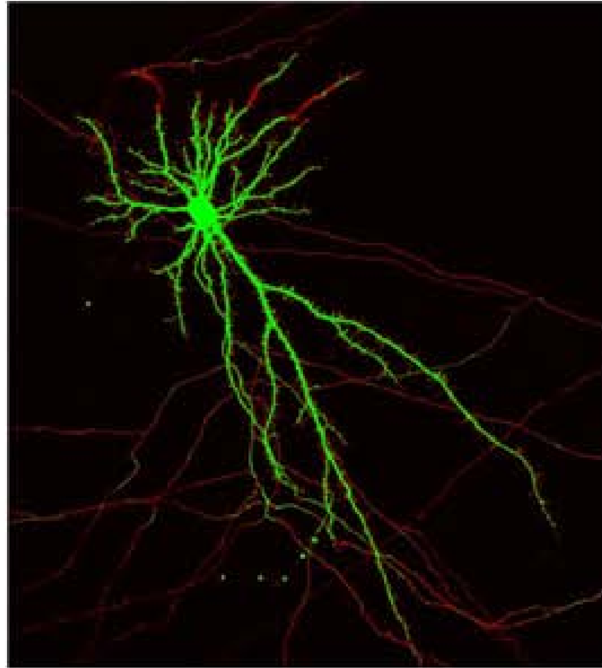

EFECTO DE LAS MANIPULACIONES GENÉTICAS Y FARMACOLÓGICAS SOBRE LA ACTIVIDAD DEL COMPLEJO GAMMA-SECRETASA

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Hospital de la Santa Creu i Sant Pau
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Laboratori Alzheimer

Efecto de las manipulaciones genéticas y farmacológicas sobre la actividad del complejo gamma-secretasa

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I ÍNDICE ***1***

II. ABREVIATURAS ***3***

III. INTRODUCCIÓN ***7***

1. ANATOMÍA-PATOLÓGICA DE LA ENFERMEDAD DE ALZHEIMER 8

1.1 Hallazgos macroscópicos 8

1.2 Hallazgos microscópicos 8

1.2.1 Placas de amiloide 8

1.2.2 Ovillos Neurofibrilares 9

1.2.3 Angiopatía amiloide 10

1.2.4 Pérdida neuronal y sináptica 11

1.2.5 Otros procesos 13

1.2.5.1 Estrés oxidativo 13

1.2.5.2 Inflamación 14

2. GENÉTICA DE LA ENFERMEDAD DE ALZHEIMER: 16

2.1 Gen de la Proteína Precursora de Amiloide (*APP*)............................... 16

2.2 Genes de las presenilinas (*PS*) 21

2.3 Gen *APOE*.. 22

2.4 Otros genes (*APOJ*, *SORL1*) 23

3. FISIOPATOLOGÍA DE LA ENFERMEDAD DE ALZHEIMER 24

3.1 Hipótesis de la cascada amiloide 24

3.1.1 Péptido β-amiloide y procesamiento de APP 26

3.2 Papel de la proteína Tau 29

3.3 Secretasas 29

3.3.1 α-secretasa 29

3.3.2 β-secretasa 30

3.3.3 γ-secretasa 31

3.3.3.1 Presenilinas 32

3.3.3.2 Nicastrina, Aph1 y Pen-2 33

3.3.3.3 Formación/ensamblaje del complejo γ-secretasa 34

3.3.3.4 Substratos de la γ-secretasa 35

3.3.3.5 Inhibidores de secretasas 36

* Inhibidores β-secretasa 36

* Inhibidores γ-secretasa 37

3.3.3.6 Moduladores γ-secretasa (AINEs) 38

3.4 Influencia del colesterol en la generación de Aβ (Estatinas) 39

IV. OBJETIVOS 43

V. MATERIAL Y MÉTODOS 47

1. Análisis por Inmunoabsorción Ligado a Enzimas (ELISA) 47

1.1 ELISA de Aβ40-42 y Aβ1-x total 47

2. Mutagénesis Dirigida 48

3. Microscopía Confocal (FRET y FLIM) 49

3.1 *Fluorescence Resonance Energy Transfer* (FRET) 50

3.2 *Fluorescence Lifetime Imaging Microscopy* (FLIM) 51

3.3 *Photobleaching* 53

3.4 Anticuerpos y protocolos inmunocitoquímica 53

VI. RESULTADOS 55

CAPÍTULO 1. *Mild cholesterol depletion reduces amyloid-β production by impairing APP trafficking to the cell surface* 57

CÁPITULO 2. *Clinical, Neuropathologic, and Biochemical Profile of the Amyloid Precursor Protein I716F Mutation* 73

VII. DISCUSIÓN 85

CAPÍTULO 1 85

CAPÍTULO 2 91

VIII. CONCLUSIONES 99

IX. BIBLIOGRAFÍA 103

X. ANEXO 145

II. ABREVIATURAS

AAC	Angiopatía Amiloide Cerebral
Aβ	β-amiloide
ADAM	*A Disintegrin And Metalloproteases*
AICD	*APP Intracellular Domain*
AINEs	Anti-Inflamatorios No Esteroideos
Aph-1	*Anterior Pharnyx defective-1*
APLP	*Amyloid β Precursor-Like Protein*
APOE	Apolipoproteína E
APOJ	Apolipoproteína J
APP	Proteína Precursora de Amiloide
BACE-1	*β-site APP cleaving enzyme*
CA	*Cornu Ammonis*
CBF1	*Centromere-Binding Factor*
CERAD	*Consortium to Establish a Registry for Alzheimer's Disease*
CHO	*Chinese Hamster Ovary cell*
CLU	*Clusterin* o Apolipoproteina J
COX	Ciclo-oxigenasa
CR1	Receptor del Complemento 1
CT-B	*Cholera Toxin Subunit B*
CTF	Fragmentos C Terminales
C83	Fragmento C terminal de 83 aminoácidos
C99	Fragmento C terminal de 99 aminoácidos
DAPT	*N-[N-(3,5-Difluoro-phenacetyl)-L-Alanyl]-(S)-Phenylglycine T-butyl ester*
DCL	Deterioro Cognitivo Ligero
DFT	Demencia Frontotemporal
DTM	Dominios Transmembrana
EA	Enfermedad de Alzheimer
EAF	Enfermedad de Alzheimer Familiar
EGF	*Epidermal Growth Factor*
ELISA	Análisis por Inmunoabsorción Ligado a Enzimas
FLIM	*Fluorescence Lifetime Imaging Microscopy*
FPP	*Farnesyl PyroPhosphate*
FRAP	*Fluorescence Recovery Acceptor photobleaching*
FRET	*Fluorescence Resonance Energy Transfer*
GGPP	*GeranylGeranyl Pyrophosphate*
GSK-3	*Glycogen synthase Kinase 3*
HCHWA-D	*Hereditary Cerebral Hemorrhage with Amyloidosis, Dutch Type*
HES-1	*Hairy/Enhancer of Split*
HMG Co-A	Hidroxi-Metil Glutaril CoA
HRP	*Horseradish Peroxidase*
I-CliPs	*Intramembrane Cleaving Proteases*
IL	Interleuquina
iNOS	Óxido Nítrico Sintasa Inducible
LCR	Líquido Cefaloraquídeo
LDL	*Low Density Lipoprotein*
LRP	*Low-density- lipoprotein- receptor- related-Protein*
LTP	*Long-Term Potentiation*

MAPT	*Microtubule Associated Protein Tau*
MVB	*Multi-vesicular bodies*
MβCDX	*Methyl-β Cyclodextrin*
NICD	*Notch IntraCellular Domain*
NINCS-ADRA	*National Institute of Neurologic and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association*
NMDA	N-Metil-D-Aspartato
NTF	*N-Terminal Fragment*
PBS	*Phosphate Buffer Saline*
PCR	Reacción en Cadena de la Polimerasa
PFs	Proteínas Fluorescentes
Pen-2	*Presenilin Enhancer-2*
PHF	*Paired Helical Filaments*
PICALM	*Phosphatidylinositol binding Clathrin Assembly protein*
PPARα	*Peroxisome Porliferator Response Element α*
PS	Presenilina
sAPP	*Soluble Amyloid Precursor Protein*
SORL1	*Sortilin Related Receptor L type 1*
Su(H)	*Supressor of Hairless*
TGF-β	*Transforming Growth Factor β*
TGN	*Trans Golgi Network*
TNF-α	*Tumor Necrosis Factor α*
TSA	*Transition-State Analogue inhibitors*
wt	*Wild Type*

INTRODUCCIÓN

III. INTRODUCCIÓN

La enfermedad de Alzheimer (EA) es la forma más común de demencia y es responsable del 50-60% de todos los casos [1]. Esta patología afecta a un 2% de la población mundial, y el riesgo de padecerla se incrementa exponencialmente a partir de los 65 años. Predicciones recientes estiman que el número de pacientes con EA se multiplicará por tres en 50 años [2]. Uno de los factores implicados en este aumento es el envejecimiento de la población de las sociedades occidentales.

La EA fue inicialmente descrita por Alois Alzheimer el año 1906, cuando presentó a la comunidad científica los resultados de la neuropatología de su paciente, Auguste D., que presentaba a los 51 años una demencia progresiva acompañada de delirios y alucinaciones [3]. Los síntomas clínicos clásicos de la EA son pérdida de memoria episódica [4, 5], deterioro del lenguaje [6], déficits visuo-espaciales [7, 8] y alteraciones conductuales. Alteraciones motoras y sensitivas, alteraciones de la marcha y convulsiones pueden presentarse en fases más avanzadas de la enfermedad [9].

En la actualidad el diagnóstico de EA está basado en los criterios desarrollados en el National *Institute of Neurologic and Communicative Disorders and Stroke-Alzheimer's Disease and related Disorders Association* (NINCS-ADRDA) [9], según los cuales el diagnóstico es clasificado como: definitivo (diagnóstico con confirmación histológica), probable (síndrome clínico sin confirmación histológica) o posible (características atípicas clínicas pero sin un diagnóstico alternativo posible y sin confirmación histológica).
No obstante, a día de hoy, se está tratando de avanzar en el diagnóstico precoz para realizar una intervención más temprana de la enfermedad [10].

1. ANATOMÍA-PATOLÓGICA DE LA ENFERMEDAD DE ALZHEIMER

1.1 Hallazgos macroscópicos

El cerebro de un enfermo de Alzheimer presenta como características macroscópicas principales la atrofia del hipocampo y de la corteza cerebral, acompañada por la dilatación de los ventrículos. Este síndrome se caracteriza por tener reducidas en tamaño áreas cerebrales responsables del aprendizaje y la memoria, como el lóbulo temporal y el frontal.

1.2 Hallazgos microscópicos

Los hallazgos microscópicos típicos de la EA son la presencia de placas de amiloide y de ovillos neurofibrilares acompañados por pérdida neuronal y reacción inflamatoria en las áreas afectadas, que son principalmente sistema límbico y córtex [11]. Aún hoy es necesaria la observación de estas lesiones durante la autopsia para el diagnóstico definitivo de la EA.

1.2.1 Placas de amiloide

La lesión mayoritaria de la EA son las placas de amiloide. Una placa de amiloide es un agregado fibroso extracelular e insoluble compuesto principalmente de β-amiloide (Aβ) de 39-43 aminoácidos con una longitud de 8-10 nm [12, 13]. En la EA existen 3 formas principales de placas de amiloide: neuriticas, difusas y algodonosas. Las placas neuríticas se caracterizan por ser una masa extracelular de fibras de amiloide rodeada por dendritas distróficas y axones, algunos con acúmulos de tau, y por microglía activada y astrocitos reactivos [14]. Estas placas neuríticas aparecen en número variable pero generalmente están altamente representadas en la capa molecular del giro dentado del hipocampo, la amígdala, las cortezas de asociación del lóbulo frontal, los lóbulos parietales y en los núcleos más internos que proyectan a todas estas regiones. La morfología microscópica de estas placas maduras sugiere un depósito central de amiloide que atrae a otras especies celulares a su alrededor.

Además de las placas neuríticas, se observan placas difusas en las cortezas límbicas y de asociación, e incluso en el estriado y el cerebelo. Estos depósitos tienen una apariencia porosa, poco compacta y granular sin la presencia de neuritas distróficas, astrocitos o microglía. Presentan placas difusas individuos sanos adultos o ya envejecidos y pacientes con síndrome de Down (trisomía 21) a partir de la segunda década de vida. Este hecho sugiere que estas placas podrían ser las lesiones precursoras a las placas neuríticas, que contienen amiloide fibrilar y se asocian a otras muchas alteraciones estructurales y funcionales, como la respuesta inflamatoria y el estrés oxidativo [15-18].
Existe un tercer tipo de placa, las conocidas como placas algodonosas (*Cotton wool plaques*) más grandes que las placas difusas y neuríticas (de más de 150 μm de diámetro), eosinófilas, con márgenes claramente definidos y pequeños infiltrados neuríticos o inflamatorios [19]. Son las placas observadas fundamentalmente en pacientes portadores de mutaciones en *Presenilina 1 (PS1)* localizadas sobretodo en los exones 8 y 9 [19-30] y, en menor frecuencia, en los exones 4 [31], 5, 6 [27], 12 [32] y el intron 8 [21]. Son placas positivas principalmente para Aβ42 y con poca señal para Aβ40 [26, 29, 31-34]. La distribución típica de estas placas es similar a la de las placas neuríticas y difusas, es decir, principalmente en el neocórtex, regiones límbicas y estriado [29, 31].

1.2.2 Ovillos Neurofibrilares

Los **ovillos neurofibrilares** son conjuntos de pares de filamentos proteicos helicoidales (*paired hellically protein filaments* PHF) de proteína tau hiperfosforilada que residen en el citoplasma de los cuerpos neuronales y en los procesos neuríticos. [35-40]. Tau es una proteína del citoesqueleto con 3 o 4 dominios de unión a microtúbulos que sufre un *splicing* alternativo. Esta asociación estabiliza los propios microtúbulos y ayuda a que se formen puentes de unión entre ellos [41]. Los PHF son característicos de varias enfermedades neurodegenerativas, entre ellas la EA [42]. Las inclusiones intraneuronales de tau aparecen inicialmente en la corteza transentorrinal desde la cual se extienden

al hipocampo y al neocórtex [43]. Algunos de los PHF, se encuentran situados alrededor de los depósitos de amiloide, pero la mayoría están dispersos en el neuropilo de las cortezas límbicas y de asociación.
El patrón de depósito de los ovillos neurofibrilares en la EA queda recogido en los criterios de Braak. Basándose en la localización anatómica y en la densidad de los ovillos neurofibrilares que cambia con la edad, Braak y sus colaboradores definieron 6 estadios diferentes en la formación de los ovillos neurofibrilares. Cuatro de estos estadios son previos al desarrollo de la demencia [43]. Los estadios I/II se localizan en la corteza entorrinal, III/IV en las regiones límbicas y, finalmente, los estadios V/VI en el neocórtex. Cada uno de estos tres estadios suelen corresponder a personas sin alteraciones cognitivas (I/II), con alteraciones cognitivas iniciales (III/IV) y, finalmente, con demencia (V/VI) [43]. Se recomienda para el diagnóstico de la EA, la utilización de la escala de Braak conjuntamente con un análisis de la distribución, tipo y número de placas de amiloide del protocolo CERAD (*Consortium to Establish a Registry for Alzheimer's Disease*). Sin embargo, ninguno de estos criterios neuropatológicos ha sido universalmente aceptado por los neuropatólogos debido a que la variabilidad y el solapamiento entre patologías puede dificultar su diagnóstico [44].

1.2.3 Angiopatía amiloide

La angiopatía amiloide cerebral (AAC) consiste en la acumulación progresiva de amiloide en la pared de los vasos sanguíneos de pequeño y mediano calibre [45]. La prevalencia de AAC en pacientes con EA y en personas mayores sin EA es, respectivamente, de >80% y de entre el 10-40%, [46, 47]. La AAC se caracteriza por la presencia de depósitos de Aβ en las capas media y adventicia de las arterias y arteriolas de la corteza y las leptomeninges. La corteza cerebral, en particular el lóbulo occipital, es la región más frecuentemente afectada por la AAC [46]. Se postula que la AAC impide el suministro de oxígeno al cerebro e induce la degeneración de los vasos [48]. Las arterias de gran calibre no se ven afectadas por depósitos de Aβ pero sí

pueden desarrollar arterioesclerosis. Además, la presencia del alelo *ε4* del gen de la Apolipoproteína E (*APOE)* es un factor de riesgo para la EA y la AAC y se ha asociado a un aumento de Aβ42 en la vasculatura [49-51]. Más aún, numerosas mutaciones en los genes de la *APP* y *PS* se han asociado también con la AAC.

1.2.4 Pérdida neuronal y sináptica

Una de las características principales del cerebro de un enfermo de Alzheimer es la degeneración de la estructura cerebral mediada por la atrofia cerebral del hipocampo y de la corteza cerebral y la dilatación de los ventrículos. La identificación de la atrofia cerebral es compleja debido a las variaciones interindividuales en la forma y tamaño cerebral y la atrofia de la sustancia blanca atribuida al envejecimiento normal (menos del 0.25% por año) [52, 53]. En individuos envejecidos con deterioro cognitivo ligero (DCL), este nivel de atrofia se multiplica por dos en el cingulado posterior, temporoparietal y regiones temporales mediales [52, 54]. Una vez la clínica de EA se ha iniciado, la atrofia progresiva y pérdida neuronal afecta particularmente al hipocampo [55-58] y se correlaciona con los estadíos de Braak [59, 60]. El avance de la atrofia cerebral para pacientes diagnosticados de EA es de un 2,4% por año, con una pérdida generalizada y simétrica de volumen [61, 62] con una atrofia más concentrada en el giro temporal, el polo temporal, giro frontal, amígdala, corteza entorrinal e hipocampo. En el hipocampo la atrofia se correlaciona con el grado de pérdida neuronal [63].

En los últimos años se consideraba que la responsable de la muerte neuronal era la precipitación de formas hiperfosforiladas de tau. De hecho, alrededor de las placas neuríticas de amiloide se observa la presencia de dendritas distróficas y axones, algunos de los cuales presentan PHF [14]. Sin embargo, parece ser que los fenómenos de apoptosis pueden ser el mecanismo primario subyacente a la neurodegenración en la EA [64]. Existen evidencias de que al menos algunas células en casos de EA esporádica fallecen por apoptosis [65-68]. Además se observan signos claros de este fenómeno en EA, por ejemplo: disfunción mitocondrial, actividad de las caspasas, alteraciones nucleares,

daño en el DNA y alteración de genes relacionados con apoptosis como p53 o Bax [66, 68, 69].

La atrofia cerebral puede ser debida a la disminución del tamaño celular y/o a la pérdida sináptica. La atrofia neuronal puede tener lugar tanto por una reducción del soporte neurotrófico o de la señalización en la EA [70] como por los cambios sinápticos inducidos por los oligómeros de Aβ [71, 72] que resultan en la degradación sináptica y en la remodelación de las espinas dendríticas. Estudios en cerebros de pacientes con EA han revelado pequeñas alteraciones de la densidad sináptica del hipocampo que pueden correlacionar con los niveles corticales de Aβ y con el nivel de afectación cognitiva [73-75]. Se ha hipotetizado que los responsables de esta disfunción sináptica son los oligómeros solubles de Aβ estudiados en pacientes con EA y modelos murinos de EA [76-78]. De acuerdo con esta hipótesis, los agregados poliméricos grandes, como las placas de amiloide, representan reservorios inactivos que están en equilibrio con los pequeños reservorios neurotóxicos de Aβ. Esto ha permitido descifrar el transcurso de la enfermedad que se inicia con la acumulación de Aβ que, a su vez, potencia la formación de los ovillos neurofibrilares [79]. La hiperfosforilación de tau de estos ovillos y su liberación provoca la desestabilización de los microtúbulos y, en consecuencia, alteraciones axonales y del metabolismo neuronal y cambios estructurales en los cuerpos neuronales y las dendritas [41, 80], lo cual conlleva muerte neuronal.

Sin embargo, existen otras hipótesis que atribuyen este protagonismo a las placas de amiloide, por ejemplo en un estudio reciente con ratones transgénicos APPswe/ PS1dE9 se ha observado que las neuronas y las dendritas próximas a las placas de amiloide mostraban una actividad neuronal menor comparado con ratones *wild-type*. Estos datos apoyarían la idea que las placas representan una lesión focal que provoca el deterioro del sistema neuronal [81]. Esta idea ha sido corroborada en varios ensayos con modelos animales [82-84].

En el caso particular de las mutaciones de *APP* y *PS1*, trabajos recientes han mostrado que los pacientes poseen atrofia marcada en regiones frontales y temporales mediales [85, 86]. [85, 87-89]. Se atribuye este efecto a la influencia

de las mutaciones de *PS1* sobre otras proteínas que interactuan con el complejo γ-secretasa, como la β-catenina, N-Cadherina, GSK-3β, tau, Bcl2, metaloproteasas y Notch [90]. Todos estos interactores están implicados en apoptosis (Bcl2), agregación proteica anormal (GSK-3β y tau) o alteraciones del ciclo celular (β-catenina).

1.2.5 Otros procesos

1.2.5.1 Estrés oxidativo

La producción de radicales libres de oxígeno altamente reactivos se conoce como estrés oxidativo. Las neuronas de los pacientes afectos de EA tienen cantidades anómalas de proteínas, lípidos y DNA oxidados y modificados. Este daño celular provocado por los radicales libres es particularmente destacado en los alrededores de las placas de amiloide y de los ovillos neurofibrilares de las neuronas, lo cual sugiere que el estrés oxidativo podría jugar un papel importante en el mecanismo fisiopatológico de esta enfermedad [91]. Algunas fuentes originarias del estrés oxidativo propuestas en la EA son la presencia de Aβ, así como de metales con potencial redox como el hierro y el cobre [91-93].

Por otro lado, durante el proceso de agregación de Aβ se genera peróxido de hidrógeno, un proceso que necesita oxígeno y que está potenciado por el hierro y el cobre [94, 95]. Esta alteración de la homeostasis iónica celular, del metabolismo energético y del estrés oxidativo asociado a la membrana pueden conducir a las neuronas más vulnerables a la excitotoxicidad y, finalmente, a la apoptosis. Además, el estrés oxidativo inducido por Aβ provoca la disfunción y degeneración de las sinapsis debido a la alteración de los transportadores de membrana de iones y de glutamato y a la alteración de la función mitocondrial [96].

1.2.5.2 Inflamación

Una característica frecuente en el cerebro de los pacientes con EA es la presencia de una reacción inflamatoria crónica en las zonas afectadas por la enfermedad. En este proceso participan más de 40 sustancias pro-inflamatorias producidas por las neuronas, los astrocitos y las células de microglía [16] y en él se observa la presencia de astrocitos y microglía alrededor de las placas de amiloide (hecho ya descrito en 1906 por Alois Alzheimer). En portadores de mutaciones en *PS1*, se observa una potente respuesta inflamatoria alrededor de las placas de amiloide [97], con un gran número de mediadores inflamatorios en los extractos solubles de cerebro [98], posiblemente debido a la pérdida de la regulación inflamatoria que lleva a cabo la propia PS1 [99]. Estas placas con un incremento de la inflamación se conocen como placas inflamatorias y están localizadas en regiones corticales de portadores de mutaciones en *PS1* y *APP* [89, 97]. Las placas inflamatorias son pequeñas (alrededor de 25 μm de diámetro), densas y bien delimitadas con microglía reactiva y astrocitos alrededor. La presencia de estas placas inflamatorias no está relacionada con una mayor pérdida neuronal [89], sin embargo, el incremento de mediadores inflamatorios específicos puede contribuir al incremento de la neurodegeneración observada en las formas genéticas de EA [98].

De hecho, se ha propuesto la neuroinflamación como el nexo de unión entre la deposición de Aβ y la formación de los ovillos neurofibrilares. Análisis de imagen cerebral muestran que la activación de las células de microglía en pacientes se inicia en fases muy tempranas de la patología [100]. La microglía activada en respuesta a una agresión puede liberar especies reactivas de oxígeno y nitrógeno y enzimas destructoras resultando dañino para el ambiente celular [101]. Los astrocitos también colaboran en la neurotoxicidad y la neuroinflamación debido a que al activarse expresan la enzima óxido nítrico sintasa inducible (iNOS), la cual puede facilitar los depósitos de péptido β-amiloide [102].

Sin embargo, no hay que olvidar el papel dual que tiene la inflamación, aunque por un lado promueve todos estos efectos adversos, por el otro desempeña

una función neuroprotectora. Así pues la microglía activada puede reducir el acúmulo de Aβ mediante un aumento de la fagocitosis o de degradación extracelular [103-105]. Está demostrado que los astrocitos en la corteza entorrinal de los enfermos con EA contienen cantidades de $A\beta_{42}$ proporcionales a la severidad de la patología [106].

Por último, otros hallazgos que destacan la aportación de los mecanismos inflamatorios a la EA son que el grado de inflamación correlaciona con la atrofia cerebral [107] y con la gravedad de la demencia [108] en los estadios iniciales de la EA.

2. GENÉTICA DE LA ENFERMEDAD DE ALZHEIMER:

Un gran porcentaje de casos de EA son esporádicos, y tan sólo un 1-3% de los casos de EA tiene una causa genética conocida. No obstante, la historia familiar es el segundo factor de riesgo más importante después de la edad. La EA familiar (EAF) es una patología genéticamente compleja y heterogénea (revisado en [109]) con herencia autosómica dominante y que generalmente aparece antes de los 65 años. La mayoría de estos casos son debidos a mutaciones en los genes de la *APP* y las *PS 1* y *2*. Gracias a los descubrimientos que han acontecido estos últimos 20 años en el campo de la genética se ha progresado mucho en el conocimiento de las alteraciones moleculares y celulares responsables de la disfunción cognitiva y neuronal que caracteriza la EA.

2.1 Gen de la Proteína Precursora de Amiloide (*APP*)

. En 1987, se publicó el primer ligamiento genético en el brazo largo del cromosoma 21 en familias con EA [110] pocos años después de la identificación del péptido Aβ como el componente principal de las placas neuríticas [13]. Ese mismo año, un estudio localizaba y caracterizaba el gen *APP* en el mismo segmento cromosómico [111]. El clonaje del gen que codificaba para la proteína APP y su localización en el cromosoma 21 [111-114] tuvo lugar en paralelo al hallazgo que asocia casi invariablemente trisomía 21 (síndrome de Down) con EA [115]. Este hallazgo sentó las bases de Aβ como elemento principal de la neuropatología de la EA. Además, la identificación de mutaciones en el gen de *APP* que provocan hemorragia cerebral hereditaria con amiloidosis mostraban que las mutaciones de *APP* (tipo *Dutch*) pueden formar depósitos de Aβ principalmente en los vasos sanguíneos [116, 117]. Las primeras mutaciones descritas que causaban EA de inicio temprano y con herencia autosómica dominante fueron las localizadas en el gen *APP* en el año 1991 [118-121]. En concreto, se describió un ligamiento genético en el cromosoma 21 en cuatro familias con EAF. Actualmente hay descritas 25 mutaciones en el gen de *APP* que producen EA.

La mayoría de ellas se encuentran agrupadas alrededor de los lugares de corte de α-, β- o γ-secretasa alterando el procesamiento o agregación normal de la proteína APP [122-125].

Las mutaciones que se localizan cerca del lugar proteolítico de la β-secretasa son: Glu665Asp, *Swedish* (670 y 671), Als673Thr, His677Arg y Asp678Asn. La mutación más estudiada de APP es la mutación *Swedish* (APP_{Swe}), localizada en el mismo lugar de escisión de la β-secretasa, en la que un doble cambio de aminoácido conduce a un incremento de la proteólisis de APP por β-secretasa [126] y, por tanto, un incremento de los fragmentos C terminales (APP CTF). Además se ha estudiado la acumulación del fragmento intracelular de APP o AICD provocada por esta mutación [127].

Otro grupo de mutaciones son aquellas que se localizan alrededor de α-secretasa, *Flemish*, *Arctic*, *Dutch*, *Italian* y *Iowa*. La mutación *Arctic* (APP_{Arc}) incrementa la agregación de Aβ, provocando formas muy tempranas y agresivas de EA [125]. Las mutaciones *Dutch* [117], *Italian* [128] y *Iowa* [129] pueden causar hemorragias cerebrales. Además las mutaciones *Dutch* (E693Q) *Flemish* (A692G)[121], *Arctic* (E693G) [125] e *Italian* (E693K) causan AAC presenil, acúmulos de amiloide en el parénquima o ambas cosas mediante el aumento de fibrilación de Aβ [130]. En el caso de la mutación *Dutch* el Aβ se acumula preferentemente en los vasos cerebrales sugiriendo un déficit en el aclaramiento de Aβ en este mutante [131]. Por otro lado, se ha demostrado que estas 4 mutaciones alargan la vida del péptido β-amiloide en el cerebro al aumentar su resistencia a la degradación por la neprilisina [132].

Finalmente, las mutaciones situadas alrededor del lugar de procesamiento de la γ-secretasa, como Ala713Thr, Ala713Val, *Iranian*, *Austrian*, *French*, *German*, *Florida*, Ile716Thr, *Indiana*, Vla717Gly, *London* y Val717Leu aumentan selectivamente $A\beta_{42}$ o $A\beta_{42/40}$ o $A\beta_{42/total}$ [118, 124, 133-137]. Adicionalmente, aquellas mutaciones localizadas en los codones 714, 716 y 717 incrementan los niveles de los APP CTF. Está demostrado que estos fragmentos, juegan un papel directo en la regulación de la expresión génica, la dinámica del citoesqueleto y apoptosis [138-141]. En cuanto al procesamiento de γ-secretasa, cada una de estas mutaciones presenta pequeñas diferencias. Las mutaciones situadas en los codones 714 (T714I) y 715 (V715A y V715M)

provocan una disminución de $A\beta_{40}$ sin un claro incremento de $A\beta_{42}$, mientras que las mutaciones en los codones 716 (I716V) y 717 (V717I y V717L) afectan predominantemente al corte γ42 e incrementan la secreción de $A\beta_{42}$ [134]. Además, en las mutaciones cercanas al corte por γ-secretasa se ha observado una correlación inversa entre la ratio $A\beta_{42/40}$ y la edad de inicio de la enfermedad en diferentes familias [134]. Estos datos apoyan que la ratio $A\beta_{42/40}$ es el mejor indicador de severidad de la enfermedad en pacientes portadores de mutaciones de *APP*.

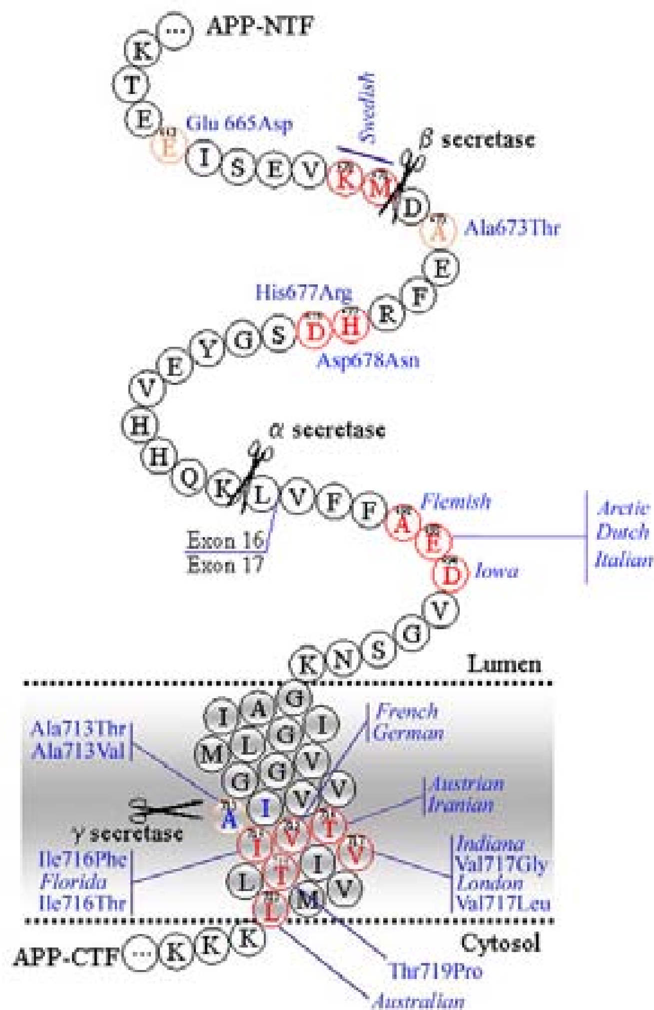

Figura 1. Localización de las mutaciones de *APP* a lo largo de la proteína. Adaptado de www.alzforum.org

★ ***Alteraciones del número de copias de un gen como causa de EA:***

La primera prueba de que una alteración del número de copias de un gen podía provocar demencia tipo Alzheimer proviene de la observación de la presencia de una copia adicional del gen *APP* en el cromosoma 21q21 en pacientes con síndrome de Down. Estos pacientes presentan una producción excesiva de APP y de depósitos de Aβ en forma de placas de amiloide que desemboca en una EA de aparición temprana [142-144]. Estudios posteriores describieron duplicaciones en el gen *APP* en 5 familias con EA autosómica dominante de inicio temprano asociada con AAC [145].

Estos estudios demuestran que la alteración del número de copias de APP conlleva una sobreexpresión protéica que produce EA.

Tabla.1 Mutaciones de *APP*

Mutación APP	Fenotipo	Edad de Inicio	Referencia
Glu 665 Asp	EA, pero puede ser no-patogénica	86?	[146]
Lys/Met670-671/Asn/Leu (*Swedish*)	EA	52 (44-59)	[119]
Ala673Thr	Normal	N/A	[147]
His677Arg	EA	55 (55-56)	[148]
Asp678Asn	EAF	60	[149]
Ala692Gly (*Flemish*)	Placas densas grandes. Asociada a AAC	40-60 pero variable	[121, 150-152]
Glu693Gly (*Arctic*)	EA, pero puede ser no-patogénica	58?	[153]
	Multiplica Aβ40 and 42 en plasma, eleva los niveles de protofibrillas	N/A	[125]
Glu693Gln (*Dutch*)	HCHWA-D (*Hereditary Cerebral Hemorrhage with Amyloidosis*)	Típicamente ~50 pero variable	[117]
Glu693Lys (*Italian*)	Hemorragia cerebral	?	[128]
Asp694Asn (*Iowa*)	EA o Hemorragia cerebral	69	[129]
Ala713Thr	EA, pero puede ser no-patogénica	59	[154]
Ala713Val	Esquizofrenia: probablemente no-patogénica	¿	[155]
Thr714Ile (*Austrian*)	Fenotipo: Afecta directamente el corte de γ-secretasa, incrementa 11X la ratio Aβ42/ Aβ40 *in vitro*. En cerebro, predominantes y abundantes placas pre-amiloides no fibrilares compuestas de Aβ42 N-truncada y asencia total de Aβ40.		[156]
Thr714Ala (*Iranian*)	EA	52 (40-60)	[157]
Val715Met (*French*)	EA	52 (40-60)	[158]

Val715Ala (*German*)	EA	47	[134, 159]
Ile716Phe	EA : placas difusas de Aβ42, ovillos neurofibrilares, cuerpos de Lewy en la amígdala.	31	[160]
Ile716Val (*Florida*)	EA	55	[161]
Ile716Thr	EA	55	[162]
Val717Phe (*Indiana*)	EA	47 (42-52)	[163]
Val717Gly	EA	55 (45-62)	[164]
Val717Ile (*London*)	EA	55 (50-60)	[118]
Val717Leu	Aβ y Tau en LCR consistente con EA; edad de inicio más temprana que otras mutaciones en 717	38	[165]
Thr719Pro	EA	46	[166]
Leu723Pro (*Australian*)	EA	56 (45-60)	[167]

2.2 Genes de las presenilinas (*PS*)

En 1993, varios trabajos mostraron ligamiento genético en una región del cromosoma 14 en diferentes famílias con EA. Estudios posteriores detectaron una mutación en la proteína PS1 en diversas familias [168]. La PS1 es una proteína con nueve dominios transmembrana que contiene el lugar catalítico de la enzima γ-secretasa. También se detectaron mutaciones en un gen que comparte un alto grado de homología con el de la *PS1*, al que llamaron *PS2*. *PS2* está localizado en el cromosoma 1 y provoca una forma de EAF [169, 170]. Únicamente se han descrito 10 mutaciones en este gen.

Las mutaciones en PS incrementan los niveles de $A\beta_{42}$ y aumentan su agregación [171-173]. Este incremento de $A\beta_{42}$ se observa también en plasma y LCR (líquido cefaloraquídeo) de portadores pre-sintomáticos de mutaciones en *PS* [171]. La presencia de una de estas mutaciones en *PS1* se manifiesta en

una forma más temprana de la EA, con edad de inicio entre 28 y 60 años [174], mientras que las mutaciones en *PS2* producen la enfermedad a una edad más tardía y variable, de 35-82 años [175]. Curiosamente las familias con mutaciones de PS1 no presentan una correlación entre la edad de inicio y la secreción de $A\beta_{42}$ [176-178] y la edad de inicio tampoco se ve influenciada por el genotipo de *APOE* [179].

Se ha sugerido que mutaciones en *PS1* pueden asociarse a demencia frontotemporal [180, 181, 182]. Sin embargo, el hallazgo de mutaciones en el gen de la *progranulina* en algunas de esas familias ha cuestionado el hallazgo de estas mutaciones.

Debido a que la enzima γ-secretasa tiene gran variedad de substratos, incluso un pequeño cambio en la especificidad por sus sustratos o una pérdida de función parcial puede resultar en un amplio espectro de efectos tóxicos. Existen datos de diferencias neuropatológicas cuantitativamente muy importantes en sujetos con la misma mutación en PS1, que incluso eran miembros de una misma familia [87].

2.3 Gen *APOE*

Hasta el momento, el único gen que ha sido consistentemente replicado como factor de riesgo genético en la EA es el de la Apolipoproteína E (*APOE*) [183]. Este gen se encuentra situado en el cromosoma 19q13, y posee 3 alelos que codifican para 3 isoformas diferentes de la APOE: E2, E3, E4. Los individuos que poseen el alelo *APOE-*ε*4* tienen un riesgo mayor de padecer la enfermedad [50, 184]. En particular, estudios de meta-análisis muestran un riesgo 3 veces mayor de padecer EA en individuos portadores de una copia de la isoforma *APOE-*ε*4* y un riesgo 15 veces mayor en individuos homocigotos para *APOE-*ε*4* [185]. Además, *APOE-*ε*4* disminuye la edad de inicio de modo dosis dependiente. Trabajos en cultivos celulares y modelos animales muestran que *APOE-*ε*4* puede influenciar la acumulación de $A\beta$ y la formación de placas [186-188], así como la agregación de tau [189-197]. Hay que destacar que existen varios trabajos que sugieren que APOE afecta a $A\beta$ y tau por mecanismos independientes. En concreto, APOE provoca un incremento en $A\beta$

mediante la disminución del proceso de aclaramiento y el incremento de la agregación [186, 187, 198] y, por otro lado, modula tau regulando la actividad de su principal quinasa, GSK3 [195, 199]. Recientemente, se ha observado incremento de Aβ fibrilar en individuos portadores de *APOE-ε4* ancianos y cognitivamente sanos [200].

2.4 Otros genes (*APOJ*, *SORL1*)

Recientes resultados de análisis de ligamiento en todo el genoma y estudios de asociación con gran número de muestras han permitido conocer mejor la etiología de la EA tardía. De este modo se han identificado algunos loci en cromosomas con genes que representan un riesgo de susceptibilidad para la EA [201]. Investigaciones de búsqueda de genes candidatos se han centrado en zonas codificantes para proteínas relevantes, por ejemplo el complejo γ-secretasa, proteínas que regulan el tráfico de APP (SORL1, [202, 203]) o involucradas en fibrilación o eliminación del péptido Aβ .

Dos estudios recientes de asociación realizados en muestras de pacientes con EA han concluido con la identificación de tres genes de riesgo para la EA: *CLU* (también conocido como *APOJ*, odds ratio = 0.86*)*, *PICALM* (odds ratio = 0.86) y CR1 (odds ratio= 1.21) [204, 205]. *CLU* codifica para clusterin o apolipoproteina J en el cromosoma 8 y CR1 codifica para el componente del receptor del complemento 1, 3b/4b, en el cromosoma 1 [204]. PICALM es una proteína ubicua en todo los tejidos con una expresión importante en neuronas involucrada en la endocitosis mediada por clatrina y en el tráfico de la proteína VAMP2, implicada en la fusión de vesículas a la membrana pre-sináptica durante la liberación de neurotransmisores. Por lo tanto, PICALM tiene un papel muy relevante en la correcta función neuronal [205]

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3. FISIOPATOLOGÍA DE LA ENFERMEDAD DE ALZHEIMER

3.1 Hipótesis de la cascada amiloide

La hipótesis más aceptada sobre la fisiopatología de la EA, es la llamada "hipótesis amiloide" [206, 207]. Según ésta el péptido Aβ juega un papel central tanto en la EAF como esporádica. Posteriormente, se ha ido modificando esta teoría y se cree que los monómeros de Aβ se convierten en oligómeros que provocan una afectación sináptica, neuronal y glial gradual que desemboca en una demencia [208, 209].

Existen cuatro argumentos que refuerzan la hipótesis amiloide: el primero que las mutaciones en el gen *MAPT* que codifica para de la proteína tau causan demencia frontotemporal mientras que las mutaciones en *APP* causan EA [210-212]. La segunda: ratones transgénicos que sobreexpresan APP y tau mutada sufren un incremento de ovillos tau-positivo, mientras que la estructura y el número de placas permanece inalterado [82] lo que sugiere que las alteraciones del procesamiento de APP probablemente potencian la agregación de tau. Tercero, el cruce de un ratón transgénico para *APP* con uno deficiente en *APOE* reduce claramente los depósitos de Aβ en los descendientes [213], demostrando el papel que ocupa la variabilidad genética del locus de *APOE* humano en el metabolismo de Aβ [50]. Cuarto, la variabilidad genética encargada del catabolismo y eliminación de Aβ puede contribuir al riesgo de padecer EA tardía [214-219]. En conjunto, estas cuatro observaciones son consistentes con el hecho de que la acumulación de Aβ precede a la presencia de tau y los ovillos [209]. No obstante, existen varios autores que discrepan debido a que algunos datos no concuerdan con la hipótesis amiloide. La objeción más importante es que el número de placas de amiloide no correlaciona con el déficit cognitivo en la EA, pero si con los ovillos neurofibrilares y pérdida sináptica. Incluso controles ancianos sin problemas cognitivos presentan depósitos de Aβ corticales similares a los de los pacientes. No obstante, los estudios más recientes implican a las formas solubles de Aβ en la génesis del deterioro cognitivo en la EA [73-75, 220]. Estos hallazgos concuerdan con la idea que en enfermedades

neurodegenerativas como EA, Parkinson y Huntington, los agregados poliméricos representan reservorios inactivos de las especies más pequeñas neurotóxicas. Otro hecho a tener en cuenta es que los principales modelos de ratones transgénicos de EA que muestran acumulación progresiva de Aβ, como tg2576 o PDAPP, no muestran una clara pérdida neuronal [221, 222] ni depósitos de tau. Y por último, la observación en estudios post-mortem de cerebros de personas sanas mostraban estadios de Braak y Braak IV y V sin síntomas de demencia aparente [223, 224].

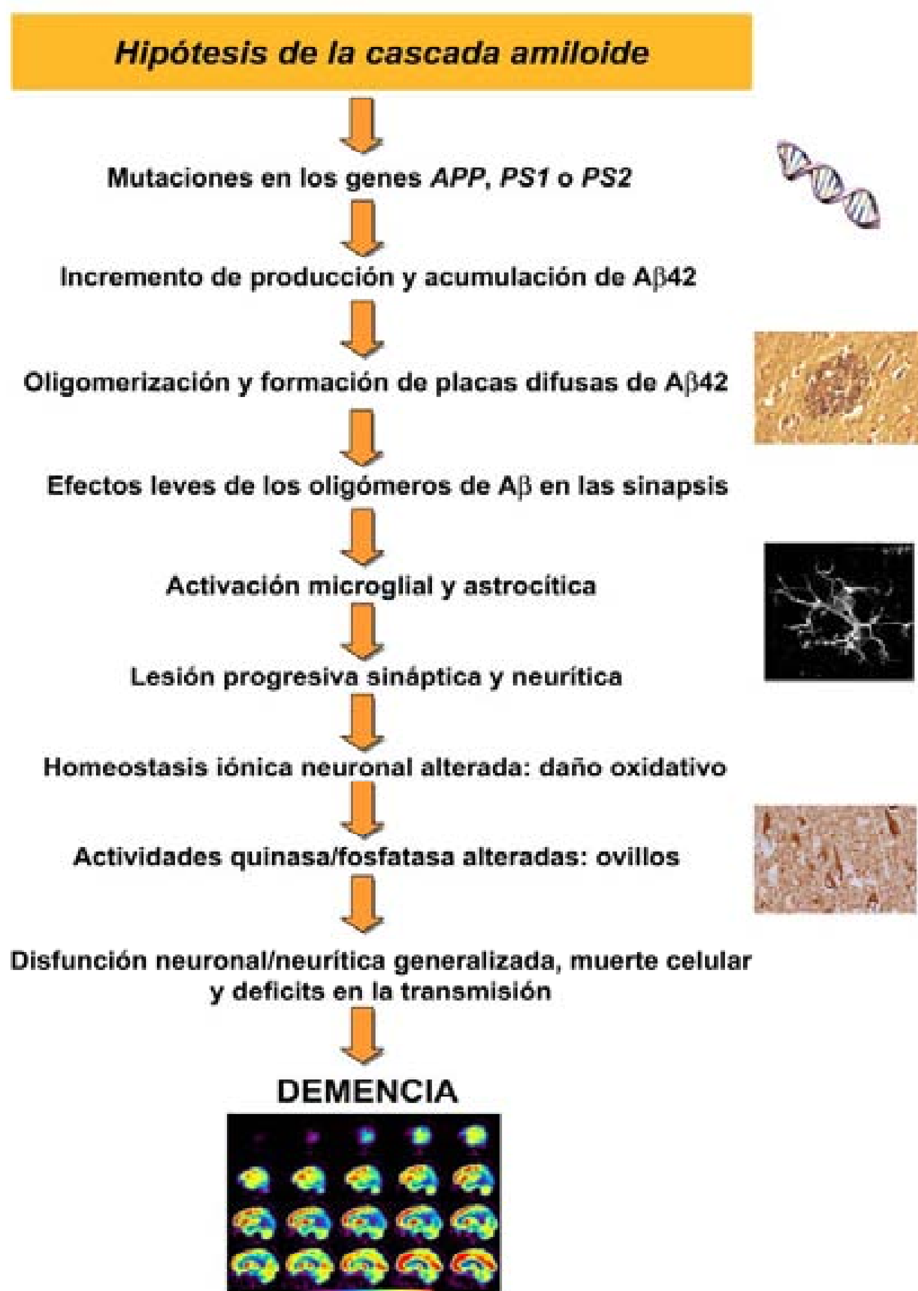

Figura 2. Hipótesis de la cascada amiloide

3.1.1 Péptido β-amiloide y procesamiento de APP

El péptido Aβ es el fragmento que resulta de la proteólisis de la APP [112], el cual se produce de manera natural durante la vida de casi todas las células mamíferas [225-227]. APP es una glicoproteína tipo I ubicua que sufre una serie de modificaciones post-traduccionales: splicing alternativo, glicosilación en N- y O-, fosforilación, sulfatación, adición de glicosaminoglicanos y una compleja proteólisis. La proteína APP se encuentra localizada en la membrana plasmática y participa en funciones de adhesión celular [228] y movimiento celular [229], pero también se encuentra en la red trans-Golgi [230], en el retículo endoplasmático, endosomas, lisosomas [231] y membranas mitocondriales [232]. El procesamiento de Aβ sigue dos vías celulares: dentro de los límites de la célula, formando el Aβ intracelular; o en la membrana plasmática, también llamada vía secretora, en la que Aβ es liberado en el espacio extracelular. Es posible que funcionen ambas vías, aunque la gran mayoría de Aβ es secretada, sugiriendo que Aβ se produce principalmente en la membrana plasmática o como parte de la vía secretora, y posteriormente es secretada al exterior de la célula. La formación de Aβ y la enzima γ-secretasa se localizan en los rafts lipídicos. Estas regiones son microdominios de membrana enriquecidos con colesterol localizados en la membrana plasmática. Por esta razón la alteración del medio lipídico membranal, mediante por ejemplo la reducción de colesterol, puede alterar el procesamiento de APP [233, 234].

En neuronas, APP se transporta anterógradamente a lo largo de los axones centrales y periféricos y durante este desplazamiento se procesa proteolíticamente [235, 236].

Aβ se libera tras varios cortes proteolíticos secuenciales de APP. El procesamiento de APP se puede dividir en dos grandes vías: la amiloidogénica y la no-amiloidogénica. En la **vía amiloidogénica** la APP es procesada por la enzima β-secretasa (BACE-1 es la principal, aunque también existe BACE-2), una aspartil-proteasa con su centro activo orientado hacia el lumen [237]. Esta primera escisión da lugar a la liberación de fragmento β soluble de APP

(sAPPβ) al espacio extracelular y al fragmento de 99 aminoácidos (C99) que permanece unido a la membrana. Seguidamente, el procesamiento por la γ-secretasa, una aspartil-proteasa intramembrana con presenilina como centro catalítico y otras tres proteínas que configuran el complejo: Nicastrina, Aph-1 y Pen-2 (presenilin enhancer 2) [238-240], da lugar al péptido Aβ. En paralelo se da la **vía no-amiloidogénica** en la que se produce el procesamiento por α-secretasas, proteínas pertenecientes a la familia de metaloproteasas ADAM (ADAM9, [241], ADAM10, [242], y ADAM17, [243, 244]), también conocidas como enzimas convertidoras del factor de necrosis tumoral). El primer procesamiento de α-secretasa ocurre a 83 residuos del extremo C terminal, dando lugar a un gran ectodominio N-terminal llamado fragmento alfa soluble de APP (sAPPα) que es secretado al medio extracelular [245]. El fragmento resultante de 83 aminoácidos (C83) es retenido en la membrana y cortado posteriormente por γ-secretasa dando lugar al pequeño fragmento hidrófobico p3 con función desconocida en células mamíferas [225, 246]. Asimismo, el corte secuencial de α-secretasas y γ-secretasas da lugar al fragmento intracelular de APP o AICD, una región de la cual se puede unir a la proteína adaptadora Fe65 y mediar en vías de señalización nuclear [247-249]. Se ha observado que una delección de APP en ratones transgénicos provoca un fenotipo no letal con alteraciones leves en el sistema nervioso central adulto y en el desarrollo neurítico [250, 251]. Esta delección no confiere un fenotipo de mayor gravedad debido a la existencia de dos proteínas APP homólogas, *Amyloid beta precursor-like protein* 1 (APLP-1) específica de neuronas y *Amyloid beta precursor-like protein* 2 (APLP-2) ubicua.

La mayoría de los péptidos de Aβ resultantes del procesamiento amiloidogénico de APP tienen una longitud de 40 aminoácidos (Aβ_{40}) y, únicamente una pequeña proporción (10%) es de 42 aminoácidos (Aβ_{42}). Esta segunda especie es mucho más hidrofóbica y tiene más facilidad para formar fibrillas que aquellas de menor longitud [133]. Estos datos son consistentes con la observación que el péptido Aβ_{42} es la especie inicial y más abundante en las placas de amiloide [252].

Se ha observado la existencia de formas intracelulares de Aβ en cerebelo, medula espinal y cerebro de individuos con y sin EA [253]. A partir de este

estudio original, muchos estudios en autopsias de pacientes con EA, síndrome de Down y cerebros de ratones transgénicos demostraron la presencia de Aβ intraneuronal, mayoritariamente del tipo $A\beta_{42}$. Más específicamente se localizó $A\beta_{42}$ en los cuerpos multi-vesiculares (MVB) de las neuronas de cerebro humano asociadas a la patología sináptica [254]. Algunos trabajos recientes sugieren que la acumulación de Aβ intracelular puede ser un fenómeno temprano en el desarrollo de la enfermedad, ya que se observa también en sujetos con DCL [255]. Del mismo modo, se ha visto que la acumulación intracelular de Aβ precede a la formación de placas extracelulares en pacientes con síndrome de Down [143, 144]. Por último, se ha observado que la inmunoreactividad de Aβ intraneuronal aparece durante el primer año de vida, aumenta durante la infancia y se estabiliza durante la segunda década de la vida, manteniéndose elevado durante la edad adulta incluso en pacientes sanos [256].

Como ya se ha explicado anteriormente, Aβ se produce en forma de monómero pero rápidamente agrega formando complejos multiméricos. Estos complejos van desde dímeros y trímeros de bajo peso molecular a protofibrillas y fibrillas de alto peso molecular. Se ha postulado que las especies oligoméricas de Aβ son las más tóxicas, en particular los dímeros que interfieren en los procesos de memoria, aprendizaje, función sináptica y potenciación a largo plazo (LTP long term potentiation) [78, 257].

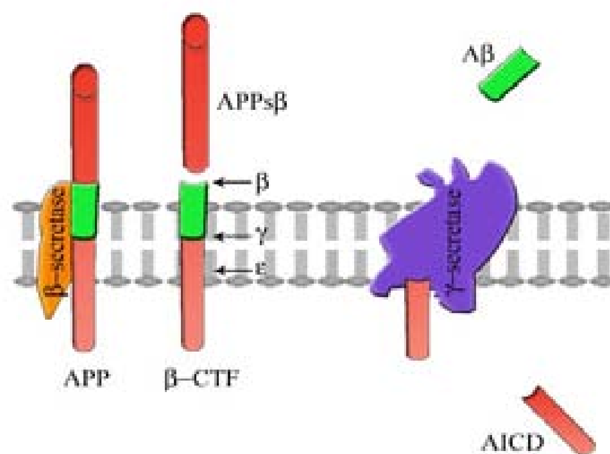

Figura 3. Procesamiento amiloidogénico de la APP

3.2 Papel de la proteína Tau

Además de los depósitos de β-amiloide, la EA cursa con ovillos neurofibrilares compuestos de proteína tau hiperfosforilada. La proteína tau es una proteína de asociación a microtúbulos (MAP) localizada abundantemente en los axones neuronales. Existen 6 isoformas de tau que se expresan en el cerebro adulto, derivadas de un proceso de *splicing* alternativo. La proteína tau posee una región C-terminal compuesta por repeticiones de un motivo de unión a microtúbulos muy conservado [258]. Las seis isoformas de tau se diferencian en el número de repeticiones de este motivo y se las conoce como isoformas 3R o 4R [259]. Estas 6 isoformas parecen ser igualmente funcionales, a pesar de que desempeñan diferentes roles fisiológicos y se expresan en diferentes fases del desarrollo. Sin embargo, las isoformas 3R y 4R se expresan en una ratio 1:1 en la mayor parte de regiones cerebrales y una variación de esta ratio es característica de diversas demencias neurodegenerativas [260].
Además de su conocido papel en la estabilización de microtúbulos, tau, interacciona con otras estructuras y enzimas [261] como la membrana plasmática [262, 263], la actina del citoesqueleto [264] y algunas tirosina quinasas [265]. Se ha propuesto que la neurodegeneración mediada por tau puede ser el resultado de una función tóxica adquirida por estos agregados o su precursor y los efectos perjudiciales que se derivan de la pérdida de la función normal de tau en este estado patológico. En condiciones patológicas, el equilibrio de tau unida a microtúbulos se encuentra perturbado y como consecuencia los niveles de tau libre (sin unir a microtúbulos) están incrementados provocando un incremento en la agregación y fibrilización de tau [266]. Este exceso da lugar a pre-ovillos sin aún conformación β-plegada [267, 268], y finalmente ovillos neurofibrilares, con estructura secundaria β-.

3.3 Secretasas

3.3.1 α-secretasa

Algunas Zinc metaloproteasas, como ADAM10 [242], pueden procesar APP en la región de Aβ entre la Lys^{16} y la Leu^{17}, un corte que impide la

generación de Aβ. Este procesamiento en la superficie celular por α-secretasa genera el ectodominio soluble sAPPα [269]. Se cree que los fragmentos de APP, sAPPα liberados por α-secretasa realizan funciones autocrinas y paracrinas. Por un lado, sAPPα induce la diferenciación de células madre neuronales a la línea astrocítica [270], por el otro, en cerebros de roedores adultos, sAPPα actúa junto con *Epidermal Growth Factor* (EGF) para estimular la proliferación de células madre neuronales con capacidad de respuesta al EGF en la zona subventricular [271]. El papel de este dominio sAPPα fue estudiado en un modelo de ratón APPV717I con sobreexpresión de ADAM10, en el que se observó una reducción de Aβ y una menor formación de placas, además de un menor déficit cognitivo [272]. Estos resultados mostraron la primera evidencia *in vivo* de que α-secretasa puede ser una buena diana terapéutica en la EA, y corroboraron la idea que una disminución de la actividad de α-secretasa contribuye al desarrollo de EA. La familia ADAM se reveló como una de las familias proteicas más extensas que median en la proteólisis de ectodominios y la liberación de dominios extracelulares. Mediante este procesamiento las células pueden regular las proteínas expresadas en su superficie celular. En muchos casos, estos ectodominios solubles, son biológicamente activos como mediadores de las funciones adscritas a sus equivalentes transmembranales [273]. Estas proteínas tipo-ADAM pueden activar factores de crecimiento y citocinas regulando vías de señalización importantes para el desarrollo y para procesos patológicos como el cáncer [274].

ADAM-10 se expresa de modo constitutivo en astrocitos de cerebro humano en condiciones normales y de inflamación [275]. ADAM10 y 17 son abundantes en microglía [276]. Asimismo células de neuroblastoma tratadas con ciertos Antiinflamatorios No Esterooideos (AINEs) (nimesulide, ibuprofen y indometacina) aumentan la secreción del fragmento no-amiloidogénico sAPPα [277].

3.3.2 β-secretasa

La β-secretasa neuronal más importante es una aspartil proteasa transmembrana de tipo I, llamada BACE-1 (del inglés, *β-site cleaving enzyme*)

[278]. BACE-1 escinde APP en la posición N-terminal de la secuencia de Aβ. Además, BACE-1 también puede cortar dentro del dominio de Aβ entre la Tyr^{10} y la Glu^{11} (*β'-cleavage site*). La deficiencia de BACE1 en ratones transgénicos inhibe la producción de Aβ. Recientemente se ha demostrado que el ratón knock-out para BACE-1 desarrolla un comportamiento parecido a la esquizofrenia, debido probablemente a la implicación de BACE-1 en el procesamiento de Neuregulin-1, proteína relacionada a ésta y a otras enfermedades psiquiátricas [279]. Este comportamiento se caracteriza por alteración de la inhibición prepulso (*prepulse inhibition*), hiperactividad inducida, hipersensibilidad a los psicoestimulantes, alteraciones en el reconocimiento social y déficits cognitivos [279].

BACE-1 está predominantemente localizada en el aparato de Golgi tardío/*trans Golgi Network* (TGN) y endosomas, donde ocurre el procesado proteolítico de APP durante la vía endocítica/ de reciclaje [280]. BACE-1 se expresa predominantemente en neuronas [278, 281, 282], pero también puede expresarse en astrocitos en condiciones de estrés crónico [281, 283, 284]. Por otro lado, en ratones transgénicos para APP jóvenes, BACE-1 neuronal está sobreexpresada cerca de la microglía activada y astrocitos [284], demostrando su implicación en los procesos inflamatorios. Varios resultados muestran incrementos en los niveles de proteína BACE-1 y su producto, β-CTF en cerebros de casos esporádicos [285, 286], en plaquetas y en LCR de pacientes con EA y DCL [287-289]. Estas alteraciones de actividad de BACE-1 indican una regulación transcripcional y/o traduccional de su expresión en el cerebro [290].

3.3.3 γ-secretasa

La enzima γ-secretasa pertenece a una familia de proteasas que procesan proteínas en el interior de la membrana (intramembrane cleaving proteases o I-CliPs) [291]. γ-secretasa está compuesta por cuatro proteínas integrales de membrana: PS, nicastrina, Aph-1 (*anterior pharnyx defective-1*) y Pen-2 (*presenilin enhancer-2*) [238-240], de las cuales la PS forma su centro catalítico. Se ha observado que las cuatro subunidades son necesarias para

que la enzima sea activa. Otros factores pueden modular la actividad de γ-secretasa, como TMP21 y CD147 [292, 293]. Aunque probablemente a un nivel diferente, el complejo γ-secretasa está implicado en muchos procesos celulares, el más estudiado es la vía de señalización de Notch. La actividad γ-secretasa tiene como substrato al receptor de Notch, y fruto de su actividad libera el fragmento intracelular de Notch (NICD) que se trasloca al núcleo y regula la transcripción de genes como HES-1, CBF1, Supressor of Hairless (Su(H)), Lag-1, Dishevelled, Deltex y Mastermind [294-297].

3.3.3.1 Presenilinas

Existen dos formas homólogas de PS en mamíferos, PS1 y PS2, con un alto grado de homología (67%) y redundancia funcional. La PS1 es una proteína transmembrana de 467 residuos y 50 KDa. La PS2 tiene 4 aminoácidos menos en el extremo N-terminal. Ambas PS poseen 9 dominios transmembrana (DTM), con el extremo amino-terminal citosólico y el carboxi-terminal orientado al lumen. [298-300]. La activación del complejo γ-secretasa requiere que PS sufra una endoproteólisis entre los residuos N292 y V293 en dos fragmentos, uno N-terminal (NTF de unos 30KDa, DTM 1-6) y otro C-terminal (CTF de unos 20 KDa, DTM 7-9). Este proceso de endoproteólisis parece ser un evento autocatalítico intramolecular que es llevado a cabo por la misma actividad γ-secretasa [301, 302]. Estos dos fragmentos permanecen unidos y juegan un papel esencial en la función de γ-secretasa [303]. Varios estudios situan el centro activo de PS en la región existente entre NTF/CTF [208, 240, 304]. Además, otros trabajos sugieren la existencia de un lugar de unión a substrato inicial en PS1 (*docking site*), diferente del centro activo [305, 306]. Es importante destacar que a la PS1 también se le han atribuido otras funciones independientes de γ-secretasa relacionadas con la homeóstasis del Calcio [307, 308], interacción y estabilización de β-catenina [309, 310] y otras [311].

3.3.3.2 Nicastrina, Aph1 y Pen-2

La PS1 requiere de otros tres componentes para formar el complejo activo de la γ-secretasa: nicastrina, Aph-1 y Pen-2. La nicastrina es una glicoproteina de membrana tipo I con un gran dominio en el lumen involucrada en el ensamblaje, maduración y activación del complejo γ-secretasa [312-314]. Aunque se ha propuesto que la región extracelular de nicastrina participa en el reconocimiento del substrato [312]; este punto permanece controvertido [314]. De hecho, recientemente se ha descrito un complejo γ-secretasa que no contiene nicastrina formado por: PS1, Pen-2 y Aph-1A. Este complejo es activo pero altamente inestable, sugiriendo que la nicastrina juega un papel en la estabilización del complejo pero no en su actividad o en el reconocimiento de substrato [315].

Aph-1 tiene una topología de siete DTM con un extremo C-terminal citosólico, mientras que Pen-2 contiene dos DTM con los dos extremos en el lumen [316-318]. La especie humana posee dos genes parálogos de *Aph1*: *Aph-1A* y *Aph-1B*, pero tres variantes de la proteína Aph1, dos variantes de splicing de Aph-1A (S y L) y Aph-1B. Todas estas variantes se incorporan de forma independiente a los complejos de γ-secretasa [319]. La isoforma Aph-1A es la mayoritaria en los complejos de γ-secretasa, de igual modo que ocurre con PS1 [320]. Por consiguiente, mientras el gen *Aph-1A* es esencial para la señalización de Notch durante la embriogénesis [320, 321], *Aph-1B* puede ser totalmente eliminado, produciendo una reducción de Aβ [322].

Teniendo en cuenta la existencia de dos formas protéicas de PS y tres de Aph-1, existen al menos 6 complejos diferentes con diferentes funciones biológicas. [319, 321, 323, 324]. Estos 6 complejos desarrollan actividades γ-secretasa distintas pero solapadas [321, 323, 325-327].

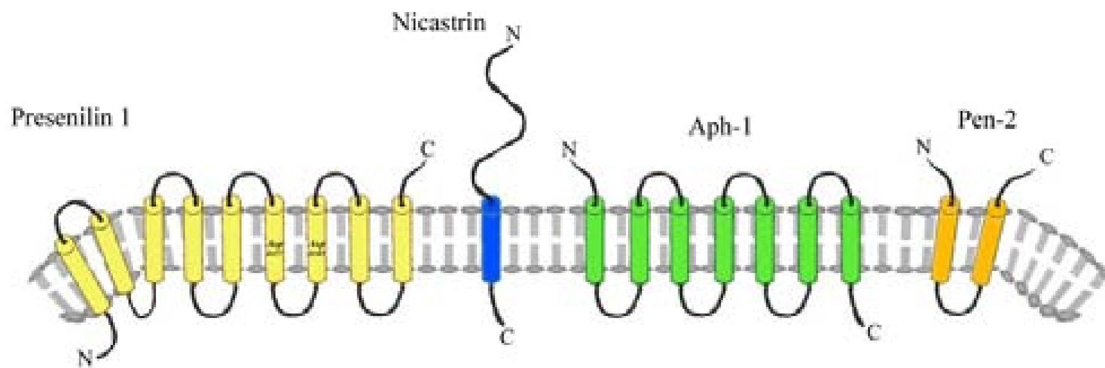

Figura 4. Subunidades proteicas que forman el complejo γ-secretasa

3.3.3.3 Formación/ensamblaje del complejo γ-secretasa

El proceso de ensamblaje de los componentes del complejo γ-secretasa ocurre en el retículo endoplasmático, donde adquiere su total funcionalidad. Este proceso se inicia con la formación de un subcomplejo entre nicastrina y Aph-1 y seguidamente la PS es incorporada para formar un complejo heterotrimérico [328]. Por último, la unión de Pen-2 permite la formación del complejo maduro y su activación tras la endoproteólisis de PS [329-332]. El conocimiento exacto de la estructura de la γ-secretasa se ha complicado por la falta de su estructura cristalizada ya que actualmente sólo se dispone de un mapa de baja resolución obtenido por microscopía electrónica [333-335]. De acuerdo con estos trabajos de microscopía electrónica se ha propuesto la existencia de tres cavidades interiores [334, 335] o un poro central [333] en el complejo γ-secretasa.

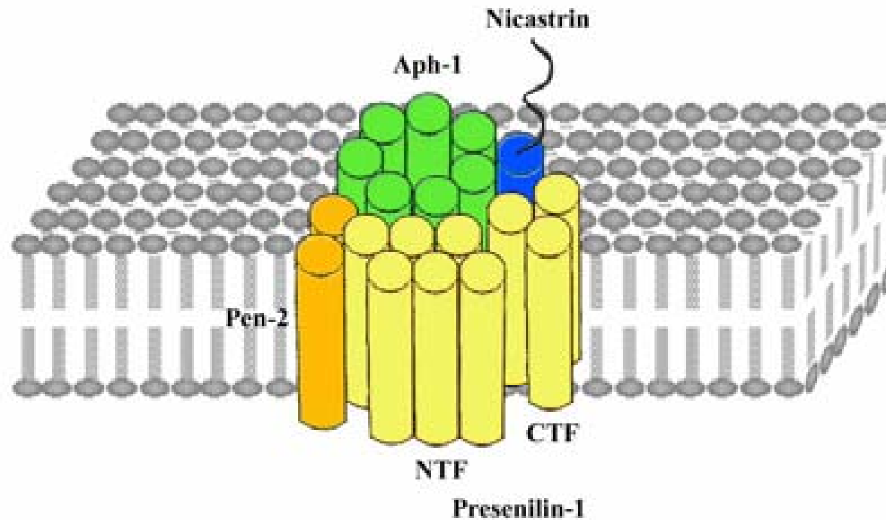

Figura 5. Disposición tridimensional de las subunidades del complejo γ-secretasa

3.3.3.4 Substratos de la γ-secretasa

Desde los estudios iniciales que mostraban el procesamiento de APP y Notch por γ-secretasa [304, 336], se han encontrado más de 70 proteínas de membrana tipo I procesadas por γ-secretasa [337]. La enzima γ-secretasa tiene una baja especificidad por sus substratos, con una secuencia de consenso no clara [336]. Sin embargo, para algunos substratos sí que se ha demostrado un procesamiento funcional evidente, ejemplo de ello son Notch, N-cadherina, ErB4, o APP. Para que una proteína sea proteolizada por γ-secretasa ha de cumplir ciertos requerimientos: tener una hélice transmembrana tipo I y poseer un ectodominio pequeño, generalmente procedente de un corte previo por otra proteasa [338]. A pesar de ello, no todas las proteínas de membrana tipo I con ectodominios cortos son procesadas por γ-secretasa, y, algunos datos sugieren que se requiere un dominio citoplasmático transmembrana permisivo para ser un substrato adecuado [338, 339]. Algunos substratos como APP, APLP-1, Notch o CD44 son procesados en varios lugares por la γ-secretasa. Por ejemplo, APP es proteolizada en el sitio-γ (cerca de los residuos 40-42), el sitio-ε (cerca del residuo 49) y en el sitio-ζ (cerca del residuo 46) [340].

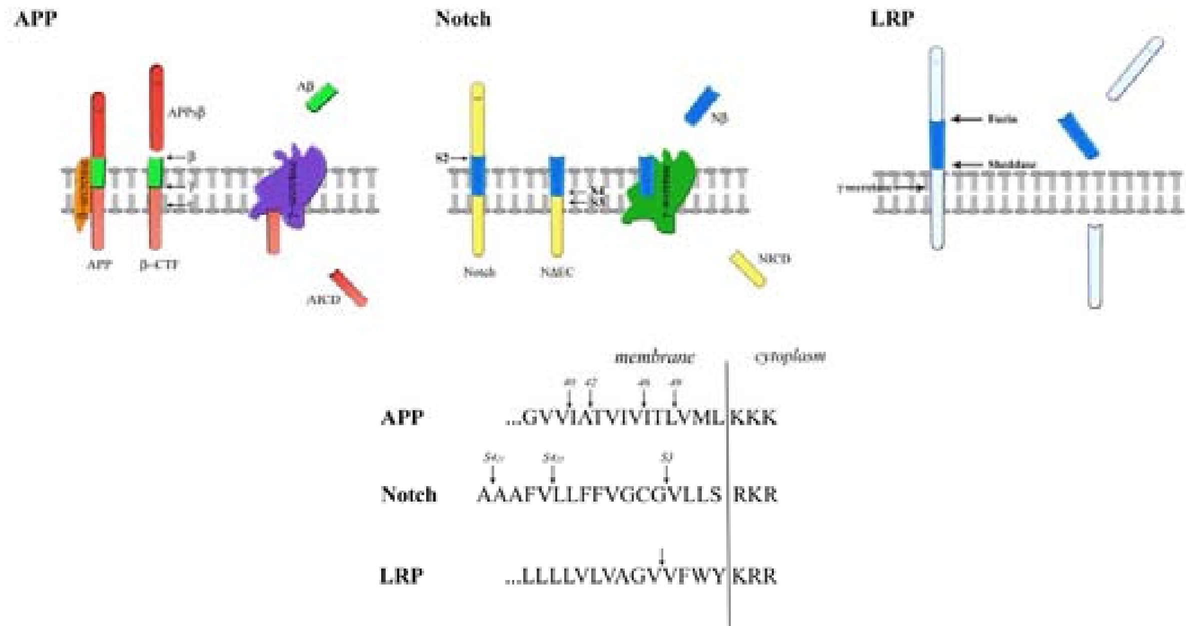

Figura 6. Procesamiento de substratos por la γ-secretasa (de izquierda a derecha: APP, Notch, LRP)

3.3.3.5 Inhibidores de secretasas

★ **Inhibidores β-secretasa**

La enzima β-secretasa o BACE-1 es una diana muy atractiva para el desarrollo de fármacos en la EA. A pesar que los animales *knock out* para BACE-1 muestran una mínima generación de Aβ [341], algunos animales presentan hipomielinización de los nervios periféricos y alteraciones axonales de las fibras aferentes, lo que plantea dudas acerca de la seguridad de la inhibición de BACE-1 como tratamiento de la EA [342].

Los inhibidores de β-secretasa han resultado difíciles de diseñar debido al gran dominio catalítico de BACE-1 [343]. Por esta razón, el avance de los inhibidores de β-secretasa hacia ensayos clínicos ha sido lento y dificultoso.

Durante los últimos 7 años, se han descrito algunos inhibidores de la β-secretasa [344]. Estos compuestos actúan como péptidos miméticos transitorios basados en las secuencias aminoacídicas del lugar de corte de

APP por β-secretasa [345, 346]. También existen algunos inhibidores no competitivos naturales como el hispidin [347] y las catequinas [348], pero su baja potencia y especificidad limitan su desarrollo farmacológico. La primera generación de inhibidores de la β-secretasa OM99-1 y OM99-2, eran muy potentes pero eran esencialmente peptídicos y no tenían características de fármaco [345]. Recientemente, se ha mostrado datos de un nuevo inhibidor de la β-secretasa que reduce Aβ40 y Aβ42 en el cerebro de ratones transgénicos Tg2576 [349]. Otra estrategia consiste en inhibir la β-secretasa mediante la immunización pasiva de ratones transgénicos, donde los anticuerpos para BACE-1 funcionan como inhibidores y mejoran la cognición [350]. Un compuesto recientemente desarrollado, el GRL-8234, tiene una excelente capacidad de inhibición de β-secretasa en células de ovario de hamster (CHO)[343]. Asimismo la administración a ratones Tg2576 reduce en un 65% la producción de Aβ40 [343]. La compañía CoMentis ha completado la fase I de un estudio clínico en humanos con la pequeña molecula CTS-21166, que abre la posibilidad de estudiar los inhibidores de β-secretasa como fármacos en humanos.

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★ Inhibidores γ-secretasa

La larga lista de substratos procesados por la γ-secretasa tiene implicaciones claras para el desarrollo de nuevas terapias para la EA, en particular para la búsqueda de inhibidores y moduladores de γ-secretasa. El objetivo principal es conseguir una inhibición parcial de γ-secretasa que consiga reducir suficientemente Aβ evitando los efectos secundarios negativos provocados por la inhibición de Notch. Sorprendentemente, la inhibición de Notch por estos fármacos ha transformado a γ-secretasa en una diana terapéutica interesante para la investigación del cáncer, y se están estudiando actualmente para el tratamiento de la leucemia o el cáncer de mama [351].

Por lo que conlleva a la EA, varios estudios han demostrado que los inhibidores de γ-secretasa reducen Aβ *in vitro* e *in vivo* [352-355]. Los primeros inhibidores diseñados, llamados *Transition-state analogue inhibitors* o TSA, fueron compuestos capaces de interaccionar con el centro activo de la proteasa, en la

región NTF/CTF de la PS [305, 356-358]. Los inhibidores TSA bloquean el procesamiento de APP y de otros substratos de la γ-secretasa, como Notch [359]. Posteriormente, se diseñaron péptidos basados en el dominio transmembrana de APP y con una conformación helicoidal que inhibían γ-secretasa [306]. El uso de estos péptidos helicoidales permitió el descubrimiento del *docking site* en la región NFT/CFT [305]. Otro de los inhibidores más estudiados en investigación básica es el DAPT (*N-N-(3,5-difluoro-phenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester*) [353], el cual se une al extremo C-terminal de PS en un sitio distinto al centro activo o al lugar de unión a substrato [358]. La administración aguda o crónica de DAPT puede reducir los niveles de Aβ42 y mejorar los déficits cognitivos en un modelo de ratón transgénico [354]. Actualmente el estudio clínico más avanzado es el del inhibidor desarrollado por Eli-Lilly LY-450139 que reduce Aβ total en el rango nanomolar (IC50= 60 nM) y es 3 veces más selectivo en inhibir el procesamiento por γ-secretasa de APP que de Notch [360, 361]. Además este fármaco es capaz de reducir los niveles de Aβ en diferentes modelos animales en plasma, LCR y cerebro [361].

3.3.3.6 Moduladores γ-secretasa (AINEs)

Un avance considerable en el campo de los moduladores de la γ-secretasa es el descubrimiento de que algunos Antiinflamatorios No Esteroideos (AINEs) de uso común, reducían selectivamente los niveles de Aβ42 en cultivos celulares y animales transgénicos, independientemente de la actividad ciclooxigenasa (COX) [362, 363]. Posteriormente se observó que los (R)-enantiómeros con baja inhibición de COX todavía retienen la habilidad por disminuir Aβ42 [362, 364]. Los AINEs provocan un desplazamiento del lugar de corte de APP por γ-secretasa, provocando la liberación de especies de Aβ más cortas, sobretodo Aβ38, pero no inhiben el procesamiento de otros substratos por γ-secretasa [362, 365, 366]. Los AINEs también desplazan el lugar de corte de Notch, pero no inhiben su vía de señalización o la liberación de su dominio intracelular [367]. En conjunto, estos resultados sugieren que los AINEs inducen un cambio conformacional en γ-secretasa por un mecanismo alostérico

[366, 368-370]. Desde los trabajos iniciales se han desarrollado numerosos análogos del flurbiprofeno [365, 371], que poseen una capacidad anti-amiloide hasta 27 veces mayor. Algunos de estos compuestos, como el CHF5074, han mostrado una gran potencia y su aplicación clínica está siendo investigada [372]. Sorprendentemente, la búsqueda de nuevos moduladores de la γ-secretasa condujo al descubrimiento de compuestos que elevaban Aβ42, como el celecoxib [373], un AINE selectivo de COX-2 o fenofibrato, un agonista de PPARα [374-376]. El descubrimiento de estos nuevos moduladores sugiere que γ-secretasa puede ser manipulada farmacológicamente de modo similar a las modificaciones genéticas que producen las mutaciones que producen EAF y elevan la producción de Aβ42 [374, 377].

Asimismo existen moduladores de la γ-secretasa no pertenecientes a la familia de los AINEs. El descubrimiento de un lugar de unión a nucleótido en el complejo γ-secretasa llevó a la síntesis de compuestos con este lugar como diana [378]. Estos compuestos se parecen a los inhibidores de las kinasas e inhiben el procesamiento de APP sin afectar a la proteólisis de Notch.

3.4 Influencia del colesterol en la generación de Aβ (Estatinas)

Estudios *in vitro* han demostrado que el colesterol es capaz de influenciar el metabolismo de APP [233, 379-381]. Asimismo, varios trabajos han mostrado que animales con dieta rica en colesterol aumentan sus niveles de Aβ cerebral y, disminuyendo el colesterol de la dieta reducen la producción de Aβ. El efecto del colesterol en el metabolismo de APP es dosis dependiente y no está mediado por el receptor de lipoproteínas de baja densidad (LDL)[379].

Del mismo modo, estudios epidemiológicos sugieren la existencia de asociación entre los niveles de colesterol y el riesgo de padecer EA [382-389], así como una menor prevalencia de EA en pacientes tratados con estatinas [390-394]. Las estatinas son fármacos inhibidores de la 3-hidroximetil glutaril CoA reductasa (HMG Co-A reductasa) que inhiben la síntesis de colesterol. Sin embargo, no se ha descrito un incremento en la síntesis de colesterol en el cerebro de pacientes con EA, de hecho, los niveles del mRNA de HMG Co-A

reductasa son constantes en cerebros de pacientes comparados con controles ajustados por edad [395].

Se han llevado a cabo varios ensayos clínicos estos últimos años para descifrar el papel de las estatinas en la EA. Las estatinas atorvastatina y simvastatina han mostrado resultados esperanzadores en EA [396, 397] y ensayos clínicos con muestras mayores están actualmente en marcha [398].

El mecanismo por el que una reducción leve de colesterol, similar a la que ocurre en humanos, [397] disminuye Aβ y el mecanismo por el cuál esto ocurre es objeto de discusión. Algunos estudios sugieren una interacción aumentada entre APP y BACE-1 [399]. Por otro lado, la observación que la producción de Aβ se da en los rafts lipídicos y que las β-ciclodextrinas, que extraen colesterol de la membrana, son capaces de inhibir la producción de Aβ abre la posibilidad que la reducción de colesterol pueden alterar el procesamiento de APP perturbando la estructura de los rafts lipídicos [233, 234].

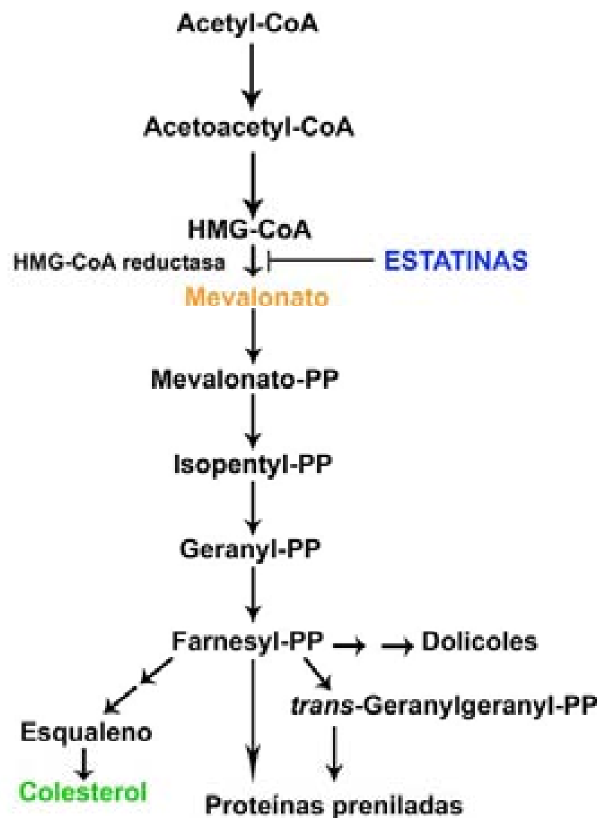

Figura 7. Vía de síntesis del colesterol

OBJETIVOS

IV. OBJETIVOS

Los objetivos de este trabajo son:

1. Estudiar los efectos de la reducción leve de colesterol de membrana sobre el procesamiento proteolítico de la APP y Notch en cultivo celular.

2. Describir en detalle las características clínicas, neuropatológicas y bioquímicas asociadas a la mutación APP I716F

MATERIAL y MÉTODOS

V. MATERIAL Y MÉTODOS

1. Análisis por Inmunoabsorción Ligado a Enzimas (ELISA)

Esta técnica bioquímica se basa en la detección de un antígeno inmovilizado sobre una fase sólida mediante anticuerpos que reaccionan hasta obtener un producto que emite color o fluorescencia y que puede ser cuantificado mediante un lector apropiado.

1.1 ELISA de Aβ40-42 y Aβ1-x total

Los niveles de Aβ se midieron en medio de cultivo 24 horas (DAPT) o 48 horas (estatinas) tras el tratamiento con fármacos o bien 24 horas post-transfección del plásmido con la mutación de interés (en el caso de las mutaciones de *APP*). Para el ELISA de Aβ40, 6E10 es utilizado como anticuerpo de captura (reconoce Aβ1-17, Chemicon, Temecula, CA, USA) y un anticuerpo policlonal Aβ40 (Chemicon) como anticuerpo de detección. Transcurridas 3 horas de incubación, los pocillos se lavan con tampón fosfato salino (PBS) y se añade un anticuerpo marcado con HRP (*horseradish peroxidase*). Los pocillos se lavan de nuevo y se revela con el reactivo Quantablue (Pierce, Rockford, IL, USA). Se realiza la lectura de la placa a 320nm en un lector de placas Victor 3 (Perkin-Elmer, Waltham, MA, USA).
Para el ELISA de Aβ42 y Aβ total (A$\beta_{1\text{-}x}$) se usaron kits comerciales(Wako, Osaka, Japan and IBL, Hamburg,Germany, respectivamente).

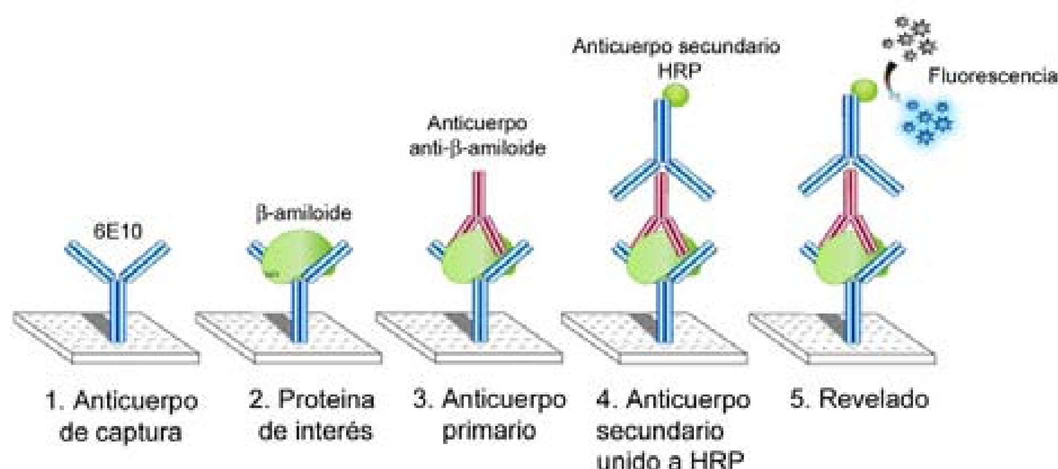

Figura 8. Técnica de ELISA

2. Mutagénesis Dirigida

La construcción de los plásmidos APPV717I y APPI716F se realizó a partir del cDNA de APP695 *wild-type* por mutagénesis dirigida (Stratagene). El protocolo de mutagénesis dirigida se utiliza para crear mutaciones puntuales, reemplazar aminoácidos y eliminar o insertar aminoácidos adyacentes únicos y múltiples. El método básico se inicia a partir de un vector de DNA *supercoiled* de doble cadena (dsDNA) que posea el inserto de interés y dos cebadores de oligonucleótidos sintéticos que también contengan la mutación de interés. Ambos cebadores, complementarios a las dos hebras del vector, son amplificados durante la reacción en cadena de la polimerasa (PCR) con una polimerasa de alta especificidad. La amplificación de los cebadores genera un plásmido mutado con espacios escalonados. Posteriormente, el producto es tratado con la enzima de restricción *Dpn* I. Esta endonucleasa es específica para el DNA metilado y hemimetilado y es utilizada para la digestión del DNA *template* parental y para la selección del DNA que ha incorporado la mutación. El DNA que ya contiene la mutación de interés es entonces transformado en células supercompetentes de *E.Coli*. La incorporación de la mutación fue confirmada por secuenciación directa en un secuenciador automático ABI 3100 (Applied Biosystems).

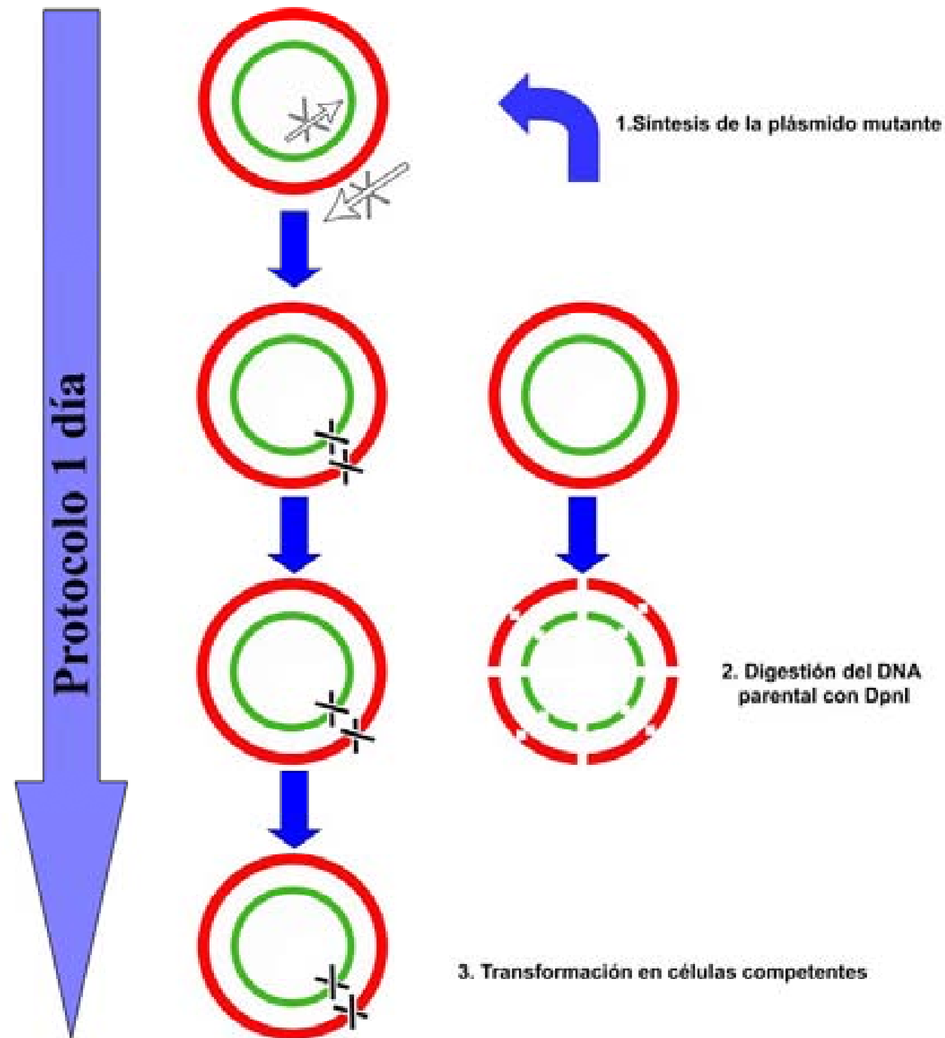

Figura 9. Técnica de mutagénesis dirigida. Esta técnica se inicia a partir de un DNA de doble cadena (dsDNA) con el inserto de interés y dos cebadores sintéticos complementarios con la mutación a las dos hebras del vector. Ambos cebadores son amplificados por PCR con una polimerasa específica generando un plásmido mutado con espacios escalonados. El producto se trata con la enzima *DpnI* y así queda seleccionado el DNA que ha incorporado la mutación de interés. Posteriormente se transforma este plásmido en células supercompetentes de *E. Coli*.

3. Microscopía Confocal (FRET y FLIM)

Recientes avances en el campo de la fluorescencia y la microscopía han permitido el descubrimiento de potentes herramientas con ventajas sustanciales respecto a los tradicionales Western Blot o ensayos de

inmunotinción. En particular, el desarrollo de nuevas proteínas fluorescentes (PFs) y biosensores codificados genéticamente son muy útiles para el estudio de cascadas de señalización, mecanismos de transducción y otros mecanismos biológicos. La microscopía confocal se llevó a cabo con un microscopio fluorescente invertido Leica (Institut Ciències Cardiovasculars de Catalunya, Leica TCD SP2-AOBS, Wetzlar, Germany). Este microscopio está equipado con un laser di-Yodo pulsátil, un detector PMC-100 (Leica, Wetzlar, Germany) y un módulo para contar fotones aislados dependiente del tiempo ((SPC730) para FRET/FLIM.

3.1 *Fluorescence Resonance Energy Transfer* (FRET)

FRET es un fenómeno físico basado en la mecánica cuántica. Esta técnica requiere de dos fluoróforos, un donante y un aceptor, asociados a proteínas u otros componentes de interés. FRET únicamente ocurre cuando estos dos componentes están próximos y la energía del donante es transferida al aceptor. Un fluoróforo puede actuar de donante de FRET si su espectro de emisión se solapa con el espectro de excitación de otro fluoróforo (>30%) que haga de aceptor. Cuando el donante y el aceptor están próximos físicamente (<10nm) con orientaciones favorables, la excitación del donante puede provocar una energía de transferencia e inducir la emisión del aceptor [400]. La distancia y la orientación relativa de los dos fluoróforos puede afectar a la eficiencia de la señal FRET, que se mide por la ratio de la emisión entre aceptor/donante. Por lo tanto, permite obtener información en el rango de nm [401, 402]. FRET ocurre casi instantáneamente y es reversible. Para la aplicación de esta técnica las células pueden ser teñidas con anticuerpos secundarios o ser transfectadas con plásmidos que codifican para la proteína de interés unida a una PFs. La aplicación de FRET puede ser en célula viva o fijada. Por lo tanto para obtener una señal FRET partimos de dos proteínas celulares de interés (donante y aceptor) marcadas con un fluoróforo, se excita el donante a una determinada longitud de onda y se registran los datos de la emisión del donante y/o aceptor. Además la señal FRET puede ocurrir inter o intramolecularmente, permitiendo el estudio de cambios conformacionales de proteínas.

Hay que tener en cuenta que las proteínas donantes y aceptoras de FRET compiten por las proteínas endógenas. Además la ratio aceptor/donante no es uniforme en los diferentes dominios subcelulares por las diferencias de expresión y localización de donantes/aceptores. Por consiguiente, la ratio de emisión de aceptor/donante no puede ser aplicada para monitorizar la eficiencia de FRET y las interacciones moleculares uniformemente en varias regiones celulares. Como resultado de la superposición de espectros (donante-aceptor) que presenta esta técnica, la señal FRET suele estar contaminada por la emisión del donante en el canal del aceptor y por la excitación del aceptor en el canal del donante. La utilización de filtros adecuados y algoritmos matemáticos es necesaria para obtener datos precisos e interpretar los resultados [400]. Por tanto, hay que ser cauto con el análisis matemático complejo que requiere esta técnica para interpretar las observaciones.

3.2 *Fluorescence Lifetime Imaging Microscopy* (FLIM)

FLIM es una técnica novedosa basada en el principio de FRET para el análisis de proximidad entre proteínas que permite visualizar la vida media de moléculas fluorescentes durante el estado de excitación. La técnica de FLIM fue inicialmente desarrollada para el estudio de imagen del comportamiento de especímenes vivos y para estudiar el movimiento dinámico desde células únicas a moléculas [403-405]. El método FLIM se basa en la reducción de la vida media de un fluoróforo donante en presencia de FRET cercano (<10nm). Esta reducción de la vida media es proporcional a la distancia entre fluoróforos a R^6. Los métodos para medir la vida fluorescente están separados en dos categorías: dominio de frecuencia y dominio de tiempo. Para el método de dominio de frecuencia, tanto la luz de emisión como de excitación tienen un formato sinusoidal con la misma frecuencia. La fase entre la excitación y la emisión es usada para calcular la vida media de la fluorescencia. Para el método de dominio de tiempo, utilizado en esta tesis, el fluoróforo es excitado con una luz pulsátil con una duración más corta que la vida media de la emisión. La duración de la intensidad de la fluorescencia emitida capturada es usada para el cálculo de la vida media.

Como FLIM es independiente de las concentraciones locales de las moléculas fluorescentes y la intensidad de excitación, este método proporciona señales más fiables que otras técnicas basadas en la intensidad de la fluorescencia. Sin embargo FLIM es muy dependiente del ambiente en el que se encuentra el fluoróforo, por ejemplo de la existencia de FRET, de los cambios de pH y temperatura y de la presencia o ausencia de iones de Calcio. Cuando una PF donante interacciona con una aceptora mediante FRET, la vida media fluorescente de la PF donante se reduce. Por tanto, FLIM permite separar la población de donantes FRET de aquellos que no interaccionan según la distribución de las vidas medias y de este modo se potencia la ratio señal/ruido de FRET [406].

Al contrario que con la técnica de FRET estándar, la combinación de FRET/FLIM ofrece algunas ventajas. La combinación de las técnicas de FRET y FLIM permite una resolución espacial (nm) y temporal (ns) mayor que la obtenida únicamente con FRET [407-409].

La medición de la vida media del donante en presencia o ausencia del aceptor, permite calcular con precisión la distancia que existe entre las proteínas-donante y aceptora. FLIM-FRET suele presentar dos picos de la vida media del donante (FRET y no-FRET) permitiendo una estimación de la distancia más precisa basada únicamente en los donantes FRET. Ambas técnicas son usadas para varias aplicaciones biológicas, como estudios estructurales de orgánulos celulares, anticuerpos conjugados, identificación citoquímica y metabolismo oxidativo [410].

En el equipo utilizado para la realización de esta tesis el hardware/ software permite medir la vida media fluorescente píxel a píxel. Los valores son ajustados a dos curvas de decaimiento exponencial que representan la población de moléculas no-FRET con una vida media más larga (t2), y la población FRET con una vida media más corta (t1)[406]. Los experimentos de FLIM se realizaron a 22ºC, usando el láser de excitación a 488 nm o a 555 nm y el objetivo de inmersión en aceite 63x.

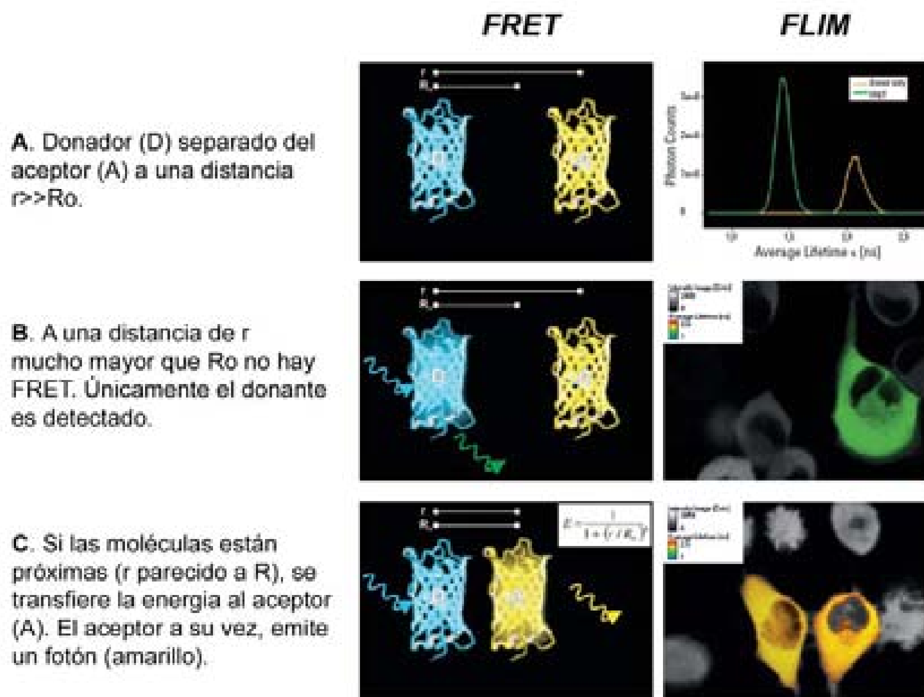

Figura 10. Técnica de FLIM basado en FRET. Adaptado de "Confocal application letter: FRET with FLIM; Quantitative In-Vivo Biochemistry June 2009. Leica Microsystems"

3.3 *Photobleaching*

El mecanismo de *photobleaching* consiste en la destrucción con energía lumínica de un fluoróforo mediante la sobreexposición de una zona celular. En microscopía confocal se usa el *photobleaching* como control de la existencia de FRET y FRAP (*Fluorescence Recovery Acceptor photobleaching*). Al destruir el fluróforo aceptor, el fluoróforo donante ya no puede hacer transferencia de energía y por tanto la señal FRET se reduce (los dos fluoróforos pierden la proximidad).

3.4 Anticuerpos y protocolos inmunocitoquímica

Para el análisis de la interacción de APP con PS1, células PS70 tratadas con lovastatina y MβCDX fueron fijadas y permeabilizadas. Se realizó la doble inmunotinción de las células con anticuerpos contra PS1 loop (aminoácidos del

275-367, Chemicon) y contra el fragmento C-terminal de APP (aminoácidos 643-695, Chemicon). Se añadió como anticuerpos secundarios Alexa 488 y Cyanina 3, respectivamente.

Para el estudio de la integridad de los rafts lipídicos se fijaron y permeabilizaron células H4. La inmunotinción se realizó con la subunidad B de la toxina colérica conjugada con Alexa 555 (CT-B, Invitrogen) o con el anticuerpo anti-Flotilina (proteína residente en los rafts lipídicos, BD Biosciences). Para la detección de APP en la membrana celular, se procedió a la inmunotinción contra APP (anticuerpo APP anti rabbit; Sigma-Aldrich) sin permeabilizar las células con Triton-X.

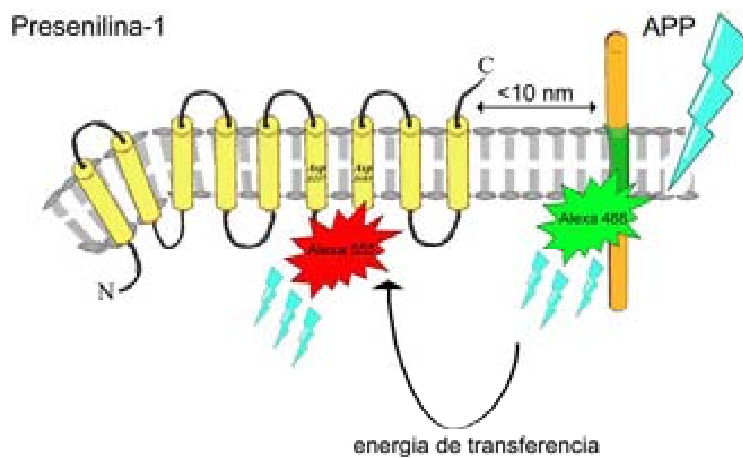

Figura 11. Técnica de FLIM entre las proteínas APP y PS1.

RESULTADOS

CAPÍTULO 1. "*Mild cholesterol depletion reduces amyloid-production by impairing APP trafficking to the cell surface*"

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Mild cholesterol depletion reduces amyloid-β production by impairing APP trafficking to the cell surface

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Abstract

It has been suggested that cellular cholesterol levels can modulate the metabolism of the amyloid precursor protein (APP) but the underlying mechanism remains controversial. In the current study, we investigate in detail the relationship between cholesterol reduction, APP processing and γ-secretase function in cell culture studies. We found that mild membrane cholesterol reduction led to a decrease in $A\beta_{40}$ and $A\beta_{42}$ in different cell types. We did not detect changes in APP intracellular domain or Notch intracellular domain generation. Western blot analyses showed a cholesterol-dependent decrease in the APP C-terminal fragments and cell surface APP. Finally, we applied a fluorescence resonance energy transfer (FRET)-based technique to study APP–Presenilin 1 (PS1) interactions and lipid rafts in intact cells. Our data indicate that cholesterol depletion reduces association of APP into lipid rafts and disrupts APP–PS1 interaction. Taken together, our results suggest that mild membrane cholesterol reduction impacts the cleavage of APP upstream of γ-secretase and appears to be mediated by changes in APP trafficking and partitioning into lipid rafts.

Keywords: Alzheimer disease, cholesterol, FLIM, FRET, presenilin, rafts, statins, γ-secretase.

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Amyloid-β protein (Aβ) is a 4-kDa peptide believed to play a central role in the pathogenesis of Alzheimer's disease (Hardy and Higgins 1992). Aβ arises from cleavage of the β-amyloid precursor protein (APP), encoded by a gene on human chromosome 21. APP undergoes a proteolytic event by α-secretase within the Aβ region creating a large secreted ectodomain (α-APPs) and a shorter α-cleaved membrane-retained carboxyl-terminal fragment (α-CTF). The resultant 10-kDa α-CTF is cleaved by a presenilin (PS)-dependent γ-secretase to generate a small fragment called p3 (non-amyloidogenic pathway). In an analogous fashion, other APP holoproteins can be cleaved by another protease, β-site APP cleaving enzyme (BACE), generating a 12-kDa CTF (β-CTF) that is cleaved by the same γ-secretase to produce Aβ. In addition to releasing Aβ or p3, the same γ-secretase activity generates an APP intracellular fragment (AICD) that may be transcriptionally active (Cao and Sudhof 2004). Although the generation of the different isoforms of Aβ and

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Abbreviations used: AICD, amyloid precursor protein intracellular domain; APP, amyloid precursor protein; Aβ, amyloid-β protein; BACE, β-site APP cleaving enzyme; BAP, biotin acceptor peptide; CHO, chinese hamster ovary cells; CT-B, cholera toxin subunit B; Cy3, cyanine 3; DAPT, *N*-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester; DLFBS, delipidated fetal bovine serum; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; H4, human neuroglioma cell line; HA, hemagglutinin; HEK, human embryonic kidney; HMG CoA, 3-hydroxyl-3-methylglutaryl coenzyme A reductase; HRP, horseradish peroxidase; MβCDX, methyl-β-cyclodextrin; NICD, Notch intracellular domain; PS1, presenilin 1; PS2, presenilin 2; TM, transmembrane domain; α-CTF, α-cleaved carboxyl-terminal fragment; β-CTF, β-cleaved carboxyl-terminal fragment.

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p3 has been intensively studied, the normal biological function of APP proteolysis remains unclear.

The γ-secretase complex is a multiprotein complex composed of at least four proteins: presenilin (presenilin 1 or presenilin 2, PS1 and PS2, respectively), which is believed to contain the catalytic site, nicastrin, Pen-2, and Aph1 (Wolfe and Kopan 2004). PS1 is a 467 amino acid nine-transmembrane domain (TM) protein that requires endoproteolysis to generate a functional heterodimer in which the C- and N-terminal fragments remain non-covalently associated (Selkoe and Wolfe 2007). This multiprotein complex is responsible for the cleavage of at least 30 different substrates, mostly type-I membrane proteins including APP and the Notch receptor among others (Lleo 2008). Notch receptor is a type I transmembrane receptor that is critically required for a variety of signaling events and cell fate decisions during embryogenesis and in adulthood (Artavanis-Tsakonas and Simpson 1991; Levitan and Greenwald 1995). Full-length Notch is cleaved in the presence of its biological ligand Delta, and the generated Notch intracellular domain (NICD) rapidly translocates to the nucleus where it acts as a transcriptional coactivator (Schroeter *et al.* 1998; Jack *et al.* 2001). Similar to APP proteolysis, Notch proteolysis is dependent on PS1 (Schroeter *et al.* 1998).

Because γ-secretase is responsible for the last step in Aβ generation, understanding how to modulate its activity is of considerable therapeutic interest. Several compounds (γ-secretase inhibitors) have been developed to decrease Aβ production by inhibiting the activity of this multiprotein complex. However, general γ-secretase inhibitors impair the cleavage of other substrates in addition to APP, giving rise to concerns about tolerability. Another therapeutic approach that has raised interest is γ-secretase modulation. For example, certain non-steroidal anti-inflammatory agents have been observed to alter the site of γ-secretase cleavage in APP, rather than inhibiting the enzymatic activity (Weggen *et al.* 2001; Lleo *et al.* 2004). We have suggested that the mechanism of action of modulatory agents is allosteric modulation of γ-secretase (Lleo *et al.* 2004).

Another pharmacological intervention investigated in Alzheimer disease (AD) has been the use of cholesterol-lowering agents. There is growing evidence that links AD and cholesterol metabolism. Epidemiological studies have shown a decreased incidence and prevalence of AD among individuals treated with statins, widely used drugs that reduce cholesterol by inhibiting 3-hydroxyl-3-methylglutaryl coenzyme A (HMG CoA) reductase (Jick *et al.* 2000; Wolozin *et al.* 2000; Rockwood *et al.* 2002; Yaffe *et al.* 2002; Zamrini *et al.* 2004). Clinical trials with atorvastatin and simvastatin in AD have shown some promising results (Simons *et al.* 2002; Sparks *et al.* 2005), and larger clinical trials are currently ongoing (http://www.clinicaltrials.gov). Another line of evidence comes from studies showing that changes in cholesterol homeostasis affect APP processing. Most studies have shown that strongly reducing cholesterol levels with statins and/or cyclodextran causes a marked reduction in Aβ levels *in vitro* and *in vivo* (Simons *et al.* 1998; Fassbender *et al.* 2001; Refolo *et al.* 2001; Ehehalt *et al.* 2003; Ostrowski *et al.* 2007). However, it remains controversial whether milder cholesterol depletion similar to that observed in humans taking therapeutic levels of statins (Sparks *et al.* 2005) lowers Aβ production and the mechanism by which this occurs. Some studies have suggested that mild cholesterol reduction may actually enhance Aβ generation by facilitating the interaction between APP and BACE 1 (Abad-Rodriguez *et al.* 2004). On the other hand, the observation that Aβ generation depends on lipid rafts and that β-cyclodextrins, which rapidly extract cholesterol directly from the plasma membrane, are able to inhibit Aβ production raises the possibility that cholesterol reduction may alter APP processing by disrupting lipid rafts structure (Simons *et al.* 1998; Wahrle *et al.* 2002).

Since γ-secretase consists of multiple transmembrane proteins, and is preferentially distributed in association with lipid rafts, we explored the mechanism by which manipulation of the membrane lipid environment might impact APP processing. In this study, we show that mild cholesterol depletion led to a reduction in secreted Aβ, APP CTFs and cell surface APP, but preserved AICD generation and the γ-secretase-dependent cleavage of Notch. We also found that membrane cholesterol depletion reduced the association of APP with lipid rafts at the cell membrane in intact cells by using a fluorescence resonance energy transfer (FRET)-based microscopy approach.

Experimental procedures

Cell lines, plasmids and transfection protocol

We used the following cell lines: naive Chinese hamster ovary (CHO) cells, CHO cells stably over-expressing wild-type human PS1 and wild-type APP (PS70, a generous gift from Dr Selkoe, Brigham and Women's Hospital, Boston, MA, USA), human neuroglioma cell line (H4) stably expressing the double Swedish APP mutation (a generous gift from Bruno Imbimbo, Chiesi Farmaceutici, Parma, Italy) or human embryonic kidney (HEK) cells stably expressing the double Swedish APP mutation. Biotin acceptor peptide (BAP)-APP construct was used for cell surface biotinylation. The construct contains a BAP on the N-terminus of APP695 and a hemagglutinin (HA) tag on the C-terminus. Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C with 5% CO_2 in a tissue culture incubator. For fluorescence resonance energy transfer (FRET)/fluorescence lifetime imaging microscopy (FLIM) and confocal microscopy, we used PS70 cells, H4 cells stably expressing the double Swedish APP mutation or CHO cells transiently transfected with wild-type APP695, or wild-type PS1 (a generous gift from Carlos Saura, Autonomous University of Barcelona, Barcelona, Spain) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

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Membrane cholesterol depletion

Different doses of lovastatin (Calbiochem, San Diego, CA, USA), or methyl-β-cyclodextrin (MβCDX, Sigma-Aldrich, St Louis, MO, USA) were used for different time periods to induce membrane cholesterol depletion. Cell toxicity was analyzed for all drugs by measuring adenylate kinase levels using Toxi-light reagent (Cambrex, Charles City, IA, USA). The γ-secretase inhibitor *N*-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester (DAPT 1 μM, Sigma-Aldrich) was used as a control. All conditions were supplemented with mevalonate (0.25 mM, Sigma-Aldrich) to support the isoprenoid pathway. Total cell cholesterol levels were measured using the Amplex Red Cholesterol Assay Kit (Invitrogen). We tested lovastatin at different concentrations and for different time periods. We found that treatment for 48 h with either 20 μM lovastatin with 0.25 mM mevalonate and delipidated fetal bovine serum (DLFBS) was able to induce mild but consistent (25–30%) cell membrane cholesterol reduction without signs of cytotoxicity (Fig. S1). We also examined the effects of methyl-β-cyclodextrin (MβCDX), which selectively extracts cholesterol from the plasma membrane in preference to other lipids. Consistent cholesterol reductions were only obtained after treating cells with 5 mM MβCDX for 10 or 60 min as treatment for 48 h induced significant cytotoxicity (Fig. S1). Therefore, we used for all experiments 20 μM lovastatin or 5 mM MβCDX for 10 or 60 min which induced a consistent cholesterol reduction.

Aβ ELISA and western blot

For human $A\beta_{1-40}$ ELISA we used 6E10 (against $A\beta_{1-17}$, Chemicon, Temecula, CA, USA) as a capture antibody and a rabbit polyclonal $A\beta_{1-40}$ (Chemicon) as a detection antibody. After incubation for 3 h, wells were washed and a horseradish peroxidase-conjugated Donkey anti-rabbit (Jackson Laboratories, West Grove, PA, USA) was added. Wells were washed with phosphate-buffered saline (PBS), Quantablue reagent (Pierce, Rockford, IL, USA) was added and samples were read at 320 nm using a Victor3 Wallac plate reader (Perkin-Elmer, Waltham, MA, USA). To measure human $A\beta_{1-42}$ and $A\beta_{1-x}$ we used sensitive ELISA kits (Wako, Osaka, Japan and IBL, Hamburg, Germany, respectively).

For the western blot analysis of Notch cleavage, CHO cells were transfected with the truncated Notch1 construct NotchΔECmyc (a generous gift from R. Kopan, Washington University, St Louis, MO, USA) and treated with lovastatin, DAPT or MβCDX for 48, 24 h or 10 min, respectively. The cellular lysate was electrophoresed in 10–20% Tris-glycine gels and transferred to a nitrocellulose membrane. The immunoblotting was performed with a mouse 9E10 anti-myc antibody (Chemicon).

For the western blot analysis of the APP C-terminal fragments (CTFs) we isolated cellular membranes, as described (Steiner *et al.* 1998), from PS70 cells treated with statins or MβCDX. Membrane preparations were electrophoresed in 5–16% Tris–Tricine gels, transferred to 0.2 μm nitrocellulose membranes, and detected by immunoblotting with a rabbit anti-APP C-terminal (Sigma) antibody. Incubation with primary antibodies was followed by detection with IR-fluorescent-conjugated antibody (LI-COR Biosciences, Bad Homburg, Germany). The bands were quantitated using Odyssey software (LI-COR Biosciences), and the values normalized to APP full-length expression.

Cell-surface APP biotinylation

Human embryonic kidney cells were transfected with the BAP-APP-HA construct using Fugene 6 reagent (Roche, Indianapolis, IN, USA) and the cells were treated with lovastatin, 5 or 10 mM MβCDX in serum-free medium for 60 min (48 h for lovastatin) at 37°C. Cell surface was biotinylated in PBS with 0.3 μM BirA and 10 μM biotin-AMP for 40 min at 30°C (Chen *et al.* 2005). The remaining biotin was removed by washing three times with PBS. The cells were lysed in 70 μL PBS containing 1% Triton X-100, 0.1% SDS and protease inhibitors. Insoluble debris were removed by centrifugation (20 000 *g* for 30 min at 4°C). The lysate was analyzed for APP expression by western blotting. The membrane was probed with streptavidin (SA)-horseradish peroxidase for cell surface APP and normalized to HA-HRP (total APP).

Cell-free AICD generation assay

AICD was generated *in vitro* from membrane preparations of PS70 cells as described (Sastre *et al.* 2001). Cells lysates were treated with vehicle, different concentrations of MβCDX or DAPT and incubated at 37°C for 2 h. As a negative control we incubated cells on ice. APP C-terminal fragments were detected as described above.

CBF1 luciferase assay

The CBF1-luciferase assay was performed as described previously (Berezovska *et al.* 2000). Briefly, CHO cells were transfected with a CBF1 luciferase reporter plasmid (a generous gift from D. Hayward) and β-galactosidase as an internal control for transfection efficiency. This assay detects activation of CBF1, a Notch1 downstream transcription factor, as a measure of Notch signaling. The treatment with lovastatin or MβCDX was begun 6 h after transfection. Luciferase activity was measured 48 h after treatment using a Victor3 Wallac plate reader (Perkin-Elmer), and results were normalized to β-galactosidase expression levels.

Antibodies and immunocytochemistry procedures

For the analysis of APP-PS1 interaction, PS70 cells were double immunostained with antibodies against PS1 loop (amino acids 275–367, Chemicon) and against the C-terminal fragment of APP (amino acids 643–695, Chemicon). Pairs of primary antibodies were labeled with Alexa 488 or cyanine 3 (Cy3)-conjugated secondary antibodies.

For the detection of lipid rafts, living H4 cells were treated with MβCDX or a vehicle control, then immunostained with red-fluorescent Alexa 555 conjugate of cholera toxin subunit B (CT-B, Invitrogen) or fixed and immunostained with a Alexa 555-labeled flotillin antibody (BD Biosciences). For the detection of cell surface APP, cells were then immunostained without permeabilization with an APP antibody (Sigma-Aldrich). CT-B binds to the pentasaccharide chain of plasma membrane ganglioside GM1, which selectively partitions into and is one of the most widely used markers for lipid rafts (Sandvig and van Deurs 2002).

Confocal microscopy and fluorescence lifetime imaging microscopy

Confocal microscopy was performed using a Leica inverted fluorescent confocal microscope (Institut Ciències Cardiovasculars de Catalunya, Leica TCD SP2-AOBS, Wetzlar, Germany). This microscope is equipped with a 405 diode pulsed laser, a PMC-100 detector (Leica, Wetzlar, Germany) and a time-correlated single photon

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counting module (SPC730) to perform FRET/FLIM. The hardware/software package allows the measurement of fluorescence lifetimes on a pixel-by-pixel basis. Values were fitted to two exponential decay curves to represent a 'non-FRETing' population with a longer lifetime (*t*2) and a 'FRETing' population with a shorter lifetime (*t*1).

FLIM has been described as a novel technique for the analysis of protein proximity (Berezovska *et al.* 2003; Lleo *et al.* 2004). The technique is based on the observation that fluorescence lifetimes of a donor fluorophore shorten in the presence of a FRET acceptor in close proximity (< 10 nm). The decrease in lifetime is proportional to the distance between the fluorophores at R^6.

For APP-PS1 FRET/FLIM experiments, cells were fixed and double-immunostained for APP and PS1 as described previously (Berezovska *et al.* 2003; Lleo *et al.* 2004). We also applied this technique to detect lipid rafts and partition of APP into rafts by double staining H4 cells with Alexa 555-CT-B conjugate or a flotillin antibody and an APP antibody (Sigma-Aldrich). As a positive control, cells were immunostained against flotillin and CT-B, labeled with Alexa 555 and Alexa 488 secondary antibodies respectively, or with equimolar concentrations of Alexa488-CT-B and Alexa555-CT-B. All samples were compared with a negative control in which the donor fluorophore (Alexa 488) fluorescence lifetime was measured in the absence of the acceptor (no FRET ~2500 ps). As positive controls, we included two additional conditions. First, Alexa 488 lifetime was measured in the presence of a FRET acceptor (Cy3) in close proximity (Cy3-labeled antibody against Alexa488). Under these conditions, we observed that Alexa 488 lifetime was shortened to ~1000 ps. Second, we performed photobleaching of the acceptor fluorophore and observed that the FRET signal was completely abolished (Fig. S2).

Statistical analysis

One-way ANOVA was performed to analyze differences in lifetime or Aβ levels followed by least significant difference *post hoc* analysis. Levene's test was also performed to determine whether variances were equal.

Results

Mild membrane cholesterol depletion reduces $A\beta_{40}$ and $A\beta_{42}$, and APP CTFs

We developed a paradigm to induce mild cholesterol depletion by treating cells with lovastatin in the presence of low doses of mevalonate and DLFBS. Supplementation of statin-treated cells with 0.25 mM mevalonate is required to rescue the normal isoprenoid levels while blocking cholesterol biosynthesis (Brown and Goldstein 1980; Goldstein and Brown 1990; Keller and Simons 1998; Simons *et al.* 1998; Fassbender *et al.* 2001; Kojro *et al.* 2001; Meske *et al.* 2003; Cole *et al.* 2005). We found that treatment of CHO or HEK cells with 20 μM lovastatin in the presence of 3% DLFBS for 48 h induced a consistent total cholesterol depletion (~30%) without signs of cytotoxicity (Fig. S1). Treatment with statins did not induce cholesterol reduction in the presence of non-delipidated FBS. We also measured the total cholesterol levels after treatment with MβCDX, which selectively extracts cholesterol from the plasma membrane in preference to other lipids. Consistent cholesterol reductions were only obtained after treating cells with 5 mM MβCDX for 10 or 60 min. Treatment with MβCDX for longer periods of time affected cell viability (Fig. S1).

We next investigated the effects of cholesterol depletion on APP processing. We treated APP/PS1 over-expressing CHO (PS70) or SweAPP over-expressing HEK cells for 48 h in the presence or absence of lovastatin and DLFBS and levels of Aβ40 and Aβ42 were measured in the conditioned media by sandwich ELISA. After 48 h of treatment both Aβ40 and Aβ42 levels in the conditioned media were significantly reduced compared with that in the vehicle control-treated cells (~50%, $p < 0.05$, Fig. 1a). The reduction was observed in both PS70 and HEK cells confirming that the effect was not cell-type specific. To ensure that this effect was due to a decrease in cellular cholesterol levels and not by any other pleiotropic action of statins, we treated cells with lovastatin in the presence of 3% FBS. The addition of cholesterol-containing serum was sufficient to reverse the reduction in Aβ levels (Fig. 1a) indicating that the observed effects on APP processing were specifically due to cholesterol depletion. As a positive control, we treated cells for 24 h with 1 μM DAPT, a commonly used γ-secretase inhibitor, and found that it reduced both Aβ40 and Aβ42 levels by ~95% consistent with a complete inhibition of γ-secretase ($p < 0.001$).

We next examined whether cholesterol reduction would lead to changes in the levels of full-length APP or APP CTFs, the direct substrates of γ-secretase. We isolated cell membranes from PS70 cells treated with lovastatin/DLFBS or MβCDX, and the lysate was subjected to western blot analysis. We found that cholesterol depletion did not alter total APP levels, but reduced the levels of both α- and β-CTF by ~30% compared with a vehicle control (Fig. 1b). Addition of serum to cells was able to restore the CTF levels confirming that the effect was cholesterol-dependent. As expected, DAPT led to a strong accumulation of APP CTFs. These data indicate that cholesterol reduction lowers Aβ, affects APP processing and APP CTF generation.

Cholesterol depletion affects APP trafficking and reduces cell surface APP

The reduction in APP CTFs observed after cholesterol reduction could reflect a possible effect on APP trafficking or an inhibition of α- and β-cleavages. To distinguish between these two possibilities we performed experiments to analyze APP trafficking by using a BAP-APP construct in HEK cells. We found that treatment with lovastatin or MβCDX in cells transfected with BAP-APP led to a reduced cell surface APP without changes in total APP levels (Fig. 1c). Combination of lovastatin and MβCDX showed an additive effect. Overall, our results suggest that cholesterol depletion reduces APP CTFs by altering APP trafficking and reducing substrate availability.

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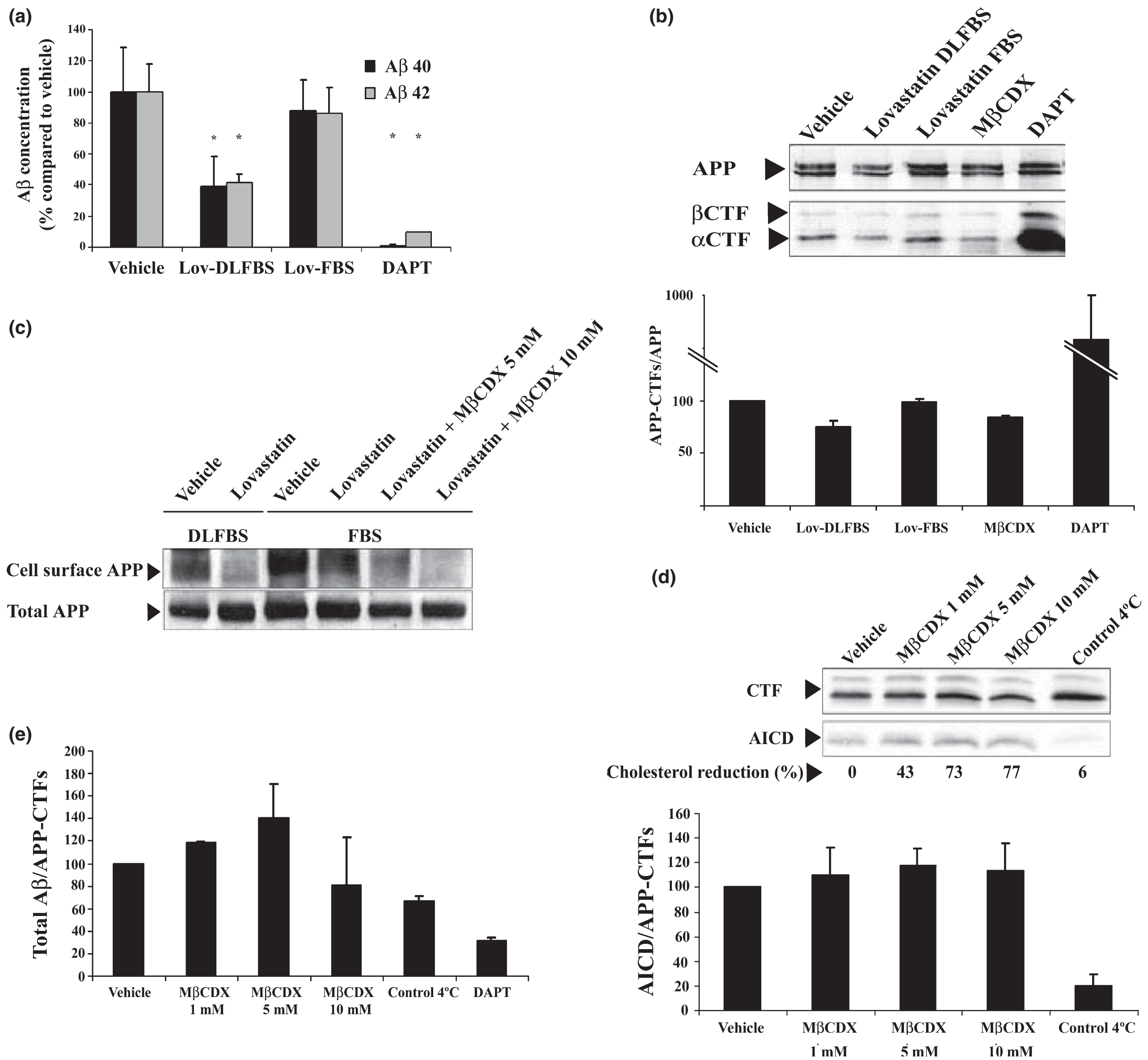

Fig. 1 Cholesterol depletion reduces Aβ, APP CTFs and cell surface APP without affecting AICD generation. (a) PS70 or HEK cells were treated with 20 μM lovastatin in the presence or absence of DLFBS for 48 h and Aβ levels were measured by sandwich ELISA. Treatment with lovastatin led to a reduction of Aβ40 and Aβ42 that is prevented by the presence of cholesterol-containing serum. (b) PS70 cells were treated with lovastatin or MβCDX in the presence or absence of DLFBS for 48 h and membrane preparations were obtained as described (Sastre *et al.* 2001). Levels of full-length APP and APP CTFs were measured by western blot. Cholesterol depletion reduced both α- and β-CTFs that can be recovered by addition of cholesterol-containing serum. Quantification from four independent experiments is shown below. (c) HEK cells were transfected with BAP-APP construct and treated with vehicle, lovastatin, 5 or 10 mM MβCDX in FBS or DLFBS. Cell surface APP was biotinylated and the cell lysate was subjected to Western blot analysis for APP expression. Treatment with lovastatin or/and MβCDX reduced cell surface APP without altering total APP expression. (d) Cell-free AICD generation assay. PS70 cells were treated with different concentrations of MβCDX and membrane preparations were incubated at 37 °C for 2 h. Extent of cholesterol reduction is indicated below. APP CTFs and AICD were measured by western blot. No changes in AICD generation were observed after cholesterol depletion. Incubation at 4°C or with DAPT inhibited the production of AICD. Average data from three independent experiments are shown below. (e) $A\beta_{1-x}$ levels were measured in the same cell-free assay and results were normalized to levels of APP CTFs. Only a slight decrease was observed at the highest MβCDX concentration. Incubation at 4°C and more markedly treatment with DAPT reduced Aβ generation. Data represent the average of two independent experiments.

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Cholesterol depletion does not impair AICD generation in a cell-free assay

This assay measures *in vitro* generated AICD which results from a preexisting β-CTF (Sastre *et al.* 2001). We incubated cell membrane preparations with different concentrations of MβCDX for 2 h at 37°C and analyzed the levels of AICD by western blot. As a control, membranes were incubated at 4°C or with DAPT and minimal amounts of AICD are detected consistent with γ-secretase inhibition (Fig. 1d). Treatment of cell membranes with different concentrations of MβCDX, which was able to reduce total cholesterol in a dose-dependent manner up to 70%, had no effect on AICD generation. Because this assay measures *de novo* AICD from preexisting CTFs (Sastre *et al.* 2001), we did not observe any effects on the levels of CTFs. We also measured in parallel the levels of total Aβ in this assay and results were normalized to levels of APP CTFs. We found that cholesterol reductions up to 70% did not reduce total Aβ (Fig. 1e).

Cholesterol depletion does not impair Notch signaling or the S3 Notch cleavage

We next examined whether cholesterol depletion altered the processing of other γ-secretase substrates. The γ-secretase cleavage of the Notch receptor was monitored by using a luciferase assay that reflects Notch signaling as previously described (Hsieh *et al.* 1996; Lleo *et al.* 2003). CHO cells were transfected with a CBF1 luciferase reporter construct or an empty vector and β-galactosidase as an internal control for the transfection efficiency. Cells were treated for 48 h with lovastatin, MβCDX or DAPT, and CBF1 luciferase activity was detected in the lysates. The results were normalized to β-galactosidase expression levels. There was no difference in the CBF1 luciferase activity after cholesterol depletion compared with that in vehicle-treated control cells (Fig. 2a). As expected, a marked reduction in CBF1 activity was observed after treatment with DAPT ($p < 0.05$).

To confirm these results, we analyzed the levels of NICD, the γ-cleaved product of Notch, by western blot. CHO cells were transfected with a constitutively active form of Notch (NΔEC) which undergoes cleavage and generation of the NICD domain. NΔEC-transfected cells were treated with lovastatin, DAPT, MβCDX or a vehicle control. After 48 h the cells were harvested and lysates were subjected to western blot analysis (Fig. 2b upper panel). After quantification, we did not observe any differences in the generation of NICD after cholesterol depletion (Fig. 2b lower panel). As expected, treatment with DAPT led to a marked reduction in the generation of NICD with accumulation of the NΔEC fragment, consistent with inhibition of γ-secretase. Taken together, these results indicate that cholesterol depletion does not impair either Notch signaling or γ-secretase-dependent Notch S3 cleavage.

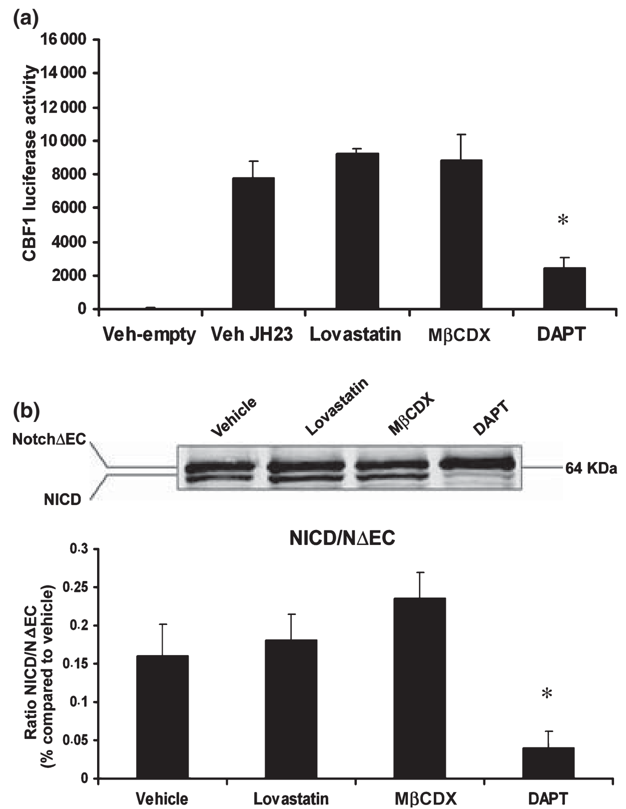

Fig. 2 Cholesterol depletion does not impair Notch signaling or S3 cleavage. (a) CHO cells were transfected with a CBF1 luciferase reporter construct or and empty vector and β-galactosidase as an internal control for transfection efficiency. Cells were treated with MβCDX, lovastatin, or DAPT and CBF1 luciferase activity was detected in the lysates as a measure of Notch signaling. We did not observed differences in CBF1 luciferase activity after cholesterol depletion compared with a vehicle control. (b) CHO cells transfected with NΔEC were treated with lovastatin, MβCDX, DAPT or a vehicle control. After 48 h the cells were harvested and the lysate was subjected to western blot analysis. After quantification, we did not observe any significant differences in the production of NICD after cholesterol depletion. As a control, cells were treated with DAPT that led to a marked reduction in the generation of NICD with accumulation of the NΔEC fragment consistent with γ-secretase inhibition. Average data from three independent experiments are shown.

Cholesterol removal decreases APP partition into lipid rafts assessed by FLIM

Since lipid rafts are one of the main sites where APP amyloidogenic processing takes place (Lee *et al.* 1998), we next explored whether cholesterol removal had any impact on raft-associated APP. We developed a novel FRET-based assay (FLIM) to measure association of APP into lipid rafts by staining cells with a flotillin antibody or CT-B-Alexa555. This FRET assay is based on the principle that when the two fluorophores are in close proximity (< 10 nm), the measured

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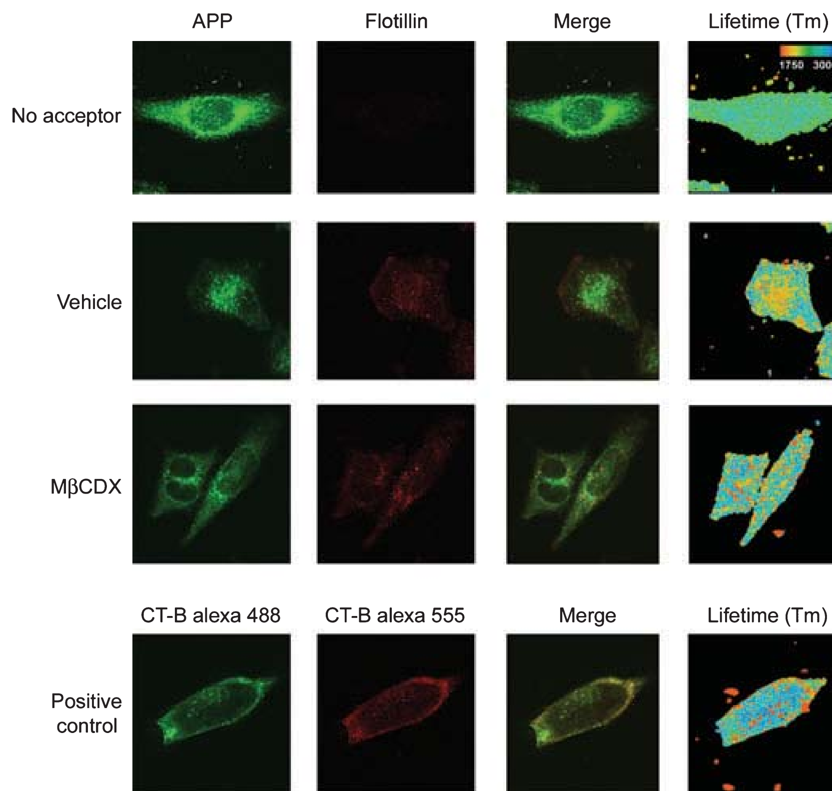

Fig. 3 A novel FRET/FLIM assay to measure lipid raft-associated APP in intact cells. We developed a novel FRET-based assay to detect lipid rafts and APP in intact cells by immunostaining H4 neuroglioma cells with Alexa488-labeled APP and flotillin -Alexa555 antibodies. The Alexa488 (donor) fluorescence lifetime under these conditions was measured on a pixel-by-pixel basis in intact cells, and *t*m values are shown in a pseudocolored image. We observed that although only small percentage of APP co-localized with rafts, donor lifetime was significantly shortened after co-immunostaining with Alexa555-flotillin probe indicating the presence of FRET. Treatment with MβCDX increased fluorescence lifetime, which is reflected by a shift towards the blue color. As a positive control, cells were immunostained with equimolar concentrations of Alexa488-CT-B and Alexa555-CT-B. As expected, we observed wide co-localization and donor lifetime was significantly shortened, which is reflected by the presence of bright red pixels near the cell surface.

Table 1 Cholesterol depletion reduces association of APP into lipid rafts

Condition	No[a]	Donor lifetime[b]	Probability [c]
Vehicle	12	100 ± 4	–
MβCDX	11	114 ± 3	$p = 0.03$
Positive control	15	78 ± 4	$p < 0.01$

We treated H4 cells with MβCDX to induce a membrane cholesterol reduction. After treatment, cells were fixed and immunostained with an alexa488-labeled APP and alexa555-flotillin (FRET acceptor) antibodies. The donor fluorophore lifetime (alexa488) was measured under different treatment conditions, and values were compared with vehicle-treated cells. In the absence of acceptor (Alexa555) Alexa488 lifetime is ~2500 ps, but in its presence is shortened by ~25% to ~1800 ps. The increased Alexa488 lifetime in cells treated with MβCDX indicates a reduced FRET signal after cholesterol depletion. Cells stained with equimolar concentrations of CT-B labeled with Alexa488 and Alexa555 were used as a positive control. Results were confirmed in three independent experiments. A confirmatory approach using Alexa555-CT-B as a FRET acceptor showed similar results (data not shown). [a]Number of cells analyzed. [b]Expressed as a percentage of vehicle. Mean ± SE. [c]Compared with vehicle control.

lifetime of the donor fluorophore (Alexa 488-APP) is shortened in proportion to the distance between the fluorophores. For these experiments we tested CHO, HEK or H4 cells and found that only the latter ones had sufficient lipid raft staining to perform FRET experiments. We observed that only a small percentage of APP co-localized with rafts on the cell surface (Fig. 3). However, this small amount was enough to shorten Alexa488 lifetime by ~25% in the presence of the acceptor probe (flotillin or Alexa555-CT-B) indicating the presence of FRET (Table 1). As a positive control, we stained cells with CT-B and flotillin or equimolar concentrations of Alexa488-CT-B and Alexa555-CT-B and observed wide co-localization as well as a further 25% reduction in fluorescence lifetime (Table 1). Therefore, this assay reflected the association of APP into lipid rafts and could be applied to measure changes under cholesterol reducing conditions. We observed that treatment with MβCDX increased Alexa488 lifetime (15%) compared with vehicle-treated cells (Fig. 3, Table 1). We interpret these data as a reduced partition of APP into lipid rafts.

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Table 2 Cholesterol depletion leads to a change in APP-PS1 interaction as assessed by FLIM

Condition	No[a]	Donor lifetime[b]	Probability [c]
Vehicle	24	100 ± 14	–
Lovastatin	23	111 ± 15	$p = 0.03$
MβCDX	6	138 ± 14	$p < 0.01$

PS70 cells stably expressing wtPS1 and wtAPP were treated with lovastatin or MβCDX to deplete membrane cholesterol. After the treatment, cells were fixed, and immunostained for PS1 and APP with alexa488- and Cy3-labeled antibodies respectively. The donor Alexa488 fluorescence lifetime was measured under different conditions, and the values were compared with vehicle-treated cells. Increased Alexa488 lifetime after treatment with lovastatin or MβCDX indicates a reduced APP-PS1 interaction. [a]Number of cells analyzed. [b]Expressed as a percentage of vehicle. Mean ± SE. [c]Compared with vehicle control.

FLIM assay shows that cholesterol depletion leads to a reduced APP-PS1 interaction

We postulated that the observed effects on APP processing and trafficking under mild cholesterol-lowering conditions might have altered γ-secretase–APP interactions. To confirm this possibility we used a FRET-based (FLIM) assay to detect APP CTFs–PS1 interactions in intact cells (Berezovska *et al.* 2003, 2005). We measured the proximity of the loop region of PS1, which is adjacent to the putative catalytic site of γ-secretase, to the C-terminus of APP (Berezovska *et al.* 2003, 2005; Lleo *et al.* 2004). We checked that treatment with lovastatin or MβCDX did not alter APP or PS1 cellular distribution in PS70 cells as assessed by confocal microscopy. Interestingly, PS1 partially co-localized with lipid rafts at the cell surface but co-localization was not affected by cholesterol depletion (Fig. S3). PS70 cells were treated with 20 μM lovastatin for 48 h or 5 mM MβCDX for 10 min. The donor fluorophore (Alexa 488-PS1) had a lifetime of ~2500 ps in the absence of a FRET acceptor. When the acceptor (Cy3-labeled APP epitope) is in close proximity to the donor, the lifetime is shortened by 40% (~1600 ps). By contrast, treatment with lovastatin or MβCDX diminished this effect and increased Alexa 488 lifetime by 11.5% and 38.5% respectively compared with the baseline conditions (Table 2, Fig. 4). We interpret these data as reflecting a reduced interaction between APP CTFs and PS1 in cells with depleted cholesterol.

Discussion

The main focus of this study was to determine whether mild membrane cholesterol depletion affects APP processing and the mechanism by which this occurs. This is a relevant question because cholesterol-lowering agents are being investigated as a possible treatment for AD. While strong reduction of the cholesterol levels has been shown to reduce Aβ production (Simons *et al.* 1998; Fassbender *et al.* 2001; Kojro *et al.* 2001; Refolo *et al.* 2001; Ehehalt *et al.* 2003; Xiong *et al.* 2008), the effect of mild cholesterol depletion on Aβ and the mechanism by which this occurs is controversial. Here we find that treatment of different cell lines with lovastatin reduced Aβ40 and Aβ42 in a cholesterol-dependent manner. The reduction of Aβ was observed in cells supplemented with mevalonate, and was reversed by addition of cholesterol-containing serum (Cole *et al.* 2005; Cordle

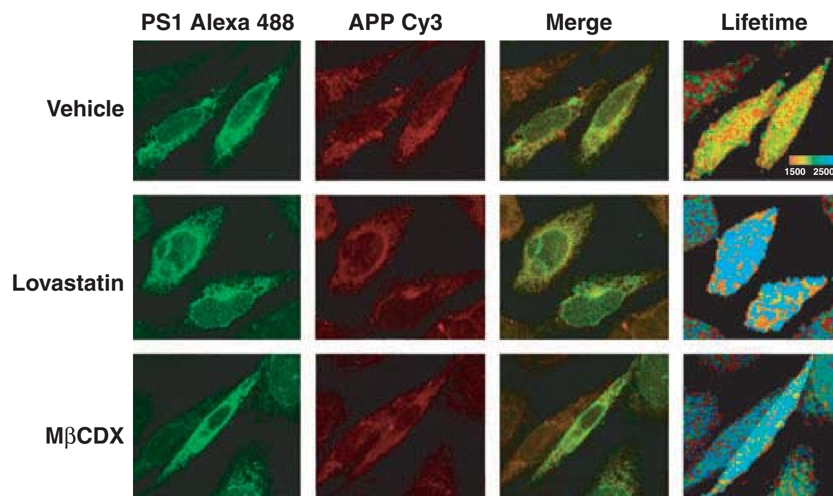

Fig. 4 Cholesterol depletion leads to a reduced APP-PS1 interaction. PS70 cells stably expressing PS1 and APP were treated with lovastatin or MβCDX to induce a membrane cholesterol reduction. After treatment, cells were fixed, and immunostained for PS1-loop and APP-C terminus with alexa488- and Cy3-labeled antibodies respectively. *t*1 values are shown in a pseudocolored image. The red pixels represent closest proximity between PS1 and APP. Treatment with lovastatin and more markedly MβCDX increased Alexa488 lifetime, which is reflected by a shift towards the blue color.

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and Landreth 2005; Ostrowski *et al.* 2007). This indicates that the observed effects are directly related to cholesterol depletion and not to any other pleiotriopic effect of statins. Although statins have also been shown to lower Aβ by inhibiting protein isoprenylation (Ostrowski *et al.* 2007), our data suggest that statins can also display the same effect by reducing membrane cholesterol.

We next investigated whether γ-secretase is sensitive to membrane cholesterol reduction. Experiments to address this issue have yielded conflicting results so far. Some authors have shown that γ-secretase depends on rafts but is not cholesterol-dependent (Wada *et al.* 2003), while others have found that indeed the enzyme can be modulated by cholesterol (Wahrle *et al.* 2002; Xiong *et al.* 2008). Our data support the notion that neither the ε-cleavage (that generates AICD) nor the overall γ-secretase cleavage are sensitive to mild cholesterol depletion. Mild cholesterol reduction did not impair the generation of AICD or the cleavage of the truncated form of Notch, NΔEC, which is the direct substrate of γ-secretase. We cannot exclude that mild cholesterol depletion had an effect on specific γ-secretase cleavages, since we did not measure all Aβ species. High cholesterol reductions are difficult to assess in cell culture models because cholesterol depletion may affect cell viability. However, in a cell-free assay of γ-secretase (Sastre *et al.* 2001) moderate reductions (< 70%) in cholesterol content were not able to reduce total Aβ production. A recent report (Ostrowski *et al.* 2007) describes a direct and potent influence of cholesterol on γ-secretase activity. However, the authors used a purified mammalian γ-secretase system that allows drastic manipulations of the lipid environment that are not achievable in living cells and therefore their results are not directly comparable to ours. We also found that cholesterol depletion reduced both APP α- and β-CTF generation, indicating a possible effect on APP trafficking or an inhibition of α- and β-cleavages. To distinguish between these two possibilities we performed experiments to study APP trafficking by using a BAP-APP construct. Our results indicate that cholesterol depletion alters APP trafficking and reduces cell surface APP. Although MβCDX is known to interfere with endocytosis, the same effects were also observed in cells treated with lovastatin alone suggesting that the effects are due to cholesterol depletion. Furthermore, the fact that mild cholesterol depletion lowers Aβ in cell culture models but not in cell-free assays supports the notion that the effect takes place upstream of γ-secretase cleavage. Overall, our results suggest that the reduction in APP CTFs could be due to reduced substrate availability.

Our work also indicates that neither the γ-secretase-dependent cleavage of Notch nor Notch signaling is affected by cholesterol depletion. This is supported by other studies that show that Notch CTFs are predominantly found in non-raft membrane domains while APP CTFs reside in lipid rafts (Vetrivel *et al.* 2005). This has implications for cholesterol-lowering strategies in AD, since these compounds may target Aβ production without interfering with Notch function.

Several lines of evidence suggest lipid rafts as the principal sites in cellular membranes where Aβ is generated (Lee *et al.* 1998; Cordy *et al.* 2003; Ehehalt *et al.* 2003; Wada *et al.* 2003; Vetrivel *et al.* 2005; Hur *et al.* 2008). Lipid rafts are highly dynamic sphingolipid- and cholesterol-rich membrane microdomains with important roles in cellular signaling and trafficking (Allen *et al.* 2007). Biochemically, rafts are characterized by their insolubility in non-ionic detergents such as Triton X-100 (Brown and Rose 1992). By this method, some proteins relevant to Aβ production, such as APP, BACE and PS1, have been shown to be present in rafts prompting the hypothesis that amyloidogenic processing of APP takes place in rafts (Parkin *et al.* 1999; Ehehalt *et al.* 2002; Cordy *et al.* 2003). However, the use of detergents to solubilize cell membranes in these studies has the potential to introduce significant artifacts (Munro 2003). To avoid this limitation, here we used FLIM, a FRET-based technique, to assess lipid rafts in intact cells. Compared with conventional FRET experiments, FLIM has the advantage that it does not depend on the fluorophore concentration, is not destructive, and is not sensitive to miss-excitation phenomenon. We used a FLIM assay to detect FRET between Alexa488-APP (donor) and Alexa555-CT-B or Alexa555-flotillin (acceptor), which are known rafts markers (Sandvig and van Deurs 2002). We found that cholesterol depletion did result in increased fluorescence lifetime, suggesting that APP is less associated to rafts under these conditions. These results combined with our biochemical data showing reduced trafficking of APP to the cell surface, and reduced APP CTFs-PS1 interaction assessed by FLIM under cholesterol-lowering conditions, would suggest that less APP CTF is available for γ-secretase, therefore decreasing both Aβ40 and Aβ42.

In summary, we report that mild cholesterol depletion impairs APP processing without affecting Notch cleavage or the APP ε- or γ-secretase cleavage. The effects of cholesterol reduction are observed upstream of γ-secretase by altering APP trafficking, reducing APP CTF generation and raft-associated APP.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Membrane cholesterol reduction.

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Figure S2. FRET-based assay (FLIM) detects APP-PS1 interactions in intact cells.

Figure S3. Cholesterol depletion does not impair PS1 subcellular distribution.

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SUPPORTING INFORMATION

Fig.S1

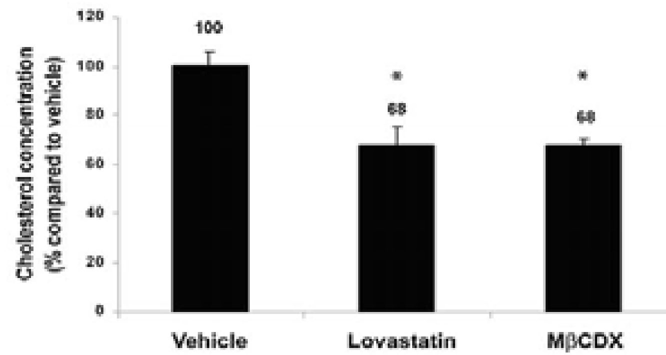

Figure S1. Membrane cholesterol reduction. We treated cells with different concentrations of lovastatin or MβCDX for different time periods. We found that treatment for 48 hours with 20 μM lovastatin, 0.25 mM mevalonate and delipidated fetal bovine serum (DLFBS) was able to induce mild but consistent (~30%) cell membrane cholesterol reduction without signs of cytotoxicity. In the case of MβCDX, consistent cholesterol reductions were only obtained after treating cells with 5 or 10 mM MβCDX for 10 or 60 min as treatment for 48 hours induced significant cytotoxicity. * One way anova, $p < 0.05$.

Fig.S2

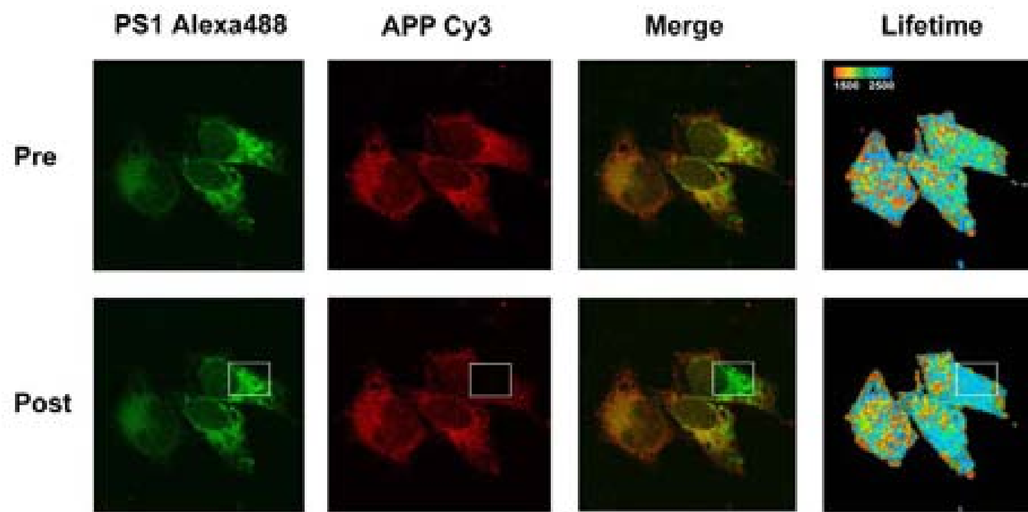

Figure S2. FRET-based assay (FLIM) detects APP-PS1 interactions in intact cells. PS70 cells were immunostained with PS1-loop and APP-C terminal antibodies labeled with Alexa488- and Cy3-donor and acceptor fluorophores, respectively. As described, these two domains remain in close proximity mainly near the cell surface, represented as red pixels in the pseudocolored image (Berezovska et al. 2003; Lleo et al. 2004). Photobleaching of the FRET acceptor in part of the cell (square) leads to an almost complete loss of the FRET signal, reflected as blue pixels in the pseudocolored lifetime image.

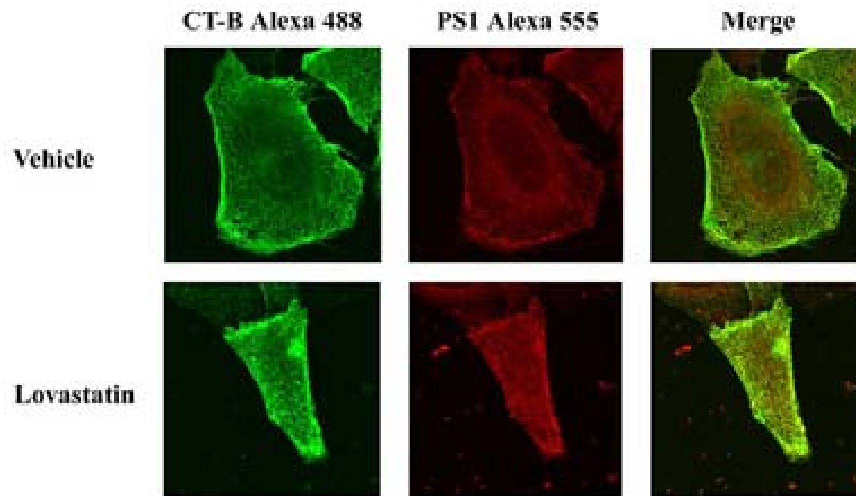

Figure S3. Cholesterol depletion does not impair PS1 subcellular distribution. H4 cells were stained with a PS1 antibody and Alexa555-CT-B. As shown, endogenous PS1 partially colocalized with lipid rafts at the cell surface. Colocalization was not affected by mild cholesterol depletion

CÁPITULO 2: "*Clinical, Neuropathologic, and Biochemical Profile of the Amyloid Precursor Protein I716F Mutation*"

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ORIGINAL ARTICLE

Clinical, Neuropathologic, and Biochemical Profile of the Amyloid Precursor Protein I716F Mutation

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Abstract

We report the clinical, pathologic, and biochemical characteristics of the recently described amyloid precursor protein (*APP*) I716F mutation. We present the clinical findings of individuals carrying the *APP* I716F mutation and the neuropathologic examination of the proband. The mutation was found in a patient with Alzheimer disease with onset at the age of 31 years and death at age 36 years and who had a positive family history of early-onset Alzheimer disease. Neuropathologic examination showed abundant diffuse amyloid plaques mainly composed of amyloid-β_{42} and widespread neurofibrillary pathology. Lewy bodies were found in the amygdala. Chinese hamster ovary cells transfected with this mutation showed a marked increase in the amyloid-$\beta_{42/40}$ ratio and APP C-terminal fragments and a decrease in APP intracellular domain production, suggesting reduced APP proteolysis by γ-secretase. Taken together, these findings indicate that the *APP* I716F mutation is associated with the youngest age of onset for this locus and strengthen the inverse association between amyloid-$\beta_{42/40}$ ratio and age of onset. The mutation leads to a protein that is poorly processed by γ-secretase. This loss of function may be an additional mechanism by which some mutations around the γ-secretase cleavage site lead to familial Alzheimer disease.

Key Words: α-Synuclein, γ-Secretase, Alzheimer disease, Amyloid, APP mutations, Genetics.

INTRODUCTION

The genes of amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) have been implicated in the pathogenesis of familial Alzheimer disease (FAD). Mutations in the *PSEN1* gene represent the most common cause of FAD (1), and more than 175 mutations have been identified to date (www.molgen.ua.ac.be/ADMutations). Mutations in the *APP* gene are rare but provide insight into the pathogenesis of FAD. All *APP* pathogenic mutations are located either in the amyloid-β (Aβ) sequence or in the vicinity of a protease cleavage site that influences APP proteolysis by different mechanisms. The Swedish *APP* mutation, adjacent to the β-cleavage site, increases the production of total Aβ by enhancing APP cleavage by β-secretase (2, 3). *APP* mutations within the Aβ sequence (e.g. the Arctic and Iowa mutations) cause severe cerebral amyloid angiopathy or Alzheimer disease (AD) (4–8) by enhancing the tendency of the Aβ peptide to aggregate (7, 9) or by increasing its resistance to proteolytic degradation (10, 11). Mutations near the γ-secretase cleavage site such as the London (V717I, V717G), Indiana (V717F), and Florida (I716V) mutations lead to an increase in the amyloidogenic $A\beta_{1-42}$ (12–17).

Amyloid precursor protein has been shown to be cleaved by γ-secretase through a series of sequential cleavage steps. First, there is ε-cleavage near the membrane-cytoplasm boundary, followed by γ-cleavage in the middle of the transmembrane domain (18). The ε-cleavage results in the release of an APP intracellular domain (AICD), whereas the γ-cleavage results in the generation of Aβ peptides (19–21). Several Aβ species consisting of 36 to 43 residues are generated and constitutively secreted. $A\beta_{40}$ is the most predominant species, and although $A\beta_{42}$ is a minor one, it predominates in the diffuse and mature plaques found in AD (22).

Most studies on *APP* mutations near γ-secretase cleavage site have focused on the ratio $A\beta_{42/40}$ as the main mechanism by which these mutations exert their pathogenic effects (15, 17, 23). Here, we describe the clinical, neuropathologic, and biochemical characteristics of the recently described *APP* I716F mutation (24). This family shows the youngest age of onset for this locus, an aggressive clinical course, and severe neuropathologic phenotype. Biochemical experiments confirmed a marked increased in $A\beta_{42/40}$ ratio and reduced APP proteolysis by γ-secretase.

MATERIALS AND METHODS

Neuropathologic Examination

The neuropathologic study was performed on formalin-fixed, paraffin-embedded samples, as previously described (25). Sections of frontal (Area 8), primary motor, primary

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sensory, parietal, temporal superior, temporal inferior, anterior cingulate, anterior insular, and primary and associative visual cortices, entorhinal cortex and hippocampus, caudate, putamen and pallidum, medial and posterior thalamus, subthalamus, nucleus basalis of Meynert, amygdala, midbrain (2 levels), pons, medulla oblongata, cerebellar cortex, and dentate nucleus were examined. Dewaxed sections (5-μm thick) were stained with hematoxylin and eosin and with Klüver-Barrera, or were processed for immunohistochemistry using the EnVision+ system peroxidase procedure (DAKO, Barcelona, Spain). After incubation with methanol and normal serum, the sections were incubated with 1 of the primary antibodies at 4°C overnight. Antibodies to glial fibrillary acidic protein (DAKO), Aβ (Boehringer, Barcelona, Spain), and ubiquitin (DAKO) were used at dilutions of 1:250, 1:50, and 1:200, respectively. Antibodies to $A\beta_{1-40}$ and $A\beta_{1-42}$ (a generous gift from Dr. Sarasa, Zaragoza, Spain) were used at dilutions of 1:50. Antibodies to α-synuclein (Chemicon, Barcelona, Spain) were used at a dilution of 1:3000. Monoclonal anti-phospho-tau AT8 (Innogenetics, Gent, Belgium) was diluted 1:50. Phospho-specific tau rabbit polyclonal antibodies Thr181, Ser199, Ser202, Ser214, Ser231, Ser262, Ser396, and Ser422 (all from Calbiochem, Torrey Pines, CA) were used at a dilution of 1:100 except for anti-phospho-tauThr181, which was used at a dilution of 1:250. Antibodies to 3R and 4R tau (Upstate, Millipore, Barcelona, Spain) were used at dilutions 1:800 and 1:50, respectively. TAR DNA binding protein was examined using a mouse monoclonal antibody (Abnova, Tebu-Bio, Barcelona, Spain) at a dilution of 1:1000, and a rabbit polyclonal antibody (Abcam, Cambridge, UK) at a dilution of 1:2000. Phospho–TAR DNA binding protein was studied by using a mouse monoclonal antibody at a dilution of 1:5000 and a rabbit polyclonal antibody at a dilution of 1:2500 (both from Cosmo Bio Co., Ltd., Koto-ku, Japan). The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% H_2O_2. Sections were counterstained with hematoxylin. Sections processed for phospho-tau immunohistochemistry were boiled in citrate buffer before incubation with the primary antibody. Sections processed for Aβ and α-synuclein were pretreated with 95% formic acid.

Cell Culture and Transfections

Chinese hamster ovary (CHO) cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum at 37°C with 5% CO_2 in a tissue culture incubator. Cells were transfected using Fugene reagent (Invitrogen) according to the manufacturer's instructions.

Plasmid Construction

Mutated cDNA constructs encoding APP V717I and APP I716F were introduced in human wild-type APP 695 cDNA by site-directed mutagenesis (Stratagene, Cedar Creek, TX).

Aβ Enzyme-Linked Immunosorbent Assay

Conditioned medium was collected 24 hours after transfection. Human $A\beta_{1-40}$ was measured by ELISA, as described (26). Briefly, antibody 6E10 (against $A\beta_{1-17}$; Chemicon, Temecula, CA) was used as a capture antibody and a rabbit polyclonal $A\beta_{1-40}$ (Chemicon) as a detection antibody. After incubation for 3 hours, wells were washed with PBS and a horseradish peroxidase–conjugated donkey anti-rabbit antibody (Jackson Laboratories, West Grove, PA) was added. Wells were washed with PBS, Quantablue reagent (Pierce, Rockford, IL) was added, and samples were read at 320 nm using a Victor3 Wallac plate reader (Perkin-Elmer, Waltham, MA). Human $A\beta_{1-42}$ and $A\beta_{1-x}$ (as a measure of total Aβ) were detected using sensitive ELISA kits (Wako, Osaka, Japan, and IBL, Hamburg, Germany, respectively).

Membrane Preparations and Cell-Free AICD Generation Assay

For Western blot analysis of the APP C-terminal fragments (CTFs), cellular membranes were isolated from CHO

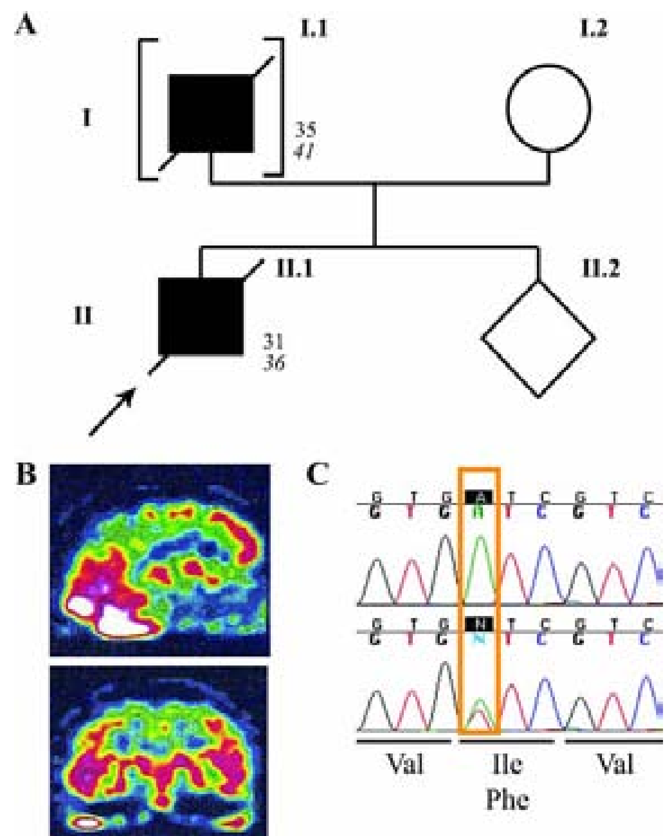

FIGURE 1. Pedigree and clinical characteristics of the family with the amyloid precursor protein (*APP*) I716F mutation. **(A)** Pedigree of the family: males are represented by squares, females by circles, shaded symbols indicate affected individuals, diagonal bands indicate deceased individuals. Ages at onset are shown below symbols. Ages at death are shown in italics below ages at onset. A blood sample was taken from the proband (II.I) for DNA analysis. **(B)** Axial and coronal ^{99m}Tc-Hexamethylpropyleneamine oxime single-photon emission computerized tomography of the proband (II.I) at age 33 years showing marked hypoperfusion (depicted in green) predominantly in parietal regions. **(C)** Proband's DNA sequence of APP exon 17 showing a missense mutation (A→T) at the first position of Codon 716, which predicts an isoleucine-to-phenylalanine substitution (p.APP I716F).

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cells transfected with wild-type, V717I, or I716F APP constructs, as described (27). For the brain samples, 100 to 200 mg of tissue was homogenized with the Proteo Extract Native Membrane Protein Extraction Kit (Calbiochem). Amyloid precursor protein intracellular domain was generated in vitro from membrane preparations of transfected CHO cells, as described (19). The samples were electrophoresed in 5% to 16% Tris-Tricine gels, transferred to 0.2-μm nitrocellulose membranes, and detected by immunoblotting with a rabbit anti-APP C-terminal (Sigma-Aldrich, St. Louis, MO) antibody.

γ-Secretase Activity

γ-Secretase activity in cell lysates was measured by a fluorometric activity Kit (R&D Systems, Minneapolis, MN).

Statistical Analysis

One-way analysis of variance was performed to analyze differences in Aβ levels, APP CTFs, and γ-secretase activity, followed by least significant difference post hoc analysis. Levene test was also performed to determine whether variances were equal.

RESULTS

Family History

The proband was a 33-year-old man who complained of a progressive history of forgetfulness and difficulties in concentrating, with the onset of symptoms at age 31 years (Fig. 1A). He had problems in remembering recent events and with abstract reasoning. Over the next 2 years, he showed

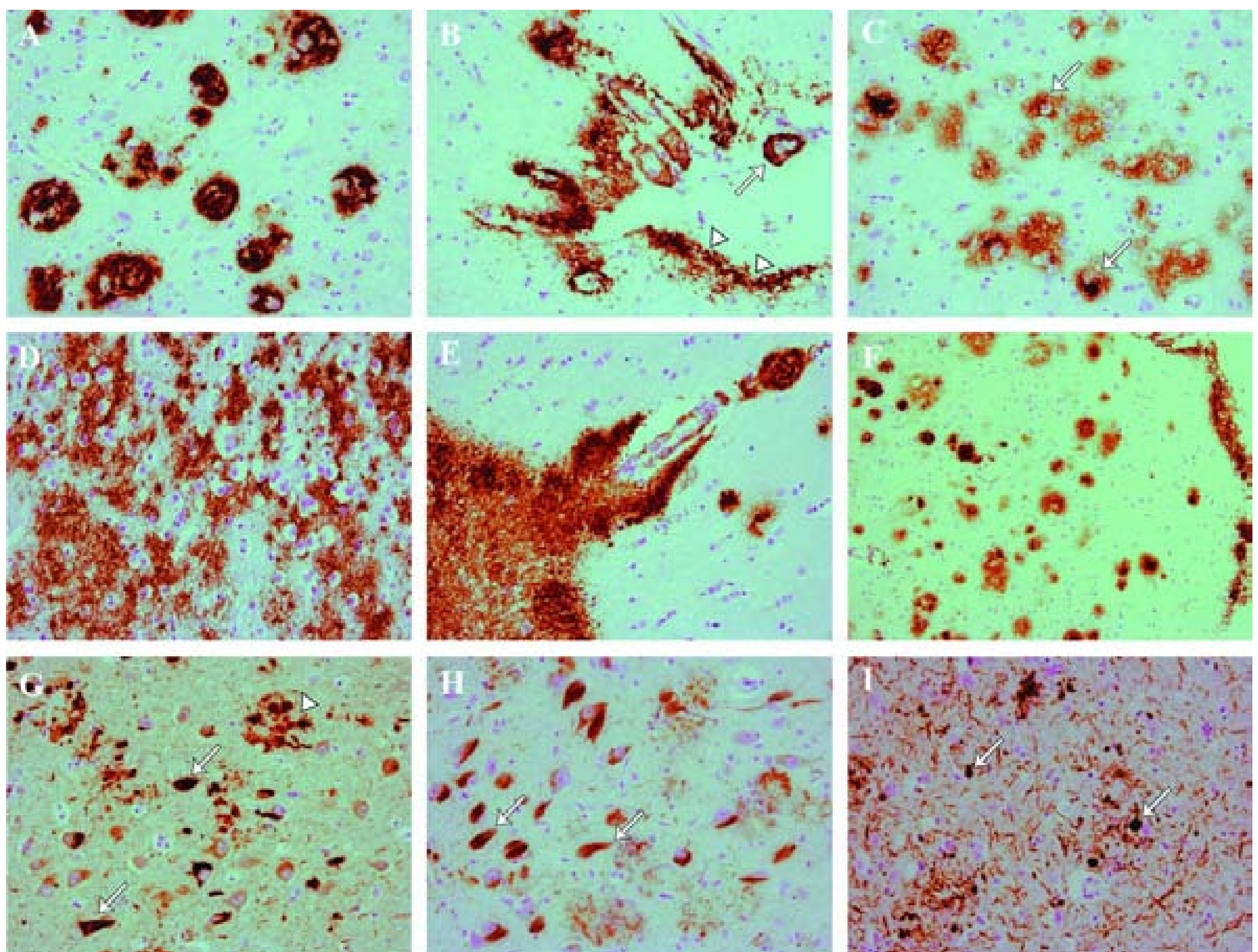

FIGURE 2. Neuropathologic findings associated with the amyloid precursor protein (*APP*) I716F mutation. **(A–F)** β-Amyloid deposits in the cerebral cortex are seen in the inner layers of the parietal cortex **(A)**, molecular layer (**B**; arrowheads, subpial deposits; arrows, meningeal blood vessels), and upper layers of the cerebral cortex (**C**; arrows) in sections stained with anti-total Aβ antibodies. Diffuse deposits, mainly perineuronal, are present in the entorhinal cortex **(D)**. Perineuronal **(D)** and subpial deposits **(E)**, and diffuse plaques **(F)** were mostly stained with anti-$A\beta_{1\text{-}42}$ antibodies. **(G, H)** Neurofibrillary tangles (arrows), dystrophic neurites (arrowheads in **G**), and neuropil threads are stained with anti-4R **(G)** and anti-3R **(H)** antibodies. **(I)** α-Synuclein-immunoreactive Lewy bodies (arrows) and aberrant neurites are restricted to the amygdala. Original magnifications: **(A, C, D, G–I)** 200×; **(B, E, F)** 100×.

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difficulties planning, using utensils, and performing fine-hand sequences that interfered with his work as a gardener. He also displayed difficulties with the sense of direction, and as a result, he had to stop working and driving. Occasionally, he complained of irregular jerks in both arms. Neuropsychologic evaluation at the age of 33 years showed deficits in verbal and visual memory, attention, and calculating with marked motor and constructive apraxia. His Mini Mental State Examination score was 21 of 30. Brain magnetic resonance imaging at the age of 33 years showed bilateral atrophy in frontoparietal regions. ^{99m}Tc-Hexamethylpropyleneamine oxime brain perfusion single-photon emission computerized tomography showed hypoperfusion in both parietal regions (Fig. 1B). The patient continued to worsen and died at the age of 36 years. The father's proband had developed dementia at the age of 35 years. A brain biopsy at the age of 39 years showed abundant diffuse and neuritic plaques, amyloid angiopathy, and neurofibrillary tangles immunoreactive for phosphorylated tau. The diagnosis made was AD, and he died at the age of 41 years. We were unable to investigate family history of dementia because he had been adopted. The proband's sibling is cognitively normal and refused genetic testing. There was no other family history of dementia. Genetic screening of the coding regions of *PSEN1*, *PSEN2*, and *APP* genes in the proband disclosed an *APP* I716F mutation (Fig. 1C; [24]). *APOE* genotype was $\varepsilon3\varepsilon3$.

Neuropathologic Examination of the Proband

Neuropathologic evaluation showed global cerebral atrophy. Microscopic examination revealed extensive diffuse and neuritic perineuronal Aβ plaques, subpial deposits, and cerebral amyloid angiopathy. Neuritic plaques, composed mainly of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, predominated in the entorhinal cortex, subiculum, hippocampus, amygdala, and inner region of the temporal and orbitofrontal cortex (Fig. 2A–C). In contrast, diffuse plaques, perineuronal plaques, and subpial Aβ deposits were mainly stained with antibodies to $A\beta_{1\text{-}42}$ (Fig. 2D–F) and were present in most of the neocortex. Cerebral amyloid angiopathy affected small arteries, arterioles, and venules of the meninges and the brain (Fig. 2B); capillaries were spared. Neurofibrillary tangles and neuropil threads were present in large numbers in the entorhinal and transentorhinal cortices, hippocampus, amygdala, nucleus basalis of Meynert, septal nuclei, and the entire cerebral neocortex, including the primary sensory and motor areas (Fig. 2G, H). Neurofibrillary tangles were also present in selected nuclei of the brainstem, including the substantia nigra, motor ocular nuclei, locus ceruleus, and reticular formation of the pons and medulla oblongata. Dystrophic neurites containing hyperphosphorylated tau were abundant in the amygdala, hippocampus, and entorhinal cortex, corresponding to AD Stage VI of Braak. All of these structures were stained with AT8, other phospho-specific anti-tau antibodies, and with anti-4R (Fig. 2G), anti-3R (Fig. 2H), and anti-ubiquitin antibodies.

α-Synuclein immunoreactivity was observed in the amygdala in the form of large numbers of Lewy bodies and aberrant neurites (Fig. 2I). TAR DNA binding protein–immunoreactive inclusions were absent. Neuronal loss, astrocytic gliosis, and microgliosis were moderate in the cerebral neocortex but were more marked in the entorhinal and perirhinal cortex, subiculum, and amygdala. Astrocytes predominated in the inner cortical layers of the neocortex, plexiform layers of the hippocampus, and around neuritic plaques. Likewise, microglia were increased in number in the cerebral cortex and white matter and more abundantly around neuritic plaques.

Biochemical Effects of the *APP* I716F Mutation

We next investigated the effects of this mutation on APP processing in transfection studies using CHO cells. Quantitative analysis of the major Aβ species in the conditioned medium from APP I716F–transfected CHO cells showed increased (~2-fold) $A\beta_{42}$, reduced (~0.5-fold) $A\beta_{40}$,

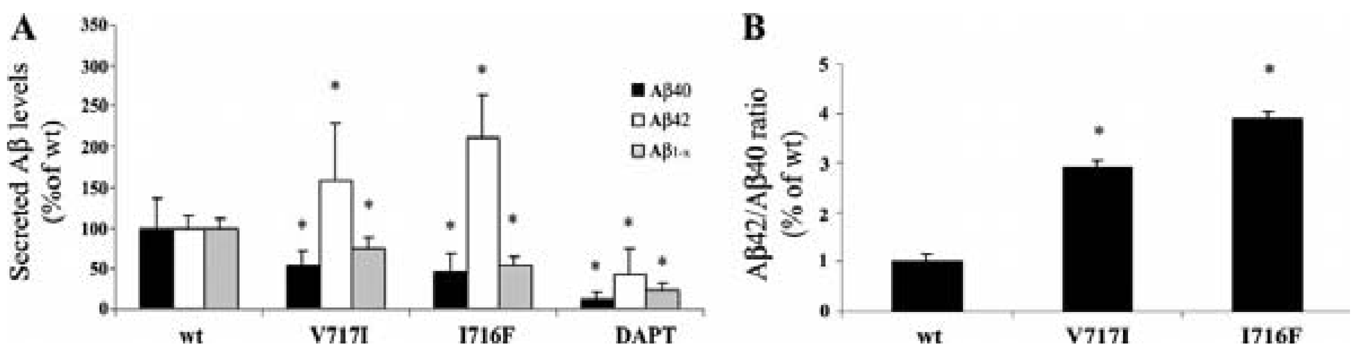

FIGURE 3. Amyloid-β (Aβ) levels from Chinese hamster ovary (CHO) cells transfected with the *APP* I716F and V717I mutations. **(A)** Amyloid-β_{40}, $A\beta_{42}$, and $A\beta_{1\text{-}x}$ (total) levels from CHO cells transfected with the *APP* I716F and V717I mutations. Amyloid-β was measured by ELISA in the conditioned medium 24 hours posttransfection. Treatment with *N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycin e-1,1-dimethylethyl ester, a potent inhibitor of γ-secretase, was used as a positive control in wild-type APP-transfected cells. Amyloid precursor protein I716F-transfected CHO cells showed increased (~2 fold) $A\beta_{42}$, reduced (~0.5 fold) $A\beta_{40}$, and total Aβ (~0.5 fold) levels compared with that of wild-type APP. Similar but milder results were found in APP V717I–transfected cells. *, One-way analysis of variance, $p < 0.05$ vs wild-type APP. Average Aβ levels are expressed as a percentage relative to wild-type. Values represent the mean ± SD of 3 independent experiments. **(B)** Amyloid-$\beta_{42/40}$ ratios were calculated for wild-type APP and the *APP* mutations. Results are expressed relative to wild-type. Amyloid-$\beta_{42/40}$ ratio was markedly increased in cells transfected with the *APP* I716F or V717I mutations. *, One-way analysis of variance, $p < 0.05$ vs wild-type APP.

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and total Aβ (~0.5-fold) levels compared with those of wild-type APP (Fig. 3A). As a result, the $A\beta_{42/40}$ ratio was markedly increased (~4-fold) in cells transfected with the *APP* I716F mutation (Fig. 3B). Results were compared with cells transfected with the adjacent *APP* V717I mutation (13), which led to similar but milder effects. Membrane preparations from APP I716F– and V717I–transfected cells showed a marked increase (2- and 2.5-fold) in APP CTFs compared with that of wild-type APP (Fig. 4A). Interestingly, the levels of APP CTFs were higher in the proband's brain homogenates

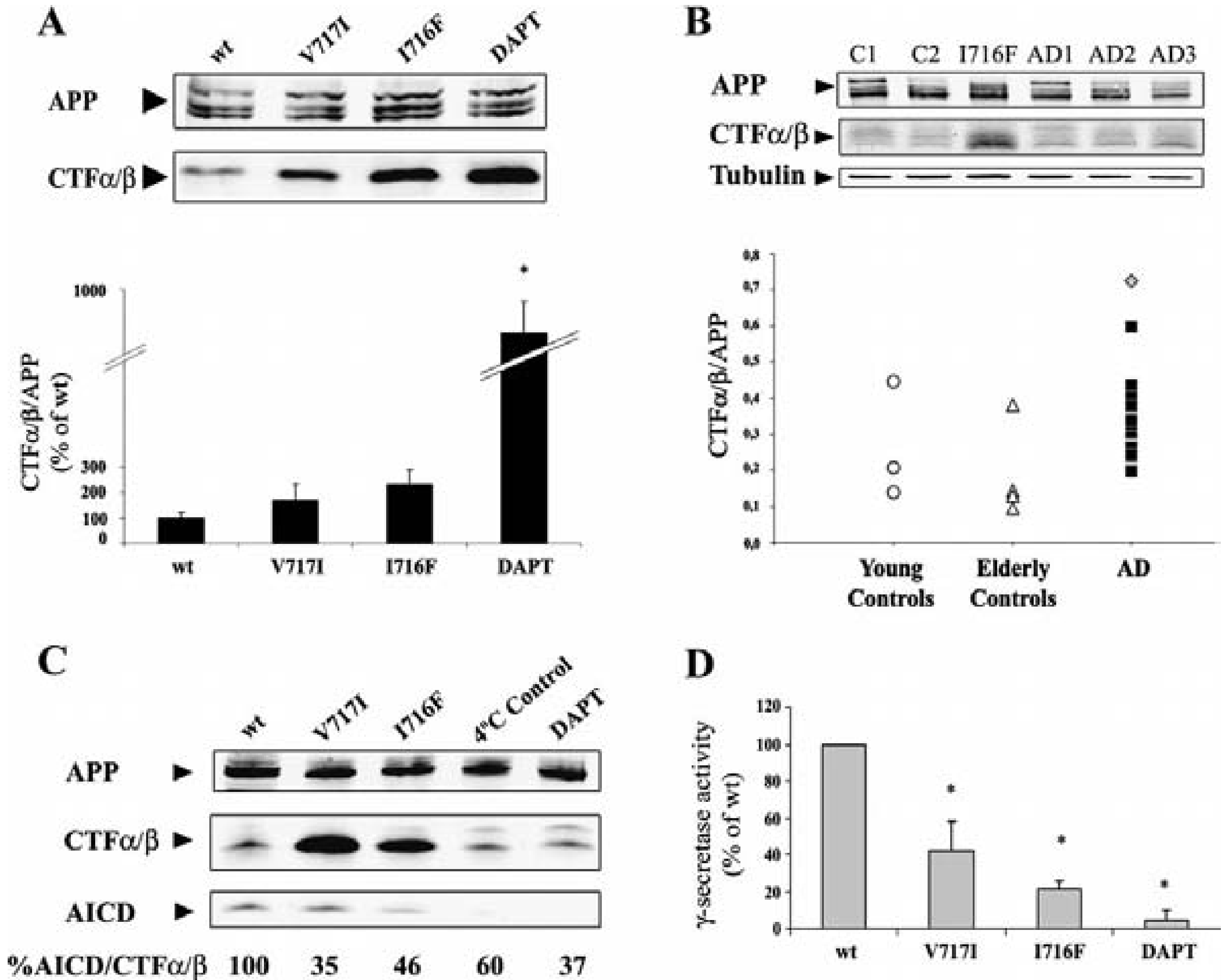

FIGURE 4. Effects of amyloid precursor protein (*APP*) I716F mutation on APP processing. **(A)** Effects on APP C-terminal fragments (CTFs). Immunoblotting from membrane preparations from Chinese hamster ovary (CHO) cells transfected with the I716F and V717I mutations. Amyloid precursor protein I716F and V717I transfected cells showed marked accumulation of APP CTFs. Treatment with the γ-secretase inhibitor *N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycin e-1,1-dimethylethyl ester was used as a positive control on wild-type APP-transfected cells. Data are expressed relative to wild-type (wt) and represent mean ± SD of 3 experiments. Quantitation is shown below. *, One-way analysis of variance, $p < 0.05$ vs wild-type APP. **(B)** Western blot of APP CTFs from brain homogenate samples of the proband (1716F), young healthy controls (C1, C2), and late-onset Alzheimer disease (AD1-3) patients. Amyloid precursor protein CTFs are higher in the proband's brain homogenates than in sporadic AD patients or young healthy controls. Quantification of the ratio CTFs/full-length APP is shown below. The proband is indicated as a gray diamond. **(C)** Amyloid precursor protein intracellular domain (AICD) was measured in a cell-free assay from CHO cells transfected with wild-type APP or the *APP* I716F and V717I mutations. Lysates from APP I716F and V717I transfected cells showed reduced AICD generation. Lysates kept at 4°C or treated with *N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycin e-1,1-dimethylethyl ester served as controls. Data are expressed relative to wild-type and represent mean ± SD of 3 experiments. **(D)** γ-Secretase activity was measured by a fluorometric assay in cell lysates from cells transfected with the *APP* I716F and V717I mutations. Cells transfected with *APP* I716F and V717I mutations showed reduced fluorogenic activity. Values are expressed as a percentage of wild-type and are the average of 2 independent experiments. *, One-way analysis of variance, $p < 0.05$ vs wild-type APP.

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than in sporadic AD patients or young and elderly healthy controls (Fig. 4B). Incubation of membrane preparations from APP I716F- and V717I-transfected cells showed a reduced production of AICD and accumulation of CTFs (Fig. 4C). Finally, lysates from cells transfected with *APP* I716F or V717I mutations showed reduced γ-secretase activity assessed by a fluorogenic kit assay (Fig. 4D).

DISCUSSION

The *APP* I716F mutation had previously been described as an artificial mutation with extreme effects on the $A\beta_{42/40}$ ratio (23, 28–30). Here, we describe the full clinical, neuropathologic, and biochemical profile of the recently reported *APP* I716F mutation (24). This FAD mutation is associated with the youngest age of onset for this locus (mean age of onset, 49 years [31]), supporting the strong inverse association between $A\beta_{42/40}$ ratio and age of onset (17). Neuropathologic study of the proband's brain revealed atypical generalized and extensive Aβ deposition in the brain and cerebral blood vessels. Amyloid-β deposition formed neuritic plaques in the inner regions of the temporal lobe and large numbers of $A\beta_{1\text{-}42}$-predominant diffuse plaques, perineuronal plaques, and subpial deposits in the neocortex. As in other families with *APP* mutations (32, 33), as well as in sporadic AD cases (34), Lewy bodies were observed in the amygdala, suggesting that α-synuclein pathology is downstream of $A\beta_{1\text{-}42}$ deposition in these families. However, the reasons for such selective involvement are not known.

Biochemical characterization of this mutation extended previously described results (23, 28–30). The *APP* I716F mutation leads to a marked increase in $A\beta_{42}$ and the $A\beta_{42/40}$ ratio and reduced $A\beta_{40}$ and $A\beta_{1\text{-}x}$ levels. As expected, the $A\beta_{42/40}$ ratio was lower than other studies that used APP C99 (which is the direct substrate for γ-secretase) for transfection studies (23, 28, 29). As for other *APP* mutations located near the γ-secretase cleavage site (17, 35), we also showed that the *APP* I716F mutation led to a prominent accumulation of APP CTFs in transfected cells. Interestingly, APP CTFs were higher in the proband's brain homogenates than in sporadic AD patients and healthy controls. Although the availability of only a single brain sample precluded an in-depth analysis of APP processing in this kindred, this increase suggests that APP CTF accumulation is not an artifact in the cellular model. However, the increase in APP CTFs does not necessarily reflect reduced γ-secretase activity because it has been observed without concomitant reduction in Aβ secretion (36). Therefore, we cannot completely exclude the possibility that this accumulation may be partially due to impaired degradation. In any case, accumulation of APP CTFs has been shown to be neurotoxic and to cause neurodegeneration in vitro and in vivo (35, 37, 38), and we cannot exclude the possibility that this can also contribute to the neurodegeneration observed in this family.

We further demonstrated a reduced AICD generation in cells expressing the APP I716F mutation. Amyloid precursor protein intracellular domain results from the ε-cleavage of APP β-CTF that occurs near the membrane-cytoplasm boundary (19–21). This initial cleavage is followed by different γ-cleavage events toward the middle of the transmembrane domain to generate different Aβ species (18). The presence of cleavage sites at every 3 residues between the γ- and ε-cleavage fits well with an α-helical model (39–42). According to this model, the cleavage sites for $A\beta_{49}$, $A\beta_{46}$, $A\beta_{43}$, and $A\beta_{40}$ are aligned on the α-helical surface of the β-CTF molecule, whereas those for $A\beta_{48}$, $A\beta_{45}$, and $A\beta_{42}$ are aligned on the other α-helical surface (39). Although we only measured the major secreted Aβ species, the reduction in γ- and ε-cleavage and APP CTF accumulation in our study suggests that the *APP* I716F mutant is poorly processed by γ-secretase. This is supported by a reduced activity measured by a fluorogenic assay. Taken together, our results suggest that a selective loss of function in APP proteolysis can be caused by an APP mutation. A similar loss-of-function mechanism has been proposed for *PSEN* mutations (43). Consistent with this notion, a FAD-associated *PSEN1* mutation was shown to slow sequential intramembrane cleavage by γ-secretase and other GXGD-aspartyl proteases, resulting in longer cleavage products (44).

Overall, this family reveals that, although the $A\beta_{42/40}$ ratio seems to be the best indicator of severity of the disease in patients with *APP* mutations, the reduced processing might also contribute to the disease process and suggests an additional mechanism by which some mutations around the γ-secretase cleavage site may lead to AD.

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DISCUSIÓN

VII. DISCUSIÓN

CAPÍTULO 1

El objetivo principal de nuestro primer estudio fue determinar cómo la reducción de colesterol de membrana afectaba al procesamiento de APP y el mecanismo implicado en esta alteración del procesamiento. La importancia de este estudio parte de trabajos recientes en los que muestran que las estatinas podrían ser beneficiosas en el tratamiento de la EA debido a su efecto reductor de Aβ [233, 411-415]. Sin embargo el mecanismo por el cual ocurre sigue siendo todavía controvertido. Nosotros confirmamos en diferentes líneas celulares tratadas con lovastatina y mevalonato el efecto reductor de Aβ40 y Aβ42 de modo colesterol-dependiente. Cuando los cultivos eran tratados con un suero rico en colesterol, se incrementaban significativamente los valores de Aβ [416-418], sugiriendo que el mecanismo subyacente era dependiente de colesterol. Está descrito que la vía de síntesis del colesterol se bifurca en sus últimas etapas en la formación de isoprenoides o de colesterol. Por esta razón, con la adición de mevalonato (precursor de los isoprenoides) al tratamiento descartamos que el efecto fuera debido a la inhibición de la vía de los isoprenoides, como también está descrito [418].

El papel de los isoprenoides en la fisiopatología de la EA sigue siendo controvertido. La bifurcación de la vía de síntesis del colesterol provoca efectos desiguales tras el tratamiento con inhibidores de la HMG-CoA reductasa como las estatinas. Incluso se ha postulado que los efectos provocados por la lovastatina en neuronas son debidos a su efecto sobre el GGPP, un isoprenoide, [419], y no a los niveles de colesterol, como nosotros observamos. Otro ejemplo es un estudio muy reciente en el que se muestra un incremento en los niveles de isoprenoides en la sustancia gris y blanca de cerebros de pacientes del género masculino con EA pero no de los niveles de colesterol comparado con controles. En el mismo estudio, al realizar un tratamiento con simvastatina en ratones *wild-type* se obtenía una reducción significativa de los isoprenoides, FPP y GGPP, y una reducción más leve de los niveles de colesterol. Esto podría indicar que los isoprenoides también están contribuyendo de manera importante a la patología [420].

Una vez habíamos establecido el papel del colesterol en la producción de Aβ, nuestro objetivo se centró en esclarecer el mecanismo por el cual aparecía esta disminución de los niveles de Aβ. Por consiguiente, centramos nuestro interés en descifrar cómo esta reducción de colesterol de membrana afectaba al complejo γ-secretasa. Algunos autores defienden que el complejo γ-secretasa es dependiente de los rafts lipídicos pero no de los niveles de colesterol [421], mientras otros aseguran que la enzima es modulada por el colesterol [234, 415]. Incluso, algunos autores sugieren que las actividades de BACE1 y γ-secretasa son estimuladas por algunos de los componentes lipídicos de los *rafts*, como los glicoesfingolípidos y el colesterol [422-425]. Existe otro estudio [426] en el que los autores sugieren que el APP CTF, C99, podría poseer un lugar de unión a colesterol localizado en su fragmento extramembranal, el cual es fundamental para la proteólisis de γ-secretasa. Este fragmento C99, y la APP total, se uniría específicamente al colesterol, lo cual sugeriría que el colesterol puede participar en el tráfico de la APP hacia los rafts lipídicos y en la modulación de las proteólisis de β- y/o γ-secretasa. Por otro lado estos autores sugieren que la APP podría actuar como un sensor/receptor de colesterol y podría regular la entrada de colesterol a la célula en condiciones de alta concentración de colesterol celular [426].

Nosotros comprobamos que ni el procesamiento ε- de la γ-secretasa ni el procesamiento total de γ-secretasa quedaba afectado por la reducción de colesterol. Asimismo, la reducción leve de los niveles de colesterol de membrana no afectaba a la generación de AICD ni al procesamiento proteolítico de Notch, sustrato directo de la γ-secretasa. Sin embargo, no podemos descartar que la reducción de colesterol afecte a otras escisiones de la γ-secretasa ya que no hemos estudiado todas las especies de Aβ. Por otro lado, una reducción de colesterol más drástica no es posible en nuestro modelo ya que comprometería la viabilidad celular. Sin embargo, en un ensayo *in vitro* de γ-secretasa [427] con una reducción moderada de colesterol de $<70\%$ tampoco conseguimos obtener una disminución de la producción total de Aβ. En contraposición, otro trabajo reciente describe la relación directa existente entre γ-secretasa y los niveles de colesterol [418]. Sin embargo, se utilizó un

sistema purificado de γ-secretasa de mamífero que permite manipulaciones de los niveles de colesterol mucho más acentuadas, y por tanto sus resultados no son comparables a los nuestros.

Los efectos de la reducción de colesterol observados en el procesamiento de APP en nuestro trabajo podrían deberse también a otros efectos, por ejemplo a la alteración del tráfico de APP o la inhibición de las secretasas α- y β-, reflejado en la disminución de los fragmentos APP CTFα y los fragmentos APP CTF-β. Es ampliamente conocido que las enzimas α- y β-secretasa compiten entre ellas por la proteólisis del ectodominio de APP. Existen evidencias que esta elección viene determinada por el tráfico intracelular de APP. Numerosos estudios han demostrado que el procesamiento de APP por α-secretasa se localiza en la membrana celular [428], mientras que la actividad de β-secretasa tiene lugar principalmente en los rafts lipídicos de las membranas endosomales. Por lo tanto, la localización preferencial de APP en los rafts lipídicos ricos en colesterol (que pueden ser internalizados en los endosomas ricos en β-secretasa) puede ser un paso determinante para la competición entre α- y β-secretasas durante la proteólisis inicial de APP [413, 429]. Para establecer cuál de estos procesos es el responsable de la alteración en la producción de Aβ, estudiamos el tráfico de APP mediante el constructo BAP-APP. Se conoce que los factores que promueven la asociación a los rafts lipídicos y su internalización aumentan la actividad β-secretasa, mientras que los factores que inhiben esta internalización o promueven la localización en la superficie celular elevan la actividad α-secretasa. Nuestros resultados indican que la reducción de colesterol altera el tráfico de APP y reduce los niveles de APP de superficie. Sabemos que el tratamiento con MβCDX altera la vía endocítica, pero los efectos fueron observados también en células tratadas con lovastatina, sugiriendo que el efecto es debido a la disminución del colesterol celular. Además el hecho de que la reducción de Aβ se observe en los cultivos celulares pero no en la muestra purificada de γ-secretasa, refuerza la idea que este efecto tiene lugar en pasos previos al procesamiento por γ-secretasa. En resumen, la reducción de los niveles de APP-CTFs puede ser debida a una menor disponibilidad de substrato. Posteriormente a la publicación de nuestro trabajo se ha confirmado esta hipótesis con la demostración que el tratamiento

con inhibidores de la enzima acyl-coA colesterol aciltransferasa (ACAT), que regula la distribución subcelular de colesterol, disminuye la producción de Aβ mediante la retención de las moléculas de APP en la vía secretora temprana (*early-secretory pathway*), limitando la disponibilidad de APP para la proteólisis mediada por secretasas [430]. Por tanto, se ha definido una nueva vía dependiente de ACAT que regula la secreción de APP, contribuyendo a una reducción de Aβ *in vivo* [430]. En nuestro estudio, la alteración del ambiente lipídico celular por la reducción de los niveles de colesterol, podría provocar efectos similares.

Alternativamente, nuestro trabajo también indica que el procesamiento de Notch por γ-secretasa y su vía de señalización no quedan afectados por la reducción de colesterol, indicando su especificidad hacia APP. Esto quedó demostrado en otros estudios que mostraban que los fragmentos Notch-CTFs se localizaban en dominios “no-rafts”, mientras los fragmentos APP-CTFs si residían en los dominios “rafts” lipídicos [431]. Esta distribución tiene implicaciones en la terapia con reductores de colesterol, ya que podrían reducir Aβ sin afectar a la función de Notch.

Algunas líneas de investigación sugieren los rafts lipídicos como los dominios en los que Aβ se genera [413, 421, 431-434]. Los rafts lipídicos son microdominios muy ricos en esfingolípidos y colesterol que participan en las vías de señalización y tráfico intracelular [435]. Son dominios insolubles en detergentes no-iónicos como el Triton-X [436]. Se conoce que proteínas como APP, BACE y PS1 residen en estos dominios y, por consiguiente, la generación del péptido amiloide también tiene lugar aquí [433, 437, 438]. Sin embargo, la utilización de detergentes para la localización de estas proteínas introduce muchos artefactos que entorpecen la técnica [439]. Para evitar esta limitación, nosotros aplicamos la técnica de microscopía confocal basada en FLIM. FLIM es una técnica basada en FRET que permite estudiar los rafts lipídicos en células intactas. Comparado con los experimentos convencionales de FRET, FLIM tiene la ventaja de no depender de la concentración del fluoróforo, no ser destructiva y no sufrir procesos de excitación anormal. Utilizamos la técnica de FLIM para la detección de FRET entre Alexa488-APP (donante) y Alexa555-CT-B (*cholera toxin* subunidad B) o Alexa555-flotillin (aceptor), que son

marcadores conocidos de rafts lipídicos [440]. Encontramos que la reducción de colesterol provoca un incremento de la vida media fluorescente, sugiriendo una menor asociación de APP en los rafts lipídicos. Por lo tanto, al reducir los niveles de colesterol estaríamos impidiendo la internalización de APP y su posterior desplazamiento a los endosomas, que son un fenómeno necesario para la proteólisis de APP [231, 280, 413].

Si combinamos estos resultados con los resultados bioquímicos, podemos concluir que el resultado final es una disminución del tráfico de APP hacia la superficie celular y una disminución de la interacción de los fragmentos APP-CTFs con PS1, sugiriendo que existen menos APP-CTFs disponibles para la enzima γ-secretasa y, por tanto, provocando una disminución de Aβ40 y Aβ42.

En resumen podemos concluir que una reducción leve de colesterol altera el procesamiento de APP sin afectar al corte de Notch o al procesamiento ε- y γ-secretasa. Los efectos de la reducción de colesterol se han observado en los pasos previos al corte de la enzima γ-secretasa provocando alteraciones del tráfico de APP, reduciendo la generación de APP-CTFs y su incorporación a los rafts lipídicos.

Algunas evidencias apuntan al posible efecto protector y sintomático beneficioso de las estatinas en la EA. La reducción de los niveles de colesterol mediante el uso de estatinas parece afectar al procesamiento de APP y a la producción de Aβ [411].

Sin embargo, estudios sobre el efecto del tratamiento con estatinas sobre las concentraciones de Aβ en suero y LCR humanos son equívocos [441]. Los estudios retrospectivos caso-control han descrito una relación positiva entre el uso de estatinas y un riesgo menor de padecer EA [390, 442], mientras algunos estudios prospectivos han mostrado resultados contradictorios [443, 444].

El beneficio potencial del tratamiento con estatinas en pacientes con EA viene apoyado por un reciente estudio [445], en el cual se investigó la utilidad del tratamiento con estatinas en ratones que sobreexpresan Aβ humana que habían sufrido un trauma cerebral. El tratamiento con simvastatina provocó una disminución de los niveles de Aβ40 y 42, redujo la lesión hipocampal, redujo la activación microglial y mejoró el comportamiento de los ratones comparado con ratones no tratados.

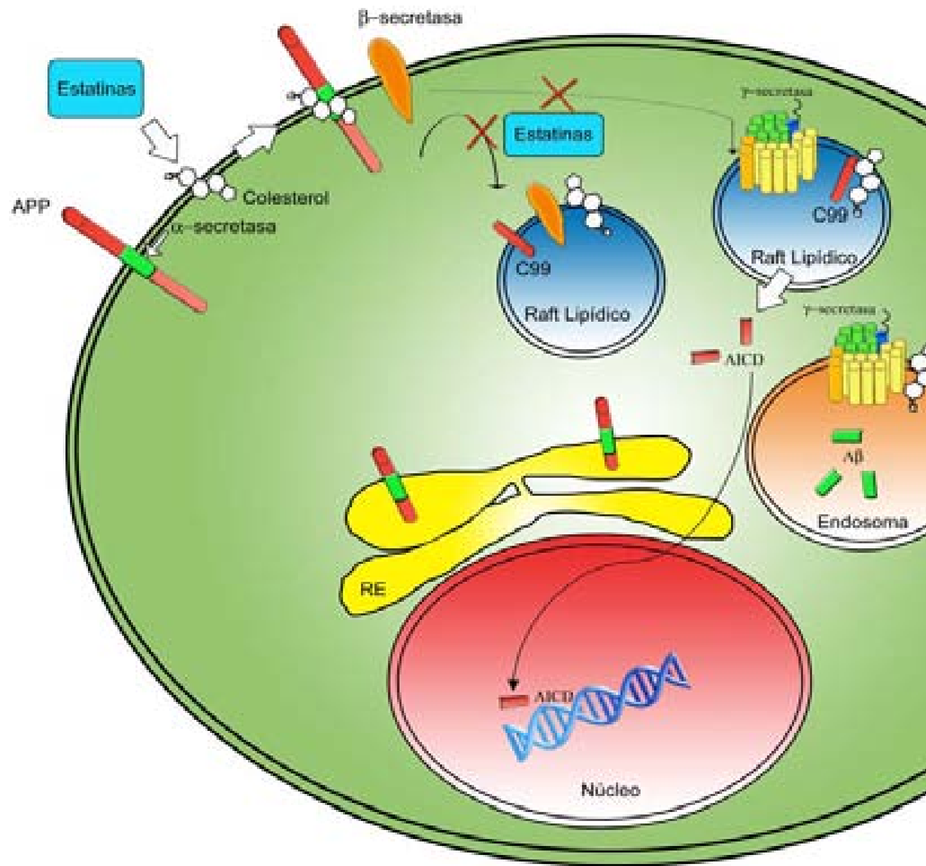

Figura 12. Figura resumen Capítulo 1. La proteína APP, coloreada en rojo, reside en la membrana plasmática. La unión inicial de APP a colesterol (estructura policíclica blanca), induce su translocación a los rafts lipídicos (sombreados en azul). APP podría actuar como un receptor o sensor de colesterol, y controlar la entrada de éste a la célula. Estos dominios lipídicos contienen la enzima BACE-1 (en naranja), existen dos posibles vías de presentación de la APP a BACE-1: por un lado puede ser presentada en los rafts lipídicos y, por otro, en pasos posteriores de la vía endocítica (datos no representados). La internalización de los rafts lipídicos que contienen APP a un ambiente más ácido, activan BACE-1 y promueven la liberación del fragmento soluble de la APP. γ-secretasa puede co-internalizarse con APP y BACE-1 o un endosoma que contiene C99 y BACE-1 puede fusionarse con otro endosoma que contenga el complejo γ-secretasa. C99 puede contener un lugar de unión a colesterol en su fracción extramembranal. A continuación, C99 es proteolizado para liberar el fragmento AICD y Aβ. AICD forma un complejo terciario que se transloca al núcleo (sombreado en rojo), donde se une a varios promotores. En resumen, el complejo γ-secretasa es dependiente de los rafts lipídicos y la actividad de BACE-1 y γ-secretasa es estimulada por algunos componentes lipídicos de los rafts (glicoesfingolípidos y colesterol). Las estatinas inhiben la formación de colesterol, disminuyen la APP de superfície celular y su endocitosis. En consecuencia, disminuyen los niveles de CTF al haber menor disponibilidad de sustrato, y las proteínas APP y PS1 interaccionan menos entre ellas en los rafts lipídicos.

CAPÍTULO 2

La mutación *APP* I716F había sido previamente descrita como una mutación artificial con efectos extremos sobre la ratio $A\beta_{42/40}$ [203, 446-448]. En este trabajo describimos el perfil clínico, neuropatológico y bioquímico de la mutación recientemente descrita en una familia por Guerreiro *et al*. 2008.[160]. Esta mutación se asocia a EA familiar con la edad de inicio más temprana para este locus (edad media de inicio de 49 años, [449]), confirmando la fuerte asociación inversa existente entre la ratio $A\beta_{42/40}$ y la edad de inicio de la enfermedad [134]. Curiosamente, esta observación contrasta con familias portadoras de mutaciones para *PS1*, en las cuales, la edad de inicio no está inversamente correlacionada con la secreción de $A\beta_{42}$ [176-178] ni con el genotipo *APOE* [179].

El estudio neuropatológico del probando reveló un depósito de $A\beta$ de forma atípica, y extensa en el cerebro y en los vasos sanguíneos cerebrales. Se observaron numerosas placas neuríticas en las regiones internas del lóbulo temporal, y un gran número de placas difusas formadas predominantemente de $A\beta_{42}$, parecido a lo que ocurre en las mutaciones de *PS1*. Asimismo se detectaron placas perineuronales y depósitos subpiales en el neocórtex.

Se conoce la existencia de una fuerte correlación entre $A\beta_{40}$ y el nivel de maduración de las placas. Las placas difusas representan el estadio más temprano de la deposición de $A\beta$ y son exclusivamente positivas para $A\beta_{42(43),}$ y completamente negativas para $A\beta_{40}$ [252]. Posteriormente aparecen las placas ricas en $A\beta_{40}$ y de tipo neurítico [450].

En nuestro caso, se observaron cuerpos de Lewy en la amígdala, del mismo modo que se observan en familias con otras mutaciones en *APP* [451, 452] y casos esporádicos, [453], sugiriendo que la patología α-sinucleína es posterior a la deposición de $A\beta_{42}$ en estas familias. Sin embargo, la razón de este depósito selectivo de α-sinucleína en amígdala es desconocida.

La caracterización bioquímica de esta mutación extiende resultados previamente publicados [203, 446-448]. La mutación *APP* I716F provoca un incremento de $A\beta_{42}$ y de la ratio $A\beta_{42/40}$ y la reducción de los niveles de $A\beta_{40}$ y $A\beta_{1\text{-}x}$. Como era de esperar, la ratio $A\beta_{42/40}$ era menor que en otros estudios que

usaban el fragmento APP C99 para estudios de transfección, el sustrato directo de γ-secretasa [446-448].

Asimismo observamos que, de modo similar a otras mutaciones que se localizan cerca del lugar de corte de la γ-secretasa [134, 454], la mutación *APP* I716F provoca una acumulación de APP CTFs en células transfectadas. Curiosamente, los fragmentos APP CTFs eran más abundantes en el homogeneizado de cerebro del probando que en pacientes con EA esporádico tardío y controles jóvenes o ancianos sanos. A pesar de la limitación de disponer únicamente de una muestra de cerebro que no permite llevar a cabo un estudio con detalle del procesamiento de APP en esta familia, este incremento de APP CTFs sugiere que no es un artefacto de nuestro modelo celular. Sin embargo en otros estudios, este incremento en APP CTFs se ha observado sin una reducción concomitante de la secreción de Aβ, como ocurre en el caso de las mutaciones de *PS1* [455], y no podemos excluir que esta acumulación pueda ser debida, en parte, a una degradación anómala. En cualquier caso, la acumulación de APP CTFs se ha descrito como neurotóxica y puede provocar neurodegeneración *in vitro* e *in vivo* [454, 456, 457], y no podemos excluir que ésto también contribuya a la neurodegeneración observada en esta familia. Recientemente ha aparecido un trabajo en el cual se observa que la toxicidad de APP y los fragmentos βCTF provoca una disfunción de la vía endocítica en síndrome de Down y EA. Este efecto lo atribuyen al incremento de la conductancia neuronal que provocan los fragmentos βCTF de modo concentración-dependiente y que pueden romper la homeóstasis iónica neuronal crítica para la supervivencia celular [458].

Por otro lado, nuestros experimentos demuestran una menor producción de AICD en células que expresan la mutación *APP* I716F. El fragmento AICD es el resultado del corte ε- del fragmento APP β-CTF que tiene lugar cerca del límite membrana-citoplasma [100, 143, 427]. Esta menor producción de AICD podría afectar al potencial efecto de este fragmento en la plasticidad sináptica sugerido en varios trabajos previos [459, 460].

La APP sufre varios cortes γ- consecutivos a lo largo del dominio transmembrana para generar varias especies de Aβ [238]. La presencia de lugares de corte cada tres residuos entre los cortes γ- y ε- encaja bien con el

modelo de la α-hélice [461, 462] [463, 464]. De acuerdo con este modelo, los lugares de corte para $A\beta_{49}$, $A\beta_{46}$, $A\beta_{43}$ y $A\beta_{40}$ se encuentran alineados en la superficie α-hélice de la molécula de β-CTF, mientras aquellas para $A\beta_{48}$, $A\beta_{45}$ y $A\beta_{42}$ están alineadas en la superficie opuesta de la α-hélice [461]. A pesar que nosotros estudiamos únicamente las especies de Aβ más abundantes, la reducción en los cortes γ- y ε- y la acumulación de APP CTFs sugiere que la mutación *APP* I716F está procesada anómalamente por γ-secretasa. La identificación de las especies largas de Aβ condujo al hallazgo de un nuevo lugar de corte proteolítico de APP, el corte ζ-, que produce $A\beta_{46}$ localizado entre los lugares de escisión γ- y ε- [340] que sería interesante analizar en este caso para corroborar el modelo de la α-hélice.

Esta reducción de la actividad γ-secretasa se confirma por una reducción de la actividad analizada mediante un ensayo fluorogénico.

Los lugares precisos de escisión γ- tienen una influencia importante en el potencial de agregación y patogénico de Aβ, ya que únicamente los péptidos largos de Aβ, en particular $A\beta_{42}$, tienen una gran tendencia a oligomerizar y agregar *in vivo*. Estos péptidos de mayor longitud se consideran más neurotóxicos a pesar que es la forma oligomérica y no la forma fibrilar y no-soluble la que parece consolidarse como la más dañina [78, 125, 465-468]. Existe una fuerte correlación entre los niveles de las especies de Aβ soluble y la extensión de la pérdida sináptica y la gravedad del deterioro cognitivo [74, 75], corroborando la hipótesis de la cascada amiloide [207]. Se han observado experimentalmente uniones directas de especies oligoméricas de Aβ a las sinapsis, provocando la disrupción de la LTP. También se ha estudiado la capacidad de Aβ de generar estrés oxidativo, alteración mitocondrial, inflamación y formación de poros en las membranas [469-472]. Estas alteraciones neuropatológicas provocadas por Aβ han sido ampliamente estudiadas en un estudio reciente mediante microscopía electrónica en ratones *wild-type* hAPP y $APP_{Swe, Ind}$, en los que observaron un aumento significativo del número de sinapsis y del número de vesículas por sinapsis en los ratones hAPP que quedaba totalmente anulado con la expresión de $APP_{Swe, Ind}$. Esto puede ser debido a un exceso de Aβ o a una reducción excesiva de la actividad

sinaptotrófica de APP (dominio extracelular de APP, sAPP) [473]. En otro artículo reciente con ratones transgénicos para APPswe/PS1dE9 se describió una reducción de la actividad neuronal en segmentos dendríticos y neuronas cercanas a las placas de amiloide comparado con ratones *wild-type*, indicando el papel iniciador de las placas de amiloide que provocarían la disfunción neuronal [81].

En resumen, nuestros resultados sugieren una pérdida de función selectiva en la proteólisis de APP causada por una mutación en *APP*. Una pérdida de función similar ha sido propuesta en las mutaciones de *PS* [372]. Consistentemente, una mutación familiar de EA en *PS* muestra un enlentecimiento en el corte secuencial intramembrana de γ-secretasa y otras G*X*GD-aspartil proteasas provocando productos de corte más largos [327].

En conjunto, esta familia muestra que, a pesar que la ratio $A\beta_{42/40}$ parece ser el mejor indicador de severidad de la enfermedad en pacientes con mutaciones en *APP*, la reducción del procesamiento puede contribuir también en el proceso de la enfermedad y sugiere un mecanismo adicional por el cual algunas mutaciones alrededor del lugar de corte de la γ-secretasa pueden provocar EA.

Considerando el conjunto de los resultados, se puede afirmar que las manipulaciones genéticas y farmacológicas del complejo γ-secretasa son un buen modelo para el estudio de las alteraciones proteicas que ocurren durante el avance de la EA. Los modelos celulares son un buen ejemplo para entender mejor las interacciones en el ámbito proteico de esta enfermedad.

★ Futuros proyectos

Este trabajo nos ha proporcionado la oportunidad de explorar dos nuevas vías, las manipulaciones genéticas y farmacológicas del complejo γ-secretasa. En el ámbito de las manipulaciones farmacológicas, nuestros estudios siguen avanzando con una nueva y prometedora generación de fármacos análogos al flurbiprofeno capaces de reducir los niveles de Aβ sin afectar a Notch.

Complementariamente, las manipulaciones genéticas mediante el estudio de la mutación APPI716F nos han generado nuevas preguntas referentes al tráfico celular y sinapsis neuronales. Hemos iniciado el estudio de tráfico intracelular de APP mediante la utilización de un constructo de APP unido a proteínas fluorescentes (APP Dendra) que nos permite su estudio mediante microscopía confocal. Por otro lado, estudiaremos la integridad de las sinapsis neuronales y su afectación con diferentes mutaciones de *APP* con estudios de espinas dendríticas en cultivos primarios neuronales y muestras de pacientes portadores de estas mutaciones.

CONCLUSIONES

VIII. CONCLUSIONES

1 Una depleción leve de los niveles de colesterol de membrana en cultivo celular reduce la generación de Aβ sin afectar a la proteólisis de Notch, ni la escisión de APP por ε- o γ-secretasa. Este efecto puede estar mediado por cambios en el tráfico de APP y una reducción en la producción de APP CTF y la fracción de APP asociada a los rafts lipídicos.

2 La mutación I716F de la APP provoca EA familiar con edad de inicio muy precoz y con abundantes depósitos de Aβ42 cerebral. En el ámbito funcional la mutación induce un aumento de la ratio Aβ42/Aβ40 y una reducción del procesamiento proteico de APP. Nuestro estudio demuestra que esta reducción del procesamiento puede representar un mecanismo patogénico adicional en la EA familiar.

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IX. BIBLIOGRAFÍA

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ANEXO

γ-Secretase as a Therapeutic Target in Alzheimer's Disease

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Abstract: γ-secretase is an intramembranous multi-protein complex that cleaves many type-I proteins with critical roles in neuronal function. In Alzheimer's disease (AD) interest in γ-secretase comes, in part, from the fact that this complex is responsible for the last cleavage step of the amyloid precursor protein (APP) that generates the amyloid-β peptide (Aβ). Aβ represents the primary component of the amyloid plaque, one of the main pathological hallmarks of AD. Over the last years, considerable efforts have been made to develop drugs to reduce Aβ production with the aim to slow AD progression. Many inhibitors of this protease have been identified, although the clinical use has been limited by concerns about the possible toxicity of these compounds. γ-secretase inhibitors have been shown to reduce Aβ *in vitro* and *in vivo*, but interference with Notch proteolysis causes immunological and gastrointestinal toxicity in animal models. The observation that some nonsteroidal anti-inflammatory drug (NSAID) derivatives are able to specifically lower $A\beta_{42}$ and the development of inhibitors with Notch-sparing selectivity has revived the interest in γ-secretase as an attractive target for drug intervention in AD. Despite the fact that all clinical trials with NSAIDs or γ-secretase modulators in AD have failed to show clinical benefit thus far, the main concern is that the Aβ-lowering potency of the tested compounds may be too low. Active efforts are being made to develop compounds able to penetrate into the brain to lower Aβ at physiological doses without interfering with the cleavage and function of other critical γ-secretase substrates. These novel inhibitors and modulators may soon offer hope in the Alzheimer's fight.

Keywords: γ-Secretase, NSAIDs, Alzheimer's disease, β-amyloid, Notch.

INTRODUCTION

Alzheimer disease (AD) is the most common form of dementia and affects over 20 million people worldwide. This number is expected to rise up to 115 million by 2050 if new preventive or curative treatments do not emerge [1]. Current treatments for AD, such as acetylcholinesterase inhibitors or memantine, offer a modest symptomatic benefit but have little or no impact on disease progression. A great effort is being made to develop novel therapies to treat this devastating condition. Most disease modifying therapies that have emerged over the last years have focussed on targets related with the main neuropathological hallmarks of AD: amyloid plaques, neurofibrillary tangles, inflammation and oxidative stress.

Amyloid plaques are mainly composed of the 4-kDa amyloid-β peptide (Aβ). Much evidence supports the view that oligomers of Aβ in high concentrations are neurotoxic and initiate a cascade of events eventually leading to synaptic and neuronal dysfunction and death [2, 3]. Aβ is proteolytically generated from a larger β-amyloid precursor protein (APP) through sequential cleavages by the enzymes β- and γ-secretase. γ-secretase cleaves the C-terminus of APP at different positions to generate Aβ peptides of different lengths. Although, $A\beta_{40}$ is the most predominant species, the longer and more amyloidogenic $A\beta_{42}$ is the one initially deposited in the brain and predominates in diffuse and mature plaques [4].

The two main anti-amyloid approaches have been to reduce the production or to increase the clearance of $A\beta_{42}$. The goal of Aβ reduction has focused on a search for molecules that inhibit β- or γ-secretase [5]. β-secretase (β-site APP-cleaving enzyme, BACE) is a single membrane spanning aspartyl protease expressed at high levels in neurons and BACE1 is the main isoform in the brain [6, 7]. Development of active inhibitors for this protease has been stimulated by the observation that brain and cerebrospinal fluid (CSF) BACE1 activity is increased in AD [8, 9]. Some effective compounds have been tested *in vitro* and in animal models and a potent inhibitor developed by CoMentis has recently completed a phase I trial [10, 11]. γ-secretase is an unusual aspartyl protease that intramembranously cleaves a wide range of type I membrane proteins in addition to APP [12, 13]. Initial enthusiasm for γ-secretase inhibitors has cooled down by the concern that these compounds may lead to adverse effects due to inhibition of Notch processing [14]. Paradoxically, this unwanted effect has made γ-secretase an attractive target in cancer research [15]. The development of inhibitors with Notch-sparing selectivity and the finding that some nonsteroidal anti-inflammatory drugs (NSAIDs) and related compounds are able to specifically lower $A\beta_{42}$, have revived the interest in γ-secretase as an attractive drug target in AD. In this review, we will summarize the current evidence that supports γ-secretase as a valid target for AD.

STRUCTURE AND ACTIVITY OF γ-SECRETASE

γ-secretase belongs to a group of proteases called intra-membrane cleaving proteases (I-CLiPs) that are membrane-embedded enzymes that hydrolyze transmembrane substrates and the residues essential to catalysis reside within the

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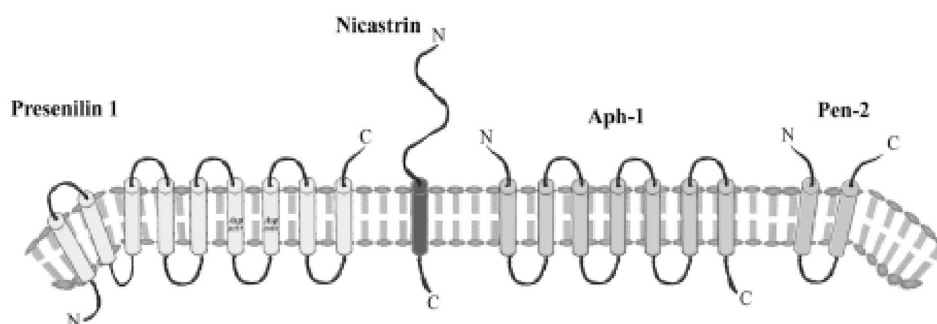
boundaries of the lipid bilayer [16]. These I-CLiPs are proposed to bend or unwind their helical substrates to make the amide bonds susceptible to hydrolysis [17]. γ-secretase is an aspartyl protease composed of four components that are required for the enzymatic activity: presenilin (PS), anterior pharynx-defective-1 (Aph-1), presenilin enhancer-2 (Pen-2) and nicastrin. [18-25]. Two mammalian PS homologues exist, PS1 and PS2, and they show a high degree of homology (67%) and functional redundancy. PS are nine-transmembrane proteins that must undergo endoproteolysis to generate active N- and C-terminal fragments (NTF and CTF) that remain closely associated [26-28]. Several studies have indicated that the PS heterodimer plays a key role in γ-secretase function. First, cells cultured from mouse embryos deficient in PS1 (PS1-/-) have a marked reduction in Aβ and accumulate APP CTFs, the direct substrate of γ-secretase [23, 29-31]. Second, the loss of PS1 and PS2 leads to the accumulation of APP CTFs and complete absence of Aβ [32, 33]. Third, PS contains a G*X*GD motif, similar to that of the bacterial aspartyl proteases type-4 prepilin peptidases (TFPPs) [34] and signal peptide peptidase (SPP) [35]. Last, mutation of either of two conserved transmembrane aspartates (Asp257 or Asp385) in PS1 abolishes γ-secretase activity and studies with transition-state analogue inhibitors of γ-secretase showed that these compounds were able to bind directly to the NTF/CTF heterodimer [21, 36, 37]. All together, these studies suggest that PS is an aspartyl protease that contains the catalytic center of the γ-secretase complex at the NTF/CTF interface. In addition, other studies have indicated the existence of an additional substrate-binding site in PS1, distinct from the active site [38, 39]. Another line of data that supports PS1 as a key player in AD pathogenesis comes from the fact that mutations in the *PSEN* genes are the most common cause of familial early-onset AD (www. molgen.ua.ac.be/ADMutations). To date, more than 175 mutations in *PSEN1* gene have been identified in familial AD, and most of them elevate the $A\beta_{42/40}$ ratio and interfere with the processing of APP and other γ-secretase substrates [40, 41]. Interestingly, *PSEN1* mutations interfere with many other functions in which PS1 is involved, such as cell adhesion, apoptosis, protein trafficking, calcium homeostasis, tau phosphorylation and β-catenin turnover among others [42]. Taken together, *PSEN1* mutations seem to result in a partial loss-of-function of the γ-secretase [43]. The same loss-of-function effect would be responsible for the gain of a toxic property causing elevated $A\beta_{42/40}$ ratio due to an incomplete Aβ cleavage.

In addition to PS1, three other subunits are necessary for active γ-secretase: nicastrin, Aph-1 and Pen-2 (Fig. **1**). In this sense, γ-secretase is a unique intramembrane protease composed by four proteins while all the others apparently work alone as single proteins [44]. Nicastrin is a type I membrane glycoprotein with a large lumenal domain involved in the assembly, maturation and activation of the γ-secretase complex [24, 45, 46]. However, whether the

A



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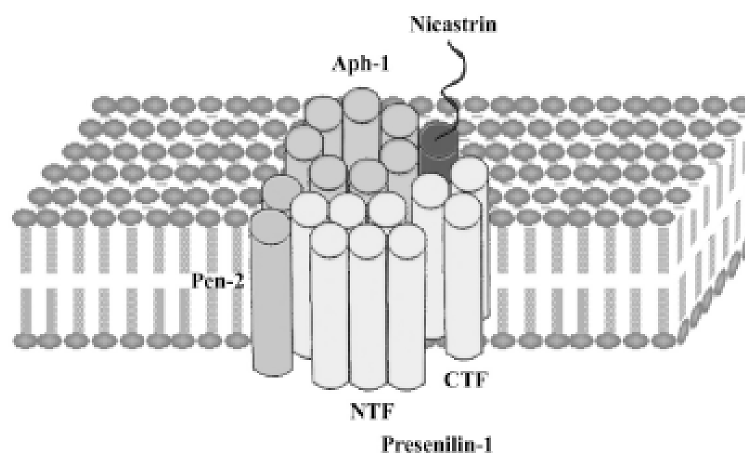

Fig. (1). Structure of the γ-secretase complex. A) Topology of the four subunits of the enzyme: presenilin, nicastrin, Aph-1 and Pen-2. **B)** 3D image of the distribution within the plasma membrane of the four γ-secretase components.

extracellular region of nicastrin plays a role in substrate recognition remains controversial [46, 47]. Nicastrin conditional knock-out mice display learning and memory deficits and age-dependent cortical neuronal loss [48]. Aph-1 is a seven-transmembrane domain (TMD) protein with a cytosolic C-terminus while Pen-2 encodes a two-TMD hairpin-like protein with both ends in the lumen (Fig. **1**) [22, 25, 49, 50]. In humans, there are two paralog *APH-1* genes (*APH-1A and B)* but three variants of the Aph-1 protein (Aph-1a with two splice variants, S and L respectively, and Aph-1b), which differentially incorporate in different γ-secretase complexes [51, 52]. Aph-1a is the major isoform present in γ-secretase complexes [53]. Therefore, while *APH-1A* gene is essential for Notch signaling during embryogenesis [53, 54], *APH-1B* can be removed completely, and this leads to a significant Aβ reduction [55]. Under these conditions Notch function is maintained by the presence of *APH-1A* [55]. Although these four subunits act as a limiting factors, other factors, like TMP21 and CD147 among others, can influence γ-secretase activity [56, 57]. The protein TMP21 has been reported to differentially modulate γ-cleavage of APP [56] while CD147 appears to play a role as a negative regulator of γ-secretase [57]. Although these proteins can modulate γ-secretase activity they do not appear to act at the γ-secretase level [58-60]. Given that there are two PS and three Aph-1 proteins, at least six different complexes exist with potentially different biological functions [51, 52, 54, 61]. Consistent with this notion, complexes containing different Aph-1 or PS proteins have been shown to display distinct but overlapping γ-secretase activities [26, 52, 54, 61-63].

All components of γ-secretase assemble in the endoplasmic reticulum in a specific manner to become fully functional. Initially, nicastrin and Aph-1 form a subcomplex and subsequently presenilin is incorporated to form a heterotrimeric subcomplex [64]. The addition of Pen-2 results in a mature complex and allows the activation of the complex by endoproteolysis of presenilin [65-68]. It is still debated the exact stoichiometry of the complex and whether oligomerization can occur *in vivo* [69]. However, this phenomenon is unlikely to have a significant impact on the activity because it has been reported that purified γ-secretase can be fully active in a monomeric form [69]. The structure elucidation of the γ-secretase complexes has been complicated by the lack of crystal structure of γ-secretase. However, according to a low-resolution map obtained by electron microscopy studies [70-72], human γ-secretase has an spherical structure with three potential interior cavities [71, 72] or, alternatively, one interior central pore [70].

SUBSTRATE SPECIFICITY OF γ-SECRETASE

Since the initial studies that demonstrated that PS1-dependent γ-secretase is essential for the processing of APP and the Notch receptor [23, 74], an increasing number of type-I membrane proteins have been shown to be cleaved by γ-secretase (Fig. **2** and Table **1**) [12, 13]. Despite the fact that more than 70 type-I integral membrane proteins are known to be cleaved by γ-secretase (Table **1**), the physio-logical function of these proteolytic events is poorly understood. γ-secretase displays poor substrate specificity with no clear consensus sequence motif which suggests a major degradative function [73]. However, a functional γ-

Table 1. Known γ-Secretase Substrates

Substrate	Function	Refs.
ADAM10	Proteolytic processing and shedding of proteins	[165]
Alcadein α, β, γ	Post-synaptic protein	[166]
ApoER2/LRP8	Lipoprotein receptor, neuronal migration, synaptic plasticity, LTP	[167, 168]
APP	Cell adhesion, migration, synaptogenesis, neuritic growth	[23]
APLP1, APLP2	Cell adhesion, migration, synaptogenesis, neuritic growth	[169]
Bri2 (Itm2b)	Type-II oriented transmembrane protein	[170]
CD43	T Cell migration and proliferation, leukocyte cell to cell interactions	[171]
CD44	Cell adhesion, metastasis, tumor growth	[172, 173]
CSF-1R	Receptor tyrosine kinase. Proliferation and differentiation	[101]
CXCL16 & CX3CL1	Transmembrane chemokine ligands	[174-177]
DCC	Netrin-1 receptor. Axonal guide	[178]
Delta 1	Receptor Notch ligand	[179-182]
DNER	Neuronal Notch receptor ligand	[84]
DSG2	Structural component of desmosomes	[84]
Dystroglycan	Member of multiprotein dystrophin-glycoprotein complex	[84]
E-Cadherin	Cell adhesion	[183]
Ephrin B1,B2	Ephrin receptor ligand. Neuritogenesis and angiogenesis	[184]
EphB2	Receptor tyrosine kinase. Neuritogenesis and angiogenesis	[185]
Erb-B4	Growth-factor-dependent receptor tyrosine kinase	[78, 186-188]
GluR3	Glutamate receptor	[189]
Growth hormone receptor	Receptor	[190]
HLA-A2	MHC Class I protein. T-cell development	[191]
IFNaR2	Subunit of the type I IFN-α receptor	[192, 193]
Insulin receptor	Receptor tyrosine kinase	[194]
Interleukin-1 type I and II receptor	Cytokine receptor	[195, 196]
Ire1 α/β	ER transmembrane protein with kinase and endoribonuclease activities.	[197]
IGF-1R	Tyrosine kinase receptor. Cell proliferation	[198]
Jagged	Notch ligand	[180, 181]

(Table 1) Contd…..

Substrate	Function	Refs.
Klotho	Regulates hormone metabolism	[199]
LAR	Phosphatase of receptor tyrosine kinase. Synapses formation and neuronal network	[200]
LDLR	Lipoprotein receptor	[84]
LRP 1	Endocytic receptor. LDL superfamily receptor.	[201-204]
Megalin/LRP 2	Endocytosis and transport	[205]
N-cadherin	Cell adhesion	[77]
NCAM-L1	Cell adhesion molecule	[206, 207]
Nectin-1α	Adherens-junction formation. Synaptic contact	[99]
Notch 1-4	Signaling receptor. Cell differentiation. Neuritic growth.	[74, 208, 209]
NPR-C	Natriuretic peptid receptor	[84]
p75^{NTR}	Neurotrophin co-receptor. Cell survival	[210, 211]
NRG-1	ErbB family ligand. Growth factor, differentiation	[212]
NRADD	Induce apoptosis in certain cell types	[213]
PLXDC2	Nervous system protein	[84]
Polyductin/ fibrocystin	Cell receptor	[214]
γ-protocadherins	Neuronal connectivity and survival.	[215]
RPTP (κ,μ)	Receptor protein tyrosine phosphatase. Cell adhesion receptors	[216]
SorLA, Sortilin, SorCS1b	Vps10p receptors. Sorting and intracellular trafficking.	[102]
Syndecan 1-3	Cell-surface proteoglycan co-receptor, cytokine receptor	[84, 217]
Tie-1	Tyrosine kinase receptor, vascular development	[218]
Tyr, Tyrp1, DCT/Tyrp2	Pigment synthesis, intracellular trafficking	[100]
Vasorin	TGF-β inhibitor	[84]
VE-Cadherin	Cell adhesion molecule	[219]
VLDL receptor	Lipoprotein receptor	[167]
Voltage-gated sodium channel β-1-4 subunits	Regulates cell adhesion and migration	[220, 221]
VEGFR-1	Growth factor receptor	[222, 223]

Abbreviations: ADAM10, A disintegrin and metalloprotease; ApoER2, Apolipoprotein E receptor-2; APP, Amyloid precursor protein; APLP, Amyloid precursor like-protein; CSF-1R, Colony-stimulating factor 1; CXCL, Chemokine (C-X-C motif) ligand; DCC, Deleted in colorectal cancer; DNER, Delta/Notch-like EGF repeat; DSG2, Desmoglein 2; GluR3, Glutamate receptor 3; HLA-A2, Human leukocyte antigen type 2; IFNaR2, Subunit of the type I IFN-α receptor; IGF-1R, Insulin-like growth factor 1 Receptor; LAR, Leukocyte-common antigen related; LDLR, Low density lipoprotein receptor; LRP1, Low-density lipoprotein receptor-related protein; NCAM-L1, Neural cell adhesion molecule L1; NPR-C, Natriuretic peptid receptor; NRG-1, Neuregulin-1; NRADD, neurotrophin receptor alike death domain protein; NTR, neurotrophin receptor; PLXDC2, Plexin domain containing 2; RPTP, receptor protein tyrosine phosphatase; VLDL, very low density lipoprotein; VEGFR-1, vascular endothelial growth factor

secretase cleavage has been clearly demonstrated for some substrates. Notch proteolysis by γ-secretase generates an intracellular domain which is essential for many cell differentiation events and neurite outgrowth (Fig. **2**) [74-76]. Proteolysis of N-cadherin leads to degradation of the transcriptional factor CBP (CREB binding protein), and cleavage of ErB4 inhibits astrocyte differentiation by interacting with repressors of astrocyte gene expression [77-79]. Cleavage of APP generates an APP intracellular domain (AICD), although its role in signal transduction remains controversial [80-82]. The general requirements to be cleaved efficiently by γ-secretase are: a type I transmembrane helix and a small ectodomain usually resulting from a prior shedding by another protease [83]. However, not all type-I transmembrane proteins with short ectodomain are processed by γ-secretase and data suggest that both a permissive transmembrane and permissive cytoplasmic domains are also necessary [83, 84]. Some substrates, such as APP, APLP-1, Notch, or CD44 are cleaved at multiple sites by γ-secretase. For example, APP is cleaved at the γ-site (near residues 40-42), at the ε-site (near residue 49) and at the ζ-site (near residue 46) [85].

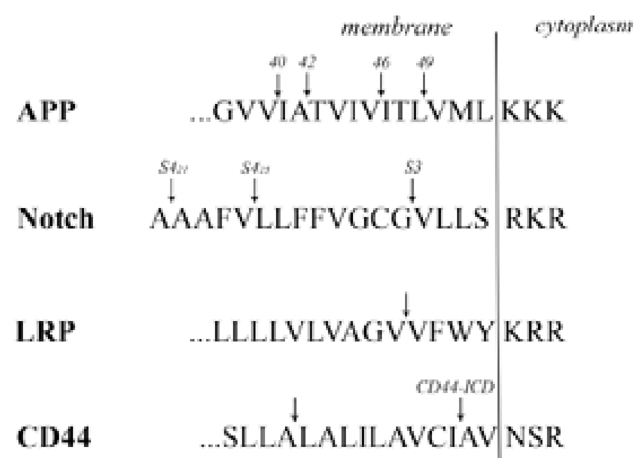

Fig. (2). Regulated intramembrane proteolysis by γ-secretase. Schematic representation of the intramembraneous cleavage sites of APP, Notch, LRP and CD44.

The long list of substrates processed by γ-secretase has clear implications for the development of new therapies for AD and, in particular, for the search of γ-secretase inhibitors or modulators. Interference with the cleavage of substrates with important cellular functions, such as Notch, has been shown to be associated with serious adverse effects in animal models [14, 86].

γ-SECRETASE AS A VALID THERAPEUTIC TARGET

Three properties make γ-secretase a highly interesting but challenging target. First, γ-secretase is an unconventional aspartyl protease that resides and cleaves its substrates within the lipid bilayer. Second, AD is believed to be caused by a progressive cerebral accumulation of Aβ, and γ-secretase cleaves APP to release Aβ. Third, γ-secretase

processes a wide range of type I membrane proteins, some of them with critical cellular functions. The challenge in AD research has been thus far to find a γ-secretase inhibitor able to selectively lower Aβ but without interfering with the cleavage of other important substrates.

Current research on γ-secretase inhibitors has mainly focussed on compounds with Notch-sparing selectivity. The working paradigm is that partial inhibition of γ-secretase will lower Aβ sufficiently while avoiding Notch-related side effects. Interestingly, a report using transgenic mice heterozygous for one or more components of γ-secretase showed that >30 % inhibition of γ-secretase induces abnormal proliferation of granulocytes and that >50 % inhibition increases tumorigenesis [87]. Therefore, it has been suggested that up to 30% γ-secretase inhibition could be an achievable target in humans to avoid Notch-related side effects. By contrast, interference with Notch has made γ-secretase an attractive target in cancer research and γ-secretase inhibitors are currently being investigated in breast cancer and leukaemia (www.clinicaltrials.gov) [15].

Although treatment with γ-secretase inhibitors has been shown to lower Aβ in plasma and in the brain, *in vivo* imaging animal studies suggest that γ-secretase inhibition could be a better strategy to prevent formation of new amyloid plaques rather than to remove existing ones [88, 89]. In agreement with these data, studies in transgenic animal models have indicated that reducing expression of APP is able to halt amyloid progression but remaining plaques may persist many months [90].

DEVELOPMENT OF γ-SECRETASE INHIBITORS AND MODULATORS

Several γ-secretase inhibitors have been shown to decrease Aβ *in vitro* and *in vivo* [36-39, 91-96]. Transition-state analogue (TSA) inhibitors, compounds designed to interact with the active site of γ-secretase the protease, were found to bind directly to PS1 NTF-CTF heterodimer [36, 37], which is the biologically active form. TSA inhibitors also block the cleavage of other γ-secretase substrates, including the Notch receptor [97]. In fact, long term treatment with γ-secretase inhibitors causes gastrointestinal toxicity and interferes with the maturation of B- and T-lymphocytes in mice, effects due to inhibition of Notch signaling [14, 98]. Inhibition of γ-secretase by these drugs can be monitored by the accumulation of the CTFs of different substrates, such as APP, nectin, sortilin receptors and tyrosinase among others [99-102].

Designed peptides based on the transmembrane domain of APP and constrained in a helical conformation have been shown to inhibit γ-secretase [39]. The use of helical peptide inhibitors as photoaffinity probes led to the localization of a substrate docking site on the PS NTF/CTF interface, at a distinct but overlapping site from that of TSA inhibitors [38]. Extension of a helical peptide inhibitor by just three additional residues resulted in a potent inhibitor capable of binding to both active and substrate docking sites [38, 103]. The close proximity of both sites suggests that only part of the transmembrane substrate might need to insert into the active site, with the rest remaining in the docking site [38]. Investigation of other proteases similar to γ-secretase has been important for the development of specific γ-secretase inhibitors. One example can be found in the study of SPP [104]. SPP is an intramembrane aspartyl protease that contains active motifs and biochemical characteristics also found in PS [35]. SPP is inhibited by helical peptides and TSA inhibitors and contains a substrate-binding site distinct from the active site [72, 104, 105]. Although these proteases bear similar active sites, SPP does not require additional protein cofactors, has opposite membrane topologies that of PS and cleaves type II transmembrane proteins.

A number of structurally diverse γ-secretase inhibitors have been described in addition to the classical TSA [106]. One of the most commonly used inhibitors in research studies is the dipeptidic compound DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester) [94]. Interestingly, DAPT has been shown to bind to the C-terminus of PS at a novel site distinct from the active or substrate-binding sites [107]. Acute or chronic γ-secretase inhibition with DAPT can lower $A\beta_{42}$ and improves cognitive deficits in a transgenic animal model [92, 94].

Current efforts are focused to develop compounds able to inhibit γ-secretase with Notch-sparing selectivity. Compounds able to inhibit γ-secretase with 10-15-fold Notch-1-sparing selectivity have been developed [96, 108]. These compounds can lower Aβ *in vivo* in transgenic mice without Notch-1 related side effects. The benzodiazepine analogue LY411575 and the benzolactam LY450139 (semagacestat), developed by Eli-Lilly, have been widely tested *in vitro* and *in vivo* [109, 110]. Semagacestat is the most well known γ-secretase inhibitor that has reached clinical testing. Semagacestat can lower $A\beta_{1-x}$ in the low nanomolar range, and it is 3-fold more selective in inhibiting APP than Notch γ-secretase cleavage [11, 109]. This compound is able to reduce Aβ levels in plasma, CSF and in the brain of different animal models [11, 109]. However, a recent study has shown that γ-secretase inhibition with DAPT or semagacestat also reduced spine density in mice *via* an APP-dependent pathway [111].

Begacestat (GSI-953), a thiosphere sulfonamide inhibitor developed by Wyeth, has also been well-characterized in preclinical studies [112]. Begacestat lowers $A\beta_{42}$ in the low nanomolar range and it is 16-fold more selective for APP over Notch cleavage. This compound is able to lower brain, plasma and CSF Aβ in a transgenic tg2576 mouse model. Begacestat is currently in clinical trials.

A considerable advance in the field of γ-secretase took place after the discovery that some commonly used NSAIDs selectively lowered $A\beta_{42}$ in cell culture and transgenic animal models, independently of cyclooxygenase (COX) activity [113, 114]. Therefore, the (R)-enantiomers that have low COX inhibitory activity still retain the ability to reduce $A\beta_{42}$ [113, 115]. NSAIDs shift the cleavage of APP to promote shorter Aβ species, in particular $A\beta_{38}$, but do not inhibit the cleavage of other γ-secretase substrates [113, 116, 117]. Interestingly, NSAIDs also seem to shift the proteolytic cleavage of Notch to generate shorter species of Nβ, the Aβ-like peptide cleaved by γ-secretase, but do not inhibit Notch signaling pathway or the release of the intracellular

domain [118]. At higher concentrations NSAIDs are able to inhibit overall γ-secretase activity [119, 120], indicating that there is a window for modulation of γ-secretase. NSAIDs are also able to modulate the cleavage site of SPP [105]. Taken together, NSAIDs appear to induce a conformational change in γ-secretase through an allosteric mechanism [117, 119-121]. Although the molecular target of NSAIDs has remained elusive, a recent study using a biotinylated photoactivable drug showed that, surprisingly, γ-secretase modulators are able to bind to the juxtamembrane region of APP [122].

Since the original description that some NSAIDs lower $A\beta_{42}$, enormous efforts have been done to develop γ-secretase modulators with enhanced $A\beta_{42}$-lowering effects but without COX inhibitory activity. Several flurbiprofen analogues with potent and selective inhibitory activity against $A\beta_{42}$ have been reported [116, 123, 124]. These compounds display improved potency against $A\beta_{42}$ in cell culture and transgenic mice and improve learning deficits in animal models of AD [124]. Interestingly, the search for novel γ-secretase modulators has encountered compounds with $A\beta_{42}$-raising properties, such as celecoxib, a COX-2 selective NSAID, or fenofibrate, a PPARα agonist [125-127]. The existence of such γ-secretase modulators suggests that γ-secretase can be pharmacologically manipulated in a way similar to the genetic modifications induced by familial AD mutations, which lead to an elevation of $A\beta_{42}$ production [127, 128].

Other non-NSAID molecules with modulatory effect on γ-secretase have been reported. The finding that γ-secretase contains a nucleotide-binding site led to the discovery of compounds able to modulate γ-secretase by targeting this site [129]. These compounds resemble kinase inhibitors and inhibit APP processing without affecting the proteolysis of Notch. In this setting, the Abl kinase inhibitor imatinib (Gleevec™) and other related compounds are being investigated as selective inhibitors of Aβ production [130].

Although the discovery of γ-secretase modulators has revived the interest in γ-secretase, the limited potency of these compounds and the poor pharmacokinetic properties have challenged its use in humans. Whereas γ-secretase inhibitors with subnanomolar activity have been reported [91, 103], the most potent NSAID-type γ-secretase modulator show potencies in the low micromolar concentration range. NSAID-analogues exhibit poor brain penetration, which has been associated with the presence of the carboxylic acid moiety. Many novel γ-secretase modulators with enhanced $A\beta_{42}$-lowering potency have been developed by companies such as Merck, Pfizer, EnVivo Pharmaceuticals or Chiesi Farmaceutici, and its clinical use is currently under investigation [116, 123, 131, 132]. In particular, Merck has developed a γ-secretase modulator (GSM-1) that selectively reduces $A\beta_{42}$ in cell culture and monkeys [11]. Chiesi has also developed a potent γ-secretase modulator (CHF5074) that preferentially lowers $A\beta_{42}$ in cell culture and attenuates Aβ pathology and behavioral deficits in a transgenic mouse model of AD [116, 123, 131, 133]. CHF5074 is moving towards clinical testing [116, 133]. Finally, EnVivo Pharmaceuticals has developed a γ-secretase modulator (EVP-0962) with enhanced $A\beta_{42}$-lowering activity that improves hippocampal dependent memory in a transgenic mouse model of AD [134].

γ-SECRETASE INHIBITORS AND MODULATORS IN HUMANS

Despite the concerns about the possible toxicity of γ-secretase inhibitors in humans, these compounds have advanced to the clinical arena without overt side effects. Nowadays, there are six ongoing clinical trials with γ-secretase inhibitors in humans (www.clinicaltrials.gov). The γ-secretase inhibitor LY450139 (semagacestat) developed by Eli-Lilly has safely completed phase 1 and 2 in healthy volunteers and AD patients [135-137]. Skin rash and hair color change were reported in the treatment group [137]. The phase 2 randomized, double-blind study demonstrated that the compound lowered plasma but not CSF Aβ levels [137]. However, a recent study using stable-isotope labeling in healthy volunteers has shown that semagacestat is able to acutely lower Aβ in CSF [138]. The compound has moved on to phase 3, and results are expected for 2012. Wyeth (GSI-953/begacestat), Merck (MK-0752), Pfizer (PF-3084014), Eisai (E2012) and Bristol-Myers-Squibb (BMS-708163) have also developed γ-secretase inhibitors and trials are currently in phase I or II [133].

One of the first evidence pointing to a potential protective role of NSAIDs for AD was the observation that subjects with arthritis have a reduced incidence of AD [139]. Since then, many retrospective epidemiological studies have shown a reduced incidence of AD among NSAID users [140-151]. A meta-analysis of nine studies showed that the use of NSAIDs was associated with a lower risk of developing AD, and the benefit was mainly observed in long-term users [152]. However, the reduced risk of AD can not be clearly associated with those NSAIDs that reduce $A\beta_{42}$ [153, 154]. This putative protective effect has not been confirmed in prospective trials. A large NIA-sponsored preventive trial (ADAPT) with celecoxib or naproxen was halted due to concerns about increased cardiovascular risk. In spite of the early termination, no cognitive benefit could be detected with any drug [155]. The possible preventive effect of NSAIDs has not been confirmed in patients with AD. Many clinical trials with different NSAIDs (Table **2**) have failed to show any efficacy in patients with AD [156-160] or mild cognitive impairment (MCI) [161, 162]. A recent placebo-controlled trial with R-flurbiprofen sponsored by Myriad could not demonstrate any efficacy [163, 164]. The failure of all these trials could be explained by the modest Aβ-lowering potency and brain penetration of the tested compounds, and the possibility that these drugs may be only effective in early phases of the disease.

Taken together, the potential protective role of NSAIDs observed in retrospective epidemiological studies has not been confirmed in prospective studies and clinical trials in AD have failed to show any benefit thus far. Therefore, NSAIDs are not currently recommended as a treatment for patients with AD or MCI. Other γ-secretase modulators with enhanced Aβ-lowering effects have been developed by Merck, Pfizer, EnVivo Pharmaceuticals or Chiesi Farmaceutici and its clinical use is being investigated [116, 123, 131, 133].

Table 2. Clinical Trials with NSAIDs other than Aspirin in AD or MCI

Study	Drug	Type of NSAID	Patients Enrolled/ Completed	Duration (Weeks)	Outcome
Rogers *et al*. [224]	Indomethacin	COX-1 mainly PPARγ agonist NF-κB inhibitor Aβ42-lowering agent	24/14	26	Small benefit
Scharf *et al*. [157]	Diclofenac/ Misoprostol	COX-1/COX-2	24/12	25	No benefit
Aisen *et al*. [160]	Naproxen	COX-1/COX-2 PPARγ agonist	118/90	52	No benefit
Aisen *et al*. [159]	Nimesulide	COX-2 selective	26/18	12 + 12	No benefit
Soininen *et al*. [225]	Celecoxib	COX-2 selective	285/278	52	No benefit
Aisen *et al*. [226]	Rofecoxib	COX-2 selective	122/89	52	No benefit
Reines *et al*. [156]	Rofecoxib	COX-2 selective	348/253	52	No benefit
Zanetti *et al*. [227]	Ibuprofen	COX-1/COX-2 PPARγ agonist	132/95	54 + 27	Non-significant slower decline at 18 months
Wilcock *et al* [164]	R-flurbiprofen	$A\beta_{42}$-lowering NF-κB inhibitor	210/160	54	Slower decline in mild AD
Thal *et al*. [162]*	Rofecoxib	COX-2 selective	1457/588	4 years	No benefit
Gómez-Isla *et al*. [161]*	Triflusal	COX-1 mainly NF-κB inhibitor	257/152	58	Lower rate of progression
De Jong *et al*. [228]	Indomethacin	COX-1 mainly PPARγ agonist NF-κB inhibitor $A\beta_{42}$-lowering agent	51/38	1 year	No benefit
Galasko *et al*. [163]	R-flurbiprofen	$A\beta_{42}$-lowering NF-κB inhibitor	48/48	21 days	Higher plasma drug concentration was related to lower $A\beta_{42}$ plasma levels

*The trial included only patients with MCI.

CONCLUSIONS

γ-secretase has reemerged as an interesting therapeutic target for drug intervention in AD. The biochemical characterization of this protease has allowed the development of novel γ-secretase inhibitors with Notch-sparing selectivity or modulators with enhanced $A\beta_{42}$-lowering properties. The lack of efficacy observed so far in clinical trials may be due to the limited potency of the tested compounds and the poor pharmacokinetic properties, in particular brain penetration. Some novel promising compounds have been developed and clinical benefit is currently being investigated. Paradoxically, interference of some of these drugs with Notch proteolysis has made this approach attractive for cancer research.

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