Universitat Autònoma de Barcelona

El papel del sistema autofágico lisosomal en las enfermedades con cuerpos de Lewy

Laia Pérez Roca

Tesis Doctoral

El papel del sistema autofágico lisosomal en las enfermedades con cuerpos de Lewy

Laia Pérez Roca

Directora de la Tesis: Katrin Beyer.
Tutor: Aurelio Ariza

Departamento de Ciencias Morfológicas
Programa de Doctorado en Cirugía y Ciencias Morfológicas
Universitat Autonoma de Barcelona
Barcelona, 2020

Pel meu pare

ÍNDICE

AMC: autofagia mediada por chaperonas
AMS-C: AMS con fenotipo cerebeloso.
AMS-P: AMS con fenotipo parkinsoniano
AMS: atrofia multisistémica
APP: proteína precursora del beta-amiloide
AS: α–sinucleína
ATG: autophagy related genes
BA: péptido β-amiloide
BECN1: Beclina
BS: B-Sinucleína
CL: cuerpos de Lewy
DCL: demencia con cuerpos de Lewy
DCLc: demencia con cuerpos de Lewy común
DCLp: demencia con cuerpos de Lewy pura
EA: enfermedad de Alzheimer
ECL: enfermedades con cuerpos de Lewy
EG: enfermedad de Gaucher
EP: enfermedad de Parkinson
EPCD: EP con demencia
EPSD: EP sin demencia
FIP200: focal adhesión kinase family-interacting protein
GCasa: enzima lisosomal glucocerebrosidasa
Hsp: heat shock proteins
LAMP2A: Lysosome-associated membrane protein 2
LC3I: microtubule-associated proteins 1A/1B light chain 3
LIMP-2: proteína de membrana lisosomal-2
mTOR: mammalian target of rapamycin
PAS: estructura pre autofagosomal
pCL: patología de cuerpos de Lewy
pCL+pEA: patología de cuerpos de lewy con EA concominante.
PE: fosfatidiletanolamina
PI: fosfatidilinositol
PI3P: fosfatidil inositol trifosfato
Tfeb: factor de transcripción EB
Ubl: ubiquitin-like proteins

1.INTRODUCCIÓN

1. Enfermedades Neurodegenerativas

Las enfermedades neurodegenerativas se caracterizan por una pérdida neuronal acompañada de una clínica de disfunción progresiva. Los avances de las técnicas de tinción durante el pasado siglo permitieron demostrar, que algunas proteínas mal plegadas se acumulan formando depósitos intra o extracelulares en los cerebros de pacientes con enfermedades neurodegenerativas [1].
Estas proteínas mal plegadas generan una disrupción de la homeostasis de la célula provocando, entre otros factores, la sobreexpresión de diversas vías celulares[2]. Dos de los sistemas de control de la acumulación proteica son la autofagia y el sistema lisosomal [3].

1.1. Clasificación

Las enfermedades neurodegenerativas se pueden clasificar según:

1. Los síntomas clínicos, determinados por la región anatómica afectada.

2. Proteínas acumuladas en las neuronas o células gliales, o de forma extraneuronal[2].

1.1.1. Clasificación Clínico-anatómica

Según la clasificación clínico-anatómica, las enfermedades neurodegenerativas se dividen en dos grandes grupos; las demencias y los trastornos del movimiento [4].

Demencia: Los síntomas clínicos son cambios cognitivos y psicológicos, causados por la alteración de las funciones del área superior del cerebro. Anatómicamente las regiones más afectadas son el hipocampo, el córtex entorrinal, el sistema límbico (amígdala, córtex olfatorio, córtex del cíngulo anterior y estructuras subcorticales) y las áreas neocorticales [4, 5].

Trastornos del movimiento: Entre los síntomas más frecuentes se encuentran la hipercinesia, la hipocinesia, la ataxia y la rigidez. Estos

síntomas están relacionados con la disfunción cerebral, encontrando afectados los ganglios basales, el tálamo, el cerebelo, la corteza y las neuronas motoras inferiores de la medula espinal[4].

En algunas formas tempranas de estas enfermedades o durante el curso clínico de ellas se observa una combinación de estos síntomas, dificultando su diagnóstico clínico, siendo éste de gran relevancia principalmente en sus estadios iniciales [6,2,3].

1.1.2. Clasificación Neuropatológica

La clasificación neuropatológica se basa en la evaluación de la pérdida neuronal, la astrogliosis reactiva y las características histológicas en función de la distribución anatómica: Estos cambios se detectan tanto por técnicas de tinciones convencionales como por técnicas de inmunohistoquímica, esta última necesaria para estudiar los depósitos proteicos[4].

La clasificación se realiza en función de la proteína predominante en los depósitos producidos, y se diferencia entre tauopatías, prionopatías, enfermedades por expansión de trinucleótidos, sinucleinopatías[2] y otras formas (Figura 1).

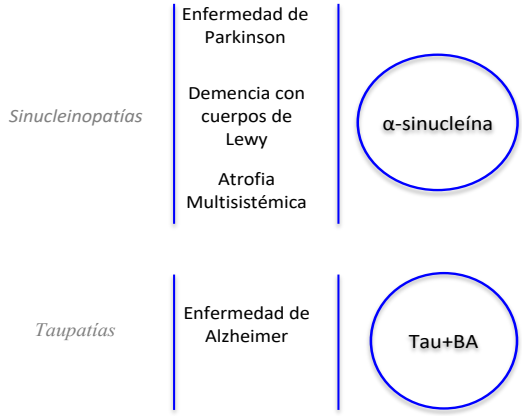

Figura 1. Esquema de las enfermedades neurodegenerativas estudiadas en esta tesis.
Clasificación, según la proteína predominante,en sinucleinopatías (proteína α-sinucleína) y taupatias(proteínas Tau y β-Amiloide)

1.2. Sinucleinopatías

Las sinucleinopatías se caracterizan neuropatológicamente por la agregación de la proteína α-sinucleína. Se clasifican en dos grandes grupos, las enfermedades con cuerpos de Lewy (ECL) y la atrofia multisistémica (AMS)[7]. Mientras que las ECL se caracterizan por la presencia de cuerpos de Lewy intraneuronales, en la AMS las inclusiones son gliales y citoplasmáticas, reciben el nombre de inclusiones gliales citoplasmáticas y se encuentran principalmente en la sustancia blanca[8] (Figura 2).

Los cuerpos de Lewy (CL) fueron descritos en 1912 por el neurólogo alemán Dr. Friedrich Lewy cuando estudiaba la neuropatología de la enfermedad de Parkinson en el laboratorio del Dr. Alois Alzheimer [4]. Pero no se dilucidó hasta la década de los 1990 que la proteína que generaba estos cuerpos de inclusión era la α–sinucleína[9] (AS). Los CL se clasifican morfológicamente en CL clásicos y corticales. Los CL clásicos se encuentran en los núcleos del tronco del encéfalo y el diencéfalo mientras que los CL corticales están presentes en el córtex cerebral y límbico, y en la amígdala[10]. Mientras que los CL clásicos son inclusiones neuronales esféricas con un núcleo denso eosinofílico rodeadas de un halo pálido, los CL corticales carecen de ese halo. Ultraestructuralmente los CL clásicos forman filamentos dispuestos de forma radial y asociados a material granular y vesicular. Mientras que los CL corticales están poco organizados y formados por filamentos de 7-27 nm de grosor[11].

1.2.1. Enfermedades con cuerpos de Lewy

Clínicamente, las enfermedades con cuerpos de Lewy se clasifican en la enfermedad de Parkinson (EP) y la demencia con cuerpos de Lewy (DCL). Las dos se caracterizan por la presencia de CL, cuyo componente principal es la AS[12] (Figura 2).

La AS fue descrita en 1988, después de clonarse a partir de muestras del pez Torpedo Californica, y se la llamó sinucleína debido a que se encontró en las terminaciones nerviosas[1]. La familia de las sinucleínas consta de tres proteínas: α,

β y γ. Son proteínas pequeñas (14-16 kDa) con secuencias de aminoácidos homologas ubicadas en distintos genes.
Hasta el momento se han descrito cuatro isoformas de la α-sinucleína, donde la mayoritaria está constituida por 140 aminoácidos (AS140), mientras que las otras se generan a partir de mRNAs resultado del splicing del exón 3 (AS 112), del exón 5 (AS 126) o de ambos (AS 98) [8,12]. Esta proteína no solamente está involucrada en el desarrollo de la EP, DCL y la AMS, sino también juega un papel importante en otras enfermedades neurodegenerativas como la neurodegeneración por acumulación cerebral de hierro tipo I[2].
La evolución neuroanatómica de las enfermedades con cuerpos de Lewy fue planteada en 1984 por Kosava, quien postuló que progresan y se disponen según un orden anatómico determinado. Así sugirió que las distintas ECL tienen su origen en unas áreas específicas para cada una de ellas y que a través de sus conexiones neuroanatómicas van expandiéndose a las distintas áreas[2]. A partir de esta premisa surgieron distintas clasificaciones que se han ido perfeccionando, analizando cada vez más áreas cerebrales para una correcta clasificación. En la actualidad las dos clasificaciones recomendadas son la de Braak y la de McKeith[13] basándose en la desarrollada inicialmente por Kosava[2,13].

1.2.1.1. Enfermedad de Parkinson.

La enfermedad de Parkinson fue descrita por James Parkinson en 1817 llamándola Parálisis Agitans. Pero no fue hasta 1861 que Jean-Martin Charcot la denominó con su nombre actual en honor a su descubridor [2].
La EP es el trastorno de movimiento progresivo más común en personas mayores de 60 años. Es una enfermedad multisistémica con una degeneración progresiva del sistema nigroestriado, dopaminérgico y una amplia extensión extranigral[2].
El diagnóstico clínico se basa en la triada bradicinesia, temblor en reposo y rigidez[8]. Debido a que la mayoría de estos síntomas están presentes en los ancianos en mayor o menor medida, el diagnóstico de la enfermedad de Parkinson es complejo y como en el resto de las enfermedades neurodegenerativas, no se confirma hasta la autopsia del paciente [2].

La edad es el mayor factor de riesgo para esta enfermedad con una media de edad del inicio de 60 años y con una incidencia que aumenta en el grupo de edad comprendido entre los 70-79 años. Su prevalencia es de 0,5-1% en la población de 65-69 años, aumentando hasta 1-3% en personas mayores de 80 años [14]. Su afectación es mayor en hombres que en mujeres siendo la neumonía la mayor causa de muerte[6]. Con el envejecimiento global de la población actual, y siendo la edad el mayor factor de riesgo se cree que la incidencia aumentará hasta el 30% en el 2030 [15].

La EP es una enfermedad multifactorial esporádica, pero de la que se conocen riesgos ambientales y genéticos[8]. Uno de los factores genéticos más importantes descritos para la EP son mutaciones del gen *GBA1*, que codifica una enzima lisosomal, la glucocerebrosidasa.

Solo entre el 10 al 15% de los casos con EP son formas puramente genéticas. Entre los genes que causan formas autosómicas dominantes se encuentran *SNCA*, gen que codifica la proteína α-sinucleína, y *LRRK2*, que es la causa del 4% de los casos familiares de EP, así como del 1% de los casos esporádicos. Las mutaciones en *PRKN6* y *PRKN2* causan las formas más comunes de la EP recesiva familiar[8]. Estos genes codifican las proteínas PINK1 (PTEN induced putative kinase 1) y parkina, respectivamente, involucradas las dos en la iniciación de la autofagia[15].

Los cambios neuropatológicos más evidentes son la pérdida selectiva de neuronas en la sustancia negra pars compacta y el locus cerúleo. Esta pérdida neuronal se manifiesta por la pérdida de pigmentación en la sustancia negra y es debida a la muerte de las neuronas dopaminérgicas que contienen neuromelanina en esta área cerebral, mientras que la pérdida de neuronas neuroadrenérgicas son las responsables de esta despigmentación en el locus cerúleo[5].

Estudios sugieren que la EP empieza en los núcleos del tronco cerebral inferior, como el núcleo motor dorsal del vago y el sistema olfatorio (estadio 1-2). Posteriormente, su progresión asciende hacia el locus cerúleo, la sustancia negra pars compacta, el núcleo subtalámico y la amígdala (estadio 3-4), y hasta el neocórtex en las fases más avanzadas de la enfermedad (estadio 5-6)[5].

Un 20-50% de los pacientes diagnosticados de EP desarrollan demencia a los 10 - 15 años de su diagnóstico [16]. Estos pacientes se clasifican como enfermedad de

Parkinson con demencia (EPCD), mientras que los que no desarrollan demencia son denominados enfermedad de Parkinson sin demencia (EPSD). La EPCD presenta una distribución de CL más generalizada con un patrón parecido a los pacientes diagnosticados con demencia con cuerpos de Lewy [17].

1.2.1.2. Demencia con cuerpos de Lewy

La demencia con cuerpos de Lewy (DCL) no fue descrita como tal hasta 1996, cuando tuvo lugar el Taller Internacional del Consorcio para la Demencia con cuerpos de Lewy y se marcaron sus criterios diagnósticos. Hasta entonces, esta enfermedad se clasificaba como EP o enfermedad de Alzheimer (EA)[2]. El Consorcio para la Demencia con cuerpos de Lewy marcó una regla, como concepto para distinguir clínicamente la DCL de la EPCD. Si el déficit cognitivo aparece junto a los signos motores durante el primer año de la enfermedad, se considera DCL, mientras que si el deterioro cognitivo aparece después del primer año del debut de los síntomas motores, se diagnostica como EPCD[5].
La DCL es una demencia degenerativa y progresiva, siendo la segunda más frecuente después de la EA. Se ha estimado que afecta a 1,4 millones de americanos y que el factor de riesgo mayoritario es la edad con una mayor afectación en hombres que en mujeres[9]. Además, progresa muy rápidamente con una supervivencia de entre 6 y 10 años [7]. Su diagnóstico es complejo debido a los rasgos clínicos que comparte con la EA, y se estima que la DCL está infradiagnosticada con una clara discrepancia entre el número de casos diagnosticados clínicamente con los que se diagnostican post-mortem[18] . Esto presenta un grave problema debido a que el tratamiento farmacológico para los síntomas conductuales y cognitivos usados para otras enfermedades produce reacciones adversas en hasta el 50% de los pacientes con DCL[6].
A nivel clínico el primer síntoma de la DCL es la demencia acompañada de signos motores al inicio de la enfermedad o durante el primer año de su diagnóstico[2] . Además del parkinsonismo su diagnóstico incluye la presencia de alucinaciones visuales, fluctuación y trastorno de conducta del sueño en fase REM (TCSR)[1, 2].

Los cambios neuropatológicos principales, como en la EP idiopática, son la perdida neuronal en la sustancia negra y el locus cerúleo con la acumulación de CL clásicos en las neuronas restantes[8]. La diseminación de los CL es parecida a la propuesta para al EP[5], y se inicia en los nervios craneales y el sistema reticular, expandiéndose por el encéfalo y el sistema límbico hasta llegar finalmente al neocórtex [8].
Neuropatológicamente la DCL se clasifica por la presencia o ausencia de patología Alzheimer concomitante, donde los casos que presentan patología Alzheimer, se clasifican como demencia con cuerpos de Lewy común (DCLc) y los casos que no la presentan como demencia con cuerpos de Lewy pura (DCLp)[19].

1.2.2. Atrofia multisistémica

La atrofia multisistémica (AMS) fue definida como tal en 1969 por Graham y Oppenheimer, englobando tres entidades neurológicas; la atrofia olivopontocerebelosa, el síndrome de Shy-Drager y la degeneración estriatonigra[l6]. El común denominador de todas ellas es la acumulación de inclusiones de α -sinucleína en el citoplasma glial.
La AMS es una enfermedad neurodegenerativa fatal caracterizada por un fallo autonómico y piramidal progresivo además de parkinsonismo. Aunque tiene una progresión motora muy rápida, queda enmascarada por la EP y por la ataxia cerebelar en las fases más avanzadas de la enfermedad[2].
La incidencia de la AMS se estima de 0,6 a 0,7 casos por 100 000. La prevalencia de esta enfermedad es de un 4.4/ 100 000 habitantes con un incremento hasta el 7,8 por 100 000 en poblaciones mayores de los cuarenta años, según los estudios realizados en EEUU en el año 2015 [2]. Todo y que su causa es desconocida, los factores ambientales no parecen representar un factor de riesgo, siendo considerada una enfermedad esporádica todo y conocerse casos donde los factores genéticos juegan un papel importante.

Los síntomas clínicos más característicos son parkinsonismo, ataxia cerebelar, fallo autonómico y parálisis laríngea, debilidad motora y un declive cognitivo progresivo [6, 20].

Se han descrito dos fenotipos clínicos diferentes de AMS que se distinguen por sus principales signos y síntomas en el momento del diagnóstico. El fenotipo parkinsoniano, conocido como AMS-P, se distingue por parkinsonismo asociado mientras que, en el fenotipo cerebeloso, AMS-C, la característica principal es la presencia de ataxia cerebelar. En los países occidentales el 80% de los casos diagnosticados presentan el fenotipo parkinsoniano, dificultando su diagnóstico diferencial con la EP[7].

La característica anatomopatológica principal es la pérdida de pigmentación en la sustancia negra, el locus cerúleo, el núcleo motor del vago, características que comparte con la EP y con la ECL. Además de una atrofia del cerebelo, del pedúnculo cerebelar y de la base del puente, se observa también astrogliosis y pérdida neuronal4. En las mismas áreas donde se encuentra esta pérdida, se encuentran los llamados cuerpos de Papp-Lantos. Su pobre visualización en la tinción de hematoxilina-eosina, siendo solo detectables con algunas tinciones de plata, dificultan su observación[2].

El diagnóstico neuropatológico de la AMS se realiza por la pérdida neuronal en las áreas del estriado, la sustancia negra, el locus cerúleo, los núcleos de la oliva inferior, la base del puente, el núcleo vago, la columna intermedia de la espina dorsal y el núcleo Onuf y de las células de Purkinje en el cerebelo [2,5].

1.3. Enfermedad de Alzheimer

La enfermedad de Alzheimer fue descrita en 1906 por el psiquiatra alemán Alois Alzheimer, cuando reportó el seguimiento clínico y una descripción de las alteraciones en las fibrillas neurales típicas de la EA por primera vez. Pero no fue hasta 1910 cuando Emil Kraepelin categorizó la EA como un subtipo de demencia senil. Durante parte del siglo XX, su diagnóstico se limitó a pacientes con demencia con edades comprendidas entre los 45 y los 67 años, mientras que los pacientes mayores de esta edad se clasificaban como demencia senil. No fue

hasta 1977, debido a sus similitudes tanto patológicas como clínicas, que estas dos entidades se agruparon bajo una misma patología independientemente de la edad del paciente[2].

La EA es un trastorno neurodegenerativo progresivo e irreversible de origen desconocido. Es la demencia más común en la población mundial mayor de 65 años, siendo de un 50 a 75% de todas las demencias diagnosticadas e incrementando su incidencia con la edad con un claro aumento en pacientes mayores de 85 años[21]. Su elevada prevalencia y el envejecimiento de la población a nivel mundial la convierten en un importante problema de salud pública, llegando a ser denominada la pandemia del siglo XXI. Los tratamientos actuales palian los síntomas, permitiendo una mejor calidad de vida, pero no son capaces de frenar la progresión de la enfermedad [22]. Aunque la mayoría de los casos de EA son esporádicos, entre un 5-10% de los casos se deben a variantes genéticas dominantes correspondientes a la proteína precursora del beta-amiloide (APP), la presenilina 1 y la presenilina 2 (PSEN1 y PSEN2). Además, se han descrito variantes alélicas en numerosos genes adicionales que conllevan un aumento de la susceptibilidad de sufrir esta patología[23].

Los síntomas clínicos incluyen una demencia progresiva caracterizada por la pérdida de memoria a corto plazo, la capacidad de orientación y de las funciones cognitivas superiores como la dificultad en el lenguaje, la perdida de atención y función visuoespacial. Así como la capacidad de resolver problemas y la agilidad mental para el razonamiento debido a una progresiva alteración funcional del lóbulo parietal. En estadios avanzados de la enfermedad y debido al extenso deterioro cortical y subcortical los pacientes se deterioran hasta quedar inmóviles y mudos[2].

A nivel neuropatológico, la EA presenta cuerpos de inclusión del péptido β-amiloide (BA) en el parénquima extracelular llamados placas neuríticas. Además, se observa la agregación de la proteína tau hiperfosorilada en forma de ovillos neurofibrilares intraneuronales. La densidad y localización de estas últimas representan uno de los criterios diagnósticos determinantes. Se ha descrito también pérdida sináptica y, en fases avanzadas de la enfermedad, de neuronas corticales[6].

Las placas neuríticas están formadas por pequeños péptidos de 39-43 aminoácidos de BA, producto de la digestión proteolítica de la proteína APP. Esta es una proteína transmembrana cuyo procesamiento puede transcurrir por dos vías opuestas, la no-amiloidogénica y la amiloidogénica. La vía no-amiloidogénica está controlada por las a- y ϒ-secretasas dando lugar a fragmentos intracelulares indispensables para el crecimiento, supervivencia y reparación de las neuritas. En la vía amiloidogénica, APP se proteoliza mediante b- y g-secretasa produciendo péptidos de AB, donde el 90% es AB40 y aproximadamente el 10% es la forma más fibrilogénica AB42. Sin embargo, en situaciones patológicas o en individuos ancianos se observa un desequilibrio en la ratio de producción de AB con un aumento significativo de los péptidos de AB42 y AB43 causando la acumulación de los mismos[21]. Estos péptidos tienden a agruparse en fibras en el exterior de la neurona, formando las llamadas placas neuríticas o placas seniles[2].

Los ovillos neurofibrilares están formados por hebras de proteína tau hiperfosforilada. La proteína tau está presente específicamente en neuronas, es parte estructural de los microtúbulos axonales y regula su estabilidad. En la EA tau se hiperfosforila, y las moléculas de proteína tau hiperfosforilada se unen entre ellas formando primero filamentos helicoidales emparejados que más tarde dan lugar a la formación de los ovillos neurofibrilares[6]. Al mismo tiempo este proceso da lugar a la desestabilización de los microtúbulos y así a cambios irreversibles en el citoesqueleto de las neuronas.

La clasificación de la EA se realiza mediante estadios según la distribución de los ovillos neurofibrilares y en fases según la distribución de los depósitos de BA. Así se observa que la primera área afectada por la acumulación de la proteína tau es el córtex transentorrinal (estadio I) expandiéndose hacia el córtex entorrinal (fase II) para llegar al giro temporal inferior y medio (fase III y IV) para afectar finalmente al córtex occipital (fase V y VI)[24] .

El desarrollo de la patología asociada a la BA se divide en cinco fases dependiendo del área cerebral afecta. En la fase I se detecta afectación isocortical, en la fase II los depósitos de BA se observan en el hipocampo y córtex entorrinal, en la fase III en los ganglios basales y el diencéfalo, en la fase IV en el tronco cerebral en y en el cerebelo en la fase V [5].

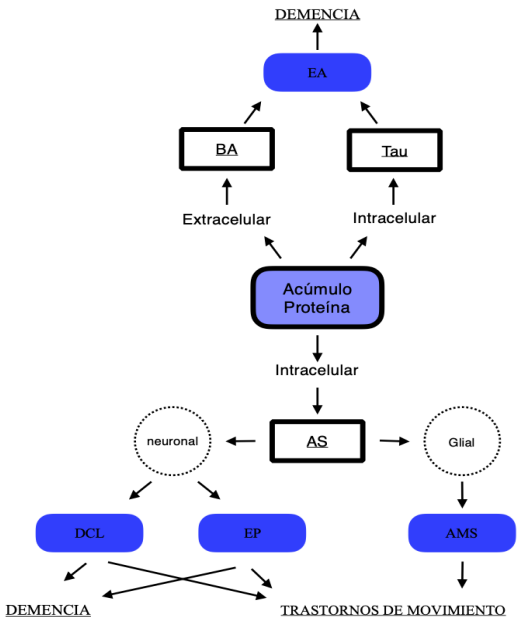

Figura 2. Esquema-resumen de las enfermedades neurodegenerativas. (adaptado de Current Concepts of neurodegenerative diseases de G. G. Kovacs, Cambridge University Press, 2015)

2. Sistema Lisosomal

El reciclaje y la eliminación de las proteínas anómalas de la célula se realizan mediante los sistemas ubiquitina-proteosoma y autofágico lisosomal[23] . Mientras que en el sistema ubiquitina-proteosoma la unión de una ubiquitina a la proteína correspondiente permite el transporte de esta hasta el proteosoma para su eliminación [25], en el sistema autofágico lisosomal, las proteínas se eliminan a través de los lisosomas[26]. En la macroautofagia, el lisosoma se une a un complejo denominado autofagosoma mientras que en la microautofagia el lisosoma sufre una invaginación en su membrana que permite la internalización de las proteínas. En el tercer tipo de autofagia, la autofagia regulada por chaperonas, las proteínas se interiorizan en los lisosomas a través de un complejo de chaperonas[26].

2.1. Lisosomas

En 1955 De Duve describió los lisosomas como organelas que desempeñan una función en la degradación de sustratos y el reciclaje [27].

Más recientemente se identificaron como un punto clave en el metabolismo y como parte activa en procesos como la regulación de algunos genes, la reparación de la membrana plasmática, la homeostasis, la respuesta inmune y el transporte de colesterol entre otros[28].

Los lisosomas son organelas compuestas por una bicapa lipídica que contiene más de 120 proteínas, con un lumen ácido (pH 6,5-4,5) donde se encuentran las hidrolasas lisosomales[26] , de las que actualmente se conocen más de 60. Las hidrolasas son responsables del catabolismo de las moléculas complejas, tanto endógenas como exógenas, y entre estas hidrolasas se encuentran nucleasas, lipasas, glicosilasas, fosfatasas, sulfatasas y proteasas. Las proteínas de la membrana lisosomal no son solamente necesarias para el funcionamiento de los lisosomas, ya que mantienen el pH ácido de su interior, sino también regulan el transporte hacia el interior de los lisosomas y permiten su fusión con las membranas de otras organelas[26].

Algunas de las mutaciones en los genes que codifican enzimas lisosomales o proteínas que participan en la maduración de los lisosomas provocan una acumulación de distintas sustancias no metabolizadas en el interior del lisosoma. Existen más de 50 distintas patologías que se denominan enfermedades de almacenamiento lisosómico [26], estando la mayoría de ellas relacionadas con patologías del sistema nervioso central [28,29,30]. De las enfermedades de almacenamiento lisosómico, la mayoritaria es la enfermedad de Gaucher[29].

2.2. Enfermedad de Gaucher

La enfermedad de Gaucher (EG) es una enfermedad autosómica recesiva provocada por un déficit en la enzima lisosomal glucocerebrosidasa (GCasa)[31], [32]. Esta es la responsable del catabolismo del glucocerebrósido en glucosa y ceramida[33], y mutaciones en el gen *GBA*, que codifica la GCasa, causan su acumulación en el interior de los lisosomas, principalmente en los macrófagos de distintos órganos[34].

La clasificación de la EG se basa en la presencia o ausencia de características neurodegenerativas y su progresión clínica. Mientras que la EG tipo I se caracteriza por hepatoesplenomegalia, anemia, trombocitopenia y lesiones óseas,

la EG tipo II y III tienen un carácter marcadamente neuronopático con un declive neurológico progresivo[35]. Tradicionalmente se consideraba la EG tipo I como no neuronopática pero este concepto cambió debido a la asociación de esta con la EP[36].

Fue en el año 2004 que se relacionaron mutaciones de *GBA* con la EP, al observarse que una parte de los pacientes con EG tipo I desarrollaban EP en la edad adulta[37]. Mientras que en la población mayor de 80 años solo el 3-4% sufren EP, en los pacientes de EG tipo I este porcentaje aumenta hasta el 10%[40]. Además, diversos estudios demostraron, que los familiares de primera generación de pacientes con la EG tipo I, tienen un mayor riesgo de sufrir EP que el resto de la población[30,36]. También se observó, que en pacientes con EP de origen judío Ashkenazi, la frecuencia de mutaciones en *GBA* es de un 15-20%, mientras que en la población sin EP es de un 3%[38]. Estos estudios postularon las mutaciones de *GBA* como un factor de riesgo a padecer la EP[34,39].

2.3. *GBA*

El gen *GBA*, se localiza en el cromosoma 1q22, se extiende a lo largo de 7.6 kb y está constituido por 12 exones. Cinco transcritos distintos son el resultado del splicing alternativo del gen *GBA* y estos dan lugar a tres isoformas proteicas[34.] La desregulación del splicing alternativo de *GBA*, se ha descrito como uno de los mecanismos de desarrollo de la EP[32]. Además de esta desregulación, se han identificado más de 300 mutaciones de este gen como mutaciones con desplazamiento del marco de lectura, mutaciones que afectan el sitio del splicing o mutaciones puntuales, además de inserciones y alelos recombinantes[33].

Los transcritos *GBAtv1*, *tv2* y *tv3* originan la misma proteína, la isoforma1 de la GCasa (GCasa-IF1) todo y tener distintos exones iniciales, mientras *GBAtv4* codifica una proteína más corta al carecer de los exones 2 y 3. Por último, *GBAtv5* no contiene el exón 5 y origina la proteína GCasaIF3 (Figura 3 y 7).

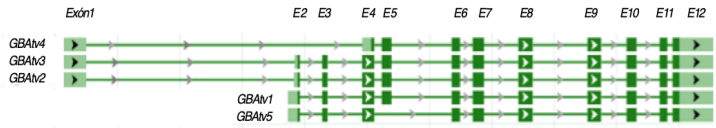

Figura 3. Representación esquemática de los transcritos de *GBA*1.
Los 12 exones están indicados con la letra E, mientras que los intrones están representados por las líneas que unen los exones de cada transcrito (https://www.ncbi.nlm.nih.gov/gene/2629).

La enzima GCasa se sintetiza en el retículo endoplasmático rugoso y es dirigido a través del aparato de Golgi hacia el lisosoma mediante su unión específica a la proteína de membrana lisosomal-2[40](LIMP-2) (Figura 8). La acumulación de glucocerebrósido en los lisosomas, característico de la EG, provoca una disfunción del sistema autofágico lisosomal[41,42], reduciendo su capacidad de eliminación de las proteínas, entre ellas AS. Se han postulado dos hipótesis que pretenden explicar la relación entre las mutaciones de *GBA* y la acumulación de AS. Según la primera, denominada modelo de pérdida de función, la reducción de la actividad de GCasa provoca una acumulación de glucocerebrósido en los lisosomas que promueve la oligomerización de AS y su acumulación en forma de fibrillas. Estas fibrillas al mismo tiempo inhibirían la actividad GCasa creando un bucle patogénico bidireccional. La segunda hipótesis, la hipótesis de ganancia de función, implica que la enzima aberrante participa directamente en la agregación de AS, o a través de una interacción bioquímica con AS, o mediante la interferencia con las vías de homeostasis de AS [23,43]. Esta alteración de las vías de eliminación de AS no solo se han encontrado en la EP sino también en la DCL [44]. Los principales actores de la regulación de GCasa son LIMP2 codificado por el gen *SCARB2* y el factor de transcripción EB (Tfeb) codificado por el gen *TFEB*.

2.4. LIMP2

El gen *SCARB2*, que codifica a la proteína LIMP2, se localiza en el cromosoma 4q21.1, contiene 12 exones y de todos los transcritos generados solo dos han sido validados, SCARBtv1 y SCARBtv2. SCARBtv2 transcribe una proteína más corta que LIMP2 denominada LIMP2-IF2 (Figura 4 y 7).

Figura 4. Representación esquemática de los transcritos de SCARB.
Los 12 exones están indicados con la letra E, mientras que los intrones están representados por las líneas que unen los exones de cada transcrito (https://www.ncbi.nlm.nih.gov/gene/950).

LIMP2 es una de las cuatro proteínas más comunes de membrana lisosomal [45,46]. Es una proteína transmembrana tipo III con dos dominios hidrofóbicos transmembrana y los extremos N- y C-terminales localizados en el citoplasma[47]. Además, LIMP2 contiene un gran dominio luminal donde se encuentra la región de unión a GCasa identificada en los aminoácidos 150-167, y la región responsable del direccionamiento hacia los lisosomas[46,48] (Figura 5).

La unión entre LIMP2 y GCasa es dependiente del pH, uniéndose en el retículo endoplasmático donde el pH es neutro y transportándose unidas por el aparato de Golgi hasta el lisosoma donde el pH ácido de estos permite su separación[48,49]. En situaciones fisiológicas normales, LIMP2 se incorpora a la membrana una vez alcanzado el lisosoma (Figura 8).

Además de jugar un rol básico en la biogénesis y el mantenimiento de los lisosomas y de los endosomas tardíos[46] LIMP2 también está involucrado en la formación de los autofagolisosomas que se generan mediante la fusión de los lisosomas y los autofogosomas[50].

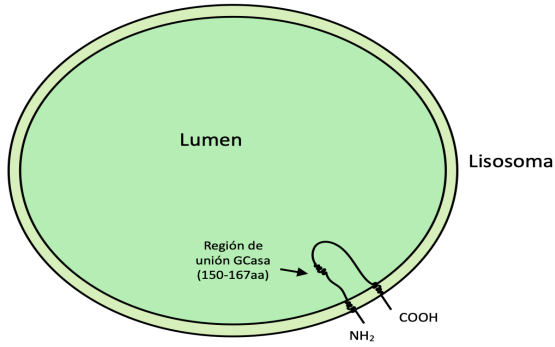

Figura 5. Representación esquemática de LIMP2.
LIMP2 es una proteína transmembrana con los extremos N y C terminal en el citoplasma mientras que en el dominio luminal se encuentra la región de unión con la GCasa.

2.5. Tfeb

El gen *TFEB* codifica a la proteína Tfeb, se localiza en el cromosoma 6p21.1. Cinco de los transcritos que se generan por splicing alternativo se han validado, y mientras que *TFEBtv1-4* incluye diferentes exones iniciales, *TFEBtv5* carece del exón 3 (Figura 6 y 7).

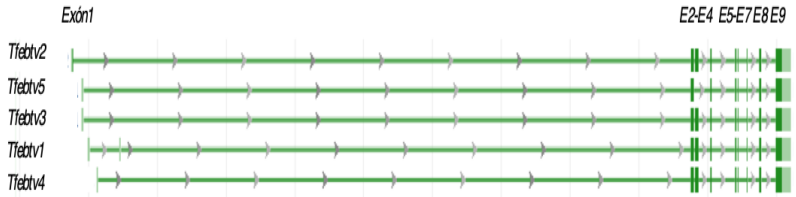

Figura 6. Representación esquemática de los transcritos de Tfeb.
Los exones están indicados con la letra E, mientras que los intrones están representados por las líneas que unen los exones de cada transcrito (https://www.ncbi.nlm.nih.gov/gene/7942).

Tfeb es uno de los reguladores de la biogénesis y función del sistema lisosomal[25]. Esta coordinación primordial de los lisosomas, tanto en situaciones fisiológicas como patológicas, ha provocado su estudio en diversidad de enfermedades entre ellas las neurodegenerativas o el cáncer [51].

Recientemente se ha descrito que Tfeb promueve la transcripción de muchos genes lisosomales entre ellos *GBA* y *SCARB2* [36]. Esta regulación se realiza mediante la unión directa de Tfeb a regiones promotoras definidas que comparten todos los genes que forman parte de la red coordinada de expresión y regulación lisosomal, o CLEAR (Coordinated Lysosomal Expression and Regulation). Estas regiones, denominadas elemento CLEAR son palindrómicas (CACGTG) y se encuentran cerca del lugar de comienzo de la transcripción pudiendo haber uno o más elementos CLEAR en cada promotor[51]. Además de estar involucrado en la regulación del sistema lisosomal, Tfeb también está relacionado con la formación de los autofagosomas y su fusión con los lisosomas[52].

Tfeb se regula a través del gen *mTOR* que, en condiciones fisiológicas normales, fosforila distintos residuos de serina de Tfeb impidiendo su migración al núcleo[53].

En situaciones de deprivación, esta inhibición desaparece permitiendo que Tfeb se traslade al núcleo de la célula donde se une a los genes que contienen elementos CLEAR iniciando su transcripción. Cuando se une a *GBA* provoca el aumento de la expresión de este, y por lo tanto, una mayor actividad GCasa[52]. Como la disfunción del sistema autofágico lisosomal contribuye a la patogénesis de las enfermedades neurodegenerativas no está esclarecido aún. Pero se sabe, que AS se degrada a través del sistema ubiquitina proteosoma y el sistema autofágico lisosomal en condiciones fisiológicas[54].

Gen	Transcritos	Proteína
GBA	*GBAtv1*	GCasa IF1
	GBAtv2	
	GBAtv3	
	GBAtv4	GCasa IF2
	GBAtv5	GCasa IF3
SCARB2	*SCARBtv1*	LIMP2-IF1
	SCARBtv2	LIMP2-IF3
TFEB2	*TFEB2tv1*	Isoforma 1
	TFEB2tv2	Isoforma 2
	TFEB2tv3	Isoforma 1
	TFEB2tv4	
	TFEB2tv5	Isoforma 3

Figura 7. Transcritos y proteínas de los genes *GBA*, *SCARB2* y *TFEB*2.

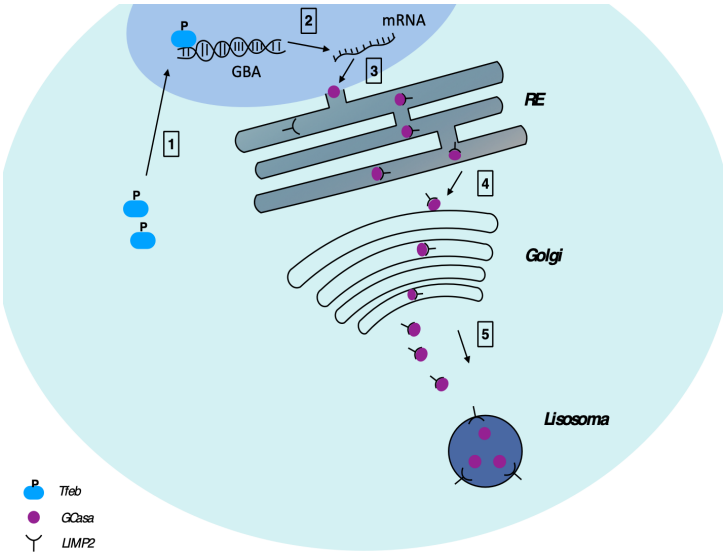

Figura 8. Representación esquemática del sistema lisosomal.
1-Tfeb fosforilado viaja al núcleo donde se une a la región CLEAR de algunos genes, como *GBA*. 2- *GBA* sintetiza la proteína GCasa. 3-Viaja por el retículo endoplasmático donde se une a LIMP2. 4/5-GCasa y LIMP2 viajan a través del aparato de Golgi hasta los lisosomas donde LIMP2 se incorpora en la membrana y GCasa es liberada al lumen.

3. Autofagia

Después del descubrimiento de los lisosomas, DeDuve describió una de las vías por la que las células digieren los constituyentes de su propio citoplasma a través de estos[55]. Este proceso fue denominado autofagia (auto=propio, phagy=comer) y aunque actualmente hay descritos distintos mecanismos y tipos, todos ellos tienen como punto común la degradación de proteínas mediante las hidrolasas lisosomales [56, 57].

La autofagia juega un rol importante en la homeostasis celular, en la degradación de proteínas de larga vida, de los agregados proteicos y de las organelas dañadas[58]. Además, actúa como respuesta celular en situaciones patológicas como la deprivación de nutrientes o las infecciones por patógenos[59]. También se ha demostrado que su desregulación o su funcionamiento alterado está relacionado

con distintas enfermedades, entre las que se encuentran las enfermedades neurodegenerativas[60,61].

La autofagia, junto con el sistema ubiquitino-proteosómico es el sistema de eliminación de proteínas mal plegadas y de organelas dañadas de la célula. En el caso de las proteínas mal plegadas, son las proteínas de shock térmico (heat shock proteins, hsp) que intervienen directamente en la reparación de los errores introducidos durante la traslación y la postraducción [62]. Cuando estos errores superan las capacidades funcionales de las hsp, las proteínas mal plegadas son eliminadas a través del sistema ubiquitino-proteosómico o por la vía de la autofagia mediada por chaperonas. Cuando estos dos sistemas no son lo suficientemente eficiente, se activa otra de las vías autofágicas, la macroautofagia. Correspondientemente, los oligómeros, agregados solubles u organelas, al ser demasiado grandes para poder eliminarse por el sistema ubiquitino-proteosómico, son siempre degradados a través de la vía autofágica [63].

La autofagia además se denomina fábrica de reciclaje celular, ya que los aminoácidos, producto de la degradación proteica son devueltos al citoplasma, donde representan una fuente para la formación de nuevas macromoléculas. Además, la autofagia promueve la eficiencia energética mediante la generación de ATP y sus niveles son una medida de control del daño celular [64].

Los tres tipos principales de autofagia mencionados en el párrafo anterior son la macroautofagia, la microautofagia y la autofagia mediada por chaperonas (AMC) [65] (Figura 9).

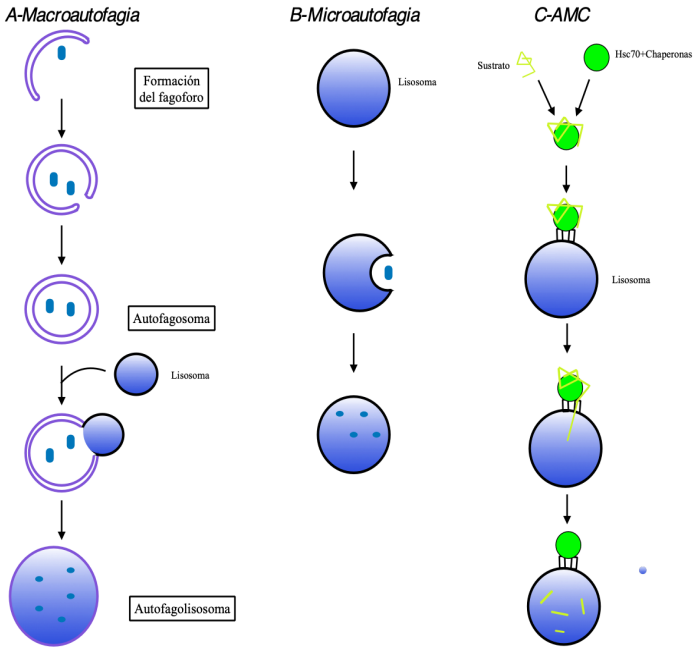

Figura 9. Esquema de las distintas vías de la autofagia.
A-La macroautofagia se inicia con la formación del fagóforo que envuelve el cargo a degradar, la doble membrana crece hasta cerrarse formando el autofagosoma que se une al lisosoma formando el autofagolisosoma donde se hidroliza el cargo. B- En la microautofagia es el lisosoma directamente el que se invagina introduciendo el cargo en su interior para la degradación de este a través de las hidrolasas. C-En la AMC, la proteína Hsc 70, reconoce la proteína a degradar transportándola hasta el lisosoma para su hidrólisis.

3.1. Macroautofagia

En condiciones fisiológicas, agregados proteicos, así como organelas dañadas se degradan mediante la macroautofagia.

La autofagia se inicia con la formación de una doble membrana denominada fagóforo de la que se desconoce el origen exacto, aunque se cree que deriva del retículo endoplasmático, la mitocondria o el aparato de Golgi[66]. A medida que avanza la formación del fagóforo, éste envuelve en su interior el cargo a eliminar, formando una estructura denominada autofagosoma[67]. Una vez cerrado el autofagosoma se une a los lisosomas formando el autolisosoma, donde el cargo se degrada mediante las hidrolasas lisosomales [65] (Figura 15).

Los autofagosomas fueron observados por primera vez a través de microscopía electrónica en el año 1968, pero no se empezaron a estudiar a nivel molecular

hasta hace una década con el análisis de distintas levaduras entre las que destaca Saccharomyces cerevisiae[68]. Fue en esta levadura dónde se identificó un grupo de genes denominado ATGs (autophagy related genes), cuyos productos están involucrados en distintos puntos clave de la autofagia. Además, la mayoría de estos genes tienen sus homólogos en los eucariotas superiores, sugiriendo que la autofagia es un mecanismo altamente conservado evolutivamente [69,70].
Existen diversas fases críticas en la macroautofagia como son la inducción, la formación del fagóforo y la elongación del autofagosoma [71].

3.1.1. Inducción

El nivel basal de la autofagia es muy bajo [72] en condiciones fisiológicas, por lo que es crucial que este sistema responda extremadamente eficiente a situaciones de estrés y otras señales extracelulares[70]. Uno de los inhibidores principales y más conocidos de la autofagia es la proteína serina/treonina kinasa mTOR (mammalian target of rapamycin)[64].

mTOR está involucrada en la mayoría de las vías reguladoras de la autofagia y es responsable del control de los cambios relacionados con la disponibilidad de los nutrientes y la energía metabólica. mTOR inhibe la autofagia a través de dos mecanismos[73]. Por un lado, actúa en las cascadas involucradas en la autofagia tanto en la traducción como transcripción[74] y por otro, actúa directa o indirectamente sobre proteínas ATG involucradas en la formación del autofagosoma[73,75].
En la autofagia participan dos complejos principales, relacionados entre si, el complejo ULK y el PI3k[76]. El primero de ellos está regulado por mTOR, que fosforila a ULK1 (Unc-51-like kinase 1) inhibiendo la unión de esta a las otras proteínas involucradas en la constitución del complejo ULK, cuya formación es indispensable para el inicio de la autofagia. Por el contrario, la inhibición de mTOR resulta en la desfosforilación de ULK1 provocando una cascada de reacciones resultando en un cambio conformacional del complejo y la inducción de la autofagia[64]. Además de la fosforilación de ULK1, mTOR, desencadena la translocación del segundo complejo proteico, PI3K[70]. También, mTOR fosforila *TFEB* inhibiendo así la biogénesis lisosomal[57] (Figura 10).

3.1.2. Formación del fagóforo

Mientras que en las levaduras la formación del fagóforo está precedida de una estructura citosólica denominada estructura pre autofagosomal (PAS), en los mamíferos no hay evidencia de esta[68].

La elongación del fagóforo se inicia con la formación de dos complejos principales mencionados en el apartado anterior, el complejo ULK y el complejo PIK3 [76].

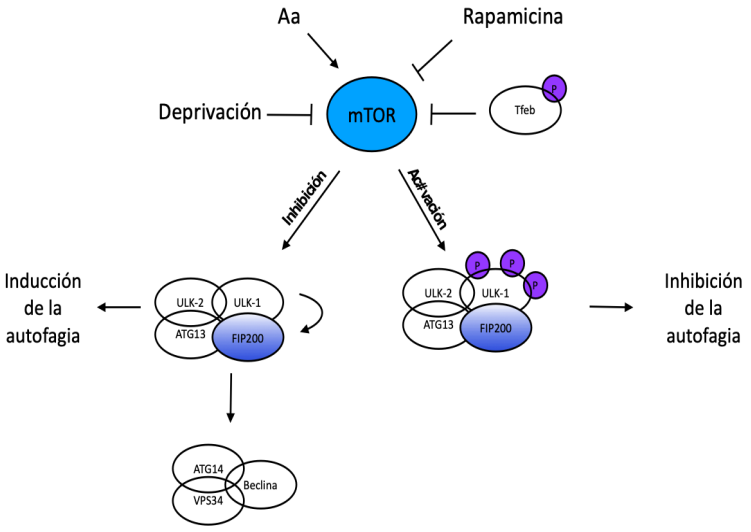

Figura 10. Esquema de la interacción de mTOR con su entorno en la autofagia. mTOR se activa o inhibe por algunos sustratos tal y como se observa en la parte superior del esquema. Esto genera la activación o inhibición del complejo ULK. Sí ULK1 es fosforilado, se inhibe la autofagia mientras que, si ULK1 no es fosfolirado, éste activa otras proteínas de su complejo que a posteriori activaran el núcleo I3K.

El complejo ULK está formado por las proteínas ULK1/2 (Unc-51-like kinase 1 y Unc-51-like kinase 2), ATG13 y FIP200 (focal adhesión kinase family-interacting protein). Este complejo está regulado principalmente por mTOR que en condiciones fisiológicas bloquea su actividad[57]. Por el contrario, en situaciones de estrés celular como por ejemplo concentraciones reducidas de aminoácidos específicos, factores de crecimiento, ATP, hipoxia, o en la presencia de ciertos tipos de agregados proteicos, mTOR se inhibe permitiendo así la desfosforilación de ULK1. Esta proteína junto con ULK2 fosforila Atg13 y FIP200 provocando un

cambio conformacional del complejo y la inducción de la autofagia, al fosforilar Beclina (BECN1)[74].

El segundo complejo involucrado en la formación del fagóforo es PI3K compuesto por Vps34 (vacuolar protein sorting 34), BECN1 y Vps15 (vacuolar protein sorting 15)[73]. Este complejo es uno de los puntos más críticos de la formación del autofagosoma, donde BECN1 tiene un papel de proteína adaptadora interaccionando con otras proteínas para estimular o inhibir la autofagia dependiendo de qué proteínas se unan a esta. Las interacciones de BECN1 se realizan en los tres dominios que conforman la proteína[76](Figura 10). En el dominio N-terminal BH3 se unen los miembros de la familia Bcl-2 y también es el lugar de formación de dímeros con una actividad principalmente inhibidora[77]. Mientras que en el dominio central CCD, se unen proteínas activadoras del complejo como Ambra1, UVRAG/Atg14L (UV radiation resistance-associated gene y autophagy related gene 14). Por último, el domino ECD está muy conservado evolutivamente [78], siendo indispensable para mediar en la autofagia u otras funciones como la inhibición de la tumorogénesis. BECN1 puede interaccionar con las proteínas en más de un dominio, como en el caso de Vps34 que se une tanto en ECD como en CCD79 (Figura 11). Es la fosforilación de BECN1 la que aumenta la actividad del complejo permitiendo el reclutamiento de otras proteínas al complejo original como UVRAG o Atg14 necesarias para la formación de la membrana[80]. Por el contrario, sí se une la proteína Rubicon se inhibe la formación de la membrana (Figura 12). Por este motivo, los niveles de expresión y las modificaciones postraduccionales de BECN1 son considerados un indicador del control de degradación autofágica[76].

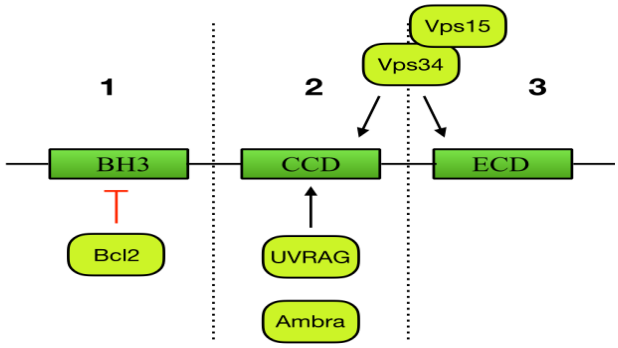

Figura 11. Esquema de la proteína Beclina 1.
Están representados los tres dominios principales (rectángulos verdes) de la proteína BECN1, BH3, CCD y ECD. Para cada uno de ellos están indicados en amarillo las proteínas que interaccionan activando o inhibiendo a BECN1.

Vps34 está involucrada en la formación de distintas membranas celulares. En el caso de la doble membrana del fagóforo su implicación es selectiva y clave para la formación del complejo con BECN1. Además, Vps34 es única entre las PI3-quinasas en el uso de fosfatidilinositol (PI) como sustrato para generar fosfatidil inositol trifosfato (PI3P)[81]. Este sustrato es indispensable para la elongación del fagóforo y para la unión de otras proteínas ATG a este. La interacción de BECN1 con Vps34 promueve la actividad catalítica de esta última e incrementa los niveles de PI3P[82].

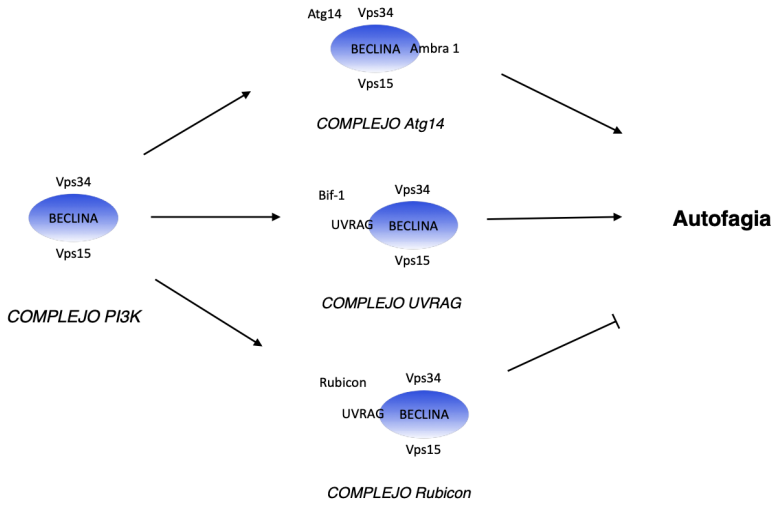

Figura 12. Esquema de los complejos formados por PI3K.
Dependiendo de qué proteínas se unan al complejo inicial PI3K se activará o inhibirá el inicio de la autofagia.

3.1.3. Elongación del autofagosoma

La extensión y el cierre del autofagosoma se realizan a través de dos sistemas de ubiquitinización relacionados entre ellos[70]. Las Ubl (ubiquitin-like proteins) son una familia de pequeñas proteínas involucradas en las modificaciones postraduccionales que modifican otras proteínas normalmente con una función reguladora.

En los sistemas de ubiquitinación participan tres enzimas mediadoras denominadas E. La enzima activadora E1 usa ATP para activar la ubiquitina para la conjugación y la transfiere a una enzima E2. Esta interactúa con la ubiquitina ligasa E3 y transfiere la ubiquitina a la proteína diana[83]. Tanto Atg12 (autophagy related gene 12) como LC3 I (microtubule-associated proteins 1A/1B light chain 3), se ubiquitinan de manera similar y ambas se activan a través de Atg7 (autophagy related gene 7) la enzima activadora E1. Atg12 es conjugada por la Atg10 (autophagy related gene10) como E2 y esta permite la unión covalente a una lisina de la proteína sustrato, Atg5[64] (autophagy related gene 5). Esta conjugación Atg12-Atg5 es constitutiva e irreversible, y aparentemente no requiere de una ligasa E3 para su proceso (Figura 13).

El complejo Atg12-Atg5 a posteriori interacciona con la proteína Atg16 (autophagy related gene 16) formando un complejo que se oligomeriza formando un tetrámero[84]. Esta asociación se considera la responsable de la curvatura del fagóforo con su reclutamiento asimétrico de LC3 II [71].

El segundo sistema de ubiquitinación involucrado en la formación de los fagóforos es un sistema constituido por la proteína LC3 I [64]. LC3 se convierte en LC3 I mediante proteólisis[85] y Atg7 activa una ubiquitina y Atg3 es la mediadora de su conjugación a la fosfatidiletanolamina (PE), uno de los fosfolípidos más abundantes en las membranas celulares[70]. La enzima E3 es el complejo Atg12-Atg5 y convierte la LC3 I en LC3 II (Figura 14). Cuando el fagóforo está cerca de su cierre, el complejo Atg5-Atg12 se disocia de la membrana mientras que LC3 II queda asociada a ella, a la cara interna como externa de los autofagosomas. En esta ubicación juega un importante rol en la selección del cargo a eliminar, siendo la única proteína especifica asociada a la membrana de los autofagosomas[85] (Figura 15).

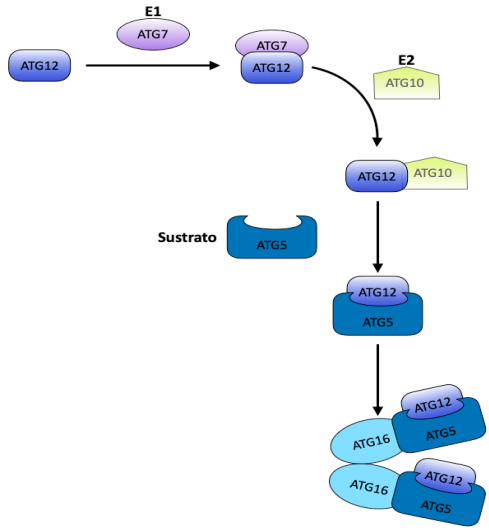

Figura 13. Esquema del sistema de conjugación Atg12-Atg5.
En este sistema Atg12 se conjuga con la enzima activadora Atg7 para unirse posteriormente a la enzima conjugadora Atg10 que permite la unión de Atg12 al sustrato Atg5. Al complejo Atg12-Atg5 se le une la proteína Atg16, formando a través de esta unión de hexámeros de Atgs.

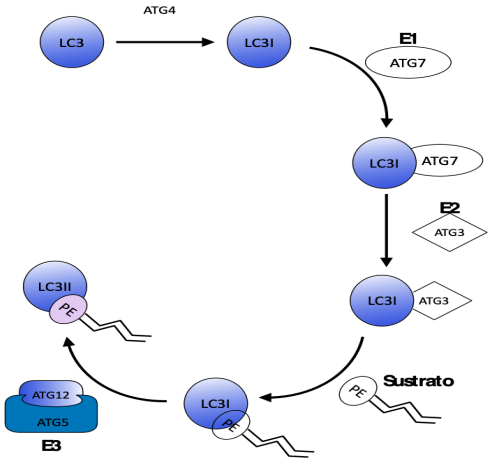

Figura 14. Esquema del sistema de conjugación LC3.
LC3 se convierte en LC3 I a través de su unión a Atg4. LC3 I se une primero a la enzima activadora Atg7 y posteriormente a la enzima conjugadora Atg3, para permitir la unión de LC3 al sustrato PE. En este sistema participa la enzima E3 (Atg5-12-16) y se transforma LC3 I en LC3 II.

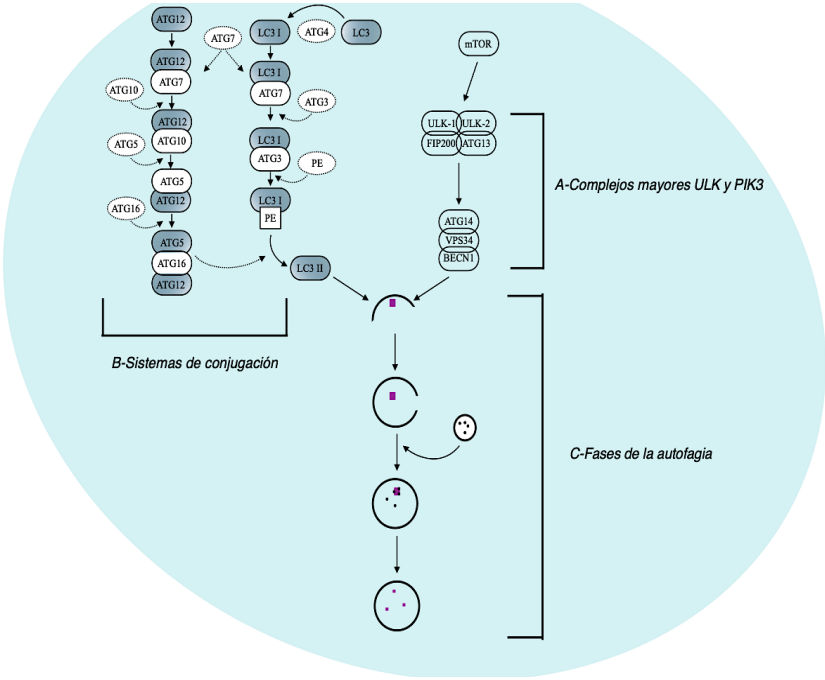

Figura 15. Representación esquemática de la macroautofagia.
A-Formación de los complejos ULK y PI3K como complejos necesarios para el inicio de la macroautofagia. B-Formación de los sistemas de conjugación Atg12-Atg5-Atg16 y LC3, necesarios para la formación y elongación del fagóforo. C-Representación de las distintas fases de la autofagia, desde el fagóforo hasta el autolisosoma donde se degrada el cargo.

3.2. Microautofagia

La microautofagia es un proceso muy similar a la macroautofagia pero en esta, los lisosomas se invaginan directamente. En el interior, el cargo es degradado por las vesículas alojadas en el lumen [71]. La microautofagia, igual que la macroautofagia, es capaz de degradar los oligómeros, agregados solubles u organelas, tanto de manera selectiva como no selectiva [86]. La degradación selectiva incluye la de organelas dañadas o senescentes. Por el contrario, la degradación no selectiva, que no se activa por deprivación nutricional ni por estrés celular[56], es una rotación continua de los constituyentes intracelulares.

3.3. Autofagia mediada por chaperonas

La autofagia mediada por chaperonas (AMC) se diferencia completamente de la macroautofagia y la microautofagia. El 30% de las proteínas citosólicas que contienen el pentapéptido especifico (KFERQ)[56] , se degradan a través de esta autofagia. Esta secuencia de aminoácidos es reconocida por la chaperona citosólica hsc70 (heat-shock protein 70), se une a ella y a otras proteínas formando un complejo y este se traslada a los lisosomas. La proteína de membrana lisosomal LAMP2A (Lysosome-associated membrane protein 2) reconoce a este complejo proteico y es responsable de su disociación y la translocación de la proteína reconocida por hsc70 al interior de los lisosomas[62]. A diferencia de la macroautofagia y la microautofagia donde el transporte de los sustratos es por invaginación o por secuestro del cargo, en la AMC este transporte es proteína a proteína[74.] Además, la AMC está activa en condiciones basales pudiendo aumentar en situaciones de estrés y en periodos de deprivación prolongados. Mientras que la macroautofagia es inducida principalmente en periodos cortos de deprivación.

2. OBJETIVOS

Objetivo general

Analizar los niveles de transcripción de los genes lisosomales *GBA* y sus reguladores *TFEB* y *SCARB2*, así como de 6 genes relacionados con la autofagia mediante de análisis de la expresión del mRNA, en cerebro y sangre de pacientes con enfermedades con cuerpos de Lewy.

Objetivos específicos

1. Analizar los niveles de expresión del transcrito mayoritario de *GBA* en cerebro y sangre de pacientes con enfermedades con cuerpos de Lewy en comparación con controles.
2. Evaluar la implicación del splicing alternativo del gen *GBA* en las distintas enfermedades estudiadas mediante análisis de expresión de los transcritos minoritarios.
3. Identificar una posible correlación entre los niveles de expresión del gen *GBA* y la edad, sexo y duración de la enfermedad.
4. Analizar la expresión de mRNA de los genes *SCARB2* y *TFEB* en cerebros de pacientes con ECL en comparación con controles.
5. Analizar el posible papel de las diferentes formas del splicing alternativo en los cambios de expresión de *SCARB2* y *TFEB*.
6. Identificar una posible correlación entre los niveles de expresión de *GBA* y sus variantes de splicing y los niveles de expresión de *SCARB2* y *TFEB* y sus respectivas variantes de splicing.
7. Identificar una posible asociación entre la desregulación de la expresión *GBA* y *TFEB* y/o *SCARB2*.
8. Analizar la expresión de mRNA de genes involucrados en diferentes puntos de la autofagia y determinar su papel en las ECL.
9. Evaluar las diferencias entre los genes autofágicos estudiados en relación con la edad, sexo y duración de la enfermedad.

.

3.ARTÍCULOS

Aging and Disease
www.aginganddisease.org
Volume 9, Number 2; 208-219, April 2018
http://dx.doi.org/10.14336/AD.2017.0505

Original Article

Glucocerebrosidase mRNA is Diminished in Brain of Lewy Body Diseases and Changes with Disease Progression in Blood

Laia Perez-Roca[1], Cristina Adame-Castillo[1], Jaume Campdelacreu[2], Lourdes Ispierto[3], Dolores Vilas[3], Ramon Rene[2], Ramiro Alvarez[3], Jordi Gascon-Bayarri[2], Maria A. Serrano-Munoz[1], Aurelio Ariza[1], Katrin Beyer[1,*]

[1]Department of Pathology, Hospital Universitari and Health Sciences Research Institute Germans Trias i Pujol, Universitat Autònoma de Barcelona, Spain
[2]Department of Neurology, Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat, Spain
[3]Department of Neurology, Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain.

[Received February 12, 2017; Revised April 26, 2017; Accepted May 5, 2017]

ABSTRACT: Parkinson disease (PD) and dementia with Lewy bodies (DLB) are Lewy body diseases characterized by abnormal alpha-synuclein deposits and overlapping pathological features in the brain. Several studies have shown that glucocerebrosidase (GBA) deficiency is involved in the development of LB diseases. Here, we aimed to find out if this deficiency starts at the transcriptional level, also involves alternative splicing, and if GBA expression changes in brain are also detectable in blood of patients with LB diseases. The expression of three *GBA* transcript variants (GBAtv1, GBAtv2 and GBAtv5) was analyzed in samples from 20 DLB, 25 PD and 17 control brains and in blood of 20 DLB, 26 PD patients and 17 unaffected individuals. Relative mRNA expression was determined by real-time PCR. Expression changes were evaluated by the ΔΔCt method. In brain, specific expression profiles were identified in the temporal cortex of DLB and in the caudate nucleus of PD. In blood, significant GBA mRNA diminution was found in both DLB and PD patients. Early PD and early-onset DLB patients showed lowest GBA levels which were normal in PD patients with advanced disease and DLB patients who developed disease after 70 years of age. In conclusion, disease group specific GBA expression profiles were found in mostly affected areas of LBD. In blood, GBA expression was diminished in LB diseases, especially in patients with early onset DLB and in patients with early PD. Age of disease onset exerts an opposite effect on GBA expression in DLB and PD.

Key words: glucocerebrosidase deficiency, Parkinson's disease, dementia with Lewy bodies, GBA mRNA expression, transcript variants

Parkinson disease (PD) and dementia with Lewy bodies (DLB) belong to the group of Lewy body diseases (LBDs) and are characterized by abnormal aggregates of alpha-synuclein, so called Lewy body pathology (LBP), in the brain [1]. Neuropathologically, two LBP forms can be distinguished: pure LBP, showing LBP only, and common LBP, with a mixture of LBP and concomitant Alzheimer disease (AD) pathology [2]. Lewy bodies are intraneuronal proteinaceous inclusions containing as a main component. They are found in the substantia nigra and other brain stem nuclei in early PD [3] and throughout almost all brain areas in DLB [4]. Usually, about 20%-

***Correspondence should be addressed to:** Katrin Beyer, PhD, Department of Pathology, Hospital Universitari Germans Trias i Pujol, 08916 Badalona, Barcelona, Spain. Email: katrinbeyer@hotmail.com

ISSN: 2152-5250

50% of PD patients develop dementia (PDD) after no less than 10 to 15 years following PD diagnosis [5].

Glucocerebrosidase (GCase) is a lysosomal enzyme responsible for the breakdown of glucocerebroside into glucose and ceramide [6]. Mutations in the GCase gene *GBA* cause GCase deficiency leading to glucocerebroside accumulation inside the lysosome. This accumulation results in Gaucher disease, the most frequent lysosomal storage disorder [6]. Since several probands with Gaucher disease present parkinsonism [7] and have *GBA* mutation-carrier relatives with PD [8], subsequent studies have revealed that *GBA* mutations are strongly associated with PD but also with DLB [9-11].

Analyses of GCase activity and expression levels in PD brains have shown that GCase activity and protein levels are diminished in sporadic PD with and without *GBA* mutations [12, 13]. Furthermore, decreased GCase activity has been found in blood of PD patients [14]. Decrease of GCase activity causes the accumulation of glucocerebroside in lysosomes, directly promoting AS oligomerization and fibrillation. At the same time, AS fibrils inhibit GCase activity creating a bidirectional pathogenic loop [15].

Over the past years, deregulation of alternative splicing has been described repeatedly as an important mechanism involved in ageing and disease development [16, 17]. In this context, we have reported that differential isoform expression changes are involved in LBD pathogenesis [18, 19]. For the GBA gene, five transcript variants (tv; http://www.ncbi.nlm.nih.gov/gene/2629) have been reported by the NCBI database. GBAtv1-3 are the result of alternative inclusion of their initial exons encoding the same protein. GBAtv4 and tv5 are the result of splicing out of exons 2 and 3 or exon 5, and bear shorter proteins.

In this study, we addressed three main questions. First, we wanted to know if GCase deficiency in LBD starts at the transcriptional level; second, if possible brain GBA expression changes are also detectable in blood of LBD patients and third, if alternative *GBA* splicing is dysregulated in these patients.

MATERIALS AND METHODS

Brain tissues

Post-mortem brain samples and their corresponding clinical and neuropathological diagnoses were provided by the Institute of Neuropathology Brain Bank and the Neurological Tissue Bank of the University of Barcelona / Hospital Clinic, Barcelona, Spain. They were obtained from 20 patients with clinical diagnosis of DLB, 25 patients with clinical diagnosis of PD, and 17 donors devoid of neurological signs or symptoms and lack of neuropathological findings. Eight of the DLB brains did not present AD-related pathology and were defined as pure DLB (pDLB), while 12 DLB brains contained concomitant AD-related pathology and were considered as common DLB (cDLB). Of the 25 PD patients, 13 developed dementia (Parkinson's disease with dementia; PDD) but 12 did not (Parkinson's disease without dementia; PDND). None of the patients included in this study carried GBA mutations. Neuropathological diagnosis was carried out as described before [20]. Two brain areas, temporal cortex and caudate nucleus, were analyzed for all disease groups, and the pons was available for PD only. Frontal cortex samples were used to estimate relative expression levels of GBA transcripts. Clinical and neuropathological characteristics of patients and controls are summarized in Table 1.

Table 1. Clinico-neuropathological characteristics of Lewy body disease cases and controls.

Disease	n	PMtime[1] (range)	ADstage[2]	Br&Br[3]	Death[4] (range)	M:F ratio[5]
pDLB[6]	8	9:30 (3:30-17:00)	0-II	A-C	74.6 (60-85)	3:1
cDLB[7]	12	10:30 (4:00-21:15)	III-VI	B-C	79.0 (74-86)	1.4:1
PD[8]	12	7:00 (3:30-14:00)	III-IV		80.8 (68-93)	1:1
PDD[9]	13	7:10 (4:00-12:20)	II-VI	A-C	78.7 (71-87)	0.9:1
CTRL[10]	17	8:40 (2:30-23:30)			69.3 (55-81)	1.4:1

[1] post-mortem time; [2] AD stages following Braak and Braak, I-VI: neurofibrillary tangles; [3] AD stages following Braak and Braak, A-C: amyloid plaques; [4] death, age at death; [5] M:F ratio, male-female ratio; [6] pDLB, dementia with Lewy bodies, pure form; [7] cDLB, common dementia with Lewy bodies; [8] PD, Parkinson disease without dementia; [9] PDD, Parkinson disease with dementia; [10] CTRL, control brain samples.

Patients

Twenty DLB patients (mean age, 73.9; mean age of onset, 68; mean disease duration, 5.9 years; male-female ratio, 1:0.5) were recruited by the Department of Neurology of the Bellvitge Hospital and were diagnosed according to the 2005 DLB Consortium criteria [21]. Twenty-six PD patients (mean age, 68.9; mean age of onset, 65.3; mean disease duration, 6.5 years; male-female ratio, 1:1.4) were diagnosed in the Department of Neurology of the Hospital Germans Trias i Pujol following UK Parkinson's disease Brain Bank clinical diagnostic criteria [22]. None of them presented dementia when blood samples were obtained, and no GBA mutation carriers had been identified. Age at onset was defined as the age when memory loss or parkinsonism was first noticed by relatives. Seventeen control individuals (mean age, 74.4; male-female ratio, 1:1.1) were devoid of neurological symptoms and familial history of neurodegenerative disease and were recruited by both Neurology departments. Written informed consent was obtained from all subjects, either directly or from their legal guardians. The study was carried out with the approval of our local Ethics Committee for Clinical Investigation.

RNA isolation, reverse transcription and assessment of mRNA stability

TRI-Reagent (MRC, Cincinnati, USA) was used for RNA isolation according to the manufacturer's protocol. RNA quantity, purity and integrity was ascertained by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Only samples with RIN values higher than 6 were stored at -80°C until use. Two g of total RNA were used for reverse transcription by Ready-to-go™ You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK). A "no RT" reaction, using water instead of RT, served as a control for the exclusion of genomic DNA contamination. cDNAs were either used immediately for PCR amplification or stored at -20ºC. Stability assessment of the transcripts analyzed in this study was carried out by defining their degradation rates at RIN values of about 6 [20].

Table 2. RNA primer sequences used for the amplification of *GBA1* isoforms, beta-actin, *GUS* and *PBGD*.

Name and NCBI[1]	Primer name	Primer sequence (5' – 3')	Size[2]
GBA1tv1	GBA1tv1U*	ATC ACA TGA CCC ATC CAC A	214 bp[3]
NM_000157.3	GBA1tv1L	ACT CAA AGG CTT GGG ACA T	
GBA1tv2	GBA1tv2U2*	TTC GCC GAC GTG GAT CCT CT	236 bp
NM_001005741.2	GBA1tv2L2	ACC GAG CTG TAG CCG AAG CT	
GBA1tv3	GBA1tv3U*	TTC GCC GAC GAG ACT CTG GA	176 bp
NM_001005742.2	GBA1tv3L	ACC TGA TGC CCA CGA CAC TG	
GBA1tv4	GBA1tv4U*	TTC TCT TCG CCG ACG GTG CC	169 bp
NM_001171811.1	GBA1tv4L	AGC TCC ATC CGT CGC CCA CT	
GBA1tv5	GBA1tv5U*	ACG GGC ACA GGA ATC GGA TA	173 bp
NM_001171812.1	GBA1tv5L	AAC TGC AGG GCT CGG TGA AT	
	b-act U2	TCT ACA ATG AGC TGC GTG TG	228 bp[4]
	b-act L2	GGA TAG CAA CGT ACA TGG CT	
	b-act U3	AAC TGG GAC GAC ATG GAG AA	178 bp[5]
	b-act L3	TAG ATG GGC ACA GTG TGG GT	
	GUS[6]-U1	ATG TGG TTG GAG AGC TCA TT	176 bp
	GUS–L2	TGT CTC TGC CGA GTG AAG AT	
	PBGD[7]_U1	ACA CAC AGC CTA CTT TCC AAG	183 bp
	PBGD_L1	TCA ATG TTG CCA CCA CAC TGT	

[1]Name and NCBI, name of the transcript variant and NCBI accession number; [2]Size, amplicon size; [3] bp, base pairs; [4]228 bp, amplicon size resulting from primer pair b-actU2 + b-actL3; [5]178 bp, amplicon size resulting from primer pair b-actU3 + b-actL2; [6]GUS, beta-glucuronidase; [7]PBGD, porphobilinogen deaminase.* These primers comprise transcript-specific sequences.

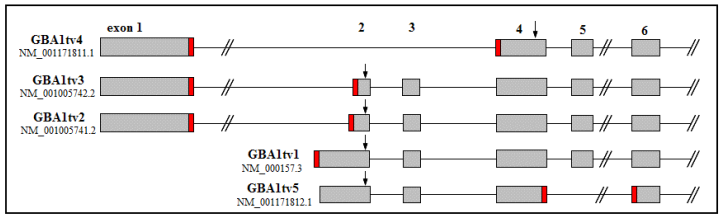

Figure 1. Schematic representation of the five *GBA* transcripts and location of forward primers. Grey boxes represent exons and the lines, introns. Narrow red rectangles at the end of some exons indicate sequences, chosen for designing isoform-specific primers.

Primer design

Primers were designed for specific amplificacition of *GBA* transcripts. Primer location is shown in Figure 1, and sequences and amplimer sizes in Table 2. The *GBA* gene bears 5 transcript variants by the inclusion of alternative 5´ initial exons and by splicing out of internal exons. All five show specific sequences (www.ncbi.nlm.nih.gov/gene/2629) permitting the design of isoform-specific primers. The design of primers for the *GBA* gene is especially challanging because of its high homology with the *GBAP1* gene [23]. Therefore not all primers could be designed to rise 150-180 bp long amplicons (Table 2). To amplify GBAtv1, the forward primer was located in exon 2 and the reverse primer in exon 3 (Fig.1). Since GBAtv5 lacks its exon 4, the forward primer spanned the boundary between exons 4 and 6, and the reverse primer was located in exon 7. Transcripts GBAtv2 and GBAtv3 have different initial sequences of their exons 2, while GBAtv4 lacks exons 2 and 3, differences that were used to design specific primers (Fig.1).

Assessment of SNCB mRNA stability

High quality RNA cannot be taken for granted when dealing with post-mortem brain samples, especially with post-mortem times larger than 5 hours [24]. In order to assure that *GBA*, *ACTB, GUSB* and *PBGD* RNAs presented similar stabilities even at RIN values of about 6, we assessed their RNA degradation rates [25]. Ten µg of RNA corresponding to two of each, temporal cortices and caudate nucleus, were incubated at 50°C. Two-µg-aliquotes were withdrawn from each sample after 15, 30, 60, 120, and 240 minutes, respectively. Of these, 1 µg was subjected to the study of RNA integrity using the Agilent 2100 Bioanalyzer and from 1 µg cDNA was obtained. Incubation time dependent RNA degradation coincided with diminishing RIN values. Real-time PCR analysis of the relative amount of the three GBA transcripts showed that none of the GBA transcripts degraded at higher rates than *ACTB, GUSB* and *PBGD* mRNAs, or vice versa.

Real time PCR

Relative expression was determined for three GBA transcript variants (GBAtv1, GBAtv2 and GBAtv5). Real-time PCR was carried out on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia). PCR was performed in 15 µl reactions with the QuantiTect SYBR Green PCR Kit (QiaGen, Hilden, Germany), containing 16 pmol of each primer and 1 µl of cDNA. To estimate relative expression changes, two housekeeping genes were analyzed in each brain region, beta-actin (*ACTB*) and beta-glucuronidase (*GUSB*) [26] , and two housekeeping genes were analyzed in blood as well, *ACTB* and porphobilinogen deaminase (*PBGD*) [27]. Primer sequences are listed in Table 2.

All assays included two replicates of each sample, were performed twice and independently to assure their reproducibility and minimize possible errors. Standard curves for the target and reference genes were generated for each run by amplifying the same serially diluted cDNA control sample [28, 29].

To assess relative gene expression, relative transcript variant expression data were obtained by the ΔΔCt method based on similar PCR efficiencies to analyze relative gene expression [26, 27].

Differences among GBAtv1, GBAtv2 and GBAtv5 expression levels, were calculated by 2^n, where n corresponds to the cycle number difference between Ct of one of the less expressing transcript and Ct of the major transcript [30].

Statistical analysis

Analyses were performed independently for both housekeeping genes and the mean obtained from both

analyses represented the final expression change of *GBA* isoforms compared to controls for which expression level is assumed to be 1. In the text, all values are given as mean values with variance estimates in brackets. According to the ΔΔCt method, values below 0.5 represented significantly decreased expression levels, while values above 1.5 corresponded to significantly increased expression levels. Nevertheless, results were accepted as significant only with 0.5 as major variance value in the case of expression decrease and with 1.5 as minor variance value in the case of expression increase. To take into account the exponential function of Ct values, in the final calculation the variance was estimated by evaluating $2^{-\Delta\Delta Ct}$ term using ΔΔCt plus the standard deviation and ΔΔCt minus the standard deviation [29].

Differences between age, disease onset and disease duration were assessed by t-test, and regression analyses was performed to evaluate the possible association between GBAtv1 expression levels and age, disease onset or disease duration. Statistical analyses were performed using the SPSS 21 (IBM, Armonk, NY, USA) software, and Statpages (http://www.statpages.org).

	GBA		
	tv1	tv2	tv5
FC	1	2	3
TC	1	2	3
Ca	3	3	2
Put	3	3	2
NBM	1	2	2
Am	1	3	1
SN	2	3	1
Pt	3	3	...
Cr	2	3	...

Figure 2. Relative *GBA* isoform expression in different brain areas. *GBA* expression in neural tissue estimated by appraising agarose gel electrophoretograms: tv, transcript variant; FC, frontal cortex; TC, temporal cortex; Ca, caudate nucleus; Put, putamen; NBM, Nucleus basalis of Meynert; Am, Amygdala; SN, Substantia nigra; Pt, pons; Cr, cerebellum. White fields correspond to lack of expression, light gray (1) to very slight expression, middle gray (2) to readily detectable expression, and dark gray (3) to high expression. The black fields represent very intense expression levels.

RESULTS

Of the five GBA transcripts, GBAtv3 and GBAtv4 showed very low expression in brain as well as in blood. Therefore, it was not possible to analyze their relative expression.

GBA transcripts expression in different brain areas

Analysis of *GBA* isoform expression in different brain areas revealed highest *GBA* levels in the caudate nucleus and putamen, with strong GBAtv1 and tv2 expression and intermediate GBAtv5 expression. In contrast, both cortical areas, frontal and temporal cortices, showed only low or intermediate GBAtv1 and tv2 expression, but strong GBAtv5 expression (Fig. 2). GBAtv1 was mostly expressed in the caudate nucleus, putamen and pons, as compared with the other brain areas analyzed. GBAtv2 showed almost uniformly high expression in all brain areas studied. On the contrary, GBAtv5 was expressed mostly in the cortex and showed only traces of expression in the pons and cerebellum (Fig.2).

GBA isoform mRNA expression changes in the temporal cortex, caudate nucleus and pons from LBD brains

All results are represented as relative expression changes compared to normal control brain areas with variance estimations in brackets. Only significant changes are shown in the text.

In the temporal cortex, GBAtv1 expression was diminished in pDLB (0.38 (0.29-0.49)), and in cDLB (0.27 (0.14-0.49)), but unchanged in both PDND and PDD. GBAtv2 did not show significant expression changes in any of the groups. GBAtv5 was almost 4-fold decreased in pDLB (0.35 (0.23-0.49)), but not in the other groups.

In the caudate nucleus, GBAtv1 was significantly diminished in PDD (0.41 (0.38-0.45)), pDLB – 0.41 (0.30-0.58) and cDLB – 0.44 (0.34-0.61). No changes in GBAtv1 expression were observed in PDND. GBAtv2 expression did not change in any of the groups, and GBAtv5 was diminished only in PDD (0.36 (0.34-0.37)).

Samples of pons were only available for PD, PDD and control brains. None of the GBA isoforms was altered in PDND or PDD in this brain area (data not shown).

Expression profiles (Fig.3) revealed the presence of disease-specific and brain-area specific expression changes of *GBA* isoforms. Disease-specific expression profiles were detected for pDLB and cDLB in the temporal cortex. Both, PD with and without dementia showed overlapping expression profiles in the temporal cortex. In contrast, both DLB groups showed overlapping

expression profiles in the caudate nucleus. On the contrary, PD groups showed differential expression profiles in this brain area (Fig. 3).

Characterization of patients by onset, duration and disease progression indicators

The influence of age at onset and disease duration on GBA expression levels in blood was studied by dividing patients into the following groups: 1. according to age at onset: (a) patients who developed the disease at age of 65 years or before and (b) patients who developed disease at the age of 66 years or later; and 2. according to the duration of disease: (a) less than 6 years or (b) 6 years or more. Table 3 shows that disease duration from onset was similar in both age-at-onset dependent DLB groups. On the contrary, in PD disease duration from onset was significantly longer in patients who developed PD at the age of 65 years or earlier when compared to patients with PD onset after 65 years.

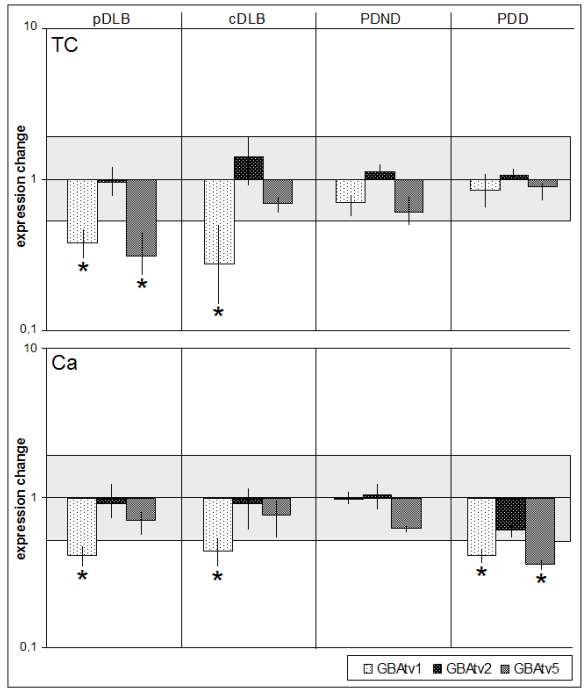

Figure 3. Expression profiles of *GBA1* isoforms in three brain areas of LBD after adjustment with controls. The included areas were temporal cortex (TC) and caudate nucleus (Ca) from the groups of pure dementia with Lewy bodies (pDLB), common dementia with Lewy bodies (cDLB), Parkinson´s disease without dementia (PDND) and Parkinson´s disease with dementia (PDD). The results are shown as relative expression changes obtained by the ΔΔCt method in comparison with normal controls and are represented in a logarithmic scale. Grey areas represent normal expression range. * Significant expression change below 0.5.

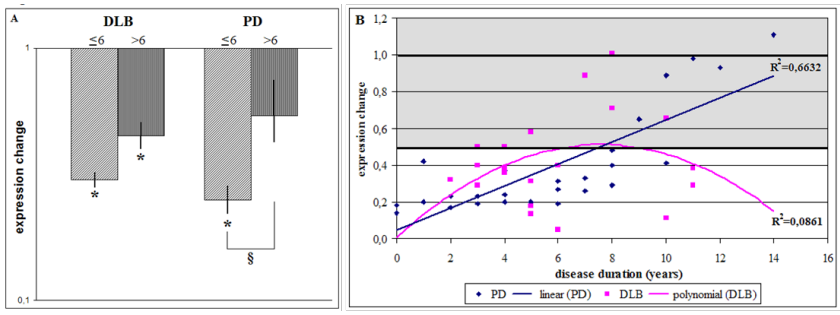

Figure 4. GBA1tv1 expression in blood of DLB and PD patients in dependency on disease duration. GBA1tv1 expression was analyzed (**A**) in two groups and (**B**) for each patient individually. For (**A**), the results are shown as relative expression changes obtained by the ΔΔCt method in comparison with control individuals. * Significant expression change below 0.5. § Significant expression change between the disease duration subgroups. For (**B**) each point corresponds to the value of the expression change of each individual obtained by the ΔΔCt method, where ΔCt of patients was determined individually and ΔCt of control individuals was the mean value of the entire control group. Grey areas represent normal expression range.

GBA mRNA expression in blood

Differential expression analysis of the three *GBA* transcripts in blood of DLB and PD patients unveiled that GBAtv1 was significantly decreased in DLB (0.41 (0.38-0.44)) and PD (0.35 (0.32-0.39)). In contrast, neither GBAtv2 nor GBAtv5 showed significant expression changes in blood of DLB or PD patients (data not shown).

To further assess the possible association between GBA mRNA expression and clinical features, data were analyzed taking into account gender, disease duration from onset and age at disease onset. No differences in GBAtv1 expression levels were found for different gender.

Expression of GBAtv1 and disease duration

Although there was a tendency of lower GBAtv1 expression (0.30 (0.29-0.32)) in blood of DLB patients with shorter disease duration from onset versus patients with longer disease duration (0.45 (0.42-0.50)), this difference (0.64 (0.58-0.73)) was not significant between both onset groups (Fig.4A). In blood of PD patients in turn, patients with early PD showed two times less GBAtv1 expression levels (0.26 (0.21-0.32)) than patients with 6 years of disease duration or more, where GBAtv1 diminution was no longer significant (0.55 (0.43-0.71); Fig.4A).

These differences between DLB and PD prompted us to analyze in depth the relation between disease duration and GBAtv1 expression in blood. As shown in Figure 4B, the effect of disease duration on GBAtv1 expression in blood was different in DLB and PD. Whereas in DLB the first years of disease were characterized by low GBAtv1 levels, GBAtv1 expression reached normal levels in patients with disease duration between 6 and 8 years and diminished again in DLB patients with longer disease duration. On the contrary, in PD a linear correlation between GBAtv1 expression and duration of disease was detected. Initial stages of the disease were characterized by most pronounced GBAtv1 diminution which levels raised to normal after about 10 years of disease progression (Fig.4B).

Expression of GBAtv1 and age at disease onset

As shown in figure 4A, when compared to controls, GBAtv1 expression was five times lower in patients in blood of DLB patients with early onset (0.21 (0.20-0.22)) and almost two times lower than in those who started after age of 65 years (0.51 (0.44-0.60)). Interestingly, GBAtv1 expression was also more than two times lower in patients with early onset when compared to later onset DLB (0.39 (0.33-0.46)). On the contrary, in PD, compared to controls, patients with earlier disease onset had two times lower GBAtv1 expression (0.39 (0.36-0.41) while patients who developed PD after the age of 65 years had four-times lower GBAtv1 (0.24 (0.17-0.38)). Early onset PD had 2-times higher GBAtv1 expression than patients who developed PD after the age of 65 years (1.90 (1.54-

2.66); Fig. 5A). Data were also plotted individually and underwent regression analysis. As shown in figure 4B, linear association between disease onset and GBAtv1 expression was observed for both DLB and PD, but with opposite effects. Whereas GBAtv1 expression was drastically diminished in DLB patients with early disease onset but was normal in patients of 72 years or older, PD patients who debuted before the age of 60 years presented normal GBAtv1 levels which were decreased in patients with later onset (Fig. 5B).

Table 3. Clinical characteristics of DLB and PD patients in the disease onset and duration groups.

DLB			
Disease onset	**≤65 years**	**>65 years**	***P*[1]**
n	6	14	
age at onset (range)	61.4 (59-65)	68.5 (66-74)	n.p.[2]
age (range)	67.6 (63-71)	73.8 (69-80)	n.p.
duration (range)	6.2 (2-10)	4.9 (2-10)	0.135
male: female ratio	1: 0.33	1: 0.4	0.765
Disease duration since onset	**≤6 years**	**>6 years**	
n	13	7	
age at onset (range)	67.1 (59-74)	63.5 (60-67)	0.238
age (range)	70.8 (63-80)	72.8 (70-77)	0.302
male:female ratio	1: 0.5	1: 0.25	0.097
PD			
Disease onset	**≤65 years**	**>65 years**	
n	12	14	
age at onset (range)	62.2 (60-64)	70.0 (68-73)	n.p.
age (range)	69.0 (61-75)	72.5 (68-75)	n.p.
duration (range)	6.8 (1-14)	2.5 (0-6)	**0.015**
male: female ratio	1: 0.8	1:01	0.827
Disease duration since onset	**≤6 years**	**>6 years**	
n	15	11	
age at onset (range)	66.9 (60-73)	61.7 (60-64)	0.105
age (range)	69.6 (61-75)	72.3 (68-75)	0.376
male: female ratio	1: 0.9	1: 0.9	1

[1] p-value, obtained by t-test; [2] n.p., does not proceed.

DISCUSSION

Expression of GBA transcripts in brain

In the present study we have analyzed the differential expression of three GBA transcript variants in the temporal cortex, caudate nucleus and pons of DLB and PD brains divided into pure and common DLB and into PD with and without dementia. Although tissue specific expression of alternative splice variants has been described earlier, it has been also shown that isoforms expression changes in different brain areas are associated with disease [31, 32]. Accordingly, we identified specific expression profiles in the temporal cortex of DLB with decreased GBAtv1 expression in cDLB and combined GBAtv1 and tv5 diminution in pDLB. The specific

expression profile of GBA transcripts in PDD caudate nucleus also showed the decrease of both GBAtv1 and tv5. In the caudate nucleus, GBAtv1 was also diminished in both DLB groups. Although these results suggest a defined role for GBAtv5 during pathogenesis of LBD, the specific function of this minor isoform remains to be determined. Our findings furthermore underline the specific involvement of cortical regions in DLB [33] and the association of the caudate nucleus with dementia in LBD. In this context, it has been shown that dopamine depletion in the caudate nucleus can be detected in DLB [34] and correlates with cognition in PD [35].

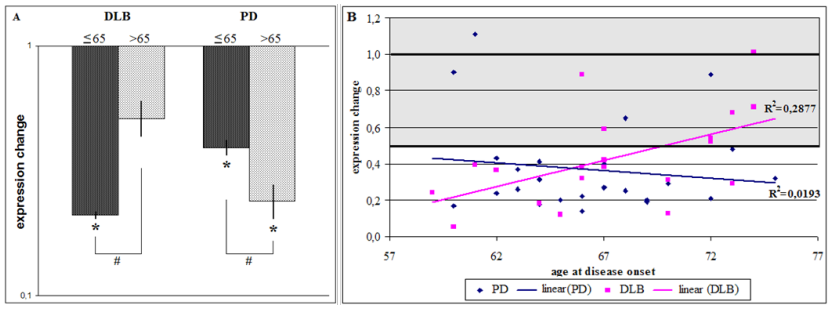

Figure 5. GBA1tv1 expression in blood of DLB and PD patients in dependency on the age of disease onset. GBA1tv1 expression was analyzed (**A**) in two groups and (**B**) for each patient individually. For (**A**), the results are shown as relative expression changes obtained by the ΔΔCt method in comparison with control individuals. *Significant expression change below 0.5. #Significant expression change between the age-at-onset dependent subgroups. For (**B**) each point corresponds to the value of the expression change of each individual obtained by the ΔΔCt method, where ΔCt of patients was determined individually and ΔCt of control individuals was the mean value of the entire control group. Grey areas represent normal expression range.

In two recent studies GCase activity and expression levels were analyzed in PD brains. Whereas Gegg and colleagues analyzed cerebellum, frontal cortex, putamen, amygdala and substantia nigra samples from patients with sporadic PD and from patients with *GBA* mutations [36], Murphy and colleagues investigated anterior cingulate cortex and occipital cortex in early and late PD stages [12]. Results revealed diminished GCase activity and protein levels in cerebellum, substantia nigra [36] and anterior cingulate cortex, a region that accumulates abnormal AS in sporadic PD [12] and additionally in putamen and amygdala of PD brains with *GBA* mutations [36]. In both studies, no changes in GCase levels or activity were found in the cortical areas. Both studies further coincided in no finding any significant GBA mRNA expression changes, indicating that in PD GCase expression changes occur at the posttranscriptional level. Our results are in concordance with those of Gegg's study showing unchanged GBA mRNA expression levels in the cortex of PD patients with and without dementia. In DLB, GCase protein levels have been found to be diminished by 20% in the frontal cortex of GBA mutation carriers [37], but no changes have been reported for no-mutation carriers, so far. However, our results show diminished GBA mRNA levels in PDD caudate nucleus and in both DLB cortex and caudate nucleus independently on the mutation status suggesting that in these cases GCase deficiency already starts at the transcriptional level.

Recently, the age-related decline of GCase activity in the ageing brain has been also found. Correspondingly, it has been proposed that this diminished related to aging may act as a predisposing factor for AS accumulation and PD [38]. Since our control group included aged individuals, such changes could not be detected in the present study.

PD patients whose brains had been included in our study presented disease for at least 8 years and no *GBA* isoforms expression changes were found in the pons. Growing evidence suggests that caudal brainstem structures are involved in PD pathology even before development of nigrostriatal pathology [20]. Those early changes would include the degeneration of nondopaminergic pathways in the pons [39] indicating that molecular changes in this brain area are to be

expected at very early stages of the disease. Therefore, the study of prodromal PD cases is necessary to find out whether GBA isoform expression changes are present before clinical manifestations appear.

Upon comparison with our earlier findings, the pDLB group analyzed here represents the molecular subgroup of DLB that is characterized by the drastic diminution of two main beta-synuclein (BS) gene (*SNCB*) transcripts in the cerebral cortex [20]. The lack of this natural AS antiaggregants [40, 41, 42] would strongly enhance AS oligomerization and aggregation in the cortex of these brains. It has been also shown that diminished GCase levels increase AS aggregation rate [43]. The joint effect of the drastic decrease of two key proteins primarily involved in maintaining soluble and functional AS could be the main cause of disease development in this DLB subgroup with pure LBP in the brain and a short and aggressive disease course.

On the other hand, in cDLB the temporal cortex shows diminution of only one of the two *SNCB* transcripts [20] and of only one *GBA* transcript. The remaining BS and GCase would be sufficient to avoid reaching high AS aggregation rates, so that in cDLB additional factors enhancing AS aggregation must participate to achieve a similar pathological effect to pDLB. Indeed, LBP is accompanied by characteristic AD changes in cDLB brains, a fact that suggests the involvement of elements other than AS-aggregation factors.

The caudate nucleus in PDD showed diminution of more than a half of GBA1 levels, including GBA1tv1 and GBA1tv5 that is accompanied by SNCB over-expression in that brain area [20]. Taken together, these findings indicate that different mechanisms trigger the AS aggregation process in the various neurological conditions displaying LBP.

Expression of GBA transcripts in blood

We also analyzed expression of all three GBA transcripts in blood obtained from DLB and PD patients. In contrast to our findings in brain, in blood only GBAtv1 exhibited expression changes corresponding to significant diminution in both DLB and PD. These results correlate with those of Alcalay and colleagues who measured GCase activity in blood of PD patients with and without *GBA* mutations, and as the major finding they observed lower GCase enzyme activity in PD patients [14]. Our results of diminished GBA mRNA in blood of LBD patients suggest that here GCase deficiency starts at the transcriptional level and could represent the peripheral response to the diminution of GCase activity in the brain.

When we further studied the impact of disease duration from disease onset and age of disease onset on GBA expression, we detected the linear correlation between disease onset from duration and GBAtv1 expression in PD. The shorter the duration from disease onset the lower GBAtv1 levels which became normal with disease progression of more than 6 years. These results must be further explored to determine if GBAtv1 mRNA could be a valid biomarker for LBD. During the past years, the determination of mRNA expression changes in blood has been established as valid biomarker for disease diagnosis and progression, including PD [44]. In this context, different mRNAs have been proposed as biomarkers to identify or monitor PD patients [45-47], and GBAtv1 should be evaluated as useful part of a corresponding diagnostic panel.

When GBAtv1 expression was analyzed by age at onset, both DLB and PD showed linear correlation between GBAtv1 expression and disease onset, but with opposite tendencies. Whereas in DLB lowest GBAtv1 levels were detected in patients with earliest onset, in PD these were seen for patients with latest onset. Age at onset plays an important role for disease progression and has been postulated to be also opposite for DLB and PD. Whereas disease progresses more slowly in patients with young-onset PD, DLB shows a more aggressive course in patients with early onset [48-49].

An additional challenge in the clinical practice is the differential diagnosis of DLB versus AD due to multiple overlapping features, especially at early disease stages [50, 51]. Although diagnostic criteria for DLB have been improved substantially over the past years [52], its differential diagnosis versus AD remains very difficult. To address this difficulty, the specific diminution of GBAtv1 in early-onset as well as early-stage DLB could be applied as biomarker to differentiate between DLB and AD. The availability of reliable biomarkers is an urgent need to enhance diagnostic competency of dementias in the clinical practice.

While promising, the results obtained in the present study must be affirmed in other populations. Furthermore, larger patient groups must be also analyzed to confirm decreased GBA mRNA in blood of DLB and PD patients. Specifically, the suitability of GBAtv1 mRNA as early diagnostic biomarker needs to be studied in blood of AD patients.

In conclusion, in brain we identified disease group specific expression profiles of GBA transcripts in the temporal cortex of DLB and the caudate nucleus of PD. Dysregulation of alternative splicing was detected in brain but not in blood, where GBAtv2 and GBAtv5 did not show altered expression. The analysis of GBAtv1 expression in blood revealed its diminution in LBD, but especially in patients with early onset DLB and in patients with early PD. Finally, an opposite tendency of GBAtv1 expression levels was found in DLB and PD when

analyzed in dependency on the age of disease onset with lowest levels in youngest DLB but oldest PD patients.

Acknowledgements

We are indebted to the Neurological Tissue Bank of the Biobank-Hospital Clinic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain for sample and data procurement. We thank the Institute of Neuropathology Brain Bank, L'Hospitalet de Llobregat, Spain. This work was supported by Spain's Ministry of Health FIS grants PI12/1702 and PI15/216, and the MaratóTV3 grant 1405/10.

References

[1] Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M (1998). Alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. Proc Natl Acad Sci USA, 95:6469-73.

[2] Braak H, Braak E (1997). Diagnostic criteria for neuropathological assessment of Alzheimer's disease. Neurobiol Aging, 18:S85-8.

[3] Jellinger KA (2009). A critical evaluation of current staging of alpha-synuclein pathology in Lewy body disorders. Biochim Biophys Acta, 1792:730-40.

[4] Ferman TJ, Boeve BF (2007). Dementia with Lewy bodies. Neurol Clin, 25:741-60.

[5] Aarsland D, Andersen K, Larsen JP, Lolk A, Kragh-Sørensen P (2003). Prevalence and characteristics of dementia in Parkinson disease: an 8-year prospective study. Arch Neurol, 60:387-92.

[6] Beutler E, Grabowski GA (2001). Glucosylceramide lipidosis–Gaucher disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited diseases. 8th edition, 3635–68.

[7] Li Y, Li P, Liang H, Zhao Z, Hashimoto M, Wei J (2015). Gaucher-Associated Parkinsonism. Cell Mol Neurobiol, 35:755-61.

[8] Goker-Alpan O, Giasson BI, Eblan MJ, Nguyen J, Hurtig HI, Lee VM, et al. (2006). Glucocerebrosidase mutations are an important risk factor for Lewy body disorders. Neurology, 67:908-10.

[9] Goker-Alpan O, Schiffmann R, LaMarca ME, Nussbaum RL, McInerney-Leo A, Sidransky E (2004). Parkinsonism among Gaucher disease carriers. J Med Genet, 41:937-40.

[10] Mata IF, Samii A, Schneer SH, Roberts JW, Griffith A, Leis BC, et al. (2008). Glucocerebrosidase gene mutations: a risk factor for Lewy body disorders. Arch Neurol, 65:379-82.

[11] Nalls MA, Duran R, Lopez G, Kurzawa-Akanbi M, McKeith IG, Chinnery PF, et al. (2013). A multicenter study of glucocerebrosidase mutations in dementia with Lewy bodies. JAMA Neurol, 70:727-35.

[12] Murphy KE, Gysbers AM, Abbott SK, Tayebi N, Kim WS, Sidransky E, et al. (2014). Reduced glucocerebrosidase is associated with increased α-synuclein in sporadic Parkinson's disease. Brain, 137:834-48.

[13] Pitcher TL, Melzer TR, Macaskill MR, Graham CF, Livingston L, Keenan RJ, et al. (2012). Reduced striatal volumes in Parkinson's disease: a magnetic resonance imaging study. Transl Neurodegener, 1:17.

[14] Alcalay RN, Levy OA, Waters CC, Fahn S, Ford B, Kuo SH, et al. (2015). Glucocerebrosidase activity in Parkinson's disease with and without GBA mutations. Brain, 138:2648-58.

[15] Mazzulli JR, Xu YH, Sun Y, Knight AL, McLean PJ, Caldwell GA, et al. (2011). Gaucher disease glucocerebrosidase and α-synuclein form a bidirectional pathogenic loop in synucleinopathies. Cell, 146:37-52.

[16] Harries LW, Hernandez D, Henley W, Wood AR, Holly AC, Bradley-Smith RM, et al. (2011). Human aging is characterized by focused changes in gene expression and deregulation of alternative splicing. Aging Cell, 10:868-78.

[17] Zhang J, Manley JL (2013). Misregulation of pre-mRNA alternative splicing in cancer. Cancer Discov, 3:1228-37.

[18] Beyer K, Domingo-Sàbat M, Humbert J, Carrato C, Ferrer I, Ariza A (2008). Differential expression of alpha-synuclein, parkin, and synphilin-1 isoforms in Lewy body disease. Neurogenetics, 9:163-72.

[19] Beyer K, Munoz-Marmol AM, Sanz C, Marginet-Flinch R, Ferrer I, Ariza A (2012). New brain-specific beta-synuclein isoforms show expression ratio changes in Lewy body diseases. Neurogenetics, 13:61-72.

[20] Beyer K, Domingo-Sàbat M, Santos C, Tolosa E, Ferrer I, Ariza A (2010). The decrease of β-synuclein in cortical brain areas defines a molecular subgroup of dementia with Lewy bodies. Brain, 133:3724-33.

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

[22] Hughes AJ, Daniel SE, Kilford L, Lees AJ (1992). Accuracy of clinical diagnosis of idiopathic Parkinson's disease. A clinico-pathological study of 100 cases. JNNP, 55:181-4. [23]

[23] Winfield SL, Tayebi N, Martin BM, Ginns EI, Sidransky E (1997). Identification of three additional genes contiguous to the glucocerebrosidase locus on chromosome 1q21: implications for Gaucher disease. Genome Res, 7:1020-6.

[24] Durrenberger PF, Fernando S, Kashefi SN, Ferrer I, Hauw JJ, Seilhean D, et al (2010). Effects of antemortem and postmortem variables on human brain mRNA quality: a BrainNet Europe study. J Neuropathol Exp Neurol, 69:70-81.

[25] Popova T, Mennerich D, Weith A, Quast K (2008). Effect of RNA quality on transcript intensity levels in microarray analysis of human post-mortem brain tissues. BMC Genomics, 9:91.

[26] Barrachina M, Castaño E, Ferrer I (2006). TaqMan PCR assay in the control of RNA normalization in human post-mortem brain tissue. Neurochem Int, 49:276-84.

[27] Fuchs J, Tichopad A, Golub Y, Munz M, Schweitzer KJ, Wolf B, et al. (2008). Genetic variability in the SNCA gene influences alpha-synuclein levels in the blood and brain. FASEB J, 22:1327-34.

[28] Schefe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H (2006). Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's C $_{T}$ difference" formula. J Mol Med, 84:901-10.

[29] Schmittgen TD, Livak KJ (2008). Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc, 3:1101-8.

[30] Rutledge RG, Côté C (2003). Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res, 31:e93.

[31] Yao P, Lin P, Gokoolparsadh A, Assareh A, Thang MW, Voineagu I (2015). Coexpression networks identify brain region-specific enhancer RNAs in the human brain. Nat Neurosci, 18:1168-74.

[32] Bagot RC, Cates HM, Purushothaman I, Lorsch ZS, Walker DM, Wang J, et al. (2016). Circuit-wide Transcriptional Profiling Reveals Brain Region-Specific Gene Networks Regulating Depression Susceptibility. Neuron, 90:969-83.

[33] Mak E, Su L, Williams GB, Watson R, Firbank M, Blamire A, et al. (2015). Progressive cortical thinning and subcortical atrophy in dementia with Lewy bodies and Alzheimer's disease. Neurobiol Aging, 36:1743-50.

[34] O'Brien JT, Colloby S, Fenwick J, Williams ED, Firbank M, Burn D, et al. (2004). Dopamine transporter loss visualized with FP-CIT SPECT in the differential diagnosis of dementia with Lewy bodies. Arch Neurol, 61:919-25.

[35] Niethammer M, Tang CC, Ma Y, Mattis PJ, Ko JH, Dhawan V, et al. (2013). Parkinson's disease cognitive network correlates with caudate dopamine. Neuroimage, 78:204-9.

[36] Gegg ME, Burke D, Heales SJ, Cooper JM, Hardy J, Wood NW, et al. (2012). Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. Ann Neurol, 72:455-63.

[37] Kurzawa-Akanbi M, Hanson PS, Blain PG, Lett DJ, McKeith IG, Chinnery PF, et al (2012). Glucocerebrosidase mutations alter the endoplasmic reticulum and lysosomes in Lewy body disease. J Neurochem, 123:298-309.

[38] Rocha EM, Smith GA, Park E, Cao H, Brown E, Hallett P, et al (2015). Progressive decline of glucocerebrosidase in aging and Parkinson's disease. Ann Clin Transl Neurol, 2:433-8.

[39] Zarow C, Lyness SA, Mortimer JA, Chui HC (2003). Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. Arch Neurol, 60:337–41.

[40] Windisch M, Hutter-Paier B, Rockenstein E, Hashimoto M, Mallory M, Masliah E (2002). Development of a new treatment for Alzheimer's disease and Parkinson's disease using anti-aggregatory beta-synuclein-derived peptides. J Mol Neurosci, 19:63-9.

[41] Wei J, Fujita M, Nakai M, Waragai M, Sekigawa A, Sugama S, et al (2009). Protective role of endogenous gangliosides for lysosomal pathology in a cellular model of synucleinopathies. Am J Pathol, 174:1891-909.

[42] Fujita M, Sugama S, Sekiyama K, Sekigawa A, Tsukui T, Nakai M, et al (2010). A β-synuclein mutation linked to dementia produces neurodegeneration when expressed in mouse brain. Nat Commun, 1:110.

[43] Blanz J, Groth J, Zachos C, Wehling C, Saftig P, Schwake M (2010). Disease-causing mutations within the lysosomal integral membrane protein type 2 (LIMP2) reveal the nature of binding to its ligand beta-glucocerebrosidase. Hum Mol Genet, 19:563-72.

[44] Chikina MD, Gerald CP, Li X, Ge Y, Pincas H, Nair VD, et al. (2015). Low-variance RNAs identify Parkinson's disease molecular signature in blood. Mov Disord, 30:813-21.

[45] Locascio JJ, Eberly S, Liao Z, Liu G, Hoesing AN, Duong K, Trisini-Lipsanopoulos A, et al. (2015). Association between α-synuclein blood transcripts and early, neuroimaging-supported Parkinson's disease. Brain, 138:2659-71.

[46] Santiago JA, Potashkin JA (2015). Blood Biomarkers Associated with Cognitive Decline in Early Stage and Drug-Naive Parkinson's Disease Patients. PLoS One, 10:e0142582.

[47] Santiago JA, Potashkin JA (2015). Network-based metaanalysis identifies HNF4A and PTBP1 as longitudinally dynamic biomarkers for Parkinson's disease. Proc Natl Acad Sci U S A, 112:2257-62.

[48] Van Rooden SM, Heiser WJ, Kok JN, Verbaan D, van Hilten JJ, Marinus J (2010). The identification of Parkinson's disease subtypes using cluster analysis: a systematic review. Mov Disord, 25:969–78.

[49] Schneider SA, Obeso JA (2015). Clinical and pathological features of Parkinson's disease. Curr. Top Behav Neurosci, 22:205-20.

[50] Fujishiro H, Iseki E, Nakamura S, Kasanuki K, Chiba Y, Ota K, et al. (2013). Dementia with Lewy bodies: early diagnostic challenges. Psychogeriatrics, 13:128-38.

[51] Mayo MC, Bordelon Y (2014). Dementia with Lewy bodies. Semin Neurol, 34:182-8.

[52] McKeith I, Taylor JP, Thomas A, Donaghy P, Kane J (2016). Revisiting DLB Diagnosis: A Consideration of Prodromal DLB and of the Diagnostic Overlap With Alzheimer Disease. J. Geriatr Psychiatry Neurol, 29:249-53.

Neuroscience Letters 706 (2019) 164–168

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Glucocerebrosidase regulators *SCARB2* and *TFEB* are up-regulated in Lewy body disease brain

Laia Pérez-Roca[a,b], Patricia Prada-Dacasa[b], Cristina Segú-Vergés[b], Ana Gámez-Valero[a,b], María A. Serrano-Muñoz[a], Cristina Santos[c], Katrin Beyer[a,b,*]

[a] Department of Pathology, Hospital Universitari and Health Sciences Research Institute Germans Trias i Pujol, Badalona, Barcelona, Spain
[b] Universitat Autònoma de Barcelona, Spain
[c] Unitat d'Antropologia Biològica, Departament de Biologia Animal, Biologia Vegetal i Ecologia, Universitat Autònoma de Barcelona, Spain

ARTICLE INFO

Keywords:
Lysosomal integral membrane protein type 2 gene – *SCARB2*
Transcription factor EB – *TFEB*
Dementia with Lewy bodies
Parkinson's disease
Differential mRNA isoform expression
Transcript variants

ABSTRACT

Mutations in the glucocerebrosidase (GCase) gene (*GBA*) and GCase deficiency are major risk factors for Lewy body diseases. Decreased GCase activity enhances alpha-synuclein aggregation and disease development. Lysosomal integral membrane protein type 2, encoded by *SCARB2*, binds GCase targeting it to lysosomes and transcription factor EB (Tfeb) regulates lysosomal proteostasis. Our aim was to find out if GCase deficiency in Lewy body diseases is accompanied by *SCARB2* and *TFEB* deregulation at the transcriptional level involving alternative splicing as well. Relative mRNA expression of two *SCARB2* and two *TFEB* transcripts was studied by real-time PCR in post-mortem brain samples of cases with pure Lewy body pathology (LBP), cases with concomitant LBP and Alzheimer disease-like pathology, and controls. *TFEB* expression was increased in the temporal cortex and caudate nucleus of LBP cases, and *SCARB2* was differentially expressed. Female-gender associated overexpression of all transcripts was found in the caudate nucleus, and disease duration associated *TFEB* expression changes in the temporal cortex. *SCARB2* and *TFEB* expression correlated negatively with *GBA* mRNA expression in the temporal cortex. Our findings show disease-specific deregulation of *TFEB* and *SCARB2* expression affecting alternative promoter usage and alternative splicing in Lewy body diseases.

1. Introduction

Parkinson disease (PD) and dementia with Lewy bodies (DLB) belong to the group of Lewy body diseases (LBD) and are characterized by Lewy body pathology (LBP) in the brain [1]. Widespread LB distribution is observed in DLB, and in PD, the most affected brain area is the substantia nigra [2]. The primary event in the pathogenesis of LBD is LB formation after abnormal alpha-synuclein (AS) oligomerization and aggregation [2,3]. One of the factors driving AS oligomerization is the lysosomal protein glucocerebrosidase (GCase) [4], and mutations in the GCase gene *GBA* are associated with PD and DLB [5,6]. Moreover, GCase activity is diminished in the affected brain areas in PD [7,8].

GCase is targeted to the lysosome by direct binding to the lysosomal integral membrane protein type 2 (LIMP2), encoded by the gene *SCARB2* [9], and its expression is regulated by the key modulator of lysosomal proteostasis, the transcription factor EB (Tfeb) [10]. Tfeb, encoded by the *TFEB* gene, regulates expression of many lysosomal genes including also LIMP2 and its activation increases GBA expression improving GCase activity [10]. In LBD, high AS levels have been linked to decreased nuclear Tfeb in nigral dopaminergic neurons [11]

Over the past years, deregulation of alternative promoter usage and alternative splicing has been repeatedly involved as important mechanisms in aging and disease development [12]. In this context, we have reported that changes in the differential expression of alpha- and beta-synuclein isoforms are implicated in LBD development [13,14]. Although 27 transcripts are predicted for *SCARB2* and 17 for *TFEB* so far, the existence of two *SCARB2* and five *TFEB* gene transcript variants (tv) has been validated (http://www.ncbi.nlm.nih.gov/gene/). *TFEBtv1-4* are the result of alternative inclusion of their initial exons and are driven by different promoters. *SCARB2tv2* is the result of splicing out of exons 3–5 and *TFEBtv5* of exon 3.

Recently, we described that GBA deficiency starts at the transcriptional level in brain and blood of patients with LBD [15]. Therefore, we wanted to find out if the expression of the *GBA* regulator genes *SCARB2* and *TFEB* is altered in these diseases too, and whether deregulation of their alternative promoter usage and splicing may also be involved. Expression of two transcript variants of each gene was analyzed in two main groups, cases with LBP only and cases where LBP was

* Corresponding author at: Department of Pathology, Hospital Universitari Germans Trias i Pujol, 08916 Badalona, Barcelona, Spain.
E-mail address: kbeyer@igtp.cat (K. Beyer).

https://doi.org/10.1016/j.neulet.2019.05.034
Received 26 September 2018; Received in revised form 16 May 2019; Accepted 17 May 2019
Available online 19 May 2019

accompanied by Alzheimer disease-like pathology (ADP). The results highlighted that the differential involvement of deregulated alternative promoter usage and alternative splicing depends on the presence of ADP and is additionally modulated by disease duration and gender.

2. Materials and methods

2.1. Source of tissues

Post-mortem brain samples and the corresponding clinical and neuropathological diagnoses were provided by the Institute of Neuropathology Brain Bank and the Neurological Tissue Bank of the University of Barcelona/Hospital Clinic, Barcelona, Spain. Temporal cortex and caudate nucleus samples were obtained from 19 brains that predominantly presented LBP, from 25 brains with both LBP and ADP, and 13 donors devoid of neurological signs or symptoms and lack of neuropathological findings (Table S1). Temporal cortex was included because of its early involvement in DLB and caudate nucleus, because of its early involvement in PD. The presence and distribution of AS related pathology was evaluated following the criteria established by the third report of the DLB consortium [16] and according to Braak and Braak [17]. AD-type pathology was assessed by using the Braak and Braak criteria [18]. The study was carried out in accordance with the requirements of the local Ethics Committee.

2.2. RNA isolation, primer design, and real-time PCR

TRI-Reagent (MRC, Cincinnati, USA) was used for RNA isolation according to the manufacturer's protocol. RNA quantity, purity, and integrity were ascertained by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). After assessing RNA degradation rates, only samples with RIN values higher than 6 were stored at −80 °C until use [14].

Two μg of total RNA was used for reverse transcription by Ready-to-go™ You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK). Specific primers (Table S2) were designed to amplify 2 *SCARB2* and 5 *TFEB* transcript variants (Fig. S1).

Since three of the five *TFEB* transcripts were expressed at no measurable levels in the brain, relative expression was determined for *TFEBtv1* and *TFEBtv3* and the two *SCARB2* transcripts. Beta-actin (*ACTB*) and beta-glucuronidase (*GUSB*) were included as housekeeping genes [15]. Real-time PCR was carried out on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia), in 15 μl reactions with the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) containing 16 pmol of each primer (Table S2) and 1 μl of cDNA. All assays were performed twice and independently. Standard curves were generated for each run by amplifying the same serially diluted cDNA control sample.

2.3. Statistical analysis

Possible differences between the pathological groups were assessed by the Mann-Whitney test, to test possible differences in quantitative variables (age at disease onset, age at death and disease duration), and Fisher's exact test to analyze qualitative variables, such as gender. Expression analyses were performed independently for both housekeeping genes, and the normalization factor was derived from the geometric mean of this data [19]. In the text, all values are given as means with standard deviation estimates in brackets. Expression data were corrected for gender and age at death by ANCOVA analyses with sex as a factor and age at death as a covariate followed by post hoc comparisons with Bonferroni correction using Jamovi 0.9.2.3 (https://www.jamovi.org). Differences between *SCARB2tv1* and *SCARB2tv2*, and *TFEBtv1* and *TFEBtv3* expression levels were calculated by 2^n (n = difference in cycle number) [14]. The percentage expression change of all transcripts was calculated using the relative expression obtained for normal brain and the fold change obtained by the ΔΔCt method. Expression changes were adjusted stepwise for age at disease onset, disease duration and gender. Correlation of *SCARB2* and *GBA*, *TFEB* and *GBA*, and *TFEB* and *SCARB2* expression data was estimated by linear regression. Pearson correlation coefficient R and its corresponding p-value were calculated at https://www.socscistatistics.com.

Table 1
Clinical characteristics of patients and controls included in the study.

Disease	Age at onset	Age at death	Duration	Female: male ratio
LBP[a]	62.8 ± 10.34	77.0 ± 8.36	13.6 ± 9.21	1 : 1.4
LBP + ADP[b]	66.6 ± 7.48	79.6 ± 3.64	12.6 ± 7.16	1 : 1.1
CTRLs	n/a[c]	69.9 ± 8.76	n/a	1 : 1.1

[a] LBP, Lewy body pathology.
[b] ADP, Alzheimer disease-like pathology.
[c] n/a, not applicable.

3. Results

3.1. Sample characteristics and pathological groups

All 44 cases included in this study presented neocortical Lewy bodies. The samples were divided into two main groups, LBP without and LBP with concomitant ADP. Additional clinical characteristics including disease onset, disease duration, and female: male ratio is shown in Table 1. Neither age at onset, age at death and duration of disease nor the female: male ratio differed significantly between disease groups, although the latter was slightly lower in the group of LBP. However, whereas 60% of LBP-cases had disease duration of fewer than ten years, only 40% of LBP + ADP cases had a short disease duration. Finally, age at death of control cases was significantly lower than of disease ($p = 0.001$). But, it must be taken into account, that patients had a disease duration of at least six years, and when comparing the age at disease onset of LBD and age at death of controls, no significant difference was found ($p = 0.153$).

3.2. Brain expression levels of SCARB2 and TFEB transcripts

To ascertain which of the *SCARB2* and *TFEB* transcripts were mostly expressed in temporal cortex and caudate nucleus of control brains, Ct values were compared after PCR efficiency and threshold corrections. Whereas the minor transcript *SCARB2tv2* was 20 times less expressed than *SCARBtv1* in the temporal cortex (5% of total *SCARB2* expression), its expression was 80 times lower in the caudate nucleus (1,2% of total *SCARB2* expression), confirming that *SCARB2tv2* is the minor *SCARB2* transcript in the brain (Fig. S2). In contrast, *TFEBtv1* containing exons 1c and 1e (Fig. S2) was five times less expressed than *TFEBtv3* (17% of total *TFEB* expression), in the temporal cortex and two times less in the caudate nucleus (33% of total *TFEB* expression), indicating that *TFEBtv3* with initial exon 1b is the major *TFEB* transcript in the brain (Fig. S2).

3.3. TFEB and SCARB2 transcripts in Lewy body diseases

The results are shown as expression profiles representing relative expression changes (Fig. 1) and in percentages (Table S3). Individual expression change values for each sample are shown in Table S4.

Expression profiles revealed that both *TFEB* transcripts were overexpressed in the LBP-group in the temporal cortex ($p = 0.001$, each). In the caudate nucleus, TFEBtv1 was overexpressed in LBP-cases in comparison with controls ($p = 0.003$), but also in comparison with LBP + ADP-cases ($p = 0.012$; Fig. 1a, b). Total *TFEB* mRNA amount was increased to 308% in the temporal cortex and 187% in the caudate nucleus of LBP cases (Table S3). In contrast, only the mayor *TFEB*

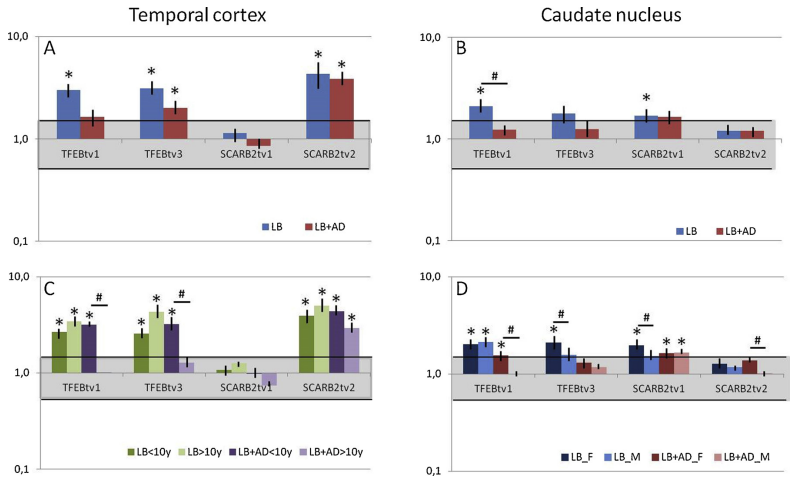

Fig. 1. Expression profiles of *TFEB* and *SCARB2* isoforms in two brain areas of LBD after adjustment with controls. (A) *TFEB* and *SCARB2* expression in the temporal cortex in two pathological groups, with LBP and LBP + ADP. (B) *TFEB* and *SCARB2* expression in the caudate nucleus in two pathological groups, with LBP and LBP + ADP. (C) *TFEB* and *SCARB2* expression in the temporal cortex in dependency on disease duration; < 10 – disease duration of fewer than 10 years; > 10 disease duration of 10 years or more. **(D)** *TFEB* and *SCARB2* expression in the caudate nucleus in dependency on gender; F – female; M – male. The results were obtained by the ΔΔCt method, and they are shown as relative expression changes for each transcript and disease group respect to controls. Error bars represent the variance estimates. The grey area represents the normal-expression range. Significant expression change ($p > 0.05$) * vs. controls, # between two disease groups.

transcript tv3 showed increased expression levels in the temporal cortex of the LBP + ADP-group ($p = 0.047$; Fig. 1a).

Whereas *SCARB2tv1* expression levels did not differ between pathological groups in the temporal cortex, the minor transcript *SCARB2tv2* was importantly increased ($p = 0.001$ for LBP, $p = 0.026$ for LBP-AD). The expression of *SCARB2tv2* that constitutes only 5% of total *SCARB2* in normal temporal cortex, was incremented to 22% in LBP and 19% in LBP + ADP (Table S3). *SCARB2tv1* was slightly increased in the caudate nucleus, raising the total *SCARB2* mRNA amount to 168% in LBP ($p = 0.01$), and 164% in LBP + ADP ($p = 0.053$).

3.4. Gender- and disease-duration-dependent expression of TFEB and SCARB2 transcripts

Further adjustment of expression data revealed that *TFEB* and *SCARB2* expression was independent of age at disease onset. However, disease duration was found to modulate *TFEB* expression in the temporal cortex and gender the expression of *TFEB* and *SCARB2* in the caudate nucleus.

In the temporal cortex of the LBP + ADP group, both *TFEB* transcripts were overexpressed in the cases with disease duration of fewer than ten years (Fig. 1c). Whereas *TFEBtv1* expression was raised from 17% to 53% ($p = 0.0013$) and *TFEBtv3* expression from 83% to 268% ($p = 0.0021$) in these cases, the expression of these transcripts remained unchanged (*TFEBtv1*, 17%; *TFEBtv3*, 106%) in LBP + ADP cases with disease duration of ten years or more.

In the caudate nucleus, gender-dependent expression change was observed. In the LBP group, the major transcripts *TFEBtv3* and *SCARB2tv1* were overexpressed in female cases, *TFEBtv3*, from 66% to 138% in female ($p = 0.007$) and 103% in male cases ($p = 0.067$); *SCARB2tv1*, from 98.8% to 196% ($p = 0.01$) in female and 151% in male cases ($p = 0.09$; Fig. 1d). The minor transcripts *TFEBtv1* and *SCARB2tv2* showed significantly higher expression in the female cases of the LBP + ADP group, *TFEBtv1*, from 33% to 51% in female ($p = 0.033$) and remaining at 33% in male cases; *SCARB2tv2*, from 1.2% to 1.6% in female ($p = 0.041$) and remaining at 1.2% in male cases (Fig. 1d).

3.5. Correlation of SCARB2, TFEB, and GBA expression

In a previous study, we have reported diminished expression levels of the major *GBA* transcript, *GBAtv1* in the temporal cortex of both pure and common DLB, which had been analyzed in the same brain samples used in the present study [15]. To find out if there was a defined relationship between *GBAtv1* and *TFEB* or *SCARB2*, or between *TFEB* or *SCARB2* transcript expression levels we performed regression analyses using the individual expression values of each sample obtained for *GBAtv1*, *SCARB2* and *TFEB* expression (Table S4). We found a negative linear correlation for expression of *GBAtv1*and *TFEBtv3* (Fig. 2A) and a negative logarithmic correlation for expression of *GBAtv1*and *SCARB2tv2* (Fig. 2B). These correlations showed that the lower GBAtv1 expression, the higher the expression of the major *TFEBtv3* transcript and the higher the expression of the minor *SCARB2tv2* transcript. Furthermore, we detected a positive linear correlation between TFEBtv3 and SCARB2tv2 expression (Fig. 2C). No correlation between *GBAtv1*and *SCARB2tv1* expression and *GBAtv1*and *TFEBtv1* expression was observed neither we found any correlation between transcript expression levels in the caudate nucleus.

4. Discussion

Since we have recently identified diminished *GBA* mRNA expression in LBD brain [15], here we analyzed the differential expression of the transcripts of two *GBA* regulatory genes *SCARB2* and *TFEB* in the same brain samples. As the main finding, we identified the up-regulation of *TFEB*, especially in LBP. *TFEB* overexpression was accompanied by an

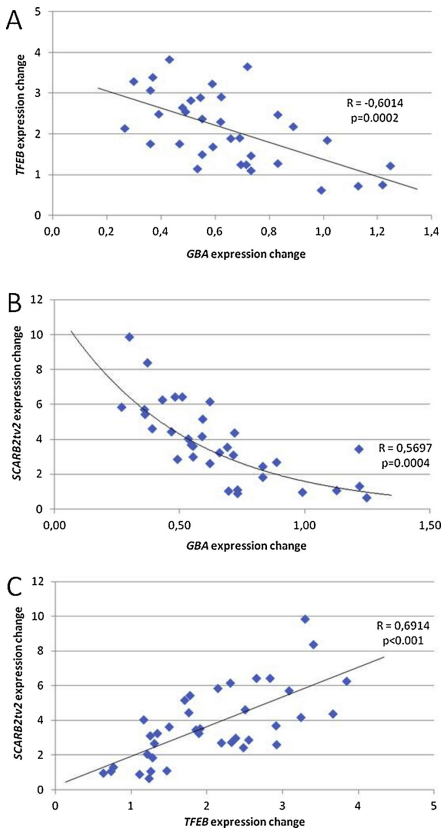

Fig. 2. Correlation between expression levels of (A) *GBAtv1* and *SCARB2tv2*, (B) *GBAtv1* and *TFEBtv3*, and (C) *TFEBtv3* and *SCARB2tv2* in the temporal cortex.

important overexpression of the minor *SCARB2* transcript tv2 in the temporal cortex of both groups.

TFEB is a key regulator of lysosomal biogenesis and function [20]. Various studies have shown that *TFEB* activation is neuroprotective in models of neurodegeneration [21] and attenuates A-beta generation and amyloid plaque formation [21]. Normally, Tfeb is located in the cytoplasm but translocates to the nucleus under stress conditions to promote the transcription of its target genes [10,20,22]. In the substantia nigra of PD brains, high AS levels not only inhibit GCase activity in neurons [4] but also the translocation of Tfeb to the nucleus by sequestering Tfeb to LBs [11]. These observations suggest that *TFEB* mRNA would be down-regulated in brain areas that are compromised by AS pathology and GCase deficiency.

On the contrary, we found *TFEB* over-expression, especially in the temporal cortex where the increase of *TFEBtv3* correlated with decreased *GBA* expression [10]. This discrepancy could be due to a brain area specific Tfeb translocation, and although it has been reported for the SN [7], the cortex could undergo other modulating mechanisms of Tfeb localization and action. Neither the presence of Tfeb in cortical LB has been reported so far, supporting the view of brain region-dependent involvement of Tfeb translocation. *SCARB2tv2* showed a negative correlation with *GBA* expression; the lower *GBA* mRNA, the higher were *SCARB2tv2* levels. These results indicate that diminished *GBA* levels in the cortex may activate *TFEB* expression, which then could raise *SCARB2* expression in this brain area.

Our results further show that alternative promoter usage and alternative splicing are deregulated in LBD. Deregulation of both mechanisms has been shown for other neurodegenerative diseases, and aging [23] and the deregulation of alternative splicing in LBD has been widely reported [13,14,24]. Alternative splicing was similarly affected in both groups, LBP and LBP + ADP, as observed by similar up-regulation of *SCARB2* in the temporal cortex. In contrast, expression of *TFEBtv1* and *TFEBtv3* was mainly up-regulated in the LBP group indicating that alternative promoter usage was especially enhanced in the LBP group. The co-development of ADP could counteract the increase of *TFEB* expression, a question to be addressed in future studies.

Additionally, both alternative promoter usage and alternative splicing were modulated by female gender in the caudate nucleus. Whereas overexpression of the main transcripts, *TFEBtv3* and *SCARB2tv1* was observed in female LBP cases, minor transcripts *TFEBtv1* and *SCARB2tv2* showed higher expression levels in female LBP + ADP cases when compared to the male cases. Female sex has been associated with a larger volume of the caudate nucleus in a recent study [25]. Although caudate nucleus volume was increased with increasing age and in different dementia types including AD, it was not in PDD-DLB. However, in female cases, the volume of the caudate nucleus was increased independently of the dementia state [25]. Also, other studies have reported larger subcortical volumes for females, and an elevated amount of sex steroid receptors found in the basal ganglia could be responsible for this enlargement [26]. If age-related enlargement observed in females correlates directly with increased *TFEB* and *SCARB2* expression should be examined independently.

The cases included in this study had a wide range of disease duration, varying from two to 30 years. We found that *TFEB* expression in the temporal cortex is modulated by disease duration in LBP + ADP. Cases suffering from the disease for fewer than ten years showed high *TFEB* expression, similar to those found for the LBP group indicating that fast development of concomitant LBP and ADP might involve a higher level of molecular changes than their slow development. Accordingly, short duration of LBD has been associated with a more aggressive disease course [27].

When analyzing the differential expression of *SCARB2* transcripts, an opposite expression pattern for both transcripts was observed. Whereas *SCARB2tv2* was overexpressed in the temporal cortex, *SCARB2tv1* was overexpressed in the caudate nucleus. These results are in concordance with the previous observation that the deregulation of alternative splicing is associated with disease development on one hand, and brain-area specific, on the other [28].

Further research is needed to confirm our findings and to determine if the diminution of *GBA* promotes the increase of *TFEB* and *SCARB2* expression as cell response to restore proteostasis, or if both events occur simultaneously. Additionally, our data provide further evidence for gender-specific differences in the development of neurodegenerative diseases.

In conclusion, both alternative promoter usage and splicing are deregulated in LBD brain. Whereas female-gender associated overexpression of all transcripts was found in the caudate nucleus, disease-duration modulated *TFEB* expression in the temporal cortex of LBP + ADP cases. Finally, *SCARB2* and *TFEB* expression correlated negatively with *GBA* mRNA expression in the temporal cortex.

Conflict of interest statement

The authors report no conflict of interest regarding the contents of

the manuscript. Neither financial conflict is to be declared.

Acknowledgments

We are indebted to the Neurological Tissue Bank of the Biobank-Hospital Clinic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain for sample and data procurement. We thank the Institute of Neuropathology Brain Bank, L'Hospitalet de Llobregat, Spain. This work was supported by Spain's Ministry of Health, the PI18/00276 projects, integrated in the National R + D + I and funded by the ISCIII and the European Regional Development Fund. This work was supported CS is part of GREAB, research group from the Generalitat de Catalunya [2017SGR1630].

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2019.05.034.

References

[1] M.G. Spillantini, R.A. Crowther, R. Jakes, M. Hasegawa, M. Goedert, Alpha-synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 6469–6473.
[2] K.A. Jellinger, A critical evaluation of current staging of alpha-synuclein pathology in Lewy body disorders, Biochim. Biophys. Acta 1792 (2009) 730–740.
[3] K.A. Jellinger, Dementia with Lewy bodies and Parkinson's disease-dementia: current concepts and controversies, J. Neural Transm. (Vienna) 125 (2018) 615–650.
[4] J.R. Mazzulli, Y.H. Xu, Y. Sun, A.L. Knight, P.J. McLean, G.A. Caldwell, E. Sidransky, G.A. Grabowski, D. Krainc, Gaucher disease glucocerebrosidase and α-synuclein form a bidirectional pathogenic loop in synucleinopathies, Cell 146 (2011) 37–52.
[5] I.F. Mata, A. Samii, S.H. Schneer, J.W. Roberts, A. Griffith, B.C. Leis, Glucocerebrosidase gene mutations: a risk factor for Lewy body disorders, Arch. Neurol. 65 (2008) 379–382.
[6] M.A. Nalls, R. Duran, G. Lopez, M. Kurzawa-Akanbi, I.G. McKeith, P.F. Chinnery, C.M. Morris, J. Theuns, D. Crosiers, P. Cras, S. Engelborghs, P.P. De Deyn, C. Van Broeckhoven, D.M. Mann, J. Snowden, S. Pickering-Brown, N. Halliwell, Y. Davidson, L. Gibbons, J. Harris, U.M. Sheerin, J. Bras, J. Hardy, L. Clark, K. Marder, L.S. Honig, D. Berg, W. Maetzler, K. Brockmann, T. Gasser, F. Novellino, A. Quattrone, G. Annesi, E.V. De Marco, E. Rogaeva, M. Masellis, S.E. Black, J.M. Bilbao, T. Foroud, B. Ghetti, W.C. Nichols, N. Pankratz, G. Halliday, S. Lesage, S. Klebe, A. Durr, C. Duyckaerts, A. Brice, B.I. Giasson, J.Q. Trojanowski, H.I. Hurtig, N. Tayebi, C. Landazabal, M.A. Knight, M. Keller, A.B. Singleton, T.G. Wolfsberg, E. Sidransky, A multicenter study of glucocerebrosidase mutations in dementia with Lewy bodies, JAMA Neurol. 70 (2013) 727–735.
[7] M.E. Gegg, D. Burke, S.J. Heales, J.M. Cooper, J. Hardy, N.W. Wood, A.H. Schapira, Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains, Ann. Neurol. 72 (2012) 455–463.
[8] K.E. Murphy, A.M. Gysbers, S.K. Abbott, N. Tayebi, W.S. Kim, E. Sidransky, A. Cooper, B. Garner, G.M. Halliday, Reduced glucocerebrosidase is associated with increased α-synuclein in sporadic Parkinson's disease, Brain 137 (2014) 834–848.
[9] D. Reczek, M. Schwake, J. Schröder, H. Hughes, J. Blanz, X. Jin, W. Brondyk, S. Van Patten, T. Edmunds, P. Saftig, LIMP2 is a receptor for lysosomal mannose-6-phosphate-independent targeting of beta-glucocerebrosidase, Cell 131 (2007) 770–783.
[10] W. Song, F. Wang, M. Savini, A. Ake, A. di Ronza, M. Sardiello, L. Segatori, TFEB regulates lysosomal proteostasis, Hum. Mol. Genet. 22 (2013) 1994–2009.
[11] M. Decressac, B. Mattsson, P. Weikop, M. Lundblad, J. Jakobsson, A. Björklund, TFEB-mediated autophagy rescues midbrain dopamine neurons from α-synuclein toxicity, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) E1817–1826.
[12] S. Pal, R. Gupta, H. Kim, P. Wickramasinghe, V. Baubet, L.C. Showe, N. Dahmane, R.V. Davuluri, Alternative transcription exceeds alternative splicing in generating the transcriptome diversity of cerebellar development, Genome Res. 21 (2011) 1260–1272.
[13] K. Beyer, M. Domingo-Sàbat, J. Humbert, C. Carrato, I. Ferrer, A. Ariza, Differential expression of alpha-synuclein, parkin, and synphilin-1 isoforms in Lewy body disease, Neurogenetics 9 (2008) 163–172.
[14] K. Beyer, M. Domingo-Sàbat, C. Santos, E. Tolosa, I. Ferrer, A. Ariza, The decrease of β-synuclein in cortical brain areas defines a molecular subgroup of dementia with Lewy bodies, Brain 133 (2010) 3724–3733.
[15] L. Perez-Roca, C. Adame-Castillo, J. Campdelacreu, L. Ispierto, D. Vilas, R. Rene, J. Gascón-Bayarri, M.A. Serrano-Muñoz, A. Ariza, K. Beyer, Glucocerebrosidase mRNA is diminished in brain of Lewy body diseases and changes with disease progression in blood, Aging Dis. 9 (2018) 208–219.
[16] I.G. McKeith, D.W. Dickson, J. Lowe, M. Emre, J.T. O'Brien, H. Feldman, J. Cummings, J.E. Duda, C. Lippa, E.K. Perry, D. Aarsland, H. Arai, C.G. Ballard, B. Boeve, D.J. Burn, D. Costa, T. Del Ser, B. Dubois, D. Galasko, S. Gauthier, C.G. Goetz, E. Gomez-Tortosa, G. Halliday, L.A. Hansen, J. Hardy, T. Iwatsubo, R.N. Kalaria, D. Kaufer, R.A. Kenny, A. Korczyn, K. Kosaka, V.M. Lee, A. Lees, I. Litvan, E. Londos, O.L. Lopez, S. Minoshima, Y. Mizuno, J.A. Molina, E.B. Mukaetova-Ladinska, F. Pasquier, R.H. Perry, J.B. Schulz, J.Q. Trojanowski, M. Yamada, Consortium on DLB, Diagnosis and management of dementia with Lewy bodies: third report of the DLB Consortium, Neurology 65 (2005) 1863–1872.
[17] H. Braak, K. Del Tredici, U. Rüb, R.A. de Vos, E.N. Jansen Steur, E. Braak, Staging of brain pathology related to sporadic Parkinson's disease, Neurobiol. Aging 24 (2003) 197–211.
[18] H. Braak, E. Braak, Neuropathological stageing of Alzheimer-related changes, Acta Neuropathol. 82 (1991) 239–259.
[19] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) RESEARCH0034.
[20] C. Settembre, R. Zoncu, D.L. Medina, F. Vetrini, S. Erdin, S. Erdin, T. Huynh, M. Ferron, G. Karsenty, M.C. Vellard, V. Facchinetti, D.M. Sabatini, A. Ballabio, A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB, EMBO J. 31 (2012) 1095–1108.
[21] B. Dehay, J. Bové, N. Rodríguez-Muela, C. Perier, A. Recasens, P. Boya, M. Vila, Pathogenic lysosomal depletion in Parkinson's disease, J. Neurosci. 30 (2010) 12535–12544.
[22] X. Guo, P. Tang, L. Chen, P. Liu, C. Hou, X. Zhang, Y. Liu, L. Chong, X. Li, R. Li, Amyloid β-Induced redistribution of transcriptional factor EB and lysosomal dysfunction in primary microglial cells, Front. Aging Neurosci. 9 (2017) 228.
[23] L.M. Pardo, P. Rizzu, M. Francescatto, M. Vitezic, G.G. Leday, J.S. Sanchez, A. Khamis, H. Takahashi, W.D. van de Berg, Y.A. Medvedeva, M.A. van de Wiel, C.O. Daub, P. Carninci, P. Heutink, Regional differences in gene expression and promoter usage in aged human brains, Neurobiol. Aging 34 (2013) 1825–1836.
[24] V. La Cognata, V. D'Agata, F. Cavalcanti, S. Cavallaro, Splicing: is there an alternative contribution to Parkinson's disease? Neurogenetics 16 (2015) 245–263.
[25] K. Persson, V.D. Bohbot, N. Bogdanovic, G. Selbaek, A. Braekhus, K. Engedal, Finding of increased caudate nucleus in patients with Alzheimer's disease, Acta Neurol. Scand. 137 (2018) 224–232.
[26] A. Király, N. Szabó, E. Tóth, G. Csete, P. Faragó, K. Kocsis, A. Must, L. Vécsei, Z.T. Kincses, Male brain ages faster: the age and gender dependence of subcortical volumes, Brain Imaging Behav. 10 (2016) 901–910.
[27] S.A. Schneider, J.A. Obeso, Clinical and pathological features of Parkinson's disease, Curr. Top. Behav. Neurosci. 22 (2015) 205–220.
[28] L.W. Harries, D. Hernandez, W. Henley, A.R. Wood, A.C. Holly, R.M. Bradley-Smith, H. Yaghootkar, A. Dutta, A. Murray, T.M. Frayling, J.M. Guralnik, S. Bandinelli, A. Singleton, L. Ferrucci, D. Melzer, Human aging is characterized by focused changes in gene expression and deregulation of alternative splicing, Aging Cell 10 (2011) 868–878.

Autophagy genes are contrarily deregulated in brains with Lewy body pathology and brains with Alzheimer pathology.

Laia Pérez-Roca[1,2], Aintzane Urbizu[1], Ana Anillo[1], María A. Serrano-Muñoz[1], Cristina Santos[3], Katrin Beyer[1,2]*

From the (1) Department of Pathology, Hospital Universitari and Health Sciences Research Institute Germans Trias i Pujol, Badalona, Barcelona, Spain; (2) Universitat Autònoma de Barcelona, Spain; (3) Unitat d'Antropologia Biològica, Departament de Biologia Animal, Biologia Vegetal i Ecologia, Universitat Autònoma de Barcelona, Spain.

Corresponding Author: Katrin Beyer, PhD, Department of Pathology, Hospital Universitari Germans Trias i Pujol, 08916 Badalona, Barcelona, Spain.
Phone: 34-93-497 88 53, e-mail: katrinbeyer@hotmail.com

Abstract

Lewy body diseases comprise Parkinson disease (PD) and dementia with Lewy bodies (DLB), and show abnormal alpha-synuclein deposition leading to overlapping pathological features. Several studies have shown that autophagy is compromised in PD and especially post-transcriptional changes of the corresponding proteins lead to its alteration. With the aim to find out, if transcriptional changes of autophagy genes also contributes to the development of Lewy body pathology (LBP), we analyzed relative expression of *BECN1*, *mTOR*, *ATG5*, *ATG12*, *ATG3* and *LC3B* in samples from 12 brains with pure LBP, from 17 with both LBP and Alzheimer pathology (ADP), from 11 brains with pure ADP, and 9 control brains. Expression changes were obtained by real-time PCR and evaluated by the ΔΔCt method. No major expression changes of autophagy-related genes were found neither in LBP, LBP+ADP or ADP. Instead, some specific changes were identified. In temporal cortices with ADP overexpression of BECN1, and with LBP+ADP diminution of ATG5 was found. In caudate nuclei with ADP, increase of ATG3 was observed compared to controls, and of BECN1 and mTOR compared to LBP+ADP. Expression analysis in dependency of disease onset and duration revealed BECN1 overexpression in ADP temporal cortex of cases with short, and diminution of mTOR in cases with long disease duration. In LBP and LBP+ADP with short disease duration, down-regulation of LC3B and ATG5, respectively, was found. In late-onset ADP BECN1 was increased, and in late-onset LBP ATG5 and LC3B, and in late-onset LBP+ADP ATG5 were diminished.

In conclusion, autophagy driven by BECN1 overexpression is increased in ADP brains. In brains with LBP, autophagy is diminished due to decreased LC3B levels in pure LBP and to reduced ATG5 levels in LBP+ADP.

1.Introduction

Parkinson's disease (PD) and dementia with Lewy bodies (DLB) are Lewy body diseases (LBD). They are characterized by Lewy body pathology (LBP)[1], including Lewy bodies and Lewy neurites formed mainly by abnormally aggregated alpha-synuclein. Neuropathologically, two DLB forms can be distinguished: pure DLB, showing LBP only, and common DLB, where a mixture of LBP and concomitant Alzheimer disease (AD) pathology (ADP) can be found [2]. Lewy bodies are intraneuronal inclusion bodies and, whereas the substantia nigra is the most affected brain area in PD [3,4], widespread distribution of Lewy bodies throughout almost all brain areas is observed in DLB [5]. About 50% of PD patients develop dementia (PDD) after no less than 10 to 15 years following PD diagnosis[6]. Despite the fact that the clinical course differs between DLB and PDD, it is challenging to detect neuropathological differences between the two conditions.

Autophagy is the key mechanism inducing cell death (autophagy-induced cell death) that, similar to apoptosis represents one of the forms of regulated cell death. At the same time, autophagy is also the primary protein degradation system responsible for the turnover of bulk cellular constituents[7]. It plays an important role in cell homeostasis preserving the balance between synthesis, degradation and subsequent recycling of cellular components.

Several subtypes of autophagy have been described and include macroautophagy, microautophagy and chaperon mediated autophagy [8].

The macroautophagy pathway initiates with the formation of the phagophore constituted by a double membrane, isolating specific cellular components. The elongation of the phagophore gives rise to the autophagosome, which contains the intracellular cargo. The autophagosome fuses with the lysosome and its content is degraded by lysosomal

hydrolases. Protein aggregates or damaged organelles are degraded through this pathway [9].

Microautophagy is very similar to macroautophagy, but the formation of an autophagosome is not taking place. Instead, cytoplasmic constituents are engulfed directly by the lysosome [10].

Chaperon mediated autophagy (CMA) is responsible for the clearance of unfolded proteins containing the motif KFERQ. These are recognized by the cytosolic chaperone heat shock cognate 70 (hsc70) and transported to the lysosome for degradation[11].

Several genes have been identified to be responsible for driving the different stages of macroautophagy. Beclin, encoded by the gene *BECN1*, is an essential element of the class III phosphatidylinositol 3-kinase (PI3K-III) complex, which also contains other vacuolar sorting proteins[12]. This complex is necessary for the formation of the phagophore[13,14], and in combination with the conjugation systems ATG12-ATG5 and MAP1LC3, PI3K-III, it regulates membrane elongation and expansion during the formation of the autophagosome [15,16].

The ubiquitin-like protein ATG12 is activated by the ubiquitin-like E1 activating enzyme ATG7[17]. Then, ATG10, a ubiquitin-like E2 enzyme, attaches ATG12 covalently to its target protein ATG5. This conjugate subsequently associates with ATG16 assembling a complex[18] , essential for the formation of the double-membrane phagophore[19]. LC3 is conjugated to the growing phagophore by the E2-like enzyme ATG3 [20], [21], and there is evidence that the ATG12-ATG5 complex has E3-like activity responsible for efficient PE-lipidation of LC3[22].

In humans, the *LC3* gene family is constituted by three members *LC3A*, *LC3B* and *LC3C*. *LC3A* and *LC3B* undergo different types of post-translational processing and two transcript variants have been identified for each. Moreover, *LC3A* and *LC3B* are

abundantly expressed in the brain while *LC3C* expression is much lower [23, 24]. Before its incorporation to the autophagosome, LC3 undergoes post-translation modification and is coverted into a shorter form, LC3-II, by proteolytic cleavage[25]. It is this form II that is present in the autophagosomes.

The mammalian target of rapamycin (mTOR) encoded by its gene *MTOR*, is a nutrient sensor and one of the key regulators of autophagy. In presence of nutrients, mTOR inhibits autophagy meanwhile the absence of nutrients inhibits mTOR[16]. Although autophagy occurs at basal levels in all cells, diverse environmental stressors and nutrient deprivation are strong inducers of this degradation machinery[8]. *MTOR* has been identified as the main component of the mTOR complex 1 (mTORC1) and accumulating evidence has revealed that it represents a master regulator of autophagy. It is not only responsible for the initiation of autophagy, but regulates also all subsequent steps including the nucleation, autophagosome elongation, autophagosome maturation and termination [26].

Over the past years, intense investigation has revealed which proteins are involved in the different stages of authophagy, but only very few studies have addressed the identification of transcriptional changes of these genes. Therefore, the present study aimed to analyze the mRNA expression at different points of autophagy and to identify possible disease-associated changes in Lewy body disorders.

2.Materials and methods

2.1. Source of tissues

Post-mortem brain samples with clinical and neuropathological diagnoses were provided by the Institute of Neuropathology Brain Bank and the Neurological Tissue Bank of the University of Barcelona/Hospital Clinic in Barcelona, Spain. These samples

were obtained from 12 brains that predominantly presented pure LBP, from 17 with both LBP and ADP, from 11 brains with only ADP, and 9 donors devoid of neurological signs or symptoms and lack of neuropathological findings. Samples from the caudate nucleus and temporal cortex were obtained in all cases. Temporal cortex was included because of its early involvement in DLB and caudate nucleus, because of its early involvement in PD. The presence and distribution of AS related pathology was evaluated following the criteria established by the third report of the DLB consortium[27] and according to Braak and Braak[28]. AD-type pathology was assessed by using the Braak and Braak criteria [29]. The study was carried out in accordance with the requirements of the local Ethics Committee.

2.2. RNA isolation, reverse transcription and real-time polymerase chain reaction

TRI-Reagent (MRC, Cincinnati, USA) was used for RNA isolation according to the manufacturer's protocol. RNA quantity, purity and integrity was ascertained by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Only samples with RNA integrity values higher than six were stored at −80°C until use. Two microgram of total RNA were used for reverse transcription by Ready-to-go™ You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Uppsala, Sweden). A 'no reverse transcription' reaction, using water instead of reverse transcription mix, served as a control for the exclusion of genomic DNA contamination.

Relative expression of *BECN1*, *mTOR*, *ATG5*, *ATG12*, *ATG3* and *LC3B* was determined by real-time PCR on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia). PCR was performed in 15 ml reactions with the QuantiTect SYBR Green PCR Kit (QiaGen, Hilden, Germany), containing 16 pmol of each primer (Table 1) and 1 ml of

cDNA. To estimate relative expression changes, beta-actin (ACTB) was included as housekeeping gene.

All assays included two replicates of each sample and were independently performed twice to assure their reproducibility and minimize possible errors. Standard curves for the target and reference genes were generated for each run by amplifying the same serially diluted cDNA control samples. Primer sequences are given in table 1.

Table 1. RNA primer sequences used for the amplification of autophagic genes.

Primer Name	Primer sequence (5'–3')
Beclin-10/11 U	CAA ATC TAA GGA GCT GCC GT
Beclin-12L	CTT GCC TTT CTC CAC ATC CA
mTOR -6U	AGA CCT TGG CCA AAG AGA AG
mTOR -7L	GCA GTA CTT GTC GTG TAC CA
ATG3-10U	TTG AGC ACA TGT ATG AAG ACA T
ATG3-11/12L	AGA AGA TAC ATA TGA ACT CC
ATG5-1U	GCC ATA GCT TGG AGT AGG TTT
ATG5-2L3	ACC ACA CAT CTC GAA GCA CA
ATG12-2U	AGT AG AGC GAC GAA CCA
ATG12-4L	CCT GAG ACT TGC AGT AAT GTA
MAP1LC3B- 1/2U	GCA CCT TCG AAC AAA GAG TA
MAP1LC3B-3L	CTC ACT CAT GTT GAC ATG GT
b-act U2	TCT ACA ATG AGC TGC GTG TG
b-act L2	GGA TAG CAA CGT ACA TGG CT

2.3. Statistical analysis

Relative mRNA expression changes were assessed by the ΔΔCt method. According to the ΔΔCt method, values below 0.5 represented significantly decreased expression

levels, while values above 1.5 corresponded to significantly increased expression levels [30,31,32]. The results were tested for significance by the Wilkoxon-Mann-Whithey test. Possible differences in quantitative variables (age at disease onset, age at death and disease duration) between the pathological groups were assessed by the Mann-Whitney test. Qualitative variables, such as gender, were tested using Fisher's exact test.

3.Results

3.1. Sample characteristics and pathological groups

The samples from diseased brains were divided into three main groups, ADP, LBP and LBP with concomitant ADP. Additional clinical characteristics including age at death, age at disease onset and disease duration are shown in table 2. Neither age at onset, age at death or disease duration differed significantly between groups. Although control subjects were significantly younger at death compared to diseased individuals ($p=0.012$), no significant differences were detected when comparing age at death of control subjects and age of disease onset of patients.

Table 2. Clinical characteristics of patients and controls included in the study.

	n	Onset disease	Death	Duration
LBP+ADP	17	66,7+-9,7	80,4+- 4,0	13,4 +- 8,6
LBP	12	61,4+-9,5	75,4+-8,2	12,9 +-9,4
ADP	11	67,0 +-8,7	76,1 +-7,3	8,9 +-2,7
CTRLs	8	n/a	61,8+- 9,6	n/a

3.2. Brain expression levels of BECN1, mTOR, ATG3, ATG5 and ATG12

All results are represented as relative expression changes compared to controls and only significant changes are given in the text and shown in Figure 1.

In the temporal cortex, *BECN1* was overexpressed in ADP in comparison with controls (relative expression change: 1,90; p=0,0062) but its expression remained unchanged in LBP and LBP+ADP. Furthermore, *ATG5* was downregulated in LBP+ADP (relative expression change: 0,45; p=0,008) and did not show changes in the other pathologies (Fig. 1). *ATG3*, *ATG12* and *LC3B* expression levels did not differ between pathological groups in this brain area.

In the caudate nucleus, *ATG3* expression was increased in ADP (relative expression change: 2,10; p=0,021). No differences in gene expression between disease groups and controls were observed in other genes. Instead, *BECN1* and *mTOR* (Fig.1) were overexpressed in ADP in comparison with LBP+ADP (p= 0,022 and p= 0,027 respectively).

3.3. Characterization of patients by disease onset and duration

The impact of age at onset and disease duration on gene expression was studied by dividing patients into the following groups: 1. according to age at onset: (a) patients who developed the disease before the age of 65 years and (b) patients who developed disease at the age of 65 years or later (Table 3); and 2. according to disease duration: (a) less than 10 years or (b) 10 years or more (Table 4).

When dividing groups by age-at-onset, patients who developed both LBP and ADP before the age of 65 years suffered almost three times longer from the disease than patients who developed disease at 65 years or later. However, the age at death was similar in both groups (Table 3). Patients who developed only LBP did not show differences in disease duration, but patients with earlier disease onset died at younger

ages (Table 3). No differences between disease-onset dependent groups were found for patients who developed only ADP, neither for disease duration nor for age at death (Table 3).

Table 3. Clinical characteristics of patients and controls in the disease onset.

		<65y	≥65y
LBP+ADP	n	6	10
	age at onset	55 (52-62)	73 (69-83)
	age	79 (76-87)	81 (77-90)
	duration	24 (21-29)	8 (2-16)
LBP	n	7	4
	age at onset	56 (45-62)	71 (65-81)
	age	71 (60-82)	80 (74-83)
	duration	15 (4-28)	9 (2-16)
ADP	n	5	5
	age at onset	59 (57-61)	75 (72-77)
	age	69 (67-72)	82 (77-85)
	duration	10 (8-14)	7 (5-11)

When dividing patients by disease duration, significant differences were found only for patients who developed LBP with concomitant ADP. Patients who suffered from disease for less than 10 years were older at disease onset than patients suffering from the disease for 10 years or more (Table 4).

Table 4. Clinical characteristics of patients and controls in duration groups.

		<10	≥10
LBP+ADP	n	7	8
	age at onset	74 (70-83)	60 (51-73)
	age	80 (77-90)	80 (76-85)
	duration	6 (2-8)	20 (11-29)
LBP	n	6	6
	age at onset	61,4(56-81)	58 (45-66)
	age	75,42 (60-88)	78 (73-82)
	duration	12,91 (2-8)	20 (14-28)
ADP	n	6	4
	age at onset	70 (58-77)	63 (57-76)
	age	76 (67-85)	74 (67-85)
	duration	7 (5-9)	12 (10-14)

3.4. Expression and disease duration

We analyzed the expression change in disease duration-dependent groups in comparison with controls and comparing both groups of each pathology. Patients with disease duration of less than 10 years were defined to have short and patients with disease duration of 10 years or more, to have long disease duration.

In ADP temporal cortices, both *BECN1* and *mTOR* were deregulated showing opposite expression. Whereas *BECN1* was upregulated in cases with short disease duration (1,94; p=0,002), *mTOR* showed decreased expression with longer disease duration (0,35; p=0,017). *ATG5* was downregulated in cases with LBP+ADP with short disease duration in comparison with controls (0,45; p= 0,035), and also in comparison with LBP+ADP cases with long disease duration (p= 0,008; Fig. 2A).

LC3B was downregulated in LBP temporal cortices compared to controls in cases with short disease duration (0,42, p=0,030; Fig. 2A), and upregulated in LBP with concomitant ADP caudate nuclei of short disease duration compared to long disease duration (p=0,014; Fig. 2B).

3.5. Expression and age at disease onset

Disease onset before the age of 65 years was defined as early disease onset and at the age of 65 or later as late disease onset. When analyzing expression in these groups, changes were detected only in the temporal cortex and only in patients with late disease onset. Whereas *BECN1* expression was increased in ADP (1,44; p= 0,006), both *ATG5* and *LC3B* were diminished in LBP (0,43; p=0,013, and 0,47; p=0,012, respectively), and *ATG5* was diminished in LBP+ADP (0,48; p=0,023).

Expression analysis of the different genes and gender could not be carried out since our patients are not well balanced between both genders.

4.Discussion

In the present study, we have analyzed the expression of six genes involved in autophagy in the temporal cortex and caudate nucleus of brains with ADP, LBP+ADP and LBP and compared it to control brains. The most affected brains were those with ADP and showed overexpression of *BECN1* in the temporal cortex and of *ATG3* in the caudate nucleus. Moreover, in the caudate nucleus, *BECN1* and mTOR were overexpressed in the same pathological group compared to brains with concomitant LBP and ADP.

Its has been shown that BECN1 forms part of a protein complex which is necessary for the initiation of the assembly of autophagosomes from phagophores[33]. Moreover, accumulating evidence suggests that BECN1 phosphorylation and ubiquitination is

responsible to maintain the equilibrium between pro-survival autophagy and pro-apoptotic responses [34]. Although post-transcriptional modification of BECN1 is mandatory for the initiation of autophagy, also the overexpression of its gene may contribute to the enhancement of autophagy.

ATG3 is an E2-like enzyme and conjugates LC3II to phosphatidylethanolamine (PE) present in the growing phagophore[35]. Since ATG3 is active only in the presence of a highly curved membrane, the conjugation takes place after initiation of autophagy, when the phagophore is enlarging[36] This observation indicates, that *ATG3* mRNA overexpression only, would not influence autophagy rate, because initiation factors are mandatory for the action of *ATG3*. However recently, *ATG3* and other autophagy genes have been involved in cellular processes different to autophagy. In this context, *ATG3* has been shown to participate in different membrane-trafficking pathways including the secretion of exosomes [37].

The development of ADP starts in the hippocampus[38] and with disease progression it spreads to the cerebral cortex, so that the temporal cortex is one of the early affected brain areas. At later stages ADP starts to affect the midbrain including the caudate nucleus[38]. Whereas *BECN1* overexpression seems to indicate enhanced autophagy in the cortex, it remains unclear if *ATG3* overexpression in caudate nucleus reflects altered autophagy or exosome secretion. Since the latter has been related to beta-amyloid spreading, increased *ATG3* expression could also enhance exosome release and contribute to disease propagation [39].

Interestingly, while transcriptional changes found in ADP corresponded to an up-regulation of mRNA expression, LBP-containing brains were, on the contrary, characterized by diminished expression of some autophagy related genes. In brains with LBP only, downregulation of LC3B was identified in temporal cortices of patients with

short disease duration and late disease onset. The cleaved form of LC3, LC3II is a main component of the phagophore[22,26]. Although posttranscriptional modification of LC3 is mandatory, its decrease at the transcriptional level may be directly responsible for its deficiency at the protein level, leading to a decreased autophagy.

ATG5 was downregulated in the temporal cortex of individuals who had developed concomitant LBP and ADP after the age of 65 years and had disease duration of less than 10 years. These clinical data indicate that specifically these patients suffered a more aggressive disease form. ATG5 is essential for the formation of autophagic vesicles and when knocked down, the downregulation or inhibition of autophagy has been observed [40]. It has been also shown that the conjugate constituted by ATG12 and ATG5 represents a key signaling molecule for the control of autophagy levels in a cell[41]. This conjugate promotes the formation of LC3II, enhancing the formation of the phagophore. When cleaved by calpain I, ATG5 dissociates from the conjugate, autophagy is downregulated and accompanied by elevated levels of apoptosis[42]. Our results together with these observations indicate that diminished efficiency of autophagy may contribute to the development of concomitant LBP and ADP, and additionally, contribute to an accelerated disease course.

Another important finding of our study is that transcriptional changes in autophagic genes are not a main factor for the development of either Lewy body diseases or AD. Instead, the extremely high intra-group variation of the expression levels of the different genes indicates that alteration of autophagy may exert a role as individual risk factor in certain percentage of the patients.

The study has two main weaknesses. On one hand the number of samples included in the ADP group is relatively small, and on the other hand, all samples were obtained

from end-disease-stage brains. If there are more important and/or generalized changes at early disease stages, remains to be determined.

However, there are two main observations that can be withdrawn. First, transcriptional changes in the expression of autophagy genes do not primarily drive the development of LBP or ADP, but may enhance the dysfunction of the authophagic system at specific points. Second, there is an urgent need to identify and characterize the different molecular subgroups of DLB, PD and AD, because in each of these, different molecular mechanisms mediate the development of the corresponding pathology. Once identified, we will be able to tackle with these diseases and to avoid their development by the use of adequate therapies in each case.

In conclusion, we found that autophagy may be increased in ADP and could be driven by *BECN1* overexpression in both the temporal cortex and caudate nucleus. Diminished autophagy levels could be associated to LBP, where diminished LC3B levels are characteristic for pure LBP containing brains, and diminished *ATG5* levels for brains with concomitant LBP and ADP.

Acknowledgements: We are indebted to the Neurological Tissue Bank of the Biobank-Hospital Clinic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain for sample and data procurement. We thank the Institute of Neuropathology Brain Bank, L'Hospitalet de Llobregat, Spain. This work was supported by Spain's Ministry of Health [grant number PI15/00216 (FIS)]. CS is part of GREAB, research group from the Generalitat de Catalunya [2017SGR1630].

Conflict of interest statement:

The authors report no conflict of interest regarding the contents of the manuscript. Neither financial conflict is to be declared.

References

1. Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M. & Goedert, M. alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6469–6473 (1998).

2. Braak, H. & Braak, E. Diagnostic criteria for neuropathologic assessment of Alzheimer's disease. *Neurobiol. Aging* 18, S85-88 (1997).

3. Jellinger, K. A. A critical evaluation of current staging of α-synuclein pathology in Lewy body disorders. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1792, 730–740 (2009).

4. Jellinger, K. A. Dementia with Lewy bodies and Parkinson's disease-dementia: current concepts and controversies. *J Neural Transm* 125, 615–650 (2018).

5. Ferman, T. J. & Boeve, B. F. Dementia with Lewy Bodies. *Neurologic Clinics* 25, 741–760 (2007).

6. Aarsland, D., Andersen, K., Larsen, J. P., Lolk, A. & Kragh-Sørensen, P. Prevalence and characteristics of dementia in Parkinson disease: an 8-year prospective study. *Arch. Neurol.* 60, 387–392 (2003).

7. Parzych, K. R. & Klionsky, D. J. An Overview of Autophagy: Morphology, Mechanism, and Regulation. *Antioxidants & Redox Signaling* 20, 460–473 (2014).

8. Li, S. & Le, W. An insight review of autophagy biology and neurodegenerative diseases: machinery, mechanisms and regulation. *Science China Life Sciences* 60, 1457–1459 (2017).

9. Cheung, Z. H. & Ip, N. Y. Autophagy deregulation in neurodegenerative diseases - recent advances and future perspectives: Autophagy deregulation in neurodegeneration. *Journal of Neurochemistry* 118, 317–325 (2011).

10. Guo, F., Liu, X., Cai, H. & Le, W. Autophagy in neurodegenerative diseases: pathogenesis and therapy: Autophagy in neurodegenerative diseases. *Brain Pathology* 28, 3–13 (2018).

11. Liu, X. *et al.* Chaperone-mediated autophagy and neurodegeneration: connections, mechanisms, and therapeutic implications. *Neurosci Bull* 31, 407–415 (2015).

12. Spencer, B. *et al.* Beclin 1 Gene Transfer Activates Autophagy and Ameliorates the Neurodegenerative Pathology in -Synuclein Models of Parkinson's and Lewy Body Diseases. *Journal of Neuroscience* 29, 13578–13588 (2009).

13. Glick, D., Barth, S. & Macleod, K. F. Autophagy: cellular and molecular mechanisms. *J. Pathol.* 221, 3–12 (2010).

14. Arroyo, D. S. *et al.* Autophagy in inflammation, infection, neurodegeneration and cancer. *International Immunopharmacology* 18, 55–65 (2014).

15. Yang, Y., Liang, Z., Gu, Z. & Qin, Z. Molecular mechanism and regulation of autophagy1. *Acta Pharmacologica Sinica* 26, 1421–1434 (2005).

16. Nixon, R. A. The role of autophagy in neurodegenerative disease. *Nature Medicine* 19, 983–997 (2013).

17. Mizushima, N., Yoshimori, T. & Ohsumi, Y. The Role of Atg Proteins in Autophagosome Formation. *Annu. Rev. Cell Dev. Biol.* 27, 107–132 (2011).

18. Suzuki, K., Noda, T. & Ohsumi, Y. Interrelationships among Atg proteins during autophagy inSaccharomyces cerevisiae. *Yeast* 21, 1057–1065 (2004).

19. Ohsumi, Y. Molecular dissection of autophagy: two ubiquitin-like systems: Ubiquitin and proteasomes. *Nature Reviews Molecular Cell Biology* 2, 211–216 (2001).

20. Tanida, I., Nishitani, T., Nemoto, T., Ueno, T. & Kominami, E. Mammalian Apg12p, but not the Apg12p.Apg5p conjugate, facilitates LC3 processing. *Biochem. Biophys. Res. Commun.* 296, 1164–1170 (2002).

21. Yamada, Y. *et al.* The Crystal Structure of Atg3, an Autophagy-related Ubiquitin Carrier Protein (E2) Enzyme that Mediates Atg8 Lipidation. *Journal of Biological Chemistry* 282, 8036–8043 (2007).

22. Radoshevich, L. *et al.* ATG12 Conjugation to ATG3 Regulates Mitochondrial Homeostasis and Cell Death. *Cell* 142, 590–600 (2010).

23. Dooley, H. C. *et al.* WIPI2 Links LC3 Conjugation with PI3P, Autophagosome Formation, and Pathogen Clearance by Recruiting Atg12–5-16L1. *Molecular Cell* 55, 238–252 (2014).

24. Koukourakis, M. I. *et al.* Autophagosome Proteins LC3A, LC3B and LC3C Have Distinct Subcellular Distribution Kinetics and Expression in Cancer Cell Lines. *PLOS ONE* 10, e0137675 (2015).

25. He, H. *et al.* Post-translational Modifications of Three Members of the Human MAP1LC3 Family and Detection of a Novel Type of Modification for MAP1LC3B. *Journal of Biological Chemistry* 278, 29278–29287 (2003).

26. Dossou, A. S. & Basu, A. The Emerging Roles of mTORC1 in Macromanaging Autophagy. *Cancers* 11, 1422 (2019).

27. McKeith, I. G. *et al.* Diagnosis and management of dementia with Lewy bodies: Fourth consensus report of the DLB Consortium. *Neurology* 89, 88–100 (2017).

28. Braak, H. *et al.* Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of Aging* 24, 197–211 (2003).

29. Braak, H. & Braak, E. Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol* 82, 239–259 (1991).

30. Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3, 1101–1108 (2008).

31. Rutledge, R. G. Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Research* 31, 93e–993 (2003).

32. Schefe, J. H., Lehmann, K. E., Buschmann, I. R., Unger, T. & Funke-Kaiser, H. Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's C T difference" formula. *J Mol Med* 84, 901–910 (2006).

33. Matsunaga, K. *et al.* Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L. *The Journal of Cell Biology* 190, 511–521 (2010).

34. Menon, M. B. & Dhamija, S. Beclin 1 Phosphorylation – at the Center of Autophagy Regulation. *Front. Cell Dev. Biol.* 6, 137 (2018).

35. Ichimura, Y. *et al.* A ubiquitin-like system mediates protein lipidation. *Nature* 408, 488–492 (2000).

36. Nath, S. *et al.* Lipidation of the LC3/GABARAP family of autophagy proteins relies on a membrane-curvature-sensing domain in Atg3. *Nat Cell Biol* 16, 415–424 (2014).

37. Levine, B. & Kroemer, G. Biological Functions of Autophagy Genes: A Disease Perspective. *Cell* 176, 11–42 (2019).

38. Braak, H. & Braak, E. Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol* 82, 239–259 (1991).

39. Sardar Sinha, M. *et al.* Alzheimer's disease pathology propagation by exosomes containing toxic amyloid-beta oligomers. *Acta Neuropathol* 136, 41–56 (2018).

40. Ye, X., Zhou, X.-J. & Zhang, H. Exploring the Role of Autophagy-Related Gene 5 (ATG5) Yields Important Insights Into Autophagy in Autoimmune/Autoinflammatory Diseases. *Front. Immunol.* 9, 2334 (2018).

41. Romanov, J. *et al.* Mechanism and functions of membrane binding by the Atg5-Atg12/Atg16 complex during autophagosome formation: Role of Atg5-Atg12/Atg16 during autophagy. *The EMBO Journal* 31, 4304–4317 (2012).

42. Xia, H.-G. *et al.* Control of basal autophagy by calpain1 mediated cleavage of ATG5. *Autophagy* 6, 61–66 (2010).

Figure legends:

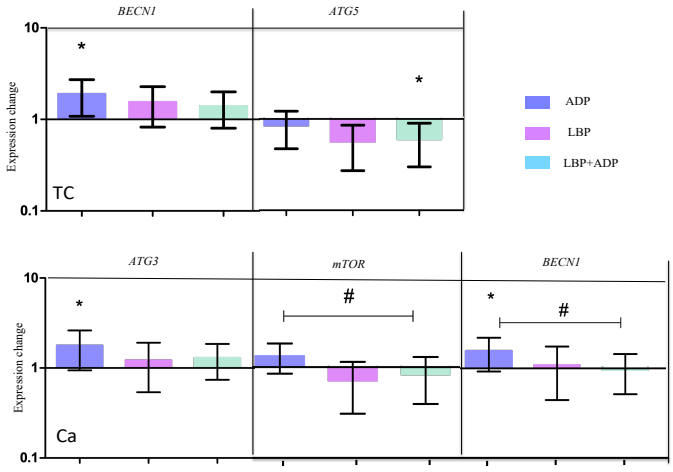

Figure 1. Expression profiles of *BECN1*, *ATG5*, *mTOR* and *ATG3* in two brain areas with ADP, LBP and LBP+ADP after adjustment with controls. The included areas were temporal cortex (TC) and caudate nucleus (Ca) from the groups of Alzheimer pathology (ADP), Lewy body pathology (LBP) and Lewy body pathology with concomitant Alzheimer (LBP+ADP). The results are shown as relative expression changes obtained by the ddCt method in comparison with normal controls and are represented in a logarithmic scale. * Significant expression change vs. CTRLs. # Significant expression change comparing two disease groups.

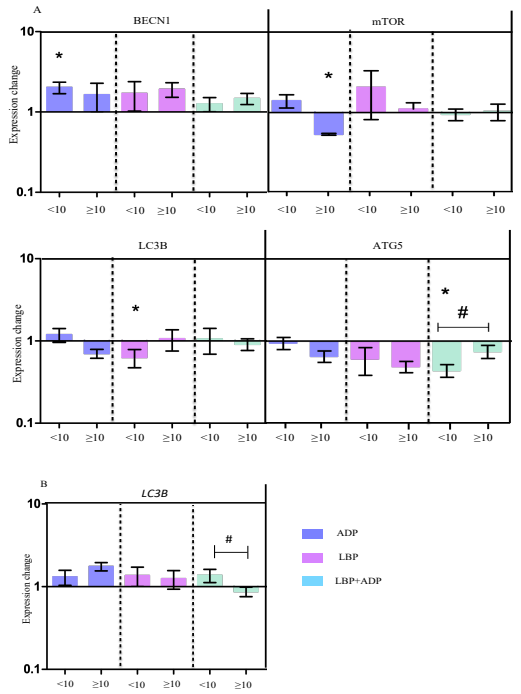

Figure 2. *BECN1*, *mTOR*, *ATG5* and *LC3B* expression in ADP, LBP and LBP+ADP brains in dependency on disease duration in (A) temporal cortex and (B) caudate nucleus. * Significant expression vs CTRLs. # Significant expression change between the disease duration subgroups.

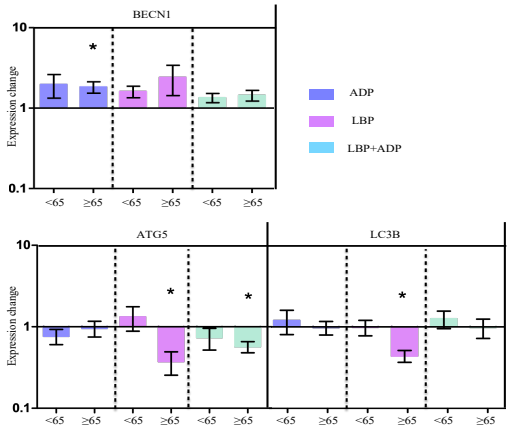

Figure 3. *BECN1*, *ATG5* and *LC3B* expression in ADP, LBP and LBP+ADP brains in dependency on the age of disease onset. The results are shown as relative expression changes comparison with control individuals. *Significant expression change vs. CTRLs. #Significant expression change between the age-at-onset dependent subgroups.

4.DISCUSIÓN

Con esta tesis hemos querido contribuir a elucidar el posible papel del sistema autofágico-lisosomal durante el desarrollo de las enfermedades con cuerpos de Lewy, con especial énfasis a la demencia con cuerpos de Lewy (DCL). Aunque esta demencia representa, después de la enfermedad de Alzheimer, la segunda causa más importante de la demencia, su diagnóstico clínico es muy complejo y hasta el 80% de todos los casos siguen siendo diagnosticados erróneamente. La causa para esta dificultad diagnóstica se encuentra a nivel neuropatológico, ya que la DCL comparte cambios característicos con la enfermedad de Alzheimer (EA), por una parte, y al ser una sinucleinopatía, con la enfermedad de Parkinson (EP), por otra. La mayoría de los pacientes con DCL se diagnostican como EA y, consecuentemente, reciben los tratamientos correspondientes. Estos tratamientos causan reacciones adversas severas en un 50% de ellos, así que la caracterización molecular de la DCL identificando mecanismos moleculares específicos para esta es de primordial importancia. Solamente identificando dichos mecanismos será posible desarrollar las terapias necesarias.

Nuestra primera pregunta se dirigía hacia el gen *GBA* que se había involucrado en el desarrollo de las enfermedades con cuerpos de Lewy por una elevada presencia de variantes raras, y un déficit de la enzima glucocerebrosidasa (GCasa) en la sustancia nigra de pacientes con EP. Queríamos saber si cambios similares se detectan por una parte también a nivel transcripcional, y por otra parte en sangre, reflejando de este modo los cambios que tienen lugar en el cerebro.

Nuestros resultados revelaron una disminución de la expresión del gen de la GCasa, *GBA*, en zonas cerebrales específicas. Mientras que la expresión de *GBA* estaba disminuida en el córtex temporal de pacientes con DCL, esta disminución fue encontrada en el núcleo caudado de pacientes con EP con demencia. Nuestros hallazgos subrayan la participación específica de las regiones corticales durante el desarrollo de la DCL [87] y la asociación del núcleo caudado con la demencia en las enfermedades con cuerpos de Lewy. En este contexto, se había demostrado que la depleción de dopamina en el núcleo caudado puede detectarse en la DCL[88] y se correlaciona con el deterioro cognitivo en la EP [89] Además, cabe destacar que una de las funciones de la GCasa es prevenir la agregación de la α -sinucleína, así que la disminución de la GCasa favorece esta agregación [36]. De este modo, nuestros resultados revelaron que la expresión de *GBA* está

disminuida en las regiones cerebrales con los niveles más elevados de α - sinucleína agregada, lo que permite asociar *GBA* directamente a la etiopatogenia de la DCL y EP. La disminución de la actividad de GCasa se había encontrado anteriormente en áreas cerebrales vulnerables en EP, incluyendo el cerebelo, la sustancia negra [90] y el giro cingulado anterior, una región que acumula α - sinucleína alterada en EP esporádica [91]. En ninguno de los dos estudios se encontraron cambios en los niveles o actividad de GCasa en las áreas corticales. En DCL, se han encontrado niveles de GCasa disminuidos en un 20% en la corteza frontal de los portadores de variantes de *GBA*[92]. Nuestros resultados en cambio indican que el déficit de GCasa no solamente se encuentra en portadores de variantes raras, sino en todos los pacientes afectados por la enfermedad y que este déficit comienza al nivel transcripcional.

No solamente detectamos la disminución de la expresión de *GBA* en cerebro, sino también en sangre de pacientes con DCL y EP. Estos resultados están acorde con estudios previos, donde la actividad de la GCasa en sangre de pacientes con EP con y sin mutaciones de *GBA* estaba disminuida[93]. Similar a lo observado en cerebro, también en sangre la deficiencia de la GCasa está causada por una alteración a nivel transcripcional y parece reflejar la patología cerebral.

Para determinar si la disminución de *GBA* está asociada a factores específicos, estudiamos su posible correlación con diferentes parámetros clínicos. Por una parte, detectamos la correlación lineal entre la duración desde el inicio de la enfermedad y la expresión de *GBA* en la EP, siendo los niveles de *GBA* más bajos en enfermos con debut reciente. Por otra parte, tanto en DCL como EP identificamos una correlación lineal entre la expresión de *GBA* y la edad de inicio de la enfermedad, pero con tendencias opuestas. Mientras que en DCL los niveles de *GBA* eran más bajos en pacientes con el inicio de la enfermedad más temprano, en la EP, *GBA* estaba más disminuido en pacientes con el inicio de la enfermedad más tardío. La edad de inicio juega un papel importante en la progresión de las enfermedades con cuerpos de Lewy y se ha postulado que también es opuesta para DCL y EP. Mientras que la enfermedad progresa más lentamente en pacientes con EP de inicio joven, la DCL muestra un curso más agresivo en pacientes con inicio temprano [94, 95].

Los cambios de expresión de un gen específico se observan generalmente en el contexto de alteraciones de vías metabólicas o de señalización. La GCasa es una enzima lisosomal, y su funcionalidad y cambios en esta dependen de factores adicionales. Por una parte, su expresión está regulada por el factor de transcripción EB (*TFEB*) que es un regulador clave de la biogénesis y función lisosomal[25] , y por otra parte, solo la unión a su receptor LIMP2 puede efectuar su internalización al lisosoma.

Por eso, nuestra segunda pregunta iba dirigida a la expresión de los genes *TFEB* y *SCARB2* (gen de LIMP2) en las mismas regiones cerebrales estudiadas para *GBA*. Los resultados obtenidos correspondían a alteraciones menos pronunciadas, que correlacionaban en este caso más con los cambios patológicos que la presentación clínica. Como hallazgo principal, identificamos una marcada sobreexpresión de *TFEB*, especialmente en la corteza temporal con patología Lewy pura. Este incremento de *TFEB* estaba acompañado de una sobreexpresión importante de uno de los transcritos de *SCARB2* en la misma área cerebral.

En su papel de regulador lisosomal, *TFEB* se ha descrito como neuroprotector en modelos de trastornos neurodegenerativos [52, 51]Además, la activación de *TFEB* en neuronas atenúa la generación de beta-amiloide y la formación de placas amiloideas [51]. Nuestros resultados podrían reflejar esta posible función de *TFEB*, ya que su sobreexpresión fue detectada en cerebros sin patología Alzheimer concomitante. Adicionalmente, encontramos una correlación entre los niveles de expresión de *TFEB* y *GBA*, correspondiendo los niveles más elevados de *TFEB* con la disminución más pronunciada de *GBA*. Estos resultados indican, que el déficit de GCasa en el sistema lisosomal podría representar una señal para la inducción de la expresión de *TFEB*, resultando en niveles elevados del mismo. También *SCARB2*tv2 presentaba una correlación negativa con la expresión de *GBA*, y mientras más bajos los niveles de *GBA*, más sobreexpresión se observaba en *SCARB2*. Estos resultados indican que la disminución de *GBA* en la corteza puede activar la expresión de *TFEB* como respuesta celular para restaurar la proteostasis lisosomal, pero la activación de *TFEB* resultaría al mismo tiempo en la sobreexpresión de otros genes lisosomales en esta área cerebral, incluyendo *SCARB2*.

Los casos incluidos en este estudio tenían un amplio rango de duración de la

enfermedad, que variaba entre los 2 y los 30 años. Teniendo en cuenta este parámetro encontramos, que la expresión de *TFEB* está modulada por la duración de la enfermedad en el córtex con patología Lewy y patología Alzheimer concomitante. *TFEB* estaba especialmente sobreexpresado en cerebros de pacientes que padecieron la enfermedad durante menos de 10 años, sugiriendo que el desarrollo rápido de la patología implica cambios moleculares más pronunciados que su desarrollo lento. Correspondientemente, la DCL se caracteriza por una progresión agresiva y duración corta[96].

Como el sistema lisosomal está estrechamente asociado con el sistema autofágico, nuestra tercera pregunta se dirigía hacia el transcriptoma del mismo (artículo pendiente de publicar). En este contexto analizamos la expresión de seis genes implicados en puntos específicos de la autofagia. Estudiamos su expresión en la las mismas áreas cerebrales, corteza temporal y el núcleo caudado, con la misma patología que en los estudios anteriores. Aparte de cerebros con patología Lewy pura y patología Lewy con patología Alzheimer concomitante, también incluimos cerebros con patología Alzheimer pura.

Los casos más afectados habían desarrollado patología Alzheimer pura y presentaban con una sobre expresión de *BECN1* en la corteza temporal y de *ATG3* en el núcleo caudado, indicando que los niveles de autofagia están incrementados en estos casos. Al contrario, los niveles de autofagia parecen estar disminuidos en cerebros con patología Lewy pura debido a la disminución de *LC3B*, y en cerebros patología Lewy con patología Alzheimer concomitante debido a la disminución de *ATG5*. En este último grupo la regulación negativa de *ATG5* se observaba especialmente en casos de debut tardío y duración corta de la enfermedad, indicando que este mecanismo está asociado a las formas más agresivas de la enfermedad.

Se ha demostrado que BECN1 forma parte de un complejo proteico necesario para la formación de autofagosomas [97], y su fosforilación y ubiquitinación regula el equilibrio entre la autofagia pro-supervivencia y las respuestas proapoptóticas[98]. Aunque la modificación postranscripcional de BECN1 es necesaria para iniciar la autofagia, también la sobreexpresión de su gen puede contribuir al incremento de la misma. ATG5 es esencial durante la formación de vesículas autofágicas [99] y ATG12 y ATG5 en forma de conjugado representan

una molécula de señalización clave para el control de los niveles de autofagia celular [100]. ATG12-ATG5 promueven la formación de LC3II, potenciando la formación del fagóforo, y niveles disminuidos de *ATG5* están asociados a la reducción de la actividad autofágica [99, 101].

El hallazgo más importante de este estudio es que cambios transcripcionales en los genes autofágicos no están primariamente involucrados en el desarrollo de enfermedades con cuerpos de Lewy. En cambio, la variación encontrada dentro de cada grupo de pacientes es muy elevada indicando que alteraciones puntuales en los mecanismos de la autofagia pueden actuar como factores de riesgo individuales.

En resumen, en esta tesis hemos descrito que cambios transcripcionales en el sistema lisosomal intervienen en el desarrollo de las enfermedades con cuerpos de Lewy, especialmente en el desarrollo de las formas más agresivas de la enfermedad. Estos cambios se observan en las áreas cerebrales especialmente vulnerables en estas enfermedades. En cambio, alteraciones en la autofagia parecen jugar un papel menos generalizado, afectando a cada paciente de forma diferencial y representando de esta manera moduladores de la enfermedad.

En conjunto nuestros resultados indican que el sistema lisosomal puede representar una diana terapéutica para las enfermedades con cuerpos de Lewy dirigiendo posibles terapias a las áreas cerebrales específicamente afectadas en cada una de ellas.

5.CONCLUSIONES

- Los perfiles de expresión de *GBA* son área-dependientes y específicos para las dos ECL, y están caracterizados por la disminución de *GBAtv1* en DCLc, y de *GBAtv1* y *tv5* en DCLp en el córtex temporal. Y de ambos transcritos en el núcleo caudado de EPCD.
- El splicing alternativo del gen GBA está desregulado en las ECL, indicando que *GBAtv5* juega un rol específico en la patogénesis de las ECL.
- En las ECL, la deficiencia de GCasa comienza a nivel transcripcional, ya que *GBAtv1* está disminuida en sangre y cerebro de estos pacientes.
- La expresión de *GBAtv1* correlaciona con la edad de inicio de la enfermedad, teniendo los niveles más bajos los pacientes con DCL de debut temprano y pacientes con EP de debut tardío.
- Los perfiles de expresión de los genes reguladores de *GBA, TFEB* y *SCARB2* son específicos para cada área cerebral y cada patología.
- La expresión de *SCARB2tv2* está correlacionada negativamente con la expresión de *GBAtv1* en el córtex temporal.
- El splicing y uso de promotor alternativos de *SCARB2* y *TFEB* están desregulados en cerebros con patología Lewy, ya sea pura o acompañada con patología Alzheimer.
- La duración de la enfermedad modula la expresión de *TFEB* en el córtex temporal de cerebros con pCL+pEA y su expresión está especialmente desregulada en casos de menos de 10 años de duración de la enfermedad.
- Los cambios de expresión de los genes involucrados en la autofagia no son primariamente responsables del desarrollo de la pCL o pEA, pero pueden potenciar la disfunción del sistema autofágico en puntos específicos de la misma.
- Los niveles de la autofagia están aumentados en pEA debido a la sobreexpresión de BECN1 tanto en la corteza temporal como en el núcleo caudado.

- Los niveles de la autofagia están disminuidos en cerebros con pCL, donde la expresión de *LC3B* está disminuida en pCL y de *ATG5* en pCL + pEA.

6.REFERENCIAS

1. David Ellison *et al. Neuropathology A reference text of CNS pathology*.
2. Kovács, G. *Neuropathology of neurodegenerative diseases: a practical guide*. (Cambridge University Press, 2015).
3. Magalingam, K. B., Radhakrishnan, A., Ping, N. S. & Haleagrahara, N. Current Concepts of Neurodegenerative Mechanisms in Alzheimer's Disease. *BioMed Research International* **2018**, 1–12 (2018).
4. Kovacs, G. G. Concepts and classification of neurodegenerative diseases. in *Handbook of Clinical Neurology* vol. 145 301–307 (Elsevier, 2018).
5. Kovacs, G. Molecular Pathological Classification of Neurodegenerative Diseases: Turning towards Precision Medicine. *International Journal of Molecular Sciences* **17**, 189 (2016).
6. *Neuropathology: a reference text of CNS pathology ; [get full access and more at ExpertConsult.com]*. (Mosby, Elsevier, 2013).
7. Beyer, K., Domingo-Sàbat, M. & Ariza, A. Molecular Pathology of Lewy Body Diseases. *International Journal of Molecular Sciences* **10**, 724–745 (2009).
8. Beyer, K. & Ariza, A. Protein Aggregation Mechanisms in Synucleinopathies: Commonalities and Differences. *Journal of Neuropathology & Experimental Neurology* **66**, 965–974 (2007).
9. Sanford, A. M. Lewy Body Dementia. *Clinics in Geriatric Medicine* **34**, 603–615 (2018).
10. Olanow, C. W., Perl, D. P., DeMartino, G. N. & McNaught, K. S. P. Lewy-body formation is an aggresome-related process: a hypothesis. *The Lancet Neurology* **3**, 496–503 (2004).

11. Shults, C. W. Lewy bodies. *Proceedings of the National Academy of Sciences* **103**, 1661–1668 (2006).

12. Beyer, K. & Ariza, A. The therapeutical potential of alpha-synuclein antiaggregatory agents for dementia with Lewy bodies. *Curr. Med. Chem.* **15**, 2748–2759 (2008).

13. McKeith, I. G. Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the Consortium on DLB International Workshop. *J. Alzheimers Dis.* **9**, 417–423 (2006).

14. Nussbaum, R. L. & Ellis, C. E. Alzheimer's Disease and Parkinson's Disease. *New England Journal of Medicine* **348**, 1356–1364 (2003).

15. Lees, A. J., Hardy, J. & Revesz, T. Parkinson's disease. *The Lancet* **373**, 2055–2066 (2009).

16. Aarsland, D., Andersen, K., Larsen, J. P., Lolk, A. & Kragh-Sørensen, P. Prevalence and characteristics of dementia in Parkinson disease: an 8-year prospective study. *Arch. Neurol.* **60**, 387–392 (2003).

17. Colom-Cadena, M. *et al.* Regional Overlap of Pathologies in Lewy Body Disorders. *Journal of Neuropathology & Experimental Neurology* (2017) doi:10.1093/jnen/nlx002.

18. Palmqvist, S., Hansson, O., Minthon, L. & Londos, E. Practical suggestions on how to differentiate dementia with Lewy bodies from Alzheimer's disease with common cognitive tests. *International Journal of Geriatric Psychiatry* **24**, 1405–1412 (2009).

19. Braak, H. & Braak, E. Diagnostic Criteria for Neuropathologic Assessment of Alzheimer's Disease. *Neurobiology of Aging* **18**, S85–S88 (1997).

20. Fanciulli, A. & Wenning, G. K. Multiple-System Atrophy. *New England Journal of Medicine* **372**, 249–263 (2015).

21. Folch, J. *et al.* Una revisión de los avances en la terapéutica de la enfermedad de Alzheimer: estrategia frente a la proteína β-amiloide. *Neurología* **33**, 47–58 (2018).

22. Chiang, K. & Koo, E. H. Emerging Therapeutics for Alzheimer's Disease. *Annual Review of Pharmacology and Toxicology* **54**, 381–405 (2014).

23. Wong, E. & Cuervo, A. M. Autophagy gone awry in neurodegenerative diseases. *Nature Neuroscience* **13**, 805–811 (2010).

24. Braak, H. & Braak, E. Evolution of the neuropathology of Alzheimer's disease. *Acta Neurol. Scand., Suppl.* **165**, 3–12 (1996).

25. Sardiello, M. *et al.* A Gene Network Regulating Lysosomal Biogenesis and Function. *Science* **325**, 473–477 (2009).

26. *Lysosomes*. (Springer US, 2005). doi:10.1007/0-387-28957-7.

27. De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60**, 604–617 (1955).

28. Bajaj, L. *et al.* Lysosome biogenesis in health and disease. *Journal of Neurochemistry* **148**, 573–589 (2019).

29. McKenna, M. C., Schuck, P. F. & Ferreira, G. C. Fundamentals of CNS energy metabolism and alterations in lysosomal storage diseases. *Journal of Neurochemistry* **148**, 590–599 (2019).

30. Kielian, T. Lysosomal storage disorders: pathology within the lysosome and beyond. *Journal of Neurochemistry* **148**, 568–572 (2019).

31. Beutler E, Grabowski GA. *The metabolic and molecular bases of inherited diseases*. vol. 67 (2001).

32. Perez-Roca, L. *et al.* Glucocerebrosidase mRNA is Diminished in Brain of Lewy Body Diseases and Changes with Disease Progression in Blood. *Aging and Disease* **9**, 208 (2018).

33. Choy, F. Y. M. & Campbell, T. N. Gaucher Disease and Cancer: Concept and Controversy. *International Journal of Cell Biology* **2011**, 1–6 (2011).

34. Westbroek, W., Gustafson, A. M. & Sidransky, E. Exploring the link between glucocerebrosidase mutations and parkinsonism. *Trends in Molecular Medicine* **17**, 485–493 (2011).

35. Zimran, A. *et al.* Demographics and patient characteristics of 1209 patients with Gaucher disease: Descriptive analysis from the Gaucher Outcome Survey (GOS). *American Journal of Hematology* **93**, 205–212 (2018).

36. Siebert, M., Sidransky, E. & Westbroek, W. Glucocerebrosidase is shaking up the synucleinopathies. *Brain* **137**, 1304–1322 (2014).

37. Lwin, A. Glucocerebrosidase mutations in subjects with parkinsonism. *Molecular Genetics and Metabolism* **81**, 70–73 (2004).

38. Gan-Or, Z. *et al.* Genotype-phenotype correlations between GBA mutations and Parkinson disease risk and onset. *Neurology* **70**, 2277–2283 (2008).

39. Zimran, A., Gelbart, T., Westwood, B., Grabowski, G. A. & Beutler, E. High frequency of the Gaucher disease mutation at nucleotide 1226 among Ashkenazi Jews. *Am. J. Hum. Genet.* **49**, 855–859 (1991).

40. Reczek, D. *et al.* LIMP-2 Is a Receptor for Lysosomal Mannose-6-Phosphate-Independent Targeting of β-Glucocerebrosidase. *Cell* **131**, 770–783 (2007).

41. Yang, Z. & Klionsky, D. J. Eaten alive: a history of macroautophagy. *Nature Cell Biology* **12**, 814–822 (2010).

42. Arias, E. & Cuervo, A. M. Chaperone-mediated autophagy in protein quality control. *Current Opinion in Cell Biology* **23**, 184–189 (2011).

43. Sardi, S. P. *et al.* Augmenting CNS glucocerebrosidase activity as a therapeutic strategy for parkinsonism and other Gaucher-related synucleinopathies. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 3537–3542 (2013).

44. Gámez-Valero, A. *et al.* GBA Mutations Are Associated With Earlier Onset and Male Sex in Dementia With Lewy Bodies: *GBA* Mutations Precipitate DLB Onset. *Movement Disorders* **31**, 1066–1070 (2016).

45. Coutinho, M. F., Prata, M. J. & Alves, S. A shortcut to the lysosome: the mannose-6-phosphate-independent pathway. *Mol. Genet. Metab.* **107**, 257–266 (2012).

46. Eskelinen, E.-L., Tanaka, Y. & Saftig, P. At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends Cell Biol.* **13**, 137–145 (2003).

47. Fujita, H. *et al.* Isolation and sequencing of a cDNA clone encoding 85kDa sialoglycoprotein in rat liver lysosomal membranes. *Biochem. Biophys. Res. Commun.* **178**, 444–452 (1991).

48. Schwake, M., Schröder, B. & Saftig, P. Lysosomal membrane proteins and their central role in physiology. *Traffic* **14**, 739–748 (2013).

49. Settembre, C., Fraldi, A., Medina, D. L. & Ballabio, A. Signals from the lysosome: a control centre for cellular clearance and energy metabolism. *Nature Reviews Molecular Cell Biology* **14**, 283–296 (2013).

50. Gleich, K. *et al.* Abnormal Processing of Autophagosomes in Transformed B Lymphocytes from SCARB2-Deficient Subjects. *BioResearch Open Access* **2**, 40–46 (2013).
51. Sardiello, M. Transcription factor EB: from master coordinator of lysosomal pathways to candidate therapeutic target in degenerative storage diseases: TFEB, a master coordinator of lysosomal pathways. *Annals of the New York Academy of Sciences* **1371**, 3–14 (2016).
52. Settembre, C. *et al.* TFEB controls cellular lipid metabolism through a starvation-induced autoregulatory loop. *Nature Cell Biology* **15**, 647–658 (2013).
53. Martina, J. A. & Puertollano, R. Rag GTPases mediate amino acid–dependent recruitment of TFEB and MITF to lysosomes. *The Journal of Cell Biology* **200**, 475–491 (2013).
54. Vogiatzi, T., Xilouri, M., Vekrellis, K. & Stefanis, L. Wild Type α-Synuclein Is Degraded by Chaperone-mediated Autophagy and Macroautophagy in Neuronal Cells. *Journal of Biological Chemistry* **283**, 23542–23556 (2008).
55. De Duve, C. The lysosome. *Sci. Am.* **208**, 64–72 (1963).
56. Mizushima, N. & Komatsu, M. Autophagy: Renovation of Cells and Tissues. *Cell* **147**, 728–741 (2011).
57. Nixon, R. A. The role of autophagy in neurodegenerative disease. *Nature Medicine* **19**, 983–997 (2013).
58. García-Arencibia, M., Hochfeld, W. E., Toh, P. P. C. & Rubinsztein, D. C. Autophagy, a guardian against neurodegeneration. *Semin. Cell Dev. Biol.* **21**, 691–698 (2010).

59. Li, S. & Le, W. An insight review of autophagy biology and neurodegenerative diseases: machinery, mechanisms and regulation. *Science China Life Sciences* **60**, 1457–1459 (2017).

60. Menzies, F. M. *et al.* Autophagy and Neurodegeneration: Pathogenic Mechanisms and Therapeutic Opportunities. *Neuron* **93**, 1015–1034 (2017).

61. Guo, F., Liu, X., Cai, H. & Le, W. Autophagy in neurodegenerative diseases: pathogenesis and therapy: Autophagy in neurodegenerative diseases. *Brain Pathology* **28**, 3–13 (2018).

62. Saftig, P., Beertsen, W. & Eskelinen, E.-L. LAMP-2: a control step for phagosome and autophagosome maturation. *Autophagy* **4**, 510–512 (2008).

63. Loeffler, D. A. Influence of Normal Aging on Brain Autophagy: A Complex Scenario. *Front. Aging Neurosci.* **11**, 49 (2019).

64. Mizushima, N. Autophagy: process and function. *Genes Dev.* **21**, 2861–2873 (2007).

65. Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* **8**, 445–544 (2012).

66. Rubinsztein, D. C., Shpilka, T. & Elazar, Z. Mechanisms of Autophagosome Biogenesis. *Current Biology* **22**, R29–R34 (2012).

67. Mizushima, N., Yoshimori, T. & Ohsumi, Y. The Role of Atg Proteins in Autophagosome Formation. *Annu. Rev. Cell Dev. Biol.* **27**, 107–132 (2011).

68. Klionsky, D. J. Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat. Rev. Mol. Cell Biol.* **8**, 931–937 (2007).

69. Nakatogawa, H., Suzuki, K., Kamada, Y. & Ohsumi, Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat. Rev. Mol. Cell Biol.* **10**, 458–467 (2009).

70. He, C. & Klionsky, D. J. Regulation Mechanisms and Signaling Pathways of Autophagy. *Annual Review of Genetics* **43**, 67–93 (2009).

71. Glick, D., Barth, S. & Macleod, K. F. Autophagy: cellular and molecular mechanisms. *J. Pathol.* **221**, 3–12 (2010).

72. Scott, R. C., Juhász, G. & Neufeld, T. P. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr. Biol.* **17**, 1–11 (2007).

73. Kim, J. & Guan, K.-L. mTOR as a central hub of nutrient signalling and cell growth. *Nat Cell Biol* **21**, 63–71 (2019).

74. Yang, Y., Liang, Z., Gu, Z. & Qin, Z. Molecular mechanism and regulation of autophagy1. *Acta Pharmacologica Sinica* **26**, 1421–1434 (2005).

75. Cardenas, M. E., Cutler, N. S., Lorenz, M. C., Di Como, C. J. & Heitman, J. The TOR signaling cascade regulates gene expression in response to nutrients. *Genes & Development* **13**, 3271–3279 (1999).

76. Russell, R. C. *et al.* ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nature Cell Biology* **15**, 741 (2013).

77. He, C. & Levine, B. The Beclin 1 interactome. *Current Opinion in Cell Biology* **22**, 140–149 (2010).

78. Liang, X. H., Yu, J., Brown, K. & Levine, B. Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. *Cancer Res.* **61**, 3443–3449 (2001).

79. Kang, R., Zeh, H. J., Lotze, M. T. & Tang, D. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death & Differentiation* **18**, 571–580 (2011).

80. Shibutani, S. T. & Yoshimori, T. A current perspective of autophagosome biogenesis. *Cell Res* **24**, 58–68 (2014).

81. Dooley, H. C. *et al.* WIPI2 Links LC3 Conjugation with PI3P, Autophagosome Formation, and Pathogen Clearance by Recruiting Atg12–5-16L1. *Molecular Cell* **55**, 238–252 (2014).

82. Bechtel, W. *et al.* Vps34 Deficiency Reveals the Importance of Endocytosis for Podocyte Homeostasis. *JASN* **24**, 727–743 (2013).

83. Ohsumi, Y. Molecular dissection of autophagy: two ubiquitin-like systems: Ubiquitin and proteasomes. *Nature Reviews Molecular Cell Biology* **2**, 211–216 (2001).

84. Shao, Y., Gao, Z., Feldman, T. & Jiang, X. Stimulation of ATG12-ATG5 Conjugation by Ribonucleic Acid. *Autophagy* **3**, 10–16 (2007).

85. Kabeya, Y. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO Journal* **19**, 5720–5728 (2000).

86. Banerjee, R., Beal, M. F. & Thomas, B. Autophagy in neurodegenerative disorders: pathogenic roles and therapeutic implications. *Trends in Neurosciences* **33**, 541–549 (2010).

87. Mak, E. *et al.* Progressive cortical thinning and subcortical atrophy in dementia with Lewy bodies and Alzheimer's disease. *Neurobiology of Aging* **36**, 1743–1750 (2015).

88. O'Brien, J. T. *et al.* Dopamine Transporter Loss Visualized With FP-CIT SPECT in the Differential Diagnosis of Dementia With Lewy Bodies. *Arch Neurol* **61**, 919 (2004).

89. Niethammer, M. *et al.* Parkinson's disease cognitive network correlates with caudate dopamine. *NeuroImage* **78**, 204–209 (2013).

90. Gegg, M. E. *et al.* Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. *Ann Neurol.* **72**, 455–463 (2012).

91. Murphy, K. E. *et al.* Reduced glucocerebrosidase is associated with increased α-synuclein in sporadic Parkinson's disease. *Brain* **137**, 834–848 (2014).

92. Kurzawa-Akanbi, M. *et al.* Glucocerebrosidase Mutations alter the endoplasmic reticulum and lysosomes in Lewy body disease: GCase mutations in Lewy body disease. *J. Neurochem.* **123**, 298–309 (2012).

93. Alcalay, R. N. *et al.* Glucocerebrosidase activity in Parkinson's disease with and without *GBA* mutations. *Brain* **138**, 2648–2658 (2015).

94. van Rooden, S. M. *et al.* The identification of Parkinson's disease subtypes using cluster analysis: A systematic review. *Mov. Disord.* **25**, 969–978 (2010).

95. Schneider, S. A. & Obeso, J. A. Clinical and Pathological Features of Parkinson's Disease. in *Behavioral Neurobiology of Huntington's Disease and Parkinson's Disease* (eds. Nguyen, H. H. P. & Cenci, M. A.) vol. 22 205–220 (Springer Berlin Heidelberg, 2014).

96. Harries, L. W. *et al.* Human aging is characterized by focused changes in gene expression and deregulation of alternative splicing: Gene expression changes in human aging. *Aging Cell* **10**, 868–878 (2011).

97. Matsunaga, K. *et al.* Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L. *The Journal of Cell Biology* **190**, 511–521 (2010).

98. Menon, M. B. & Dhamija, S. Beclin 1 Phosphorylation – at the Center of Autophagy Regulation. *Front. Cell Dev. Biol.* **6**, 137 (2018).

99. Ye, X., Zhou, X.-J. & Zhang, H. Exploring the Role of Autophagy-Related Gene 5 (ATG5) Yields Important Insights Into Autophagy in Autoimmune/Autoinflammatory Diseases. *Front. Immunol.* **9**, 2334 (2018).

100. Romanov, J. *et al.* Mechanism and functions of membrane binding by the Atg5-Atg12/Atg16 complex during autophagosome formation: Role of Atg5-Atg12/Atg16 during autophagy. *The EMBO Journal* **31**, 4304–4317 (2012).

101. Xia, H.-G. *et al.* Control of basal autophagy by calpain1 mediated cleavage of ATG5. *Autophagy* **6**, 61–66 (2010).

7.AGRADECIMIENTOS

Mi primer agradecimiento es para la persona que me introdujo en el mundo de las enfermedades neurodegenerativas, Katrin. Porque su energía y capacidad de sacrificio ha hecho de esta tesis un proceso duro pero fascinante. Voy a echar mucho en falta nuestras charlas sobre la vida.
A mi madre, que ha sufrido, reído, animado y nunca desfallecido en el proceso. Y que cuando ni yo misma creo en mí, ella continúa convencida de ello. Con más mujeres como ella el mundo iría mucho mejor. Sin olvidarme del resto de mi familia, uno de mis pilares. Una familia que han aguantado las vicisitudes de la tesis con el carácter que caracteriza a los Roca Valls, el estoicismo. Y por estar siempre al pie del cañón. También a Àlex, mi compañero de viaje en este suplicio. La persona que ha renunciado a fines de semana y a proyectos comunes para que pudiera realizar mi sueño. Porque sus "tot anirà bé" han acabado desquiciándome a la vez que convirtiéndose en un mantra para mí.
A mi amigo Oriol, que conocí con 14 años y me enseño que tus amigos pueden ser polos opuestos a ti y que simplemente los tienes que querer como son. Una de las lecciones más valiosas de mi vida y porque después de tantos años está a mi lado hasta en la distancia.
Gracias a muchos de los compañer@s de anatomía que de una manera u otra me han hecho sentir parte de su equipo. Porque probablemente sin saberlo han ayudado a crecer a la que escribe. No puedo olvidarme de mis compañeras del Banco de Tumores, Irian y Christel que han sido y son mi mano derecha, izquierda y muchas veces la cabeza. Y mis compañeras del Biobanco. Unas súper guerreras con una resiliencia a prueba de bombas.
Por último, hay cuatro personas con las que he tenido el placer de trabajar y lo más importante aún, de compartir vida. Napoleón y Laia fueron mi sustento al entrar en anatomía, juntos crecimos profesionalmente y personalmente. Y cuando veo en lo que se han convertido me siento extremadamente orgullosa de formar parte de su pasado y de su futuro. Laura entró en mi vida de una manera más casual y es que comer con tupper une mucho. Su humor, sentido común y monólogos me arrancan sonrisas y risas diarias convirtiendo mi estancia en anatomía en una experiencia como poco hilarante. Y por último, Ame con la que comparto gusto literario y ese puntito friki nos unió, resultando no ser el único punto en común en la vida.

Gràcies a tots!