitor cocktail (Roche Diagnostics, Barcelona, Spain), sonicated and centrifuged at 5000 × *g* for 10 min at 4 °C. The pellet was discarded and the supernatant was ultracentrifuged at 100,000 × *g* for 1 h at 4 °C to separate the PBS-soluble fraction (PBS-fraction) from the pellet (P1). All the following centrifugation steps were carried out at 4 °C and 100,000 × *g* for 1 h. The P1 pellet was re-suspended in a solution of PBS, pH 7.0, containing 0.5% sodium deoxycholate, 1% Triton and 0.1% SDS. After another ultracentrifugation, the supernatant was kept as deoxycholate-soluble fraction (Dxc-fraction). The corresponding pellet was extracted with a solution of SDS 2% in PBS and maintained at room temperature for 2 h and ultracentrifuged at 25 °C. The resulting supernatant was kept as SDS-soluble fraction (SDS fraction), and the corresponding pellet was re-suspended and sonicated in a buffer containing 8 M urea and 5% SDS and left at room temperature for 1 h to allow the complete dissolution of the material. The same volume of each fraction was mixed with reducing sample buffer and loaded in 12% polyacrylamide gels.

2.7. Gel electrophoresis and Western blotting

Mouse brain tissues were manually homogenized using a glass homogenizer in ice with 10 volumes of RIPA buffer with a complete protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain), 1 mM PMSF (phenylmethylsulfonylfluoride) and 2 mM orthovanadate (Sigma, Madrid, Spain). Total homogenates were centrifuged at 10,000 × *g* for 5 min at 4 °C, pellets were discarded and the protein content of resulting supernatants was determined by the Bradford Assay (Sigma, Madrid, Spain). Total homogenates were mixed with reducing sample buffer at a final concentration of 1 μg/μl, heated at 97 °C for 3 min, aliquoted and stored at −20 °C until use. Equal amounts of each sample (20 μg) were loaded on SDS-PAGE gels for electrophoresis (20 mA/gel) and then transferred to nitrocellulose membranes (100 V for 75 min). Membranes were stained with Ponceau Solution (Sigma) to test quality control and only those with uniform staining were subsequently blocked with TBS-T (100 mM Tris-base, 1.4 M NaCl and 0.1% (v/v) Tween 20, pH 7.4) with 5% skimmed milk for 30 min to 1 h at room temperature. Then, the membranes were incubated at 4 °C with one of the primary antibodies diluted in TBS-T with bovine serum albumin (BSA) (Sigma) at 3% overnight. Human α-synuclein monoclonal antibody (Novocastra) was used at a dilution of 1:1000; Ab-1 (87-110) monoclonal antibody (Neomarkers) was diluted 1:2000; and α-synuclein rabbit polyclonal antibody (Chemicon) was used at a dilution of 1:4000. Monoclonal antibody to β-actin (Sigma) diluted 1:10,000 was used as a control of protein loading. Immediately afterwards, the membranes were washed with TBS-T and incubated with the corresponding secondary antibody labelled with horseradish peroxidase (Dako) at a dilution of 1:1000 in the same buffer (TBS-T with 5% skimmed milk) for 45 min at room temperature. Protein bands were detected by the chemiluminescence ECL method (Amersham Biosciences, Barcelona, Spain). β-Actin (45 kDa) or the mitochondrial porin (31 kDa) were used as protein loading controls. The densitometric quantification of Western blot bands was carried out with Total Lab v2.01 software and the obtained data were analyzed using Statgraphics Plus v5.1 software. Differences between wild type and transgenic mice samples were analyzed with ANOVA and LSD post-test.

2.8. Bi-dimensional gel electrophoresis, Western blotting, in-gel dissection and mass spectrometry

Proteins from total homogenates of 12-month-old mice were precipitated with acetone by mixing 200 μl of total homogenate with 800 μl of acetone and keeping them on at −20 °C. The next day samples were centrifuged at 15,000 × *g* for 10 min and supernatants were removed. Pellets were dried, re-suspended by adding 400 μl of 2D lysis buffer (40 mM Tris-base, 9 M urea, 2 M thiourea, 4% (w/v) CHAPS (Bio-Rad) and a mix of protease inhibitors containing 1 mM PMSF, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, all from Sigma). Finally, protein concentration was measured by Bradford Assay (Sigma), and 125 μg of total protein of each sample in a final volume of 125 μl with 0.8% Bio-Lyte 3/10 Ampholites (Bio-Rad), 2 mM TBP (tributylphosphine) (Sigma) and 0.0004% bromophenol blue was prepared to re-hydrate the IPG strips. Immobilized 7 cm

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pH 4–7 linear gradient ReadyStrip IPG Strips (Bio-Rad) were used to run the first dimension electrophoresis by re-hydrating strips actively for 12 h at 50 V. Subsequently, proteins were focused at 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 1 h. Finally, the voltage was kept at 8000 V till reaching a total of 24 kV/h. Before running the second dimension, IPG strips were equilibrated for 2× 15 min in equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.0002% bromophenol blue) with 1% DTT (dithiothreitol, Sigma) and, subsequently, with 4% iodoacetamide (Bio-Rad). Then, the strips were placed on 12% SDS-PAGE and electrophoresis was run at 35 mA/gel for 1.5 h. Two bi-dimensional electrophoresis of each sample was run in parallel. One gel was stained with Bio-Safe Colloidal Coomassie Blue G-250 Stain (Bio-Rad) as described by the manufacturer, or with PlusOne Silver Staining Kit Protein (GE Healthcare, Uppsala, Sweden), and the other gel was transferred to a nitrocellulose membrane (20 V and 50 mM/gel for 70 min). Membranes were blocked with TBS-T with 5% skimmed milk for 30 min at room temperature and then incubated with one of the primary antibodies. The mouse monoclonal anti-α-synuclein (Novocastra) was used at a dilution of 1:500 in TBS-T with 5% skimmed milk at 4 °C overnight. The membranes were incubated with the corresponding secondary antibody labelled with horseradish peroxidase (Dako) at a dilution of 1:1000 in the same buffer for 45 min at room temperature, and protein bands were detected by chemiluminescence ECL method (Amersham). Selected spots corresponding to those labelled with anti-α-synuclein antibodies were manually excised from the gels stained with Bio-Safe Colloidal Coomassie Blue G-250 or with silver stain, and proteins were in-gel digested with trypsin (Sequencing grade modified, Promega, Barcelona, Spain) in the authomatic Investigator ProGest robot of Genomic Solutions. Briefly, gel excised spots were washed sequentially with ammonium bicarbonate buffer and acetonitrile. Then, proteins were reduced and alkylated for 30 min each, 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 peptids were extracted from the gel matrix with 10% formic acid and acetonitrile. The extracts were pooled and dried in a vacuum centrifuge. Once the proteins were digested, they were 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 μl of 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, Netherlands). The eluted peptides were ionized via coated nano-ES needles (PicoTipTM, 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 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, 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 200 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 $10^{(-\text{Mowse score}/10)}$. In the statistical analysis of these spots, mowse scores greater than 52 corresponding with a $p < 0.05$ were considered to be of high confidence of identification.

2.9. Immunohistochemistry

Cryoprotected sections from transgenic animals and corresponding age-matched wild littermates ($n = 15$; 2 or 3 for each time-point) were obtained at the following ages: 6, 9, 12, 15, 18 and 24 months, and processed in parallel for immunohistochemitry. After pre-treatment with formic acid and incubation with methanol and H_2O_2 in PBS and normal serum, the sections were incubated with one of the primary antibodies at room temperature overnight. Rabbit polyclonal anti-α-synuclein antibody (Chemicon, Barcelona, Spain) was used at a dilution of 1:500; mouse monoclonal anti-human α-synuclein (Novocastra, Newcastle, UK) was used at a dilution of 1:500; and Ab-1 (87–110) mouse anti-α-synuclein (LabVision/Neomarkers, Cheshire, UK) was diluted 1:500. Some sections were incubated with anti-ubiquitin antibody (Dako, Barcelona, Spain) diluted 1:100. Other sections were processed for *Lycopericum sculentum* lectin histochemistry for microglia. Following incubation with the primary antibody, the sections were incubated with EnVision + system peroxidase for 15 min at room temperature. The peroxidase reaction was visualized with diaminobenzidine and H_2O_2. Control of the immunostaining included omission of the primary antibody; no signal was obtained following incubation with only the secondary antibody. Finally, de-waxed 4-μm-thick paraffin-embedded sections were stained with haematoxylin and eosin, and for immunohistochemistry to glial fibrillary acidic protein for astrocytes, and phosphorylated neurofilament epitopes following current methods.

2.10. Quantification of glial cells

The number of GFAP-immunoreactive and lectin-positive cells was counted in the parietal cortex (Pars 1 and Pars 2). Four fields at a magnification of 400× per section in four non-consecutive sections were examined for every mouse. The number of astrocytes and microglia was expressed as the number of cells per field ± SD.

2.11. Statistics

Data were analyzed by one-way ANOVA. After the ANOVA, the Duncan test was performed for comparisons between pairs of groups. The minimum level of statistical significance was set at $p < 0.05$ for all the analyses.

3. Results

3.1. Human α-synuclein mRNA and protein expression in animals bearing mutant α-synuclein DNA in tail samples

Founders and all of their progeny were genotyped from the tail, and the whole colony was positive for the mutated transgene (Fig. 1A). RT-PCR analysis showed mutant α-synuclein mRNA in SNCA+ mice (Fig. 1B). The antibody used to detect human α-synuclein expression was Ab-1 from Neomarkers (clone syn204) raised against the specific 87–110 epitope of the human protein. It disclosed the presence of human α-synuclein in SNCA+ positive mice (Fig. 1C).

3.2. α-Synuclein levels in brain

Transgenic mice killed at 6, 12, 15 and 24 months did not show significant variations in the amount of human α-synuclein as revealed on total brain homogenates and Western blots. Similar levels were observed in different regions throughout the life span (Fig. 1D). Western blots using antibodies that recognize both human and mouse α-synuclein disclosed that the total amount of protein was higher in transgenic mice when compared with wild littermates The total amount of α-synuclein measured in the frontoparietal cortex was 4-fold higher in transgenic mice when compared with controls (Fig. 1E).

3.3. Low molecular weight isoforms

Using Ab-1 antibody or the Novocastra antibody that recognize only the human form, a band below the monomeric band at 17 kDa was revealed in brain homogenates. This band has a molecular weight of about 12 kDa and, although it was present in all the regions examined, the expression levels were higher in the cerebral cortex (Fig. 2).

Bi-dimensional gel electrophoresis and Western blotting disclosed one large spot of about 17 kDa and two small spots of low molecular weight when processed with the Novocastra antibody (Fig. 2). Corresponding silver-stained spots in gels run in parallel were excised, and mass spectrometry processing disclosed α-synuclein as the primary option with a very high grade of confidence (Table 2).

3.4. α-Synuclein aggregates

α-Synuclein aggregates were examined in the cerebral cortex and cerebellum in transgenic mice at 6, 12 and 24 months in PBS, deoxycholate and SDS fractions, using the anti-α-synuclein Chemicon antibody. Bands of about 17 kDa were observed in the PBS, Dxc and SDS fractions in the two regions at every age. In addition, bands of higher molecular weight, particularly of about 40 kDa were found in the cerebral cortex and cerebellum being more marked in the cerebral cortex than in the cerebellum (Fig. 3).

α-Synuclein aggregates were not seen in the cerebral cortex and striatum in wild mice processed in parallel. In contrast, α-synuclein-immunoreactive bands of about 40 kDa were recovered in the PBS and SDS fractions in transgenic mice aged 7 and 23 months. These bands were equally revealed with Chemicon and

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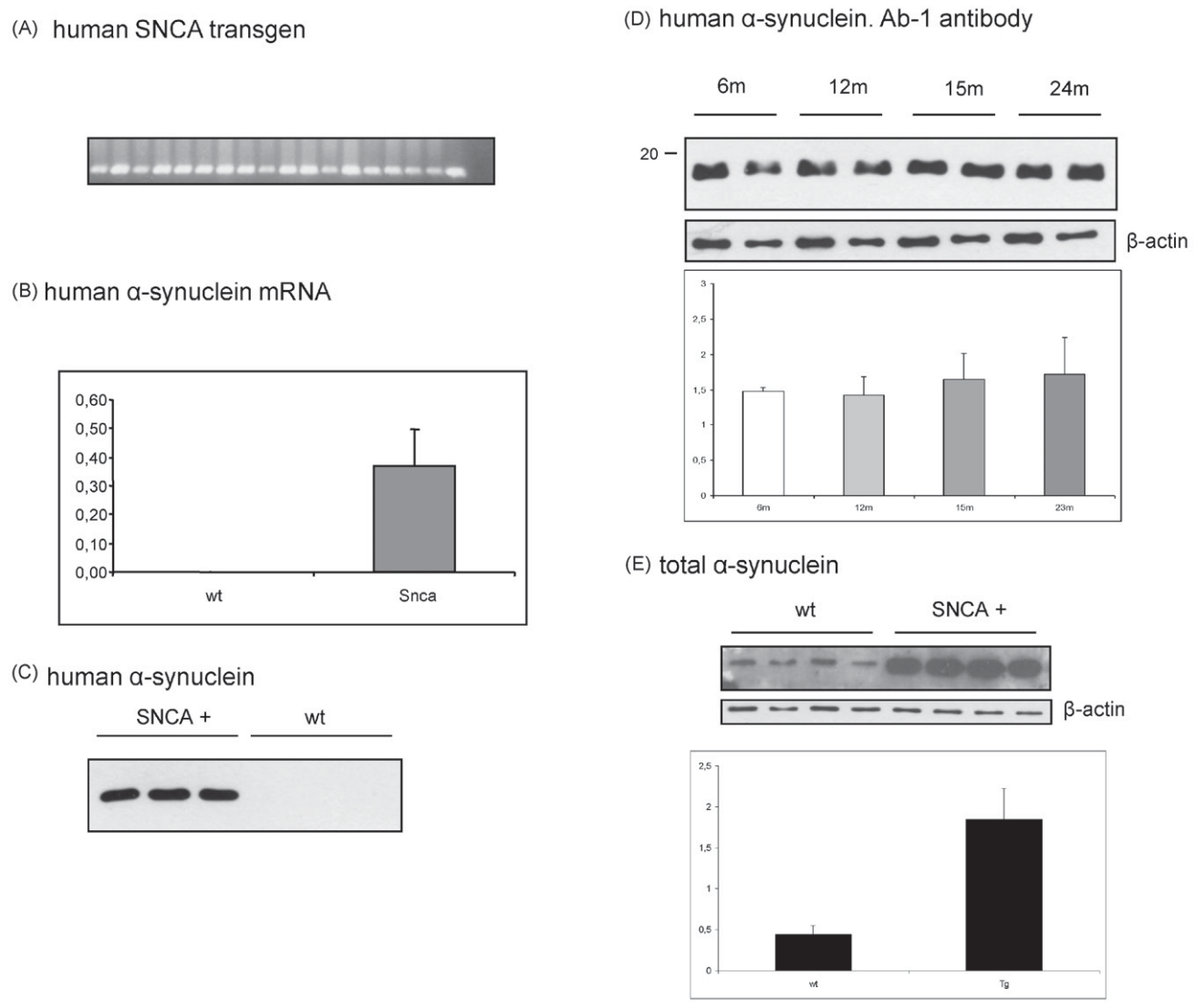

Fig. 1. Characterization of α-synuclein expression in A53T transgenic mice. (A) Expression of human *SNCA* transgen as revealed by PCR. (B) Human α-synuclein mRNA expressed in SNCA-positive mice. (C) Human α-synuclein protein expression in SNCA-positive mice and in wild type (Wt) animals. (D) Total expression levels of human α-synuclein do not increase with age in transgenic mice. (E) Total α-synuclein levels (mouse + human α-synuclein) represent more than 4-fold increase in transgenic mice when compared with wild type.

Table 2
Identification of proteins in-gel digested from bi-dimensional gels analyzed in parallel with membranes blotted for α-synuclein. Spot numbers correspond to the numbers indicated in Fig. 3. Truncated α-synuclein was identified in the spots of low molecular weight. Matched peptides for truncated α-synuclein are shown in bold letters. Regions matched: 11–21, 35–43 and 59–96.

Spot	Calculated pI	Nominal mass Da	Protein	Score	Sequence coverage	Number of peptides matched	gi accession
20.0	4.74	14476	synuclein, alpha [Mus musculus]	380	40%	10	gi|6678047
	4.59	14474	NACP/alpha-synuclein [Homo sapiens]	316	42%	8	gi|1230575
20.1	4.59	14474	NACP/alpha-synuclein [Homo sapiens]	589	50%	20	gi|1230575
	4.74	14476	synuclein, alpha [Mus musculus]	513	48%	11	gi|6678047
20.2	4.59	14474	NACP/alpha-synuclein [Homo sapiens]	644	50%	28	gi|1230575
	4.74	14476	synuclein, alpha [Mus musculus]	542	47%	23	gi|6678047
20.3	4.59	14474	NACP/alpha-synuclein [Homo sapiens]	677	51%	36	gi|1230575
	4.74	14476	synuclein, alpha [Mus musculus]	632	62%	24	gi|6678047
20.4	4.59	14474	NACP/alpha-synuclein [Homo sapiens]	620	50%	27	gi|1230575
	4.74	14476	synuclein, alpha [Mus musculus]	572	60%	20	gi|6678047
17.1	4.74	14476	synuclein, alpha [Mus musculus]	390	40%	12	gi|6678047
	4.59	14474	NACP/alpha-synuclein [Homo sapiens]	374	41%	14	gi|1230575

1 MDVFMKGLSK **AKEGVVAAAE K**TKQGVAEAA GKTK**EGVLYV GSK**TKEGVVH
51 GVATVAEKTK EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA ATGFVKKDQL
101 GKNEEGAPQE GILEDMPVDP DNEAYEMPSE EGYQDYEPEA

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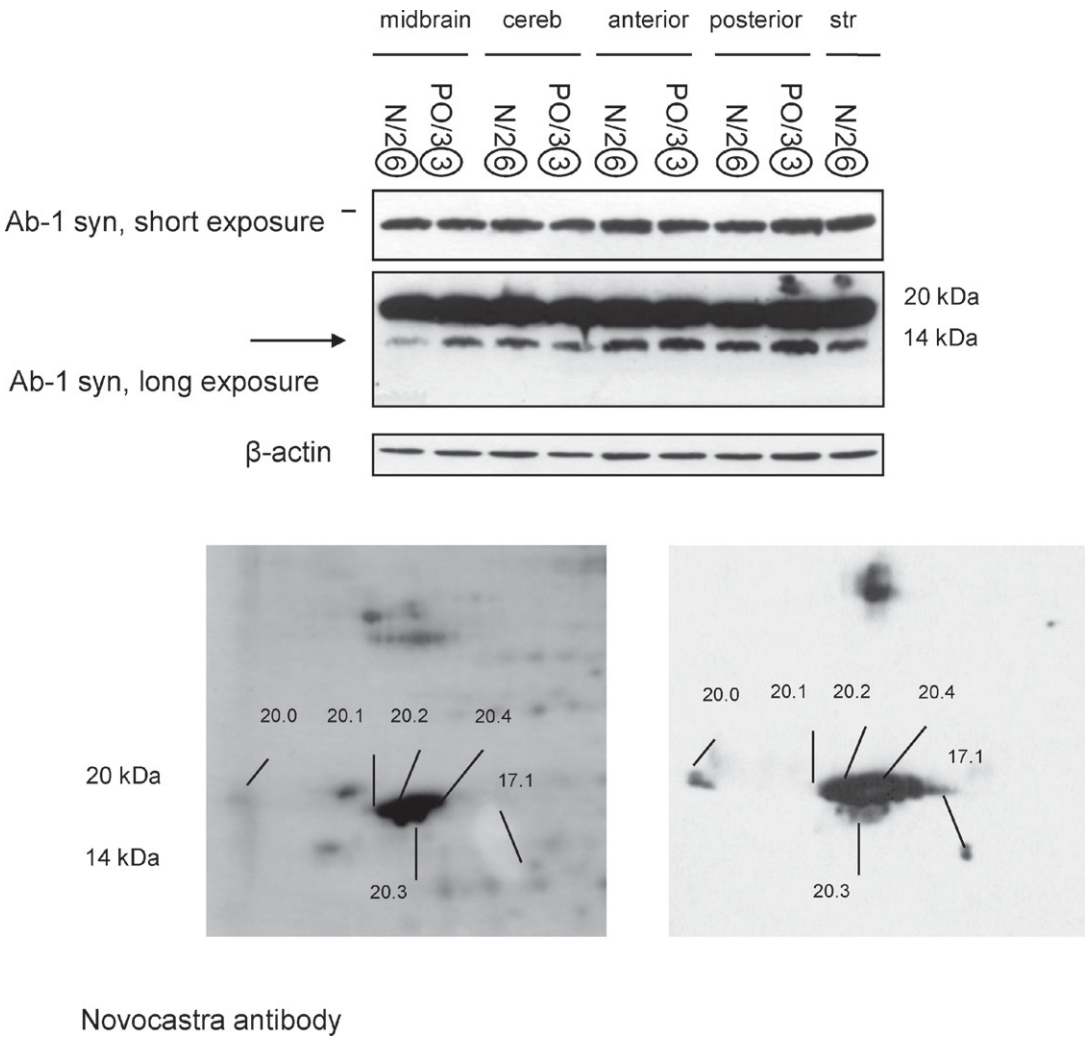

Fig. 2. Truncated forms of human α-synuclein in transgenic mice. Brain homogenates of the midbrain, cerebellum (cereb), anterior and posterior cerebral cortex, and striatum (str) blotted with the Ab-1 syn antibody, which recognizes only human α-synuclein, show similar levels of human α-synuclein (about 17 kDa) in the different regions, in accordance with immunohistochemical findings. Long exposures reveal a lower band of about 14 kDa, the expression of which is more pronounced in the cerebral cortex than in midbrain and cerebellum. Bi-dimensional gel electrophoresis shows different spots which are labelled with anti-α-synuclein antibodies in blotted membranes processed in parallel. Spots were in-gel dissected and analyzed with mass spectrometry. Identification of the spots is shown in Table 2.

Neomarkers antibodies which were raised against different α-synuclein epitopes, thus supporting the α-synuclein nature of these aggregates (Fig. 3).

3.5. Histological characterization

Coronal sections stained with anti-human-α-synuclein antibodies revealed diffuse synaptic-like human α-synuclein staining in transgenic mice, whereas negative immunostaining occurred in age-matched littermates (Fig. 4A). Microscopic examination revealed large axons in the medulla oblongata and pons filled with human α-synuclein but intracytoplasmic accumulations in the form of Lewy bodies or similar inclusions were absent (Fig. 4B). This was further examined with anti-ubiquitin antibodies; no inclusions reminiscent of those characteristics of Lewy pathology were seen in transgenic mice. Axons in transgenic mice and corresponding fibres in age-matched wild littermates were positive with antibodies to phosphorylated neurofilament epitopes. No evidence of axonal swellings was seen in transgenic mice.

Besides these differences, no morphological abnormalities were observed in transgenic mice at different ages up to 24 months. No astrogliosis or microgliosis was found in transgenic mice when compared with age-matched littermates (Fig. 4C).

3.6. Fatty acid composition in the brain of mice following long-term diets depleted and enriched in fatty acids

Fatty acid profiles of brain cortex of transgenic mice aged 12 months under different diets are shown in Table 3. Lower *n*-3 content in diet led to significantly decreased content of linolenic (18:3*n*-3), *n*-3 docosapentaenoic (22:5*n*-3) and DHA (22:6*n*-3) fatty acids, as expected. This depletion in long chain fatty acids was compensated for by a 10-fold increase in the content of docosapentaenoic acid of the *n*-6 series (22:5*n*-6). Nevertheless, total content of PUFA was significantly diminished, mainly due to *n*-3 depletion, leading to significantly lower double bond and peroxidizability indexes.

The addition of DHA to the depleted *n*-3 diet increased brain DHA levels even to a higher content than in control. This manipulation did not prevent decreased levels of linolenic (18:3*n*-3) and docosapentaenoic (22:5*n*-3) acids, as would be expected when adding the final compound of a metabolic pathway. DHA addition also normalized docosapentaenoic acid of the *n*-6

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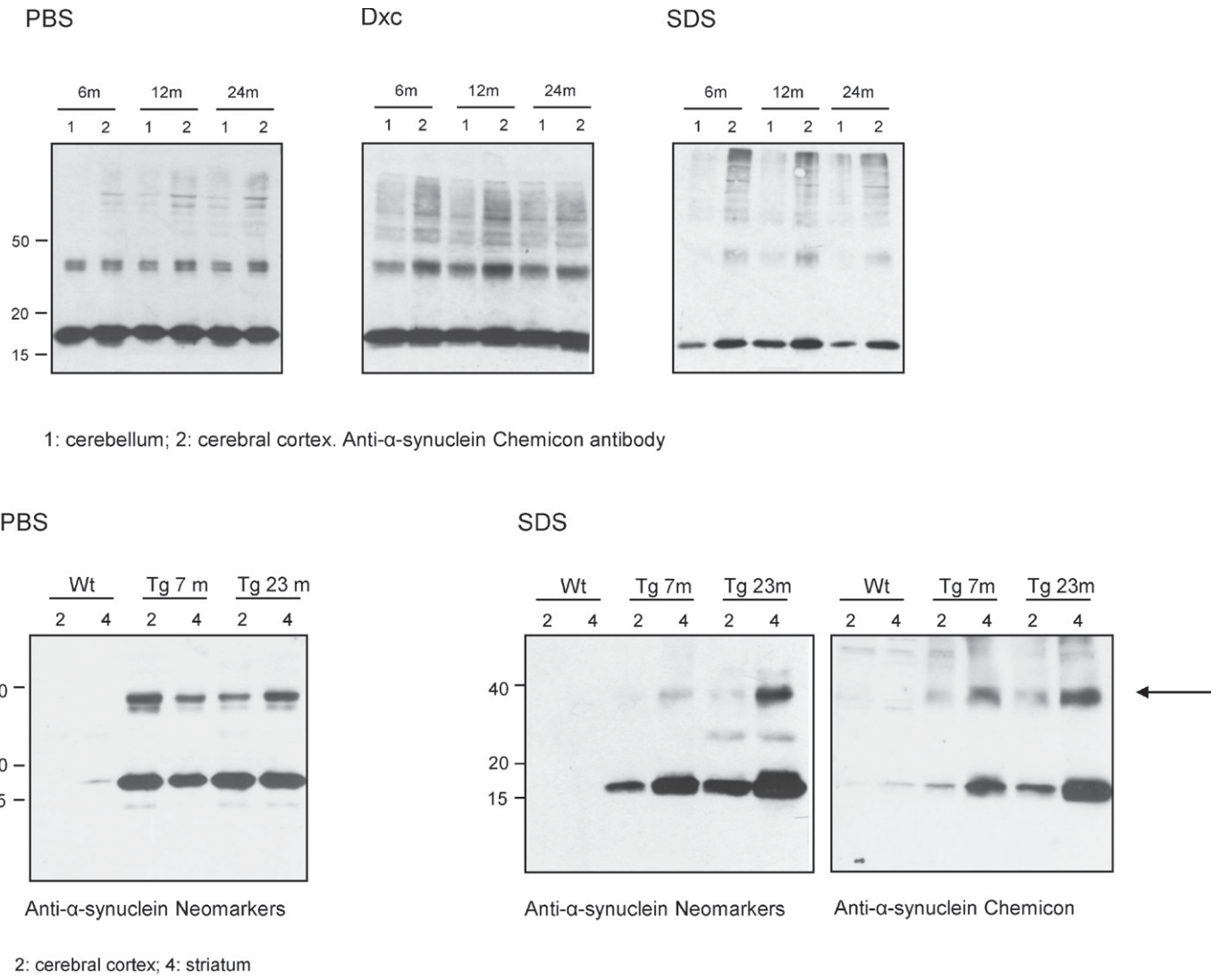

Fig. 3. (A) Solubility and aggregation of α-synuclein in the cerebellum (1) and cerebral cortex (2) of transgenic mice aged 6, 12 and 24 months. A band of about 17 kDa is observed in PBS, deoxycholate (Dxc) and SDS fractions. Bands of higher molecular weight are visible in PBS, Dxc and SDS fractions corresponding to α-synuclein aggregates. Membranes were blotted with the anti-α-synuclein Chemicon antibody. B. Comparison between wild type 20 months old (Wt) and transgenic mice aged 7 and 23 months in the cerebral cortex (2) and striatum (4) as revealed with Neomarkers and Chemicon antibodies. No aggegated α-synuclein is observed in in the PBS and SDS fractions in Wt but a band of about 17 kDa corresponding to non-aggregated α-synuclein. However, a band of about 40 kDa (arrow) is detected in Tg mice aged 7 and 23 months in the PBS and SDS fractions. Similar bands are seen by using two different anti-α-synuclein antibodies raised against different epitopes, thus supporting the α-synuclein nature of this band and, therefore, the formation of synuclein aggregates only in Tg mice. The band of 25 kDa with the Neomarkers antibody (raised in mouse) corresponds to the molecular weight of immunoglobulins and has been inerpreted as non-specific.

series (22:5*n*-6) to control levels. The decrease in the contents of adrenic acid (22:4*n*-6) in the DHA supplemented animals could be an adaptive response to increased DHA levels, in order to try to maintain *n*-6/*n*-3 ratios and acyl chain length. This is also reinforced by diminished percentages of other *n*-6 fatty acids, such as arachidonic acid or other fatty acids. Thus, globally, DHA addition led to increases in the content of PUFA *n*-3 that were offset by significant reductions in PUFA *n*-6 content. This led to normalization in the double bond and peroxidizability indexes.

3.7. Oxidative markers in the brains of mice following long-term diets

Mass spectrometric measurements of different pathways of protein oxidation revealed that *n*-3 depletion (even with DHA addition) had profound effects in oxidative damage. Thus levels of the oxidation markers glutamic and aminoadipic semialdehydes were significantly diminished (ca. 25–30%), and the levels of mixed glyco- and lipoxidation marker CEL (ca. 25–30%) were also diminished. The levels of lipoxidation markers CML and MDAL were more affected, leading to significant decreases, in the 40–60% range, with reference to control diets (Table 4).

3.8. α-Synuclein in the brains of mice following long-term diets

Despite changes in fatty acid composition, Western blot analyses revealed no significant differences in α-synuclein expression, neither after dietary treatments depleted in *n*-3 or after DHA addition. This refers to total expression levels of α-synuclein, human α-synuclein and to the presence of truncated forms. Similarly, no modifications in α-synuclein solubility and aggregation were found in the three groups of mice treated with control diets, diets impoverished in *n*-3, and diets impoverished in *n*-3 but supplemented with DHA (data not shown).

Histological examination revealed no significant differences in the numbers of astrocytes and microglial cells in animals subjected to diets impoverished in *n*-3 and to diets low in *n*-3 and

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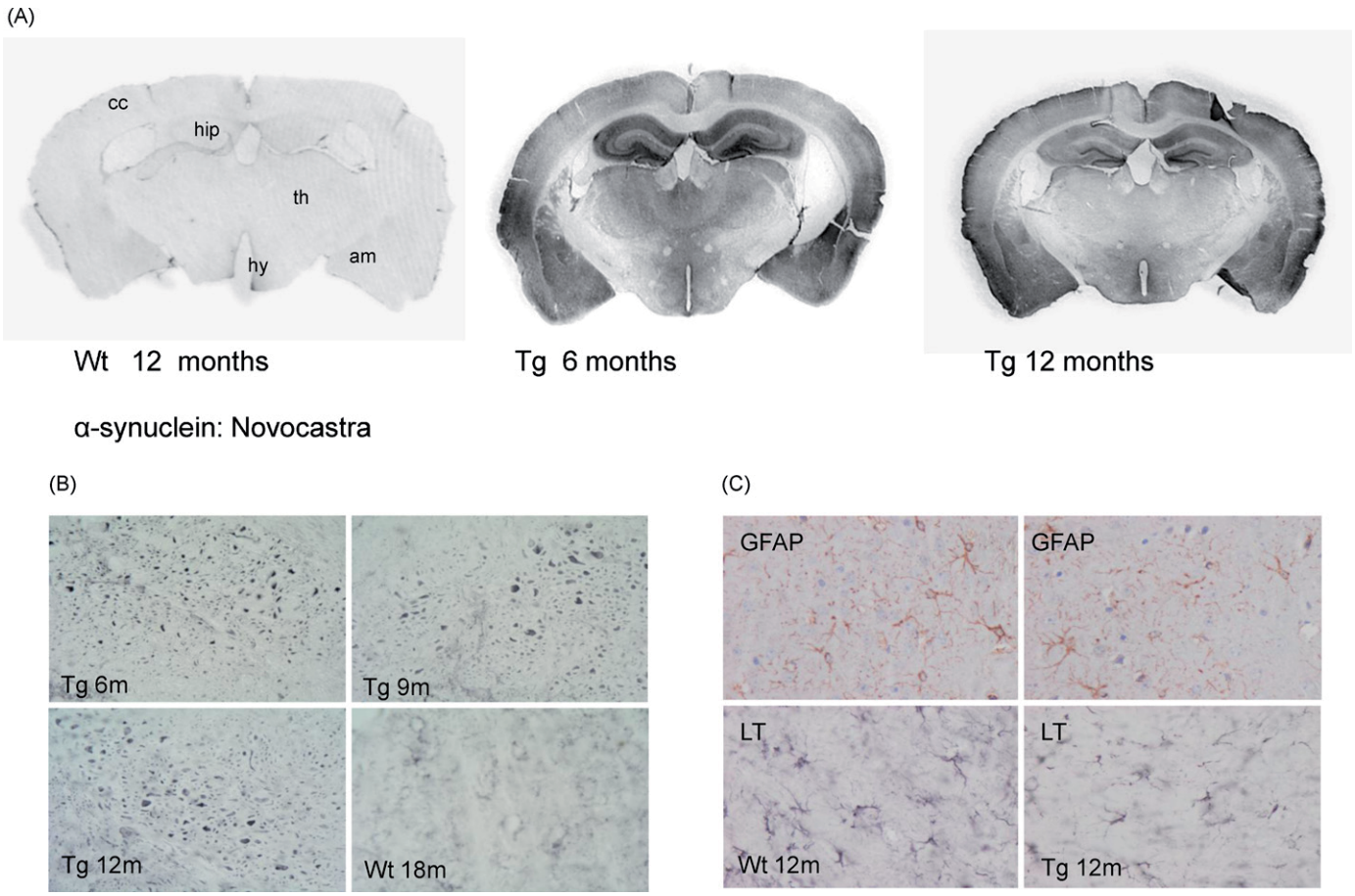

Fig. 4. (A) Distribution and localization of abnormal α-synuclein in transgenic mice (Tg) when compared with wild type (Wt) of the same age. Human α-synuclein, as revealed with the Novocastra antibody, is widely expressed in the cerebral cortex (cc), hippocampus (hip), thalamus (th), amygdala (am) and hypothalamus (hy) in transgenic mice. (B) Large axons containing α-synuclein (Novocastra) in the brain stem in Tg mice at different post-natal ages but not in wild littermates. (C) Astrocytes (GFAP) and microglial cells (LT) in transgenic mice (Tg) when compared with age-matched littermates (Wt) aged 12 months.

supplemented with DHA when compared with age-matched mice fed with control diets (Table 5).

4. Discussion

Mutant α-synuclein mRNA and protein are expressed in the present transgenic mice as revealed by RT-PCR and with antibodies recognizing only human α-synuclein. This is accompanied by several modifications of α-synuclein, including abnormal α-synuclein solubility and aggregation as revealed in sub-fractionation studies, and increased α-synuclein truncation with bands of low molecular weight. Similar increased truncation of α-synuclein has been noted in the same transgenic mice as well as in human PD cases bearing α-synuclein mutations (Lee et al., 2002; Li et al., 2005). Previous studies in A53T α-synuclein transgenic mice showed accumulation of α-synuclein in intracytoplasmatic Lewy body-like inclusions, with LB-like morphology, widely distributed in the brain in animals ranging from 8 to 16 months (Giasson et al., 2002; Lee et al., 2002). However, no similar inclusions were observed in the present study in spite of formic acid treatment of sections to enhance α-synuclein immunoreactivity. Together, the present findings are closer to those reported in more recent studies showing a less severe phenotype in the same transgenic line (Gispert et al., 2003). In any case, this mild phenotype is useful as α-synuclein abnormalities are similar to those encountered in PD (Baba et al., 1998; Hashimoto and Masliah, 1999; Iwatsubo, 2003), and, therefore, these mice are prone to develop α-synucleinopathy.

However, certain aspects must be considered. First, aggregated α-synuclein mediates neurotoxicity under particular circumstances *in vivo* (Periquet et al., 2007) but certain thresholds have to be reached in order to manifest universal toxic effects. Second, in spite of the deleterious consequences of truncated α-synuclein (Michell et al., 2007; Wakamatsu et al., 2008), no evidence of cell damage was seen in the present transgenic mice when compared with controls. Together, these observations point that α-synuclein aggregation and α-synuclein truncation are not sufficient to cause Lewy body formation, at least in the presence of appropriate concomitant amounts of non-altered α-synuclein.

Neuroprotective effects of DHA have been suggested in several models (Barceló-Coblijn et al., 2003; Favrelière et al., 2003). However, other studies have not provided evidence of such beneficial effect but rather dietary overdoses of DHA producing high levels of protein modifications resulting from enhanced lipid peroxidation (Liu et al., 2007, 2008; Long et al., 2008; Tanito et al., 2008), with a pathogenic potential. Moreover, diets enriched in DHA at an early age may be harmful to the development of the nervous system (Haubner et al., 2002, 2007).

Due to the role of α-synuclein in PUFA metabolism, we assumed that α-synuclein transgenic mice were more sensitive to *n*-3 depletion, and, therefore, dietary manipulation could be better tested by using this paradigm. The results of dietary manipulation of fatty acid profiles in mouse brain (lower linolenic and compensatory increases in docosapentaenoic acids) revealed its effectiveness. As expected, DHA addition fully reversed DHA

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Table 3
Effect of dietary treatment on brain fatty acid profile (mol%).

	Control	↓*n*-3	↓*n*-3 + DHA	*p* value (Control vs. ↓*n*-3)	*p* value (Control vs. ↓*n*-3 + DHA)	*p* value (↓*n*-3 vs. ↓*n*-3 + DHA)
14:0	0.30 ± 0.07	0.34 ± 0.04	0.31 ± 0.07	n.s.	n.s.	n.s.
16:0	22.96 ± 0.82	26.23 ± 0.88	25.21 ± 0.63	0.012	0.067	n.s.
16:1*n*-7	0.43 ± 0.02	0.49 ± 0.02	0.61 ± 0.05	n.s.	0.006	0.039
18:0	21.90 ± 0.48	22.34 ± 0.36	21.96 ± 0.26	n.s.	n.s.	n.s.
18:1*n*-9	21.36 ± 0.27	21.60 ± 0.33	21.57 ± 0.32	n.s.	n.s.	n.s.
18:2*n*-6	0.64 ± 0.02	0.87 ± 0.06	0.82 ± 0.02	0.003	0.014	n.s.
18:3*n*-3	1.77 ± 0.63	0.02 ± 0.008	0.03 ± 0.01	0.005	0.006	n.s.
18:4*n*-6	0.54 ± 0.11	0.29 ± 0.05	0.32 ± 0.04	0.034	0.065	n.s.
20:0	0.40 ± 0.08	0.25 ± 0.01	0.24 ± 0.03	0.067	0.052	n.s.
20:1*n*-9	1.47 ± 0.06	1.65 ± 0.12	1.43 ± 0.14	n.s.	n.s.	n.s.
20:2*n*-6	0.86 ± 0.28	0.18 ± 0.01	0.25 ± 0.09	0.018	0.032	n.s.
20:3*n*-6	0.12 ± 0.04	0.04 ± 0.02	0.04 ± 0.02	n.s.	n.s.	n.s.
20:4*n*-6	9.46 ± 0.46	9.18 ± 0.14	8.72 ± 0.27	n.s.	n.s.	n.s.
20:5*n*-3	0.05 ± 0.008	0.03 ± 0.004	0.14 ± 0.02	n.s.	0.001	0.001
22:0	0.56 ± 0.18	0.22 ± 0.05	0.25 ± 0.03	0.057	n.s.	n.s.
22:4*n*-6	2.68 ± 0.25	2.33 ± 0.11	1.50 ± 0.13	n.s.	0.001	0.006
22:5*n*-6	0.18 ± 0.01	1.85 ± 0.19	0.07 ± 0.02	0.001	n.s.	0.001
22:5*n*-3	0.31 ± 0.04	0.18 ± 0.02	0.14 ± 0.01	0.014	0.003	n.s.
22:6*n*-3	13.33 ± 0.32	11.24 ± 0.15	15.70 ± 0.32	0.001	0.001	0.001
24:0	0.07 ± 0.019	0.04 ± 0.008	0.03 ± 0.007	n.s.	0.054	n.s.
24:5*n*-3	0.04 ± 0.01	0.01 ± 0.003	0.04 ± 0.01	n.s.	n.s.	n.s.
24:6*n*-3	0.44 ± 0.03	0.52 ± 0.05	0.50 ± 0.06	n.s.	n.s.	n.s.
ACL	18.48 ± 0.03	18.34 ± 0.04	18.43 ± 0.03	0.017	n.s.	n.s.
SFA	46.22 ± 0.60	49.45 ± 0.62	48.02 ± 0.59	0.003	0.058	n.s.
UFA	53.77 ± 0.60	50.54 ± 0.62	51.97 ± 0.59	0.003	0.058	n.s.
MUFA	23.28 ± 0.28	23.74 ± 0.44	23.63 ± 0.42	n.s.	n.s.	n.s.
PUFA	30.49 ± 0.64	26.80 ± 0.59	28.34 ± 0.43	0.001	0.020	0.079
PUFA*n*-6	14.51 ± 0.46	14.75 ± 0.41	11.75 ± 0.43	n.s.	0.001	0.001
PUFA*n*-3	15.98 ± 0.41	12.04 ± 0.20	16.58 ± 0.35	0.001	n.s.	0.001
DBI	168.52 ± 1.55	154.40 ± 2.72	167.59 ± 2.35	0.001	n.s.	0.001
PI	170.59 ± 2.30	155.75 ± 3.29	176.23 ± 2.83	0.003	n.s.	0.001

Values: mean ± SEM; $n = 5 \times$ group. n.s: not significant. For other abbreviations, see Section 2.

Table 4
Effect of dietary treatment on brain oxidative protein damage.

	Control	↓*n*-3	↓*n*-3 + DHA	*p* value (control vs. ↓*n*-3)	*p* value (control vs. ↓*n*-3 + DHA)	*p* value (↓*n*-3 vs. ↓*n*-3 + DHA)
GSA	10894.46 ± 1622.04	7796.64 ± 736.76	7677.78 ± 743.41	0.05	0.05	n.s.
AASA	185.76 ± 18.21	141.80 ± 18.74	120.38 ± 27.52	n.s.	0.05	n.s.
CEL	240.93 ± 14.19	171.45 ± 15.84	166.41 ± 5.29	0.004	0.004	n.s.
CML	494.59 ± 110.46	169.77 ± 14.13	241.07 ± 21.05	0.003	0.02	n.s.
MDAL	2168.83 ± 219.57	961.50 ± 204.87	1549.96 ± 342.61	0.007	0.13	n.s.

Values: mean ± SEM; n.s: not significant Units: μmol/mol lysine; $n = 5 \times$ group.

depletion in *n*-3 restricted diet. Those changes strongly influenced the peroxidizability potential of lipids in the brains of treated mice. Accordingly, levels of oxidative and glycoxidative (as evidenced by diminished GSA and CEL concentrations) and, especially, lipoxidative protein damage (as noted by lowered CML and MDAL concentrations), were diminished after *n*-3 depletion, a situation maintained even after the addition of the highly peroxidizable DHA. Thus, *n*-3 restriction would have a double-edged sword effect: on the one hand it would diminish lipid peroxidizability, but on the other it would also diminish the levels of neuroprotective DHA.

Table 5
Numbers of astrocytes and microglial cells in the parietal cortex (Pars 1 + Pars 2) in mice fed with impoverished *n*-3 diets (*n*-3) or with impoverished *n*-3 diets supplement with docosahexaenoic acid (DHA) compared with controls. Values represent number of cells per field ± SD as revealed with glial fibrillary acidic protein (GFAP) immunohistochemistry and *Lycopericum sculentum* lectin histochemistry.

	Controls	↓*n*-3	↓*n*-3 + HDA
Astrocytes	6.90 ± 0.02	6.90 ± 0.80	7.04 ± 0.42
Microglia	8.72 ± 1.02	8.60 ± 0.82	8.58 ± 1.14

As a potential limitation of the present study, it may be argued that the methods used for fatty acid analyses did not allow us to account for changes in the content of the ether-bound fatty acids in the sn-1 position of plasmalogens. However, accounting that their usual composition are 16:0, 18:0 and 18:1 chains, their potential change by dietary status would not induce marked changes in the peroxidizability or double bond indexes. Furthermore, it is known that dietary changes in *n*-3 content affect preferentially amounts of diacyl phosphatidylethanolamine molecular species containing DHA without changing those present in plasmalogens (Kitajka et al., in press).

The lack of an observable effect in α-synuclein from those diets may be explained by the incomplete loss of DHA levels: even though the diet was almost depleted of its precursor, fatty acid neuronal homeostasis managed to maintain DHA levels at only 20% below the control values. Globally, these data support the concept that the brain is extremely efficient in maintaining DHA levels in adverse conditions. The low levels of 20:5*n*-3 and, perhaps more relevantly, 18:3*n*-3 present in this work should be a result of the essential *n*-3 desaturase-elongase activities in brain or other sources, such as liver. Thus, brain DHA levels are maintained even

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at expenses of other organs. As previously stated by pioneering work of Holman and cols in the early 60s (Mohrhauer and Holman, 1963). Thus, it may be suggested that a stronger or longer dietary stress is needed to change α-synuclein expression in a pathological way in this experimental model. Alternatively, the present observations suggest that wild mouse α-synuclein has a protective effect when expressed together with the human α-synuclein mutation (Cabin et al., 2005).

On the other hand, polyunsaturated acids are regulators of inflammation and, therefore, potential therapeutic or preventing agents in inflammatory-mediated disorders (Fetterman and Zdanowicz, 2009; Bouwens et al., 2009; Calder, 2009; Riediger et al., 2009). For example, reduction in dietary omega-6 polyunsaturated fatty acids minimizes atherosclerotic lesions and inflammatory responses in appropriate environments (Wang et al., 2009). Regarding the nervous system, DHA attenuates microglial activation and delays retinal degeneration in retinoschisin-deficient mice (Ebert et al., 2009) whereas peroxidation of DHA is a potent chemoattractant for microglial migration in the retina (Saraswathy et al., 2006). APP/PS1 transgenic mice fed with diets supplemented with DHA show reduced β-amyloid plaque burden and decreased microglial activation (Oksman et al., 2006).

Yet no modifications in the number of astrocytes and microglia have been found in the cerebral cortex in A53T transgenic mice subjected to diets impoverished in *n*-3 fatty acids and in mice with diets low in *n*-3 and enriched in DHA when compared with mice with controls. Negative results of DHA supplementation may be related with the *n*-3 impoverished background or with the particular characteristics of these transgenic mice.

Together, the present observations in a relatively mild model of α-synucleinopathy show that diets impoverished in *n*-3 modify the expression of brain lipids with lower indexes of peroxidizability. Such metabolic changes do not appear to interfere with phenotype in animal models with a genetic susceptibility to develop α-synucleinopathy, and are not accompanied by significant modifications in the number of astrocytes and microglial cells in A53T transgenic mice.

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4. El truncament i la fosforilació de l'α-sinucleïna són fenòmens comuns en el cervell.

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Neurobiology of Aging (en revisió)

L'α-sinucleïna és una proteïna clau en les malalties amb cossos de Lewy (LBDs) i el component més important dels cossos de Lewy i altres inclusions anòmales en el citoplasma i les neurites. Les diferències regionals en l'expressió de l'α-sinucleïna s'han associat amb la mort neuronal selectiva que es dóna en les LBDs, juntament amb la seva fosforilació (en la serina 129) i el seu truncament. En aquest estudi es demostra una disminució consistent dels nivells d'α-sinucleïna en la substància negra i el nucli de Meynert del cervell humà, comparat amb altres regions independentment de l'edat i la patologia. A més, l'α-sinucleïna fosforilada es troba incrementada de forma natural en aquestes regions, que correlaciona de forma inversa amb l'expressió de l'α-sinucleïna sense modificar.

També s'han identificat varies formes truncades de l'α-sinucleïna de manera natural tant en cervells afectats per una α-sinucleïnopatia, com en els que no, i la seva expressió correlaciona amb els nivells d'α-sinucleïna total. Una de les bandes truncades resulta d'un truncament depenent de l'activitat del lisosoma i la seva formació es pot reduir inhibint-ne la seva funcionalitat. En aquest estudi, s'exposa que tot i que les variants fosforilades i les truncades formen part dels cossos de Lewy, aquests esdeveniments es poden considerar constitutius tant en cervells control com en els afectats.

Title: α-synuclein phosphorylation and truncation are normal events in the adult human brain.

Abbreviated title: Total, phosphorylated and truncated α-synuclein levels in human brain.

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Abstract

α-synuclein is a key protein in Lewy body diseases (LBDs) and a major component of Lewy bodies and related aberrant cytoplasmic and neuritic inclusions. Regional differences in α-synuclein levels have been associated with selective neuronal vulnerability to Lewy pathology. Furthermore, phosphorylation at serine 129 (Ser129), and α-synuclein truncation have been considered crucial in the pathogenesis of Lewy inclusions. The present study shows consistent reduction in α-synuclein protein expression levels in the human substantia nigra and nucleus basalis of Meynert compared with other brain regions independently of age and pathology. Phosphorylated α-synuclein at Ser129 is naturally increased in the same regions thus inversely correlating with the total amount of α-synuclein. In contrast different truncated α-synuclein forms are naturally observed in control and diseased brains and correlating with the total amount of α-synuclein. Some of these truncated α-synuclein results from a lysosomal-dependent cleavage. Although accumulation of truncated α-synuclein variants and phosphorylated α-synuclein occurs in Lewy bodies, α-synuclein phosphorylation and truncation can be considered constitutive in control and diseased brains.

Keywords: α-synuclein; Lewy body diseases; truncated α-synuclein; phosphorylated α-synuclein; substantia nigra; Parkinson's disease.

1. Introduction:

α-synuclein is an abundant neuronal protein with natively unfolded conformation, and it is enriched in synaptic terminals (Murphy et al., 2000). α-synucleinopathies include Parkinson's Disease (PD), Dementia with Lewy Bodies (DLB) and Multisystemic atrophy (MSA). All of these disorders share a common pathological feature, namely aggregation of α-synuclein leading to the formation of intracellular inclusions in a selective population of neurons and glial cells.

Point mutations in α-synuclein gene (A30P, E46K and A53T) and duplication or triplications of the gene are associated with familial autosomal dominant PD and DLB (Polymeropoulos et al., 1997; Kruger et al., 1998; Singleton et al., 2003; Zarranz et al., 2004).

Whether the amount of α-synuclein plays a role in the development of PD and DLB has been a matter of controversy. In the rat brain, dopamine-producing areas have the lowest protein levels while areas that do not degenerate in PD express the highest levels of α-synuclein (Wersinger et al., 2004). Other studies in rats have shown lower levels in the cerebellum, a region not vulnerable to PD (Adamczyk et al., 2005). Studies in normal human brain are puzzling since it is not clear whether the substantia nigra and striatum had the lowest (Rockenstein et al., 2001) or the highest levels (Solano et al., 2000) of α-synuclein. Regarding PD, comparative studies of control and diseased brains are not illuminating. α-synuclein mRNA levels in the PD substantia nigra have been reported to be non-modified, increased, or decreased when compared with age-matched controls (Rockenstein et al., 2001; Wirdefeldt et al., 2001; Kingsbury et al., 2004; Chiba-Falek et al., 2006). Finally, α-synuclein expression levels in the substantia nigra have been reported to decrease with age (Adamczyk et al., 2005).

α-synuclein can be posttranslationaly modified by phosphorylation, oxidation, nitration and truncation, thus facilitating misfolding and triggering oligomerization and fibrillation (Hashimoto et al., 1999; Uversky et al., 2005; Anderson et al., 2006). Cleaved (truncated) fragments of α-synuclein are prone to aggregate *in vitro* and have been considered prime components in Lewy bodies (LBs) (Murray et al., 2003). Over-expression of truncated fragments in cellular models also involves the aggregation of the full-length α-synuclein (Li et al., 2005), suggesting that C-truncated forms promote the aggregation of α-synuclein.

The origin of truncated α-synuclein is not clearly understood, several proteases have been used to cleave α-synuclein *in vitro* such as neurosin (Kasai et al., 2008), calpain I (Mishizen-Eberz et al.,

2003) and metalloproteinases (Sung et al., 2005; Levin et al., 2009). It has been suggested that truncated species can originate from the incomplete degradation of α-synuclein by the proteasome (Liu et al., 2005) and lysosomes, with cathepsin D being the main lysosomal enzyme degrading its C-terminal extreme (Sevlever et al., 2008).

The present study was designed aimed to examine a) the regional expression levels of full-length, phosphorylated and truncated α-synuclein in human control and diseased brains; b) the nature of the cleaved products of α-synuclein generated in brain; and c) the mechanism by which these fragments are generated.

2. Materials and methods

2.1. Human brain samples: The brains of thirteen patients were obtained at autopsy following informed consent of the patients or their relatives, and the approval of the local ethics committee. Eight different areas were dissected, frozen on dry ice and stored at -80ºC until use (see *Supporting Methods)*.

2.2. Mice: A53T α-synuclein transgenic mice express both murine α-synuclein and human α-synuclein with the pathogenic A53T mutation (Giasson et al., 2002). Mice were purchased from Jackson Labs (ref. B6;C3-Tg(Prnp-SNCA*A53T)83Vle/J). (see *Supporting Methods)*.

2.3. Cell culture: cDNA of human wild-type, A53T, and A30P α-synuclein were sub-cloned into pcDNA 3.1 vector (courtesy of Dr. Wolozin, BU, Boston MA) and transfected in SH-SY5Y cells. Stable cell lines were obtained after selection with geneticin. Cells were maintained in DMEM medium containing 10% fetal bovine serum, and supplemented with geneticin (400μg/mL) at 37°C in 5% CO_2 atmosphere. Cells were treated with 20 mM ammonium chloride and 100 μM leupeptine for 20 hours to inhibit lysosomal activity and with 10 mM lactacystin for 20 hours to inhibit proteasome activity.

2.4. Isolation of sub-cellular fractions: Sub-cellular fractions were isolated from tissue and cellular culture by differential centrifugation and flotation in metrizamide gradients as described (Cuervo et al., 1997, Kaushik et al., 2006). (See *Supporting Methods).*

2.5. Limited proteolysis: For limited proteolysis, recombinant α-synuclein (Calbiochem) was incubated at different times with matrix lysosomal extracts from wild-type mouse or human samples at different times. (See *Supporting Methods).*

2.6. Double and triple labelling immunofluorescense and confocal microscopy: Double and triple labelling immunofluorescense was performed with tissue section as described before (Terni et al. 2007) with antibodies AB9 mouse monoclonal anti-NAC (non-amyloid-component) domain (5C2, ATGen, Montevideo, Uruguay), AB2 Rabbit polyclonal anti-N-terminal domain (Abcam, Cambridge, MA, USA), and AB8 Guinea-pig polyclonal anti-C-terminal domain (Calbiochem-Merck, Darmstadt, Germany). Other sections were incubated with anti-α-synuclein (AB3, Zymed, mouse) and anti-phosphorylated α-synuclein Ser129 (AB5p, Epitomics, rabbit).

3. Results

3.1. α-synuclein levels in human samples:

Total levels of α-synuclein were studied by western blotting in eight regions of thirteen human cases using four different antibodies directed against different α-synuclein amino acid sequences: AB1, AB2, AB3 and AB4 (Table II and supplementary Fig. 1). Fig. 1A shows representative western blots of controls, DLB cases and AD cases and quantification of α-synuclein expression in each region, as an average of densitometric values obtained with all the antibodies. α-synuclein was present in all regions and in all brains, but with consistent regional differences in the expression levels. Higher expression levels were found in frontal cortex, temporal cortex, caudate nucleus, amygdala and putamen, whereas lower levels of α-synuclein were always seen in the substantia nigra and nucleus basalis of Meynert. α-synuclein levels in the cerebellum were, on average, similar to those seen in the striatum.

The differences between the expression levels in the substantia nigra and nucleus basalis of Meynert in comparison with the other brain regions were significant, one-way ANOVA analysis revealed a significant effect of brain region in the levels of α-synuclein ($F_{(7,364)}$=82,98, $p<0.001$). It is important to remark that these variations in these regions are not associated to changes in protein solubility or a possible α-synuclein aggregation event because western blots of RIPA-insoluble fraction (P1) collected after RIPA solubilization and centrifugation revealed the same pattern as the soluble fraction (S1) (Fig. 1B).

Additional western blots were carried out running the same region from the 13 different cases in parallel in order to learn about differences related with pathology and differences related with age. A significant progressive reduction in the expression levels of α-synuclein with age was demonstrated in the frontal cortex and substantia nigra (Fig. 1C and 1D), whereas these modifications lacked

significance, in spite of the clear trend, in the nucleus basalis of Meynert, due to individual variations (Fig. 1D). Yet no differences in α-synuclein expression were found with age in the temporal cortex, caudate, putamen, amygdala or cerebellum (data not shown). Reproducibility of these regional variations in the series of cases is shown in Fig. 1E. Remarkably, no significant differences were found in each brain region according to pathology (Fig 1D, lower graphs).

3.2. Phosphorylated α-synuclein:

Specific antibody against phosphorylated α-synuclein at Ser129 (AB5p in Table II) showed the presence of phosphorylated α-synuclein in the substantia nigra and nucleus basalis of Meynert, whereas low or absent expression was found in other regions (Fig. 2A). This pattern was highly reproducible in the different cases (Fig. 2B). The band of the phosphorylated protein showed an apparent slight increase in the molecular weight of about 2 kDa when compared with the unmodified protein, probably because of the structural modifications originated by the phosphorylation and the SDS-PAGE conditions used. Due to this particular shift observed in the phosphorylated protein when using antibodies detecting total α-synuclein (AB1 and AB2), we were able to detect both the unmodified α-synuclein and the phosphorylatedα-synuclein, especially enriched in MN and SN regions, after over-exposure of the film (Fig. 2A). One-way ANOVA analysis revealed a significant effect of these two brain regions in the levels of phosphorylated α-synuclein ($F_{(7,95)}$=11,59, p<0.01), but once more, no significant differences were observed according to the pathology (Fig. 2B) . The mean values and the standard errors based on Bonferroni's multiple comparison procedure are shown in Fig. 2C.
Bi-dimensional gel electrophoresis and western blotting of parallel membranes disclosed a slight modification to the acid pH of the spot corresponding to the phosphorylated α-synuclein (antibody AB5p) when compared with the spot detected with the antibody AB1 directed to non-phosphorylated α-synuclein (Fig. 2D). Such a shift is the expected according to the phospho-sites predictor modifications of pI (Obenauer et al., 2003) (Table IIIA). The specificity of the phosphorylated antibody was further supported by the lack of recognition of recombinant unmodified α-synuclein and by the lack of signal in the putamen sample when compared with substantia nigra. This was in contrast when blotting with the AB1 antibody which recognized recombinant α-synuclein and unmodified α-synuclein in the substantia nigra and putamen (Fig. 2E). The further study the specificity of the AB5p antibody, de-phosphorylation assays were carried out (Fig. 2F). Thus, we observed a marked reduction of phospho-α-synuclein

immunoreactivity after incubation of a representative MN sample with λ-phosphatase while the signal of non-phosphorylated α-synuclein was not modified. As a positive control, the anti-phosphorylated p38 antibody was used to check the effectiveness of λ-phosphatase activity (Fig. 2F).

Double-labelling immunofluorescence and confocal microscopy with AB3 and AB5p antibodies of tissue sections of the substantia nigra pars compacta from DLB disease patients further confirmed the accumulation of phosphorylated α-synuclein in Lewy bodies, as described previously in other studies (Fujiwara et al., 2002; Saito et al., 2003; Anderson et al., 2006) (Supplementary Fig. 2).

3.3. Truncated α-synuclein in total homogenate:

One α-synuclein immunoreactive band corresponding to a molecular weight of about 15 kDa was observed in western blots of total homogenates after longer exposure of the film in control and diseased cases (Fig. 3A). In order to identify a possible site of truncation, antibodies directed against different amino acid sequences of α-synuclein were employed in a wide range covering all the brain regions of the study. As shown in Fig. 3B, antibodies directed against the amino terminus (AB2) and middle region of α-synuclein (AB1) revealed the lower band, whereas antibodies directed to the C-terminus (AB3, directed against amino acids 115-122; and AB8, directed against amino acids 123-140) were negative, suggesting that the cleavage of α-synuclein occurred at the C-terminus. The AB4 (111-131) antibody revealed the same pattern of truncated band in these human samples, suggesting that the truncation must have occurred in the final region of this C-end epitope. Interestingly, truncated α-synuclein was detected in all the regions examined although with variable intensity of the band depending on the total amount of the protein (Fig. 3B and 3C). Thus, the substantia nigra and the nucleus basalis of Meynert had lower values when compared with the other brain regions (Fig. 3C). Also important was the observation that truncated α-synuclein was not disease-dependent (Fig. 3D), control and pathological cases had truncated α-synuclein, and the levels of truncated α-synuclein were consistent with the total levels of the protein.

3.4. Truncated α-synuclein is not the result of post-mortem delay:

In order to learn whether truncated α-synuclein could be an artefact of tissue processing due to post-mortem delay, the same tissue samples were obtained following a progressive artificial post-mortem delay (Ferrer et al., 2007). Samples from the eight regions were immediately frozen after brain removal

(2 h after death, time 0) or stored at room temperature (20º C) for 3 h, 6 h, 9 h, 12 h and 24 h and frozen at - 80º C until use. The level of the full-length protein was maintained during the first 12 hours (Supplementary Fig. 3A). Expression levels of truncated α-synuclein were also maintained until 12 h of post-mortem delay, declining after this time (Supplementary Fig. 2 B). Since the samples used in the present study had post-mortem delays from death to tissue processing between 2 h 30 min and 6 h 10 min, the present results strongly indicate that truncated α-synuclein is not the result of post-mortem delay.

3.5. Truncated α-synuclein is not the 112 α-synuclein isoform:

α-synuclein alternative splicing has been proposed by different groups suggesting three putative mRNA variants: α-synuclein 112 (syn112), syn126 and syn98 (Ueda et al., 1994; Beyer et al., 2008). An antibody raised against the predicted spliced isoform syn112 (lacking exon 3) was generated to check whether truncated α-synuclein observed in human brain homogenates could indeed be variant syn112.

Antibody syn112 can recognize recombinant α-synuclein lacking exon 3 (Supplementary Fig. 4 B, lane 4) but not full-length α-synuclein (data not shown). In addition, the antibody can recognize other bands in total homogenate from mice and human brain samples (Supplementary Fig. 4A). To determine if the band around 14 kDa corresponds to the isoform 112, we performed a competition assay. Pre-incubation of the antibodies with the antigenic peptide abolished the reactive band on western blots of the recombinant protein but not the bands in human samples, thus supporting the non-specific nature of these bands (Supplementary Fig. 4B). These findings show that the putative α-synuclein isoform 112 (syn 112) is apparently not detected by the present method in the adult human brain cortex and other regions. Furthermore, AB1 antibody is not able to recognize syn112 recombinant protein since its epitope is targeting in part exon 5 (missing in the syn112 variant) and in contrast is able to recognize the 15k Da truncated bands from human brains (Fig 3A) conformimng that the 15k Da product corresponds to a truncated variant.

3.6. Tissue fractionation:

In order to study the origin of the truncated α-synuclein, we performed tissue fractionation from human tissue to isolate the different sub-cellular compartments (homogenate, postnuclear pellet, cytosol and

lysosome-enriched fractions). Full-length α-synuclein was recovered in all sub-cellular fractions and although is particularly enriched in the cytosolic fractions, it was also present in the lysosomal-enriched fraction and the post-nuclear pellet fraction (Fig. 3E). As expected, a band of about 15 kDa was recognized in the lysosomal fraction by using the antibodies AB1, AB2, AB4 and AB9 (Fig. 3E, black arrow). In addition, the AB2 antibody, directed against the N-terminal region, identified bands of truncated α-synuclein of about 10-12 kDa in the cytosolic fraction (white arrow). The same band at 12 KDa, but not the lower (10 KDa band), was detected using the AB9 antibody, suggesting that this truncation may occur within the AB9 epitope. Interestingly, antibodies directed against the C-terminal like AB8, AB7 and AB4 among others, disclosed the presence of a band of about 16 kDa mainly in the cytosolic fraction corresponding to an N-terminal truncation (gray arrow).

These results are consistent with previous findings indicating the presence of truncated α-synuclein at the C-terminal domain, but adding the observation of a possible α-synuclein truncation very close to the N-terminal domain. In order to verify the presence of such fragments of α-synuclein lacking the N-terminal domain, cytosolic fractions from human brain were processed with IEF mini-rotofor to isolate proteins according to their pI. Theoretically, α-synuclein pI increases when the protein is truncated at the C-terminal domain due to the large number of acidic amino acids present in this domain, while the pI slightly decreases when the digestion occurs at the N-terminal domain (Table III B). IEF mini-rotofor-isolated fractions were blotted against the AB8 and AB2 antibody (supplemental Fig. 5A). Western blots with AB8 demonstrated the presence of a faint band of α-synuclein of about 16 kDa in fractions corresponding to an acidic pI while when using AB2 antibody, bands with lower molecular weight were detected in high pI fractions. To confirm the α-synuclein nature of these bands, first fractions 2 and 3 from IEF Mini-rotofor were processed for bi-dimensional gel electrophoresis, transferred to membranes and blotted with the AB8 antibody (supplemental Fig. 5B, left). Three spots were clearly observed; the largest presumably corresponding to the full-length α-synuclein, whereas the spot with a molecular weight of 16 kDa was identified as the fragmented α-synuclein at the N-terminal. In addition, mass spectrometry confirmed α-synuclein in one of the spots on the acidic pole of the gel (Match to: gil4507109, sequence coverage: 50%, Score: 339), thus supporting additional α-synuclein truncation at the N-terminal. Fractions 6 to 9 from the IEF Mini-rotofor were also processed for bi-dimensional gel electrophoresis, transferred to membranes and blotted with the AB2 antibody, confirming, as

described previously, the presence of low molecular weight bands (between 15 and 10 approximately) corresponding to truncated C-terminal α-synuclein forms (supplemental Fig. 5B, right).

Additionally, tissue sections of the substantia nigra pars compacta from patients with Parkinson disease were processed for triple immunofluorescence and confocal microscopy for the purpose of examining the presence of different truncated species in the pathological inclusions of Lewy body disease cases (Fig 3F). Antibodies used were directed to the middle region (AB9), N-terminal (AB2) and C-terminal (AB8). The three antibodies largely stained punctuate α-synuclein deposits and Lewy bodies and neuritis. Although the three antibodies recognized α-synuclein deposits, co-localization of all three antibodies is found in a percentage of them (white in the merge construction) whereas some of them are mainly composed of C-terminal or N-terminal fragments indicating that different α-synuclein fragments are separately deposited in punctuate inclusions and also at early stages of deposition in vulnerable neurons (Fig. 3F, upper panels). Although most Lewy bodies were immunostained with all three antibodies, many small peripheral inclusions were differentially recognized by one of them, for example with antibodies directed to the C-terminus but not with the others (Fig. 3F, middle panels). Interesting, N-terminal-truncated fragments are mainly encountered in the halo but not in the center of certain Lewy bodies where C-terminal truncated fragments are enriched (Fig. 3F, lower panels). Together, these observations indicate that punctuate α-synuclein inclusions and Lewy bodies are not homogeneous but rather constitute variable amounts of (probably) full-length and fragmented α-synuclein residues.

3.7. Limited proteolysis and inhibition of protein degradation pathways :

Since α-synuclein degradation has been related to autophagy (Webb et al., 2003; Cuervo et al., 2004; Martinez-Vicente et al., 2008), we next studied the possible implication of the lysosomal system in the formation of truncated α-synuclein. We subjected recombinant α-synuclein to limited proteolysis using lysosomal proteases from isolated murine liver and brain lysosomes. While isolated lysosomes from liver were very effective at degrading recombinant α-synuclein *in vitro*, brain lysosomes were less effective at degrading the same amount of protein (Fig. 4A). Since degradation with liver lysosomes was very fast, we reduced the time of incubation with lysosomes within a range from 30 sec to 10 min. As seen in Fig. 4B, there were at least two different truncated bands, one at about 16 kDa and the other at around 15 kDa. The initial input condition already presented a band of 16 kDa that was

recognized with all the antibodies except the AB2, thus suggesting a spontaneous cleavage occurring at the N-terminal domain of α-synuclein (Fig. 4B).

Yet the rapid appearance of a new band ranging 15 kDa (not present at initial conditions) detected with AB1 and, to a lesser extent, with AB2 antibodies indicating that this cleavage lacks the C-terminal domain occurred when incubating recombinant α-synuclein with liver lysosomes. Since this band was not detected with the antibodies that recognize the C-terminal domain as AB3, AB7 and AB8 (Fig. 4B), these findings further support the truncation of α-synuclein at the C-terminal.

To gain understanding about the nature of the proteolytic cleavage of 15 kDa truncated α-synuclein, we tested proteolysis in combination with different inhibitors. Aprotinin (which inhibits numerous serine-proteases), pepstatin A (which inhibits reversibly aspartyl proteases like cathepsin D, one of the main lysosomal proteases) and EGTA/EDTA (which inhibits protease activity at low salt concentrations) efficiently inhibited the formation of the C-terminal-truncated band of 15 kDa, while PMSF (which irreversibly inhibits serine-proteases) and leupeptin (which inhibits serine and cysteine-proteases) were unable to stop the truncation process at the concentrations and conditions used (Fig. 4C). Increasing the pH of the reaction to pH10 blocked the cleavage (data not shown), suggesting that the proteolytic cleavage was done by a pH-dependent protease.

To further study the origin of the truncated forms, stable SHSY-5Y cell lines expressing human α-synuclein were used to analyze the role of cellular proteolytic systems (the lysosomal and the proteasome systems) in the generation of α-synuclein truncated species. Sub-fractionation of cell lysates revealed the presence of a band of 15 kDa (in addition to full-length α-synuclein) in the mitochondrial-lysosomal (ML) fraction (Fig. 4D). Treatment with leupeptin and NH_4Cl for 20h, a combination which effectively blocks all types of lysosomal degradation (autophagy) as it reduces the activity of all lysosomal proteases (Salvador et al., 2000), resulted in a complete disappearance of the truncated band in the ML fraction. This further supports the idea that the production of the 15 kDa truncated band is carried out by the lysosome (Fig. 4D). Since leupeptin *per se* was not able to inhibit this truncation *in vitro* (see Fig. 4C), it can be assumed that the intralysosomal acidic pH (increased by addition of NH_4Cl) is necessary for α-synuclein truncation. Confirming the role of the lysosome in the truncation of α-synuclein at the C-terminal, cells treated with lactacystin (ubiquitin proteasome inhibitor) for 20 hours did not show any modification in the band of 15 kDa, suggesting that the ubiquitin-proteasomal system was not involved in this process (Fig. 4E).

4. Discussion:

The present results have shown that α-synuclein protein levels are lower in the substantia nigra and nucleus basalis of Meynert, which are more susceptible than other more resistant regions to Lewy body diseases. This is in agreement with previous α-synuclein mRNA studies (Rockenstein et al., 2001), but differs from other α-synuclein protein reports (Neystat et al., 1999; Kingsbury et al., 2004; Chiba-Falek et al., 2006). Such differences may be explained by different sampling in human studies, as proteins are variably vulnerable to post-mortem delay and tissue processing (Ferrer et al., 2007). Considering strict sampling conditions and the use of several antibodies directed against different α-synuclein epitopes, we have seen that the regional pattern of α-synuclein distribution is essentially maintained in control and diseased brains with aging, however we observed a tendency to decrease α-synuclein levels even though is only significant in frontal cortex and substantia nigra, as some other authors recently pointed (Mak et al., 2009).

Vulnerable regions also have the distinctive trait of higher expression levels of phosphorylated α-synuclein at Ser129. This observation, is very reproducible from one case to another in control and diseased brains. Identification of phosphorylated α-synuclein has been shown by using specific antibodies and confirmed by de-phosphorylation assays and by bi-dimensional gel electrophoresis. This is an important point, as phosphorylation of α-synuclein at Ser129 has been described as a characteristic lesion in Lewy body diseases and a hallmark of aberrant protein aggregation and fibril formation (Fujiwara et al., 2002; Saito et al., 2003; Anderson et al., 2006). Our findings show that vulnerable regions bear α-synuclein modifications that may facilitate abnormal protein conformation, although the relationship between this change and neurodegeneration is poorly understood.

Cleavage or truncation of α-synuclein has been proposed as playing a major role in the pathogenesis of Lewy body diseases (Liu et al., 2005). Thus, over-expression of human full-length or C-terminally truncated α-synuclein in animal models recapitulates some aspects of PD-like pathology (Masliah et al., 2000; Tofaris et al., 2006; Wakamatsu et al., 2008). In contrast, generation of fragments near or within the middle region of α-synuclein are unable to form fibrils and prevent the self-aggregation of full-length protein (Mishizen-Eberz et al., 2005). Moreover, several studies have shown truncated α-synuclein only in pathological cases and in correlation with the number of Lewy bodies (Tofaris et al., 2003; Li et al., 2005). The present study does not support the uniqueness of truncated α-synuclein in

pathological conditions since a major truncated band of about 15 kDa is present in total homogenates in all regions examined in control and diseased brains. The present observations, rather, indicate that truncated α-synuclein is common in all brain regions studied and largely dependent on the total amount of α-synuclein, as some authors have suggested before (Campbell et al., 2001). Divergences between the present findings and previous studies could be methodological. Truncated α-synuclein species in pathological brains described in other studies were obtained from insoluble fractions enriched in α-synuclein aggregates, yet this approach is not suitable to the study of α-synuclein in normal brains where α-synuclein is not aggregated. We confirmed that truncated α-synuclein is not the result of post-mortem protein degradation as there is not increased appearance of α-synuclein truncated species with post-mortem delay, but rather a progressive reduction in the expression levels due to protein degradation. Finally, predicted α-synuclein 112 protein isoform (syn 112) is apparently not expressed in human brain, or at least it cannot be detected by currently used methods, confirming that truncated α-synuclein observed is not the syn112 variant. Together these observations show that truncated α-synuclein is a general event present in normal and diseased brains.

More importantly, the present study has also shown that there is no single truncated α-synuclein, as several species are recognized on specific western blots after cellular sub-fractionation. The most common form has a molecular weight of about 15 kDa and is recognized with antibodies raised against the N-terminal but not with antibodies raised against the C-terminal, suggesting that truncation occurs at the C-terminal. Another major band representing α-synuclein has a molecular weight of 16 kDa and is recognized with antibodies directed to the C-terminal but not with antibodies raised against the N-terminal, thus suggesting that this form is truncated at the N-terminal. Finally, bands of lower molecular weight (10-12 kDa), which are recognized mainly with antibodies against the N-terminal, are recovered after cell fractionation in the cytosolic fraction. All these truncated forms are encountered equally in cases with LBD and without Lewy body pathology.

The origin of these bands has been explored by using cell sub-fractionation, limited proteolysis on isolated lysosomes, and by the inhibition of proteolysis using lysosome and ubiquitin-proteasome inhibitors. The main findings include the presence of the 15 kDa truncated α-synuclein in the lysosomal fraction in human brain samples, furthermore the appearance of a band of 15 kDa is observed when incubating recombinant α-synuclein with isolated human and mice lysosomes. We could also observe the abolition of this truncated α-synuclein when treating the preparations in vitro and in vivo with

inhibitors of the lysosomal but not when adding inhibitors of the ubiquitin-proteasome system, pointing to the lysosomal origin of the 15 kDa truncated α-synuclein.

Since truncated α-synuclein of 16 kDa and α-synuclein-immunoreactive bands of 10-12 kDa are mainly present in the cytosolic fraction, it has been hypothesized that proteolysis of α-synuclein may also occur in other sub-cellular compartments and carried out by cytosolic proteases and/or the ubiquitin-proteasome system as suggested before (Mishizen-Eberz et al., 2003; Tofaris et al., 2003; Liu et al., 2005; Sung et al., 2005; Levin et al., 2009).

We observed that α-synuclein phosphorylation and α-synuclein truncation are normal events in the adult human brain. The present findings do not contradict a putative role of phosphorylated α-synuclein Ser129 in Lewy body formation and the enrichment of truncated α-synuclein in abnormal protein aggregates in Lewy body diseases. As previously reported, and further confirmed here, Lewy bodies are enriched in phosphorylated α-synuclein Ser 129. Moreover, Lewy bodies and other aberrant α-synuclein inclusions in Lewy body diseases contain variable amounts of truncated α-synuclein; some inclusions are enriched in C-terminal and others in N-terminal fragments. Although lysosomes play an important role in α-synuclein truncation (Webb et al., 2003; Cuervo et al., 2004; Martinez-Vicente et al., 2008), the role of lysosomal-derived 15 kDa α-synuclein in the genesis of Lewy bodies remains to be elucidated.

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Table I: Human brain samples used.

Sample	Gender	Post-mortem	Age	Neuropathology
1	♀	5h 20	67	ADI
2	♀	5h	64	C
3	♂	4h 20	66	ADI
4	♀	4h	85	LBD4 + ADIV
5	♀	2h 30	82	ADIII
6	♂	2h 45	38	C
7	♂	2h 45	36	C
8	♀	4h 15	59	ADII
9	♂	5h	66	ADII
10	♀	4h 40	70	LBD4 + ADII
11	♂	4h	83	LBD4 + ADII
12	♂	3h	66	C
13	♀	4h 5	88	ADIV + AGD
14	♂	4h 25	76	LBD4
15	♂	6h 10	71	AGD + ADII
16	♂	5h 15	90	AGD
17	♂	13h	84	ADIII+AGD

Table I: Human brain samples used: Samples from 1 to 13 were dissected in 8 different brain regions in order to study α-synuclein levels. All postmortem delays were between 2h 45 minutes and 5h 20 min. Samples 13 to 17 were used for tissue fractionation experiments and rotofor fractionation. AD: Alzheimer disease, I-III Braak stages: I: entorhinal, II: transentorhinal; III: hippocampus; LBD, Lewy body disease, 4: stage 4 of Braak involves substantia nigra and limbic regions; AGD: argyrophilic grain disease; C: control (no neurological symptoms, no neuropathological lesions).

Table II: α-synuclein antibodies

Name	Manufacturer	Clone	Host	Epitope
AB1	Neomarkers	syn204	mouse	87-110
AB2	Abcam	-	rabbit	11-26
AB3	Zymed	LB509	mouse	115-122
AB4	Chemicon	-	rabbit	111-131
AB5p	Epitomics	-	rabbit	Ser129p
AB6	Novocastra	KM51	mouse	-
AB7	Neomarkers	syn211	mouse	121-125
AB8	Calbiochem	-	guinea-pig	123-140
AB9	ATGen	5C2	mouse	61-90

Table II: α-synuclein antibodies: list of antibodies against α-synuclein used in this study.

Table III: Predicted pI of phosphorylated and truncated α-synuclein:

Table IIIA:

Phospho sites	Molecular Weight	Predited pI
0	14451.9339	4.67
1	14529.8979	4.59

Predicted molecular weight and isoelectric point (pI) of unmodified and phosphorylated (1 residue) α-synuclein according to platform Scansite (Obenauer 2003).

Table IIIB:

Starting residue at N-end	Ending residue at C-end	Predited pI
1	140	4.67
1	130	5.01
1	120	5.87
1	110	8.83
1	100	9.40
10	140	4.57
20	140	4.58
30	140	4.45
40	140	4.31

Predicted pI of truncated α-synuclein according to the sequence of the α-synuclein protein using ExPASy Proteomic Server (www.expasy.ch/)

Figure legends:

Figure 1:

α-synuclein quantification in different brain regions. A: α-synuclein quantification of S1 fractions from different brain regions: Cases 11 (LBD 4), 8 (AD II) and 12 (C: control), representative of each group, were blotted against α-synuclein using four different antibodies (AB1, AB2, AB3 and AB4). The bar diagram expression of α-synuclein levesl in each patient as mean values of all four antibodies. FC: frontal cortex, TC: temporal cortex, PT: putamen, MN: nucleus basalis of Meynert, SN: substantia nigra, A: amygdala, CN: caudate nucleus, C: cerebellum. LBD 4: Lewy body disease stage 4 of Braak for PD-related pathology staging; AD II, Alzheimer disease stage II of Braak; CTR: control. Reduced expression levels of synuclein were seen in the nucleus basalis of Meynert and substantia nigra in every case when compared with the other regions. B: α-synuclein quantification in different brain regions of RIPA-insoluble fractions from a representative case 2 blotted against α-synuclein using antibodies AB4. Bar diagram shows the expression of α-synuclein as mean values of four antibodies. C: Representative western blots of three regions: frontal cortex (FC), substantia nigra (SN) and nucleus basalis of Meynert (MN) analyzed with AB1 antibody in cases 1 to 13. D, upper panel: Bar diagram representation of α-synuclein protein levels according to the age of patients (less than 60 years, between 60 and 80 years and more than 80 years old). Significant differences in α-synuclein expression were observed in the substantia nigra and frontal cortex with aging, * ($p \leq 0.05$), ** ($p < 0.01$). D, lower panels: Bar diagram representation of α-synuclein protein levels according to the pathology of cases, control (C), tauopathies (T; including AD and AGD cases), Lewy body disease (LBD). No significant differences in α-synuclein expression were observed in relation with pathology. E: Representation of the expression in all brain regions using all antibodies. Squares correspond to independent values in each region using different antibodies against α-synuclein (AB1, AB2, AB3 and AB4) in all thirteen cases. Mean values are represented as a horizontal line (-).

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Figure 2:

Phosphorylated α-synuclein levels. A: Phosphorylated α-synuclein levels in different brain regions from case 13 (AD IV+AGD) using an antibody that recognizes only phosphorylated α-synuclein Ser129 (syn 129P) (upper panel) in comparison with other antibodies that recognize different epitopes, AB2 (directed against the N-terminal) and AB1 (raised against an epitope near the C-terminal region). B:

Quantification of phosphorylated α-synuclein levels according to the pathology: C (control, n = 4), LBD (Lewy body diseases, n = 3) and T (tauopathies, n = 6). FC: frontal cortex, TC: temporal cortex, PT: putamen, MN: nucleus basalis of Meynert, SN: substantia nigra, A: amygdala, CN: caudate nucleus, C: cerebellum. Anova test demonstrates statistical differences (p-value < 0.01) depending on the region studied (§), but not on the pathology of the case. C: Plot graph representing all the series of cases and showing 99 percent of confidence levels. The intervals currently displayed are based on Bonferroni's multiple comparison procedure. D: Representative bi-dimensional gel of the nucleus basalis of Meynert of a representative case using AB1 and AB5p antibodies. The left arrow indicates the position of phosphorylated α-synuclein and the right arrow the unmodified α-synuclein. E: Recombinant α-synuclein (S, lane 1), substantia nigra (SN, lane 2) and putamen (PT, lane 3) from patient 5 detected with antibodies AB5p and AB1. F: De-phosphorylation assay with λ-phosphatase (PPase) using sample of the nucleus basalis of Meynert as a substrate. Input (i) (lane 1), dephosphorylation assay with (+, lane 2) and without λ-phosphatase (-, lane 3). Dephosphorylation of phospho-p38 was used as a control of phosphatase activity. β-actin was used as a control of protein loading.

Figure 3:

Truncated α-synuclein expression and cell sub-fractionation. A: Truncated α-synunclein was detected with AB1 antibody in the homogenate of different representative cases: Control (C), Lewy Body Disease (LBD), and Alzheimer's disease stage V (AD V). B: Representative case (patient 5) was blotted with antibodies AB1, AB2, AB3, AB4 and AB8 against α-synuclein. Truncated α-synuclein is marked with an arrow. C: Mean values of full-length compared with truncated α-synuclein levels of all patients plotted together. D: truncated α-synuclein levels in 8 different brain regions from 13 patients plotted according to the pathology. There is no significance in any selected region according to the pathology. E: Representative human brain sub-fractionation detected with antibodies AB1, AB2, AB3, AB4, AB7, AB8 and AB9. Fractions were total homogenate (TH), post-nuclear pellet (PNP), cytosolic fraction (C) and lysosomal fraction (Lys). Different truncated bands are indicated by arrows: the gray arrow corresponds to a band of 16 kDa; the black arrow corresponds to a band of 15 kDa; and the white arrow marks bands of molecular weight between 10 and 12 kDa. F: Triple-labelling immunofluorescence and confocal microscopy of tissue sections of the substantia nigra pars compacta

from patients with Parkinson disease using antibodies directed to the NAC region (AB9, green), N-terminal (AB2, red) and C-terminal (AB8, blue). Bar = 25 μm

Figure 4:

Limited proteolysis and proteolysis inhibition in vivo and in vitro: A: Limited proteolysis in vitro of recombinant α-synuclein with murine brain (upper) and liver (lower) isolated lysosomes at 0, 5, 10 and 30 minutes. B: Limited proteolysis of recombinant α-synuclein using mice liver lysosomes at different times and detected with antibodies AB1, AB2, AB3, AB4, AB7 and AB8. Arrow indicates new truncated bands originated by proteolysis. C: Limited proteolysis *in vitro* using mice liver lysosomes and different inhibitors incubated during 30 seconds. Western blots against AB1 and AB2 were represented. Arrow indicates new truncated bands originated by proteolysis. Competent inhibition of the generation of new bands was marked with an asterisk (*). D: Levels of α-synuclein in total homogenate (H) and mitochondrial-lysosomal enriched fraction (ML) from SH-SY5Y cells expressing human WT α-synuclein detected with AB1, AB2, and AB8 antibodies before and after treatment with lysosomal inhibitiors (PI). E: SH-SY5Y cells expressing human WT α-synuclein cells treated with the proteasome inhibitor (PI) lactacystin during 20 hours and detected with AB1 antibody.

1. Supplemental material and methods:

Human brain samples: Areas dissected from patients after autopsy were: frontal cortex (area 8), temporal cortex (anterior superior gyrus), caudate (head), putamen (medial), nucleus basalis of Meynert, amygdala, substantia nigra, and cerebellum (upper vermis). Both genders were equally represented (6 male and 7 female); age range was between 36 and 88 years (mean age 67 years), and the average time between death and tissue processing was 4 h. The main clinical and pathological characteristics of cases are summarized in Table I. The neuropathological study was carried out in 4% buffered formalin-fixed, paraffin embedded randomized brain sections which were processed with well-established histological and immunohistochemical methods. Protocols of tissue selection, preservation and processing, as well as neuropathological diagnoses, were within the context of those proposed by the European Brain Bank Network (Ferrer et al., 2007; Alafuzoff et al., 2008; Ferrer et al., 2008; Alafuzoff et al., 2009).

Mice: A53T α-synuclein transgenic mice were housed three or four per cage in a temperature-controlled room under a 12h light/dark cycle with free access to food and water. Animal experiment protocols were approved by the Animal Care and Ethics Committee of the University of Barcelona, and all efforts were made to minimize the number of animals used and their suffering. Transgenic mice were identified routinely after weaning from tail samples by PCR analysis using the primers: IMR1772: 5'- TGT AGG CTC CAA AAC CAA GG -3', and IMR3560: 5'- TGT CAG GAT CCA CAG GCA TA -3'. Immediately after killing, the brain was removed, dissected and prepared for tissue fractionation at 4ºC.

Mono-dimensional gel electrophoresis and western blotting: 0.2 g of each human brain region was homogenized in a glass homogenizer, in 0.5 ml of RIPA Buffer (5mM Tris-HCl pH 7.4 and 1mM EGTA, 250mM sucrose) with complete protease inhibitor cocktail (Roche Molecular Systems, Almeda, USA), 1mM phenylmethanesulphonylfluoride (PMSF), 1mM sodium ortovanadate and phosphotase inhibitors (2.5mM NaF and 2mM β-gycerophosphate). After centrifugation at 10,000 g, the RIPA-soluble fraction (S1) and the RIPA-insoluble fraction (P1) were kept. The protein of this resulting supernatant was quantified by BCA method.

Aliquots at 1µg/µl were stored at -80º C with reducing Laemmli sample buffer 4x (250 mM Tris-HCl pH6.8, 20% glycerol, 10% SDS, 20% 2-mercaptoethanol, 0.004% bromophenolblue). For western blot studies, 30 µg of each fraction sample was processed for 12% or 14% SDS-PAGE electrophoresis and then transferred to nitrocellulose membranes. Different antibodies against specific epitopes of α-synuclein were used as detailed in Table II. Mouse monoclonal anti-β-actin (Sigma, Madrid, Spain) was used as loading control. All secondary antibodies were from Dako (Dakopats, Barcelona, Spain). The densitometric quantification of western blot bands was carried out with Total Lab v2.01 software (Pharmacia, Sunnyvale, CA, USA). Expression values were normalized using β-actin levels.

Bi-dimensional gel electrophoresis, western blotting and mass spectrometry: samples with the same protein concentration were re-suspended in a final volume of 150 µl with Lysis Buffer (40 mM Tris-base, 9 M Urea, 2 M thiourea, 4% (w/v) CHAPS (Bio-Rad, Hercules, CA, USA) and a mix of protease inhibitors containing 1 mM PMSF, 1 µg/ml pepstatin A, 10 µg/ml leupeptin, 10 µg/ml aprotinin, all from Sigma-Aldrich, St Louis, MO, USA) with 0.8% Bio-Lyte 3/10 Ampholites (Bio-Rad Hercules, CA, USA), 2 mM TBP (tributylphosphine) and 0.0004% bromophenol blue, prepared to re-hydrate the IPG strips.

Immobilized 7cm pH 4-7 or pH 3-10 linear gradient ReadyStrip IPG Strips (Bio-Rad, Hercules, CA, USA) were used to run the first dimension electrophoresis by re-hydrating strips actively for 12 h at 50 V. Subsequently, proteins were focused at 200 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, and 8,000 V for 1 h. Finally, the voltage was kept at 8,000 V till reaching a total of 20 kVh. IPG strips were equilibrated for 2x15 min in equilibration buffer (50 mM Tris-Hcl pH 8.8, 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.0002% bromophenol blue) with 2% dithiothreitol (DTT) and, subsequently, with 2.5 % IodoAcetamide (Bio-Rad, Hercules, CA, USA). Strips were submitted to 12% SDS-PAGE. Gels were stained with Sypro Ruby Protein Gel Stain Kit (Bio-Rad Hercules, CA, USA) or transferred to a nitrocellulose membrane and immunodetected. Selected spots corresponding to those labelled with anti-α-synuclein antibodies were manually excised from the gels stained with Sypro Ruby, and proteins were in-gel digested and analyzed by mass spectrometry, as described before (Muntane et al., 2006).

Generation of antibodies to spliced 112 α-synuclein variant: the generation of the antibody against the putative spliced 112 α-synuclein variant was carried out using the immunization

program developed by Cultek SL (Madrid, Spain). The antigenic peptide was VLYVVAEK which was designed to recognize the spliced α-synuclein variant lacking transcription of exon 3. After three months of the immunization, the rabbits were killed and the serum was tested with the pre-immune serum to confirm the detection of the spliced variant.

De-phosphorylation assay: 300 µg of total homogenate from brain samples was mixed with 1X λ-PPase Reaction Buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EGTA, 2 mM dithiothreitol and 0.01% Brij 35 at pH 7.5), 2 mM $MnCl_2$ and 5 µL of Lambda Protein Phosphatase (all from New England BioLabs, Ipswich, MA, USA). The resultant mixture was incubated for 3h at 30ºC. Reaction was stopped by adding reducing sample buffer and heating the sample to 100ºC. 40 µg of protein was processed for gel electrophoresis and immunodetection.

Isolation of sub-cellular fractions :

Tissue: Fresh frontal cortex (6 g) of four patients was rapidly dissected at autopsy, avoiding freezing. The tissue was homogenized in buffered sucrose (4mM Hepes, 150 mM sucrose, pH 7.4), and lysosomes were isolated from a light mitochondrial-lysosomal fraction in a discontinuous metrizamide density gradient following the procedure described elsewhere (Cuervo et al., 1997). The cytosolic fraction (C) was obtained after 100,000 xg centrifugation from the previous soluble fraction called C+ER which includes cytosol and endoplasmic reticulum. In addition, lysosomes from wild mice liver and brain were obtained and prepared as described for human frontal brain samples.

Cells: A fraction enriched in lysosomes and mitochondria (ML) was isolated, collecting cells washed in sucrose 0.25M (pH 7.2). The pellet was re-suspended in 1.5 mL of 0.25M sucrose and cells were disrupted in a nitrogen cavitation chamber (Kontes Glass Company, NJ, USA); this was followed by homogenization in a Teflon-glass homogenizer and centrifuging (2,500 x g for 15 min; 17,000 x g for 10 min). The cytosolic fraction was obtained after centrifuging at 100,000 x g for 1 h at 4°C of the supernatant from the ML fraction (Kaushik et al., 2006).

Micro-Rotofor fractionation: Micro-Rotofor Cell (Bio-Rad) is a preparative isoelectric focusing apparatus (IEF) to fractionate protein samples according to pI in a liquid-base medium. The pI fractionation was done as described elsewhere (Sugiyama et al., 2006). Briefly, the sample was re-suspended in the IEF buffer (7 M urea, 2 M thiourea, 5 mM DTT, 4% (w/v) CHAPS, and 1% (v/v) Bio-Lyte ampholytes 3/10) and was loaded into the rotofor chamber for initial fractionation

in a pH gradient (pH 1-10), and then electrophoresed according to the instructions of the supplier for 2.5 h. After electrophoresis, protein fractions from each compartment (200 µL) were harvested and pH values determined using a pH indicator paper 1-14 (Merck, Darmstadt, Germany). The protein concentration of each fraction was determined by the Bradford method, and aliquots of equal amounts of proteins were analyzed by western blot analysis.

Limited proteolysis: For limited proteolysis, recombinant α-synuclein (Calbiochem) was incubated at different times with matrix lysosomal extracts from wild-type mouse or human samples at different times. Lysosomal fractions without protease inhibitors from mouse and human liver were subjected to 7 cycles of freeze/thaw in order to break lysosomal membranes; centrifugation at 100,000 x g for 1h at 4°C allowed separation of membranes and lysosomal matrix. In a total volume of 40 µL of reaction, 0.25 µg of recombinant α-synuclein was mixed with 1-2 µg of lysosomal extract in a lysosomal buffer (50 mM MOPS, 2 mM DTT, pH 5.5).

Additionally, we carried out limited proteolysis of α-synuclein using lysosomal extracts from wild-type mouse incubated in the presence of different protease inhibitors such as Aprotinin (100 µg/µl), EGTA plus EDTA (10 mM each), leupeptin (100 µM), pepstatin A (10 µM), PMSF (0.2 mM) and Roche complete inhibitor cocktail (1X).

Double and triple labelling immunofluorescense and confocal microscopy: Cryostat sections, 8-µm-thick, were blocked for 30 min at room temperature with 10% foetal bovine serum diluted in 1 × PBS (Phosphate Buffered Saline) to avoid nonspecific binding. Sections were incubated overnight at 4°C with a combination of three primary anti-α-synuclein antibodies: AB9, AB2, and AB8. Other sections were incubated with anti-α-synuclein AB3 and AB5p. After washing with PBS, the sections were incubated in a cocktail of secondary antibodies in the same vehicle solution for 3 h at room temperature. Secondary antibodies were anti-mouse Alexa 488, anti-rabbit Alexa 546 and anti-guinea-pig Alexa 647 (Molecular Probes, Carlsbad, CA, USA) at a dilution of 1:400. Subsequently, the sections were mounted with Fluorescent Mounting Medium (Dako, Glostrup, Denmark), sealed and dried overnight at 4°C. Sections were examined with a Leica TCS-SL confocal microscope. To rule out non-specific reactions, some sections were incubated only with the secondary antibodies.

2. <u>Supplementary figures legends</u>:

Supplementary figure 1:

α-synuclein atibodies. Graphic representation of the epitopes that recognize antibodies against α-synuclein summarized in table II.

Supplementary Figure 2:

Phosphorylated a-synuclein by confocal microscopy. Double-labeling immunofluorescence and confocal microscopy of tissue sections of the substantia nigra pars compacta from patients with Parkinson disease with antibody AB3 against α-synuclein (green) and AB5p against phosphorylated α-synuclein at Ser129 (red). Co-localization (merge, yellow) is observed in Lewy bodies, intracytoplasmic diffuse α-synuclein deposits, aberrant neurites and the majority of, but not all, threads in the substantia nigra in Lewy body disease. Bar = 40 μm.

Supplementary figure 3:

Artificial post-mortem delay. A: Representative Western Blot of a-synuclein protein levels (full-length and truncated) in Caudate Nucleus from patient 8 detected with AB1 antibody. Truncated band is indicated with an arrow. B: Levels of full-length (upper panel) (n=2) and truncated α-synuclein (lower panel) (n=1) during artificial post-mortem delay in eight different brain regions.

Supplementary figure 4:

α-synuclein isoforms expression. A: Detected bands using the anti-syn112 antibody, lane 1: total homogenate from transgenic mouse overexpressing the A53T cDNA, lane 2: human control brain homogenate, lane 3: human PD brain homogenate. B: Competition assay with peptide VLYVVAEK. Three different human brain samples (lane 1-3) were tested with syn112 antibody using recombinant syn112 protein (lane 4) as positive control. Samples were incubated without (upper panel) or with the peptide (lower panel) before immunodetection.

Supplementary Figure 5:

Isolation and identification of C-terminal and N-terminal truncated forms by IEF micro-rotofor and 2D-SDS. A: Cytosolic fraction from human frontal cortex after IEF micro-rotofor isolation detected with AB8 (left) and AB2 (right) antibodies. Several fractions were obtained artificially, labeled 1 to 9. B, left panel: fractions 2 and 3 from IEF micro-rotofor were collected and subjected to bi-dimensional gel electrophoresis (pH 3-6), membrane transfer and western blotting with the antibody AB8. Several spots are detected: the large one is identified as fullα-synuclein whereas the small spot of about 15 kDa was in gel digested and analyzed by mass spectrometry. The spot was identified as α-synuclein. (http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20091019/FtmtCaSSO.dat&hit=gi|4507109&px=1&ave_thresh=67&_sigthreshold=0.05&_server_mudpit_switch=0.001). B, right panel: fractions 6 to 9 from IEF micro-rotofor were collected and subjected to bi-dimensional gel electrophoresis (pH 7-10), membrane transfer and western blotting with the antibody AB2.

3. Supplemental references:

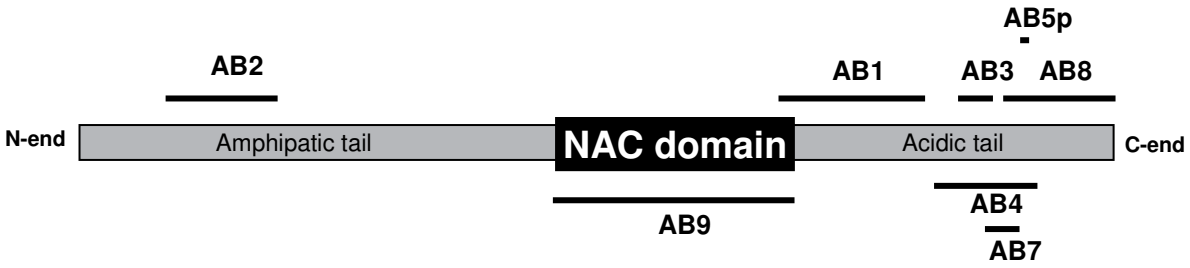
Alafuzoff I, et al. (2008) Assessment of alpha-synuclein pathology: a study of the BrainNet Europe Consortium. J Neuropathol Exp Neurol 67:125-143.

Alafuzoff I, et al. (2009) Staging/typing of Lewy body related alpha-synuclein pathology: a study of the BrainNet Europe Consortium. Acta Neuropathol 117:635-652.

Ferrer I, Martinez A, Boluda S, Parchi P, 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.

Muntane G, Dalfo E, Martinez A, Rey MJ, Avila J, Perez M, Portero M, Pamplona R, Ayala V, 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.

Sugiyama Y, Sueyoshi N, Kameshita I (2006) Two-dimensional expression pattern analysis of protein kinases after separation by MicroRotofor/SDS-PAGE. Anal Biochem 359:271-273.



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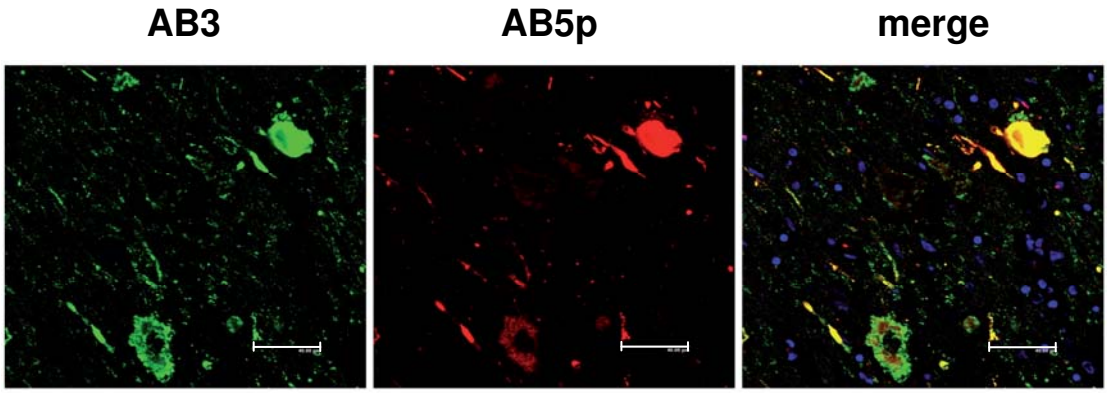
Supplementary Fig. 1
Muntane *et al.*



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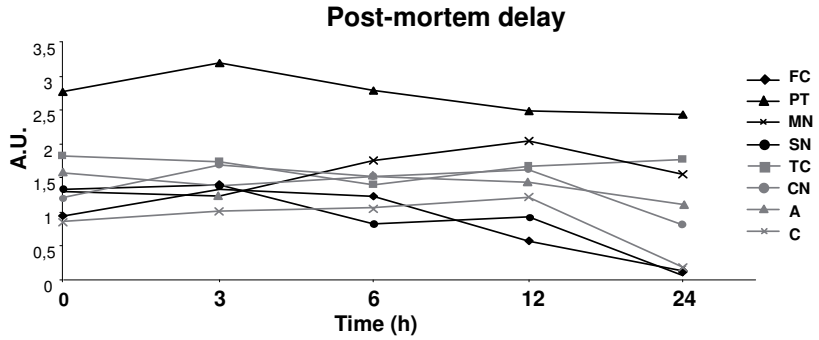
Supplementary Fig. 2
Muntane *et al.*

A



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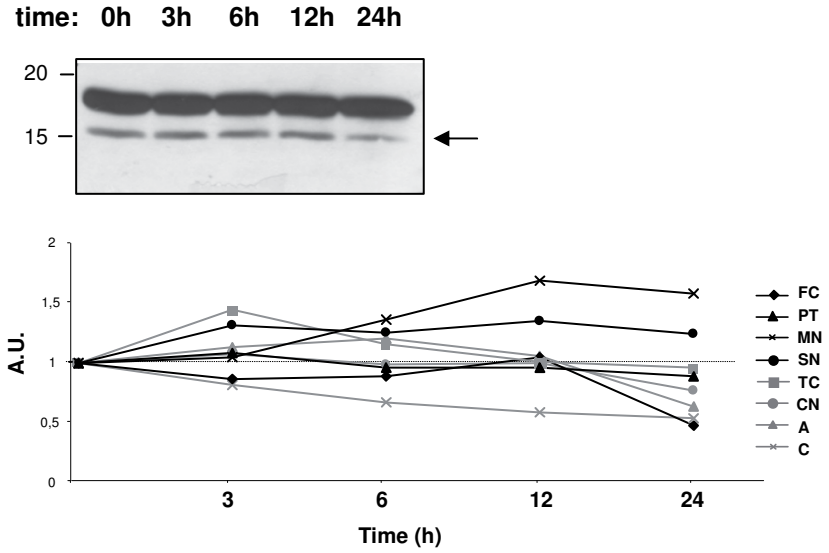

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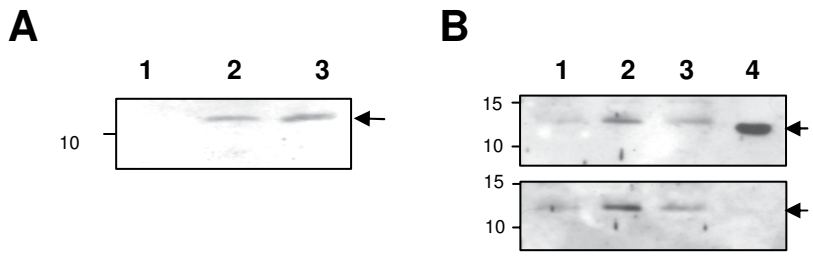
Supplementary Fig. 3
Muntane *et al.*



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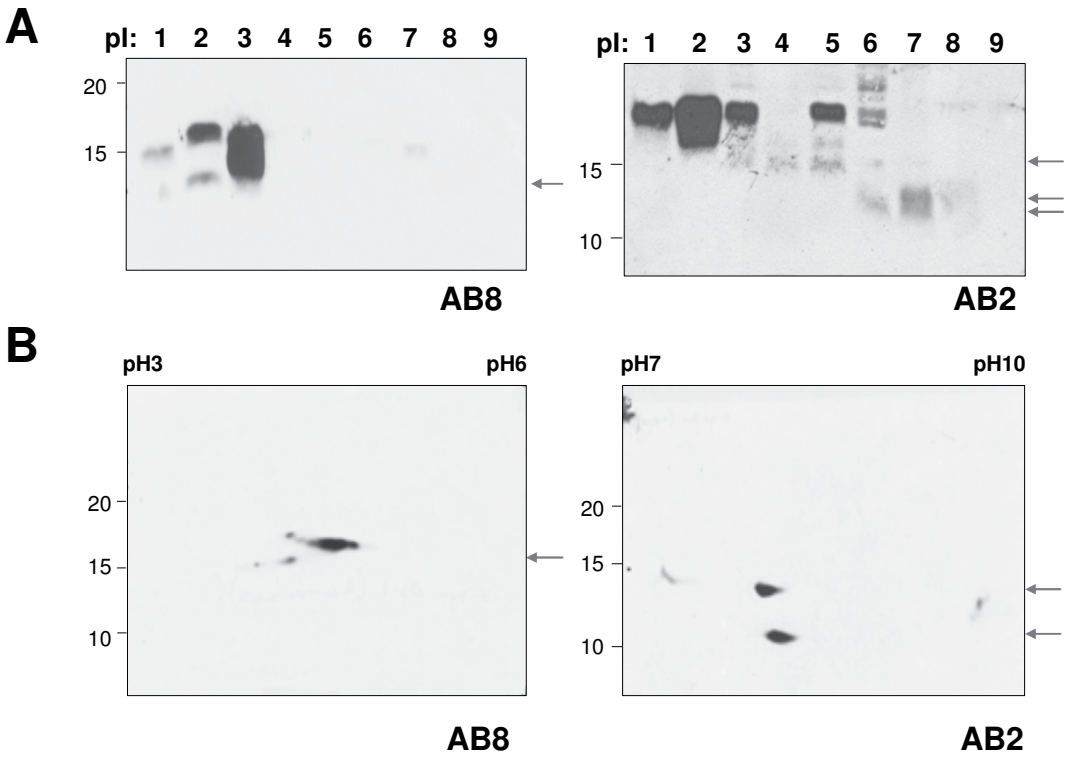
Supplementary Fig. 4
Muntane *et al.*



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Supplementary Fig. 5
Muntane *et al.*

VI. Discussió

En aquesta tesi, un dels temes que vam abordar és la relació existent entre les taupaties i les α-sinucleïnopaties i les característiques que comparteixen. Aquests dos grups de malalties han estat considerats clàssicament com dues entitats clarament diferenciades, on cadascuna manté els seus aspectes clínics i neuropatològics independents. Però, mica en mica, s'han anat suggerint certs solapaments entre els dos grups tant a nivell clínic, com genètic o bioquímic.

A partir dels anys 80 ja es va començar a considerar l'existència de signes extrapiramidals motors en moltes de les taupaties, fins llavors considerades demències [193-195]. També es va descobrir que la prevalença de demència en malalts de Parkinson era sis vegades superior a la observada en individus sense cap malaltia [196]. Evidentment, aquest solapament es fa patent en la malaltia amb cossos de Lewy (DLB), on els pacients desenvolupen demència després d'una clínica amb simptomatologia motora.

Posteriorment, aquests trets compartits s'han estès a l'abordatge neuropatològic. S'ha reportat la presència d'estructures clàssicament definitòries d'una taupatia o una α-sinucleïnopatia coexistint en un mateix pacient amb una probabilitat més alta de l'esperada. Així, s'han descrit LB reactius per α-sinucleïna en l'AD [186, 197], en PSP [198] o en les demències frontotemporals [199]. Per altra banda, també s'ha trobat l'existència de NFT amb marcatge de tau en PD [200] i en DLB on aquest solapament arriba fins al 87% [201].

Tenint en compte tot aquest conjunt de troballes, un dels objectius del nostre treball ha estat estudiar els canvis comuns que es donen entre aquestes malalties des d'un punt de vista bioquímic, fixant-nos sobretot en els canvis post-traduccionals que pateixen tant la tau com l'α-sinucleïna.

1. L'α-sinucleïna en la malaltia de Pick (Dalfó et al. 2006)

Agregació de l'α-sinucleïna en taupaties

Un dels aspectes més importants en el dany causat per l'α-sinucleïna és la formació d'oligòmers. Aquests oligòmers són unes estructures solubles transitòries que es troben a mig camí entre l'estat de l'α-sinucleïna com a monòmer i la formació de les fibres insolubles que conformen els agregats. A més, aquestes estructures oligomèriques cada cop guanyen més pes com les causants de la neurodegeneració i la seva presència és indiscutible en les α-sinucleïnopaties [202].

Partint d'aquesta evidència, nosaltres vam contrastar la presència d'aquestes estructures en una taupatia com la malaltia de Pick (PiD). Tot i que en la malaltia d'Alzheimer hi ha molts treballs que la vinculen a la presència de canvis propis de les α-sinucleïnopaties, en la PiD són molt escassos i sovint contradictoris. Mentre que clàssicament els autors no descriuen cap evidència d'estructures amb α-sinucleïna agregada [20], altres han descrit la presència de dipòsits d'α-sinucleïna en els cossos de Pick característics d'aquesta malaltia [203-204] i en forma de cossos de Lewy en l'amígdala [205]. Així, que vam considerar que era un bon model per intentar estudiar la presència d'estructures oligomèriques d'α-sinucleïna, perquè en alguns casos s'havia trobat formant agregats en algunes zones del cervell, però l'escorça frontal, que era la més afectada per la patologia tipus tau, no havia estat estudiada.

L'aproximació que vam triar per estudiar la presència d'aquests oligòmers va ser mitjançant tècniques bioquímiques com el Western Blot, metodologia que ja s'havia utilitzat en altres treballs que detectaven aquestes estructures en models d'α-sinucleïnopaties [115, 123, 171, 206-207].

Aquest estudi ens va permetre observar per primera vegada la presència d'oligòmers d'α-sinucleïna en l'escorça frontal dels casos amb PiD. Aquest fet suggeria un paper d'aquestes espècies tòxiques en les taupaties, i un mecanisme comú amb les α-sinucleïnopaties on el paper tòxic dels oligòmers n'és un tret característic

Ja que en l'escorça frontal dels casos amb PiD havia estat descrit un increment de l'estrès oxidatiu [208-209], vam pensar que aquest fet, ja fos resultat o causa del procés patològic, també podria jugar un paper en l'oligomerització de l'α-sinucleïna.

La nitració de l'α-sinucleïna en condicions d'estrès oxidatiu és un esdeveniment que s'ha descrit tant en models cel·lulars [130] com en les malalties amb cossos de Lewy (LBD) [132]. Ara bé, no quedava massa clar si aquest canvi era posterior o anterior a

l'agregació perquè el que suggerien els estudis anteriors *in vitro* era que l'α-sinucleïna nitrada era capaç d'oligomeritzar [210], però mai donava pas a agregats. Com que els nostres resultats indiquen l'existència d'agregats visibles per immunohistoquímica, és possible que la nitració, en aquests casos, sigui un esdeveniment posterior a la formació dels agregats. No obstant els nostres resultats sostenen un paper de la nitració en l'agregació de l'α-sinucleïna en les taupaties, en condicions *in vivo*, ja que trobem la presència d'α-sinucleïna nitrada tant en la forma monomèrica, com en oligòmers o en dipòsits compactes insolubles.

La nitració de l'α-sinucleïna, tot i ser un dels canvis post-traduccionals més freqüents en les LBDs [43, 130, 132], encara no havia estat descrit com un dels canvis que pot patir la proteïna en les taupaties. Aquests resultats reforcen la idea de l'existència de mecanismes comuns entre les taupaties i les α-sinucleïnopaties, ja que en una taupatia com la PiD, l'α-sinucleïna també pot tenir un paper important en el desencadenant de la malaltia. Mitjançant la nitració l'α-sinucleïna formaria oligòmers estables, que nosaltres hem detectat en aquestes mostres i resultarien patogènics d'una manera similar a la que podrien estar-ho fent en les LBDs.

No obstant, els nostres resultats no ens permeten concloure si aquests canvis per nitració són previs a la formació dels agregats i els oligòmers, o si per contra, són aquestes estructures un cop formades les que queden modificades per la nitració.

2. L'α-sinucleïna i la tau en les sinapsis (Muntané et al. 2008)

Les sinapsis són un dels processos claus en la viabilitat neuronal i la seva desaparició és un dels aspectes més importants en la neurodegeneració. En les malalties neurodegeneratives, aquest fet es pot corroborar ja que es produeix una pèrdua sinàptica important juntament a la disminució de molts dels marcadors sinàptics [211-215].

Per altra banda, l'α-sinucleïna és una proteïna que es localitza en les sinapsis on es proposa que realitza la seva hipotètica funció. A més, una falta de l'α-sinucleïna produeix una reducció dels marcadors sinàptics i en la dopamina del nucli estriat com s'ha comprovat en diversos models [111, 216].

Tenint en compte aquest fet, nosaltres ens vam centrar en la implicació de les sinapsis en diferents malalties neurodegeneratives com la AD i les LBD. Vam utilitzar l'escorça frontal dels pacients perquè en aquesta regió podríem apreciar l'efecte de l'evolució de la malaltia. Aquestes malalties progressen des d'estructures basals fins a estructures corticals i el Banc de Teixits ens oferia la possibilitat de treballar amb mostres de cada un dels estadis de la malaltia i així valorar quina era l'afectació de les sinapsis en cada un d'ells.

2.1. Modificacions de l'α-sinucleïna

L'α-sinucleïna és una proteïna sinàptica que es troba en estreta relació amb les vesícules sinàptiques en condicions normals, però que en condicions patològiques es troba formant part dels LB en el soma neuronal.

Malgrat els resultats obtinguts *in vitro*, el pas de l'α-sinucleïna monomèrica soluble a agregada encara no queda clar on es dóna en un cervell *in vivo*. Es proposa que els oligòmers solubles serien les estructures que causarien realment el dany a la neurona, però es desconeix quina és la seva localització en la neurona. S'ha proposat un model on l'α-sinucleïna en un procés de fibril·lació podria adoptar estructura anular i en contacte amb membranes lipídiques generar forats en les vesícules sinàptiques que causarien l'alliberament del seu contingut [116-117]. Seguint aquesta hipòtesi, la nostra aproximació consistia en estudiar aquests canvis en les sinapsis.

Els nostres resultats suggerien una pèrdua de l'α-sinucleïna soluble en les fraccions sinàptiques de forma depenent de la patologia. En estadis on l'escorça frontal estava

afectada (AD V-VI i DLB) l'α-sinucleïna es deslocalitzava de les fraccions sinàptiques i en els estadis entremitjos sense afectació cortical (LBD 1-4 i AD I-IV) ja s'hi intuïa una reducció. Podria ser que aquesta desaparició mantingués una relació amb la pèrdua sinàptica que es produeix en aquestes malalties, però era massa dràstica tenint en compte que els marcadors sinàptics es mantenien. Llavors vam pensar que podria ser un canvi post-traduccional com la fosforilació el que provoqués aquesta disminució en la proteïna soluble. Aquesta és una modificació amb gran capacitat patogènica, ja que, com ja s'ha esmentat abans, la fosforilació de l'α-sinucleïna afavoreix l'agregació de la proteïna *in vitro* [44, 127], i aquesta és un constituent important dels LB.

Vam observar que l'α-sinucleïna es fosforilava en les sinapsis i que al seu torn formava oligòmers, de forma que quan disminuïen els nivells d'α-sinucleïna (no modificada) en els processos sinàptics, augmentaven els nivells d'α-sinucleïna fosforilada en la serina129. Com que l'α-sinucleïna fosforilada és més procliu a la fibril·lació i nosaltres detectàvem aquests oligòmers en les fraccions sinàptiques, es reforça la idea que les estructures oligomèriques es podrien formar al voltant de les sinapsis com suggereixen altres estudis publicats [40, 217].

Aquestes observacions proposen la fosforilació de la proteïna com a factor clau en la patogènesi tant de taupaties com α-sinucleïnopaties, evocant mecanismes comuns entre malalties ben diferenciades com són la AD i la PD. A més, nosaltres anem una mica més enllà i proposem les regions sinàptiques com el lloc on convergiria la patogenicitat fruit de l'α-sinucleïna tant en taupaties com en α-sinucleïnopaties, produint-se la fosforilació i l'oligomerització de l'α-sinucleïna, tal i com es suggereix en un altre estudi que utilitza assajos de filtració [40]. Aquestes conclusions es poden fer extensives fins i tot a estadis de la malaltia on encara no s'evidencia afectació cortical, suggerint que la fosforilació i l'oligomerització de l'α-sinucleïna són esdeveniments precoços. Aquest resultat està en consonància amb altres que indiquen canvis primerencs en la relació d'àcids grassos o al dany oxidatiu en àrees del cervell on la neuropatologia encara no s'ha fet evident [133, 218].

2.2. Modificacions de la tau

Ja que era en les regions sinàptiques on es donaven les modificacions de l'α-sinucleïna, vam hipotetitzar un funcionament semblant per a la proteïna tau. La tau i l'α-sinucleïna tenen moltes similituds i comparteixen un gran nombre de semblances i mecanismes comuns [184] i la presència d'una de les dues potencia la fibril·lació de l'altra de forma sinèrgica, i a la inversa [191]. En aquest escenari, el nostre propòsit va

ser estudiar la fosforilació de la tau en el fraccionament de l'escorça frontal tant d'α-sinucleïnopaties com de taupaties en diferents estadis.

La presència de tau fosforilada en posició 396 i no en altres residus en totes les mostres que presentaven una α-sinucleïnopatia (tinguessin o no afectació cortical) ens va fer pensar que aquest era un dels canvis primerencs que podien derivar-se de la potenciació de la patologia tipus-tau per part de l'α-sinucleïna. Aquests resultats corroboraven els resultats anteriors obtinguts en el model murí que expressa l'α-sinucleïna humana mutada A30P. Aquests ratolins, presenten un increment en la fosforilació de tau en els residus 396/404 i en el 202 de forma concomitant a l'agregació de l'α-sinucleïna [172]. En un altre model on s'utilitzen cultius de neuronals que expressen l'α-sinucleïna, també s'ha observat un increment en la fosforilació de tau 396/404 després d'un insult amb MPTP sense canvis en les fosforilacions en altres residus com el 202 o el 262. En canvi, això no es produïa al tractar amb MPTP un cultiu nul per l'α-sinucleïna [219-220]. Aquests resultats, en conjunt, suggereixen la fosforilació en la serina 396 com una de les primeres modificacions de la tau, associada a l'efecte de l'α-sinucleïna.

La fosforilació en la serina 396 que nosaltres observàvem no es trobava acompanyada d'hiperfosforilació de la tau ni de NFT en l'escorça frontal en cap dels casos, excepte en els casos d'AD V-VI. En aquests, també es trobava marcatge amb l'anticòs AT8 (que marca la doble fosforilació als residus 202 i 205) en les fraccions sinàptiques. A més, aquests resultats els vam corroborar en el model murí que sobreexpressa l'α-sinucleïna mutada A53T, on també observàvem la tau fosforilada en 396 en les fraccions enriquides en marcadors sinàptics.

En contradicció amb aquests resultats hi ha els estudis clàssics que estudien el patró de fosforilació de tau i la seva deposició en els agregats. Aquests estudis suggereixen que les primeres fosforilacions que es donarien en la tau serien en els residus 153, 231 i 262, mentre que la fosforilació en la serina 396 es trobaria en un punt molt tardà de la formació dels agregats [221]. Però aquests estudis estan realitzats en escorça temporal i els nostres en escorça frontal, a més en el nostre cas, molts dels casos no presenten NFT a la zona analitzada. En canvi, si que estan en concordança amb estudis que es fixen en els canvis produïts a la tau per part de l'α-sinucleïna, on tots ells presenten la fosforilació en la posició 396 com un pas desencadenat per la presència de l'α-sinucleïna [219-220].

Proposem doncs, que aquest podria ser un canvi post-traduccional de la tau molt primerenc en el desenvolupament de la malaltia i que a més es donaria en els processos sinàptics, suggerint un cop més, que la patologia associada a l'α-sinucleïna podria estimular canvis primerencs en la tau.

2.3. Associació a vesícules

La següent pregunta que ens vam realitzar era si la tau podia trobar-se associada a les vesícules sinàptiques o si tan sols es localitzava en aquelles fraccions però sense interacció directa. Amb aquesta finalitat vam aïllar vesícules sinàptiques del còrtex frontal de cervells afectats d'AD V i vam veure que la tau fosforilada en posició 396 es trobava interactuant amb les vesícules sinàptiques. Així doncs, la transmissió sinàptica podria quedar alterada degut a aquestes interaccions anòmales entre la tau fosforilada i les vesícules sinàptiques.

Hi ha nombrosos treballs que suggereixen la unió de la tau a diferents membranes lipídiques a través de la regió rica en repeticions [222-223], i també es pensa que podria tenir un paper en la funció i la transmissió sinàptica [224-226].

El fet de trobar la tau fosforilada associada a les vesícules sinàptiques no té, ara per ara, una interpretació massa clara, però és interessant observar que l'extrem N-terminal de la proteïna s'ha proposat que podria mitjançar la unió a membranes [227]. Aquest podria ser un altre punt de convergència amb l'α-sinucleïna que al fibril·lar s'associa a les vesícules formant estructures anulars [98], a més, en processos neurodegeneratius la interacció de l'α-sinucleïna amb proteïnes de la membrana de la vesícula sinàptica es veuen alterades [115, 228].

3. A53T com a model d'estudi (Muntané et al. 2009)

3.1. Canvis en l'α-sinucleïna

Com a aproximació a l'estudi de les α-sinucleïnopaties vam triar un model murí que sobreexpressa l'α-sinucleïna humana mutada A53T. Curiosament, en els rosegadors, en la posició 53 de l'α-sinucleïna ja hi ha una treonina enlloc d'una alanina. Aquest model, havia estat descrit com un bon acostament a la patologia, ja que recopilava gran part del fenotip de la malaltia humana [169]. No obstant, en contra del que havia estat descrit fins a la data, el model del que disposàvem no presentava inclusions tot i que tots el ratolins eren portadors del transgen. Aquest fet però, no és tan estrany, ja que s'han descrit altres models amb el mateix constructe que tampoc tenen estructures semblants als LB [229].

Cabin i col., van observar que en un model amb la mateixa construcció que també desenvolupava un fenotip més o menys benigne, l'eliminació de l'expressió de la pròpia α-sinucleïna del ratolí, feia que es transformés en un de molt més sever que podia acabar en paràlisi als 16 mesos d'edat [230]. Així, la curta vida d'una ratolí podria no ser suficient per produir una patologia amb l'α-sinucleïna A53T en un fons genètic amb una α-sinucleïna en ple rendiment (la murina). Aquest conjunció podria estar-se donant en el nostre model que conserva l'expressió de la proteïna del ratolí. De totes formes, tot i ser un fenotip lleu i suau, hem caracteritzat el model per tal d'utilitzar-lo en l'estudi d'altres mecanismes específics de les α-sinucleïnopaties.

En la gran majoria d'àrees del cervell hem comprovat que es troben oligòmers d'α-sinucleïna, tot i ser més presents en l'estriat i menys en el cerebel. A més, sembla que es formen de manera edat independent, essent més abundants als 23 que als 7 mesos i la proteïna que conforma aquests oligòmers és la provinent del transgen humà i no de la murina.

Per una altra banda, també s'ha pogut detectar en el model A53T uns nivells basals d'un producte reconegut amb l'anticòs d'α-sinucleïna que té un pes molecular més baix de l'esperat. Aquesta banda baixa, que ronda els 15 kDa i que ja es fa patent als 3 mesos d'edat, s'assumeix que és un producte del truncament de la proteïna, que a més, ja havia estat descrit tant en mostres humanes, com en models que expressen l'α-sinucleïna [149, 156-157, 170, 231]. Curiosament, aquest producte del truncament de

l'α-sinucleïna no sembla que augmenti amb l'edat, contràriament al que ocorre amb el increment d'oligòmers que si s'observa lligat a l'envelliment.

Així doncs, tots aquests resultats indiquen que en contra de la descripció clàssica del model, en els ratolins que nosaltres utilitzem no es dóna un fenotip gens sever, ni s'observen les clàssiques estructures semblants als LB, que si que s'observen en la descripció del model. Tot i això, aquest ratolí conserva la sobreexpressió de l'α-sinucleïna humana mutada en totes les estructures del cervell. Podem suggerir també que ni la mutació A53T per si sola, ni l'oligomerització ni el truncament de l'α-sinucleïna no són suficients per a desenvolupar inclusions citoplasmàtiques de l'estil dels LB (almenys en un fons genètic amb la proteïna homòloga no alterada), ni per causar un fenotip típic de neurodegeneració. Això dóna suport a la teoria de que per desenvolupar una α-sinucleïnopatia completa s'ha de superar un llindar complex en el que podrien convergir múltiples factors.

Tanmateix, aquest és un model que permet estudiar els primers estadis en la fibril·lació de l'α-sinucleïna així com les àrees del cervell més sensibles. I per altra banda, també pot ser útil per identificar els fragments truncats que es generen en condicions normals i estudiar els mecanismes que intervenen en la proteòlisi de l'α-sinucleïna.

3.2. Efectes del canvi en els lípids de la dieta

Aprofitant que disposàvem d'aquest model, vam estudiar els canvis en l'α-sinucleïna en relació a la ingesta de dietes amb diferent composició lipídica.

Ja hi havia fins aleshores suficients evidències per pensar que l'α-sinucleïna podia influir en el tràfic de lípids en el cervell i regular-ne el metabolisme [232-233], així com regularitzar el percentatge de lípids neutres [234]. A més, també s'ha suggerit que un perfil lipídic ric en àcid docosahexaenoic (DHA), un àcid gras poliinsaturat, pot actuar com a neuroprotector en diferents models [235-237].

Després de l'administració d'aquestes dietes vam comprovar que el perfil lipídic del cervell mantenia una concordança amb la dieta subministrada tot i que els nivells de DHA es mantenien més o menys constants (tan sols un 20% per sota del control) en el cervell tot i ser absent en la dieta. Sembla doncs, que el cervell manté un mecanisme per produir nivells de DHA en absència del seu precursor, fet que ja s'havia suggerit en un treball clàssic [238].

Nosaltres esperàvem una millora en el fenotip del model en quant a la inflamació o a la fibril·lació de l'α-sinucleïna, però no va ser així. Possiblement degut a que ens

trobàvem davant d'un model amb una patologia molt suau, aquest canvi dietari no influïa en el fenotip, tot i que els nivells de dany lipooxidatiu disminuïen després de subministrar les dietes pobres en n-3 (àcids grassos insaturats omega-3). Aquests canvis oxidatius segurament no afectaven a l'α-sinucleïna ni a les demés proteïnes i tan sols involucraven els propis lípids del cervell.

4. Modificacions de l'α-sinucleïna en el cervell (Muntané et al. 2010, en revisió)

L'α-sinucleïna és, per ara, la proteïna que té una implicació més gran en la patogènia de les LBD i tan sols la seva sobreexpressió ja pot causar un fenotip de PD en humans i en models animals [68, 167]. Aquestes malalties segueixen un patró temporal d'afectació, essent les estructures basals les més vulnerables i les corticals les més resistents i les que degeneren més tard [30-31]. S'ha suggerit que l'expressió anòmala de l'α-sinucleïna i alguns canvis post-traduccionals en la proteïna selectius en cada àrea podrien ser els causants del desencadenament de la patologia. Entre aquests canvis s'han proposat: la fosforilació, el truncament o l'oxidació de la proteïna [44, 101, 134, 149].

Gràcies a que en el nostre laboratori tenim accés a mostres humanes congelades de cervell de diferents malalties neurodegeneratives, vam contrastar tots aquests canvis que es proposen en les mostres humanes. El fet de poder comparar entre diferents pacients de diferents edats i malalties i entre varies regions cerebrals d'un mateix individu, ens va permetre tenir una visió més àmplia del grau d'aquests canvis i de la seva importància.

4.1. Validesa de les mostres post-mortem

Per analitzar els nivells de proteïna en mostres postmortem cal tenir en compte la possible degradació dels components cel·lulars, essent un dels grans problemes als que ens enfrontem a l'estudiar el cervell humà. Degut a aquesta dificultat, el temps postmortem del material humà en limita molt la seva utilització i cal fer un examen previ on s'avaluï el grau de degradació en les mostres utilitzades [239]. Altres vegades per eliminar aquest inconvenient, es pot utilitzar material provinent de models [100, 240].

En el nostre anàlisi cal tenir en compte la degradació de l'α-sinucleïna ja que és especialment sensible al interval postmortem [239]. Per estudiar-ho vam realitzar un postmortem artificial en 8 regions del cervell de dues mostres independents i vam comprovar que fins a les 12 hores, l'expressió de l'α-sinucleïna es mantenia constant en totes les regions estudiades. Així doncs, com que les nostres mostres obtingudes durant l'autòpsia estaven dins del rang de les 6 hores postmortem, les podíem utilitzar per al nostre estudi.

Per una altra banda, ens vam proposar estudiar el truncament de l'α-sinucleïna en el cervell. Aquest truncament podria ser el resultat d'una proteòlisi postmortem com a resultat de la degradació de la mostra. Però vam descartar-ho degut a que aquestes bandes ja existien al inici del interval artificial i que la seva expressió no augmentava ni disminuïa amb temps postmortem relativament curts. A més, quan utilitzem models cel·lulars i murins on la limitació del temps és molt menor i la degradació a causa del postmortem inexistent, les bandes truncades d'α-sinucleïna també hi són presents.

Així doncs, vam concloure que, tal i com ja s'havia proposat abans [239], l'α-sinucleïna és una proteïna sensible al temps postmortem, però que aquest obstacle es podia resoldre utilitzant mostres per sota de les 12 hores després de la mort.

4.2. Nivells d'α-sinucleïna en el cervell

Degut a què hi ha poblacions selectives de neurones que moren durant els processos neurodegeneratius, és interessant estudiar si aquesta mort regional es deguda a canvis en l'expressió de l'α-sinucleïna. L'estudi d'aquests canvis esdevé important al conèixer la dependència de la càrrega proteica en alguns casos de PD, com queda palès en les formes de PD familiars provocades per duplicacions i triplicacions del gen de l'α-sinucleïna [68] o en ratolins que simplement sobreexpressen la proteïna humana [203].

Nosaltres ens vam proposar analitzar els nivells d'expressió de l'α-sinucleïna en cervells humans per intentar esbrinar si existeix aquesta relació entre la dosi i la mort neuronal selectiva en les LBDs esporàdiques. De fet, ja hi ha molts estudis en aquesta línia, però la gran majoria analitzen l'expressió a nivell de mRNA, duent-los molt sovint a resultats contradictoris [101, 103-104, 107].

Els primers abordatges es van dur a terme utilitzant la tècnica d'hibridació *in situ.* Mentre que alguns autors no trobaven cap relació entre la presència de LB i l'expressió del mRNA de l'α-sinucleïna [107], altres trobaven una davallada de l'expressió en les neurones dopaminèrgiques de la substància negra (riques en LB) i en l'escorça frontal dels malalts [103].

De forma similar, els resultats obtinguts analitzant l'ADN amb la tècnica de PCR també conduïen a resolucions contradictoris. Alguns treballs suggerien una disminució en l'expressió de l'mRNA en la substància negra dels casos amb PD [102], altres proposaven un increment de l'expressió en la substància negra i en el còrtex temporal dels casos afectats [101, 104], mentre que d'altres no trobaven variacions en els nivells comparant cervells de pacients control amb MSA [106].

En vista d'aquests resultats oposats, nosaltres ens vam plantejar estudiar l'expressió de l'α-sinucleïna a nivell proteic tenint en compte la degradació postmortem. Ja que era probable que la variació observada en aquests treballs fos resultat dels diferents temps postmortem de cada cervell i de la falta de controls interns per contrastar la validesa de l'estudi amb mRNA.

Els nostres resultats indicaven que tots els casos exhibien una expressió més baixa en la substància negra i el nucli basal de Meynert (justament les àrees que degeneren abans en el transcurs d'una PD), mentre que l'expressió era més alta en les àrees corticals, el nucli caudat, el putamen i l'amígdala. Aquesta expressió reduïda la trobàvem a nivell de proteïna soluble, però ignoràvem si un canvi en la solubilitat de la proteïna ens podria estar encobrint aquesta disminució. En tot cas, els casos sense patologia Lewy haurien de presentar un augment en l'α-sinucleïna soluble respecte als que sí la presenten, però no és així ja que els casos joves ja presenten aquesta distribució. Igualment, analitzant els nivells de la proteïna en la fracció insoluble es conservaven els mateixos nivells d'expressió, eliminant la possibilitat d'un canvi en la solubilitat de la proteïna.

Les nostres observacions suggerien doncs, que les àrees més proclius a degenerar en les LBDs (tant la substància negra com el nucli basal de Meynert) tenien els nivells més baixos d'α-sinucleïna en tots els casos. La qual cosa estava en desacord amb estudis previs realitzats utilitzant la hibridació *in situ* [241], però en avinença amb els estudis realitzats a nivells de proteïna en cervell de rata [99].

El nostre treball és el primer que es realitza a nivell de proteïna en cervell humà. Tenint en compte que s'han utilitzat diversos anticossos per precisar-ne l'expressió, que s'ha testat la validesa de les mostres postmortem i que aquestes variacions són robustes en totes les mostres, suggerim que difícilment el desencadenant de la patologia esporàdica en les àrees vulnerables del cervell pot ser un increment de l'expressió de l'α-sinucleïna. Primer, perquè les regions on l'expressió és relativament major, resulten ser les més resistents a degenerar, i segon, perquè es dóna una disminució en aquestes zones amb dependència de l'edat i independència de la malaltia. Aquest últim fet ha estat confirmat recentment en el cervell del ratolí en altres treballs [100, 240]. Aquesta disminució lligada a l'edat (i no un augment de l'expressió) podria ser un més dels factors de risc implicats en el desenvolupament de la malaltia en un escenari on múltiples factors conflueixen en la patogenicitat de l'α-sinucleïna.

4.3. Fosforilació de la serina 129

Ja s'ha comentat anteriorment que la fosforilació de l'α-sinucleïna en la posició 129 és una modificació que promou la formació d'oligòmers que estan implicats en la patologia [44], així com en la neurotoxicitat [128].

Nosaltres ens vam proposar estudiar els nivells totals d'α-sinucleïna fosforilada i la seva relació amb la distribució en el cervell, ja que hi ha unes estructures (com la substància negra o el nucli de Meynert) que són les més sensibles a desenvolupar agregats en el progrés normal de la malaltia. Estudiant la distribució de l'α-sinucleïna en el cervell vam observar que les àrees on l'expressió d'α-sinucleïna era més baixa, concordaven amb les que es trobava uns nivells d'α-sinucleïna fosforilada més alts. Així les zones on la fosforilació era més evident eren la substància negra i el nucli basal de Meynert. Sorprenentment, aquesta observació era evident fins i tot en els casos que no presentaven patologia i en els casos joves, el que suggeria que en aquestes zones, molt avanç de les manifestacions clíniques, ja s'hi poden donar unes certes predisposicions, com per exemple la fosforilació de l'α-sinucleïna. En canvi, les altres estructures mantenien uns nivells baixos o inexistents d'α-sinucleïna fosforilada.

Aquests resultats no contradiuen un paper patològic de l'α-sinucleïna fosforilada en la serina 129, ja que com ha estat descrit anteriorment [44, 127, 182], nosaltres també la trobem enriquida en els LB dels casos afectats. Però el fet que es trobi enriquida en LB o no, es proposa com un fet depenent de la patologia. Ara bé, si aquesta fosforilació juga un paper fisiològic en aquestes regions i no en les altres és un tema encara per estudiar. Si més no, l'elevada propensió a agregar-se de l'α-sinucleïna fosforilada [127-128], pot condicionar que aquestes àrees esdevinguin altament susceptibles als mecanismes tòxics de l'α-sinucleïna.

Aquestes evidències donen suport als múltiples resultats que indiquen canvis en quant a l'oxidació [51, 242], a la resposta mitocondrial [243] o la fosforilació [244], associats a àrees cerebrals que serien futuribles dianes de la patologia.

4.4. L'α-sinucleïna truncada en el cervell humà

El truncament de l'α-sinucleïna ha estat estudiat per molts grups i la seva existència és quasi inqüestionable, però el seu paper en la neurodegeneració encara no es coneix prou bé. Per una banda, s'ha proposat que té un paper en la formació dels agregats proteics [143, 149, 159], i per altra banda es considera un esdeveniment normal [156-

157]. S'ha suggerit que aquests fragments, trobats en individus sense afectació, podrien ser un producte de degradació de la mostra durant el procés d'obtenció, però sembla poc probable que les mostres es degradin només per l'extrem C-terminal. A més, l'existència d'aquest fragment queda corroborada en altres estudis, tant amb models murins com cel·lulars, que expressen l'α-sinucleïna i presenten truncaments en la proteïna, eliminant així la manipulació postmortem [138, 143, 149, 244]. Anant una mica més enllà, en el nostre estudi podem descartar-ho gràcies a la validació de la degradació de la mostra.

El gran repte és arribar a identificar aquests fragments truncats en les mostres humanes, però la baixa expressió d'aquestes en el cervell en dificulta molt la tasca. Tot i així, nosaltres ens vam plantejar acotar les regions on podrien ocórrer els truncaments *in vivo* mitjançant el reconeixement amb anticossos dirigits contra diferents epítops.

En aquest treball hem descrit l'existència d'un fragment truncat al voltant dels 15 kDa en els homogenats de totes les regions estudiades independentment de la patologia i de l'edat de cada cas. Aquest truncament es produiria per l'extrem C-terminal entre els residus 111 i 122. La troballa d'un fragment truncat per l'extrem C-terminal està en consonància amb el que ja ha estat descrit anteriorment [143, 149, 157]. Però en contra del que descriuen tots els treballs realitzats, els nivells d'expressió d'aquest fragment truncat en homogenats, es trobarien en relació directa amb els nivells d'α-sinucleïna en forma soluble. Les àrees on menys s'expressa serien, de nou, la substància negra i el nucli basal de Meynert, i les que més α-sinucleïna tenen expressarien nivells més alts d'aquesta la forma truncada. La qual cosa indica que els nivells totals de les formes fragmentades no correlacionen amb la malaltia. Primer, perquè les regions no afectades mostren els nivells més alts, i segon, perquè tant les mostres control com les afectades conserven nivells similars en totes les àrees d'estudi.

Aquest resultat contradiu els treballs que suggereixen un paper de la forma truncada en la formació dels agregats, però aquests treballs utilitzen fraccions molt insolubles (les que s'utilitzen per aïllar fibril·les) [143, 149]. Com que els agregats són inexistents en els casos control és obvi que tampoc s'hi trobaran fragments en aquestes fraccions, mentre que en casos amb cossos de Lewy s'hi podria mantenir la mateixa relació entre l'α-sinucleïna sencera i la truncada (proposada en un 15% per Liu i col. [149]). No obstant, l'aproximació que suggerim no ens és vàlida per estudiar el que ocorre en una neurona en concret (la que es troba en un procés de mort neuronal), sinó que ens indica que tenint en compte grans grups de neurones, no es donen canvis perceptibles en l'expressió de l'α-sinucleïna ni en el truncament, tot i que podria ser que aquests canvis

es donessin de forma subtil en petites poblacions susceptibles i que fossin imperceptibles utilitzant els nostres mètodes.

Tenint en compte la possible existència de diferents isoformes de l'α-sinucleïna en el cervell humà, ens vam proposar generar un anticòs que reconegués la syn112 per tal de veure si aquest fragment que detectàvem a 15 kDa corresponia al *splicing* alternatiu de l'α-sinucleïna. Tot i que l'anticòs era competent reconeixent la proteïna syn112 recombinant, no reconeixia cap producte en les mostres humanes, la qual cosa assenyalava que el producte de 15 kDa corresponia a una banda truncada. A més, utilitzant anticossos en mig de l'exó 5 de l'α-sinucleïna (que faltaria en la forma syn112) també es reconeix aquest producte, eliminant la possibilitat que provingui de *splicing* de l'exó 5.

Gràcies a les tècniques per fraccionar el teixit i enriquir-lo en determinades estructures, hem proposat l'existència d'altres fragments truncats, ja sigui per l'extrem carboxi-terminal com per l'amino-terminal, en les fraccions enriquides en citosol. Aquests fragments s'observen independentment de la patologia que mostren els casos d'estudi, el que reforça la possibilitat que aquests fragments puguin tenir una importància *in vivo* i que no necessariament haurien d'estar associat a desencadenar la fibril·lació de l'α-sinucleïna. Mentre que el fragment de 15 kDa es concentra sobretot lligat en la fracció rica en lisosomes, trobem 2 fragments de pesos menors que provenen d'un truncament major en l'extrem C-terminal, el més gran dels quals encara conté el fragment NAC. Però, es desconeix si aquests fragments C-terminals es generen com a conseqüència del primer fragment o, en canvi, es produeixen directament a partir de la proteïna sencera. Per altra banda, també s'ha identificat un fragment més gran tallat per l'extrem N-terminal en fraccions citosòliques. Ja que aquests fragments estan presents en tots els casos observats i no presenten canvis a nivell soluble en funció de la patologia (almenys utilitzant aquesta aproximació), suggerim que no són completament necessaris per al desenvolupament de la malaltia i reforcen la naturalesa multifactorial de la malaltia on és necessari sobrepassar un llindar on conflueixen tots els factors (dany oxidatiu, truncament, fosforilació, edat, etc.) per desencadenar la mort neuronal.

4.5 Formació dels fragments truncats

Un cop s'havien descrit l'existència d'una sèrie de fragments truncats en les mostres de cervell humà, vam proposar-nos buscar el mecanisme pel qual es formarien i en quines condicions. Hi ha treballs que utilitzen diverses proteases per fragmentar l'α-sinucleïna en condicions *in vitro* i d'aquests estudis s'extreu que poden tallar-la les

metal·loproteinases, la neurosina, el proteasoma, la calpaïna I i/o la catepsina D [138-141, 245].

La nostra hipòtesi era que la formació del fragment més abundant de 15 kDa podia ser deguda a una proteòlisi incompleta a càrrec lisosomes, ja que es localitzava en fraccions enriquides en aquests orgànuls tant en humans com en ratolins, mentre que els fragments que es troben en el citosol es podrien produir mitjançant proteases citosòliques, que ja s'ha descrit que podrien tallar l'α-sinucleïna per ambdós costats [139, 245].

Els lisosomes estan relacionats amb la degradació de qualsevol component cel·lular per a la seva posterior reutilització i el fet que estiguin implicats en la formació de fragments proteics, és un fet que ja ha estat suggerit: el truncament de l'α-sinucleïna per l'extrem C-terminal a través d'un enzim lisosomal ja havia estat proposat en un treball mitjançant de la catepsina D [141]. A més, recentment, un estudi ha descrit un procés pel qual la tau truncada per l'extrem N-terminal es transporta als lisosomes, però aquesta tau no es degrada completament, sinó que es trunca per l'extrem C-terminal sense arribar a entrar-hi i es torna a alliberar al citosol on rep un clivellament posterior formant un pèptid amb una capacitat més alta d'agregar-se [246-247].

Les nostres observacions secunden la importància d'aquest mecanisme utilitzant lisosomes aïllats i un model cel·lular. A més, el fet que la formació d'aquest fragment s'inhibeixi afegint Pepstatina A o augmentant el pH proposa també un paper de la catepsina D lisosomal en el truncament de la proteïna. Per altra banda, la resta de talls que nosaltres trobem en les fraccions citosòliques de mostra humana no es generarien per mitjà dels lisosomes, ja que no s'observa l'aparició d'aquestes bandes quan s'incuben lisosomes aïllats amb l'α-sinucleïna.

Com que s'havia suggerit anteriorment un paper del proteasoma en el clivellament de l'α-sinucleïna *in vitro* [149], ens vam proposar avaluar-ne la seva importància en un model cel·lular. Tanmateix, els resultats obtinguts plantegen que el proteasoma no seria l'encarregat de generar-los, ja que la seva inhibició selectiva en un model cel·lular no comporta la formació ni la desaparició de noves bandes.

El rol del sistema UPS en la degradació de l'α-sinucleïna encara no està clar del tot, en alguns estudis s'ha descrit una implicació, però en d'altres s'ha proposat que la seva participació en la degradació seria molt baixa en condicions normals o fins i tot nul·la. Aquest fet contrasta amb el que s'esperaria d'una proteïna citosòlica que en condicions patològiques es troba mal plegada i que a més es troba ubiqüitinada, totes elles característiques per ser degradada via UPS [248]. Tot i que els nostres resultats no ens donen cap evidència clara respecte una posició o l'altra, proposem que el proteasoma no estaria involucrat en la generació de fragments d'α-sinucleïna de baix pes molecular,

i sí la degradació mitjançada per lisosomes, contrastant amb el mecanisme proposat per Liu i col. que suggereix el mal funcionament del proteasoma com el culpable de la formació d'aquests fragments *in vitro* [149].

4.6. L'α-sinucleïna truncada en els agregats

En la malaltia d'Alzheimer, s'ha descrit que els fragments truncats de tau formen part del nucli dels PHF [137, 249]. Aquesta evidència confereix als fragments truncats un rol important en la patogènesi d'aquesta malaltia. Com que l'α-sinucleïna també és susceptible de ser modificada mitjançant el truncament, es podria pensar en un mecanisme similar. De fet, és la hipòtesi imperant actualment. S'ha vist que en els agregats d'α-sinucleïna (GCI) presents en la MSA, mantenen un nucli resistent a proteïnasa K, format pel fragment 31-109 [158]. Aquest fragment conté la regió 71-82, que s'ha descrit com la regió mínima i necessària de l'α-sinucleïna per autoagregar-se [92].

En el nostre treball ens vam preguntar per la localització d'aquests fragments truncats de l'α-sinucleïna, però a falta de anticossos que detectin específicament les formés truncades, vam utilitzar un panell d'anticossos generats contra diferents zones de la proteïna: la part N-terminal, la part central NAC i la part C-terminal.

Tot i que tots els anticossos reconeixen l'α-sinucleïna, tan sols marquen les mateixes estructures en alguns dels casos. En estadis primerencs de la formació dels LB s'observen petits dipòsits neuronals de la proteïna que es marquen separadament amb aquests anticossos. En alguns d'aquests dipòsits es detecta marcatge amb l'anticòs N-terminal, mentre que d'altres es marquen només amb el C-terminal. Curisament, cap dipòsit és reconegut tan sols per l'anticòs contra el fragment NAC.

Quan s'observen estructures com els LB es veu un marcatge general comú de tots tres anticossos, o sigui, que en aquestes estructures trobem en gran part l'α-sinucleïna sencera. Tot i així, s'observen varies inclusions petites i perifèriques que es marquen tan sols amb l'anticòs C-terminal, així com el nucli del LB, que també es troba enriquit en fragments truncats per l'extrem C-terminal mentre que l'halo que l'envolta es troba enriquit en fragments truncats per l'N-terminal. Aquest model concorda amb el descrit per Murray i col. [159] on l'extrem N-terminal i el NAC serien indispensables per l'agregació i formarien el nucli resistent del LB.

Els nostres resultats suggereixen que el nucli del LB està format per fragments truncats per l'extrem C-terminal, i evoca un paper d'aquests fragments en la formació dels LB. Sembla doncs, que de la mateixa forma com ja s'ha comentat en l'apartat 4.3

en referència a l'α-sinucleïna fosforilada, el truncament per si sòl no pot iniciar el procés d'agregació, ja que aquests mateixos fragments són presents en individus que no tenen agregats. Però com que el clivellament de la part C-terminal, és tan important en l'estabilitat de la proteïna, podria ser que els fragments truncats esdevinguessin un perill en la potencialitat patogènica de l'α-sinucleïna degut a l'alliberament i exposició del fragment NAC [161]. Així, aquests fragments es convertirien en els primers indicadors tòxics i la cèl·lula els amagaria formant el centre (i la part més primerenca) dels LB.

VII. Conclusions

1. L'α-sinucleïna es troba nitrada en oligòmers i formant agregats en l'escorça frontal de pacients amb malaltia de Pick, conjuntament amb els agregats característics de tau. En canvi, aquestes estructures no es detecten en l'escorça occipital.

2.1. En les fraccions sinàptiques de l'escorça frontal de pacients amb malaltia d'Alzheimer i de malalties amb cossos de Lewy (PD i DLB), s'observa un increment en la fosforilació de l'α-sinucleïna.
2.2. La fosforilació de la proteïna tau en la serina 396 és una alteració present en l'escorça frontal tant en AD com en LBDs. A més, aquesta modificació anòmala es troba també en les fraccions sinàptiques i acoblada a les vesícules sinàptiques en la malaltia d'Alzheimer.

3.1. En el model murí que sobreexpressa l'α-sinucleïna humana mutada A53T s'observa una espècie de pes molecular baix corresponent a un truncament de la pròpia α-sinucleïna humana. Conjuntament, es detecta un increment en l'agregació de l'α-sinucleïna en relació a l'edat. Cap d'aquests canvis però, és suficient per produir la formació d'inclusions proteiques en les neurones o en les cèl·lules glials.
3.2. L'administració de dietes pobres en àcids grassos insaturats modifica l'expressió dels lípids poc peroxidables del cervell en el model A53T, però no interfereix en el fenotip dels animals.

4.1. Les mostres de cervell obtingudes just després de l'autòpsia són vàlides per estudiar l'α-sinucleïna, sempre i quan el temps postmortem no sigui superior a les 12 hores, moment en què l'α-sinucleïna es comença a degradar.
4.2. Els nivells d'α-sinucleïna en el cervell són més baixos en la substància negra i en el nucli basal de Meynert en comparació amb altres zones del cervell com el nucli caudat, l'escorça frontal, l'amígdala, l'escorça temporal, el putamen o el cerebel. Aquestes diferències són independents de l'edat i la patologia de cada un dels casos.
4.3. L'expressió d'α-sinucleïna en les regions estudiades és independent de la patologia que presenten els casos, però en canvi, disminueix de manera edat-depenent en l'escorça frontal i la substància negra.
4.4. La fosforilació de l'α-sinucleïna en la serina 129 és una modificació present en la substància negra i el nucli basal de Meynert de tots els casos estudiats, sense mantenir cap relació amb l'edat o la patologia.
4.5. El truncament més abundant de l'α-sinucleïna per l'extrem C-terminal és un esdeveniment comú en totes les àrees del cervell i els seus nivells d'expressió es troben

en relació amb els de la proteïna sencera. Tampoc s'observa cap alteració en la seva expressió en dependència de la patologia ni l'edat. Per altra banda, existeixen altres fragments menors truncats tant per l'extrem C-terminal com per el N-terminal.

4.6. La formació del principal fragment de l'α-sinucleïna és un procés que es pot reproduir en lisosomes aïllats i es pot aturar amb inhibidors lisosomals.

4.7. Tots els anticossos contra l'α-sinucleïna reconeixen majoritàriament els cossos de Lewy madurs i els que estan en formació, però algunes estructures (com per exemple el nucli dels agregats) tan sols conserven els epítops C-terminals o els N-terminals.

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IX. Resultats annexos

Resultats annexos:

1. **Muntané G***, Dalfó E*, Martínez A, Rey MJ, Avila J, Pérez M, Portero M, Pamplona R, Ayala V, Ferrer I. *Glial fibrillary acidic protein is a major target of glycoxidative and lipoxidative damage in Pick's disease*. J Neurochem. 2006 Oct;99(1):177-85.

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2. La proteïna GFAP (Glial fibrillary acidic protein) és una diana important d'oxidació en la malaltia de Pick.

Muntané G, Dalfó E, Martínez A, Rey MJ, Avila J, Pérez M, Portero M, Pamplona R, Ayala V, Ferrer I. J Neurochem. 2006 Oct;99(1):177-85.

La malaltia de Pick (PiD) és una demència frontotemporal caracteritzada per una atròfia severa dels lòbuls frontals i temporals degut a una marcada pèrdua neuronal acompanyada d'una gliosi astrocítica enriquida en GFAP. Les neurones que romanen vives, presenten inclusions citoplasmàtiques anomenades cossos de Pick, compostes per tau hiperfosforilat, a la vegada que es troba tau hiperfosforilat també en astròcits i en oligodendròcits.

En aquest estudi s'ha utilitzat l'electroforesi en gel i el western blot utilitzant marcadors de glicooxidació (*advanced glycation end products*, N-carboxymethyl-lysine i N-carboxyethyl-lysine: AGE, CEL, CML, respectivament) i de lipooxidació (4-hydroxy-2-nonenal: HNE, and malondialdehyde-lysine: MDAL) en mostres d'escorça frontal i occipital en tres casos de PiD i en controls. S'ha observat un augment d'unes bandes a 50 kDa reactives per AGE, CML, CEL, HNE i MDAL en l'escorça frontal, però no en l'escorça occipital en els casos amb PiD amb associació amb el increment de reactivitat per la GFAP. Utilitzant la tècnica de gels bidimensionals s'han revelat uns nivells augmentats de GFAP i un augment en les seves isoformes en l'escorça frontal de malalts de PiD. A més, els estudis usant anticossos contra oxidació han mostrat la glicooxidació (amb l'anticòs CEL) i la lipooxidació (utilitzant l'anticòs HNE) d'almenys 3 isoformes de la GFAP.

Com a conclusió, aquest estudi demostra que la GFAP és una diana d'estrès oxidatiu en l'escorça frontal de la PiD.

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Glial fibrillary acidic protein is a major target of glycoxidative and lipoxidative damage in Pick's disease

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Abstract

Pick's disease is a subset of fronto-temporal dementia characterised by severe atrophy of the temporal and frontal lobes due to marked neuronal loss accompanied by astrocytic gliosis enriched in glial acidic protein. The remaining neurones have intracytoplasmic inclusions composed of hyperphosphorylated tau, called Pick bodies, in addition to hyperphosphorylated tau in astrocytes and oligodendrocytes. Gel electrophoresis and western blotting using markers of glycoxidation (advanced glycation end products, N-carboxyethyl-lysine and N-carboxymethyl-lysine: AGE, CEL, CML, respectively) and lipoxidation (4-hydroxy-2-nonenal: HNE, and malondialdehyde-lysine: MDAL) were used in the frontal and occipital cortex in three Pick's disease cases and three age-matched controls. In Pick's disease, increased AGE, CML, CEL, HNE and MDAL bands of about 50 kDa were observed in the frontal cortex (but not in the occipital cortex) in association with increased density of glial acidic protein bands. Bi-dimensional gel electrophoresis and western blotting also disclosed increased amounts and numbers of glial acidic protein isoforms in the frontal cortex in Pick's disease. Moreover, redox proteomics showed glycoxidation, as revealed with anti-CEL antibodies and lipoxidation using anti-HNE antibodies, of at least three glial acidic protein isoforms. The present results demonstrate that glial acidic protein is a target of oxidative damage in the frontal cortex in Pick's disease.

Keywords: glial fibrillary acidic protein, glycoxidation, hydroxy-2-nonenal, lipoxidation, oxidative stress, Pick's disease.
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Pick's disease is a subset of fronto-temporal dementia beginning at between 50 and 60 years of age, often but not always with inappropriate behaviour and personality changes, and dementia (McKhann *et al*. 2001; Hodges *et al*. 2004). Pick's disease is characterised by cerebral atrophy, mainly affecting the frontal and temporal lobes, whereas the occipital lobes are less affected (Kril *et al*. 2005). This is accompanied by marked neuronal loss and proliferation of astrocytes in vulnerable regions, expressing glial acidic protein, together with hyper-phosphorylated tau deposition in neurones, some of them comprising round inclusions called Pick bodies. In addition, phospho-tau inclusions are found in astrocytes and oligodendrocytes. Pick bodies, which are key markers of Pick's disease, are principally localised in the dentate gyrus, CA1 region of the hippocampus, amygdala, septal nuclei, and upper layers of the entorhinal cortex and isocortex (Dickson 1998; Komori 1999; Arai *et al*. 2001; Bergeron *et al*. 2003). Gel electrophoresis and western blotting of fractions

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Abbreviations used: AGE, advanced glycation end products; CML, N-carboxymethyl-lysine; CEL, N-carboxyethyl lysine; GFAP, glial fibrillary acidic protein; HNE, 4-hydroxy-2-nonenal; MDAL, malondialdehyde-lysine; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

enriched with abnormal filaments have shown two main bands of 55 and 64 kDa, consisting mostly of 3-repeat tau but also significant amounts of 4-repeat tau (Buée *et al*. 2000; King *et al*. 2001; Zhukareva *et al*. 2002; Arai *et al*. 2003).

Stress-activated protein kinase, c-Jun N-terminal kinase (SAPK/JNK) and p38 kinase (p38) are activated in Pick's disease (Atzori *et al*. 2001; Ferrer *et al*. 2001). Phosphorylated, active p38 (p38-*p*) immunoreactivity is localised in neurones and glial cells with phospho-tau deposition (Atzori *et al*. 2001; Ferrer *et al*. 2001; Hartzler *et al*. 2002). Moreover, subcellular fractions enriched in abnormal fibrillar proteins and containing p38-*p* have the capacity to phosphorylate specific substrates and recombinant tau (Puig *et al*. 2004). Similar properties can be ascribed to SAPK/JNK-*p* and tau deposits (Ferrer *et al*. 2005). Together, these observations demonstrate a link between oxidative stress, SAPK/JNK and p38 activation, and tau phosphorylation in Pick's disease (Hartzler *et al*. 2002; Ferrer *et al*. 2005).

Levels of peroxiredoxin I (Prx I), Mn superoxide dismutase (SOD2) and glutathione-S-transferase omega 1 are not modified in Pick's disease when compared with controls (Krapfenbauer *et al*. 2003). Yet Prx II is markedly increased and Prx III decreased in Pick's disease, indicating differential regulation of antioxidant enzymes (Krapfenbauer *et al*. 2003). Finally, immunohistochemical studies have shown heme-oxygenase-1 and advanced glycation-end products in Pick bodies in Pick's disease (Castellani *et al*. 1995; Kimura *et al*. 1996; Sasaki *et al*. 1998), whereas 4-hydroxy-2-nonenal (HNE) modified proteins are exceptionally expressed in Pick bodies (Montine *et al*. 1997; Zarkovic 2003).

In spite of these specific findings on antioxidant responses suggesting increased oxidative damage, practically nothing is known about possible targets of oxidative damage in Pick's disease. To gain further understanding of the extent of oxidative stress damage, the present study examined the expression levels of selected markers of oxidation, glycoxidation and lipoxidation in Pick's disease. For this purpose, gel electrophoresis and western blotting of frontal and occipital cortex homogenates were carried out in three cases of Pick's disease and three age-matched controls. Antibodies to advanced glycation-end products (AGEs), N-carboxymethyl-lysine (CML) and N-carboxyethyl-lysine (CEL) were used as markers of glycoxidation and carbonyl production. Antibodies to HNE, an end-product lipid peroxidation, and malondialdehyde-lysine (MDAL) were used as markers of lipoxidation. As these methods disclosed the presence of glycoxidative and lipoxidative adducts, bi-dimensional gel electrophoresis, western blotting and mass spectrometry were used to identify putative protein targets of oxidative damage in Pick's disease.

Materials and methods

Samples

Brain samples were obtained from the Institute of Neuropathology and University of Barcelona/Hospital Clinic Brain Banks following the guidelines of the local ethics committee. The brains of three patients with Pick's disease and three age-matched controls were obtained 1–6 h after death, and were immediately prepared for morphological and biochemical studies. The cases with Pick's disease were two men and one woman aged 65, 68 and 66 years old, with sporadic progressive behavioural impairment followed by fronto-temporal dementia and accompanied by severe fronto-temporal lobar atrophy on neuro-imaging studies (CT and MRI). The fresh brain weights were 850, 1000 and 900 g. At autopsy, half of each brain was fixed in formalin, while the other half was cut in coronal sections 1 cm thick, frozen on dry ice and stored at − 80 °C until use. For diagnostic morphological studies, the brains were fixed by immersion in 10% buffered formalin for 2 or 3 weeks. The neuropathological study was carried out on 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 globus pallidus; medial and posterior thalamus; subthalamus; Meynert nucleus; amygdala; midbrain (two levels), pons and medulla oblongata; and cerebellar cortex and dentate nucleus. The neuropathological examination was carried out on tissue fixed in formalin for no less than 3 weeks and the tissue then embedded in paraffin. De-waxed sections, 5 μm thick, were stained with haematoxylin and eosin, and with Klüver Barrera, or processed for immunohistochemistry following the streptavidin LSAB method (Dako, Dakopats, Barcelona, Spain). After incubation with methanol and normal serum, the sections were incubated with one of the primary antibodies at 4 °C overnight. Antibodies to phosphorylated neurofilaments of 170 kDa or 200 kDa (clones BF10 and RT97, Boehringer-Mannheim, Barcelona, Spain) were used at dilutions of 1 : 100 and 1 : 50, respectively. Antibodies to glial fibrillary acidic protein (GFAP, Dako), β-amyloid (Boehringer-Mannheim) and ubiquitin (Dako) were used at dilutions of 1 : 250, 1 : 50, and 1 : 200, respectively. Antibodies to α-synuclein (Dako) were used at a dilution of 1 : 100. Antibodies to pan-tau (Sigma, Madrid, Spain) were used at a dilution of 1 : 100. In addition, the following antiphospho-specific tau rabbit polyclonal antibodies were used: Thr181, Ser199, Ser202, Ser214, Ser231, Ser262, Ser396 and Ser422 (all of them from Calbiochem, Barcelona, Spain). These antibodies were used at a dilution of 1 : 100, except antiphospho-tauThr181, which was used at a dilution of 1 : 250. Following incubation with the primary antibody, the sections were incubated with LSAB for 1 h at room temperature. The peroxidase reaction was visualised with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. Sections were counterstained with haematoxylin. Sections processed for phospho-tau immunohistochemistry were boiled in citrate buffer prior to incubation with the primary antibody. Sections processed for βA4-amyloid and α-synuclein were pretreated with 95% formic acid. Tissue from two of the three patients had previously been used for neurotransmitter studies and enzymatic assays (Puig *et al*. 2004; Dalfó *et al*.

2005b). Control cases were two men and one woman (70, 62 and 68 years old) with no neurological disease. The neuropathological examination was carried out in similar sections and using the same immunohistochemical methods.

Mono-dimensional gel electrophoresis and western blotting

Brain samples (0.2 g) of the frontal (area 8) and occipital cortex (area 17 and 18) from Pick's disease and control cases were homogenised separately in a glass homogeniser in 1 mL of homogeniser 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, Almeda, Spain). After a brief centrifugation at 15 000 *g* (4 °C for 5 min), the pellet discarded and the concentration of the resulting supernatant determined by the 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% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (400 mA, 90 min). Immediately afterwards, 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% bovine serum albumin (Sigma) at 4 °C overnight. The mouse monoclonal anti-AGE, anti-CEL and anti-CML antibodies (TransGenic, Kumamoto, Japan) were used at a dilution of 1 : 1000. The goat polyclonal anti-MDAL (BioMedical, Houston, TX, USA) and the rabbit polyclonal anti-HNE (Calbiochem, Barcelona) were used at a dilution of 1 : 1000. Subsequently, the membranes were incubated for 45 min at room temperature with the corresponding secondary antibody labeled with horseradish peroxidase (Dako, Glostrup, Denmark) at a dilution of 1 : 1000, and washed with TBS-T for 30 min. Protein bands were visualised with the chemiluminescence ECL method (Amersham, Barcelona, Spain). The monoclonal antibody to β-actin (Sigma), diluted 1 : 5000, was used to control protein loading.

Bi-dimensional (2D) gel electrophoresis

Samples of the frontal cortex (area 8) in Pick's disease cases and controls were homogenised in lysis buffer (40 mM Tris pH 7.5 containing 7 M urea, 2 M thiourea and a cocktail of protease and phosphatase inhibitors), and centrifuged at 9000 *g* for 10 min. The pellet was discarded and the concentration of protein from 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, 50 μL 8 M urea and bromophenol blue in a final volume of 300 μL.

In the first dimension electrophoresis, 300 μL of sample solution was applied to an immobilised 17 cm pH 3–10 non-linear 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% (w/v) SDS, 30% (v/v) glycerol and 2% dithiothreitol, and then re-equilibrated for 10 min in the same buffer containing 2.5% iodacetamide. The strips were placed on 10% polyacrylamide gels and electrophoresed at 50 V overnight. An MS-modified silver staining method (Amersham) was used for gel staining following the manufacturer's instructions.

Two bi-dimensional electrophoreses were run in parallel in each case, one for the silver staining method 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 anti-CEL (Transgenic), anti-HNE (Calbiochem) or anti-GFAP (Dako) antibodies, used at dilutions of 1 : 1000, 1 : 1000 and 1 : 2000, respectively, as 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 dithiothreitol solution for 30 min and alkylated 100 mM solution of iodine acetamide for 15 min, 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 mass spectrometry and MS/MS spectra

Proteins manually excised from the 2D gels were digested and analysed by CapLC-nano-ESI-MS-MS mass spectrometry. The tryptic digested peptide samples were analysed 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 μL of 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 ionised via coated nano-ES needles (PicoTip$^{TM.}$, 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 25–35 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, 1 missed cleavage, carbamidomethyl (C) as fixed modification and oxidised (M) as variable modification, and mass tolerance of 150–250 p.p.m.

A 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 50 were considered to indicate a high confidence of identification.

Results

General neuropathological findings

The three cases of Pick's disease yielded the same neuropathological findings. Marked neuronal loss accompanied by severe astrocytic gliosis was found in the frontal and temporal cortices. Reduced myelin and myelin pallor, as seen with Klüver-Barrera, was found in the white matter of the frontal and temporal lobes. Changes were more marked in cases with severe atrophy (cases with brain weights of 850 and 900 g), whereas neurone loss and gliosis was less pronounced in the second case (brain weight 1000 g). Pick bodies, as visualised with immunohistochemistry to phosphorylated neurofilament epitopes, pan-tau and ubiquitin antibodies, were observed in the dentate gyrus, CA1 area of the hippocampus, subiculum, entorhinal cortex, and upper and inner layers of the frontal and temporal cortex. Phospho-tau inclusions were better visualised with specific antiphospho tau antibodies including Pick bodies, and diffuse deposits in the cytoplasm of neurones, neuropil threads, astrocytic inclusions, and coiled bodies in oligodendrocytes. No phospho-tau deposits were seen in the occipital cortex (primary visual cortex and neighboring association areas) in two cases, whereas rare tau-immunoreactive ballooned neurones and phospho-tau-immunoreactive astrocytes were seen in the third case. Special attention was paid to rule out the presence of neurofibrillary tangles and β-amyloid deposits in these cases. Lewy bodies, as revealed with anti-α-synuclein antibodies, were absent. This may explain the limited number of cases in this study, as no cases with combined pathology were included in the present series.

Age-matched control cases showed no morphological abnormalities. Hyper-phosphorylated tau inclusions, α-synuclein deposits and α-amyloid plaques were absent in all control cases.

Mono-dimensional gel electrophoresis and western blotting to glycoxidised and lipoxidised products

A thick band of about 50 kDa was detected, using anti-AGE, anti-CEL and anti-CML antibodies, in lysates in the frontal cortex but not in the occipital cortex in Pick's disease cases, in comparison with controls. Similar bands were seen in membranes blotted for anti-MDAL, whereas at least two bands in the same molecular weight range were recovered with anti-HNE antibodies. The intensity of these bands varied from one case to another, in one case being less pronounced than in the two others. The intensity of the bands correlated with the amount of glial fibrillary acidic protein in the same cases, as shown in parallel membranes blotted with anti-GFAP antibodies (Fig. 1). Differences were not related to differences in protein loading as results were similar in samples of the frontal and occipital cortex in control and diseased brains. Therefore, an increase in GFAP could be related to the relative increase due to tissue compaction resulting from a loss of neurones or to the absolute increase in the amount of GFAP in astrocytes.

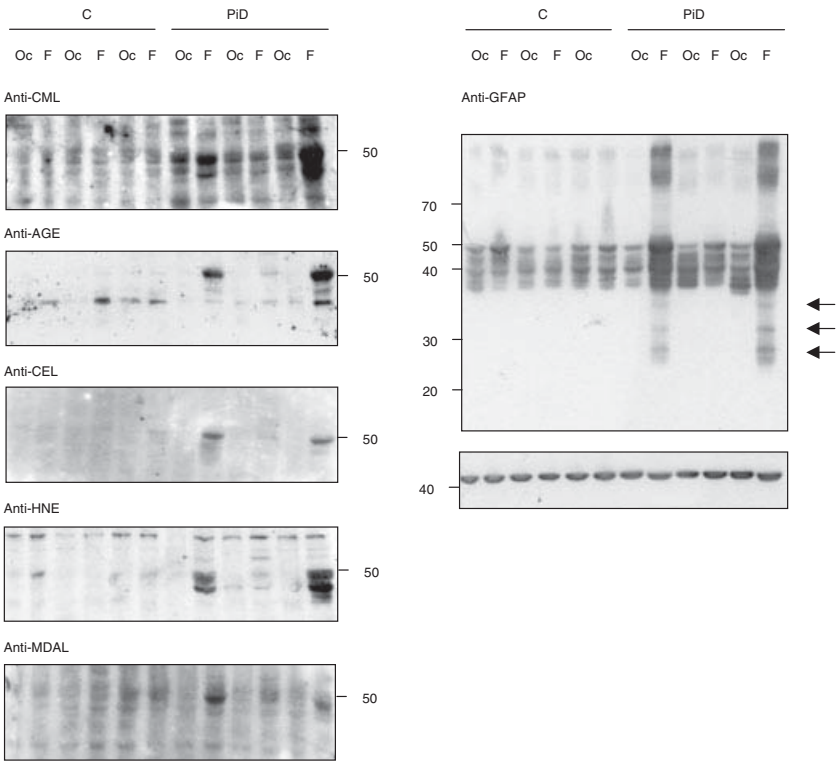

Fig. 1 Gel electrophoresis and western blotting to CML, AGE, CEL, HNE and MDAL in the occipital (Oc) and frontal (F) cortex in three control cases (C) and three cases with Pick's disease (Pick's disease) (left panel). Strong bands of about 50 kDa are found in the frontal cortex (but not in the occipital cortex) in Pick's disease when compared with age-matched controls. The bands are more intense in the two cases with severe frontal atrophy. This corresponds with the more intense bands in the frontal cortex in the two severe Pick's disease cases in membranes immunoblotted for GFAP when compared with control cases (right panel). Note the presence of several bands between 35 and 50 kDa and several lower bands of about 28, 30 and 33 kDa in the frontal cortex of two Pick's disease cases (arrows).

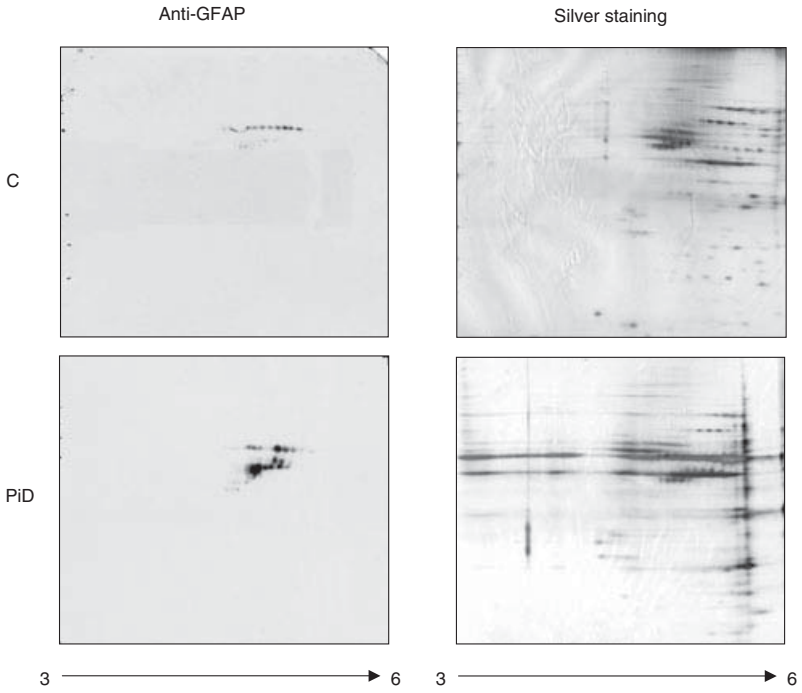

Fig. 2 Bi-dimensional gel electrophoresis and western blotting of membranes stained with anti-GFAP antibodies on the left panel disclose a strong increase in the amount of GFAP and in the number of GFAP isoforms in the frontal cortex in Pick's disease when compared with an age-matched control (C). Bi-dimensional gels processed in parallel and stained with silver are shown on the right panel for comparison.

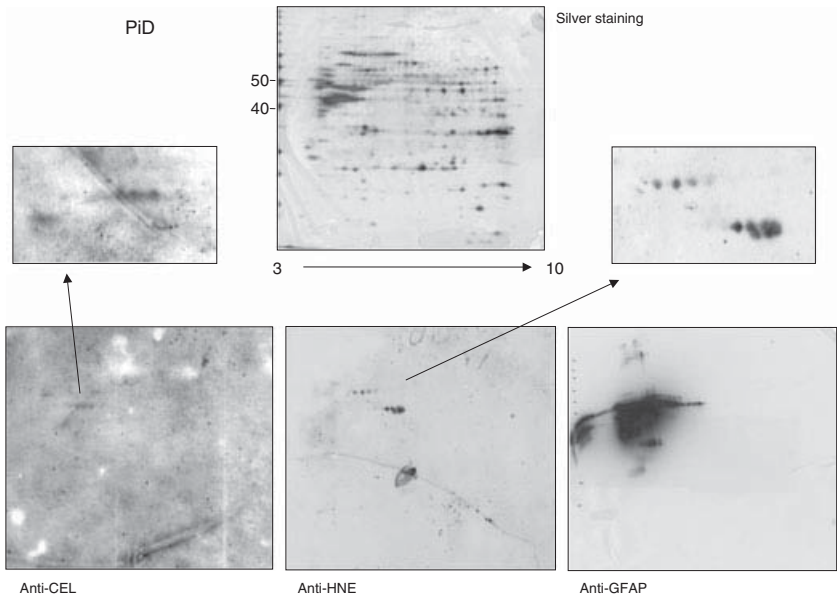

Fig. 3 Bi-dimensional gel electrophoresis and western blotting stained with anti-CEL, anti-HNE and anti-GFAP antibodies disclose oxidised spots in pH 3 → 10 strips at about 50 kDa and pH 5 in the frontal cortex in Pick's disease. These spots were not seen in control cases. Oxidised spots were identified in silver-stained bi-dimensional gels processed in parallel. Bi-dimensional gel processed in parallel and stained with silver was used to obtain the spots for mass spectrometry analysis.

Table 1 CEL-modified proteins from the frontal cortex in Pick's disease

Spot	Calculated PI	KDa Nominal mass	Protein	Score coverage	No. of peptides matched	G1 accession
1	5.42	49907	Glial fibrillary acidic protein	651 Sequence: 26%	12	AAH13596
2	5.42	49907	Glial fibrillary acidic protein	668 Sequence: 26%	12	AAH13596
3	5.42	49907	Glial fibrillary acidic protein	576 Sequence: 27%	12	AAH13596

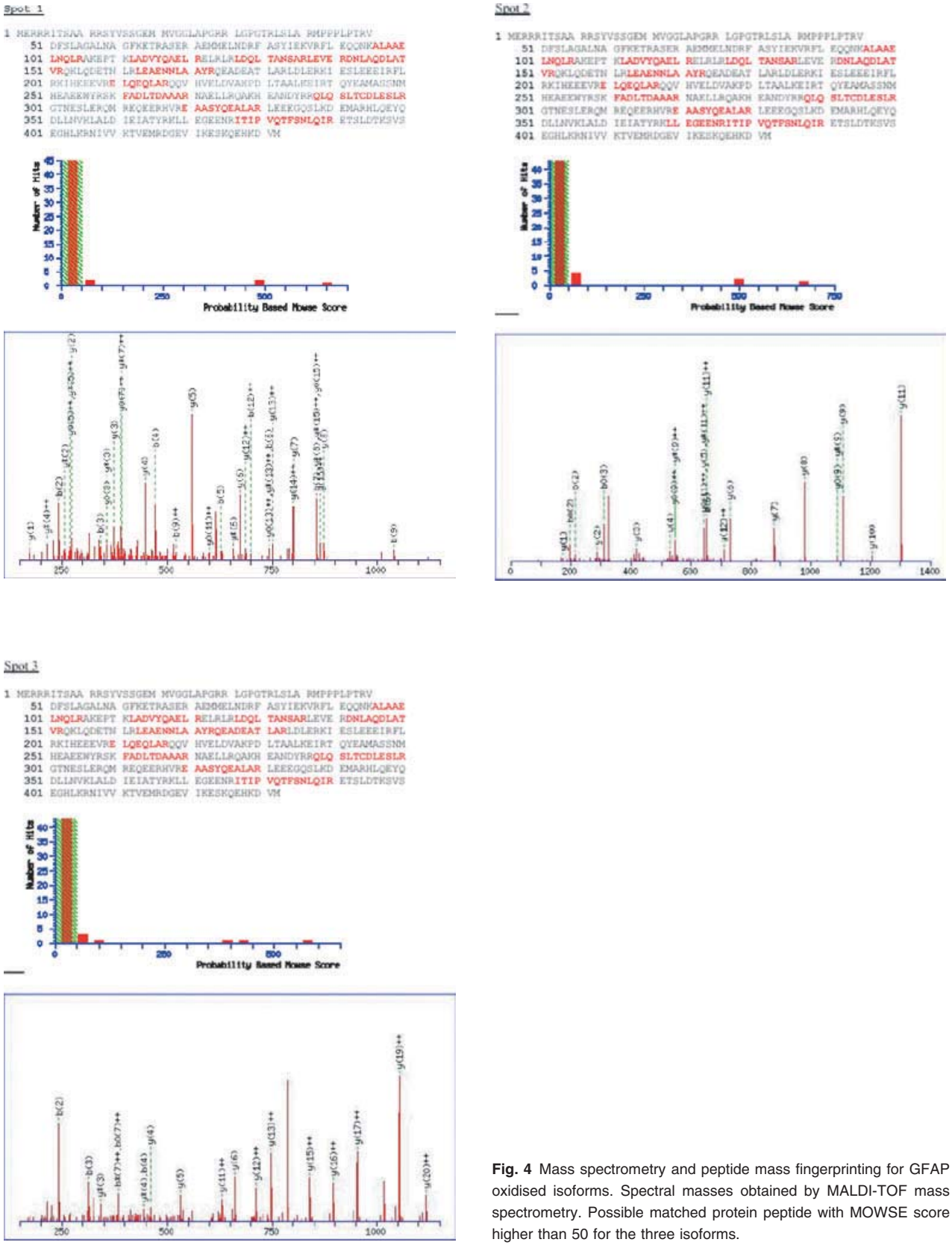

Fig. 4 Mass spectrometry and peptide mass fingerprinting for GFAP oxidised isoforms. Spectral masses obtained by MALDI-TOF mass spectrometry. Possible matched protein peptide with MOWSE score higher than 50 for the three isoforms.

Interestingly, mono-dimensional western blotting to GFAP showed several protein bands ranging of 35–50 kDa in homogenates from Pick's disease and control brains. In addition, three lower bands of about 33, 30 and 28 kDa were found only in the frontal cortex in the most severe Pick's disease cases (Fig. 1).

2D gels and GFAP blotting

Bi-dimensional gel electrophoresis and immunoblotting with anti-GFAP antibodies disclosed a strong increase in the amount of GFAP and in the number of GFAP isoforms in the frontal cortex in Pick's disease when compared with age-matched controls (Fig. 2).

2D gels, western blotting for oxidised proteins and protein characterisation

Bi-dimensional gels of the frontal cortex in Pick's disease immunostained with anti-HNE and anti-CEL antibodies showed oxidised spots in pH 3 → 10 strips at about 50 kDa and pH 5 (Fig. 3). These spots were not seen in control cases. Oxidised spots were identified in silver-stained bi-dimensional gels processed in parallel. Dissection of the spots and analysis with MALDI-TOF/TOF mass spectrometry revealed three glial fibrillary acidic protein isoforms as targets of oxidation (Table 1). Proteins showed MOWSE scores greater than 50 (Fig. 4), indicating a significant match and therefore high confidence of identification.

Discussion

Increased glycoxidation, as revealed by increased anti-CEL and anti-CML immunoreactivities, as well as lipoxidative damage, manifested as increased MDAL and HNE modification of proteins, were observed in the frontal cortex but not in the occipital cortex in Pick's disease when compared with corresponding regions in age-matched controls. Less intense abnormalities in the occipital cortex, however, cannot be ruled out with the present methods.

A major advance in the understanding of the effects of oxidative stress in neurodegenerative diseases, particularly in Alzheimer's disease and Parkinson's disease, has been derived from the application of redox proteomics. Several proteins are primary targets of lipoxidative and glycoxidative damage in Alzheimer's disease and Pick's disease even at very early stages of the disease (Aksenova *et al*. 1999; Butterfield and Kanski 2001; Beal 2002; Choi *et al*. 2004, 2005; Dalfo *et al*. 2005a; Pamplona *et al*. 2005; Sultana *et al*. 2005).

Here we have focused the study on the strong bands of about 50 kDa which could be related to oxidised glial fibrillary acidic protein. Mono-dimensional and bi-dimensional gel electrophoresis and western blotting showed increased GFAP expression in the frontal cortex in Pick's disease cases. In normal brain, GFAP presents several isoforms ranging from 35 to 50 kDa (Eng *et al*. 2000; Korolainen *et al*. 2005). In addition, lower isoforms ranging from 25 to 40 kDa have been observed only in the frontal cortex in Pick's disease. Proteolytic cleavage of GFAP is documented in a mouse model of amyotrophic lateral sclerosis, suggesting that these lower isoforms participate in neurodegeneration (Fujita *et al*. 1998). It has also been suggested that GFAP proteolytic fragments may be produced by the activation of μ-calpain in prion-infected mice (Gray *et al*. 2006). Whether the increase in GFAP isoforms is related to the degeneration and apoptosis of astrocytes described in Pick's disease (Broe *et al*. 2004) is not known.

Moreover, bi-dimensional gel electrophoresis and western blotting followed by analysis with CapLC-nano-ESI-MS-MS mass spectrometry of selected silver spots, corresponding to CEL- and HNE-modified proteins in nitrocellulose membranes processed in parallel, disclosed that at least three GFAP isoforms are modified through glycoxidation and lipoxidation in the frontal cortex in Pick's disease.

Whether GFAP oxidation is associated with loss of function, as demonstrated for certain oxidised proteins (Aksenov *et al*. 1997; Aksenova *et al*. 1999; Perry *et al*. 2002) is not known. Whether oxidation of GFAP is the result of tau phosphorylation in astrocytes can be discussed in more detail. Recent studies have shown that GFAP is highly modified by oxidation in Alzheimer's disease in which no hyper-phosphorylated tau is deposited in astrocytes (Korolainen *et al*. 2005; Pamplona *et al*. 2005). Moreover, GFAP is greatly modified by oxidative stress in aceruloplasminaemia brain (Kaneko *et al*. 2002). Interestingly, lipid peroxidation and increased levels of GFAP in diabetic retina, which is not associated with phospho-tau deposition, are prevented by melatonin supplementation (Baydas *et al*. 2004). Together, these findings suggest that GFAP modifications by glycoxidation and lipoxidation are not secondary to tau phosphorylation, and that GFAP is a major target of glycoxidative and lipoxidative damage in Pick's disease.

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