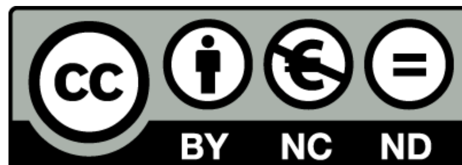
Universitat de Barcelona

Efecto del resveratrol en modelos murinos de envejecimiento y enfermedad de Alzheimer

David Porquet Costa



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FACULTAD DE FARMACIA

DEPARTAMENTO DE FARMACOLOGÍA Y QUÍMICA TERAPÉUTICA

EFECTO DEL RESVERATROL EN MODELOS MURINOS DE ENVEJECIMIENTO Y ENFERMEDAD DE ALZHEIMER

DAVID PORQUET COSTA

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INVESTIGACIÓN, DESARROLLO Y CONTROL DE MEDICAMENTOS

EFECTO DEL RESVERATROL EN MODELOS MURINOS DE ENVEJECIMIENTO Y ENFERMEDAD DE ALZHEIMER

Memoria presentada por David Porquet Costa para optar al título de doctor por la Universidad de Barcelona.

Directora — Doctorando

Mercè Pallàs Lliberia — David Porquet Costa

DAVID PORQUET COSTA

2014

“Somos la memoria que tenemos y la responsabilidad que asumimos. Sin memoria no existimos y sin responsabilidad quizá no merezcamos existir.”

José Saramago (1922-2010)

Me gustaría agradecer en primer lugar a la Dra. Mercè Pallàs, por acogerme en su grupo, ofrecerme una beca y permitirme trabajar en un proyecto tan interesante como éste. También quiero agradecerle toda la ayuda que me ha prestado con los experimentos, los artículos y las correcciones de la tesis y de la presentación. Gracias Mercè por animarme cuando las cosas no salían y recordarme que siempre hay que ver el vaso medio lleno.

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ÍNDICE ... 1
ABREVIATURAS ... 3
INTRODUCCIÓN ... 7
1. EL ENVEJECIMIENTO ... 9
1.1. Generalidades ... 9
1.2. Teorías del Envejecimiento ... 10
2. ENFERMEDAD DE ALZHEIMER ... 13
2.1. Historia ... 13
2.2. Generalidades ... 13
2.3. Tipos de enfermedad de Alzheimer ... 15
2.4. Mutaciones en genes de la enfermedad de Alzheimer ... 16
2.5. Procesamiento de APP ... 18
2.6. Proteasas amiloidogénicas: β-secretasa ... 20
2.7. Proteasas amiloidogénicas: γ-secretasa ... 21
2.8. La proteasa no amiloidogénica: α-secretasa ... 23
2.9. Degradación del APP ... 23
2.10. Péptido Aβ y placas seniles ... 24
2.11. Acumulación de Tau ... 24
2.12. Relación entre el péptido Aβ y la patología de Tau ... 26
2.13. Alteración sináptica ... 27
2.14. Inflamación ... 27
3. RESVERATROL ... 26
3.1. Generalidades ... 26
3.2. Propiedades antioxidantes ... 30
3.3. Propiedades anti-inflamatorias ... 31
3.3. Resveratrol: un activador de SIRT1 ... 31
3.4. Resveratrol y restricción calórica ... 33
3.5. Resveratrol y autofagía ... 34
4. MODELOS EXPERIMENTALES ... 35
4.1. Senescence accelelated mouse prone 8 (SAMP8) ... 35
4.2. Ratones transgénicos APP/PS1 ... 38

OBJETIVOS 41

RESULTADOS 45

Publicación 1: Low-dose pterostilbene, but not resveratrol, is a potent neuromodulator in aging and Alzheimer's disease 47

Publicación 2: Dietary resveratrol prevents Alzheimer's markers and increases life span in SAMP8 61

Publicación 3: Neuroprotective role of trans-resveratrol in a murine model of familial Alzheimer's disease 81

Publicación 4: Amyloid and tau pathology of a familial Alzheimer's disease APP/PS1 mouse model in a senescence phenotype background (SAMP8) 97

DISCUSIÓN 131

Efectos del resveratrol en los ratones SAMP8 133

Efecto del resveratrol en el modelo de EA familiar APP/PS1 138

Caracterización de un nuevo modelo de EA 142

CONCLUSIONES 147

BIBLIOGRAFÍA 151

ABREVIATURAS

Aβ: amyloid β

ADAM: A Disintegrin and metalloproteinase

AICD: APP intracellular domain

AMP: adenosine monophosphate

AMPK: adenosine monophosphate kinase

AP-1: activator protein -1

APH-1: anterior pharynx defective

ApoE: Apolipoprotein E

APP: amyloid precursor protein

ARE: antioxidant response element

BACE: β-site APP cleaving enzyme

CAMKKβ: calcium/calmodulin-dependent protein kinase

cAMP: cyclic adenosine monophosphate

CDK5: cyclin-dependent kinase 5

CTF: carboxi-terminal fragment

DNA: deoxyribonucleic acid

EA: enfermedad de Alzheimer

EAe: enfermedad de Alzheimer esporádica

EAf: enfermedad de Alzheimer familiar

FOXO: forkhead box protein O

GFAP: glial fibrillary acidic protein

GSK3β: glycogen synthase kinase 3β

HIF1α: hypoxia-inducible factor-1α

IDE: insulin degrading enzyme

IκB: inhibitor of κB

IL1β: interleukin 1β

iNOS: inducible nitric oxide synthase

JNK: c-jun amino-terminal kinase

LKB: liver kinase B1

LPS: lipopolisacarido

LRP-1: low density lipoprotein receptor-related protein 1

MCIV: mitocondrial complex IV

MnSOD: manganese superoxide dismutase

mRNA: messenger ribonucleic acid

mTOR: mammalian target of rapamycin

NAD: nicotine adenine dinucleotide

NCT: nicastrin

NFκB: nuclear factor κB

NFT: neurofibrillary tangles

NORT: novel object recognition test

NRF2: nuclear factor erythroid derived2-like 2

NTF: amino-terminal fragment

PARP: poly ADP ribose polymerase

PEN-2: presenilin enhancer-2

PGC1α: peroxisome proliferator-activated receptor gamma coactivator 1-α

PPARα: Peroxisome proliferator-activated receptor α

PS: presenilin

PSD-95: postsynaptic density protein-95

RAGE: receptor for advanced glycation endproducts

RAR: retinoic acid receptor

RC: restricción calórica

ROS: reactive oxygen species

SAM: senescence accelerated mouse

SAMP: senescence accelerated mouse prone

SAMR: senescence accelerared mouse resistant

SIRT1: sirtuina 1

SNAP-25: synaptosomal-associated protein 25

TACE: tumor necrosis factor-α-converting enzyme

TMD: transmembrane domain

TNFα: tumor necrosis factor-α

tRNA: transfer ribonucleic acid

PRÓLOGO

Debido al aumento de la esperanza de vida y a la disminución de la tasa de fecundidad, la proporción de personas mayores de 60 años está aumentando con rapidez. El envejecimiento de la población puede considerarse un éxito de las políticas de salud pública y desarrollo socioeconómico, pero también constituye un reto para la sociedad, que debe adaptarse a ello para mejorar al máximo la salud y la capacidad funcional de las personas mayores.

Según la OMS, el número de personas con 60 años o más en todo el mundo se ha duplicado desde 1980, y se prevé que alcance los 2000 millones de aquí a 2050. Entre 2000 y 2050, la proporción de la población mundial con más de 60 años de edad se duplicará, ya que pasará de aproximadamente el 11% al 22%. En 2050 habrá en el mundo cerca de 400 millones de personas con 80 años o más.

Por esta razón es importante prevenir y tratar las enfermedades neurológicas crónicas asociadas a la edad, ya que a partir de los 85 años, entre un 30 un 45% de las personas padece algún tipo de enfermedad neurodegenerativa, entre las cuales la más común es la enfermedad de Alzheimer.

INTRODUCCIÓN

1. EL ENVEJECIMIENTO

1.1. Generalidades

El envejecimiento, desde el punto de vista biológico, es el conjunto de modificaciones inevitables e irreversibles que se producen en un organismo con el paso del tiempo (Viña et al., 2007). Estos cambios, provocan una disminución gradual del funcionamiento de los órganos, y finalmente conducen a la muerte.

Características del envejecimiento:

- **Universal:** es propio de todos los seres vivos.
- **Irreversible:** no puede detenerse ni revertirse.
- **Heterogéneo e individual:** la velocidad de envejecimiento varía entre especies, entre individuos pertenecientes a una misma especie, e incluso entre distintos órganos de un mismo individuo.
- **Deletéreo:** lleva a una progresiva pérdida de función.
- **Intrínseco:** no es debido a factores ambientales modificables.

Todavía no se conoce verdadera naturaleza del envejecimiento, aunque hay diversas teorías: estocásticas, que consideran que el envejecimiento es consecuencia de alteraciones que ocurren de forma aleatoria y se acumulan a lo largo del tiempo; y no estocásticas, que consideran que el envejecimiento está predeterminado, siendo una continuación del proceso de desarrollo y diferenciación, y correspondería a la última etapa dentro de una secuencia de eventos codificados en el genoma.

1.2. Teorias del envejecimiento

Teorías estocásticas:

- **Teoría del error catastrófico:** propone que con el paso del tiempo se produciría una acumulación de errores en la síntesis proteica, causando un daño en la función celular (Orgel, 1963). Se sabe que se producen errores en los procesos de trascripción y traducción durante la síntesis de proteínas, pero no hay evidencias de que estos errores se acumulen en el tiempo. Una crítica a esta teoría, es que no se observan cambios en la secuencia de aminoácidos en las proteínas de animales viejos respecto a los jóvenes, y por otro lado, tampoco aumenta la cantidad de RNA de transferencia (tRNA) defectuoso con la edad.

- **Teoría del entrecruzamiento:** postula que se generarían enlaces o entrecruzamientos entre las proteínas y otras macromoléculas de la célula, lo que determinaría el envejecimiento y el desarrollo de enfermedades dependientes de la edad.

- **Teoría del desgaste:** propone que cada organismo estaría compuesto de partes irremplazables, y que debido a la acumulación de daño en estas partes, se podría producir la muerte de las células, tejidos, órganos y finalmente la muerte del propio organismo. Un ejemplo de esto podría ser el DNA, ya que la capacidad de reparación de éste se correlaciona positivamente con la longevidad de las diferentes especies. A pesar de esto, estudios en animales no han demostrado una disminución en la capacidad de reparación de DNA en los animales que envejecen.

- **Teoría de los radicales libres:** una de las más populares. Propone que el envejecimiento sería el resultado de una inadecuada protección contra el daño producido en los tejidos por los radicales libres (Harman, 1956).

  El oxígeno ambiental es básico para el metabolismo celular, produciendo energía a través de la cadena respiratoria mitocondrial. Como la utilización del O_2 no es perfecta, se producen radicales libres, entre ellos el radical superóxido. Los radicales libres son moléculas inestables y altamente reactivas con uno o más electrones no apareados, que producen daño a su alrededor a través de reacciones oxidativas. Este tipo de daño podría

causar alteraciones en los cromosomas y otras macromoléculas como colágeno, elastina, mucopolisacáridos, lípidos, etc.

Existen numerosos estudios que dan soporte a la teoría de los radicales libres. Se ha demostrado una buena correlación entre los niveles de superóxido dismutasa (MnSOD) y la longevidad de diferentes primates (Tolmasoff et al., 1980). Además, hay estudios que demuestran que ciertos antioxidantes pueden prevenir la aparición de enfermedades asociadas a la edad y aumentar la esperanza de vida en diferentes especies (Popov et al., 2006; Marchal et al., 2013)

Es ampliamente conocido papel que juega el daño producido por la liberación de radicales libres en ciertas patologías relacionadas con el envejecimiento, tales como patologías cardiovasculares (Maxwell and Lip, 1997), cáncer (Valko et al., 2006), y diversas enfermedades neurodegenerativas como la enfermedad de Alzheimer (Markesbery, 1997).

Por tanto, si logramos disminuir la generación de radicales libres o neutralizamos su daño, lograríamos reducir la incidencia de estas enfermedades, y entonces, seríamos capaces de prevenir una muerte precoz por estas patologías.

Teorías no estocásticas:

- **Teoría del marcapasos:** los sistemas inmune y neuroendocrino actuarían como marcadores intrínsecos del envejecimiento. Su degeneración estaría genéticamente determinada para ocurrir en momentos específicos de la vida.

- **Teoría genética:** el factor genético es un importante determinante del proceso de envejecimiento, aunque no se conocen exactamente los mecanismos involucrados.

Hay innumerables estudios que respaldan la teoría genética. Un ejemplo, es que cada especie animal tiene un patrón de envejecimiento distinto (de Magalhães, 2003), por otro lado se sabe que existe mucha mejor correlación en la supervivencia entre gemelos monocigóticos, que entre hermanos (Hjelmborg et al., 2006), además la supervivencia de los padres tiene una buena correlación con la de los hijos, que en parte, se debe a la heredabilidad de los telómeros (Njajou et al., 2007).

La mayoría de células están programadas para un número determinado divisiones. Cada cromosoma posee en sus extremos una serie de secuencias altamente repetidas y no codificantes denominadas telómeros. Debido al mecanismo de replicación del DNA, los telómeros se van acortando con las sucesivas divisiones, y esto se ve atenuado por la existencia de una enzima llamada telomerasa, que realiza la replicación telómerica. Sin embargo, la actividad telomerasa funciona en células embrionarias, pero se inactiva en células somáticas, lo que conlleva un acortamiento progresivo de los telómeros; cuando el tamaño de los telómeros llega a un mínimo, se desencadenan mecanismos que conducen a la muerte de la célula. Por esta razón, el acortamiento telómerico se ha asociado con el proceso de envejecimiento celular. De esta forma, la longitud de los telómeros representaría una especie de reloj genético que determinaría el tiempo de vida de las células.

Otro hecho que reafirma la influencia genética en el proceso de envejecimiento, es que existen enfermedades, como la progeria o envejecimiento prematuro, que son causadas por mutaciones, por ejemplo, el síndrome de Hutchinson-Gilford, que es debido a una mutación en el gen *LMNA* (Kudlow et al., 2007). Otras enfermedades de envejecimiento acelerado, entre ellas el síndrome de Werner, el sindrome de Cockayne, o *Xeroderma pigmentosum*, son causadas por mutaciones en genes que codifican proteínas implicadas en la reparación del DNA (Lehmann, 2003).

De todas las teorías anteriores, las más aceptadas son la teoría de los radicales libres y la teoría genética, y es posible que ninguna de ellas por sí sola pueda explicar el proceso del envejecimiento en su totalidad, más bien parece ser que el envejecimiento es un proceso extremadamente multifactorial en el que hay un conjunto de interacciones de origen intrínseco (genético), extrínseco (ambiental) y estocástico (aleatorio), y que por lo tanto, las primeras teorías que defienden una causa única deberían ser descartadas.

A pesar de que en los últimos años se ha observado un aumento progresivo en la esperanza de vida de la población, ya que se ha logrado prevenir y tratar mejor las enfermedades, y se han mejorado los factores ambientales, el tiempo de vida máximo que una persona puede vivir se mantiene fijo alrededor de los 118 años, siendo Jeanne Calment, quien murió el 4 de agosto de 1997 a la edad de 122 años y 164 días, la persona más vieja documentada.

Sin embargo, hay una clara diferencia entre la esperanza de vida de la población de países ricos y pobres, en 2013 el país con mayor esperanza de vida fue Mónaco con una media de 89.63 años, y el de menor el Chad con 49.07 años, lo cual indica que aunque el factor genético es determinante, en la mayoría de los casos depende en gran medida de los factores ambientales.

En los países desarrollados, la población tiene una mayor esperanza de vida, sin embargo, no se ha conseguido frenar la aparición de ciertas enfermedades asociadas a la edad como la enfermedad de Alzheimer (EA), que a día de hoy sigue siendo incurable. Todos los tratamientos disponibles en la actualidad son paliativos o ineficaces.

2. ENFERMEDAD DE ALZHEIMER

2.1. Historia

Alois Alzheimer (1864-1915) describió el primer caso de esta enfermedad en el año 1907 en Auguste D., una mujer de 51 años que presentaba un deterioro rápido de la memoria junto con alteraciones psiquiátricas. Alzheimer realizó la autopsia del cerebro y utilizó una tinción de plata para visualizar la presencia de neuronas. De esta forma, encontró unas formaciones inusuales, que ahora se conocen como placas seniles y ovillos neurofibrilares. Alzheimer hipotetizó que estas alteraciones podían ser la causa o el efecto de esta enfermedad, que más adelante fue llamada enfermedad de Alzheimer (EA). A partir de entonces se empezó a diferenciar esta patología de otros tipos de demencia que hasta la fecha se pensaba que formaban parte de un proceso natural ligado al envejecimiento. A pesar de que cada vez está más aceptado que la EA es una enfermedad, el envejecimiento es uno de los principales factores de riesgo junto a ciertos polimorfismos como la APOE ε4, enfermedades cardiovasculares y cerebrovasculares, hipertensión y obesidad, entre otros.

2.2. Generalidades

La EA es una enfermedad neurodegenerativa, incurable y terminal que se caracteriza por la presencia de dos marcadores histopatológicos: las placas seniles extracelulares y los ovillos neurofibrilares intracelulares. Ambos marcadores se encuentran predominantemente en la corteza y en el hipocampo, donde además se produce una perdida sináptica que genera los síntomas

típicos de esta enfermedad como la demencia. Sin embargo, debe diferenciarse de otras causas de demencia como la demencia vascular, demencia con cuerpos de Lewy, enfermedad de Parkinson con demencia, demencia frontotemporal y demencias reversibles (Castellani et al., 2010).

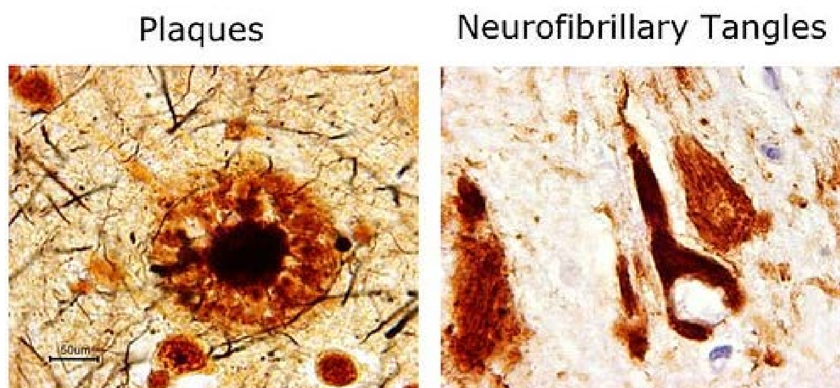

Figura 1. Alteraciones histopatológicas de la enfermedad de Alzheimer (www.coloradodementia.org).

Se manifiesta con un deterioro cognitivo progresivo y una alteración conductual. Al inicio comienza a verse afectada la memoria a corto plazo, pero a medida que progresa la enfermedad, aparecen confusión mental, irritabilidad y agresión, cambios del humor, trastornos del lenguaje y pérdida de la memoria a largo plazo. Debido a la gran pérdida neuronal, hay un límite en el que las funciones esenciales no pueden mantenerse, y el individuo acaba muriendo.

Dentro de las demencias, la EA es una de las más comunes junto con la demencia vascular, representando un 60-70% del total (Fratiglioni et al., 2007), aunque existen casos en los que ambas coexisten. La incidencia y la edad de aparición varía dependiendo del país y de su grado de desarrollo, en España suele afectar a personas mayores de 65 años, aumentando exponencialmente su incidencia desde 1,5 casos / 1000 personas-año, llegando a 69,2 casos / 1000 personas-año en mayores de 90 años (Bermejo-Pareja et al., 2008).

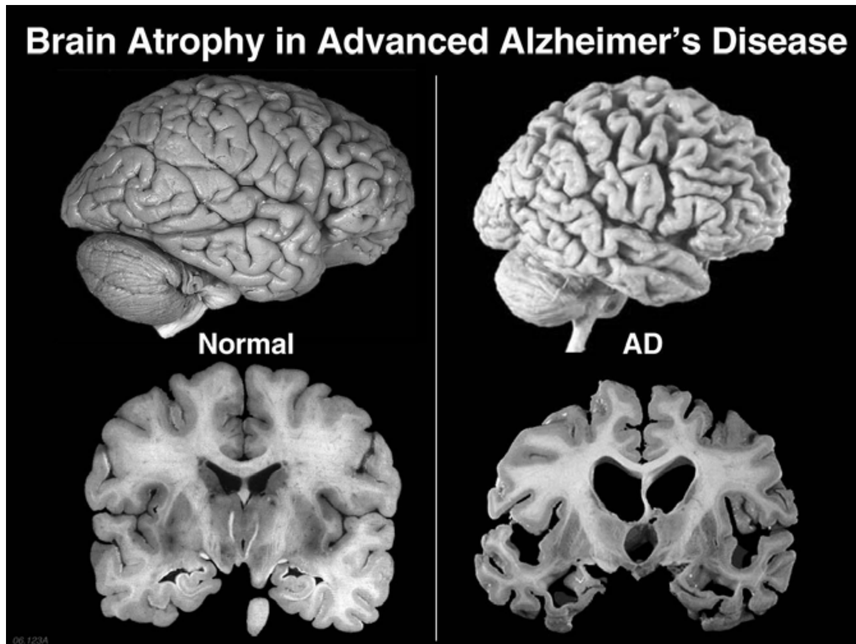

Figura 2. Atrofia cerebral debida a la pérdida neuronal en la enfermedad de Alzheimer (www.brightstarcare.com).

2.3. Tipos de enfermedad de Alzheimer

Enfermedad de Alzheimer esporádica

La enfermedad de Alzheimer esporádica (EAe) suele aparecer en personas de edad avanzada (65 años o más), y constituye 95% de los casos de EA. Su causa es todavía desconocida, y los enfermos no tienen por qué tener antecedentes familiares. Los principales factores de riesgo de esta enfermedad son el envejecimiento, las enfermedades cardiovasculares y cerebrovasculares, los polimorfismos genéticos como la ApoE ε4, defectos mitocondriales, diabetes de tipo 2 y factores ambientales como la dieta, entre otros.

Tiene múltiples factores etiológicos, incluyendo la genética, los factores ambientales y el estilo de vida (Kalaria et al., 2008). Los marcadores fisiopatológicos incluyen la acumulación de la proteína beta-amiloide (Aβ) en forma de placas seniles y la formación de depósitos intracelulares de la proteína asociada a microtúbulos tau en forma de ovillos neurofibrilares.

Enfermedad de Alzheimer familiar

Aproximadamente un 13% de los casos de EA temprana, es decir, que aparece antes de los 65 años de edad, son casos de enfermedad de Alzheimer familiar (EAf). La EAf es una forma poco frecuente de EA que aparece a una edad temprana, y únicamente representa el 5-10% del total de casos de EA (Campion et al., 1999). Tiene un patrón de herencia mendeliano autosómico dominante, y puede ser causado por mutaciones en los genes *APP* (*amyloid precursor protein*), *PSEN1* (*presenilin 1*) o *PSEN2* (*presenilin 2*), haciendo que la β-secretasa y la γ-secretasa tengan mayor actividad, dando lugar una mayor formación de Aβ (Mattson, 2004; Reddy and Beal, 2005), y por lo tanto, a su acumulación en forma de placas seniles.

2.4. Mutaciones en genes de la enfermedad de Alzheimer

Mutaciones en el gen *APP*

Las mutaciones en el gen *APP,* que codifica la proteína precursora de amiloide (APP, del inglés *amyloid precursor protein*), es ampliamente utilizada para generar modelos de EAf que reproduzcan el mayor número de marcadores y síntomas característicos de esta enfermedad. Existen diversas mutaciones de esta proteína, una de ellas, la mutación sueca (APPSwe), en la cual se produce un cambio en dos aminoácidos en las posiciones 670/671 cerca del lugar de corte de la β-secretasa, lo que promueve un mayor procesamiento del APP por parte de ésta (Haass et al., 1995). Las mutaciones en las posiciones 692 y 693, las cuales se encuentran cerca del lugar de corte de la α-secretasa, corresponden a la mutación Flamenca y Holandesa respectivamente, y producen hemorragias cerebrales con amiloidosis y EA. Las mutaciones en la zona de corte de la γ-secretasa, como la Florida en la posición 716 o la London en 717, también incrementan la formación de Aβ.

Otra mutación ampliamente utilizada es la Ártica, que se encuentra en el dominio perteneciente al péptido Aβ, y produce un aumento de su agregación (Nilsberth et al., 2001). Hay dos tipos de mutación Ártica, por una parte la 693G, que promueve la formación de protofibrillas; y la 693δ, que produce un aumento de los niveles de oligómeros sinaptotóxicos (Nilsberth et al., 2001; Tomiyama et al., 2008).

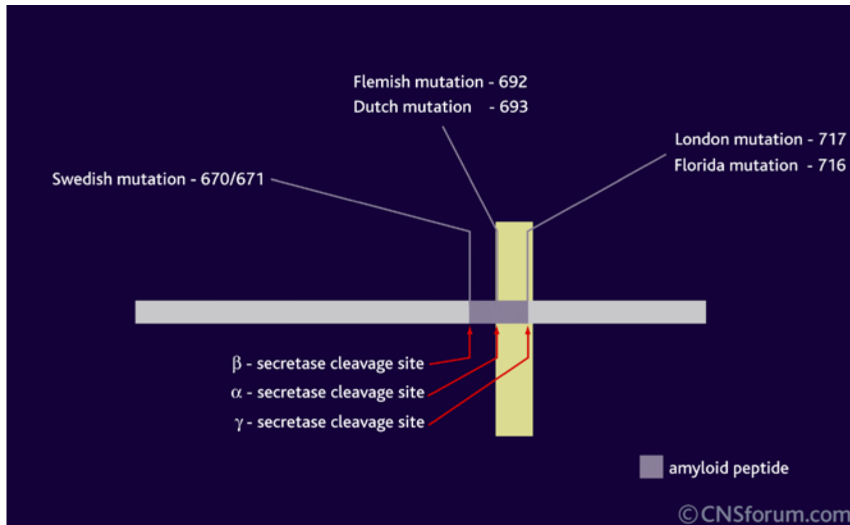

Figura 3. Mutaciones en la proteína APP (www.cnsforum.com).

Mutaciones en los genes *PSEN1* y *PSEN2*

Los genes *PSEN1* y *PSEN2* codifican para las proteínas presenilina 1 (PS1) y presenilina 2 (PS2), respectivamente. Las presenilinas son proteínas que forman parte del complejo γ-secretasa junto con otras proteínas. A pesar de que estas dos presenilinas tienen un 67% de homología entre su secuencia de aminoácidos, la especificidad para PS1 y PS2 radica en dos regiones homólogas situadas en el extremo N-terminal o en el bucle hidrofílico formado entre las regiones transmembrana 6 y 7 (De Strooper et al., 1997). Las mutaciones en los genes de las presenilinas representan un 90% de las mutaciones presentes en la EAf, y en concreto las mutaciones en *PSEN1* conforman un 70% de las mutaciones patogénicas, siendo el gen más involucrado. Existen gran cantidad de mutaciones contrasentido en *PSEN1* localizadas en las regiones transmembrana 2, 3, ó 6, y una mutación puntual en el aceptor de corte y empalme (*splicing*) que puede dar lugar a una delección del exón 9 (Sherrington et al., 1995), mientras que en PSEN2 las mutaciones se encuentran únicamente en la región transmembrana 2.

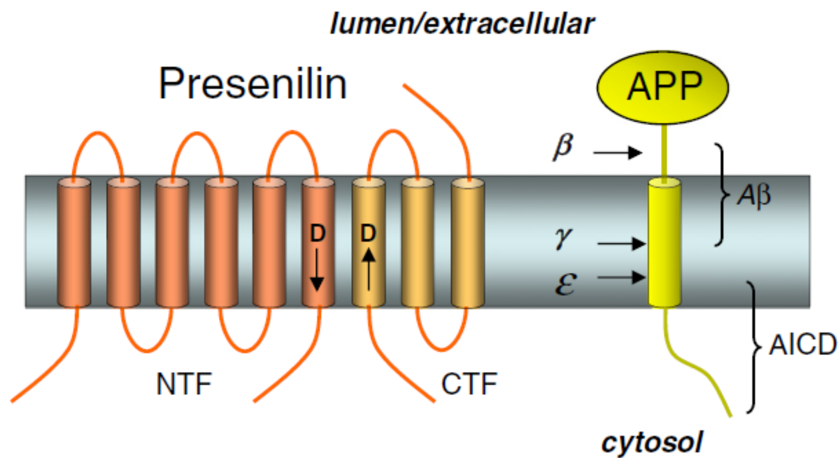

Figura 4. Estructura de las presenilinas (Michael S Wolfe, 2013).

2.5. Procesamiento de APP

El péptido Aβ está formado por 37-42 aminoácidos y es generado por una proteólisis de APP a través de un proceso fisiológico normal (Haass and Selkoe, 1993). APP es una proteína transmembrana de tipo I con el extremo amino-terminal orientado hacia el espacio extracelular y el extremo carboxi-terminal hacia el citosol (Kang et al., 1987; Dyrks et al., 1988). Aunque los productos iniciales de APP son secretados, APP no solo se proteoliza en la membrana plasmática, sino que también se puede proteolizar en otros compartimentos subcelulares (Weidemann et al., 1989). Existen tres tipos de actividad proteasa: α-, β- y γ-secretasa que están involucradas en fases específicas del procesamiento de APP (Haass et al., 2004). El nombre de "secretasas" viene por la secreción de los sustratos proteolíticamente procesados.

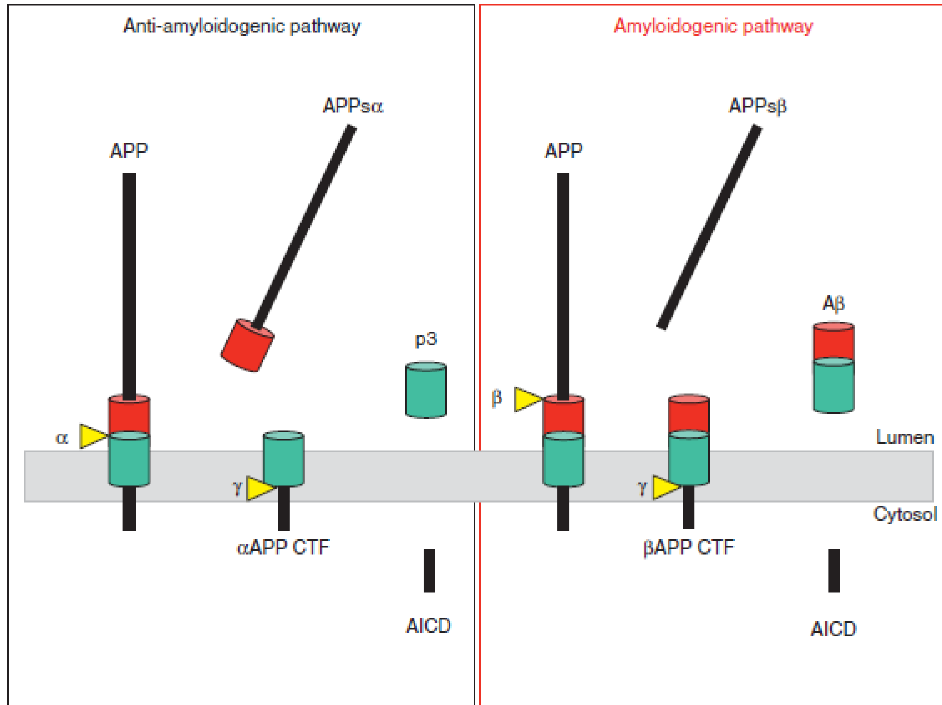

Figura 5. Vía no amiloidogénica y vía amiloidogénica de procesamiento de APP

(Haass et al., 2012).

Existen dos vías principales de procesamiento de APP: la vía amiloidogénica, que genera el péptido Aβ; y la vía no amiloidogénica, que previene la formación de éste. Aβ es producido por la vía amiloidogénica por la acción consecutiva de β- y γ-secretasa (Haass et al., 2004). La actividad β-secretasa inicia la generación de Aβ liberando una parte grande del ectodominio de APP (APPsβ) y generando un fragmento de APP carboxi-terminal (β-CTF o C99), que seguidamente es escindido por γ-secretasa. Debido a esta última escisión realizada por γ-secretasa se libera Aβ, y por ello se puede encontrar en fluidos extracelulares como el plasma o el fluido cerebroespinal (Seubert et al., 1992). En la vía no amiloidogénica, APP es escindido por la parte central de la región Aβ mediante la actividad α-secretasa (Esch et al., 1990; Sisodia et al., 1990). Este procesamiento genera otro tipo de fragmento de APP carboxi-terminal (α-CTF o C83), que carece de la porción del dominio Aβ. Posteriormente C83 es escindido por γ-secretasa, liberando un péptido llamado p3 (Haass et al., 1993), que aparentemente es patológicamente irrelevante. γ-secretasa no solo libera Aβ (a partir de C99) y p3 (a partir de C83), sino que también genera un dominio intracelular de APP

(AICD, del inglés *amyloid intracellular domain*) (Gu et al., 2001; Sastre et al., 2001; Weidemann et al., 2002), que es liberado al citosol y puede tener funciones a nivel de señalización nuclear (Cao and Südhof, 2001; Von Rotz et al., 2004).

Ambas vías, amiloidogénica y no amiloidogénica compiten entre ellas al menos en algunos compartimentos subcelulares, ya que incrementando la actividad α-secretasa en modelos animales de EA o en cultivos de células reduce la producción de Aβ e incluso la formación de placas seniles (Nitsch et al., 1992; Postina et al., 2004).

2.6. Proteasas amiloidogénicas: β-secretasa

La actividad β-secretasa es la que inicia y actúa de factor limitante en la generación de Aβ (Vassar, 2004). La clonación y la purificación bioquímica del enzima han dado lugar a la identificación de una única β-secretasa (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Hussain et al., 2000; Lin et al., 2000). Aunque originalmente se utilizaron muchas denominaciones para describir esta actividad, como memapsina, aspartil proteasa 2 o BACE1 (beta-site amyloid precursor protein cleaving enzyme 1), BACE1 está aceptado actualmente como termino para referirse a la enzima que posee actividad β-secretasa. BACE1 es una aspartil proteasa de unión a membrana que posee un sitio activo en el espacio extracelular y tiene similaridades con la familia de las pepsinas (Hong et al., 2000). Además de BACE1, se identificó una proteasa homologa llamada BACE2 (Vassar, 2004). Sin embargo, BACE2 no está relacionada en la amiloidogénesis e incluso podría ejercer una acción antiamiloidogénica en células no neurales de forma similar a α-secretasa (Bennett et al., 2000; Farzan et al., 2000; Fluhrer et al., 2002; Basi et al., 2003). BACE1 es la única β-secretasa, ya que el *knockout* bloquea completamente la producción de Aβ (Cai et al., 2001; Roberds et al., 2001; Luo et al., 2003). Esta proteasa se expresa de forma ubicua, con niveles de expresión más elevados en cerebro y páncreas; la relevancia fisiológica de esta elevada expresión en páncreas todavía es desconocida. Debido a que APP también tiene niveles muy altos de expresión en cerebro, junto con niveles elevados de BACE1, generan una gran producción de Aβ, y por lo tanto, explica por qué la EA es una enfermedad cerebral a pesar de que APP se expresa de forma ubicua. Por todo esto, BACE1 es una importante diana terapéutica (Citron, 2004), porque su inhibición no solo reduce los niveles de Aβ, sino que también previene la acumulación de β-CTF (β carboxi-terminal fragment), que contienen el dominio Aβ completo y actúa como sustrato para la producción más Aβ. Esto es un problema importante, ya que la acumulación de estos β-CTF puede

causar efectos tóxicos adicionales. Ha habido un gran progreso en la generación de inhibidores de BACE1, incluso se han realizado varios estudios clínicos (Citron, 2004; Schenk et al., 2012). Sin embargo, hay que tener en cuenta que esta aproximación farmacológica también inhibe la función fisiológica de BACE1. Hasta la fecha, solo se han validado unos pocos sustratos de BACE1 con una clara función biológica. Los ratones *knockout* de BACE1 son viables, fértiles y no presentan déficits graves a nivel comportamental, morfológico o de desarrollo (Cai et al. 2001; Roberds et al. 2001; Luo et al. 2003). Sin embargo, estos animales parecen más tímidos y exploran menos de lo normal (Harrison et al., 2003; Dominguez et al., 2005). También se ha demostrado que la expresión postnatal alta de BACE1 (Willem et al., 2006) está relacionada con el proceso de mielinización, un proceso que ocurre después del nacimiento. De hecho, todos los *knockouts* disponibles de BACE1 muestran un fenotipo de hipomielinización en el sistema nervioso periférico (Hu et al., 2006; Willem et al. 2006). Parece que el proceso de mielinización en el sistema nervioso central también se encuentra bajo el control de BACE1 (Hu et al., 2006), pero todavía hacen falta más estudios que lo corroboren, y este hecho se encuentra todavía en debate. Por otro lado, también se ha relacionado BACE1 con la regulación de los canales de sodio dependientes de voltaje (Kim et al., 2007a).

2.7. Proteasas amiloidogénicas: γ-secretasa

La liberación de Aβ a partir de su precursor APP está mediada en última instancia por γ-secretasa, y se realiza en su dominio transmembrana (TMD, del inglés *transmembrane domain*). La estructura de γ-secretasa ya se ha discutido anteriormente. γ-secretasa es un complejo con actividad proteasa que consta de cuatro subunidades (Steiner et al., 2008). La presenilina (PS) 1 o PS2 contienen los dos residuos aspartil en sus TMDs 6 y 7, que forman parte del dominio catalítico de la actividad aspartil proteasa de este complejo (Wolfe et al., 1999). Los otros tres componentes son NCT (nicastrin), APH-1 (anterior pharynx defective) y PEN-2 (presenilin enchancer-2) (Yu et al., 2000; Francis et al., 2002). Estos cuatro componentes son necesarios y suficientes para que el complejo tenga actividad (Edbauer et al., 2003). Las funciones biológicas de NCT, APH-1 y PEN-2 son poco conocidas. NCT parece que selecciona el sustrato por su tamaño (Shah et al., 2005; Dries et al., 2009). PEN-2 posiblemente facilita la endoproteólisis de PS, dando lugar a su estado heterodimérico activo y estabilizando PS en el complejo (Hasegawa et al., 2004; Prokop et al., 2004).

El procesamiento de APP por γ-secretasa no está restringido a un único lugar de escisión, y la última escisión puede ocurrir en condiciones fisiológicas entre los aminoácidos 37 y 43 del dominio Aβ. Esta diferencia en los aminoácidos de escisión es de gran importancia en lo que se refiere a la patología de la EA, ya que las formas de Aβ de 42 aminoácidos agregan mucho más, y parecen ser las responsables de la generación de oligómeros, los cuales afectan a la memoria y a la viabilidad celular (Haass and Selkoe, 2007). Aparentemente, cuando APP se une a la zona activa del complejo γ-secretasa, se realiza una proteólisis (Fluhrer et al., 2006; Fluhrer et al., 2008; Fluhrer et al., 2009) que empieza con la escisión del enlace carboxipeptídico después de los aminoácidos 49 ó 48 (*cleavage 1*). Seguidamente se produce otra lisis después de los aminoácidos 46 ó 45 (*cleavage z*), y finalmente la escisión después de los aminoácidos 42 ó 40 (*cleavage γ*), aunque también puede suceder después de los aminoácidos 37, 38, 39 y 43. De esta forma se generan dos tipos de procesamiento: uno que genera predominantemente $A\beta_{42}$ (que empieza con el *cleavage 1* después del aminoácido 48, seguido por una escisión después de los aminoácidos 45 y 42); y el otro que genera predominantemente $A\beta_{40}$ (empezando con el *cleavage 1* después del aminoácido 49, seguido una escisión después de los aminoácidos 46 y 43). Actualmente se está debatiendo la posibilidad de que estas escisiones puedan ser moduladas terapéuticamente para prevenir la producción selectiva de $A\beta_{42}$ (Schenk et al., 2012).

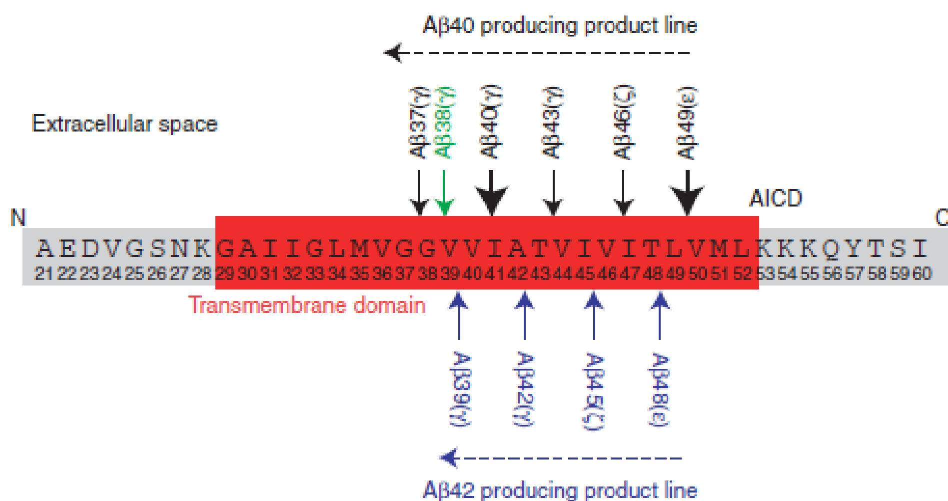

Figura 6. Puntos de escisión de APP por la acción γ-secretasa (Haass et al., 2012).

2.8. La proteasa no amiloidogénica: α-secretasa

El procesamiento no amiloidogénico del APP sucede entre los residuos Lys^{16} y Leu^{17} del dominio Aβ Leu^{17} (Esch et al., 1990; Sisodia et al., 1990; Wang et al., 1991) y da lugar a la secreción de un dominio APP amino terminal de gran tamaño y a la generación de α-CTF (C83). Este corte es realizado por un conjunto de proteasas de unión a membrana plasmática (Sisodia, 1992) denominadas α-secretasas. Varias metaloproteasas dependientes de zinc y miembros de la familia de las disintegrinas A y metaloprotesas como ADAM9, ADAM10, TACE/ADAM17 y ADAM19 pueden tener función α-secretasa (Allinson et al., 2003). Evidencias recientes sugieren que en neuronas ADAM10 es la que ejerce la principal actividad constitutiva α-secretasa (Kuhn et al., 2010). Aparte de APP, los ligandos y los receptores Notch, TNFα, cadherinas, el receptor de IL-6, el receptor de EGF y varios tipos de proteínas transmembrana de tipo I son escindidos por α-secretasas, liberando sus dominios extracelulares. Por todo esto, la selectividad proteolítica de α-secretasa parece ser independiente de la secuencia aminoacídica. Como mínimo el corte de α-secretasa en APP está determinado por la conformación de hélice α y la distancia (12-13 residuos) a la membrana del enlace hidrolizado (Sisodia, 1992). La vía amiloidogénica parece ser la más favorecida en neuronas debido a la abundancia de BACE1, mientras que la vía no amiloidogénica es predominante en todos los otros tipos celulares. La sobreexpresión de ADAM10 en ratones transgénicos reduce el procesamiento de APP a través de BACE1 y la deposición de amiloide (Postina et al., 2004), por lo que podría ser una diana terapéutica. Se ha visto que una disminución de los niveles de colesterol en células en cultivo estimulan el corte de APP por α-secretasa mediante mecanismos que implican una disminución de la endocitosis de APP y un aumento de los niveles basales de ADAM10 (Kojro et al., 2001). El efecto producido por la depleción de colesterol no afecta únicamente al corte de APP, sino también al corte del receptor de la IL-6 por ADAM-10 y TACE/ADAM17, que también es estimulado por estas condiciones (Matthews et al., 2003).

2.9. Degradación del APP

Existen formas alternativas de procesamiento del APP que no implican la acción de secretasas. APP tiene un tiempo de vida media muy corto, y no todo el APP es secretado en forma de APPs, lo que sugiere que existen estas vías de procesamiento independientes de las secretasas (Weidemann et al., 1989). Además, el APP es degradado en los lisosomas dando lugar a fragmentos amiloidogénicos o no amiloidogénicos (Golde et al., 1992; Haass et al., 1992). Por último, APP

también es un sustrato de caspasas (Weidemann et al., 1999; Lu et al., 2003), pero el impacto de este tipo de procesamiento en la producción de Aβ y/o en la patología de la EA es poco relevante (Harris et al., 2010).

2.10. Péptido Aβ y placas seniles

Las placas seniles contienen una mezcla de varias isoformas de Aβ, por una parte existen las formas N-truncadas (Liu et al., 2006), que empiezan en el aminoácido 11 y finalizan entre el aminoácido 39 y el 42. Por otra parte, las formas más comunes empiezan en el aminoácido 3 o en el 11, que contienen glutamato, y éste al deshidratarse intramolecularmente puede hacer que se formen especies de Aβ que contienen piroglutamato en estas dos posiciones (Harigaya et al., 2000). También existen las formas no truncadas que comienzan en el aminoácido 1 y acaban en el animoácido 40-42. Las isoformas que terminan en el aminoácido 42 contienen dos aminoácidos adicionales en el extremo C-terminal, que lo hace más hidrofóbico y más propenso a precipitar en soluciones acuosas. Las mutaciones en los genes del APP, y de la presenilina 1 y 2 se han asociado con un aumento en la producción de Aβ y de la ratio $A\beta_{42}$/ $A\beta_{40}$. Además, el péptido Aβ tiene tendencia a oligomerizar, y estos oligómeros podrían ser las especies tóxicas (Lambert et al., 1998), además parece ser que se generan en el interior de la célula (Takahashi et al., 2004). El péptido Aβ puede ser degradado por varias enzimas, entre ellas la neprilisina y la enzima degradadora de insulina (IDE), que probablemente sean las más importantes. Por otro lado, es eliminado del parénquima cerebral a través del fluido intersticial y el espacio perivascular de Virchow-Robin, que hace una función similar a la de un vaso linfático (Weller et al., 2009). También interacciona con el receptor relacionado con lipoproteínas de baja densidad (LRP-1) (Shibata et al., 2000) o con la glicoproteína P (Cirrito et al., 2005) para atravesar la barrera hematoencefálica. Pero también existe un flujo de péptido Aβ desde de sangre al parénquima cerebral mediado por el receptor de compuestos de glicosilación avanzada (RAGE) (Deane et al., 2003).

2.11. Acumulación de Tau

Los ovillos neurofibrillares (NFT, del inglés *neurofibrillary tangles*) fueron descritos por primera vez por Alois Alzheimer, que descubrió una acumulación de material argirofilico en el cuerpo celular de neuronas mediante la tinción de plata de Bielschowsky. El termino neurofibrillar viene de neurofibrillas, un constituyente de las neuronas que también se tiñe con tinciones de plata. Las

neurofibrillas están compuestas por neurofilamentos, y los neurofilamentos parecían estar formando parte de estos NFT (Anderton et al., 1982). Sin embargo, más tarde se descubrió que los NFT no estaban compuestos por neurofilamentos ni por tubulinas (Grundke-Iqbal et al., 1985), estaban formados de proteína tau (Brion, 2006). Tau es una fosfoproteína que contiene una región de unión a microtúbulos que contiene entre 3 y 4 regiones repetidas (tau 3 R y tau 4 R) dependiendo del splicing de su mRNA. Ambas isoformas 3 y 4 R están presentes en pacientes de EA. Las formas de tau que se acumulan en los NFT son formas anormalmente fosforiladas (Delacourte and Defossez, 1986; Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b) en las que hay un aumento en los reduos fosforilados (como Thr^{231} y Ser^{262}), específico de un proceso fosforilación anormal (Wang et al., 2007). Además la tau presente en los NFT está truncada por su región N-terminal (Horowitz et al., 2004). La proteólisis de tau también podría ser importante para la formación de los NFT (García-Sierra et al., 2008). Está acumulación de tau en la neurona está asociada con marcadores del ciclo celular como la ciclina B (Nagy et al., 1997; Mosch et al., 2007) o la cinasa dependiente de ciclina 5 (CDK5) (Takahashi et al., 2000). Estas proteínas están relacionadas con la re-entrada en el ciclo celular, que puede conducir a la muerte neuronal. En ratones transgénicos que expresan tau humana se ha encontrado signos de re-entrada en el ciclo celular, por lo tanto podría ser que la tau fuera la causa y no la consecuencia de este fenómeno (Andorfer et al., 2005). La acumulación de tau en la neurona parece tener consecuencias muy severas en lo que respecta a las funciones celulares. Las neuronas que tienen NFT presentan microtubulos anormales (Flament-Durand and Couck, 1979; Gray et al., 1987) y una disminución de tubulina estable (Hempen and Brion, 1996). En la EA la acumulación de tau se produce exclusivamente en las neuronas, sin embargo, existen algunos astrocitos tau positivos, también llamados astrocitos con forma de espina, que pueden estar presentes en pacientes de EA, pero su prevalencia y su densidad están más ligadas a la edad (Schultz et al., 2004) que a la patología. Existen numerosos anticuerpos dirigidos contra varios epítopos de tau que marcan los NFT. Unos de los más utilizados es el AT8, que reconoce los epítopos fosforilados en Ser^{202} y Thr^{205}, o en Ser^{205} y Ser^{208} (Porzig et al., 2007).

Por otra parte, los NFT extracelulares presentan una forma similar a la de una neurona, y existen claras evidencias de que la muerte neuronal es el punto final de la degeneración neurofibrilar (Cras et al., 1995).

Algunos, pero no todos los NFT son ubiquitinizados (Perry et al., 1987; He et al., 1993) y marcados por anticuerpos que reconocen p62 (Kuusisto et al., 2002). La ubiquitinización es probablemente unos de los eventos más tardíos de la formación de los NFT. También se marcan con anticuerpos dirigidos contra varias quinasas como CDK5, y la forma activa de la glicógeno sintasa quinasa β (GSK3β) (Leroy et al., 2007). Ultraestructuralmente, los NFT parecen filamentos helicoidales apareados (Kidd, 1963) o, como se sugirió posteriormente, lazos retorcidos (Pollanen et al., 1994). Los filamentos están formados por tau en configuración β cruzada (Berriman et al., 2003). La relación entre los NFT y los depósitos de amiloide todavía se desconoce.

2.12. Relación entre el péptido Aβ y la patología de Tau

Ya que las mutaciones en APP y las presenilinas causan EAf, que incluye patología tau, mientras que las mutaciones de tau causan degeneración fronto-temporal sin presencia de acumulación de Aβ (Munoz and Ferrer, 2008), se ha sugerido que la alteración del metabolismo del APP, que induce un aumento de la producción de Aβ es el iniciador de una serie de mecanismos que conducen finalmente a la patología de tau y a la muerte neuronal. Descubrimientos recientes han demostrado que los oligómeros de Aβ promueven la fosforilación de tau en cultivos primarios de neuronas hipocampales y en células de neuroblastoma (De Felice et al., 2008), y por lo tanto sugieren que la patología tau es secundaria.

Sin embargo, diversos hechos indican que esta hipótesis no se aplica en los casos esporádicos. En un estudio a gran escala que analizó la prevalencia de la patología amiloide y de tau en función de la edad, Braak et al. Demostró que la patología tau precedía la patología amiloide en varias décadas (Duyckaerts and Hauw, 1997), en otras palabras, la patología tau no parecía ser secundaria, sino la causa inicial. Los transgénicos de APP no producen NFT a no ser que no esté presente un gen mutado de tau (Oddo et al., 2003). Existe una interacción física entre tau y el péptido Aβ, la cual se ha postulado como el paso inicial para su posterior fosforilación y agregación (Guo et al., 2006). Cuál es la relación entre tau y el péptido Aβ es una de las mayores preguntas por resolver en la actualidad. Las placas seniles, donde se deposita el Aβ y se detecta tau (procesos tau positivos), es probablemente un lugar importante para su interacción: el dominio citoplasmático de APP, fosforilado en Thr^{668}, puede ser el intermediario, ya que parece estar asociado con tau y Aβ (Shin et al., 2007), aunque también está descrito que tau y Aβ se encuentran en la sinapsis (Fein et al., 2008).

2.13. Alteración sináptica

La patología sináptica en la EA presenta dos aspectos: que las sinapsis participan en las placas seniles (Gonatas et al., 1967) y que el número de sinapsis en el cerebro de los enfermos de EA disminuye con el tiempo. La pérdida de las sinapsis se ha descrito ultraestructuralmente y mediante inmunohistoquímica (DeKosky and Scheff, 1990; Scheff and Price, 1993; Scheff et al., 1996), sin embargo las sinapsis remanentes tienen un mayor tamaño. Además, el número de sinapsis se encuentra disminuido en la CA1 en un estado temprano de la enfermedad (Scheff et al., 2007). Marcadores presinápticos (sinaptofisina o SNAP-25) o postsinápticos (PSD-95) se han utilizado para analizar alteraciones sinápticas. Se ha descubierto que la sinaptofisina disminuye en estadios tempranos de la enfermedad (Masliah et al., 2001), llevando a la conclusión de que la pérdida sináptica es el marcador que mejor se correlaciona con los déficits cognitivos (Terry et al., 1991).

2.14. Inflamación

Las evidencias que indican que existe un proceso inflamatorio alrededor de las placas seniles (Mandybur and Chuirazzi, 1990; Eikelenboom et al., 2008) es la presencia de microglía activada (McGeer et al., 1993), componentes tempranos de la cascada del complemento (McGeer et al., 1989) y citocinas proinflamatorias (Dickson et al., 1993). Todavía existe discrepancia sobre si este proceso inflamatorio es tóxico o, por el contrario, es protector (Boche and Nicoll, 2008). Los enfermos de EA presentan astrogliosis en la corteza (Schechter et al., 1981), y la concentración de GFAP llega a estar aumentada hasta 10 veces en la corteza, el talamo, tallo cerebral e incluso el cerebelo, en el que la patología está limitada a la deposición de Aβ en los casos más severos (Delacourte, 1990). La astrogliosis correlaciona con la densidad de NFT y con los depósitos de Aβ (Cairns et al., 1992). Los astrocitos que rodean las placas pueden contener gránulos que son marcados con anticuerpos que detectan Aβ (Akiyama et al., 1999), probablemente debido a la endocitosis.

3. RESVERATROL

3.1. Generalidades

El resveratrol (3,5,4'-trihidroxi-trans-estilbeno) es un polifenol natural no flavonoide perteneciente a la familia de los estilbenos que se produce en 72 especies de plantas en respuesta a un factor exógeno como la radiación UV o patógenos como bacterias u hongos. Está formado por dos anillos aromáticos unidos por un puente metileno (Catalgol et al., 2012). Respecto a sus propiedades físico-químicas, es un polvo blanquecino con formula molecular $C_{14}H_{12}O_3$, un peso molecular de 228,25 g/mol y un punto de fusión de 253-255ºC. Es un compuesto soluble en lípidos, etanol y dimetil sulfóxido, pero prácticamente insoluble en agua. Sin embargo, es altamente permeable y por ello es considerado un compuesto de clase II según el BCS (Biopharmaceutics Classification System), y por tanto, se caracteriza por una alta permeabilidad y baja solubilidad, lo que hace que sea bien absorbido en el intestino. Tiene dos isómeros geométricos: el trans-, que es biológicamente más activo, y el cis-, más inestable, que no está comercializado, y que se puede generar a partir del trans- por la exposición a radiación ultravioleta.

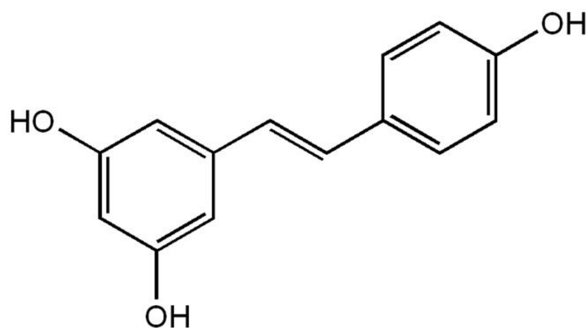

Figura 7. Estructura química del resveratrol.

Las principales fuentes de resveratrol en la dieta son el vino tinto, la uva roja (sobretodo la piel), la granada, la soja, las moras, los arándanos, los cacahuetes, el chocolate negro y el cacao. De todos los anteriores, el que mayor concentración presenta es el vino tinto. Esto es debido a que las uvas, al ser infectadas por *Botrytis cinérea,* producen una síntesis de resveratrol exclusivamente en la piel. En la elaboración del vino blanco se fermenta únicamente el mosto, sin embargo, para la elaboración de vino tinto se fermenta el prensado del mosto y la piel, y por ello el vino tinto tiene

una alta concentración de resveratrol (Catalgol et al., 2012). Además de resveratrol, se han identificado gran variedad de compuestos en el vino tinto, incluyendo flavonoides como la miricetina, el kaempferol y la quercetina, entre otros.

El resveratrol fue aislado por primera vez de la raíz del eléboro blanco (*Veratrum grandiflorum*) en 1940, posteriormente en 1960 fue aislado de la raíz de *Polygonum cuspidatum*, comúnmente conocido como hierba de Santa María, una planta utilizada en la medicina tradicional oriental (Vastano et al., 2000), y fue caracterizado como una fitoalexina (Nakata et al., 2012). Años más tarde, en 1976 fue aislado de la uva (*Vitis vinifera*), y no fue hasta 1992 que fue identificado en el vino tinto y se le atribuyó un efecto cardioprotector. El resveratrol parece ser, entre otros, el responsable de un hecho nutricional llamado "Paradoja francesa", ya que los franceses son los europeos que ingieren más grasas saturadas, y sin embargo, presentan menos riesgo de sufrir enfermedades cardiovasculares que otros europeos, como los ingleses, que al igual que los franceses siguen una dieta rica en grasas saturadas. Diversos estudios muestran una correlación entre un consumo bajo o moderado de vino con una menor mortalidad por enfermedades cardiovasculares y cerebrovasculares. Sin embargo, un consumo elevado de vino, debido a su contenido en alcohol, aumenta la prevalencia infarto, cardiomiopatía, arritmias, hipertensión, shock hemorrágico y muerte súbita, lo que confirma que este tipo de consumo es perjudicial para el sistema cardiovascular (Catalgol et al. 2012). A partir de estos estudios iniciales, estudios posteriores han demostrado que el resveratrol puede prevenir o enlentecer la progresión de cáncer, enfermedades cardiovasculares y el daño por isquemia. Además, se ha observado que aumenta la resistencia al estrés, mejora diversos marcadores de envejecimiento y aumenta el tiempo de vida medio y máximo, tanto en especies inferiores como levaduras (Howitz et al., 2003), gusanos, moscas (Bass et al., 2007) y peces (Valenzano et al., 2006), como en mamíferos (Baur et al., 2006; Porquet et al., 2013).

En términos de toxicidad y seguridad, la información disponible es limitada. Una dosis oral de 300 mg/kg no tiene efectos tóxicos en ratas, sin embargo, una dosis de 1 g/kg comienza a tener efectos adversos como deshidratación y pérdida de peso. Una dosis de 3 g/kg comienza a tener una alta toxicidad, produciendo además daño hepático, daño renal y anemia (Crowell et al., 2004). Una dosis de 18 g/kg es letal en la mayoría de los animales estudiados. En humanos, dosis únicas de hasta 10 g de resveratrol son bien toleradas (Knutson & Leeuwenburgh, 2008).

La limitación del resveratrol es su baja biodisponibilidad debido a su rápida metabolización a formas conjugadas: glucuronadas y sulfonadas (Albani et al., 2010; Timmers et al., 2012). Uno de los principales factores que afectan a la biodisponibilidad del resveratrol es el metabolismo hepático, ya que un estudio en humanos reveló que una hora después su administración intravenosa, solo se encuentran formas glucuronadas y sulfonadas en plasma (Walle et al., 2004), y por lo tanto, se piensa que estos metabolitos pueden ser parcialmente responsables de los efectos biológicos del resveratrol. No obstante, se necesitan dosis elevadas para llegar a la concentración activa descrita en modelos animales. Otra característica importante del resveratrol es su capacidad para cruzar la barrera hematoencefálica. Cuando es administrado intraperitonealmente aumenta la actividad de las enzimas antioxidantes en el cerebro de ratas sanas (Mokni et al., 2007). Además, un estudio en jerbos demostró que el resveratrol alcanza un pico de concentración en el cerebro cuatro horas después de su administración por vía intraperitoneal (Wang et al., 2002).

3.2. Propiedades antioxidantes

Hace tiempo que se conoce que el resveratrol es un antioxidante efectivo (Fauconneau et al., 1997). Al ser un estilbeno, los dos anillos fenol hacen que pueda actuar como *scavenger*, eliminando gran variedad de radicales libres como los derivados de la peroxidación lipídica, radicales libres de carbono y especies reactivas de oxígeno. Los efectos neuroprotectores del resveratrol debido a su actividad antioxidante están ampliamente descritos. Por ejemplo, el tratamiento con resveratrol disminuye los marcadores de daño por estrés oxidativo en modelos *in vivo* e *in vitro* de hipoxia-isquemia, en los cuales se produce gran cantidad de radicales libres. También protege las neuronas de cultivos primarios de la cepa SAMP8 frente a su vulnerabilidad al estrés oxidativo (Cristòfol et al., 2012) y el daño oxidativo por Aβ en cultivos de células PC12 (Sun et al., 2001). Este efecto antioxidante del resveratrol puede ser debido, en parte, a su capacidad de inducir la expresión de enzimas antioxidantes como MnSOD y glutatión peroxidasa en SAMP8 (Liu et al., 2012); y la hemo-oxigenasa 1 en un modelo de EA en rata (Huang et al., 2011). Por otro lado, induce PGC1α, un regulador importante del estrés oxidativo y del metabolismo mitocondrial, en un modelo de Parkinson en ratón (Mudò et al., 2012) y regula a la alza la expresión de Nrf2 en un modelo de isquemia en rata (Ren et al., 2011). Nrf2 activa la transcripción de varios genes cruciales para la protección frente al estrés oxidativo. El tratamiento con resveratrol también aumenta las expresión y la translocación nuclear de FOXO3a en células dopaminérgicas (Wu et al.,

2013), siendo los genes FOXO la primera línea de defensa frente al estrés oxidativo. Por último, hay que mencionar que el efecto neuroprotector frente al estrés oxidativo del resveratrol está, al menos parcialmente, mediado por la activación de la vía de Sirtuina 1.

3.3. Propiedades anti-inflamatorias

La inflamación puede ser causada por variedad de estímulos: interleucinas como la IL-1, TNF, productos bacterianos o víricos como el LPS y estímulos apoptóticos y necróticos como radicales libres de oxígeno, radiación ultravioleta y radiación γ. Todo esto promueve la activación de NFκB, el principal regulador del proceso inflamatorio (May and Ghosh, 1998). Diversos estudios demuestran que el resveratrol media la regulación a la baja de varios marcadores inflamatorios como TNFα (Prabhakar, 2013), COX2 (Annabi et al., 2012), iNOS (Centeno-Baez et al., 2011), produciendo una disminución de los niveles de óxido nítrico, y diversas interleucinas como la IL-6 (Zaky et al., 2013). Además de producir una disminución de la expresión de COX2, el resveratrol también actúa como inhibidor directo de éste (Gentilli et al., 2001). Por otro lado, puede evitar la translocación de p65 al núcleo, ya que previene la degradación de IκB (Cianciulli et al., 2012), y por ello, bloquea la acción proinflamatoria de NFκB en diferentes tejidos. Además, el resveratrol ejerce un efecto antiinflamatorio en la microglia y la astroglia inhibiendo diferentes citocinas proinflamatorias y proteínas se señalización claves como NFκB y AP-1 (Lu et al., 2010).

3.3. Resveratrol: un activador de SIRT1

Las sirtuinas son una familia de desacetilasas dependientes de NAD^+ bien conservadas a lo largo de la evolución. Los mamíferos expresan hasta siete sirtuinas (SIRT1-7), cada una con funciones distintas y diferente localización subcelular. En concreto, SIRT1 puede translocarse del citoplasma al núcleo, donde desacetila histonas en residuos específicos y contribuye al mantenimiento de los telómeros (Palacios et al., 2010), lo cual podría explicar, al menos en parte, el aumento que produce en la longevidad de distintos organismos. Por otra parte, SIRT1 desacetila otros sustratos, por ejemplo componentes de la maquinaria de reparación del DNA como Ku70 (Cohen et al., 2004) y PARP (Rajamohan et al., 2009), varios factores de transcripción relacionados con el metabolismo energético como PGC1α (Rodgers et al., 2007), HIF1α (Lim et al., 2010) y PPARα (Purushotham et al., 2009), y otros factores de transcripción como p53 (Hasegawa & Yoshikawa, 2008; Kim et al.,

2007), FOXO (Brunet et al., 2004) y NFκB (Yeung et al., 2004), que participan en la apoptosis, la resistencia al estrés oxidativo y la inflamación, respectivamente.

Mediante un ensayo de fluorescencia (Fluor de Lys) se ha demostrado que el resveratrol activa SIRT1 directamente (Borra et al., 2005; Kaeberlein et al., 2005). Sin embargo, otros estudios han cuestionado estos hallazgos y afirman que medir la actividad SIRT1 mediante este ensayo puede generar falsos resultados (Beher et al., 2009), ya que el resveratrol solo activa SIRT1 si el sustrato está unido a un fluoroforo. Por otro lado, estudios recientes demuestran que el resveratrol inhibe la PDE4, lo cual produce un aumento de los niveles de cAMP, que a su vez incrementa la actividad AMPK, esto genera un aumento de NAD+, que conduce finalmente a un aumento de la actividad SIRT1 (Park et al., 2012b).

Respecto al aprendizaje, la memoria y la plasticidad sináptica, se ha demostrado que la ausencia de SIRT1 empeora las habilidades cognitivas asociadas a los efectos en la plasticidad sináptica. Por el contrario, la sobreexpresión de SIRT1 en cerebro genera una plasticidad sináptica y una memoria normal (Michán et al., 2010).

Aun así, el resveratrol tiene otros efectos beneficiosos a parte de los derivados de la activación de SIRT1. Por ejemplo, varios estudios demuestran que es un potente activador de AMPK en líneas celulares neurales, cultivos primarios y cerebro (Dasgupta and Milbrandt, 2007), y que este efecto es independiente de SIRT1. Además, muchos de los efectos del resveratrol, incluyendo la biogénesis mitocondrial y el crecimiento neurítico, dependen de la presencia de un complejo AMPK funcional y su regulador LKB1 (Dasgupta and Milbrandt, 2007).

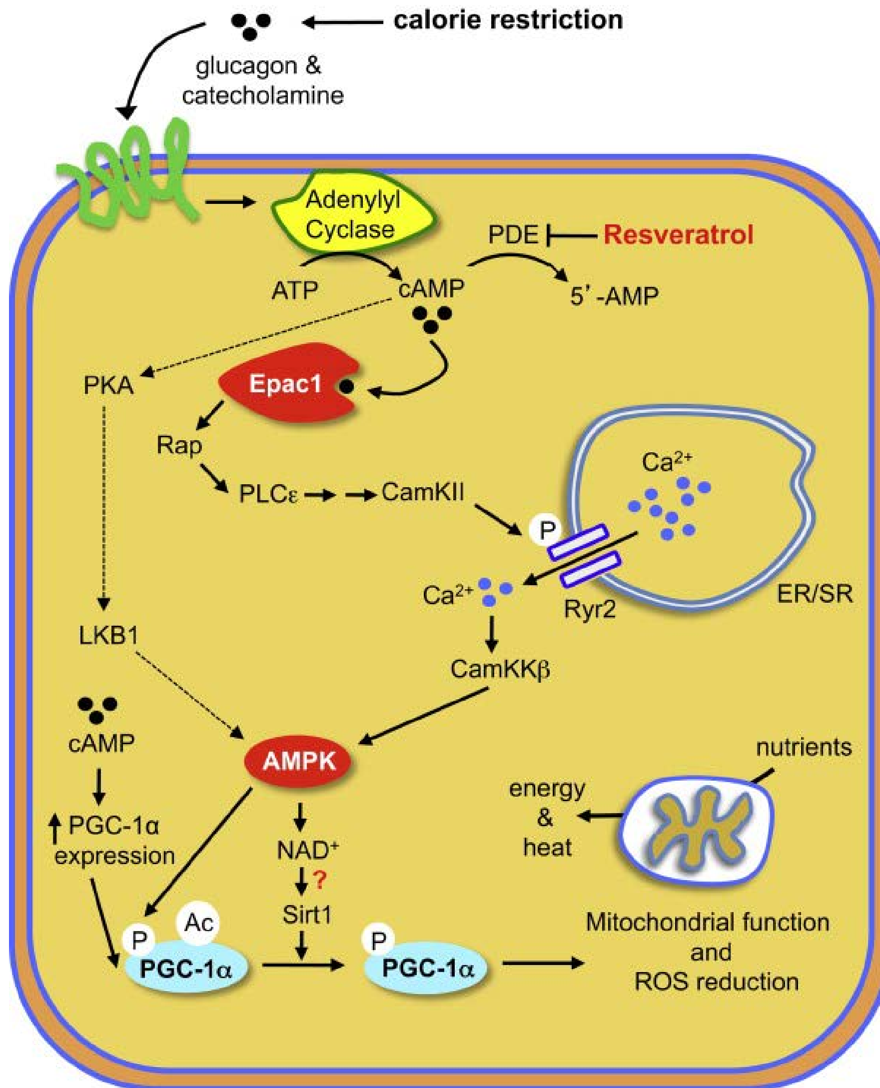

Figura 8. Efecto del resveratrol en la vía AMPK/SIRT1 (Park et al., 2012b).

3.4. Resveratrol y restricción calórica

La restricción calórica (RC) tiene efectos beneficiosos sobre la salud de los mamíferos, aumentando la esperanza de vida y retrasando la aparición de enfermedades asociadas a la edad como el cáncer, la diabetes y las enfermedades cardiovasculares en primates (Colman et al., 2009). A pesar de esto, existen estudios recientes en primates en los que no se ha observado un aumento en la esperanza de vida (Mattison et al., 2012). Para explicar las discrepancias entre estos estudios, los

autores afirman que las diferencias entre los efectos sobre la salud, morbilidad y mortalidad pueden ser debidas al diseño del estudio, la cría de animales y la composición de la dieta, que pueden modificar el efecto de la RC sobre la esperanza de vida.

El resveratrol mimetiza varios aspectos de la RC (Barger et al., 2008; Pearson et al., 2008), y ésta parece ser, al menos en parte, dependiente de SIRT1 (Cohen et al., 2004). Un hecho que refuerza esta afirmación, es que otros activadores de SIRT1 como el SRT501 y el SRT1720 producen efectos similares a la RC y activan las mismas vías de señalización (Smith et al., 2009; Dai et al., 2010). Es más, Ratones transgénicos para SIRT1 tienen fenotipos similares a los causados por la RC (Bordone et al., 2007). Estos ratones son más delgados, más activos metabólicamente y muestran una reducción los niveles sanguíneos de colesterol, adipocinas, insulina y glucosa en ayuno, así como una mayor tolerancia a esta última. Además, se desenvuelven mejor en el rotarod y tienen un retraso en el periodo de reproducción.

3.5. Resveratrol y autofagía

La autofagia es un proceso catabólico intracelular involucrado en la degradación de orgánulos y proteínas por la vía lisosomal (Salminen and Kaarniranta, 2009). Su función principal es mantener la calidad celular, eliminando los residuos o el exceso de proteínas y orgánulos. Una disminución de la autofagia favorece la aparición de enfermedades relacionadas con la edad, como el cáncer o las enfermedades neurodegenerativas (Rezzani et al., 2012). La eficiencia del sistema autofágico-lisosomal disminuye con la edad, lo que compromete el mantenimiento de la salud de la célula y puede conducir a proteinopatias y procesos neurodegenerativos típicos del envejecimiento. Vías de resistencia al estrés y supervivencia, como la vía de la SIRT1 (Lee et al., 2008), pueden regular la autofagia, y por ello activadores de SIRT1 como el resveratrol pueden inducirla. Este hecho está asociado con la regulación negativa de SIRT1 sobre mTOR (Ghosh et al., 2010), que al inhibirse induce el proceso autofágico.

4. MODELOS EXPERIMENTALES

4.1. Senescence accelelated mouse prone 8 (SAMP8)

En la actualidad, prácticamente todos los modelos de EA están restringidos a animales transgénicos que sobreexpresan genes mutados relacionados con la EAf, y estos representan solo un 5% de los casos de EA. Hasta la fecha, no hay ninguna cepa de ratón que se pueda considerar un modelo de EAe, asociado a la edad. Sin embargo, las cepas de ratón SAM (senescence-accelerated mouse), en particular la cepa SAMP8, puede ser un buen modelo para estudiar los cambios tempranos asociados con la EA.

La cepa SAMP, fue originada a partir de la cepa AKR/J en la Universidad de Kyoto por el Dr. Takeda (Takeda, 1999). Mediante cruces endogámicos convencionales de la cepa AKR/J, varias camadas mostraron características indicativas de un envejecimiento acelerado, que incluía pérdida de pelo, lordocifosis, transtornos perioftálmicos, pérdida de actividad y una menor esperanza de vida. Los ratones que presentaban estos fenotipos se cruzaron con hermanos que compartían el mismo fenotipo, dando lugar a la cepa conocida como SAMP, quienes tenían un tiempo de vida medio de 9.7 meses. Las camadas que no tenían este fenotipo de envejecimiento acelerado, fueron cruzadas entre hermanos de la misma forma, y se les denominó SAMR (senescense-accelerated mouse resistant), los cuales tenían un tiempo de vida medio de 16.3 meses. Los SAMR a pesar de que viven más que los SAMP, viven mucho menos que cualquier otra cepa endogámica, siendo lo normal 28 meses. Existen hasta nueve sub-cepas SAMP y tres sub-cepas SAMR, que tienen diferentes alteraciones fenotípicas.

Fenotipos patológicos en SAM

Cepa	Fenotipo
SAMP1	Amiloidosis senil, riñón contraído, respuesta inmune disminuida, pulmones hiperinsuflados, problemas de oído, hipertensión.
SAMP2	Amiloidosis senil y secundaria, riñón contraído, respuesta inmune disminuida, cataratas, perdida del hueso alveolar.
SAMP3	Enfermedad degenerativa de la articulación temporomandibular.
SAMP6	Osteoporosis senil, amiloidosis secundaria.
SAMP7	Linfoma linfoblástico en el timo, amiloidosis senil.
SAMP8	Déficits en el aprendizaje y la memoria, respuesta inmune disminuida, ritmos circadianos anormales.
SAMP9	Cataratas, linfona linfoblástico en el timo, amiloidosis senil.
SAMP10	Atrofia cerebral, déficits en el aprendizaje y la memoria.
SAMP11	Riñón contraído, amiloidosis senil.

Sin embargo, una característica en común de todos los SAMP es que tienen un desarrollo y una maduración reproductiva normal, seguida de una temprana manifestación de un fenotipo de senescencia acelerada. Mientras que ningún modelo animal consigue mimetizar todas las características presentes en el envejecimiento, la cepa SAMP8 parece adecuada para estudiar la transición entre el envejecimiento y la EA, ya que comparte muchos marcadores neuropatológicos, neuroquímicos y sobretodo trastornos cognitivos que se encuentran también presentes en pacientes con EA.

SAMP **SAMR**

Figura 9. Fenotipo de las cepas SAMP y SAMR.

La cepa utilizada para este estudio ha sido la SAMP8. Ésta presenta déficits en el aprendizaje y la memoria (Flood and Morley, 1998). Se ha demostrado que comienzan a presentar problemas de aprendizaje espacial a los 3 meses, y a los 5 meses ya empiezan a tener problemas de memoria espacial (Chen et al.,2004). En referencia a la memoria asociativa no espacial, a los 2 meses ya presentan déficits (Miyamoto et al., 1986; Miyamoto et al., 1992).

Los SAMP8 presentan una disminución en varias enzimas relacionadas con el estrés oxidativo como MnSOD (Kurokawa et al., 2001), catalasa (Sato et al., 1996), y glutatión peroxidasa (Okatani et al., 2002). Además, con únicamente 2 meses de edad presentan un aumento de ROS (Yasui et al., 2003) y de peroxidación lipídica (Petursdottir et al., 2007).

De hecho, estudios previos han demostrado que los SAMP8 presentan un aumento de la expresión tau (Wei et al., 1999). Además, estudios más recientes han demostrado la presencia de varias formas de tau fosforilada en este ratón; (Canudas et al., 2005; Caballero et al., 2008) y que esta

fosforilación está mediada por mecanismos relacionados con la EA como un incremento en la proteína CDK5 (Canudas et al., 2005).

Respecto a la patología amiloidea, esta cepa presenta con la edad un aumento en los péptidos Aβ 1-40 (Takemura et al., 1993) y Aβ 1-42 (Fukunari et al., 1994), junto a un incremento en la expresión de APP (Nomura et al., 1996; Kumar et al., 2000; Morley et al., 2000) en el hipocampo, que es la primera región afectada en la EA.

Además, diversos estudios han demostrado que produciendo una disminución de la expresión de APP mediante un oligonucleótido antisentido específico para el mRNA de APP, revierte los déficits cognitivos (Kumar et al., 2000; Kumar et al., 2001) y disminuye los marcadores de estrés oxidativo (Poon et al., 2004). De forma similar, el mRNA de otros marcadores asociados con la dinámica del amiloide y la EA como la ApoE y la PS2 se encuentran alterados en esta cepa (Wei et al., 1999a; Wei et al., 1999b).

Estudios todavía más recientes han demostrado que la administración de anticuerpos contra Aβ por vía intracerebral (Morley et al., 2002) o intravenosa (Banks et al., 2007), mejoran la función cognitiva de estos animales. Además, el déficit de memoria también parece estar relacionado con una disminución de los niveles de sinaptofisina y del número de espinas dendríticas (Del Valle et al., 2012).

Todo ello en su conjunto, sugiere que la expresión anormal de genes relacionados con la EA puede estar generando el deterioro cognitivo característico de la cepa SAMP8.

En referencia a procesos inflamatorios cerebrales característicos de la EA, se ha descrito que presentan astrogliosis y microgliosis moderada en comparación con sus controles SAMR1 (Nomura et al., 1996; Sureda et al., 2006).

4.2. Ratones transgénicos APP/PS1

Los ratones transgénicos APP/PS1 expresan una proteína quimérica ratón/humano de APP que contiene la mutación Sueca (Mo/HuAPP695swe) y una proteína mutante humana PS1-dE9, siendo ambas causantes de la EAf. Esta mutación de PS1 contiene una deleción del exon 9 que genera un aumento de la forma más agregante de Aβ, que consta de 42 residuos, y por ello denominada $A\beta_{42}$

(Citron et al., 1997), respecto a $A\beta_{40}$, una forma de 40 residuos mucho menos agregante. A los 4 meses de edad comienzan a presentar placas seniles tanto en la corteza como en el hipocampo (Garcia-Alloza et al., 2006), y entre los 6 y los 15 meses presentan problemas de aprendizaje y memoria (Savonenko et al., 2005; Minkeviciene et al., 2008; Huang et al., 2011). Sin embargo, no sufren otras alteraciones de comportamiento, como la ansiedad (Webster et al., 2013). Estos animales tienen un aumento de la actividad de BACE1 (Torres et al., 2012), una disminución de la expresión de ADAM10 (Reinhardt et al., 2014) y una disminución de sinaptofisina sinaptosomal (Pérez-González et al., 2014). En lo que se refiere a marcadores de inflamación cerebral, muestran niveles elevados de IL-1β (Yan et al., 2013) y TNFα (Wang et al., 2013).

OBJETIVOS

El envejecimiento y la EA son dos de los procesos que están implicados, en diferente grado, en el deterioro cognitivo asociado a la edad. La prevención del deterioro cognitivo, y por tanto el incremento de la calidad de vida del individuo, es uno de los retos de la biomedicina. El resveratrol y la acción pleiotrófica descrita para este polifenol es una de las estrategias farmacológicas que se están desarrollando para prevenir los efectos deletéreos del envejecimiento y el deterioro asociado a la EA. En este contexto, el objetivo general de este trabajo ha sido estudiar el efecto del resveratrol en modelos animales de envejecimiento y de EA. Se han planteado los siguientes objetivos concretos:

1. Estudiar los efectos de una dosis baja de resveratrol (20 mg/kg), equivalente al consumo humano presente en dos copas de vino diarias, a corto plazo (2 meses) en ratones con envejecimiento acelerado (SAMP8).

   - Evaluar el efecto del polifenol sobre la función cognitiva de los ratones tratados.
   - Analizar la vía de SIRT1 y su modulación por resveratrol.
   - Determinar la acción del resveratrol en los procesos de hiperfosforilación de tau.
   - Examinar las enzimas relacionadas con el estrés oxidativo.

2. Estudiar los efectos de una dosis alta de resveratrol (160 mg/kg), equivalente al consumo humano de suplementos con resveratrol, a largo plazo (7 meses) en ratones SAMP8.

   - Analizar el efecto del resveratrol en la longevidad.
   - Evaluar el efecto del polifenol en a función cognitiva de los ratones tratados.
   - Estudiar la acción del resveratrol en la vía de SIRT1.
   - Determinar si la administración de resveratrol induce cambios en marcadores histopatológicos de la EA relacionados con el envejecimiento, como la acumulación y agregación en el espacio extracelular de Aβ o la fosforilación de tau. Analizar el efecto del polifenol en las enzimas implicadas en la producción de Aβ y en la fosforilación de tau en SAMP8.

3. Estudiar los efectos del resveratrol (160 mg/kg) durante 10 meses en ratones transgénicos APP/PS1, un modelo de EA familiar.

    - Evaluar la función cognitiva de los ratones tratados.
    - Analizar la vía de SIRT1.
    - Determinar si existen cambios en marcadores histopatológicos típicos de EA, en este caso las placas seniles.
    - Analizar las enzimas implicadas en este proceso, así como los precursores y fragmentos polipeptídicos generados como consecuencia de éste.
    - Estudiar los marcadores y las vías relacionadas con el estrés oxidativo y la inflamación a nivel cerebral.

4. Generar una cepa de ratón que presente un fenotipo de envejecimiento acelerado junto a la presencia de marcadores típicos de EA, como la acumulación de Aβ en forma de placas seniles.

    Comparar esta nueva cepa a diferentes edades (3, 6, 9 y 12 meses) con las cepas progenitoras: la que ha proporcionado el fondo génico de senescencia (SAMP8) y la que ha proporcionado los genes con las mutaciones humanas (APP/PS1) a nivel de:

    - Función cognitiva.
    - Presencia de placas seniles y vía amiloidogénica.
    - Patología tau y cinasas implicadas.
    - Enzimas y marcadores histopatológicos de estrés oxidativo e inflamación cerebral.

RESULTADOS

Publicación 1:

Low-dose pterostilbene, but not resveratrol, is a potent neuromodulator in aging and Alzheimer's disease

Chang J., Rimando A., Pallàs M., Camins A., Porquet D., Reeves J., Shukitt-Hale B., Smith M.A., Joseph J.A. and Casadesús G.

Neurobiology of Aging 33 (2012)

Resumen

Estudios recientes han implicado al resveratrol, y su derivado el pterostilbeno, en la protección frente a enfermedades relacionadas con el envejecimiento, como la enfermedad de Alzheimer (EA). Sin embargo, el mecanismo responsable de los efectos beneficiosos del resveratrol en el cerebro todavía no están claros, y la información disponible sobre la comparación directa entre estos análogos es escasa. Por ello, el objetivo de este estudio fue comparar la eficacia de la suplementación en la dieta con una dosis baja resveratrol respecto a la misma dosis de pterostilbeno en la mejora de déficits funcionales y de la patología de la EA en los ratones SAMP8, un modelo de senescencia acelerada cada vez más validado como modelo de EA esporádica. Además, tratamos de determinar los mecanismos de acción responsables de la mejora funcional observada estudiando el estrés celular, la inflamación y marcadores patológicos alterados en la EA. Únicamente los 2 meses de dieta con pterostilbeno, pero no con resveratrol, mejoraron la función cognitiva en SAMP8. Ni el resveratrol ni el pterostilbeno activaron la vía de Sirtuina 1. Sin embargo, los marcadores de estrés celular, inflamación y EA fueron modulados positivamente por el pterostilbeno, pero no por el resveratrol, y estuvieron asociados con un aumento de la expresión de PPARα.

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Low-dose pterostilbene, but not resveratrol, is a potent neuromodulator in aging and Alzheimer's disease

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Abstract

Recent studies have implicated resveratrol and pterostilbene, a resveratrol derivative, in the protection against age-related diseases including Alzheimer's disease (AD). However, the mechanism for the favorable effects of resveratrol in the brain remains unclear and information about direct cross-comparisons between these analogs is rare. As such, the purpose of this study was to compare the effectiveness of diet-achievable supplementation of resveratrol to that of pterostilbene at improving functional deficits and AD pathology in the SAMP8 mouse, a model of accelerated aging that is increasingly being validated as a model of sporadic and age-related AD. Furthermore we sought to determine the mechanism of action responsible for functional improvements observed by studying cellular stress, inflammation, and pathology markers known to be altered in AD. Two months of pterostilbene diet but not resveratrol significantly improved radial arm water maze function in SAMP8 compared with control-fed animals. Neither resveratrol nor pterostilbene increased sirtuin 1 (SIRT1) expression or downstream markers of sirtuin 1 activation. Importantly, markers of cellular stress, inflammation, and AD pathology were positively modulated by pterostilbene but not resveratrol and were associated with upregulation of peroxisome proliferator-activated receptor (PPAR) alpha expression. Taken together our findings indicate that at equivalent and diet-achievable doses pterostilbene is a more potent modulator of cognition and cellular stress than resveratrol, likely driven by increased peroxisome proliferator-activated receptor alpha expression and increased lipophilicity due to substitution of hydroxy with methoxy group in pterostilbene.
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1. Introduction

Aging poses the greatest risk factor in the development of Alzheimer's disease (AD). With an ever-increasing population, AD incidence in the United States will jump from 4 million individuals currently affected with the disease to 14 million by 2050 (Larson et al., 1992). Of concern, despite valiant effort by the scientific field to understand the molecular underpinnings of this insidious disease, little progress has been made with regard to mechanisms, diagnostic tests, or treatments.

Research to identify mechanisms associated with AD and new therapies is currently being carried out in rodent models of AD. However, despite that 95% of AD cases are age-related, a mouse model of late-onset and/or age-related AD does not exist. Instead, current studies are carried out in mouse models which overexpress AD-related pathology

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(amyloid-beta plaques and tau hyperphosphorylation inclusions [tangles]) associated with specific mutations present in early-onset AD (< 5% of total AD cases; Pallas et al., 2008a; Teruel, 2004). On the other hand, the SAMP8 model has many of the histopathological and behavioral indicators of AD (increased levels of oxidative stress [OS], hyperphosphorylation of tau, cognitive decline, amyloid-beta levels; Castillo et al., 2009; Pallas et al., 2008a). Importantly, this mouse is a model of accelerated aging, therefore it provides an excellent model to study the chronology of neurodegenerative changes associated with AD development and therapeutic opportunities from an aging perspective.

Over the years polyphenols, endogenously produced by plants as protection against predation, have been a source of interest due to their many beneficial effects on health and disease (Casadesus et al., 2004; Joseph et al., 2005). These biochemicals have shown numerous protective properties including antibiotic, anti-inflammatory, antioxidant, and anticarcinogenic amongst others, both in vivo and in vitro (Joseph et al., 2008; Rimando and Suh, 2008). One popular polyphenol is resveratrol, which is found in grapes and red wine has shown to have neuroprotective and cognitive enhancing properties (Bhavnani, 2003; Valenzano et al., 2006; Wang et al., 2006) and to induce apoptosis in cancer cells (Rimando and Suh, 2008), however only at high doses. In vitro, resveratrol is a potent activator of sirtuin 1 (SIRT1) (Borra et al., 2005; Howitz et al., 2003), thought to provide protection through downstream pathways including forkhead box (FOXO) proteins and manganese superoxide dismutase (MnSOD) modulation (Brookins Danz et al., 2009). In this context, increasing SIRT1 has been found to protect cells against amyloid-beta-induced reactive oxygen species (ROS) production and DNA damage, thereby reducing apoptotic death (Della-Morte et al., 2009; Kim et al., 2007). In vitro effects of resveratrol, through SIRT1 activation (Yeung et al., 2004), also include inhibition of proinflammatory nuclear factor-kappa B (NFκB) transcription (Holmes-McNary and Baldwin, 2000; Jang et al., 1997; Manna et al., 2000). Moreover, it has been demonstrated that AD neurons are rescued by the activation of SIRT1, through the administration of resveratrol (Camins et al., 2010; Della-Morte et al., 2009; Sun et al., 2010).

Pterostilbene is a phenolic compound chemically similar to resveratrol. Initially isolated from sandalwood, it is also found in fruits including grapes and blueberries, known for their beneficial effects on cognition and neuronal function during aging (Casadesus et al., 2004). Pterostilbene is a potent antioxidant and anti-inflammatory agent shown to have beneficial effects in the aging brain (Joseph et al., 2008; Remsberg et al., 2008; Rimando et al., 2002). Interestingly, in vitro, it has higher potency at inducing apoptosis in cancer cells than resveratrol (Mikstacka et al., 2007; Tolomeo et al., 2005), and shows powerful agonistic properties on the peroxisome proliferator-activated receptor (PPAR) alpha receptor (Rimando et al., 2005), a receptor complex that is intimately associated with fatty acid metabolism, inflammation, and oxidative stress regulation (Pyper et al., 2010).

To date, little is known about the biochemical and molecular mechanisms associated with pterostilbene's effects on neuronal function and cognitive function and whether this compound has protective effects in age-related pathological events. Given that the effects of resveratrol on neuronal function and SIRT1 activation have often been observed only when administered at high doses, the goal of this study was to (1) determine and evaluate the effectiveness of resveratrol at diet-achievable dose on cognition and neuronal function in a model of pathological aging and/or early AD while directly comparing it to pterostilbene; and (2) determine the mechanisms associated with the observed changes in both supplementation groups.

2. Methods

2.1. Animals and diet preparation

Five-month-old male and female SAMP8 were fed with either resveratrol or pterostilbene at an identical dose (120 mg/kg of diet) for 8 weeks or control diet, 120 mg/kg of diet equated to the content of resveratrol of in 2 glasses of wine. Animals were kept on a 12-hour light and 12-hour dark cycle with free access to food and water. Pterostilbene dose was kept identical to that of resveratrol to determine potency differences. In addition, an age-matched control SAMR1 group was included to be able to determine the magnitude of improvement produced by our experimental diets. Resveratrol (ChemPacific Corporation, Baltimore, MD, USA) and pterostilbene (synthesized according to Joseph et al., 2008), both nuclear magnetic resonance pure, were incorporated, separately, into Irradiated ProLab IsoPro RMH 3000 (TestDiet, Richmond, IN, USA). Compound incorporation was carried out by Harlan Teklad (Madison, WI, USA) at low drying temperature to prevent any degradation of the compounds. Body weight and diet consumption were tracked twice across the study to ensure that there were no diet intake related differences (i.e., diet taste preference).

2.2. Radial arm water maze

The radial arm water maze is a spatial learning and memory task that involves the use of distal visual cues to locate a hidden platform in 1 of 6 arms. Behavioral testing was carried out during the light cycle. Briefly, the test was carried out within a pool (120 cm diameter) with 6 swim arms; water temperature was kept constant at 24 °C for the duration of the testing sessions. One constant goal arm with a platform was used for the duration of the training and was randomized across animals to avoid spatial preference confounds. Animals were introduced into the water maze from different arms at every trial. On Day 1, 12 trials occurred (1-minute periods), alternating between a visible and hidden goal platform, with the exception of the last 3 trials where

the platform is hidden each time. Day 2 consisted of 12 trials using the hidden platform each time. Entering the nongoal arms during the trial was considered an error and was the dependent variable of this task. Successful learning of the task is considered 2 errors or less within the 1-minute trial. This particular protocol has been shown to be very sensitive in detecting spatial memory deficits in AD transgenic mice (Alamed et al., 2006). Data are reported as blocks of 3 trials for Day 1 (block 1–5) and Day 2 (block 6–10). Statistical analyses were carried out using a 1-way analysis of variance (ANOVA) followed by post hoc analysis using the Bonferroni multiple comparisons test.

2.3. Tissue processing

Animals were deeply anesthetized with a lethal dose of Avertin (10g tribromoethanol, 10mL tert amyl alcohol) (Acros Organics, Geel, Belgium) (500 mg/kg) and hippocampal and cortical tissue was collected and flash-frozen with liquid nitrogen and stored at −80 °C until homogenization. Samples were later lysed with 1x lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na_2 ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1% triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin) (Cell Signaling, Beverly, MA, USA). Samples were centrifuged (10,000 rpm for 10 minutes) and the supernatant was collected. The protein concentration of the samples was determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

2.4. Western blotting

Aliquots of tissue containing 20–40 μg of protein were loaded on 8%–10% acrylamide gels. Gels were then transferred to a polyvinylidenedifluoride membrane (Millipore, Billerica, MA, USA) overnight. Membranes were blocked in 10% nonfat dry milk in tris buffered saline (TBS), 0.1% Tween-20 for 1 hour at room temperature and probed with primary antibodies diluted in TBS, 0.1% Tween-20 overnight at 4 °C (see Supplementary Appendix S1 for antibodies and dilutions). After incubation with primary antibody (see Supplementary Table S1), membranes were washed (3 times for 10 minutes in TBS, 0.1% Tween-20) and probed with horseradish peroxidase (HRP) species-specific secondary antibodies in TBS, 0.1% Tween-20 for 1 hour at room temperature. Membranes were then washed (3 times for 10 minutes in TBS, 0.1% Tween-20) and bands were visualized using an ECL chemiluminescence-based detection kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on Kodak film (Kodak, Rochester, NY, USA).

2.5. Extraction of resveratrol and pterostilbene in serum

Serum was collected during sacrifice following standard procedures and kept in −80 °C until analysis. Serum samples were thawed in ice and two 150-μL aliquots were taken and transferred to 2 separate Eppendorf tubes. To 1 tube was added 60 μL of β-glucuronidase, 5000 U/mL potassium phosphate buffer (75 mM, pH 6.8 at 37 °C). To the other tube, only potassium phosphate buffer (60 μL) was added. The samples were vortexed then incubated at 37 °C while shaking at 750 rpm for 20 hours. Thereafter, ice-cold High Performance Liquid Chromatography-grade acetonitrile was added in both tubes, vortexed, and centrifuged for 5 minutes at 5000 rpm, and 4 °C. The supernatant was collected, and dried under a stream of nitrogen for gas chromatography-mass spectrometry (GC-MS) analysis.

2.6. Extraction of resveratrol and pterostilbene in brain tissue

The brain tissues kept in −80 °C were thawed in ice. Three sample tissues were combined in an Eppendorf tube, and considered as 1 sample. To the tube was added 300 μL sodium phosphate buffer, and the tissues were homogenized manually for 2 minutes. Equal portions of the homogenates were transferred to 2 separate Eppendorf tubes. To 1 portion, 50 μL β-glucuronidase (5000 U/mL potassium phosphate buffer) was added. To the other portion 50 μL potassium phosphate buffer (without enzyme) was added. The samples were vortexed, and incubated (37 °C, 20 hours) while shaking (750 rpm). The samples were then centrifuged (15 min, 7000g, 4 °C). The supernatant was collected and partitioned with ethyl acetate (200 μL, twice). The ethyl acetate layers were combined, dried under a stream of nitrogen, and used for GC-MS analysis.

2.7. Quantification and analysis of Western blots

Western blots were quantified by densiometric measurements using Quantity One software Version 4.4 (Bio-Rad, Hercules, CA, USA) after appropriate background subtraction. All results are shown as mean average ± standard error of the mean. All results including behavioral data were analyzed with 1-way ANOVA and post hoc multiple comparisons to determine significant differences between groups at $p < 0.05$.

2.8. Analysis of serum and brain tissue by GC-MS

The nitrogen-dried samples were treated with 30 μL of a 1:1 mixture of N,O-*bis*[trimethylsilyl] trifluoroacetamide (BSTFA) and dimethylformamide (DMF) (both from Pierce Biotechnology, Inc., Rockford, IL, USA) and heated at 70 °C for 40 minutes. The derivatized samples were analyzed for levels of pterostilbene and resveratrol on a JEOL GCMate II Instrument (JEOL, USA Inc., Peabody, MA, USA) using a J&W DB-5 capillary column (0.25 mm internal diameter, 0.25 μm film thickness, and 30 mm length; Agilent Technologies, Foster City, CA, USA). The gas chromatography temperature program was: initial 190 °C held for 1 minute, increased to 244 °C at 30 °C per minute rate and held at this temperature for 0.5 minutes, increased to 246 °C at the rate of 0.2 °C per minute and held at this temperature for 0.5 minutes, then finally increased to 300 °C at the rate of 30 °C per minute and held at this temperature for 1 minute. The carrier gas was ultrahigh purity helium, at 1 mL per minute flow rate. The injection port was kept at

250 °C, the GC-MS interface at 230 °C, and the ionization chamber at 230 °C. The volume of injection was 2 μL (splitless injection). The mass spectrum was acquired in selected ion-monitoring mode, electron impact 70 eV. GC-MS analyses were in duplicates. The retention time of pterostilbene was 11.6 minutes, and resveratrol 13.7 minutes.

For the quantization of pterostilbene mass-to-charge ratio 328, 313, and 297 were used; for resveratrol mass-to-charge ratio 444, 429, and 341 were used. Quantitation was done using external standards of commercial samples of resveratrol (Sigma-Aldrich, St. Louis, MO, UA) and a synthetic sample of pterostilbene.

3. Results

3.1. Cognitive function

Analysis using a 1-way ANOVA with a repeated measures factor (day) indicated a significant difference in learning across groups ($p < 0.05$). Specifically, nontreated SAMP8 made significantly more errors in this task compared with pterostilbene-fed SAMP8 ($p < 0.05$) and SAMR1 controls ($p < 0.05$), suggesting that pterostilbene can normalize cognitive function to SAMR1 control levels. Interestingly, resveratrol-fed animals showed improved learning compared with nonfed SAMP8 animals but this difference was not statistically significant ($p = 0.07$) suggesting that pterostilbene is a more potent modulator of cognitive function compared with resveratrol (Fig 1). There were no significant changes in body weight and food intake between the groups.

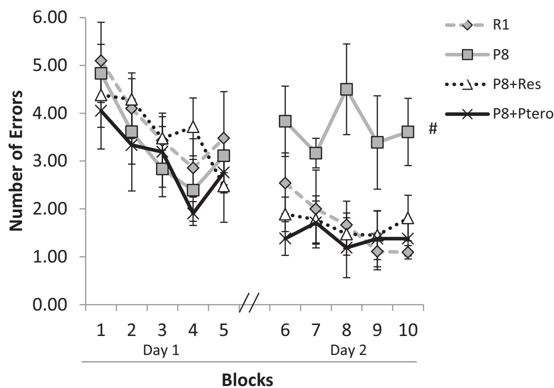

Fig. 1. Number of errors made over 2-day radial arm water maze test. SAMP8 mice were fed resveratrol, pterostilbene, and control diets. Statistical difference was found between groups by 1-way analysis of variance (ANOVA) with a repeated measures factor ($p < 0.05$). Post hoc comparisons between groups showed significant improvement in the SAMR1 (n = 9) and pterostilbene groups (n = 6) from the SAMP8 group (n = 8) ($^{\#} = p < 0.05$). Resveratrol (n = 6) was not significantly improved compared with SAMP8 but was not significantly different from SAMR1 and pterostilbene groups.

3.2. Bioavailability of pterostilbene and resveratrol

To test the bioavailability of pterostilbene in comparison with resveratrol, we measured the levels of both compounds in the serum as well as brain tissue homogenate. Previous studies have shown that pterostilbene shows a much higher bioavailability than resveratrol (Kapetanovic et al., 2011). Supporting these results, our data shows that pterostilbene was more abundant in the serum compared with resveratrol. Importantly, we were able to detect pterostilbene in brain tissue homogenate but not resveratrol (Supplementary Fig. S1).

3.3. Activation of SIRT1 and downstream targets

In order to determine the ability of dietary achievable doses of pterostilbene and resveratrol to modulate makers previously indicated to be significantly increased by resveratrol, we determined the levels of SIRT1 expression in all 4 groups. Our data indicate that SIRT1 expression was not significantly different between groups (Fig. 2B). In order to verify our SIRT1 results we also measured acetylation of p53, known to be inhibited by SIRT1 (Solomon et al., 2006). Our data show that acetylated p53 was not significantly changed by any of our treatments ($p = 0.19$) (Fig. 2C).

3.4. Upregulation of MnSOD through pterostilbene

Both pterostilbene and resveratrol have shown antioxidant properties in vivo and/or in vitro (Mikstacka et al., 2007). Furthermore, MnSOD expression has been shown to be increased by resveratrol at higher doses (Brookins Danz et al., 2009). To address the comparative effectiveness of these 2 chemically similar compounds in addition to determining their effectiveness at dietary-achievable doses, we measured changes in MnSOD expression. The groups were found significantly different through ANOVA ($F = 8.258$; $p < 0.01$). Our findings indicated that MnSOD levels significantly decreased in the SAMP8 group compared with the SAMR1 control group ($p < 0.01$) (Fig. 3C). Notably, pterostilbene rescued the levels of MnSOD back to the SAMR1 levels, an effect that was absent in the resveratrol-fed group.

3.5. PPAR-α rescue

Peroxisome proliferator-activated receptor alpha have been found to increase levels of MnSOD levels in the brain (Wang et al., 2010). Therefore, given in vitro data suggesting that pterostilbene is a potent PPAR-α agonist, we sought to determine whether changes in MnSOD observed in the pterostilbene-treated animals were due to upregulation of PPAR-α. Our results indicated a significant difference across groups ($F = 4.534$; $p < 0.05$) (Fig. 3B). Post hoc analysis revealed that PPAR-α is significantly decreased in the SAMP8 group compared with the SAMR1 control group ($p < 0.05$). Importantly, while resveratrol treatment had no

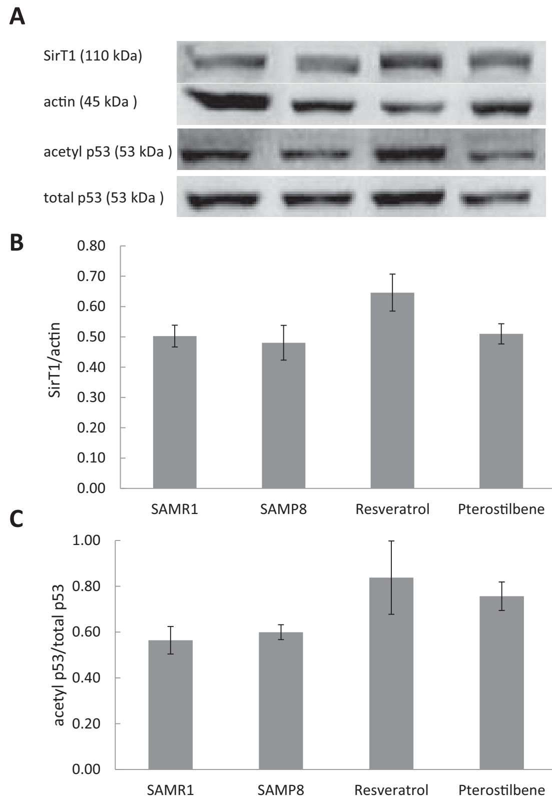

Fig. 2. (A) Western blot of sirtuin 1 (SIRT1) (with β-actin control) and acetyl p53 expression (with total p53 control) in mouse hippocampus. (B) SIRT1/actin ratio. Results are representative of protein expression from each group. No statistical difference was found between groups by 1-way analysis of variance (ANOVA). (C) Acetyl p53/total p53 ratio. No statistical difference was found between groups by 1-way ANOVA.

significant effect on PPAR-α expression, pterostilbene rescued PPAR-α expression to SAMR1 control levels.

3.6. NF$\kappa\beta$ activation

To further confirm the PPAR alpha changes observed in the SAMP8 mouse and the effectiveness of pterostilbene in normalizing these effects, we measured NF$\kappa\beta$, also known to be inhibited by PPAR α activation (Nunn et al., 2007). NF$\kappa\beta$ p65 level comparisons across groups revealed a significant group difference ($F = 4.534$; $p < 0.05$). Post hoc analyses indicated that NF$\kappa\beta$ p65 levels in SAMP8 mice were significantly higher than those in the control SAMR1 group ($p < 0.01$). This increased expression was not rescued by resveratrol treatment indicated by a significant increase of NF$\kappa\beta$ p65 levels in the resveratrol-treated group compared with the SAMR1 control group ($p < 0.05$). Pterostilbene treatment did not inhibit NF$\kappa\beta$ p65 levels significantly compared with SAMP8 but showed a strong trend toward significance ($p = 0.06$). Moreover, pterostilbene was not statistically different from SAMR1 control levels indicating at least a partial rescue by pterostilbene dietary supplementation (Fig. 3D).

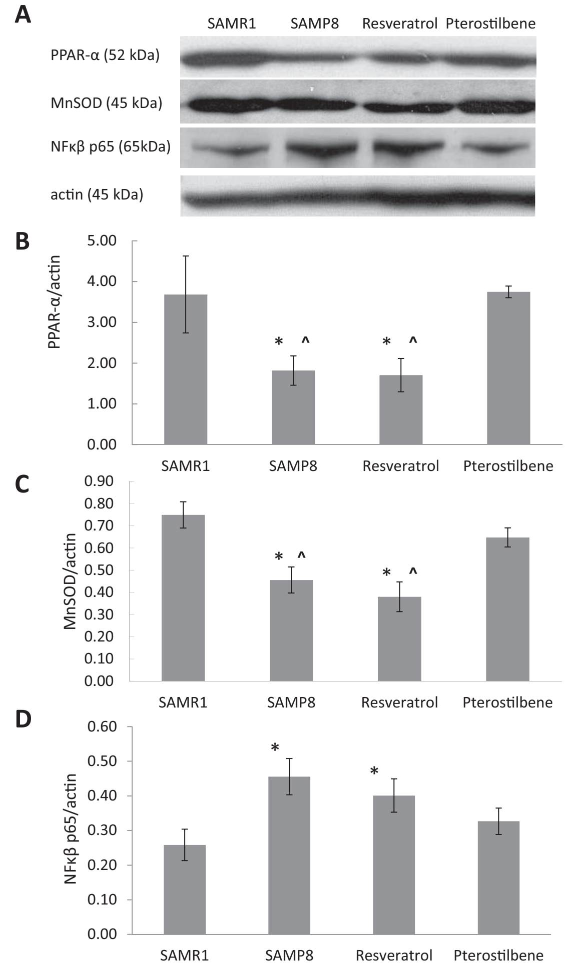

Fig. 3. (A) Western blot of peroxisome proliferator-activated receptor (PPAR)-α, manganese superoxide dismutase (MnSOD), and nuclear factor-kappa B (NFκB) p65 (with β-actin control) in mouse hippocampus. (B) PPAR-α/actin ratio. Results are representative of protein expression from each group. Groups were found to be statistical different by 1-way analysis of variance (ANOVA) ($p < 0.05$). Post hoc comparisons showed significant decrease in SAMP8 ($n = 8$) compared with SAMR1 ($n = 9$) (* = $p < 0.05$) and was reversed by pterostilbene treatment ($n = 6$) (^ = $p < 0.05$) but not resveratrol ($n = 6$). (C) MnSOD/actin ratio. Statistical significance by ANOVA ($p < 0.05$). Post hoc comparisons showed significant decrease in SAMP8 compared with SAMR1 (* = $p < 0.05$) and was increased by pterostilbene treatment (^ = $p < 0.05$). (D) NF$\kappa\beta$ p65/actin ratio. Statistical significance found by ANOVA ($p < 0.05$). Post hoc comparisons showed a significant increase in NF$\kappa\beta$ p65 levels in both SAMP8 and resveratrol-fed groups in comparison with SAMR1 control (* = $p < 0.05$).

3.7. Decreased levels of phosphorylated JnK

One signaling network dedicated to cellular maintenance under stress conditions involves stress-activated protein kinases (SAPKs), also known as Jun NH2-terminal kinases (JNKs). Increased JNK phosphorylation has been intimately associated with oxidative stress and inflammatory processes (Joseph et al., 2010; Liu et al., 2010) and well described in AD (Zhu et al., 2001a, 2001b). In this regard, we found a significant difference across groups ($F = 6.89$; $p < 0.01$) (Fig. 4B). We determined that SAMP8 had a high phosphorylation of JNK in comparison with SAMR1 control group ($p < 0.05$). Resveratrol-fed animals did not show a reduction in JNK phosphorylation and remained significantly elevated compared with SAMR1 controls. Importantly, pterostilbene-fed animals showed a reduction in JNK phosphorylation levels compared with SAMP8 groups ($p < 0.05$), similar to levels of SAMR1 control animals.

3.8. Reduced phosphorylation of tau (PHF)

Hyperphosphorylation of tau is intimately associated with cellular stress processes including JNK phosphorylation (Melov et al., 2007; Su et al., 2010; Zhu et al., 2002). We found a group difference in tau phosphorylation levels ($F = 3.318$; $p < 0.05$). Specifically, SAMP8 nontreated animals were found to have a significantly higher level of PHF-1 expression compared with the SAMR1 control group ($p < 0.05$) (Fig. 4C). Resveratrol was not effective at downregulating PHF-1 expression but pterostilbene supplementation was able to restore levels to SAMR1 controls.

4. Discussion

Alzheimer's disease (AD) poses an ever increasing threat with an aging population. SAMP8 mice have many of the histopathologic and behavior markers of AD including cognitive decline. It has been shown in several studies that many polyphenols such as resveratrol have antioxidant properties that may help the degradation that occurs with AD and modulate cascades associated with aging (Brisdelli et al., 2009). However, these studies often use doses not achievable through the diet. In our study we show that dietary supplementation of pterostilbene or resveratrol improved cognitive function in the SAMP8 model. Nevertheless, pterostilbene showed significant improvement over resveratrol. Furthermore, pterostilbene was more potent in activating protective signaling cascades and downregulating stress cascades at doses same as resveratrol, independent of SIRT1 regulation. This was shown by the lack of differences across groups in SIRT1 expression or its downstream targets such as acetylated p53 (Solomon et al., 2006). Importantly, the fact that resveratrol tended to increase both SIRT1 and acetylated p53 but was unable to upregulate more indirect targets such as acetylation of p53 suggests that while active, diet achievable doses were not sufficient to drive robust SIRT1 signaling. Previous studies have

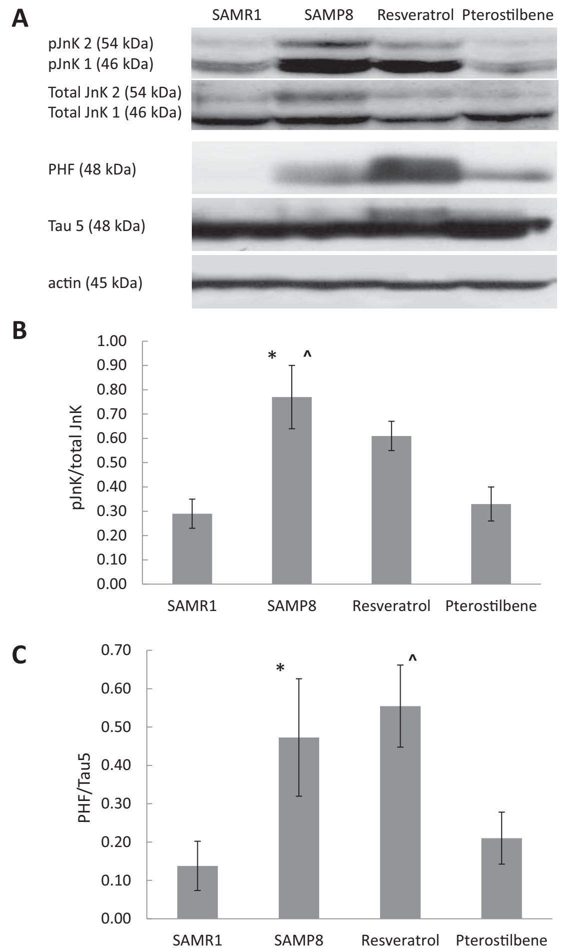

Fig. 4. (A) Western blot of phosphorylated jun NH2-terminal kinases (pJNK) (with total JNK control) and phosphorylation of tau (PHF) (with Tau5 control) in mouse hippocampus. (B) pJNK/total JNK ratio. Results are representative of protein expression from each group. Statistical difference was found between groups by 1-way analysis of variance (ANOVA) ($p < 0.05$). Post hoc comparisons between groups showed significant difference SAMP8 ($n = 8$) group and SAMR1 ($n = 9$) control (* = $p < 0.05$) and resveratrol ($n = 6$) and pterostilbene ($n = 6$) groups (^ = $p < 0.05$). (C) PHF/tau 5 ratio. Statistical difference was found between groups by 1-way ANOVA ($p < 0.05$). Post hoc comparisons between groups showed significant difference between SAMP8 group and SAMR1 control (* = $p < 0.05$) and resveratrol and pterostilbene groups (^ = $p < 0.05$).

shown that SIRT1 expression is decreased in the SAMP8 mouse compared with SAMR1 controls (Pallas et al., 2008b). However, analyses were carried out in older animals suggesting that the lack of differences between R1 and P8 mice observed in our study may be due to the age of the experimental groups.

Previously, studies have shown that cognitive improvements are likely not to be fully dependent on SIRT1 (Julien et al., 2009; Kim et al., 2007). Therefore we looked into

downstream mechanisms associated with aging and AD and shown to be important to neuronal and cognitive function, such as oxidative stress and inflammation (Castellani et al., 2008, 2009; Joseph et al., 2007; Shukitt-Hale et al., 2008, 2009). In this regard, our findings demonstrate that SAMP8 mice fed the control diet show low levels of MnSOD, an endogenous antioxidant defense, compared with SAMR1 controls and that these decreases in MnSOD was reversed only by pterostilbene. This supports previous data demonstrating powerful antioxidant effects of pterostilbene (Mikstacka et al., 2010). Here we show that, at least partially, these antioxidant effects are driven through the upregulation of endogenous antioxidant systems. Interestingly, resveratrol was not able to increase MnSOD activity in our study. Previous data demonstrate the ability of resveratrol to modulate MnSOD expression via the SIRT1/FOXO pathway both in vitro (Danz et al., 2009) and in vivo (Kao et al., 2010; Pfluger et al., 2008), however, at high doses. Furthermore, the fact that MnSOD was upregulated by pterostilbene in the absence of changes in the expression of SIRT1, suggests that the effects of pterostilbene on MnSOD were driven through an alternative mechanism.

One modulator of MnSOD is PPAR alpha (Ding et al., 2007). Importantly, PPAR alpha agonists have been shown to be neuroprotective after stroke (Ouk et al., 2009) and to increase hippocampal neurogenesis, a molecular event associated with cognition (Ramanan et al., 2009). Given that pterostilbene has been shown to be a powerful agonist of the PPAR alpha receptor in vitro (Rimando et al., 2005), we measured changes in PPAR alpha protein expression in our groups. Our findings demonstrate that PPAR alpha protein expression is downregulated in the SAMP8 mouse and is normalized by pterostilbene. Furthermore, it has been shown that ligand binding to the PPAR alpha stabilizes it by decreasing ubiquitinization and degradation (Blanquart et al., 2002). Therefore, 1 possibility is that pterostilbene, through its potent PPAR agonist properties, decreases the degradation of the protein resulting in high protein expression of PPAR alpha observed in our study.

To further elucidate the relationship between PPAR alpha modulation by pterostilbene we determined the ability of this stilbenoid to modulate NF$\kappa\beta$ expression. PPAR alpha has been shown to form physical inactive complexes with NF$\kappa\beta$ p65 hence reducing the ability to drive transcriptional activity (Delerive et al., 1999; Dragomir et al., 2006; Nunn et al., 2007). Our data demonstrates that SAMP8 mice show increases in NF$\kappa\beta$ expression compared with SAMR1 controls and pterostilbene treatment, but not resveratrol, is able to normalize these levels, consistent with upregulation of PPAR alpha activation.

Oxidative stress is a well established pathogenic factor in aging and AD (Markesbery, 1997; Perry et al., 1998; Smith et al., 1995b) and the association of oxidative stress with tau abnormalities is well known (Calingasan et al., 1999a, 1999b; Smith et al., 1995a; Takeda et al., 2000). These changes are also observed in the normal aging process as well as along the length of the axon (Wataya et al., 2002) suggesting that the oxidative modification of cytoskeletal proteins is under tight regulation. Interestingly, both tau and neurofilament protein appear uniquely adapted to oxidative attack due to their high content of lysine-serine-proline (KSP) domains and exposure of these domains on the protein surface is affected by extensive phosphorylation of serine residues resulting in an oxidative sponge of surface-modifiable lysine residues (Wataya et al., 2002). Because phosphorylation plays this pivotal role in redox balance, it is perhaps not surprising that oxidative stress, through activation of various cell signaling pathways including SAPK/JNK, leads to phosphorylation of tau (Ekinci and Shea, 2000a, 2000b; Joseph et al., 2010; Ramiro-Puig et al., 2009; Zhu et al., 2000, 2001a). Also of note is the fact that inhibition of JNK phosphorylation can be modulated by PPAR alpha agonists (Martínez de Ubago et al., 2009) and has also been shown to repress cognitive decline (Waetzig and Herdegen, 2005). Here we showed that phosphorylated JNK was significantly higher in SAMP8 mice compared with SAMR1 controls, an increase that was rescued by pterostilbene but not resveratrol. Importantly, tau phosphorylation pathology showed a similar trend. As previously reported in older mice, SAMP8 mice show increased phosphorylation of tau in comparison with the SAMR1 controls (Tajes et al., 2008; Tomobe and Nomura, 2009). In our study we show that increased tau phosphorylation was rescued by pterostilbene but not resveratrol.

In summary, while resveratrol shows important protective effects these may be restricted to when given at higher doses (Bhavnani, 2003; Sönmez et al., 2007). Our data demonstrate that pterostilbene is a more potent effector of beneficial molecular and functional events than resveratrol in the SAMP8 mouse. Importantly, these benefits are independent of SIRT1 activation and are likely driven through PPAR alpha regulation known to influence MnSOD expression (Ding et al., 2007), NF$\kappa\beta$ transcription (Delerive et al., 1999; Dragomir et al., 2006; Nunn et al., 2007), and JNK phosphorylation (Martínez de Ubago et al., 2009), all shown to be significantly improved by pterostilbene. In turn, all of these events are known to modulate tau pathology, as such it is not surprising that tau phosphorylation at sites associated with AD pathology were downregulated by pterostilbene. One potential explanation that can account for our findings is related to the chemical structure differences between these 2 compounds. In this regard, the substitution of the hydroxy group of resveratrol with a methoxy group in pterostilbene makes this molecule more lipophilic (Cichocki et al., 2008). This change may lead to better bioavailability of pterostilbene and consequently a more potent neuroprotective effect in the brain. This is supported by the fact that while diet consumption and weights did not vary in our studies, pterostilbene was found at higher doses both in serum and brain tissue compared with resveratrol, which

was found at low levels in the serum and undetectable in the brain tissue. While it is yet to be determined whether the cognitive improvements induced by pterostilbene in the SAMP8 model can be applied to humans, recent reports demonstrate that fruits containing pterostilbene such as blueberries ameliorate cognitive function in aged humans (Joseph et al., 2008) and that PPAR alpha agonists afford central nervous system protection (Hanyu et al., 2010). Therefore, use of pterostilbene may become an effective, natural, therapeutic strategy to improve cognitive function in aging and potentially a strategy to slow down the development of AD.

Disclosure statement

Drs. Casadesus, Lee, Camins, Pallas, Rimando, and Shukitt-Hale have no conflict of interest or disclosures to provide. Dr. Smith and Dr. Joseph recently passed away. Dr. Joseph had no conflict of interest or disclosures and Dr. Mark Smith was a consultant for Anavex Life Sciences Corporation, Eisai, Medivation, Neurotez, and Takeda Pharmaceuticals; owned stock options in Aria Neurosciences, Neurotez, Panancea, and Voyager, and received lecture fees from GSK, Medivation, and Pfizer. Dr. Zhu was also a consultant for Medivation. None of these companies are directly or peripherally implicated in the work hereby submitted. Jaewon Chang and David Porquet are students with no conflicts or disclosures to provide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2011.08.015.

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Publicación 2:

Dietary resveratrol prevents Alzheimer's markers and increases life span in SAMP8

Porquet D., Casadesús G., Bayod S., Vicente A., Canudas A.M., Vilaplana J., Pelegrí C., Sanfeliu C., Camins A., Pallàs M. and Del Valle J.

Age (2013) 35

Resumen

El resveratrol es un polifenol que se encuentra principalmente en la uva y el vino tinto. Además se ha demostrado que es un mimético de la restricción calórica a través de la activación de SIRT1. Diversos estudios indican que la restricción calórica, y por consiguiente el resveratrol, puede incrementar la longevidad. El resveratrol aumenta la tasa metabólica, la sensibilidad a insulina, la biogénesis mitocondrial, la resistencia física y reduce la acumulación de grasas en ratones. Asimismo, el resveratrol puede ser un fármaco efectivo para prevenir la neurodegeneración asociada a la edad y mejorar los déficits cognitivos en la EA. En este estudio hemos analizado el efecto de una dosis alta y crónica de resveratrol administrada a través de la dieta en ratones SAMP8, un modelo de envejecimiento acelerado que presenta marcadores relacionados con la EA. Los resultados indicaron que el resveratrol aumenta la esperanza de vida en SAMP8 y en su cepa control SAMR1. Además, observamos un aumento en la activación de las vías de AMPK y SIRT1, junto con una reducción del déficit cognitivo, de la carga de amiloide y de la hiperfosforilación de tau.

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Dietary resveratrol prevents Alzheimer's markers and increases life span in SAMP8

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Abstract Resveratrol is a polyphenol that is mainly found in grapes and red wine and has been reported to be a caloric restriction (CR) mimetic driven by Sirtuin 1 (SIRT1) activation. Resveratrol increases metabolic rate, insulin sensitivity, mitochondrial biogenesis and physical endurance, and reduces fat accumulation in mice. In addition, resveratrol may be a powerful agent to prevent age-associated neurodegeneration and to improve cognitive deficits in Alzheimer's disease (AD). Moreover, different findings support the view that longevity in mice could be promoted by CR. In this study, we examined the role of dietary resveratrol in SAMP8 mice, a model of age-related AD. We found that resveratrol supplements increased mean life expectancy and maximal life span in SAMP8 and in their control, the related strain SAMR1. In addition, we examined the resveratrol-mediated neuroprotective effects on several specific hallmarks of AD. We found that long-term dietary resveratrol activates AMPK pathways and pro-survival routes such as SIRT1 in vivo. It also reduces cognitive impairment and has a neuroprotective role, decreasing the amyloid burden and reducing tau hyperphosphorylation.

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Introduction

Resveratrol (trans-3,4′,5-trihydroxystilbene), a naturally occurring polyphenol mainly found in grapes and red wine, has been reported as a caloric restriction (CR) mimetic with potential anti-aging and anti-diabetogenic properties. Resveratrol increases metabolic rate, insulin sensitivity, mitochondrial biogenesis and physical endurance, and reduces fat accumulation in mice (Lagouge et al. 2006; Baur et al. 2006). The most widely accepted mechanistic hypothesis is that resveratrol's effects, in the same way as CR, are driven through Sirtuin 1 (SIRT1) regulation (Chung et al. 2010). Although there has been major controversy about whether resveratrol can be an activator of SIRT1, as its ability to interact directly with SIRT1 has been questioned (Beher et al. 2009; Pacholec et al. 2010), it now seems clear that resveratrol activates SIRT1 indirectly (Villalba et al. 2012). It is widely accepted that resveratrol benefits are mediated through AMPK activation (Zang et al. 2006; Baur et al. 2006; Price et al. 2012). Thus, resveratrol leads to increases in the NAD-to-NADH cell ratio, which results in activation of AMPK in vivo, initiating a signaling process that regulates insulin sensitivity and recruits mediators of oxidative metabolism and mitochondrial biogenesis, including PGC1α, PPARδ, and others (Um et al. 2010; Ruderman et al. 2010).

Several findings support the view that longevity can be promoted by CR in mice (Weindruch & Walford, 1988; Selman et al. 2008), along with CR's broad anti-aging activity (Park et al. 2009). In recent years, interesting studies in nonhuman primates have reported that CR also extended their life span (Colman et al. 2009), but in a very recently published study of the same species CR was not able to do so (Mattison et al. 2012). Though unlikely, the possibility that CR may extend maximum life span has still not been ruled out. Similarly, resveratrol treatment has a range of beneficial effects in mice, but up to now has failed to increase the longevity of ad libitum-fed animals when started midlife (Baur and Sinclair 2006), although in combination with other anti-aging strategies such as CR, it increased mean and maximal life span compared to control animals (Pearson et al. 2008). In addition, dietary resveratrol mimics the effects of CR in insulin-mediated glucose uptake in muscle in aged animals, and gene expression profiling suggests that both CR and resveratrol may retard some aspects of aging through alterations in chromatin structure and transcription (Halagappa et al. 2007; Barger et al. 2008).

Several in vitro and in vivo studies also support the hypothesis that resveratrol may be a powerful agent in preventing age-associated neurodegeneration (Vingtdeux et al. 2008). In in vitro models, resveratrol markedly lowers the levels of secreted and intracellular amyloid-beta (Aβ) peptides (Marambaud et al. 2005). Similarly, with a grape seed polyphenolic extract administered orally to Tg2576 mice, a murine model of Alzheimer's disease (AD) (Hsiao et al. 1996) improves cognitive deficits. These effects correlate with reductions in the amounts of high molecular weight Aβ assemblies in the brain (Wang et al. 2008). Similar findings have been observed in animals after moderate consumption of red wines (Wang et al. 2006; Ho et al. 2009). Recently, it was shown that resveratrol selectively remodels soluble oligomers, fibrillar intermediates, and amyloid fibrils into alternative aggregated species that are nontoxic (Ladiwala et al. 2010). These studies and others support the theory that resveratrol or polyphenol derivatives could be useful therapeutic agents for AD (Ono et al. 2008). Nevertheless, it is unknown whether resveratrol has similar effects in age-related models of AD.

To this end, we used the age-accelerated mouse (SAMP8). This strain is characterized by deficits in learning and memory (Takeda et al. 1981; Miyamoto et al. 1986; Takeda 2009), emotional disorders such as reduced anxiety-like behavior (Miyamoto et al. 1992; Markowska et al. 1998), impaired immune response, etc. (Yagi et al. 1988; Flood & Morley 1998). More importantly, this strain is increasingly being recognized as a model of age-related AD (Pallas et al. 2008; Morley et al. 2012) as, in addition to age-related learning and memory impairments, the mice show with aging an AD-related pathology such as increases in Aβ (del Valle et al. 2010) and other protein aggregates (Manich et al. 2011), alterations in APP processing by secretases (Morley et al. 2000, 2002), cerebral amyloid angiopathy (del Valle et al. 2011) and increases in tau hyperphosphorylation (Canudas et al. 2005).

Therefore, in this study we sought to clarify the role of dietary resveratrol in the SAMP8 mouse. Previous results in SAMP8 demonstrated that low doses and

short-term administration of pterostilbene (polyphenolic derivative of resveratrol) show positive effects on behavior, reductions in tau phosphorylation (Chang et al. 2012) and regulation of cascades associated with PPAR alpha. Based on these encouraging findings, we determined the effects of long-term administration of resveratrol on longevity and signaling cellular processes activated by this polyphenol, namely the SIRT1 pathway and AMPK system. We also extended these studies by examining the resveratrol-mediated neuroprotective mechanism in several specifically AD hallmarks present in SAMP8, such as, Aβ accumulation and tau phosphorylation.

Methods

Animals and resveratrol diet

A total of 216 male SAMP8 and SAMR1 animals were used for the survival study. The animals received a standard diet (2018 Teklad Global 18 % Protein Rodent Maintenance Diet, Harlan) or the same diet supplemented with trans-resveratrol (1 g/kg, Mega Resveratrol, Candlewood Stars, Inc., CT, USA), starting at 2 months of age and divided into four groups of 50 to 60 individuals: SAMR1 control (n=54), SAMR1 resveratrol (n=52), SAMP8 control (n=50), and SAMP8 resveratrol (n=60). For the neurodegeneration studies, two groups of 10–12 SAMP8 mice were fed with the standard diet or the resveratrol diet, starting the supplements at 2 months and killing the animals to obtain tissue samples at 9 months of age. All the animals had food and water ad libitum and were kept in standard conditions of temperature (22±2 °C) and 12:12-h light–dark cycles (300 lux/0 lux). Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona.

Object recognition test

Nine-month SAMP8 control (P8ctl) and SAMP8 resveratrol (P8rsv) animals were placed in a 90° two-arm, 25-cm-long, 20-cm-high, 5-cm-wide black maze. The 20-cm-high walls could be lifted off for easy cleaning. The light intensity in the middle of the field was 30 lux. The objects to be discriminated were made of plastic (5.25-cm high, object A and 4.75-cm high, object B). For the first 3 days, the mice were individually habituated to the apparatus for 10 min. On the 4th day, the animals were submitted to a 10-min acquisition trial (first trial) during which they were placed in the maze in the presence of two identical novel objects (A+A or B+B) placed at the end of each arm. A 10-min retention trial (second trial) occurred 2 h later. During this second trial, objects A and B were placed in the maze, and the time that the animal explored the new object (tn) and the old object (to) were recorded. A discrimination index (DI) was defined as (tn−to)/(tn+to). In order to avoid object preference biases, objects A and B were counterbalanced so that half of the animals in each experimental group were first exposed to object A and then to object B, whereas the other half saw first object B and then object A. The maze and the objects were cleaned with 96° ethanol between different animals, so as to eliminate olfactory cues.

Brain processing

One day after the object recognition test, 9-month animals were intracardially perfused after being anesthetized with 80 mg/kg of sodium pentobarbital. Afterwards, brains were dissected and separated sagitally in two hemispheres, one for immunohistochemistry and the other for protein extraction. Immunohistochemistry brains were frozen by immersion in isopentane, chilled in dry ice and stored at −80 °C until sectioning. Thereafter, frozen brains were embedded in OCT cryostat-embedding compound (Tissue-Tek, Torrance, CA), cut into 20-μm-thick sections on a cryostat (Leyca Microsystems, Germany) at −18 °C and placed on slides. Slides containing brain sections were fixed with acetone for 10 min at 4 °C, allowed to dry at room temperature and then frozen at −20 °C until further staining. The cortex and hippocampus of the other hemisphere were dissected and stored at −80 °C until protein extraction.

Immunohistochemistry

Slides were allowed to defreeze at room temperature and then rehydrated with phosphate-buffered saline (PBS) for 5 min. Then, brain sections were blocked and permeabilized with PBS containing 1 % bovine serum albumin (BSA, Sigma-Aldrich) and 0.1 % Triton X-100 (Sigma-Aldrich) for 20 min. After two

5-min washes in PBS, the slides were incubated with the primary antibody for Aβ_{40}, Aβ_{42}, (see Table 1) overnight at 4 °C. They were then washed again and incubated for 1 h at room temperature in the dark with Alexa Fluor secondary antibody. After washing again, nuclear staining was performed by incubating slides in Hoechst (H-33258, Fluka, Madrid, Spain) at 2 μg/mL in PBS for 10 min at room temperature in the dark. Finally, slides were washed, mounted using Prolong Gold (Invitrogen) anti-fade medium, allowed to dry overnight at room temperature and stored at 4 °C. Image acquisition was performed with a fluorescence laser microscope (BX41, Olympus, Germany).

Protein extraction

Cortex and hippocampus were micronized through freezing with liquid nitrogen and grinding with a mortar. For total protein extraction, lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, pH 7.4) containing complete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche, Mannheim, Germany), and Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich, St. Louis, MO, USA) were added to micronized tissue and left on ice for 30 min. Then, samples were centrifuged at 10,000×g for 10 min and a supernatant with total protein content was collected. All the protein extraction steps were carried out at 4 °C. Protein concentration was determined by the Bradford protein assay.

Western blot

For Western blot analysis, 20 ug of protein were denatured at 95 °C for 5 min in sample buffer (0.5 M Tris–HCl, pH 6.8, 10 % glycerol, 2 % sodium dodecyl sulfate (SDS), 5 % β-mercaptoethanol, 0.05 % bromophenol blue), separated by SDS-PAGE on 10 % polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4 °C with

Table 1 List of antibodies and dilutions

Antibody (clone)	Catalog reference	Dilution (1:)	Provider
Acetyl-P53 (acetyl-K382)	ab37318	500	Abcam, Cambridge, UK
ADAM-10	ab39177	1,000	Abcam, Cambridge, UK
Beclin-1	ab16998	1,000	Abcam, Cambridge, UK
Cdc2 p34 (17)	sc-54	1,000	Santa Cruz, Santa Cruz, CA, USA
Cdk5 (C-8)	sc-173	1,000	Santa Cruz, Santa Cruz, CA, USA
GSK-3β (27C10)	#9315	1,000	Cell Signaling, Danvers, MA, USA
LC3B	#2775	1,000	Cell Signaling, Danvers, MA, USA
p35/p25 (C64B10)	#2680	1,000	Cell Signaling, Danvers, MA, USA
p53 (1C12)	#2524	1,000	Cell Signaling, Danvers, MA, USA
Phospho-cdc2 (Tyr15)	#9111	1,000	Cell Signaling, Danvers, MA, USA
Phospho-GSK-3β (Ser9)	#9336	1,000	Cell Signaling, Danvers, MA, USA
Phospho-SAPK/JNK (Thr183/Tyr185)	#9251	1,000	Cell Signaling, Danvers, MA, USA
Phospho-Tau (pS396)	44752G	1,000	Invitrogen, Carlsbad, CA, USA
SAPK/JNK	#9252	1,000	Cell Signaling, Danvers, MA, USA
SIRT1 (SIR11)	ab50517	1,000	Abcam, Cambridge, UK
Tau (Tau-5)	AHB0042	1,000	BioSource, Camarillo, CA, USA
β-Actin (AC-15)	A5441	20,000	Sigma-Aldrich, St. Louis, MO, USA
Aβ40	ab10147	50	Abcam, Cambridge, UK
Aβ42 (12F4)	SIG-39142	100	Covance, CA, USA
Alexa Fluor 488 donkey anti-mouse IgG	A-11001	250	Invitrogen, Carlsbad, CA, USA
Alexa Fluor 546 donkey anti-rabbit IgG	A-11035	250	Invitrogen, Carlsbad, CA, USA
Donkey ECL anti-Rabbit IgG, HRP linked	NA934V	1,000	GE Healthcare, UK
Goat Anti-Mouse HRP Conjugate	#170-5047	1,000	Bio-Rad, Hercules, CA, USA

the primary antibodies (see Table 1) diluted with Tris-buffered saline containing 0.1 % Tween 20 (TBS-T) and 5 % BSA. Membranes were then washed and incubated with secondary antibodies (see Table 1) with TBS-T for 1 h at room temperature. Protein bands were visualized using a chemiluminescence detection kit (Amersham Biosciences). Band intensities were quantified by densitometric analysis and values were normalized to β-actin.

Statistical analysis

Results were analyzed statistically by GraphPad Prism software. Kaplan–Meier survival curve comparison was performed with the log-rank (Mantel–Cox) test. The other data are presented as mean±SEM, and means were compared with two-tailed, unpaired Student's *t* test or ANOVA following Tukey's Multiple Comparison Test when necessary. In the object recognition test (ORT) a one-sample *t* test was used to examine whether single columns were different from zero ones. Statistical significance was attained when *P* values were <0.05.

Results

Increase in life expectancy due to resveratrol

The survival curves were plotted using the Kaplan–Meier estimator. A shift to the right for the resveratrol groups revealed an increased expectancy of life for animals that had been eating the resveratrol diet. The comparison of the groups using the Mantel–Cox log-rank test indicated that there was a significant difference between the survival curves of the control group vs. the resveratrol group, not only in SAMP8 mice (Fig. 1a, $P<0.0001$ among groups, Mantel–Cox log-rank test), but also in SAMR1 animals (Fig. 1b, $P<0.01$ among groups, Mantel–Cox log-rank test). In addition, the median life expectancy of our control mice was 10.4 months for SAMP8 mice, significantly lower than the 17.8 months of SAMR1 mice (Fig. 1c) in previous studies (Takeda 2009). However, the SAMP8 resveratrol group showed a life expectancy of approximately 14 months, with an increased life expectancy of more than 33 % over the SAMP8 control mice (Fig. 1c). Furthermore, SAMR1 mice fed with resveratrol also showed a median life span of 21.8 months, 22 % more than SAMR1 control mice (Fig. 1c). In addition, maximum life span is the mean of the final 20 % of mice surviving in each group, as determined by the Kaplan–Meier analysis. In comparison with the control groups, both SAMP8 and SAMR1 animals fed with resveratrol significantly increased their maximum life span (Fig. 1d).

Resveratrol decreases cognitive impairment in SAMP8

We investigated the effects of a 7-month resveratrol food supplement on 9-month-old SAMP8 mice. This is an age when several alterations such as amyloid deposition or cognitive impairment have been reported (Pallas et al. 2008). We found that, in the ORT, control mice had an impaired memory, as their DI was close to or not different from zero (Fig. 2, $P=0.4665$, one-sample *t* test), revealing that there was no preference for the novel object. On the other hand, resveratrol mice had a positive DI different from zero (Fig. 2, $P<0.05$, one-sample *t* test), revealing that their memory was not impaired as they showed greater preference for the novel object than the one already presented. Furthermore, a comparison of the two groups revealed a more protective effect of resveratrol on their memory than in age-matched SAMP8 mice (Fig. 2).

Resveratrol increases both SIRT1 and AMPK levels while it decreases P53 acetylation

Western blot analysis of the cortex and hippocampus of the two groups revealed higher levels of SIRT1 (Fig. 3a, b) in the animals that had been eating a diet supplemented with resveratrol than in animals eating standard food (control group). In accordance with this observation, the substrate of SIRT1, p53, shows a decrease in its acetylation in these brain areas (Fig. 3c, d). In addition, higher levels of phosphorylated AMPK (p-AMPK) were found in the cortex of the resveratrol group (Fig. 3e) while no modifications were seen in the AMPK levels (Fig. 3g). However, while no increment of p-AMPK levels was found in the hippocampus of the resveratrol mice (Fig. 3f), there were higher AMPK basal levels in these animals than in SAMP8 control mice (Fig. 3h).

Resveratrol reduces amyloid deposition and favors the non-amyloidogenic pathway in the hippocampus of SAMP8 mice

Immunohistochemistry was performed on brain sections with specific antibodies directed against the

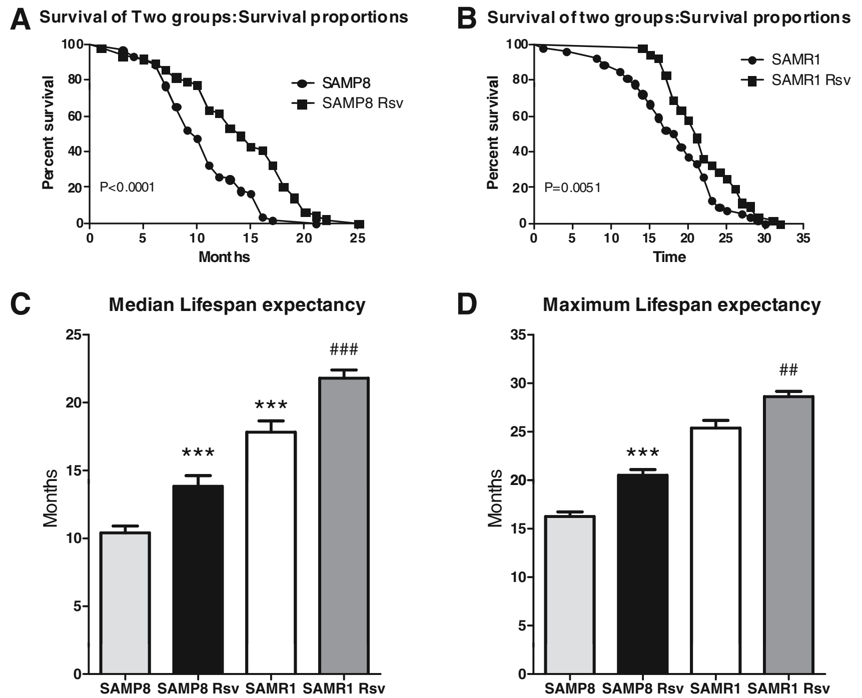

Fig. 1 Kaplan–Meier plot with data expressed as percentage of individuals alive (**a**, **b**) and median life span of the four groups studied (**c**). Mantel–Cox log-rank test analysis reveals a shift to the right for the resveratrol group in SAMP8 (**a**, $P<0.0001$) and SAMR1 (**b**, $P=0.0051$). In the median life span comparison (**c**) and maximum life span comparison considered as the mean of the final 20 % of mice surviving in each group (**d**), results are expressed as mean±SEM; $^{***}P<0.001$ vs. SAMP8, $^{\#\#}P<0.01$ vs. SAMR1, $^{\#\#\#}P<0.001$ vs. SAMR1

$A\beta_{42}$ and $A\beta_{40}$ to assess whether there were differences between the two groups. Visual analysis revealed amyloid clusters limited only to the hippocampal area, as described before (del Valle et al. 2010). Figure 4 shows that almost no Aβ granules were present in the resveratrol group while several clusters of $A\beta_{42}$ and $A\beta_{40}$ granules appeared in the control group (Fig. 4a). Furthermore, we quantified the amount of amyloid clusters that were present in the hippocampus of the two groups. We found that resveratrol decreased the amount of both $A\beta_{42}$ and $A\beta_{40}$ accumulations in SAMP8 animals in comparison with SAMP8 control mice (Fig. 4b, c). In addition, Western blot analysis quantified the levels of two enzymes responsible for the amyloidogenic/non-amyloidogenic processing of APP, the α- (ADAM10) and β- (BACE) secretases. We found that while no alterations were seen in the pro-amyloidogenic BACE enzyme (Fig. 5a, b), an increase in the non-amyloidogenic ADAM-10 enzyme was found in both the cortex (Fig. 5c) and the hippocampus (Fig. 5d) of the resveratrol group.

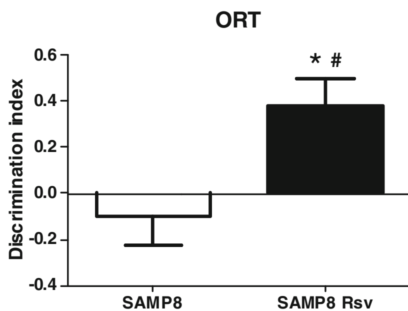

Fig. 2 Discrimination index of both groups of SAMP8 animals. Only Rsv group values are positive and different from zero ($^{*}P<0.05$). There is a higher DI of Rsv animals than of SAMP8 control mice ($^{\#}P<0.05$ vs. SAMP8 mice). *Bars* represent mean ±SEM

Resveratrol lowers tau hyperphosphorylation at serine 396 and has a differential effect on kinases of the cortex and the hippocampus

The levels of phosphorylated tau (pTau) at Ser^{396} have been described as a reliable marker of the severity of AD (Hu et al. 2002). Thus, we evaluated the effect of resveratrol on tau phosphorylation levels in the cortex and hippocampus extracts by Western blot, using a tau antibody that detects only the pTau at Ser^{396}. As can be seen in Fig. 6, not only the cortex but also the hippocampus of animals fed with resveratrol showed lower levels of pTau (Fig. 6a, b). In addition, we investigated the levels of CDK5 and the ratio of its

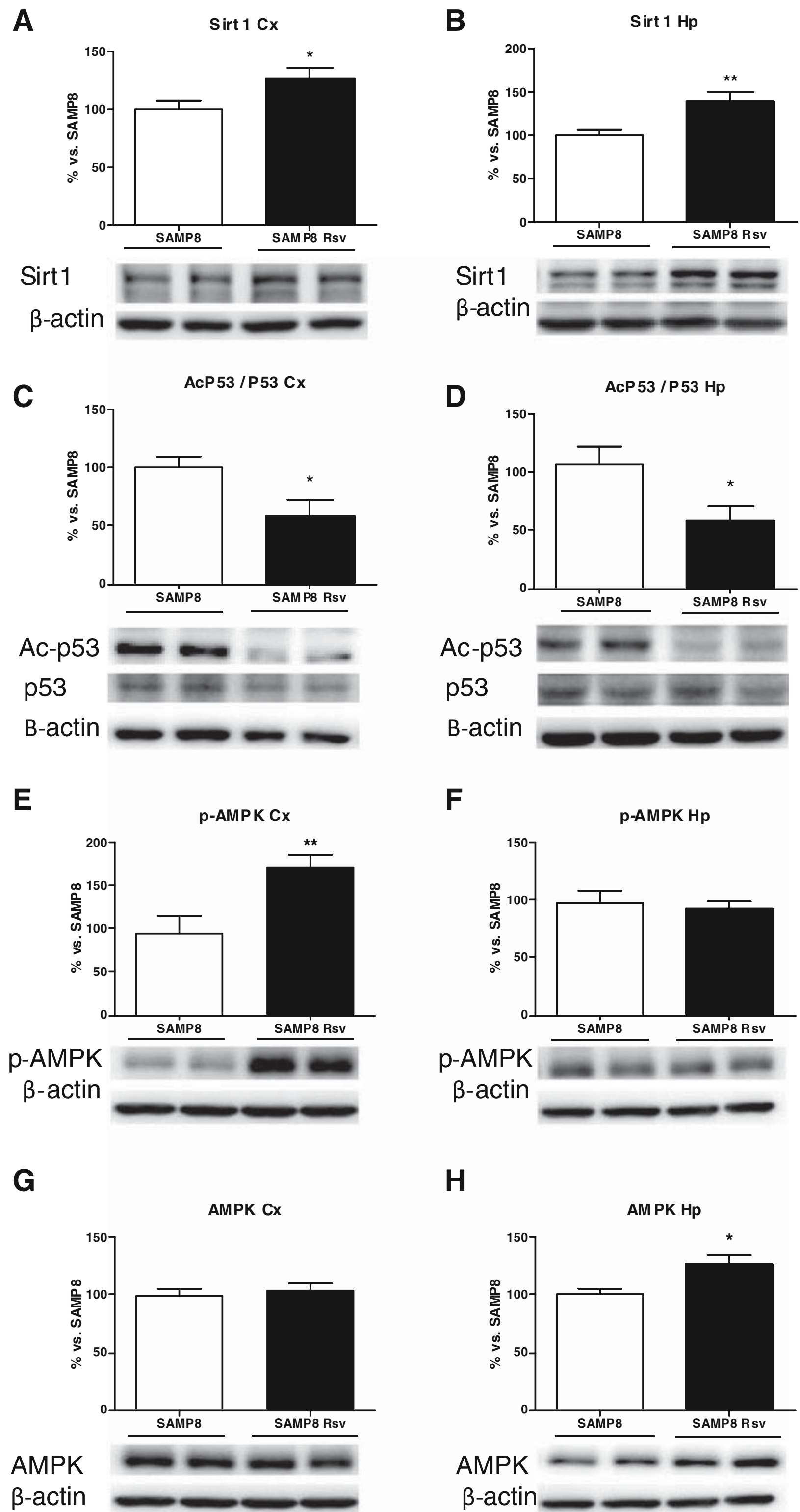

Fig. 3 Levels of sirtuin 1 (**a**, **b**), its acetylated substrate p53 (**c**, **d**), p-AMPK (**e**, **f**), and AMPK (**g**, **h**). *Bars* represent mean±SEM and values are adjusted to 100 % for levels of SAMP8 control mice. Student's paired *t* test; **P*<0.05; ***P* <0.01 vs. SAMP8. Cortex (*Cx*), hippocampus (*Hp*)

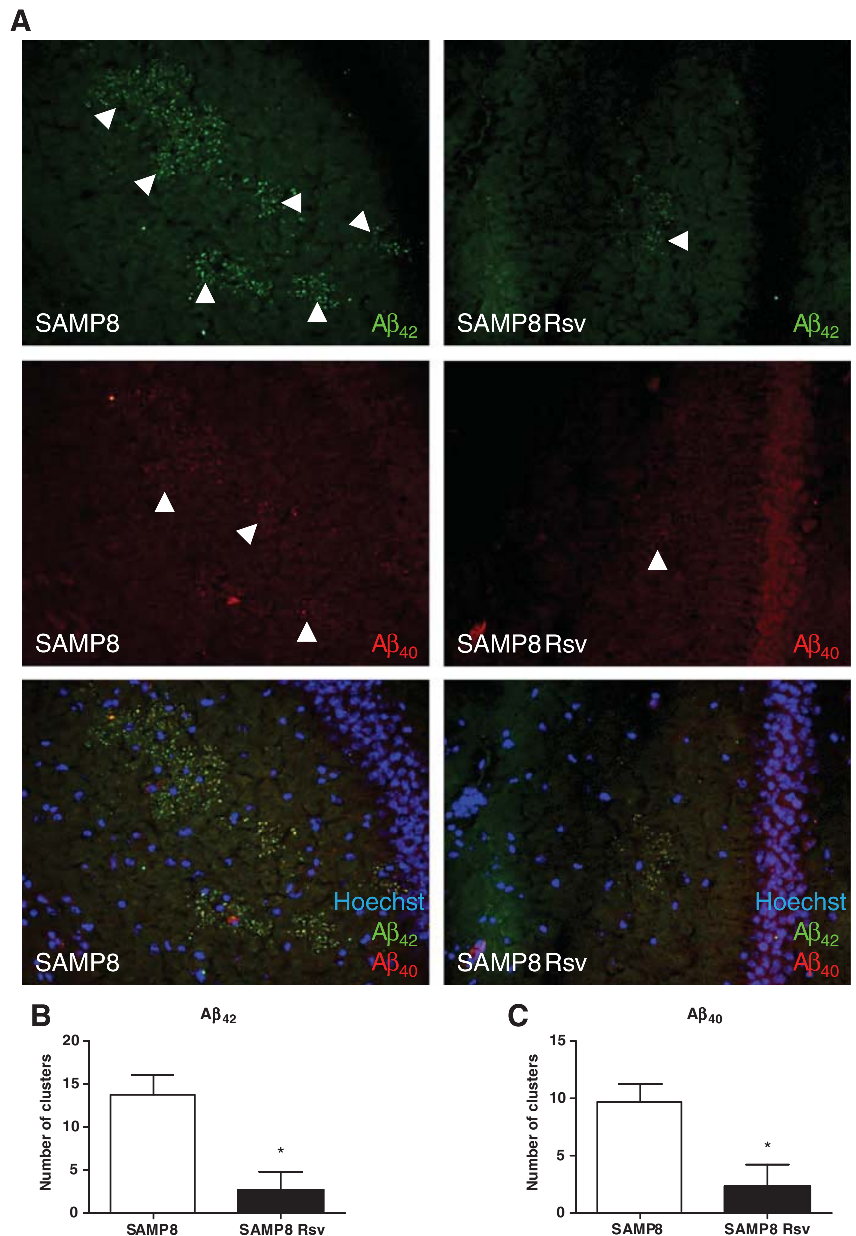

Fig. 4 Representative hippocampal images of SAMP8 and SAMP8 Rsv animals (**a**), *arrowheads* ($A\beta_{40}$ and $A\beta_{42}$) indicate some clusters of amyloid granules in both groups. Quantification of the amount of $A\beta_{42}$ (**b**) and $A\beta_{40}$ (**c**) clusters in the hippocampus of the two groups. *Bars* represent mean±SEM; values in **d**–**g** are adjusted to 100 % for levels of SAMP8 control mice. Student's paired *t* test; $*P<0.05$ vs. SAMP8. Cortex (*Cx*), hippocampus (*Hp*)

activator p25 to the precursor p35, as well as the phosphorylated levels of GSK3β, CDC2, and JNK. A drop in CDK5 protein levels (Fig. 6c), together with a decrease in the p25/p35 ratio (Fig. 6e), revealed inactivation of this kinase in the cortex of resveratrol animals. In addition, an increase in the levels of phosphorylated GSK3β at Ser^9 can be seen (Fig. 7a), which also correlates with the reduced pTau levels, as this enzyme is deactivated when phosphorylated at this residue. However, no modifications were detected in the levels of phosphorylated CDC2 (Fig. 7c) or in the levels of phosphorylated JNK (Fig. 7e). Conversely, there were no changes between resveratrol-treated SAMP8 hippocampus and age-matched SAMP8 control mice in the kinases studied (Figs. 6d, f and 7b, d, f).

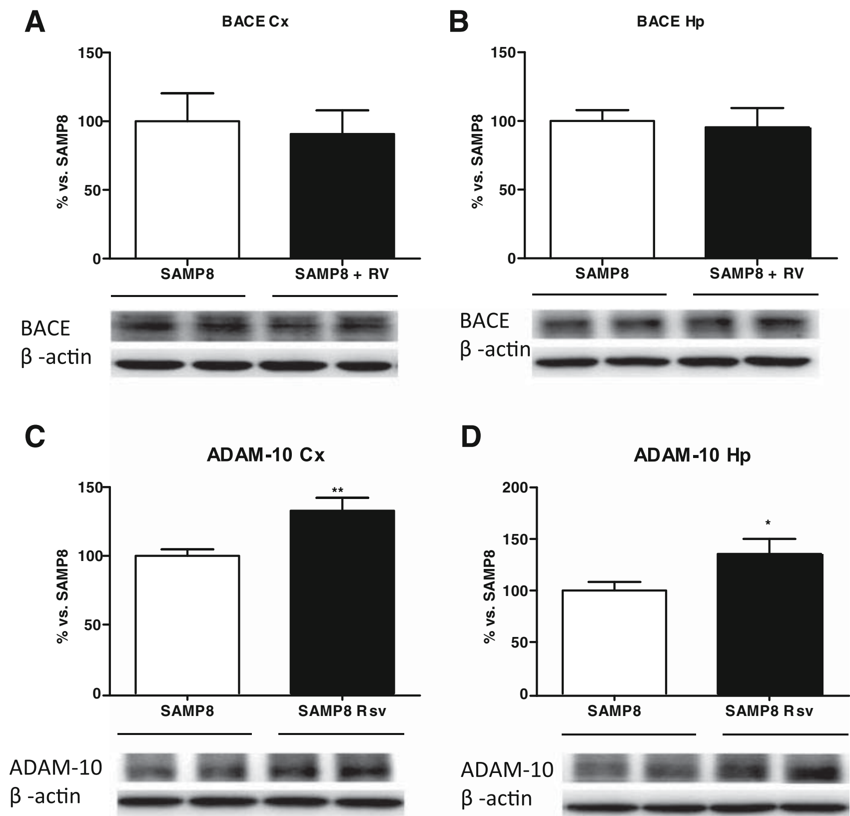

Fig. 5 Cortex and hippocampal levels of BACE (**a**, **b**) and ADAM-10 (**c**, **d**) of SAMP8 and SAMP8 Rsv animals. *Bars* represent mean±SEM; values in **a**–**d** are adjusted to 100 % for levels of SAMP8 control mice. Student's paired *t* test; *$P<0.05$; **$P<0.01$ vs. SAMP8. Cortex (*Cx*), hippocampus (*Hp*)

Discussion

The results reported here confirm the positive effect of resveratrol on extending mean and maximum life span, memory, and neurodegenerative markers in the SAMP8 mice.

It has been reported that SIRT1 activation by resveratrol increases the life span of *Saccharomyces cerevisiae* (Howitz et al. 2003), *Caenorhabditis elegans* (Viswanathan et al. 2005), *Drosophila melanogaster* (Wood et al. 2004), and the short-lived seasonal fish *Nothobranchius furzeri* (Valenzano et al. 2006). However, discrepancies between labs remain unexplained. The influence of factors such as interspecies differences in metabolism, genetic variation, diet, physical activity, disease, and mental health should not be underestimated when extrapolating from rodent models (for a review, see Agarwal & Baur 2011). Then, further experimental evidence is needed to clarify the importance of SIRT1 and other mechanisms in the effects of resveratrol.

Here we demonstrate that resveratrol can extend life span in mice. Resveratrol supplement in the diet resulted in a significant increase in mean life expectancy and in maximum life span, in both SAMP8 and SAMR1. At present, resveratrol was reported to prevent early mortality in mice fed with a high-fat diet (Baur et al. 2006) but failed to affect survival significantly in old mice (Miller et al. 2011). A growth hormone releasing hormone antagonist has been shown to extend SAMP8 mice's median life span (Banks et al. 2010), which was associated with decreased brain oxidative stress. Melatonin has also been reported to increase life span and longevity in SAMR1 and SAMP8 mice (Rodríguez et al. 2008). These authors conclude that the underlying effects of this indoleamine rely on mitochondrial physiology improvement, involving a decrease in reactive oxygen species generation. As old rodents produce more reactive oxygen species than young ones and the rate of mitochondrial reactive oxygen species production is inversely proportional to species' maximum life span, it would be reasonable to expect that an agent that lowered reactive oxygen species might extend life span (Sohal et al. 1989).

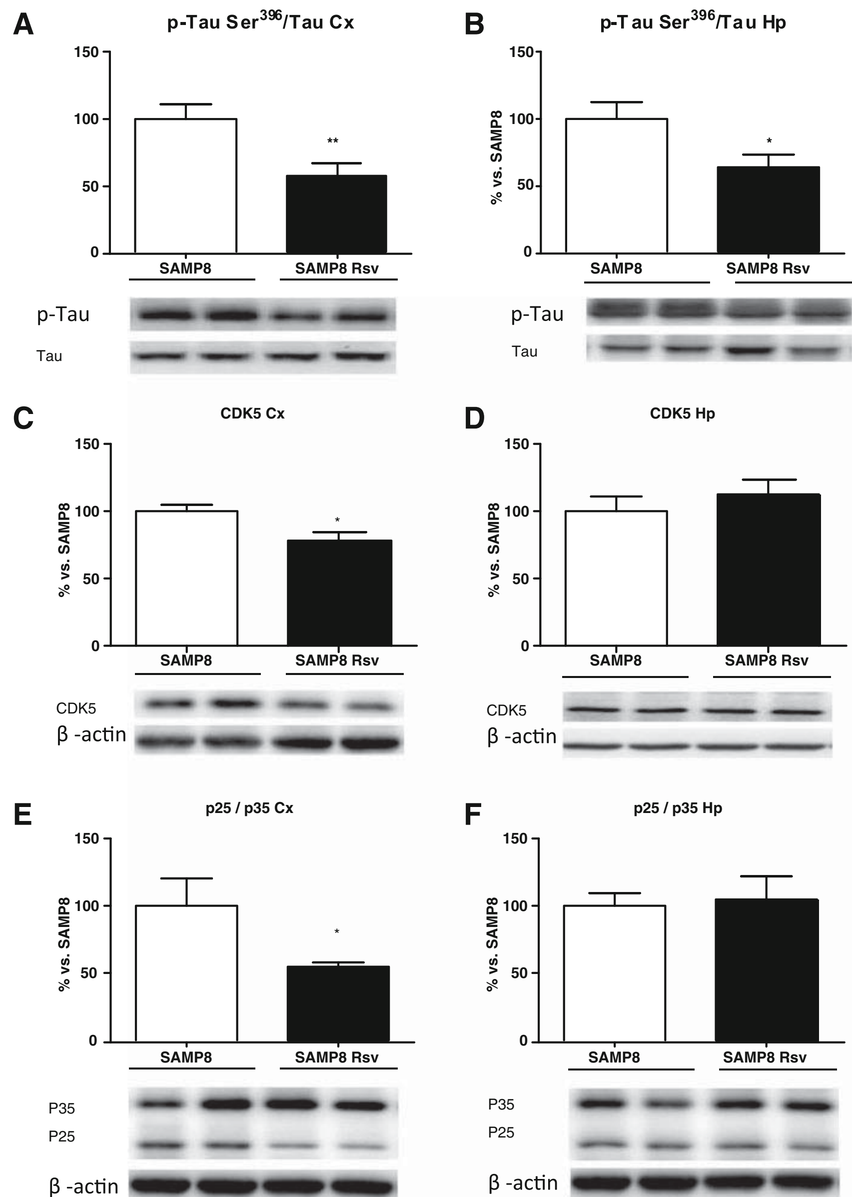

Fig. 6 Levels of phosphorylated tau (*pTau*) at Ser396 in the cortex (**a**) and hippocampus (**b**) of SAMP8 and SAMP8 Rsv groups. Cortex and hippocampal levels of CDK5 (**c**, **d**), P25/P35 ratio (**e**, **f**). Bars represent mean ±SEM and values are adjusted to 100 % for levels of SAMP8 control mice. Student's paired *t* test; *$P<0.05$; **$P<0.01$ vs. SAMP8. Cortex (*Cx*), hippocampus (*Hp*)

Sirtuins are deacetylases that show anti-aging properties in several animal models and can protect from stress (Donmez et al. 2010). SIRT1 plays a role in regulating different cell processes through deacetylation of important substrates such as p53, FOXO transcription factors, PGC-1α, NFκB, and others, which are closely linked to some age-related diseases (Saunders et al. 2010). SIRT1 activation may play an important role in the life-extending effects of CR (Cohen et al. 2004), and it has been postulated that resveratrol mimics the effect of CR. In this study, we demonstrated an increase in SIRT1 levels in SAMP8 treated with resveratrol in the two brain areas studied, which correlated with a diminution in acetylated forms of p53, one of the main substrates of deacetylase. In addition, SIRT1 pathways are closely related to AMPK signaling as a sensor of energy availability. AMPK is activated by phosphorylation of Thr-172 by LKB1 complex in response to an increase in the AMP/ATP ratio and by calmodulin-dependent protein

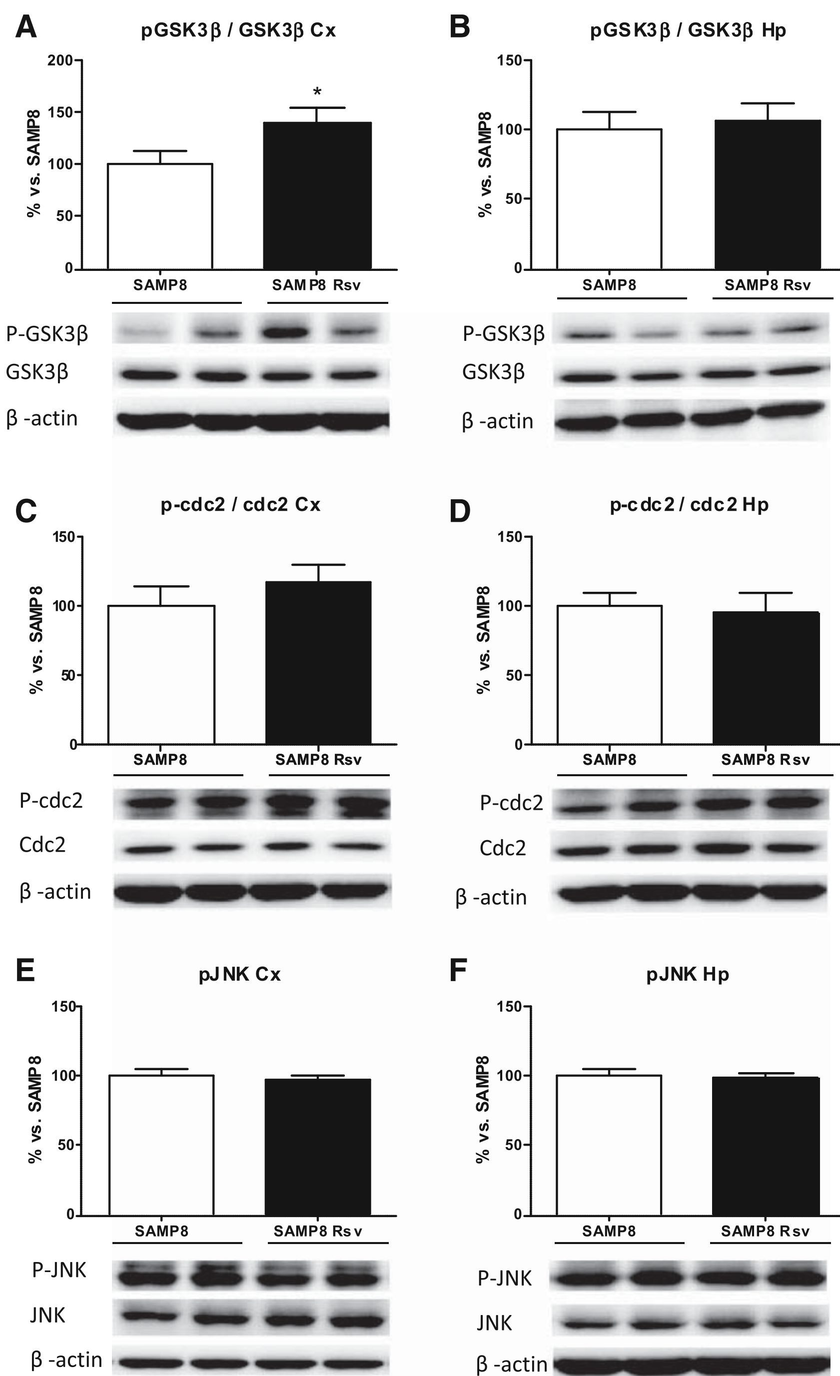

Fig. 7 Cortex and hippocampal levels of p-GSK3ß (phosphorylated in Ser[9]) (**a**, **b**). p-cdc2 (phosphorylated in Tyr[15]) (**c**, **d**) and JNK (phosphorylated in Thr[183]/Tyr[185]) (**e**, **f**). *Bars* represent mean±SEM and values are adjusted to 100 % for levels of SAMP8 control mice. Student's paired *t* test: **P*< 0.05 vs. SAMP8. Cortex (*Cx*), hippocampus (*Hp*)

kinase kinase-beta (CamKKβ) in response to high Ca^{2+} levels, which contributes to regulating Aβ generation. It has been reported that activation of deacetylase and AMPK are linked through LKB and, when SIRT1 is activated, AMPK is phosphorylated and also activated. Moreover, it has been recently demonstrated that resveratrol's effects on SIRT1 activation are mediated via the CamKKβ–AMPK pathway by

inhibition of cAMP-specific phosphodiesterases (Park et al. 2012a, b). Our results showed that resveratrol activation of SIRT1 in SAMP8 mice correlated with changes in the levels or in the phosphorylation of AMPK, demonstrating again that resveratrol modifies the SIRT1 pathway.

Furthermore, a link between SIRT1 activation, AMPK, and AD is increasingly evident (Gan 2007). Tau phosphorylation and β-amyloid production are sensitive to AMPK inhibition (Greco et al. 2011; Park et al. 2012a, b). SIRT1 activation prevents several signs of neurodegeneration (Bayod et al. 2011), protects against axonal degeneration (Araki et al. 2004), reduces polyglutamine toxicity (Parker et al. 2005), and diminishes microglia-mediated Aβ toxicity (Chen et al. 2005). AD and Aβ accumulation are inextricably linked with oxidative damage (Smith et al. 1995). Diet supplements with mulberry (a resveratrol-rich fruit) improved not only memory impairment and decreased Aβ accumulation in SAMP8 but also increased antioxidant capacity via the antioxidant response element (ARE)-Nrf2 pathway in the liver and brain (Shih et al. 2010). Furthermore, resveratrol has been reported to improve memory alterations as it preserved cognitive function in aging mice (Oomen et al. 2009) and in transgenic AD mice (Kim et al. 2007). However, although some conflicting results have been obtained on SAMP8 memory alterations (Spangler et al. 2002), we found memory-related deficits at 9 months of age and that resveratrol was able to revert the memory impairment detected.

Part of the beneficial effects described for SIRT1 on Aβ accumulation is the modulation of α-secretases. Transcription of ADAM10 is positively controlled by retinoic acid receptors (RAR), which are activated by their ligand retinoic acid or through deacetylation by SIRT1. Using SIRT1-transgenic and SIRT1-deficient mice, this protein was found to activate the RARb transcription factor, which in turn increased ADAM10 expression (Lichtenthaler 2011). In addition, SIRT1 activation reduced amyloid pathology in a mouse model of AD, and crossing SIRT1 knockout mice with these mice dramatically increased the Aβ burden (Donmez et al. 2010). Moreover, decreased SIRT1 expression has been found in patients with AD, and this decrease correlates with tau and Aβ levels (Julien et al. 2009). Modulation of ADAM10 expression by SIRT1 has also been demonstrated (Gutierrez-Cuesta et al. 2008; Donmez et al. 2010). In our experimental paradigm, we found that resveratrol reduces the Aβ burden in treated SAMP8 brain concomitantly with increases in ADAM10 expression. This effect can be considered specific because no changes were observed in the expression of other secretases, such as, BACE (Donmez et al. 2010). Thus, resveratrol, through SIRT1 activation, specifically induced the non-amyloidogenic processing of non-mutated APP, reducing the presence of previously described amyloid deposits (del Valle et al. 2010).

Furthermore, tau hyperphosphorylation, another hallmark of AD, is mediated by several kinases in the brain. We and others have demonstrated the aberrant phosphorylation of tau in the brain of SAMP8 that is accomplished by activation of several tau kinases such as CDK5, GSK3β, or JNK (Canudas et al. 2005; Chang et al. 2012). Our data show that in the cortex of SAMP8 mice, a diminution in CDK5 and GSK3β activity, both main tau kinases in AD, is induced by resveratrol treatment, and the inhibition of these tau kinases prevented tau phosphorylation in Ser^{396}.

On the other hand, no clear changes in JNK were found. Conversely, with low doses and only 2 months of treatment with pterostilbene, a resveratrol derivative, JNK inhibition was observed in SAMP8, but no changes in tau hyperphosphorylation (measured through PHF antibody) were observed in the cortex (Chang et al. 2011). All these discrepancies are probably due to the different resveratrol doses and also to the long-term treatment by resveratrol that we applied in the present study.

With regard to the hippocampus, although resveratrol was able to prevent tau phosphorylation, we were unable to find changes in the kinases studied. It is plausible to hypothesize that, although long-term treatment by resveratrol prevents tau hyperphosphorylation, detectable by specific phospho-antibodies, the inhibition of intermediate signals under these conditions is lost because of the chronicity of the treatment. On the other hand, oxidative stress is a well-established pathogenic factor in AD (Smith et al. 1995; Markesbery 1997; Perry et al. 1998), and the association of oxidative stress with tau abnormalities is well-known. As such, the resveratrol-driven reductions on tau phosphorylation in the hippocampus could be mediated by the well-known antioxidant effects of this polyphenol rather than through its inhibitory effect on tau kinases. Therefore, our results allow us to conclude that resveratrol inhibits tau phosphorylation in both the cortex and hippocampus.

Finally, we cannot discard the possibly beneficial antioxidant effect of resveratrol in the parameters

studied here. More studies should be conducted in different AD models in order to clarify the role of resveratrol in SIRT1 and AMPK pro-survival pathways and other oxidative stress routes such as ARE-Nrf2. However, taking everything into account, in this study we demonstrate that resveratrol alone not only increases mean and maximum life span, and favors AMPK pathways and pro-survival routes such as SIRT1 activation, but also has a neuroprotective role, reducing cognitive impairment in AD and other neurodegenerative parameters such as the amyloid burden and tau hyperphosphorylation.

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Publicación 3:

Neuroprotective role of trans-resveratrol in a murine model of familial Alzheimer's disease

Porquet D., Griñan C., Ferrer I., Camins A., Sanfeliu C., Del Valle J. and Pallàs M.

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Resumen

La EA es una enfermedad neurodegenerativa e incurable que produce un deterioro cognitivo severo y se caracteriza por la presencia de dos marcadores histopatológicos: las placas seniles, formadas por la acumulación y agregación del péptido Aβ; y los ovillos neurofibrilares, formados por la proteína tau hiperfosforilada. Los ratones transgénicos APP/PS1 expresan una proteína quimérica ratón/humano de APP que contiene la mutación Sueca (Mo/HuAPP695swe) y una proteína mutante humana PS1-dE9, siendo ambas causantes de la EAf. El resveratrol es un polifenol que activa diferentes vías neuroprotectoras, como la vía de AMPK/SIRT1. El objetivo de este estudio fue estudiar el efecto de una dosis alta y crónica de resveratrol administrada a través de la dieta en ratones APP/PS1. El tratamiento con resveratrol produjo la activación de la vía AMPK/SIRT1, previno la pérdida de memoria, redujo el número de placas seniles e incrementó el complejo IV de la cadena respiratoria mitocondrial. Asimismo, se observó un incremento en la expresión de los genes de IL1β y TNFα, indicando que el resveratrol promueve cambios en los procesos inflamatorios, aunque no se detectaron cambios en las vías relacionadas con el estrés oxidativo.

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Neuroprotective Role of Trans-Resveratrol in a Murine Model of Familial Alzheimer's Disease

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Abstract. The amyloid-β protein precursor/presenilin 1 (AβPP/PS1) mouse model of Alzheimer's disease (AD) has provided robust neuropathological hallmarks of familial AD-like pattern. AD is a neurodegenerative process that causes severe cognitive impairment; it is characterized by the accumulation of amyloid-β (Aβ) and hyperphosphorylated tau forms and by oxidative and inflammatory processes in brain. Currently, efforts are made to understand biochemical pathways because there is no effective therapy for AD. Resveratrol is a polyphenol that induces expression and activation of several neuroprotective pathways involving Sirtuin1 and AMPK. The objective of this work was to assess the effect of oral resveratrol administration on AβPP/PS1 mice. Long-term resveratrol treatment significantly prevented memory loss as measured by the object recognition test. Moreover, resveratrol reduced the amyloid burden and increased mitochondrial complex IV protein levels in mouse brain. These protective effects of resveratrol were mainly mediated by increased activation of Sirtuin 1 and AMPK pathways in mice. However, an increase has been observed in *IL1*β and *TNF* gene expression, indicating that resveratrol promoted changes in inflammatory processes, although no changes were detected in other key actors of the oxidative stress pathway. Taken together, our findings suggest that resveratrol is able to reduce the harmful process that occurs in AβPP/PS1 mouse hippocampus, preventing memory loss.

Keywords: AMPK, inflammation, mitochondria, resveratrol, sirtuin 1

INTRODUCTION

A central issue in cognitive neuroscience of research on aging is pinpointing precise neural mechanisms that determine cognitive outcome in late adulthood, as well as identifying early markers of neurodegeneration and preventive strategies to impede cognitive impairment in aging. Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder associated with age and characterized by senile plaques and neurofibrillary tangles. Senile plaques are formed by the accumulation of amyloid-β $(A\beta)_{1-40}$ and $A\beta_{1-42}$ peptides, which result from the sequential cleavage of amyloid-β protein precursor (AβPP) by β-secretase (BACE) and γ-secretase. The production of Aβ peptides is prevented by alternative cleavage of AβPP by α-secretase followed by γ-secretase. Donmez et al.

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[1] have reported that production of Aβ and plaques in the 3xTg-AD transgenic mouse model of AD is reduced by the overexpression of Sirtuin 1 (SIRT1) and is increased by knocking out SIRT1 in brain. SIRT1 directly activates transcription of the gene encoding α-secretase, the *ADAM10* gene. Additionally, SIRT1 deacetylates and coactivates the Retinoic acid receptor β, a known regulator of *ADAM10* transcription.

It is noteworthy that in AD, mitochondrial function is affected. Reduction of mitochondrial complex IV (MCIV) activity [2, 3] and increased reactive oxygen species production [4, 5] was reported elsewhere. Accumulation of mitochondrial DNA changes might increase reactive oxygen species production and reduce mitochondrial adenine triphosphate (ATP) in an age-dependent manner. Increases of somatic mitochondrial DNA in aging might contribute to AD development [6].

An early-onset form of AD, familial AD (defined for individuals <65 years of age), accounts for 5% of all cases and is directly linked with highly penetrant autosomal dominant mutations in one of three different genes: the presenilin 1 (PS1) gene; the PS2 gene, or the AβPP gene.

Resveratrol, a natural polyphenolic compound found in grapes and red wine, increases metabolic rate, insulin sensitivity, mitochondrial biogenesis, and physical endurance, and also reduces fat accumulation in mice and, at the brain level, is postulated as an option to prevent AD [7–9]. Although it is thought that resveratrol targets SIRT1, this remains controversial because resveratrol also activates 5-Adenosine monophosphate (AMP)-activated protein kinase (AMPK), which also regulates insulin sensitivity and mitochondrial biogenesis. Um et al. [10] demonstrated that the metabolic effects of resveratrol are dependent on AMPK using knockout mice. In addition, resveratrol, during food deprivation (caloric restriction), causes an increase in the cellular AMP/ATP ratio, resulting in the activation of AMPK, which initiates a signaling process that recruits mediators of oxidative metabolism and mitochondrial biogenesis including PGC1-α, the peroxisome proliferator-activated receptor-gamma (PPAR-δ), and others [11].

Double-transgenic AβPP/PS1 mice express a chimeric mouse/human AβPP bearing the Swedish mutation (Mo/Hu AβPP695swe) and a mutant human PS1-dE9, both causative of familial AD. Transgenic mice show senile plaques in cortical and hippocampal areas starting at 4 months [12] and impaired memory and learning performance between 6 and 15 months [13–15]. However, these mice have no alterations in motor function or anxiety-related behavior [16]. Moreover, these animals have increased BACE activity [17], decreased ADAM10 expression [18], and decreased synaptosomal synaptophysin [19]. In reference to inflammatory markers AβPP/PS1 mice present elevaled levels of IL-1β [20] and TNF [21].

The aim of this work was to elucidate the interplays between the amyloidogenic pathway, SIRT1 and AMPK signaling, and resveratrol as a preventative agent in the mouse familial AD model AβPP/PS1. The generation of this knowledge would afford us new clues to confront neurodegenerative disorders with abnormal AβPP processing, such as familial AD, and to help in preventing the processes linked with this illness.

METHODS

Animals and resveratrol feeding

All animal protocols were conducted and approved by the Ethic Committee for Animal Use from the University of Barcelona in accordance with the Generalitat de Catalunya guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize animal numbers and distress.

AβPPswe/PS1dE9 male mice (2 months of age) that were treated and age-matched control littermates were both fed with mouse chow (Harlam Diet) containing 1% resveratrol [22] or the same chow without resveratrol ($n = 10$ in each group) for 10 months. Daily resveratrol consumption was calculated to be 16 mg/kg/day (mean of food intake of 4 g/animal/day).

Novel object recognition test (NORT)

Changes in cognition were tested with an object-recognition task as described elsewhere [23]. Animals were placed in a 90°, two-arm, 25-cm-long, 20-cm-high, and 5-cm-wide black maze. Light intensity in the middle of the field was 30 lux. The objects to be discriminated were made of plastic (object A, 5.25 cm in height, and object B, 4.75 cm in height). For the first 3 days, the mice were individually acclimatized to the apparatus for 10 min. On day 4, the animals were submitted to a 10-min acquisition trial (first trial), during which they were placed in the maze in the presence of two identical novel objects (A+A or B+B), which were localized at the end of each arm. A 10-min retention trial (second trial) occurred 2 h later. During this second trial, objects A and B were placed in the maze, and the time that the animal explored the new object (t_n) and the old object (t_o) were recorded. A Discrim-

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143 ination index (DI) was defined as $(t_n–t_o)/(t_n+t_o)$. In
144 order to avoid object preference biases, objects A and
145 B were counterbalanced so that one half of the ani-
146 mals in each experimental group were first exposed to
147 object A and then to object B, whereas the remaining
148 one half first saw object B and then object A. The maze
149 and the objects were cleaned with 96° ethanol between
150 experiments to eliminate olfactory cues.

151 *Brain processing*

152 Animals were anesthetized with 80 mg/kg of sodium
153 pentobarbital and intracardially perfused with saline
154 serum. Afterward, brains were dissected and separated
155 sagitally into two hemispheres: one for histological
156 staining, and the other, for protein and RNA extraction.
157 Hemispheres for histological staining were frozen by
158 immersion in isopentane, chilled on dry ice, and stored
159 at –80°C until sectioning. Thereafter, the frozen brains
160 were embedded in Optimal cutting temperature (OCT)
161 cryostat-embedding compound (Tissue-Tek; Torrance,
162 CA, USA), cut into 20-μm-thick sections on a cryostat
163 (Leyca Microsystems, Germany) at –18°C, and placed
164 on slides. Slides containing brain sections were fixed
165 with acetone for 10 min at 4°C, allowed to dry at room
166 temperature, and then frozen at –20°C until further
167 staining. The remaining hemispheres were dissected
168 and stored at –80°C until protein or RNA extraction.

169 *Thioflavin S staining*

170 Slides were allowed to defrost at room tempera-
171 ture and then were rehydrated with Phosphate-buffered
172 saline (PBS) for 5 min. Later, the brain sections were
173 incubated with 0.3% Thioflavin S (Sigma-Aldrich) for
174 20 min at room temperature in the dark. Subsequently,
175 these were submitted to washes in 3-min series, specif-
176 ically with 80% ethanol (2 washes), 90% ethanol (1
177 wash), and 3 washes with PBS. Finally, the slides were
178 mounted using Fluoromount (EMS), allowed to dry
179 overnight at room temperature in the dark, and stored
180 at 4°C. Image acquisition was performed with an epi-
181 fluorescence microscope (BX41; Olympus, Germany).
182 For plaque quantification, similar and comparable his-
183 tological areas were selected, focusing on having the
184 hippocampus and the whole cortical area positioned
185 adjacently.

186 *Protein extraction*

187 Brains were micronized by freezing with liquid
188 nitrogen and grinding with a mortar. For total pro-
189 tein extraction, lysis buffer (50 mM Tris-HCl, 150 mM
190 NaCl, 5 mM EDTA, 1% Triton X-100, pH 7.4), EDTA-
191 free Protease inhibitor cocktail (Roche, Mannheim,
192 Germany), and Phosphatase inhibitor cocktail 1
193 (Sigma-Aldrich, St. Louis, MO, USA) were added to
194 micronized tissue and left on ice for 30 min. Then, the
195 samples were centrifuged at 10,000× *g* for 10 min and
196 a supernatant with total protein content was collected.
197 All of the protein extraction steps were carried out
198 at 4°C. Protein concentration was determined by the
199 Bradford protein assay.

200 *Western blot*

201 For western blot analysis, 20 μg of protein were
202 denatured at 95°C for 5 min in sample buffer (0.5 M
203 Tris-HCl, pH 6.8, 10% glycerol, 2% Sodium dodecyl
204 sulfate [SDS], 5% β-mercaptoethanol, 0.05% bro-
205 mophenol blue), separated by Sodium dodecyl sulfate-
206 Polyacrylamide gel electrophoresis (SDS-PAGE) onto
207 8–12% polyacrylamide gels and transferred onto
208 Immobilon polyvinylidene difluoride membranes
209 (Millipore, Billerica, MA, USA). The membranes
210 were incubated overnight at 4°C with the following
211 primary antibodies: anti-β-actin (Sigma; 1:10,000);
212 anti-Sirt1 (Abcam, 1:1,000); anti-p53 (Abcam, 1:500);
213 anti-acetyl p53 (L382) (Millipore, 1:500); anti-AMPK
214 (Cell Signaling, 1:1,000); anti-pAMPK (Cell Signal-
215 ing, 1:1,000); OXPHOS cocktail and porin (1:500;
216 MitoSciences); catalase (1:2,000; Calbiochem), and
217 superoxide dismutase (SOD) (1:3000; Calbiochem)
218 diluted with Tris-buffered saline containing 0.1%
219 Tween 20 (TBS-T) and 5% Bovine serum albumin
220 (BSA). Membranes were then washed and incubated
221 with secondary antibodies and diluted with TBS-T for
222 1 h at room temperature. Protein bands were visual-
223 ized using a chemiluminescent horseradish peroxidase
224 substrate (Millipore) and ChemiDoc XRS + (Bio-
225 rad). Band intensities were quantified by densitometric
226 analysis using Image Lab software and values were
227 normalized to β-actin.

228 *RNA extraction and gene expression determination*

229 Total RNA isolation was carried out by means of Tri-
230 zol reagent following the manufacturer's instructions.
231 RNA content in the samples was measured at 260 nm,
232 and the purity of the samples was determined by the
233 A260/280 ratio in a NanoDrop ND-1000 (Thermo Sci-
234 entific). Samples were also tested in a Bioanalyzer
235 2100B (Agilent Technologies) to determine the RNA
236 integrity number.

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237 Reverse transcription-polymerase chain reaction (RT-PCR) was performed as follows: 2 μg of messenger RNA (mRNA) was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Then, qPCR was performed using TaqMan gene expression assays (Applied Biosystems), doing triplicates for each gene and cDNA sample in 96-well optical plates. The TaqMan probes used were Interleukin-1β *(Il1b)* (Mm00434228_m1), Interleukin-6 *(Il6)* (Mm00446190_m1), Tumor necrosis factor *(Tnf)* (Mm00443260_g1), Nuclear factor erythroid 2-related factor 2 *(Nfe2l2)* (Mm00477784_m1), Heme oxygenase-1 *(Hmox)* (Mm00516005_m1), and Jun *(Jun)* (Mm00495062), with TATA-binding protein *(Tbp)* (Mm00446971_m1) as housekeeping. For each 20 μl of TaqMan reaction, 9 μl cDNA (25 ng) was mixed with 1 μL 20x probe of TaqMan Gene Expression Assays and 10 μl of 2x TaqMan Universal PCR Master Mix. The reactions were carried out using the following parameters: 50°C for 2 min; 95°C for 10 min, 40 cycles at 95°C for 15 s, and at 60°C for 1 min using the StepOnePlus Real-Time PCR System (Applied Biosystems). Finally, all TaqMan PCR data were normalized to TBP using the delta-delta Ct method.

262 *Statistical analysis*

263 Results were analyzed statistically by GraphPad PRISM (GraphPad Software, Inc.) software. Data are presented as mean ± Standard error of the mean (SEM), and means were compared with two-tailed, unpaired Student *t*-test. In the Object recognition test (ORT), a one-sample *t*-test was used to examine whether single columns were different from zero. Statistical significance was reached when *p* values were <0.05. All the experiments and its statistics were conducted with 7 to 10 animals for each experimental group.

274 **RESULTS**

275 *Resveratrol ameliorated short-term memory in AβPP/PS1 mice*

277 To date, it has been found that transgenic mice that overexpress AβPP/PS1 show loss of memory [13–15]. ORT demonstrated that at 12 months, AβPP/PS1 mice fed with resveratrol chow exhibited significantly improved memory capabilities (Fig. 1A) in comparison to AβPP/PS1 control mice.

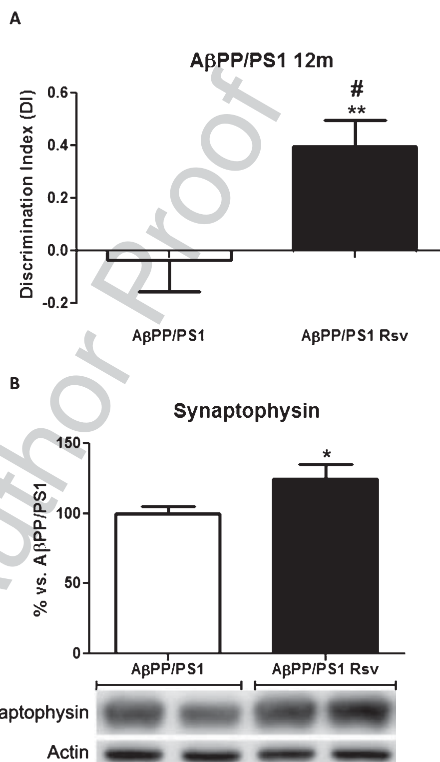

Fig. 1. A) Object recognition test analysis for AβPP/PS control and resveratrol-fed mice. Bars represent discrimination index, mean ± Standard error of the mean (SEM). One sample *t*-test $^{**}p < 0.01$ from zero. Student *t*-test versus control; $^{\#}p < 0.05$. B) Western blot analysis for synaptophysin in AβPP/PS1 control and resveratrol-fed mice. Results are represented as mean ± SEM. Student *t*-test versus control; $^{*}p < 0.05$.

283 The previously mentioned beneficial effects of resveratrol were supported by an increase in synaptophysin, a presynaptic protein that reflects an improvement in synaptic activity in resveratrol-fed mice (Fig. 1B).

288 *Resveratrol treatment reduced plaque pathology but did not alter AβPP and its carboxy terminal fragments (CTF) in AβPP/PS1 mice*

291 To test the effect of resveratrol on amyloid plaque pathology, thioflavin S staining was used. Resveratrol markedly reduced thioflavin S-positive compact plaques compared with control mice (Fig. 2). Quantification of plaques revealed a significant reduction in plaque counts and plaque burden in hippocampus and

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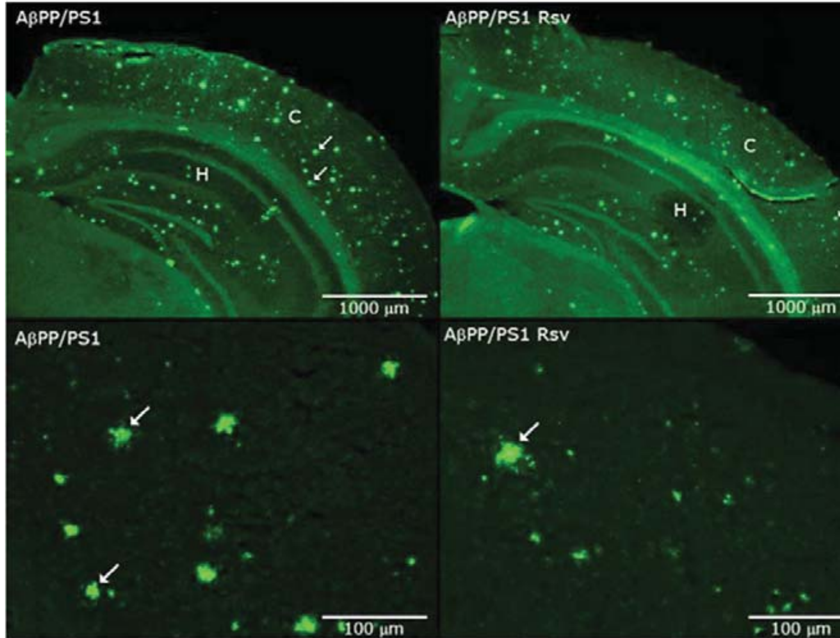
A



B

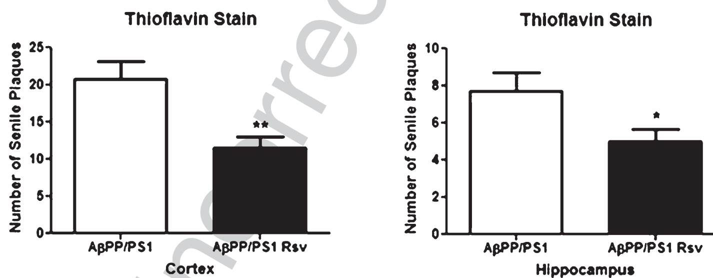


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Fig. 2. A) Thioflavin S staining of Aβ plaques in mouse brains. Representative images of histopathological brain state of AβPP/PS1 control and resveratrol-fed mice. White arrows are representative indicators of the presence of Aβ plaques in the studied areas, cortex (C), and hippocampus (H). Bar chart showing quantification of the number of amyloid plaques in the cerebral cortex (B) and hippocampus (C) of AβPP/PS1 control and resveratrol-treated animals. For quantification parameters, see Materials and Methods. Bars represent mean ± Standard error of the mean (SEM). Student *t*-test $*p < 0.05$; $**p < 0.01$ versus control.

297 medial cortex of resveratrol-fed mice compared with
298 controls (Fig. 2A-C).
299 To investigate the mechanism responsible for
300 the resveratrol-induced reduction in plaque counts,
301 enzymes that mediate AβPP cleavage were deter-
302 mined. We found no changes in PS1, but a significant
303 reduction in BACE and ADAM 10 protein levels were
304 observed (Fig. 3A–C).
305 Moreover, full-length AβPP and AβPP cleavage
306 products β CTF (C99) and α CTF (C83) levels were
307 determined by western blot using AβPP G369 anti-
308 body (against the AβPP cytoplasmic tail). Resveratrol

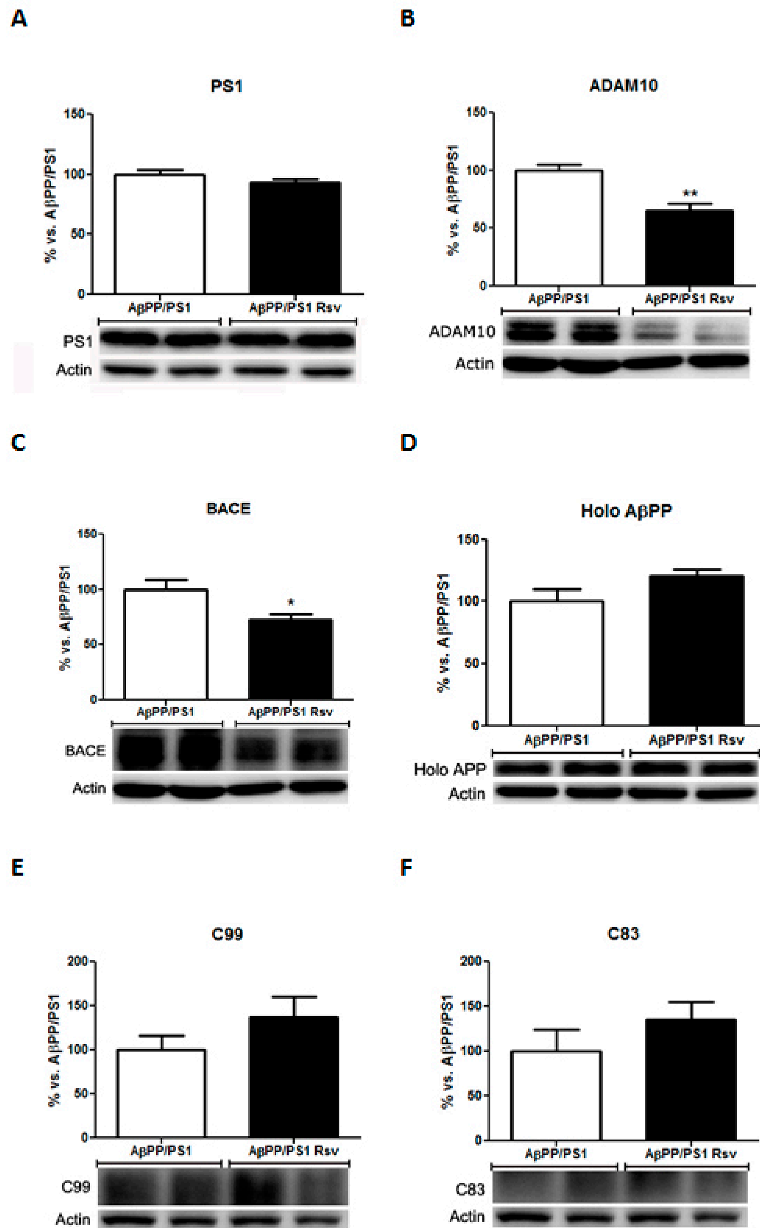

Fig. 3. Levels of presenilin 1 (PS1) (A), ADAM10 (B), β-secretase (BACE) (C), AβPP (D), C99 (E), and C83 (F) in AβPP/PS1 control and resveratrol-fed mice. Bars represent mean ± Standard error of the mean (SEM). Student *t*-test: *$p < 0.05$; **$p < 0.01$ versus control.

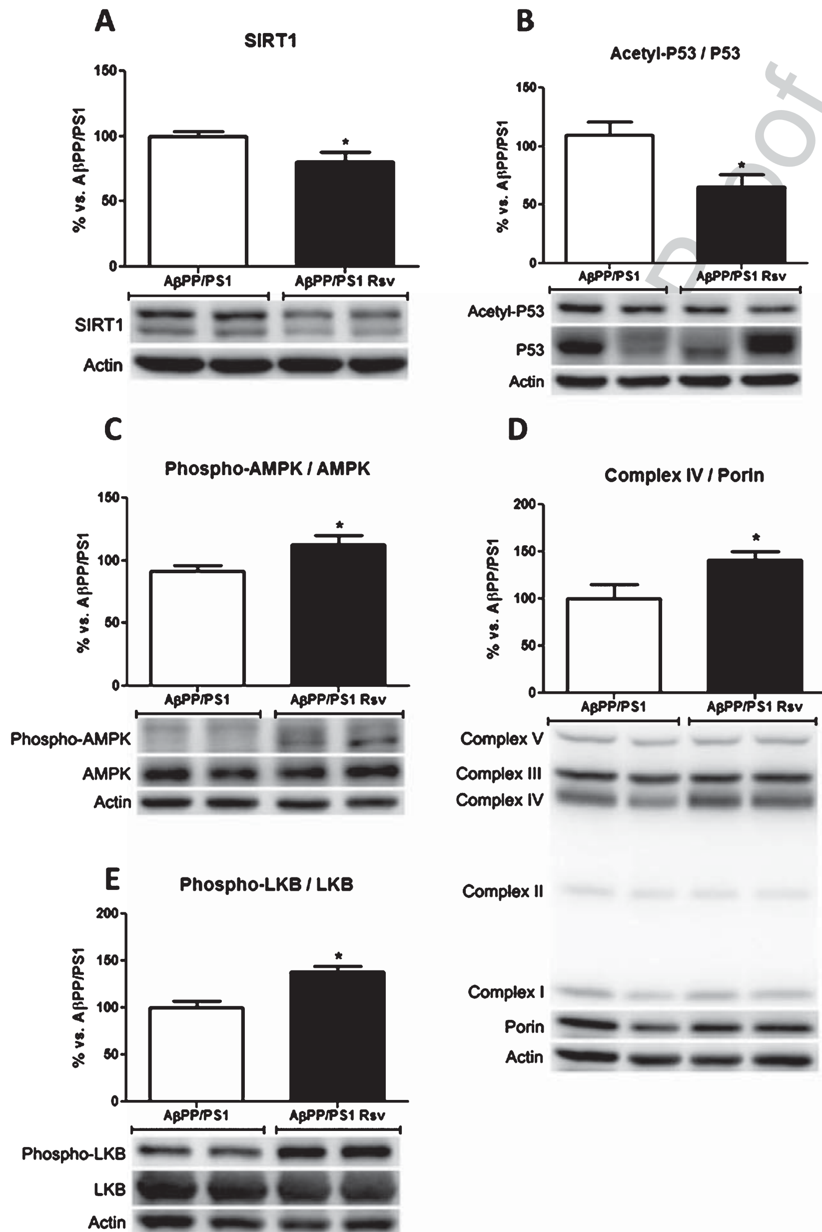

Fig. 4. Western blot analysis for Sirtuin 1 (SIRT1) (A), acetylated-p53 (B), phospho-5-Adenosine monophosphate (AMP)-activated protein kinase (p-AMPK) (C), mitochondrial complex IV (MCIV) (D), and serine–threonine kinase liver kinase B (LKB) (E). Bars represent mean ± Standard error of the mean (SEM); Student *t*-test: $^{*}p < 0.05$ versus control.

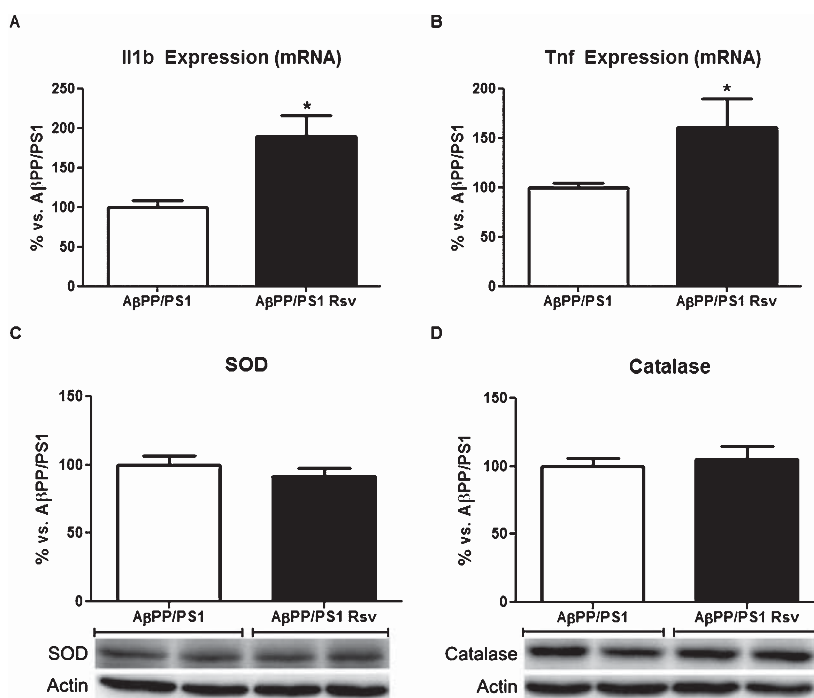

Fig. 5. Increases in gene expression for Interleukin-1β (IL1β) (A) and tumor necrosis factor (TNF) (B). Western blot analysis for protein levels of superoxide dismutase (SOD) and catalase (CAT) (C, D). Bars represent mean ± Standard error of the mean (SEM); Student *t*-test: $*p < 0.05$ versus control.

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309 did not alter levels of high-molecular-weight AβPP
310 (holo AβPP) (Fig. 3D), β CTF (C99) (Fig. 3E), or α
311 CTF (C83) (Fig. 3F).

312 *Resveratrol treatment increases the*
313 *phospho-AMPK (p-AMPK)/LKB pathway in*
314 *AβPP/PS1 although SIRT1 levels are decreased*

315 Because previous studies demonstrated that resver-
316 atrol activates the nicotinamide adenine dinucleotide
317 (NAD)-dependent protein deacetylase SIRT1, we
318 tested whether the SIRT1 protein level was upregulated
319 in AβPP/PS1 mice fed with resveratrol. As demon-
320 strated in Fig. 4, resveratrol treatment did not increase
321 SIRT1, but a significant diminution in acetyl-p53, one
322 of the main substrates of SIRT1, was observed (Fig. 4A,
323 B). Moreover, p-AMPK and also p-LKB were signif-
324 icantly increased in mice fed resveratrol (Fig. 4C–E),
325 indicating modulation by this polyphenol on phospho-
326 rylation, thus on the activity of these two kinases. In
327 reference to mitochondria and according to stimulation
328 of AMPK, an increase in MCIV expression, but not
329 in other complexes, was determined after resveratrol
330 treatment (Fig. 4D).

331 *Effect of resveratrol treatment on cytokine gene*
332 *expression and catalase/superoxide dismutase*
333 *(CAT/SOD) protein levels in hippocampus*

334 To test the effect of resveratrol on inflammation
335 and oxidative stress, different markers for these were
336 determined. Resveratrol feeding increased Il1b and Tnf
337 mRNA expression (Fig. 5A, B). In contrast, no changes
338 were determined in Il6, Nfe2l2, Hmox, and Jun expres-
339 sion (data not shown). Protein levels of CAT and SOD
340 were also unchanged (Fig. 5C, D).

341 DISCUSSION

342 AβPP/PS1 mice express a chimeric mouse/human
343 amyloid-β protein precursor (Mo/Hu AβPP695swe)
344 and a mutant human presenilin 1 (PS1-dE9) that lead to

altered AβPP and PS1 proteins. Both cause Aβ plaques from 3 months onward, and they increase in number and distribution with disease progression in parallel with increased levels of brain-soluble $A\beta_{1-42}$ and $A\beta_{1-40}$, but also with a reduced $A\beta_{1-42/1-40}$ ratio with age. Amyloid deposition in plaques is accompanied by altered mitochondria and increased oxidative damage, post-translational modifications, and the accumulation of altered proteins at the dystrophic neurites surrounding plaques [24]. This model is taken as a familial AD murine model. Resveratrol has been described as a natural compound with pleiotropic neuroprotective activities, linked with antioxidant properties and the modification of activity of different enzymatic pathways, such as the SIRT1 pathway, AMPK activation, or cellular protein degradation machinery [25].

AβPP/PS1 mice fed with resveratrol showed a significant decrease in the number and intensity of amyloid plaques, when were measured by specific S-thioflavine staining. Reduction in plaque pathology did not appear to be due to altered AβPP processing toward the non-amyloidogenic pathway, because high-molecular-weight AβPP (holo AβPP), C99 (Carboxy terminal fragment beta, CTFβ), and C83 (Carboxy terminal fragment alpha, CTFα) were unaltered; this is in agreement with a study conducted by Karuppagounder and coworkers [26] on Tg19959 fed with resveratrol. However, the significant decrease in BACE levels would ultimately have altered the amyloidogenic pathway, leading to a reduction in amyloid burden found in AβPP/PS1 fed for a lengthy period with resveratrol. Evidence is compelling that a decrease in proteasome activity occurs in AD brains [27, 28] and in AD murine models, including AβPP/PS1, a familial AD model [24, 29], and *in vitro*, resveratrol promotes Aβ clearance by increasing intracellular proteasomal activity without affecting Aβ- producing enzyme activities (β-secretase and γ-secretase) [30]. This mechanism on the proteasomal system exerted by resveratrol could be synergistic to BACE reduction, explaining the decrease in plaques observed with resveratrol treatment, albeit than a decrease in ADAM10 was also determined.

Improvement of short-term memory in these mice was evaluated by the novel object recognition test (NORT), a test widely used to evaluate learning and memory [24, 31]. The brain areas involved in this test are mainly hippocampus and perirhinal cortex. Moreover, NORT requires no external motivation, reward or punishment, thus avoiding the anxyogenic role in cognition impairment, although AβPP/PS1 mice do not present anxiety behavior [32, 33]. Synaptophysin, an integral membrane glycoprotein present in presynaptic vesicles, was used to quantify synapse number. In our case, AβPP/PS1 mice resveratrol fed increased synaptophysin levels, correlating with short-term memory improvement and reduction in amyloid plaques.

Several reports suggest that resveratrol possesses antioxidant properties [34]; thus, a tantalizing alternative speculation is that resveratrol-induced reduction in plaques may be occurring through its effect on the cellular antioxidant machinery. We measured protein levels of CAT and SOD, but there were no changes observed. In relation to the expression of several genes related with oxidative stress and inflammation, there were no changes found in the *Nfe2l2* and *Hmox1* genes, which are implicated in neuroprotection mechanisms [35, 36], whereas cytokines linked with inflammation processes, such as IL1β and TNFα, were increased. In AD, abnormal accumulation of Aβ is thought to be intimately linked with the immune system [37]. The majority of efforts have been carried out in order to understand the role of activated astroglia and microglia, which secrete cytokines that act in favor of phagocytosis and AβPP and Aβ peptide clearance, giving rise to the hypothesis that slight increases in cytokines will be protective by removing Aβ and reducing the plaque burden in brain. Our results indicate that at this time of treatment, there is no effective action of resveratrol on the inflammation and oxidative machinery of AβPP/PS1 mice; the antioxidant effect could have occurred at an earlier treatment time, as described for another AD murine model by Karuppagounder and coworkers [26]. However, long-term resveratrol treatment allows fine control of antioxidant machinery by increasing IL1β and TNFα, leading to a hormetic process that ultimately yielded a reduction in plaque number and the beneficial effect on memory, as we observed clearly in this familal AD mouse model at a late age. This fact cannot rule out the possibility that resveratrol exerts an earlier antioxidant effect at younger ages, acting as a preventive more than as a curative agent.

In addition, resveratrol has been postulated to have its beneficial effects on the lifespan, neurodegeneration, and memory improvement by activation of SIRT1 [38, 39]. Because SIRT1 activation is one of the main targets defined for the pharmacological effects of resveratrol, the levels of this deacetylase were determined. Here we observed a diminution in SIRT1 protein levels, but a decrease in acetyl-p53 was determined concomitantly. These results are in agreement

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with the literature in other transgenic models and in *in vitro* experiments where a reduction in plaque pathology without increase in sirtuin levels [26] but decrease in PGC1α acetylation was found [39]. The decline in SIRT1, together with p53 deacetylation status, could reflect decreased synthesis of this deacetylase, but increased activity mediated by resveratrol [40–42] or by crossregulation with the AMPK/LKB pathway [34, 43, 44].

Cantó and Auwerx [45] demonstrated that activation of AMPK stimulated the functional activity of SIRT1 by increasing the intracellular concentration of NAD+. Interestingly, SIRT1 was able to deacetylate LKB1 kinase, which subsequently increased its activity [46]. Because LKB1 is an upstream activator of AMPK, this signaling pathway stimulates the activation of AMPK. This positive feedback loop between SIRT1 and AMPK can also potentiate the function of the other AMPK-activated signaling pathways [47]. In the current study, the surprising resveratrol-induced decline in SIRT1 levels is consistent with AMPK/LKB activation.

The AMPK and SIRT1 signaling pathways are highly conserved energy sensors of increased levels of AMP and NAD+, respectively, and AMPK signaling is involved in the regulation of energy metabolic homeostasis [48]. In line with this, resveratrol also normalized mitochondrial function and enhanced mitochondrial biogenesis in the spinal cord of $SOD1^{G93A}$ amyotrophic lateral sclerosis mice [49]. The majority of the pathways mentioned previously are involved in mitochondrial function, and in this particular organelle, the AβPP/PS1 life-long treatment with resveratrol induced an increase in complex IV. MCIV is considered a marker of mitochondrial functionality [4, 5], which in turn is known to be decreased in AD. In our study, resveratrol increased MCIV, because this action can be taken in account for delineating the neuroprotective role of resveratrol in AβPP/PS1 mice.

In conclusion, this study supports some unexplored pathways responsible for the neuroprotective effects of resveratrol in AβPP/PS1 mice, namely, the equilibrium among SIRT1 and AMPK signaling, mitochondrial status, and inflammatory changes. In addition to other reports [26, 50], our findings indicate that the onset of this neurodegenerative disease may be delayed or mitigated employing dietary resveratrol, which is able to protect against Aβ plaque formation and cognitive loss. Further studies need to be conducted to elucidate the precise regulatory mechanisms that can be modulated by resveratrol in the AβPP/PS1 mouse model.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=2320).

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Publicación 4:

Amyloid and tau pathology of a familial Alzheimer's disease APP/PS1 mouse model in a senescence phenotype background (SAMP8)

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Pendiente de publicación

Resumen

El modelo murino de EA APP/PS1 presenta marcadores neuropatológicos de esta enfermedad a edades tempranas, mientras que el modelo de senescencia acelerada SAMP8 tiene un fenotipo de envejecimiento con características similares a la EA. El objetivo de este estudio fue la caracterización del estado cognitivo y de los marcadores neuropatológicos de la EA en un nuevo modelo que combina las características de los ratones transgénicos APP/PS1 con un fondo génico de senescencia acelerada proveniente de los ratones SAMP8. Los resultados muestran que este nuevo modelo presenta alteraciones en las vías estudiadas, demostrando que el fenotipo de senescencia acelerada exacerba la patología amiloide y tau a edades tardías manteniendo los déficits cognitivos presentes en APP/PS1 y SAMP8.

Amyloid and tau pathology of a familial Alzheimer disease APP/PS1 mouse model in a senescence phenotype background (SAMP8)

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ABSTRACT

The Amyloid precursor protein/Presenilin 1 (APP/PS1) mouse model of Alzheimer's disease (AD) has provided robust neuropathological hallmarks of familial AD-like pattern at early ages, whereas Senescence-accelerated mouse prone 8 (SAMP8) has a remarkable early senescence phenotype with pathological similarities to AD. The aim of this study was the investigation and characterization of cognitive and neuropathological AD markers in a novel mouse model that combines the characteristics of the APP/PS1 transgenic mouse model with a senescence-accelerated background of SAMP8 mice. Initially, significant differences were found regarding amyloid plaque formation and cognitive abnormalities. Bearing these facts in mind, we determined a general characterization of the main AD brain molecular markers, such as alterations in amyloid pathway, neuroinflammation, and hyperphosphorylation of tau in these mice along their lifetimes. Results from this analysis revealed that APP/PS1 in SAMP8-background mice showed alterations in the pathways studied in comparison with SAMP8 and APP/PS1, demonstrating that a senescence-accelerated background exacerbated amyloid pathology and maintained the cognitive dysfunction present in APP/PS1 mice. Changes in tau pathology, including activity of CDK5 and GSK3β, differs, but not in a parallel manner, with amyloid disturbances.

KEYWORDS: familial Alzheimer's disease (fAD), aging, β-amyloid, tau hyperphosphorylation, cognitive impairment.

1. Introduction

Aging-associated processes, neurodegenerative diseases the most prevalent of these, results mainly from the deleterious effects of time on cellular mechanisms (Agarwal et al., 2003) (Smith et al., 1996). The main neurodegenerative diseases are Alzheimer's (AD), Parkinson's (PD), Huntington's (HD), and Amyotrophic lateral sclerosis (ALS) (Zhu et al., 2007, 2004), being AD the most prevalent and one of the most studied of these and characterized by mild cognitive impairment, deficits in short-term memory, spatial memory loss, and subtle emotional imbalances (Enserink, 1998), and ultimately causing death (Pimplikar, 2009).

With regard to histopathology, changes in AD brains include neuronal and synaptic loss and the appearance of two basic hallmarks: Senile plaque (SP) formation by accumulation of extracellular of β-amyloid protein (Aβ) (Braak and Braak, 1998, 1991; Glenner and Wong, 1984), and intracellular neurofibrillary tangles (NFT) formation by accumulation of hyperphosphorylated tau protein (Bancher et al., 1989; Braak et al., 1995).

Over time, AD could be divided into two clinical phases, depending on age of onset (Koedam et al., 2010). On the one hand, it is possible to find a familial Alzheimer's Disease (fAD), which was usually defined for individuals <65 years of age, accounting for 5% of all cases (Harvey, 2003) and directly linked with highly penetrant autosomal dominant mutations in one of three different genes: the Presenilin 1 (*PS1*) gene, the Presenilin 2 (*PS2*) gene, or the Amyloid precursor protein (*APP*) gene (Campion et al., 1999). On the other hand, it is possible to find sporadic Alzheimer's disease (sAD), which accounts for approximately 95% of all AD cases and which likely results from the complex interplay of molecular, environmental and genetic factors (Feng and Wang, 2012; Guimerà et al., 2002; Koedam et al., 2010).

Due to the high degree of variability and its scattered etiology, many problems have had to be overcome in the last two decades of research on aging and associated neurodegenerative diseases. The models usually used are mice that are genetically modified by human gene insertions with the main proteins associated with AD (APP, PS1, PS2, and tau) or tau kinase overexpression (Cyclin-dependent kinase 5 [CDK5] or Glycogen synthase kinase 3 β [GSK3β]) (Lucas et al., 2001; Piedrahita et al., 2010). However, none of the numerous animal models reliably reflect what occurs in humans, because AD should be considered a polyetiologic disease (Itzhaki, 1994) that is

caused by disruption of a wide range of molecular mechanisms. At the same time, should be borne in mind that the mutations introduced into transgenic models are related with mutations linked with fAD cases; thus, animal models will not be representative of the vast majority of cases of sporadic Alzheimer type AD (Feng and Wang, 2012; Guimerà et al., 2002; Koedam et al., 2010).

It is noteworthy that advanced age is the most important risk factor for the development of sAD (Wisniewski et al., 1996). It was thought that acquiring a more representative model of the sAD type, thus better understanding of the relationship among changes in homeostasis in senescence and AD, at this stage Lok and coworkers (2013 a; 2013b) presented a combined model of AD (APP/PS1) with Senescence-accelerated mouse prone 8 (SAMP8) ,a representative model of aging, to obtain a new model that displays the characteristics of both.

On the other hand, we decided to use the double-transgenic APP/PS1 mice as an animal model of AD, which can contribute cerebral pathologic changes similar to those in humans, and which can be used to simulate neuropathologic changes such as Senile plaque (SP) formation, neuroglial cell proliferation and decrease of synapses and dendrites (Codita et al., 2006; Games et al., 2006; Irizarry et al., 1997). APP/PS1 mice express a chimeric mouse/human APP bearing the Swedish mutation (Mo/HuAPP695swe) (Borchelt et al., 1996; Dewachter et al., 2000), exhibiting a marked elevation in Aβ protein level and Aβ deposition in the cerebral cortex and hippocampus and developing similar neuropathological hallmarks to those observed in AD brains (Games et al., 1995), and a mutant human PS1-ΔE9 (Borchelt et al., 1996; Campion et al., 1999) that displays an increased Aβ42 peptide formation, potentiating amyloid deposition through superactivation of the PS1 catalytic form (McGowan et al., 1999).

SAMP8 is characterized by deficits in learning and memory (Miyamoto et al., 1986; Takeda, 2009; Takeda et al., 1981), emotional disorders such as reduced anxiety-like behavior (Markowska et al., 1998; Miyamoto et al., 1992), impaired immune response, etc. (Flood and Morley, 1998; Yagi et al., 1988) at an earlier age. More importantly, this strain is increasingly being recognized as a model of age-related AD (Morley et al., 2012; Pallas et al., 2008), because it shows an AD-related pathology with aging such as alterations in APP processing by secretases (Morley et al., 2000, 2002), increases in Aβ (Del Valle et al., 2010) and in other protein aggregates (Manich et al., 2011), cerebral amyloid angiopathy (del Valle et al., 2011), and increases in tau hyperphosphorylation (Canudas et al., 2005).

The goal of the present work is to examine key biochemical and functional characteristics of this disease, including the study of memory impairment, and amyloid and tau pathologies, combining the features of the accelerated-senescence background of SAMP8 mice and the APPswe/PS1-ΔE9 mutations of APP/PS1 mice, this mouse was APP/PS1/SAMP8 and denominated SHAP.

2. Materials and methods

2.1. Animals

SAMP8 and APP/PS1 mouse strains were commercially available (*Harlan Laboratories)*. The generation of APP/PS1 mice expressing human mutated forms of APP and PS1ΔE9 has already been described (Borchelt et al., 1996). New model strains, SHAP(+) and SHAP(-), were obtained through crossing the male APP/PS1 (*n* = 5) and the female SAMP8 (*n* = 13). All mice have been back-crossed to their parental strain for at least five generations, Once the progeny was obtained (*n* = 92), the animals were genotyped by conventional Polymerase chain reaction (PCR), following the protocol proposed by *Jackson Laboratory* for APP/PS1, resulting in 57.6% SHAP(+) (*n* = 53), with both transgenes, and 42.4% SHAP(-) (*n* = 39), without any transgene.

Experimental procedures were carried out with the following four mice strain groups at 3, 6, 9, and 12 months (12 experimental groups): SAMP8 (*n* = 12, 3 for each age; APP/PS1 (*n* = 16, 4 for each age); SHAP(+) (*n* = 16, 4 for each age), and SHAP(-) (*n* = 12, 3 for each age).

All of the animals were fed a standard chow (2018 *Teklad Global* 18% Protein Rodent Maintenance Diet, *Harlan*) and water ad libitum and were maintained under standard temperature conditions (22 ± 2°C) 12-h light/12-h dark cycles (300 lux/0 lux) and at a relative humidity of 55%. Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals (European Communities Council Directive 86/609/EEC) established by the Ethical Committee for Animal Experimentation at the University of Barcelona.

2.2. Object recognition test (ORT)

Nine- and twelve-month-old SAMP8, APP/PS1, SHAP(+), and SHAP(-) animals were placed in a 90°, two-arm, 25-cm-long, 20-cm-high, and 5-cm-wide black maze. Light intensity in the middle of the field was 30 lux. The objects to be discriminated were made of plastic (object A, 5.25 cm in height

and object B, 4.75-cm in height). For the first 3 days, the mice were individually acclimatized to the apparatus for 10 min. On day 4, the animals were submitted to a 10-min acquisition trial (first trial), during which they were placed in the maze in the presence of two identical novel objects (A+A or B+B), which were localized at the end of each arm. A 10-min retention trial (second trial) occurred 2 h later. During this second trial, objects A and B were placed in the maze, and the time that the animal explored the new object (t_n) and the old object (t_o) were recorded. A Discrimination index (DI) was defined as $(t_n-t_o)/(t_n+t_o)$. In order to avoid object preference biases, objects A and B were counterbalanced so that one half of the animals in each experimental group were first exposed to object A and then to object B, whereas the other one half saw first object B and then object A. The maze and the objects were cleaned with 96° ethanol between experiments to eliminate olfactory cues.

2.3. Brain processing

Animals were anesthetized with 80 mg/kg of sodium pentobarbital and intracardially perfused with saline serum. Afterward, brains were dissected and separated sagitally in two hemispheres: one for immunohistochemistry, and the other, for protein extraction. Immunohistochemistry brains were frozen by immersion in isopentane, chilled on dry ice, and stored at -80°C until sectioning. Thereafter, frozen brains were embedded in OCT cryostat-embedding compound (*Tissue-Tek*, Torrance, CA, USA), cut into 20-µm-thick sections on a cryostat (*Leyca Microsystems*, Germany) at -18°C, and placed on slides. Slides containing brain sections were fixed with acetone for 10 min at 4°C, allowed to dry at room temperature, and then frozen at -20°C until further staining. The remaining hemispheres were dissected and stored at -80°C until protein extraction.

2.4. Thioflavin-S staining

Slides were allowed to defreeze at room temperature and then were rehydrated with Phosphate-buffered saline (PBS) for 5 min. Later, the brain sections were incubated with 0.3% Thioflavin-S (*Sigma-Aldrich*) for 20 min at room temperature in the dark. Subsequently, these were submitted to washes in 3-min series, specifically with 80% ethanol (2 washes), 90% ethanol (1 wash), and 3 washes with PBS. Finally, the slides were mounted using Fluoromount (*EMS*), allowed to dry overnight at room temperature in the dark, and stored at 4°C. Image acquisition was performed with an epifluorescence microscope (BX41; Olympus, Germany).

For plaque quantification, similar and comparable histological areas were selected, focusing on having the hippocampus and the whole cortical area positioned adjacently.

2.5. Quantification of soluble Β-amyloid

Frozen samples of cortex and hippocampus of SHAP(+), SHAP(-), APP/PS1 and SAMP8 mice aged 3, 6, 9 and 12 months were wet mass determined (120 mg) and homogenized in ice-cold guanidine buffer (5 M guanidine hydrochloride/50 mM TrisCl, pH 8.0). The detection and measurement of b-amyloid 1–40 and b-amyloid 1–42 was carried out by enzyme-linked immunoabsorbent assay (ELISA) with the corresponding detection kits (Invitrogen, Carlsbad, CA, USA), following the instructions of the supplier and after optimizing the reaction with increasing amounts of known concentrations of b-amyloid 1–40 and b-amyloid 1–42. A ratio of the b-amyloid 1–42 levels with respect to those of b-amyloid 1–40 was calculated.

2.6. Immunohistochemistry

Slides were allowed to defreeze at room temperature and then rehydrated with PBS for 5 min. Then, brain sections were blocked and permeabilized with PBS containing 1% Bovine serum albumin (BSA; *Sigma-Aldrich*) and 0.1 % Triton X-100 (*Sigma-Aldrich*) for 20 min. After two, 5-min washes in PBS, the slides were incubated with the primary antibody for IBA-1 (WAKO, 1:500) and GFAP (*Abcam*, 1:500) (see Table 1) overnight at 4°C. They were then washed again and incubated for 1 h at room temperature in the dark with Alexa Fluor secondary antibody. Finally, the slides were washed, mounted using Fluoromount (EMS), allowed to dry overnight at room temperature, and stored at 4°C. Image acquisition was performed with an epifluorescence microscope (BX41, Olympus, Germany).

2.7. Protein extraction

Brains were micronized by freezing with liquid nitrogen and grinding with a mortar. For total protein extraction, lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, pH 7.4), EDTA-free Protease inhibitor cocktail (*Roche*, Mannheim, Germany), and Phosphatase inhibitor cocktail 1 (*Sigma-Aldrich*, St. Louis, MO, USA) were added to micronized tissue and left on ice for 30 min. Then, samples were centrifuged at 10,000 × *g* for 10 min and a supernatant with

total protein content was collected. All of the protein extraction steps were carried out at 4°C. Protein concentration was determined by the Bradford protein assay.

2.8. Western blot

For Western blot analysis, 20 µg of protein were denatured at 95°C for 5 min in sample buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, 2% Sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 0.05% bromophenol blue), separated by Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) onto 8–12% polyacrylamide gels and transferred onto Immobilon polyvinylidene difluoride membranes (*Millipore*, Billerica, MA, USA). The membranes were incubated overnight at 4°C with the primary antibodies (see Table 1) diluted with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% BSA. Membranes were then washed and incubated with secondary antibodies (see Table 1) and diluted with TBS-T for 1 h at room temperature. Protein bands were visualized using a chemiluminescent HRP substrate (*Millipore*) and ChemiDoc XRS+ (*Biorad*). Band intensities were quantified by densitometric analysis using Image Lab Software and values were normalized to β-actin, except for C-terminal fragments (CTF) of APP (14 kDa), also named C99, which were normalized by APP protein.

2.9. Statistical analysis

Results were analyzed statistically by SPSS 19.0 (*SPSS Inc.,* Chicago, IL, USA) software and GraphPad PRISM (*GraphPad Software, Inc*.) software. Data are presented as mean ± Standard error of the mean (SEM), and means were compared with two-tailed, unpaired Student *t* test or one-way Analysis of variance (ANOVA) followed by Tukey's multiple comparison test when necessary. In the Object recognition test (ORT), a one-sample *t* test, was used to examine whether single columns were different from zero. Statistical significance was attained when p values were <0.05.

3. Results

3.1. SHAP mice characteristics across aging

Throughout the study, animal weight monitoring was conducted weekly. From the results (Fig. 1), it can be observed that at early ages, weights of the novel mice obtained did not show significant weight differences among themselves, but did in comparison with parental strains APP/PS1 and

SAMP8. When age, differences among strains were maintained, and weight gain patterns were similar in SHAP and APP/PS1 model, increasing over time, but in turn and as expected, SAMP8 presented a very weak growth, with significantly lower weight than other experimental groups.

3.2. Object Recognition Test (ORT)

At late age (9 and 12 months), ORT demonstrated that SAMP8, APP/PS1, and SHAP(+) mice showed impaired memory, while SHAP(-) did not (Fig. 2). To date, it has been found that transgenic mice that SHAP(+) maintain loss of memory, but preservation of memory in comparison with SAMP8 occurs when transgenes are not present in SHAP(-) mice .

3.3. Histopathologic brain state

Plaque histopathologic state was analyzed in all experimental groups and at each age through Thioflavin-S staining. No senile plaques (SP) were determined in any mouse strains at 3 months (data not shown), as described previously for APP/PS1 and SAMP8 mice (Aso et al., 2012; Butterfield and Poon, 2005). Strains expressing APP and PS1 transgenes showed a burden of Aβ depositions that increased with age (Figs. 3G–L), whereas SAMP8 and SHAP(-) strains, both without transgene, did not (Figs. 3A–3F). Specifically, APP/PS1 showed early deposition at 6 months (Fig. 3J), always exhibiting higher levels of Aβ deposition in cortex than in hippocampus areas (Fig. 4). Conversely, SHAP(+) mice demonstrated a delayed initial deposition time. At 9 months, SHAP(+) presented SP (Fig. 3H) and displayed higher levels in comparison with APP/PS1 at this age (Figs. 3Kand 4A). At 12 months, SHAP(+) showed increased levels of burden Aβ deposition in all studied areas (Figs. 3I-3L and 4A), in comparison with APP/PS1 (Figs. 3L and 4L). In reference, to soluble Aβ-40/42, we found significant higher lelves in both transgenics strains in reference to SAMP8 and SHAP(-)(Fig 4 B-C). Increasing quantities of Aβ-42 were determined in SHAP(+) in front of APP/PS1, only at 3-months-old.

Gliosis, linked with SP formation, was assessed by Glial fibrillary acidic protein (GFAP) immunohistochemistry at the final stage (12 months). Results showed astroglial activation in APP/PS1 (Figs. 5N and 5P) and SHAP(+) (Figs. 5J, 5L), but not in SAMP8 (Figs. 5B and 5D) and SHAP(-) (Figs. 5F and 5H). Double staining with Thioflavin-S and GFAP demonstrated the co-localization of astroglial reactivity with β-amyloid deposition in APP/PS1 and SHAP(+), confirming that glial activation is related with the presence of amyloid protein aggregates both in cortical (Figs. 5J and

N) and hippocampal areas (Figs. 5L and P). Conversely, when microglial activation was determined by IBA-1 immunohistochemistry, mice with APP/PS1 double transgene, SHAP(+), and APP/PS1 exhibited some degree of microglial activation in cortical regions (Figs. 5I and 5M) and hippocampus (Figs. 5K and 5O).

3.4. Amyloid pathology in the APP/PS1/SAMP8

On observing changes in β-amyloid deposition in the newly generated strain, we decided to assess the effect of age on the level of BACE-1, PS1, and known intermediates and endproducts of APP processing.

Highest levels in BACE1 were found in SAMP8 and in newly generated mice SHAP(+) and SHAP(-) in reference to APP/PS1 at up to 9 months (Fig. 6). In contrast, at 12 months APP/PS1, achieve the highest levels of BACE1 compared with SAMP8 and SHAP mice (Fig. 6). An increase in 48 kDa fragment, corresponding to the catalytic core of PS1, was detected only in SHAP(+) at the oldest age tested (12 months) (Fig. 7). In reference to the proteolytic fragment (18 kDa) of constitutive PS1, we determined a higher level in SAMP8 in reference to mice carrying the human PS1 mutation, APP/PS1 and SHAP(+) according to the nature of this mutation (Fig. 6), which do not require cleavage activation of PS1, being constitutively active. Surprisingly, APP/PS1 exhibited an increase in this fragment at 12 months, indicating possible endoproteolysis of the endogenous murine PS1 isoform.

Levels of APP and C99 fragment were significantly increased in SHAP(+) and APP/PS1 in comparison with SHAP(-) and SAMP8 mice aged 3 and 6 months, respectively (Fig. 8). These facts are in agreement with the presence of the APP transgene and higher Aβ deposition in these mice, but not in SHAP(-) and SAMP8.

3.5. Characterization in Tau pathology

Different tau phosphoepitopes were studied, including Tau Ser199, Tau Tyr205, Tau Ser396, and Tau Ser404. Along the aging process studied in this novel strain, no changes were observed in the levels of the different phospho-tau studied, which presented high variability in the levels of each epitopes studied, except for APP/PS1 at the oldest age tested, where we observed higher levels of phospho-tau in comparison to SAMP8 (Fig. 9).

GSK3β (Phospho-Tyr279) levels, the active forms of these tau kinases, were higher in SHAP(+) than APP/PS1 and SAMP8 (Fig. 10). At the same time, higher levels of CDK5 were determined in SHAP(+) at 12 months in comparison with SAMP8 and APP/PS1, concurrently with an increase in the co-activator p25, indicating a possible enhanced process of phosphorylation in these animals (Fig. 9-10). Also, phosphor-SapK/JNK was raised in SHAP(+) in comparison with reference to SAMP8 and APP/PS1 (Fig. 10).

3.6. Oxidative enzymatic machinery

Levels of SOD1, catalase, and iNOS were determined by Western blot to detect changes in the oxidative machinery in the new generated strain, that is SHAP(+). Results showed no changes in any of the enzymes linked with Oxidative stress (OS) (data not shown).

4. Discussion

Current mouse models of AD are restricted to the expression of AD-related pathology associated with specific mutations present in early-onset fAD, thus representing <5% of AD cases. To date, there are no mouse lines that model continuum development and late-onset/age-related AD, which accounts for the vast majority; therefore, the chronology of events that lead to the disease in the aged population is difficult to establish based on current animal models. Moreover, published data show that (SAMP8), a model of accelerated aging, displays many features in brain that are known to occur in AD (i.e., increased OS, Aβ alterations, and tau hyperphosphorylation) (Morley et al., 2012; Takeda, 2009). Therefore, SAMP8 mice may offer an excellent model for studying the earliest neurodegenerative changes associated with AD and provide a more encompassing picture of the disease, considering AD as a syndrome triggered by a combination of age-related events. On the other hand, APP/PS1 are double transgenic mice that express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human Presenilin 1 (PS1-ΔE9). The presence of amyloid burden in cortex and hippocampus (CA3 and CA1) were demonstrated in APP/PS1 transgenic mice available at our laboratory (Aso et al., 2012). To date, the leading hypothesis, the Aβ hypothesis, which is based on mutations in either APP or PS1/2 that affect the processing of APP and contribute to Aβ accumulation in neurons and to the consequent formation of senile plaques (Perry et al., 2000), has come under increased scrutiny. In fact, it is becoming increasingly discussed that Aβ deposition could be a consequence, rather than

an initiator, of the pathophysiological cascade (Swomley et al., 2013). Other mechanisms of the disease, such abnormally hyperphosphorylated bundles of tau protein found in neurofibrillary tangles, OS, metal ion deregulation, inflammation, and age must be take into account in order to delineate the complete picture of the disease (Swomley et al., 2013; Verri et al., 2012).

Recently, Lok et al. (2013a, 2013b) crossed APP/PS1 mice with the SAMP8 generating a new AD mouse model, expressing human APP/PS1 genes on a senescence background, covering a phenotypical analysis by sudying working memory, spatial memory, motor performance and anxiety-like behavior, as well as changes neuronal cell death and amyloid deposition. We applied the same strategy in the present work, crossing APP/PS1 with SAMP8, delivering a double transgenic mouse in SAMP8 background, named SHAP(+). The goal was to better characterize at biochemical and molecular level a this mouse with neuropathological traits of human AD in a senescence environment.

We showed that SHAP(+) had similar short term memory disturbances to those of SAMP8 and APP/PS1 at old ages, accordingly with impairment in spatial memory or working memory reported earlier (Lok et al., 2013a, 2013b). Short-term memory lost was accompanied by a significant increase in SP, these being highest at 12 months of age compared with SAMP8 and APP/PS1.

The Swedish mutations (K595N/M596L) included raised the amount of Aβ produced by favoring the processing through the secretase pathway. Levels of APP were higher in SHAP(+) in mice aged up to 9 months old, probably related with SAMP8 genotype (Kumar et al., 2009) but at 12 months, these were lower than in APP/PS1, probably due to higher cleavage to the amyloid peptides that accumulate in the plaques that, as previously mentioned, were significantly increased at this stage. Analysis of BACE1, a β-secretase, revealed that there were sparse and no consistent changes in SHAP(+) in comparison to SAMP8. In SAMP8 and SHAP(-), where the transgenic mutant human Presenilin protein (PS1-ΔE9) and human APP were not expressed, there were no amyloid depositions; therefore, the expression of human PS1 and APP in APP/PS1 in SHAP(+) is the key factor for amyloid accumulation in these two strains, to a greater degree than the presence and levels of BACE1.

In reference to PS1 function, it is known that uncleaved PS1 holoprotein (full-length PS) (48-52 kDa) is an inactive zymogene that must be activated by endoproteolytic cleavage (Fukumori et al.,

2010; Li et al., 2000). There is compelling evidence that full-length PS1 is subject to endoproteolytic processing, generating N-terminal (NTF) (27-28 kDa) and C-terminal fragments (CTF) (16-18 kDa) (Chávez-Gutiérrez et al., 2008; Thinakaran et al., 1996). Once PS1 is cleaved, N-terminal fragments (NTF) (27–28 kDa) and C-terminal fragments (CTF) (16–18 kDa) form a biologically active NTF-CTF heterodimer, and the γ-secretase substrate can reach the catalytic site (Ahn et al., 2010; Chávez-Gutiérrez et al., 2008; Wolfe, 2013). Removal of exon 9 in the PS1-ΔE9 mutation allows access to the catalytic site, resulting in the constitutively active form of the enzyme as holoprotein, without the need for the cleavage process (Knappenberger et al., 2004). Accordingly, we found higher levels of active fragment in SAMP8 and SHAP(-) mice than in mice supporting the mutation, at early ages. However, levels of 18 kDa PS1 normalized with age. In contrast, holoprotein PS1 levels were similar in all experimental groups up to the age of 9 months, but was found significantly increased in SHAP(+) at 12 months of age in comparison with APP/PS1, in agreement with higher Aβ deposition in these mice at the same age. The greater participation of PS1 in amyloid pathology, described in APP/PS1 and in SHAP(+), would be of importance when new drugs are studied for AD treatment, in that will be more interesting to focus on PS1 inhibitors rather than on BACE inhibitors (Gandy and Dekosky, 2013).

Increases in tau hyperphosphorylation also participate in exacerbating plaque formation; thus, the presence of a higher hyperphosphorylation processes could be the cause of the increase in amyloid pathology (Guo et al., 2013). Therefore, to determine a possible link between amyloidosis and tau pathology in SHAP(+) mice, we studied tau phosphorylation in several characteristic serine and tyrosine epitopes in these mice in comparison with SAMP8 and APP/PS1. As described elsewhere, both SAMP8 and APP/PS1 possess an alteration in the tau phosphorylation process (Aso et al., 2012; Canudas et al., 2005). Total tau content was raised in APP/PS1 in comparison with that of SAMP8, whereas in SHAP(+) levels, tau content was nearer to that of the senescence model (data not shown). Four different phosphorylated epitopes were studied here. No changes were found and there is no clear correlation between phosphorylation levels and the mouse strain at early and medium age. At 12 months, the oldest age studied, APP/PS1 and SHAP(+) exhibited higher phosphorylation in Tau Ser199, Tau Ser396, and Tau Ser404. Moreover, the data showed no specific correlation between tau and amyloid pathology up to 12 months, where amyloid pathology was well established, as our results and the broad bibliography published on APP/PS1 or in SAMP8 demonstrates (Lok et al., 2013a, 2013b).

The lack of difference in phosphorylated tau epitope patterns studied in early ages among the strains is in agreement with the no-differential activation of tau kinases CDK5 and GSK3β until 9 months, at least in SHAP(+). Instead, at 9 and at 12 months, SHAP(+) displayed an altered kinase pattern that correlates with tau hyperphosphorylation, which shows higher phosphorylation levels in Ser199 and Ser404 epitopes. This fact is in agreement with the literature, which describes tau as a substrate for CDK5 and GSK3β (Casadesús et al., 2012; Iqbal et al. 2011). In turn, this pattern also correlates with the overexpression of CDK5 and p25, which results in increased tau phosphorylation at specific sites (Currais et al., 2013).

JNK has been implicated in tau phosphorylation in APP/PS1 and SAMP8 mice (Díaz-Moreno et al., 2013). Levels of JNK are also increased in SHAP(+) at 12 months of age in comparison with SAMP8 or APP/PS1.

Oxidative stress (OS) is a well-established pathogenic factor in AD, and the association of OS with amyloid and tau abnormalities is well-known (Mondragón-Rodríguez et al., 2013). A redox imbalance has been described in both SAMP8 and APP/PS1. The newly generated mice, SHAP(+), did not differ in proteins levels related with homeostatic control of OS, synch as catalase, Superoxide dismutase (SOD), or Nitric oxide (NO) synthase. On the other hand, immunohistochemical studies showed that microgliosis and astrogliosis were detectable in APP/PS1 and SHAP(+) in hippocampus and cortex, indicating that the OS described for APP/PS1 was present also in the SHAP(+). In reference to astrogliosis, and as expected, reactive astroglia are localized near the plaques both in APP/PS1 and SHAP(+), indicating that OS and a harmful effect occur mainly surrounding amyloid aggregation.

5. Conclusion

In conclusion, overexpression of APP and PS1 in a senescence background of SAMP8 generates a model of AD linked with senescence, with exacerbation of the amyloidogenic process at late ages, and maintains some of the histopathological hallmarks present in AD, and also learning and memory disturbances expected by the effect of aging and disease.

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8. Figures

Figure 1.

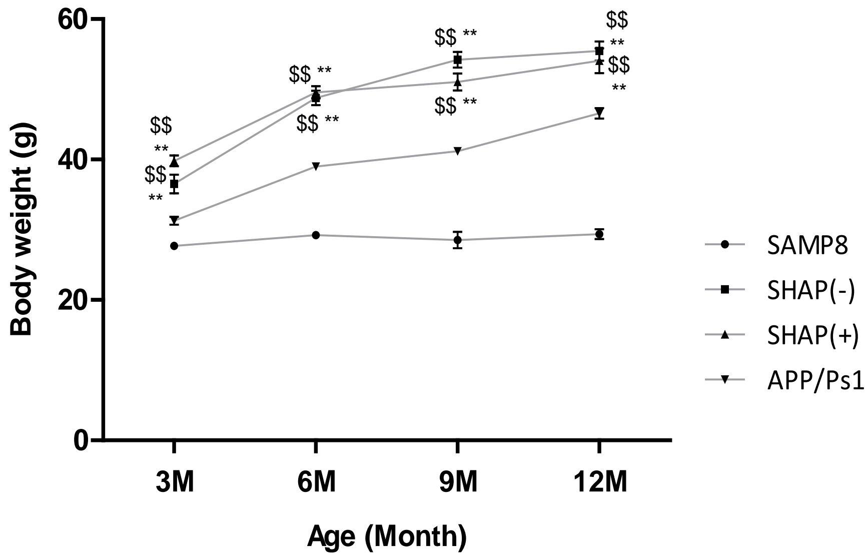


Figure 1. Body weight (BW, in grams [g]) of the studied models in function of age (months). Analysis of variance (ANOVA) following Tukey's multiple comparison test. **P* <0.05; ***p* <0.01 vs. SAMP8; $^{\$}p$ <0.05; $^{\$\$}p$ <0.01 vs. Amyloid precursor protein/Presenilin 1 (APP/PS1).

Figure 2.

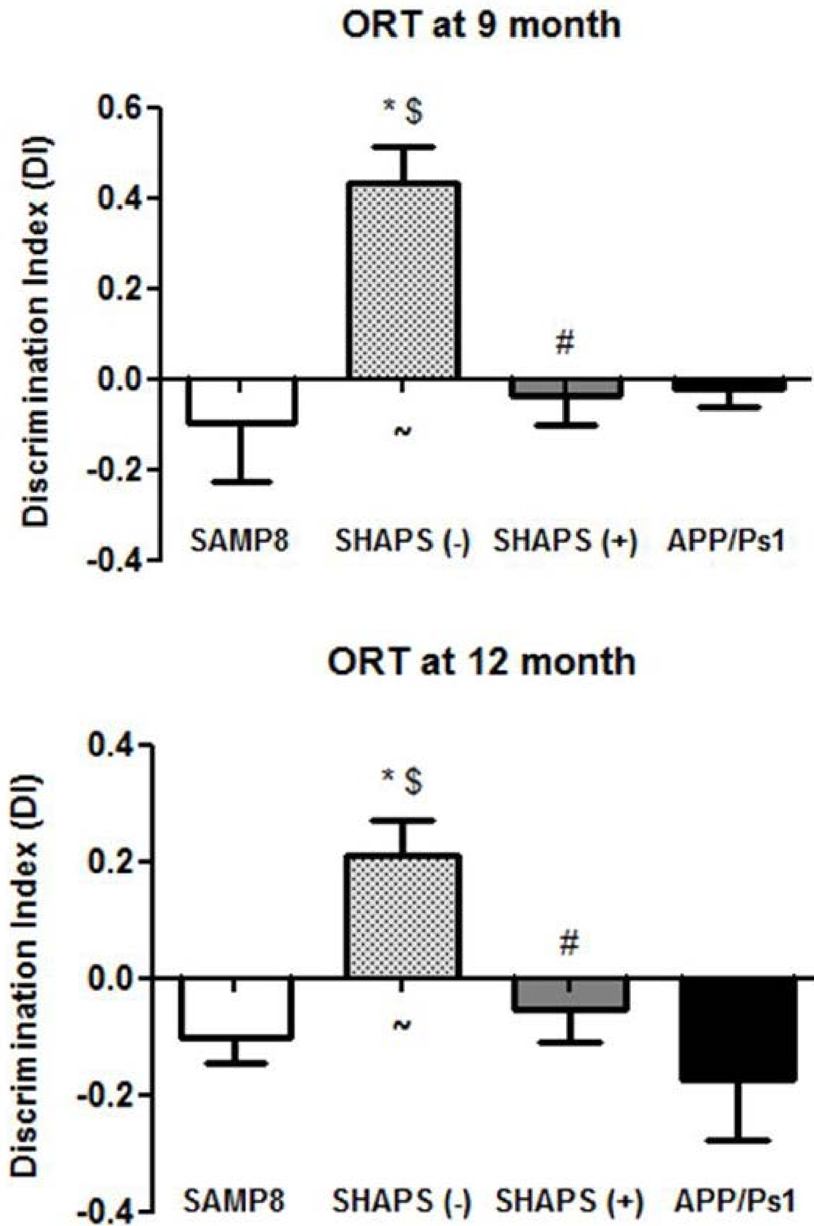


Figure 2. Discrimination index (DI) of different groups of Senescence-accelerated mouse prone 8 (SAMP8) animals at 9 and 12 months of age. There is a higher DI in SHAP(+) in reference to SAMP8, SHAP(-), and Amyloid precursor protein/Presenilin 1(APP/PS1). Bars represent mean ± Standard error of the mean (SEM). Analysis of variance (ANOVA) following Tukey's multiple comparison test: One sample *t* test: **P* <0.05 vs. SAMP8; $^{\$}p$ <0.05 vs. APP/PS1; $^{\#}p$ <0.05 vs. SHAP(-).

Figure 3.

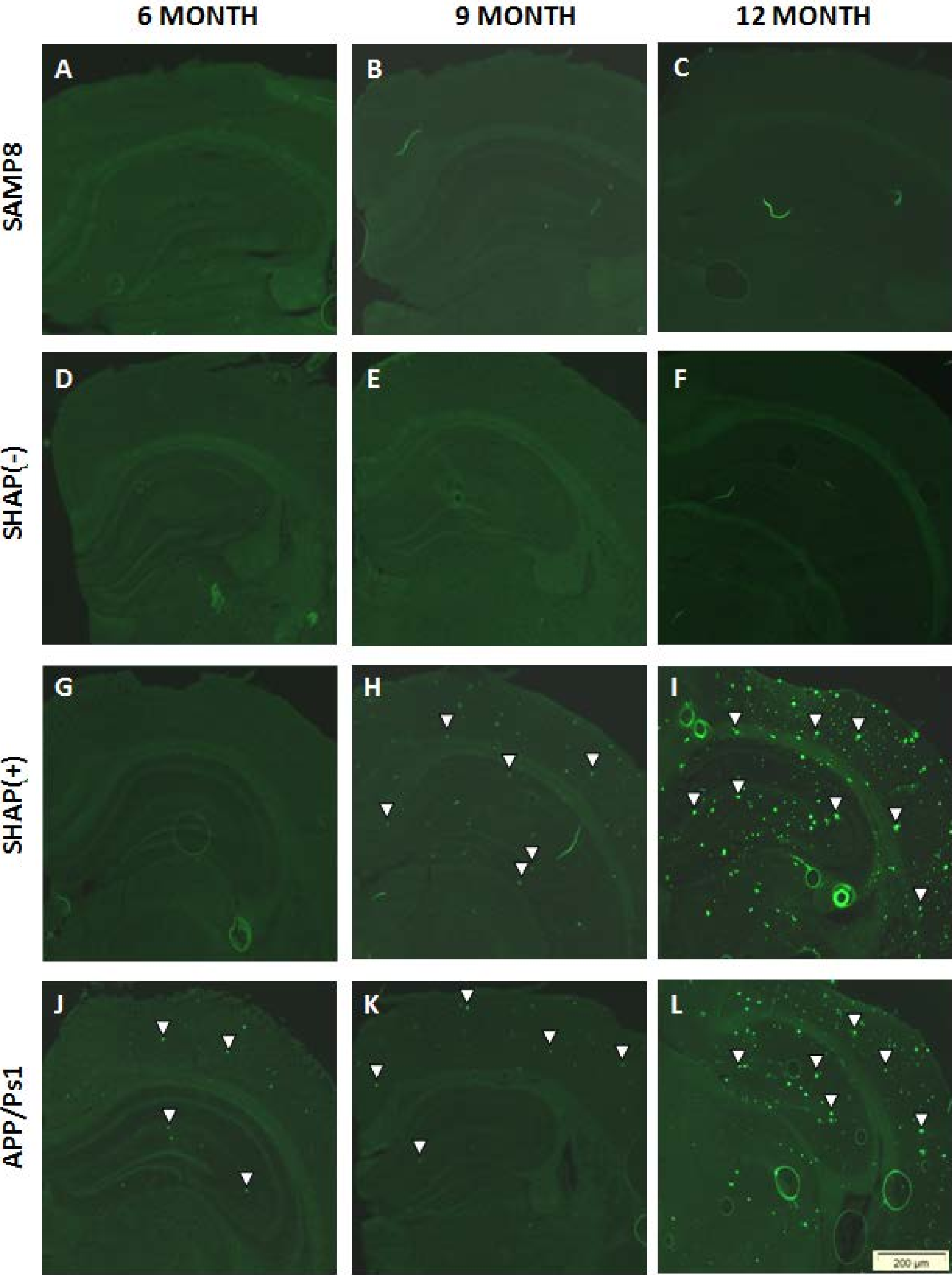
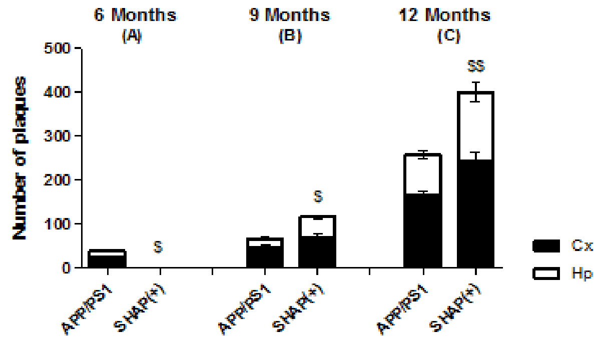


Figure 3. Thioflavin-S staining of β-amyloid plaques in mouse brains. Representative images of histopathological brain state of Senescence-accelerated mouse prone 8 (SAMP8), SHAP(+), SHAP(-), and Amyloid precursor protein/Presenilin 1 (APP)/PS1 at different times in life (6, 9, and 12 months). White arrows are representative indicators of the presence of β-amyloid plaques in the areas studied: Cortex (Cx), and Hippocampus (Hp).

Figure 4.

A



B

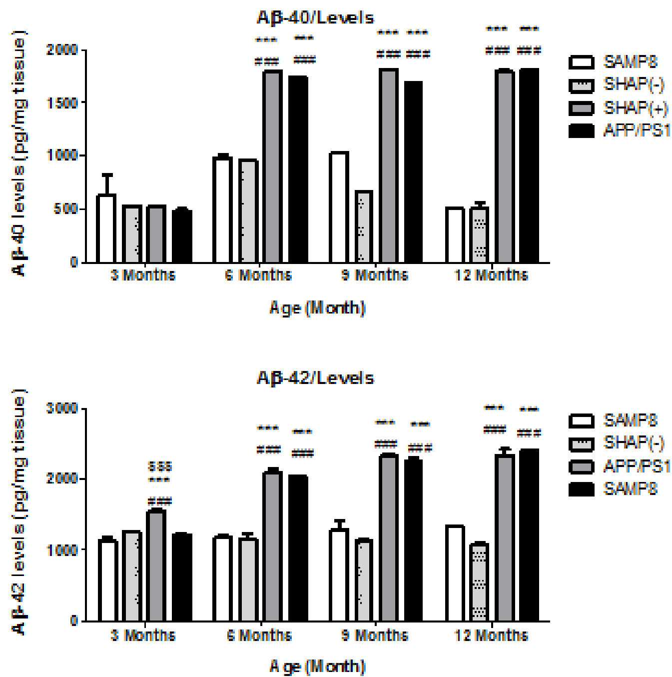


Figure 4. A: Quantification of number of β-amyloid plaques at 6, 9, and 12 months of age in the Cerebral cortex (Cx) and Hippocampus (Hp) of Amyloid precursor protein/Presenilin 1 (APP/PS1) and SHAP(+). **B:** Levels of soluble Aβ-40 and Aβ-42 respectively at 3, 6, 9, and 12 months. For quantification parameters, see Materials and Methods. Bars represent mean ± Standard error of the mean (SEM). Analysis of variance (ANOVA) following Tukey's multiple comparison test: ***p <0.001 vs. SAMP8 and SHAP(-); $p <0.05; 0 0$^{\$}$*P* <0.05; $^{\$\$}$*p* <0.01; $^{\$\$\$}$ *p*< 0.001vs. APP/PS1.

Figure 5.

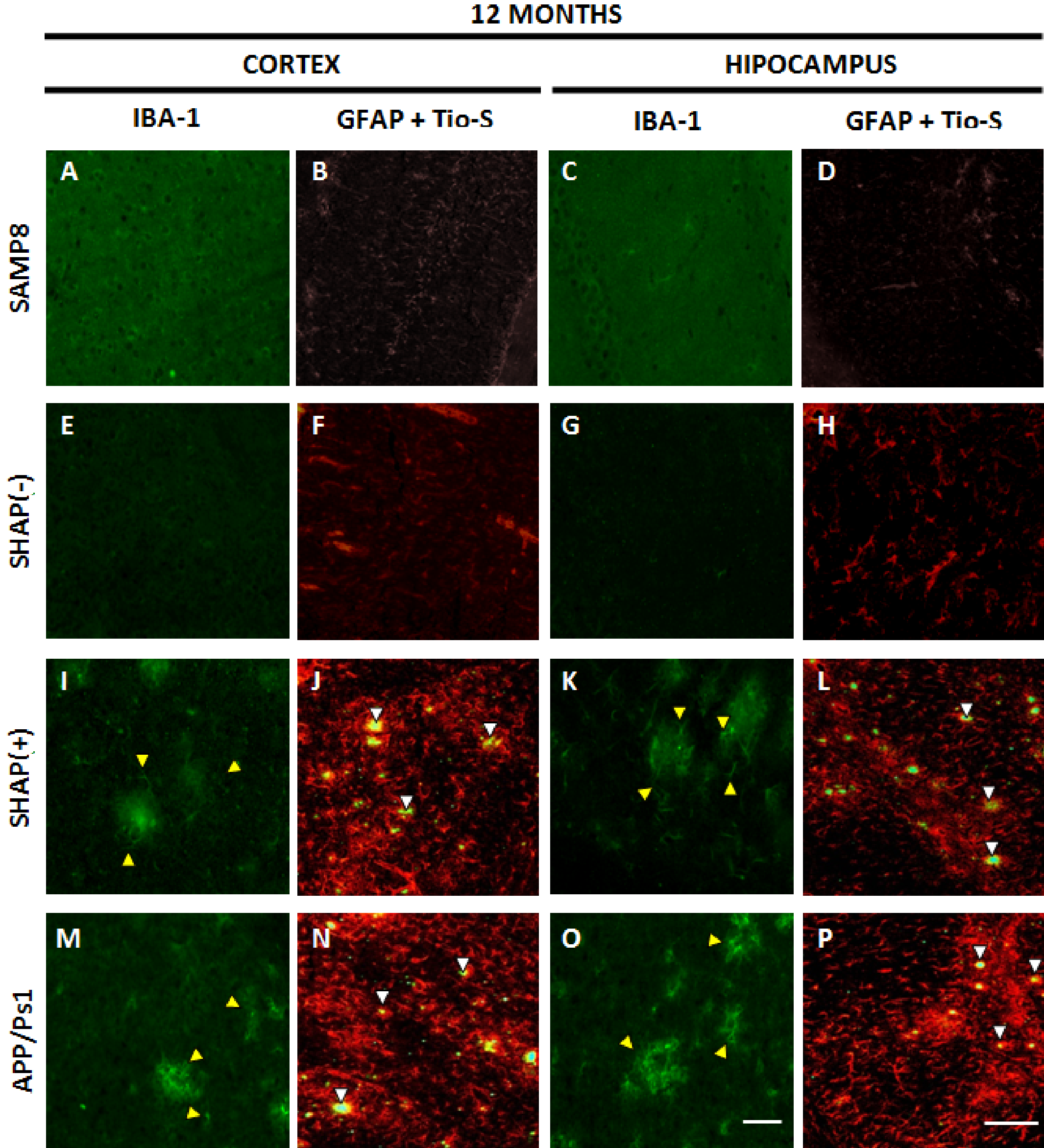


Figure 5. Immunohistochemical (cortex and hippocampus) staining of IBA-1 (green) and GFAP (red). Representative images of glial state of Senescence- accelerated mouse prone 8 (SAMP8), SHAP(+), SHAP(-), and Amyloid precursor protein/Presenilin 1 (APP/PS1) mice at 12 months of age. Yellow arrows are representative indicators of microglial activation in studied areas, whereas white arrows are representative indicators of β-amyloid plaques and Glial fibrillary acidic protein (GFAP) staining. Scale bar for Iba-1 images is 100 µm, for GFAP+Tio-S is 200 µm. (C)

Figure 6.

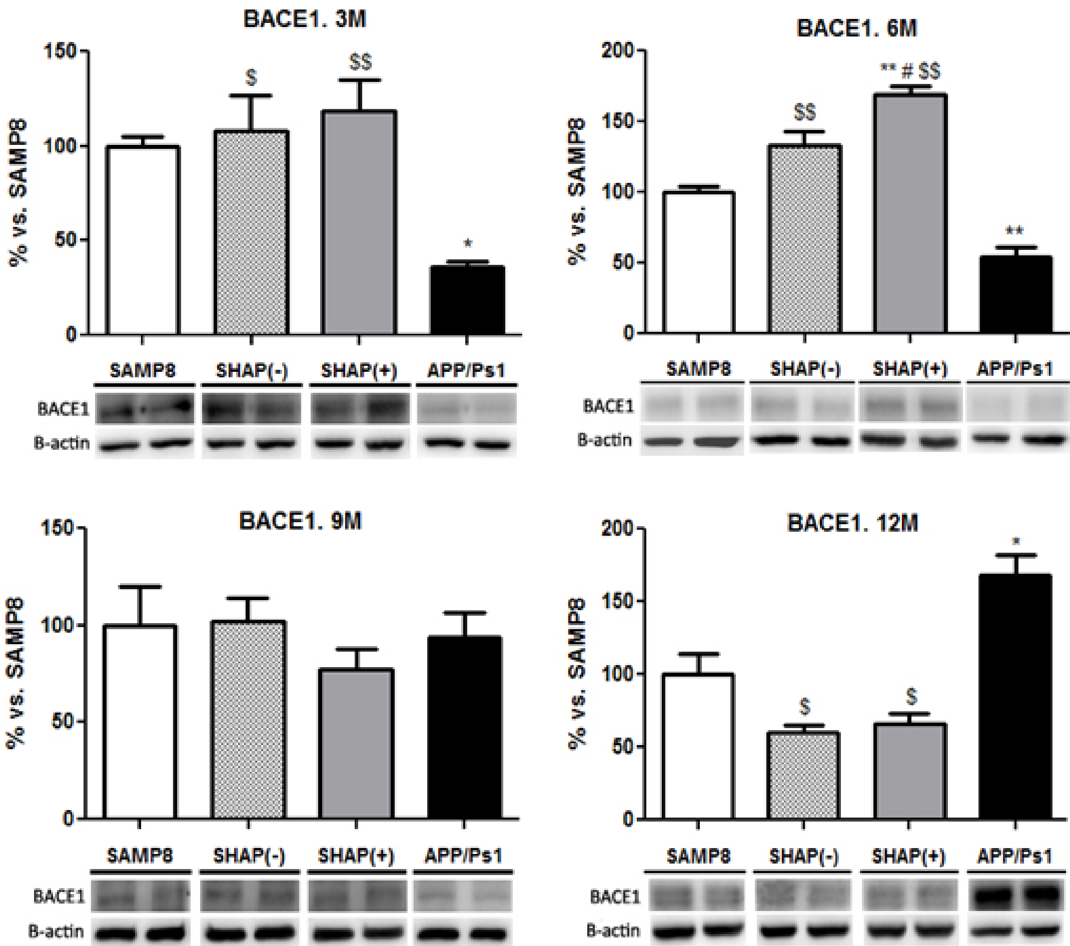


Figure 6. Levels of BACE of Senescence-accelerated mouse prone 8 (SAMP8), SHAP(-), SHAP(+), and Amyloid precursor protein /Presenilin 1 (APP/PS1). Bars represent mean ± Standard error of the mean (SEM); values are adjusted to 100% for levels of SAMP8. Analysis of variance (ANOVA) following Tukey's multiple comparison test: $^{*}P < 0.05$; $^{**}p < 0.01$ vs. SAMP8; $^{\$}p < 0.05$; $^{\$\$}p < 0.01$ vs. APP/PS1; $^{\#}p < 0.05$ vs. SHAP(-).

Figure 7.

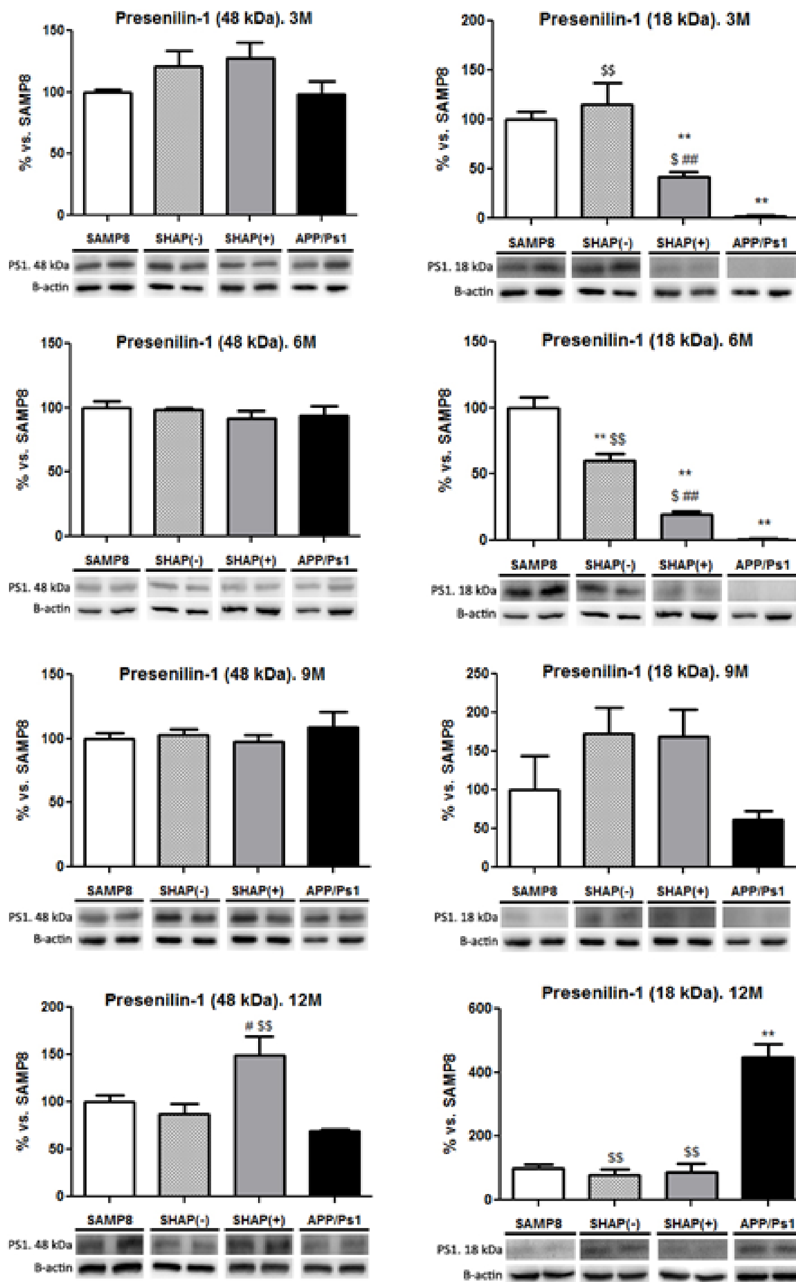


Figure 7. Levels of Presenilin 1 (PS1), holoprotein (48 kDa), and CTF fragment (18 kDa) of Senescence-accelerated mouse prone 8 (SAMP8), SHAP(-), SHAP(+), and Amyloid precursor protein/Presenilin 1 (APP/PS1) mice. Bars represent mean ± Standard error of the mean (SEM); values are adjusted to 100% for levels of SAMP8. Analysis of variance (ANOVA) following Tukey's multiple comparison test: **P* <0.05; ***p* <0.01 vs. SAMP8; $^{\$}p$ <0.05; $^{\$\$}p$ <0.01 vs. APP/PS1; $^{\#}p$ <0.05 vs. SHAP(-).

Figure 8.

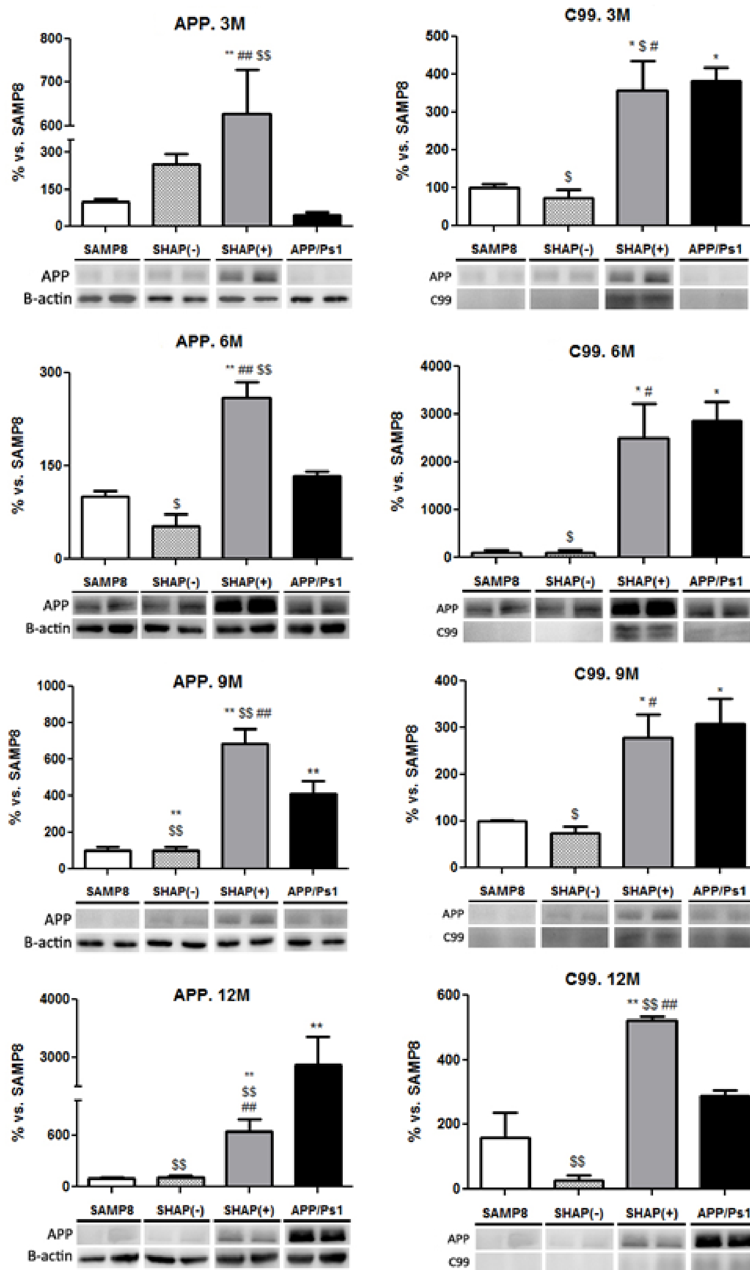


Figure 8. Western blot analysis for Amyloid precursor protein (APP) of Senescence-accelerated mouse prone 8 (SAMP8), SHAP(-), SHAP(+), and APP/Presenilin 1 (PS1) mice. Upper panels present the analysis of full APP levels at the studied ages. Lower panels present C-terminal beta fragment (CTFß/APP ratio. Bars represent mean ± Standard error of the mean (SEM); values are adjusted to 100% for levels of SAMP8. Analysis of variance (ANOVA) following Tukey's multiple comparison test: **P* <0.05; ***p* <0.01 vs. SAMP8; $^{\$}p$ <0.05; $^{\$\$}p$ <0.01 vs. APP/PS1; $^{\#}p$ <0.05 vs. SHAP(-).

Figure 9.

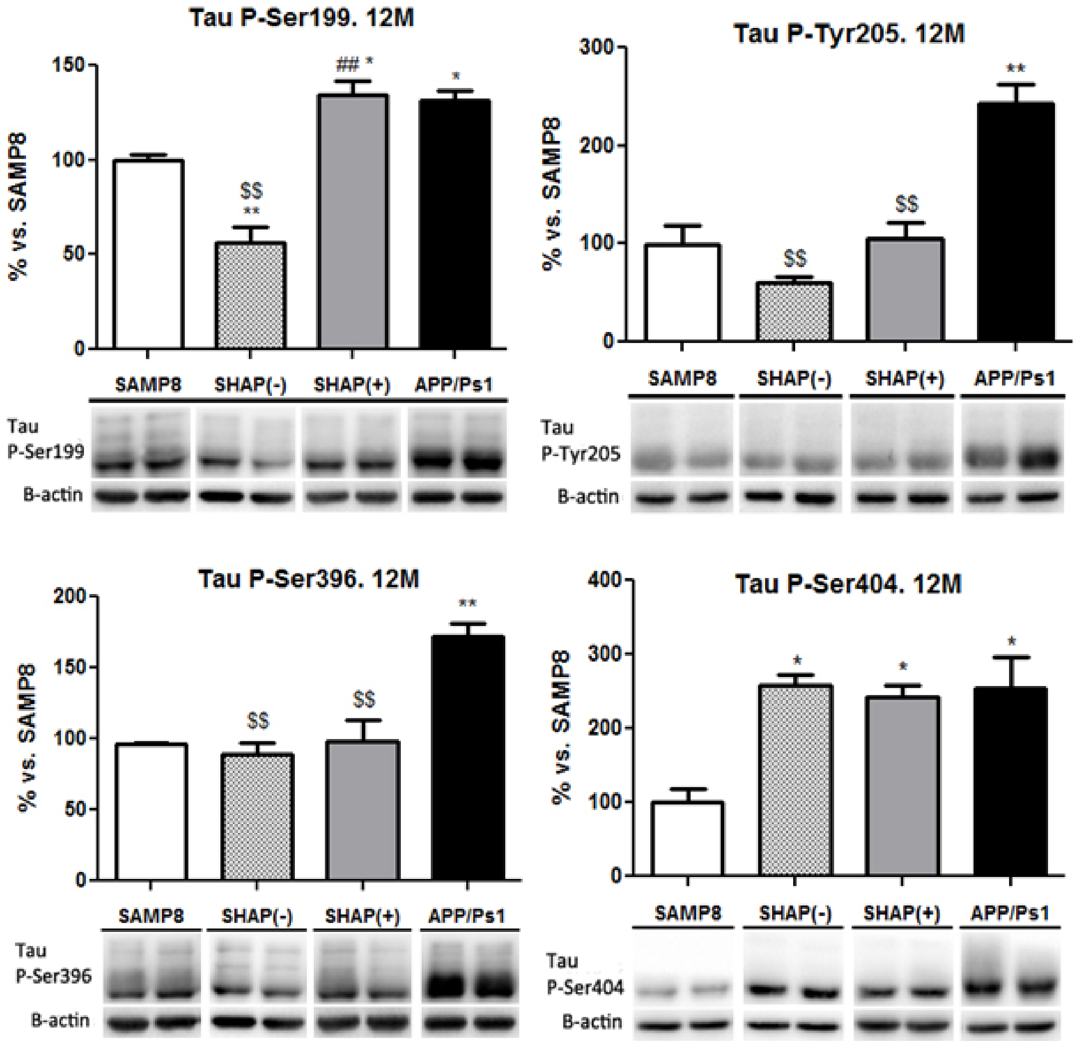


Figure 9. Levels of phosphorylated tau at Ser199, Tyr205, Ser396, and Ser404 epitopes. Bars represent mean ± Standard error of the mean (SEM) and values are adjusted to 100% for levels of Senescence-accelerated mouse prone 8 (SAMP8) mice. Analysis of variance (ANOVA) following Tukey's multiple comparison test: **P* <0.05; ***p* <0.01 vs. SAMP8; $^{\$}p$ <0.05; $^{\$\$}p$ <0.01 vs. APP/PS1; $^{\#}p$ <0.05; $^{\#\#}p$ <0.05 vs. SHAP(-).

Figure 10.

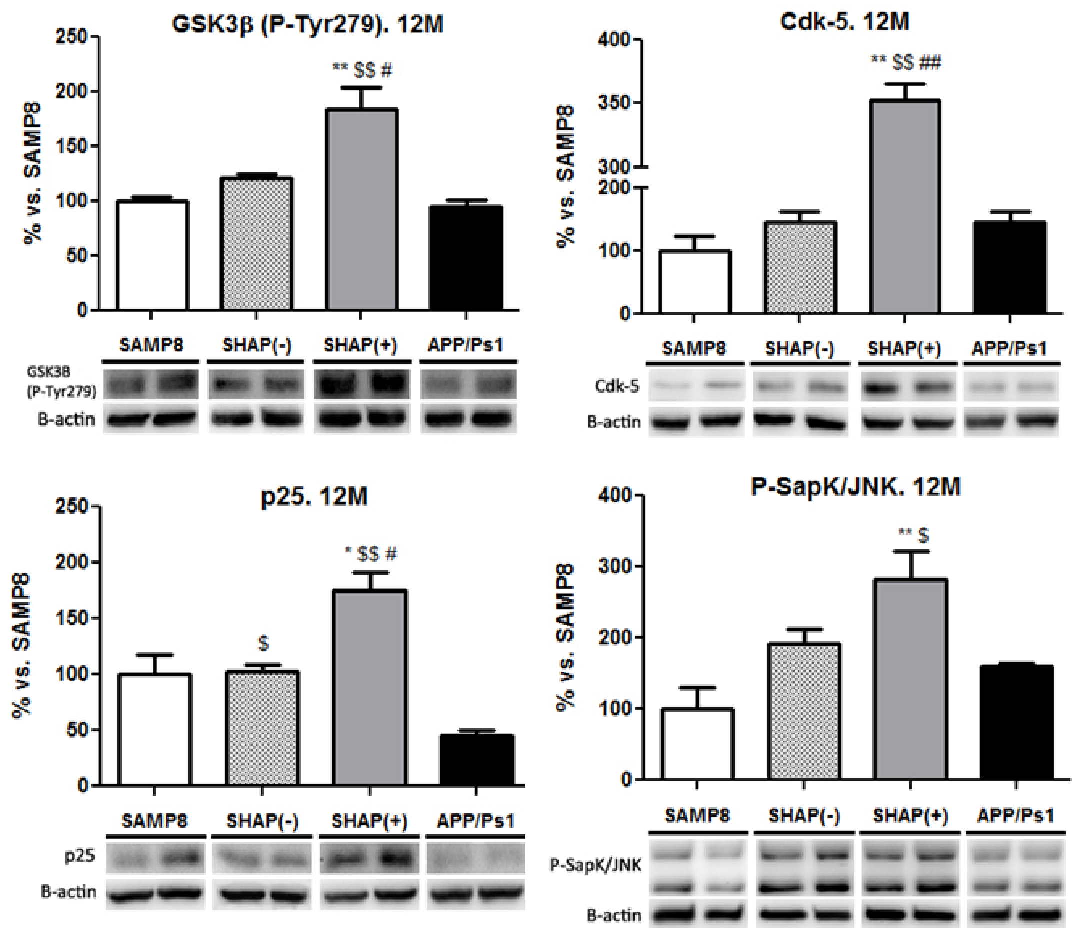


Figure 10. Levels of CDK5, p25, GSK3β, and SAPK/JNK. Bars represent mean ± Standard error of the mean (SEM) and values are adjusted to 100% for levels of Senescence-accelerated mouse prone 8 (SAMP8) mice. Analysis of variance (ANOVA) following Tukey's multiple comparison test: **P* <0.05; ***p* <0.01 vs. Senescence-.accelerated mouse prone 8 (SAMP8); $^{\$}P$ <0.05; $^{\$\$}p$ <0.01 vs. Amyloid precursor protein/Presenilin 1 (APP/PS1); $^{\#}p$ <0.05; $^{\#\#}p$ <0.05 vs. SHAP(-).

DISCUSIÓN

La enfermedad de Alzheimer (EA) supone una amenaza en alza en una población que cada vez tiene una mayor esperanza de vida, y por lo tanto, una población cada vez más envejecida. Encontrar respuestas y profundizar sobre los mecanismos fisiopatológicos implicados en el proceso de envejecimiento cerebral, y en particular los relacionados con la EA, es uno de los retos de la investigación que la sociedad moderna demanda, como se desprende de algunos de los programas de investigación y desarrollo (I+D) de países, fundaciones e instituciones.

Para ello, la investigación básica dispone de diferentes herramientas y sustratos biológicos. En este estudio hemos utilizado dos modelos murinos como herramientas de estudio: SAMP8 y APP/PS1. Los ratones SAMP8 tienen varios de los marcadores histopatológicos y de comportamiento de la EA, incluyendo el deterioro cognitivo. Por otro lado, los APP/PS1 también presentan varios de los marcadores histopatológicos y moleculares de la EA, así como también de comportamiento, pero a diferencia de los ratones SAMP8, éstos presentan placas seniles formadas por Aβ humano.

Muchos estudios demuestran que compuestos polifenólicos como el resveratrol tienen, además de propiedades antioxidantes, capacidad de actuar sobre otros mecanismos moleculares que pueden ayudar a prevenir la degeneración típica de la EA y modular las vías asociadas al envejecimiento (Brisdelli et al., 2009). De este modo, el resveratrol gracias a su acción farmacológica sobre los procesos celulares y moleculares característicos del envejecimiento cerebral, y ligados a la EA, nos permitirá avanzar en los objetivos planteados al inicio de este trabajo.

Efectos del resveratrol en los ratones SAMP8

En esta parte del proyecto se realizaron dos tipos de tratamiento con resveratrol en SAMP8: uno en animales adultos a corto plazo y a dosis bajas (Chang et al., 2012) y otro en animales jóvenes a largo plazo y a dosis altas. Los resultados obtenidos demuestran que una dosis baja de resveratrol (20 mg/kg/día) durante 8 semanas, no consigue mejorar el deterioro cognitivo presente en ratones SAMP8, ni tampoco modifica las vías moleculares que se relacionan con los efectos beneficiosos del resveratol, como la vía de SIRT1. Sin embargo, una dosis alta (160 mg/kg/día) tuvo efectos positivos aumentando la esperanza de vida, mejorando los parámetros cognitivos evaluados y reduciendo los marcadores de envejecimiento y de EA.

En concreto, la dosis alta de resveratrol suplementada en la dieta produjo un incremento en la esperanza de vida tanto en ratones SAMP8 como en ratones SAMR1. Este efecto del resveratrol sobre la longevidad en SAMP8 y SAMR1, tampoco se había descrito en ningún otro tipo de cepa de ratón, por lo tanto, es el primer estudio que demuestra que el resveratrol aumenta la longevidad en ratones normales. Sin embargo, estudios previos han demostrado que el resveratrol previene la mortalidad temprana que sufren los ratones cuando ingieren una dieta alta en grasas, es decir, bajo un estrés metabólico (Baur et al., 2006), pero no afecta a la supervivencia en ratones viejos (Miller et al., 2011).

En organismos inferiores ya se había descrito con anterioridad este incremento de la longevidad por la acción del resveratrol, en concreto por su efecto sobre la actividad de las sirtuinas. Se ha demostrado que la activación de Sir2 (silent information regulator 2), o de su homólogo en vertebrados SIRT1, mediante resveratrol aumenta la esperanza de vida de *Saccharocyces cerevisiae* (Howitz et al., 2003), *Caenorhabditis elegans* (Viswanathan et al., 2005), *Drosophila melanogaster* (Wood et al., 2004) y *Nothobranchius furzeri* (Valenzano et al., 2006).

A pesar de la diversidad de estudios que demuestran un efecto positivo del resveratrol sobre la longevidad, existen discrepancias sobre los mecanismos responsables en organismos de diferente complejidad que todavía no se han explicado. La influencia de factores como el metabolismo interespecífico, variación génica, dieta, actividad física, presencia de enfermedades o estado de salud mental, deben tenerse muy en cuenta cuando se extrapola a diferentes modelos animales, incluyendo roedores (Agarwal and Baur, 2011).

Ya que la vía de las sirtuinas está implicada en los efectos sobre la longevidad previamente descritos, analizamos los niveles proteicos de SIRT1 y el estado de acetilación de uno de sus principales sustratos. La dosis baja durante 8 semanas no produjo cambios en la vía de SIRT1. Ya que según la teoría de los radicales libres, estos son los responsables del envejecimiento y además están relacionados la acumulación de Aβ (Smith et al., 1995) y la generación de procesos inflamatorios, analizamos varias proteínas relacionadas con el estrés oxidativo y la inflamación como MnSOD, PPARα o NFκB p65, pero no se produjeron cambios en ninguna de ellas. Sin embargo, cuando se administraba una dosis alta de resveratrol de forma crónica, se produjo un aumento de los niveles de SIRT1 en SAMP8, tanto en la corteza como en el hipocampo, y esto

correlacionó con una disminución de los niveles de acetilación de p53, uno de los principales sustratos de esta desacetilasa, demostrándose una mayor actividad de SIRT1.

Para profundizar en los cambios observados en la vía de SIRT1, estudiamos otras vías y proteínas que modulan la activación de ésta, en concreto el sensor de disponibilidad energética AMPK. AMPK se activa por fosforilación en Thr^{172} mediada por la cinasa LBK1 en respuesta a un incremento del ratio AMP/ATP o por CAMKKβ en respuesta a niveles elevados de Ca^{2+}. La activación de AMPK genera un aumento de los niveles de NAD^{+}, que actúa como cofactor de SIRT1 incrementando su actividad. Además, se ha descrito que cuando SIRT1 se activa, AMPK se fosforila y también se activa. Recientemente, se ha aportado mucha más información sobre la relación del resveratrol con esta vía, demostrando que la activación de la vía de CamKKβ-AMPK, mediada por resveratrol, se produce a través de la inhibición de fosfodiesterasas específicas de cAMP (Park et al., 2012a; Park et al., 2012b). En nuestro estudio, la dosis alta de resveratrol produjo un aumento de AMPK en el hipocampo y de su forma fosforilada activa en la corteza, lo que indica un efecto positivo sobre la activación de la vía de SIRT1 en ambas áreas.

Numerosos datos apoyan el diseño del estudio y nuestros resultados, ya que cada vez existe una mayor evidencia de una relación entre la activación de SIRT1, AMPK y la EA (Gan, 2007). Por ejemplo, la fosforilación de tau y la producción de Aβ aumentan con la inhibición de AMPK (Greco et al., 2011; Park et al., 2012a, b). Además, está descrito que la activación de SIRT1 previene diversos marcadores de neurodegeneración en ratas (Bayod et al., 2011), protege frente a la degeneración axonal en ratones (Araki et al., 2004), reduce la toxicidad por poliglutamina en *C. elegans* (Parker et al., 2005) y células de neuroblastoma humano (Shin et al., 2013) y disminuye la toxicidad de Aβ mediada por la microglia en cultivo (Chen et al., 2005). Por último, existen estudios en humanos que relacionan SIRT1 con la EA, ya que los pacientes que sufren esta enfermedad presentan una disminución de la expresión de SIRT1, y esta disminución correlaciona con los niveles de hiperfosforilación de tau y de carga de Aβ (Julien et al., 2009).

Además, existen otros estudios que demuestran la eficacia de la suplementación en la dieta con resveratrol sobre marcadores de envejecimiento y EA en SAMP8. En concreto, suplementos dietéticos de mora, una fruta que contiene gran cantidad de resveratrol, mejoran el deterioro cognitivo y disminuyen la acumulación de Aβ en SAMP8, pero también incrementan la capacidad antioxidante en estos animales a través la vía del elemento de respuesta antioxidante (ARE)-Nrf2

en hígado y cerebro (Shih et al., 2010). También se ha descrito que el resveratrol mejora los problemas de memoria y preserva la función cognitiva en ratones viejos (Oomen et al., 2009) y en modelos transgénicos de EA y ELA (Kim et al., 2007). En nuestro estudio, y en concordancia con los estudios previos mencionados, la dosis alta de resveratrol previno el deterioro cognitivo característico de la cepa SAMP8. Cabe mencionar, que a pesar de que se hayan publicado resultados contradictorios en lo que respecta a las alteraciones de memoria en los SAMP8 (Spangler et al., 2002), muestro estudio ha demostrado la presencia de estos déficits cognitivos a los 9 meses de edad en SAMP8, y el resveratrol ha sido capaz de prevenirlos.

Estos efectos beneficiosos del resveratrol sobre la memoria pueden ser debidos, en parte, una disminución de la acumulación de Aβ, al menos en nuestro modelo. La explicación de este hecho es que SIRT1 es capaz de disminuir la acumulación de Aβ mediante la modulación de las α-secretasas (ADAM10). La transcripción de ADAM10 está regulada por el receptor de ácido retinoico (RAR) que es activado por su ligando, el ácido retinoico, o mediante desacetilación a través de SIRT1. Esto se ha demostrado en una amplia diversidad de estudios. Por ejemplo, mediante transgénicos y *knockouts* de SIRT1 se ha demostrado que esta proteína activa el factor de transcripción RARb, y éste hace que aumente la expresión de ADAM10 (Lichtenthaler, 2011). También se ha demostrado que la activación de SIRT1 reduce la patología amiloide en modelos de EA, y cruces de *knockouts* de SIRT1 con estos ratones incrementa la carga de Aβ (Donmez et al., 2010). En nuestro paradigma experimental, una dosis alta de resveratrol redujo la carga de Aβ en el cerebro de los ratones SAMP8 y aumentó los niveles de ADAM10, probablemente debido a un aumento en su expresión. Este efecto parece específico sobre la vía no amiloidogénica, ya que no se han observado cambios en otras secretasas como β-secretasa (BACE1). De este modo el resveratrol, a través de la activación de SIRT1, induce la activación de la vía no amiloidogénica de procesamiento de APP murino, reduciendo los depósitos de amiloide previamente descritos por Del Valle et al., (2010).

Por último, la hiperfosforilación de tau, otro marcador de la EA, está mediada por varias cinasas. Diversos autores han demostrado que la fosforilación aberrante de tau en el cerebro de ratones SAMP8 es producida por la activación de cinasas como CDK5, GSK3β y JNK (Canudas et al., 2005; Chang et al., 2012). Nuestros resultados demuestran que una dosis alta de resveratrol produce una disminución de la actividad CDK5 y GSK3β, las principales tau cinasas implicadas en la EA, en la corteza de los ratones SAMP8. En congruencia con esto, también hemos observado una

disminución de la fosforilación de tau en Ser^{396}, uno de los epítopos que fosforilan las cinasas mencionadas anteriormente y que está implicado en la EA, las taupatías y el envejecimiento. Sin embargo, ni esta dosis, ni tampoco la dosis baja de resveratrol produjo cambios en JNK. La dosis baja de resveratrol, durante 8 semanas en ratones jóvenes, no produjo una reducción de la hiperfosforilación de tau, cuantificada en este caso con el marcador PHF (del inglés *paired helical filaments,* y analizado mediante un anticuerpo anti-PHF-1, que reconoce fosforilaciones en los residuos Ser^{396} y Ser^{404}). Probablemente, estas discrepancias se deben a las diferentes dosis de resveratrol y al tiempo de tratamiento (corto y largo plazo) de los dos estudios. De hecho, el pterostilbeno, un derivado del resveratrol, a una dosis baja en ratones SAMP8 adultos durante 8 semanas de tratamiento sí produjo una inhibición de JNK, a pesar de no haberse observado cambios en la hiperfosforilación de tau en la corteza (Chang et al., 2012). La explicación a este hecho, es que pterostilbeno puede haber presentado una mayor eficacia por su mejor biodisponibilidad a nivel cerebral, que implicaría que a una misma dosis administrada, la concentración en cerebro de pterostilbeno sea mayor que la concentración de resveratrol a la misma dosis.

En el hipocampo, aunque la dosis alta de resveratrol fue capaz de disminuir la fosforilación de tau, no encontramos cambios en las cinasas estudiadas. Una hipótesis plausible sería que la actividad de estas cinasas haya estado inhibida a tiempos intermedios del tratamiento, y que por la cronicidad de éste se haya sobrepasado la capacidad del resveratrol de inhibirlas en estadíos de senescencia tan avanzada. A pesar de esto, en el momento del sacrificio todavía se observaron los efectos de la inhibición previa de estas cinasas por el resveratrol sobre la fosforilación de tau. Tampoco cabe descartar la posibilidad de que estuvieran implicadas otras cinasas o fosfatasas que no han sido objeto de este estudio.

En conclusión, una dosis baja de resveratrol en la dieta, administrada en un corto plazo de tratamiento en animales adultos, no produce una mejora cognitiva, no activa la vía de SIRT1 y no mejora marcadores de la EA como la hiperfosforilación de tau. Además, tampoco modula las vías implicadas en el estrés oxidativo y la inflamación. Sin embargo, una dosis alta administrada en edades tempranas y a largo plazo, aumenta la esperanza de vida, previene el deterioro cognitivo y mejora la patología amiloide y tau presente en los ratones SAMP8.

Efecto del resveratrol en el modelo de EA familiar APP/PS1

En esta parte del proyecto se realizó un tratamiento con resveratrol en ratones APP/PS1 adultos a largo plazo y a una dosis alta (160 mg/kg/día), dado que fue el protocolo de tratamiento que había dado mejores resultados en la cepa SAMP8.

Los ratones APP/PS1 son un modelo transgénico, aceptado y ampliamente descrito de EA, que expresan una proteína quimérica murina/humana mutada de APP (Mo/Hu APP695swe) y una PS1 humana mutada (PS1-dE9). Ambas producen placas seniles a partir de los 3 meses e incrementan en número y distribución con el paso del tiempo, llevando a una rápida progresión de la enfermedad. También producen un aumento de los niveles de $A\beta_{1\text{-}42}$ y $A\beta_{1\text{-}40}$ solubles en cerebro, aumentando la ratio 1-40/1-42 conforme avanza la edad. La deposición de amiloide en placas viene acompañada por alteraciones en la mitocondria, aumento del estrés oxidativo y modificación post-traduccional aberrante de proteínas, que provocan en última instancia su acumulación en las neutritas distróficas que rodean las placas (Aso et al., 2012).

Los resultados indicaron, de la misma forma que en los ratones SAMP8 tratados con una dosis alta resveratrol, una mejora de la memoria a corto plazo determinada mediante el NORT, un test ampliamente descrito que se utiliza para evaluar el aprendizaje y la memoria (Aso et al., 2012; Antunes and Biala, 2012). Las zonas del cerebro involucradas en este test son principalmente el hipocampo y la corteza perirrinal. Además, el NORT no requiere de ninguna motivación externa, recompensa o castigo, evitando así interferencias debido a la ansiedad que puedan sufrir los ratones como consecuencia del test.

Dado que el resveratrol prevenía la pérdida cognitiva en los APP/PS1, se valoraron los niveles de sinaptofisina como medida de preservación neuronal. La sinaptofisina, una glicoproteína integral de membrana presente en las vesículas presinapticas, es utilizada habitualmente como marcador de sinapsis. En nuestro caso, los APP/PS1 tratados con resveratrol presentaron un aumento de los niveles de sinaptofisina, indicando una posible acción neuroprotectora sobre las neuronas y las terminaciones sinápticas en particular, que correlaciona con la mejora de la memoria a corto plazo.

La menor pérdida neuronal observada podría ser debida a la disminución de placas seniles, que como es sabido, generan radicales libres, quimiocinas y otros factores que causan la muerte neuronal. Por esta razón, se cuantificó la presencia de placas seniles mediante la tinción de tioflavina. Los APP/PS1 tratados con resveratrol mostraron una disminución en el número de placas seniles, que como hemos mencionado anteriormente, podría explicar la prevención de la pérdida cognitiva y la preservación neuronal descrita en estos ratones tras el tratamiento con resveratrol. Esta reducción en la patología de placas no parece ser debida a una alteración de la vía de procesamiento del APP a través de la vía no amiloidogénica, ya que no se han encontrado cambios ni en APP, ni en su fragmento C83. Esto concuerda con los resultados obtenidos por Karuppagounder y colaboradores (2009) en ratones Tg19959 tratados con resveratrol. Sin embargo, sí se produjo una disminución de los niveles de BACE1, lo que en principio conlleva una alteración de la vía amiloidogénica y una consecuente reducción de la carga de amiloide en los APP/PS1 tratados con resveratrol.

A pesar de que la alteración de la vía de las secretasas es un factor importante en la producción de amiloide, y en consecuencia en la formación de placas, también existen mecanismos de eliminación que podrían estar actuando de forma simultánea, favoreciendo la reducción de placas. Diversos estudios avalan este hecho, por ejemplo, hay evidencias que indican una disminución de la actividad proteosomal tanto los cerebros de pacientes de EA (Keller et al., 2000; López Salon et al., 2000) como en cerebros de modelos murinos de EA, incluyendo los ratones transgénicos APP/PS1 (Aso et al., 2012; Van Tijn et al., 2012). En nuestro paradigma experimental podría haber un incremento de la actividad proteosomal que estuviera ayudando a reducir el número de placas, ya que está descrito que el resveratrol promueve la eliminación de Aβ *in vitro* incrementando la actividad proteosomal sin afectar a las enzimas productoras (β-secretasa y γ-secretasa) (Marambaud et al., 2005). Por lo tanto, este mecanismo de degradación proteosomal inducido por el resveratrol puede haber actuado de forma sinérgica junto a la reducción de BACE1 (a pesar de que también se observó una reducción de ADAM10), explicando así la disminución de placas observada en el tratamiento con resveratrol.

Otro de los factores que promueve la generación y agregación de Aβ es el estrés oxidativo. Es ampliamente conocido que el resveratrol tiene propiedades antioxidantes (Dasgupta and Milbrandt, 2007), por lo tanto, es posible que el resveratrol pudiera reducir las placas seniles a través de su efecto sobre la maquinaria antioxidante de la célula. Por ello, se cuantificaron los

niveles proteicos de MnSOD y catalasa, dos de los enzimas relacionados con la detoxificación de radicales libres, pero no los únicos. Aunque no se observaron cambios en ninguno de ellos, no se puede descartar que la acción neuroprotectora del resveratrol, en el protocolo de tratamiento utilizado, no implique su conocida acción antioxidante. Respecto a esto, tampoco se observaron cambios en la expresión de genes relacionados con el estrés oxidativo como *Nfe2l2* y *Hmox*, genes que además están implicados en mecanismos de neuroprotección (Son et al., 2013; Quincozes et al., 2014). Por lo tanto, nuestros resultados indican que a este tiempo de tratamiento no se observa una acción del resveratrol sobre la maquinaria antioxidante de estos ratones. Sin embargo, la acción antioxidante del resveratrol puede haberse producido a tiempos más tempranos, como ya ha sido descrito por Karuppagounder y colaboradores (2009), y por tanto los efectos protectores hayan perdurado.

Por último, la inflamación, otro factor que además está relacionado con el estrés oxidativo, también promueve la generación y agregación de Aβ. Por ello, se valoraron algunos genes proinflamatorios como *Il1b* y *Tnf*, los cuales codifican para las citocinas IL1β y TNFα. Sorprendentemente, se determinó un ligero aumento de la expresión de ambos, ya que lo esperado hubiera sido una reducción de este tipo de citocinas. Sin embargo, este resultado no es descabellado, ya que diversos estudios indican que la acumulación de Aβ está íntimamente relacionada con el sistema inmune, y puede ser modulada por éste (Serpente et al., 2014) a través de la activación de la astroglia y la microglia. Ambas producen citocinas que favorecen la fagocitosis y la eliminación de Aβ y APP. Esto da pie a la hipótesis de que un pequeño incremento en las citocinas proinflamatorias puede resultar beneficioso, ya que eliminaría el péptido Aβ y consecuentemente reduciria la carga de placas seniles en el cerebro. En resumen, el tratamiento a largo plazo con resveratrol parece inducir un control preciso de la inflamación, incrementando ligeramente los niveles de IL1β y TNFα, y dando lugar a un proceso hormético que produce una reducción en el número de placas seniles y una mejora de la memoria.

No hay que olvidar que el resveratrol es un activador de SIRT1, y por ello, todos los efectos anteriormente descritos pueden haber sido mediados por la activación de esta vía. En este estudio, y a diferencia de lo observado en los SAMP8 tratados con resveratrol, se observó una disminución de los niveles de SIRT1, que sin embargo iba acompañada de una disminución de la acetilación de su principal sustrato p53. Este resultado se encuentra en consonancia con resultados de otros grupos en los que han utilizado modelos transgénicos y experimentos *in vitro* y

han observado una reducción de la patología de placas sin producirse un incremento en los niveles de SIRT1 (Karuppagounder et al., 2009), pero produciéndose una desacetilación de sustratos como PGC1α (Lagouge et al., 2006). La disminución de los niveles proteicos SIRT1, junto con la desacetilación de p53, parece indicar una reducción de la síntesis de esta desacetilasa, pero produciéndose un incremento de su actividad por interacción directa con el resveratrol (Kaeberlein et al., 2005; Della-Morte et al., 2009; Du et al., 2013) o por la regulación de la vía LKB/AMPK (Zang et al., 2006; Dasgupta and Milbrandt, 2007; Hwang et al., 2007). De hecho, en nuestro estudio el resveratrol disminuyó los niveles proteicos de SIRT1, pero activó la vía de LKB/AMPK, lo cual es consistente con lo mencionado anteriormente. En resumen, es probable que el resveratrol aumente la actividad de SIRT1 y reduzca sus niveles proteicos debido a un mecanismo compensatorio que trata de mantener la actividad SIRT1 en niveles basales.

La mayoría de las vías mencionadas anteriormente están relacionadas con la función mitocondrial, tal y como se ha presentado en la introducción de esta memoria. Además, AMPK y SIRT1 actúan como sensores energéticos de AMP y NAD^+, respectivamente, y AMPK en concreto está involucrada en la regulación energética de la homeostasis metabólica (Hardie et al., 2012). De acuerdo con esto, el mismo patrón de administración de resveratrol normaliza la función mitocondrial y aumenta la biogénesis mitocondrial en la medula espinal de ratones $SOD1^{G93A}$, un modelo de esclerosis lateral amiotrofica (Mancuso et al., 2014). Por todo ello, decidimos analizar la acción del resveratrol sobre la funcionalidad mitocondrial en los ratones APP/PS1. Un marcador de funcionalidad mitocondrial, que además está disminuido en la EA, es el complejo IV de la cadena respiratoria mitocondrial (MCIV) (Nunomura et al., 2006; Sultana et al., 2011). Los resultados revelaron un incremento en el MCIV en los APP/PS1 tratados con resveratrol. Este resultado apunta otro posible mecanismo a través del cual el resveratrol ejerce su efecto neuroprotector en estos ratones.

En conclusión, este estudio nos indica las posibles vías responsables de la acción neuroprotectora del resveratrol en ratones APP/PS1, un modelo de EA familiar, entre ellas la vía de SIRT1 y AMPK, junto con la funcionalidad mitocondrial y el proceso inflamatorio. Nuestro estudio, en concordancia con otros estudios (Wang et al., 2006; Karuppagounder et al., 2009), demuestra que la aparición de la EA puede ser retrasada o mitigada utilizando suplementos con resveratrol en la dieta, ya que es capaz de proteger contra la formación de placas seniles y retrasar o incluso evitar el deterioro cognitivo.

Caracterización de un nuevo modelo de EA

Los modelos murinos de EA están restringidos a la expresión de proteínas humanas mutadas relacionadas con la EA familiar, que representa únicamente el 5% de los casos de EA. Hasta la fecha, no hay modelos murinos extrapolables a la EA esporádica, la más frecuente, y que además está asociada con la edad. Por ello, utilizando los modelos actuales es difícil establecer la cronología de eventos que hacen desencadenar esta enfermedad. Conseguir un modelo que refleje tanto los marcadores característicos de la EA, como la cronología de eventos ligados al envejecimiento que la producen, puede suponer un gran paso a la hora de elaborar terapias preventivas para una enfermedad que hasta la fecha sigue siendo incurable. La combinación de modelos murinos ya existentes mediante su cruce podría ser una estrategia útil y sencilla para generar nuevos modelos con características de ambos modelos progenitores.

Uno de los posibles modelos a utilizar sería la cepa de ratón SAMP8, ya que muestran varias características a nivel cerebral similares a las de la EA, por ejemplo, un aumento del estrés oxidativo, alteraciones de Aβ, hiperfosforilación de tau y déficits cognitivos (Takeda, 2009; Morley et al., 2012). Por lo tanto, los ratones SAMP8 pueden ser un excelente modelo para estudiar cambios previos relacionados con la EA y dar una visión más amplia de la enfermedad. Sin embargo, los SAMP8 no presentan acumulación de Aβ en forma de placas seniles, que es una de las características más importantes que define a la EA en humanos. Por esto, otro de los posibles modelos a utilizar serían los ratones APP/PS1, ya que presentan esta acumulación de Aβ en forma de placas seniles (García-Alloza et al., 2006).

Recientemente, Lok y colaboradores (2013) cruzaron ratones APP/PS1 con ratones SAMP8, generando un nuevo modelo de EA que expresa las proteínas humanas mutadas APP y PS1 en presencia de un fondo génico de senescencia acelerada. En nuestro trabajo hemos aplicado la misma estrategia, generando una nueva cepa a la que hemos denominado SHAP(+). El objetivo fue caracterizar mejor esta nueva cepa a nivel bioquímico y molecular en cuanto a marcadores neuropatológicos de la EA.

Nuestros resultados indican que los SHAP(+) tienen déficits en la memoria a corto plazo similares a los que presentan los SAMP8 y los APP/PS1 a edades avanzadas, y que concuerda con el deterioro en la memoria espacial y de trabajo descrito por Lok y colaboradores (2013). Además, a edades

avanzadas, los SHAP(+) presentaron un incremento de placas seniles en comparación con los APP/PS1. También se observó un aumento en los niveles de APP en los SHAP(+) hasta los 9 meses de edad, probablemente debido al genotipo SAMP8, en el que se ha descrito alteraciones en la transcripción y procesamiento de APP (Kumar et al., 2009). Sin embargo, a los 12 meses los niveles de APP fueron inferiores respecto a los APP/PS1, probablemente a causa de una mayor escisión y a su acumulación en forma de placas seniles, que como se ha mencionado anteriormente, incrementaron en los SHAP(+).

Debido a que encontramos una exacerbación de la patología de placas y cambios en los niveles APP en los SHAP(+), se estudiaron las secretasas implicadas en el procesamiento amiloidogénico del APP. Los SHAP(+) no presentaron cambios en los niveles de BACE1 respecto a los SAMP8. Por lo tanto, el factor clave en la formación de placas en los SHAP(+) y los APP/PS1 es la expresión de los transgenes, más que la presencia de niveles elevados de BACE1. En lo que respecta a PS1, su forma no proteolizada (48-52 kDa) es un zimógeno que se activa por endoproteólisis (Fukumori et al., 2010; Li et al., 2000), generando un fragmento N-terminal (NTF) (27-28 kDa) y otro C-terminal (CTF) (16-18 kDa), los cuales forman un heterodímero NTF-CTF enzimáticamente activo (Chávez-Gutiérrez et al., 2008; Ahn et al., 2010; Wolfe, 2013). La eliminación del exón 9 en la mutación PS1-DE9 no requiere la endoproteolisis de PS1 para su activación, ya que a diferencia de la forma no mutada, el centro catalítico de la enzima ya está expuesto (Knappenberger et al., 2004). En consecuencia, encontramos un aumento de uno de los framentos activos (CTF) en los ratones SAMP8 y SHAP(-) respecto a los ratones que presentaban la mutación. Sin embargo, los niveles de este fragmento se normalizaron con la edad. Por lo contrario, los niveles de la holoproteína PS1 fueron similares en todos los grupos experimentales hasta los 9 meses, pero aumentó en los ratones SHAP(+) a los 12 meses en comparación con los ratones APP/PS1, lo que concuerda con la mayor deposición de Aβ en forma de placas seniles. Todo esto indica que PS1 puede ser una diana importante a la hora de estudiar nuevos fármacos para tratar la EA, ya que sería más interesante centrarse en inhibidores de PS1 en vez de en inhibidores de BACE1 (Gandy and DeKosky, 2013).

Por otra parte, la hiperfosforilación de tau es otro de los factores que también puede exacerbar la formación de placas seniles. De esta forma, la presencia de una mayor fosforilación puede ser la causa de un aumento de la patología amiloide (Guo et al., 2013). Por lo tanto, para determinar un posible nexo entre la amiloidosis y la patología tau en los ratones SHAP(+), estudiamos la fosforilación de tau en varios epítopos en comparación con los ratones SAMP8 y APP/PS1, ya que

ambos presentan una alteración en los procesos de fosforilación de tau (Aso et al., 2012; Canudas et al., 2005). Por ello, hemos analizado cuatro epítopos fosforilados de tau, sin embargo, no se han observado cambios ni correlación entre los niveles de fosforilación y las diferentes cepas a edades tempranas y medias. Por el contrario, a 12 meses de edad los ratones APP/PS1 y SHAP(+) presentaron una mayor fosforilación en tau Ser^{199} y Ser^{404} en comparación con los SAMP8. En resumen, los resultados no mostraron una correlación específica entre tau y la patología amiloide hasta los 12 meses, donde la patología ya está bien establecida como demuestran nuestros resultados y la amplia bibliografía publicada sobre APP/PS1 y SAMP8 (Lok et al., 2013a, b).

Para confirmar la ausencia de diferencias en el patrón de fosforilación entre las distintas cepas a edades tempranas, y dar explicación a la hiperfosforilación observada en los SHAP(+) a los 12 meses de edad, se realizó un estudio de las cinasas implicadas en la fosforilación de tau en los epítopos estudiados. En concordancia con esto, se observó una ausencia de activación diferencial de CDK5, GSK3β y JNK hasta los 9 meses. Sin embargo, a 12 meses de edad los SHAP(+) mostraron cambios en el patrón de activación de estas cinasas, que correlacionan con la hiperfosforilación de tau observada a esta edad. Este hecho concuerda con la literatura, que describe a tau como sustrato de CDK5, GSK3β y JNK (Iqbal et al., 2011; Casadesús et al., 2012; Díaz-Moreno et al., 2013). Además, se observó un aumento en los niveles de un activador de CDK5 llamado p25, y que como está descrito, aumenta la fosforilación de tau en epítopos específicos mediada por la sobreactivación de esta cinasa (Currais et al., 2014).

Por último, estudiamos el estrés oxidativo, ya que es un factor patogénico bien establecido en la EA, y la asociación de éste con la patología amiloide y tau es bien conocida (Mondragón-Rodríguez et al., 2014). Es más, está descrito que ambos modelos utilizados en este estudio como progenitores presentan un desequilibrio redox. Los resultados indicaron que los SHAP(+) no presentan diferencias en los niveles de algunas de las proteínas relacionadas con la homeostásis del estrés oxidativo como catalasa, MnSOD y óxido nítrico sintasa (NOS). Sin embargo, mediante técnicas inmunohistoquímicas observamos astrogliosis y microgliosis tanto en ratones APP/PS1 como en SHAP(+) en corteza e hipocampo, indicando que tanto los ratones APP/PS1 como los SHAP(+) pueden tener cierto grado de estrés oxidativo. Además, como era de esperar, la astroglia reactiva se localizó cerca de las placas seniles tanto en APP/PS1 como en SHAP(+), indicando el que el estrés oxidativo u otro tipo de daño sucede principalmente alrededor de las placas.

En conclusión, la sobreexpresión de las proteínas humanas mutadas APP y PS1 en presencia de un fondo génico de senescencia acelerada, genera un modelo de EA ligado a la senescencia. En este modelo la activación de PS1 tiene más relevancia en la patología amiloide que la de BACE1. Además, estos animales presentan una exacerbación del proceso amiloidogénico y un aumento de la actividad de las cinasas implicadas en la hiperfosforilación de tau a edades tardías, manteniendo el deterioro cognitivo y algunos de los marcadores patológicos típicos de la EA.

CONCLUSIONES

1.- Una dosis baja de resveratrol en ratones SAMP8 adultos no produce una mejora en la memoria a corto plazo, pero sí lo hace el pterostilbeno, un derivado más biodisponible.

2.- El tratamiento a dosis bajas de resveratrol o pterostilbeno en ratones SAMP8 adultos no modifica los niveles proteicos o la actividad de SIRT1.

3.- El tratamiento a dosis bajas de resveratrol en ratones SAMP8 adultos tampoco mejora la taupatía, pero sí lo hace el pterostilbeno.

4.- El tratamiento a dosis bajas de pterostilbeno, pero no de resveratrol, modifica la expresión de marcadores de estrés oxidativo e inflamación en ratones SAMP8 adultos.

5.- Una dosis alta y crónica de resveratrol aumenta la esperanza de vida en ratones SAMP8 y SAMR1.

6.- Una dosis alta y crónica de resveratrol en ratones SAMP8 y APP/PS1 jóvenes produce una mejora en la memoria a corto plazo y una activación de la vía de SIRT1, aumentando la actividad AMPK y reduciendo la carga amiloide.

7.- En la corteza de ratones SAMP8 tratados a largo plazo con una dosis alta de resveratrol, se produce una disminución de la taupatía, que correlaciona con una menor activación de CDK5 y GSK3β. Sin embargo, a pesar de que en el hipocampo también se produce una reducción de la taupatía, no se observaron cambios en ninguna de las cinasas estudiadas a esa edad.

8.- Una dosis alta y crónica de resveratrol en ratones APP/PS1 produce un incremento moderado de la expresión de IL1β y TNFα, acompañado de un aumento en los niveles proteicos del complejo IV de la cadena respiratoria mitocondrial, que puede deberse a una acción beneficiosa del resveratrol por reajuste homeostático.

9.- El modelo murino SHAP(+) generado presenta una exacerbación de la patología amiloide y tau, manteniendo el déficit en la memoria a corto plazo presente en ambas cepas progenitoras.

10.- El incremento de la patología amiloide en los SHAP(+) está acompañado de una mayor activación de PS1 respecto a su progenitor APP/PS1.

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