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MODIFICACIONES DE LA VÍA DE SEÑALIZACIÓN WNT DURANTE EL ENVEJECIMIENTO Y SU RELACIÓN CON LA FOSFORILACIÓN DE LA PROTEÍNA TAU. IMPLICACIONES PARA LA ENFERMEDAD DE ALZHEIMER.

TESIS

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Nos permitimos informarle que el Comité Académico de **DOCTORADO EN CIENCIAS BIOMÉDICAS**, en su reunión **351** del 27 de agosto del 2014, designó el siguiente jurado para examen de grado de **DOCTORA EN CIENCIAS** de **ANA PAMELA SALCEDO TELLO,** con número de cuenta **402115883**, con la tesis titulada: **"MODIFICACIONES DE LA VÍA DE SEÑALIZACIÓN WNT DURANTE EL ENVEJECIMIENTO Y SU RELACIÓN CON LA FOSFORILACIÓN DE LA PROTEÍNA TAU. IMPLICACIONES PARA LA ENFERMEDAD DE ALZHEIMER"** dirigida por la Dra. Ana Brígida Clorinda Arias Álvarez

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"POR MI RAZA HABLARÁ EL ESPÍRITU"
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Salcedo-Tello P., Heras-Sandoval D. y Arias C. (2010) Papel central de las vías de señalización que inducen crecimiento celular (INSULINA y Wnt) en envejecimiento y longevidad. En Instituto de Geriatría *ENVEJECIMIENTO HUMANO Una Visión Transdisciplinaria* (pag. 85-92) México: Secretaría de Salud.

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Salcedo-Tello P, Hernández-Ortega K, Arias C. (2014) Susceptibility to GSK3β-induced tau phosphorylation differs between the young and aged hippocampus after Wnt signaling inhibition. J Alzheimers Dis. 39(4):775-85

RESUMEN

La enfermedad de Alzheimer (EA) es la forma mas común de demencia y el envejecimiento es el principal factor de riesgo. Histopatológicamente se caracteriza por la presencia de placas amiloideas compuestas de péptido β-amiloide y marañas neurofibrilares formadas de agregados de tau. La proteína tau modula la estabilidad y el ensamblaje de los microtúbulos. Tau se regula principalmente por su grado de fosforilación, la hiperfosforilación disminuye su capacidad de ensamblaje y su unión a microtúbulos. La fosforilación la llevan a cabo diferentes proteínas cinasas y es revertida por proteínas fosfatasas. La cinasa GSK3β y la proteína fosfatasa 2A (PP2A) son enzimas clave involucradas en la regulación de tau, y la evidencia sugiere que participan en la fosforilación patológica y la agregación en la EA. La activación de la vía Wnt/β-catenina inhibe la actividad de GSK3β. El antagonista endógeno de esta vía, Dkk-1 se ha encontrado incrementado en modelos transgénicos y en pacientes con EA, mostrando que la alteración en la señalización Wnt puede contribuir a dicha patología. En este trabajo empleando un modelo de rebanadas hipocampales metabólicamente activas encontramos que la inhibición de la actividad basal de GSK3β disminuye la fosforilación en los epitopes: fosfo-Ser199/202/396 y 404 en ratas jóvenes y viejas. Mostrando que la fosforilación de estos sitios es directamente dependiente de la actividad basal de GSK3β. A diferencia del epitopo fosfo-Ser214 el cual no se vio alterado por la inhibición de GSK3β. Encontramos también que la inhibición de la vía Wnt/β-catenina con Dkk-1 no muestra efecto en la fosforilación de tau en ninguno de los sitios evaluados en rebanadas hipocampales de ratas jóvenes. Pero el Dkk-1 incrementa la fosforilación de tau en Ser199/202, Ser396/404 en ratas viejas. De manera interesante encontramos también un incremento en la fosfo-Ser214. En este modelo de rebanadas hipocampales encontramos también que la inhibición de la vía Wnt reduce la actividad y la metilación de la fosfatasa PP2A en ratas viejas pero no en ratas jóvenes. Estos resultados sugieren que en individuos jóvenes existe un balance óptimo entre la actividad de cinasas/fosfatasas capaz de contrarrestar los efectos de la activación de GSK3β sobre la fosforilación de tau. Parece ser que en el envejecimiento, este balance se encuentra afectado y la disminución de la actividad de la PP2A es un factor crítico para incrementar la fosforilación de tau en una situación de inhibición de la vía canónica Wnt.

ABSTRACT

Alzheimer´s disease (AD) is the most common cause of dementia and aging is the main risk factor. AD is histopathologically characterized by the presence of amyloid plaques composed of amyloid β peptide and neurofibrillary tangles comprised of aggregates of tau. Tau protein promotes assembly and stabilizes microtubules. It is predominantly regulated by phosphorylation; hyperphosphorylation decreases tau affinity for microtubule and assembly. Various kinases and phosphatases regulate tau phosphorylation. GSK3β and protein phosphatase 2A (PP2A) are key in tau regulation, evidence suggest the involvement of both enzymes in pathological phosphorylation and aggregation in AD. Activation of Wnt/β-catenin signaling pathway inhibits GSK3β activity. Wnt antagonist, Dkk-1 has been found increased in transgenic models and AD patients, showing that Wnt signaling alteration could contribute to AD pathology. In the present work, employing a model of metabolically active hippocampal slices, we found that basal GSK3β activity inhibition decreases tau phosphorylation in phospho-epitopes Ser199/202/396 and 404 in young and aged rats. Suggesting that phosphorylation on these sites is strongly dependent of GSK3β basal activity. In contrast phospho-epitope Ser214 remained unchanged after GSK3β inhibition. We also found that Wnt/β-catenin inhibition showed no effect on tau phosphorylation in young rats. Interestingly, Dkk-1 increased tau phosphorylation in all analyzed phospho-epitopes, including phospho-Ser214. We also found that Wnt signaling inhibition decrease methylation and PP2A activity in aged rats. Thus, our results suggest that in young individuals the optimal balance between kinases and phosphatases activity is able to counteract the effects of GSK3β activation on tau phosphorylation. During aging, this balance may be affected, and the decrease in PP2A activity is a critical factor for increasing tau phosphorylation induced by canonical Wnt signaling inhibition

ABREVIATURAS

Aβ	Péptido β amiloide
ACSF	Líquido cefalorraquídeo artificial
ApoE	Apolipoproteina E
APP	Proteína precursora del amiloide
CAMKII	Calcio calmodulina cinasa II
CDK5	Cinasa dependiente de ciclina 5
CK-1	Caseina cinasa 1
Dkk-1	Dickkopf-1
Dvl	Proteína adaptadora Dishevelled
EA	Enfermedad de Alzheimer
EAE	Enfermedad de Alzheimer esporárido
EAF	Enfermedad de Alzheimer familiar
FTDP-17	Demencia frontotemporal con parkinsonismo asociado al cromosoma 17
GSK3	Cinasa glucógeno sintasa 3
LCMT-1	Leucina carboxil metiltransferasa 1
NFT	Marañas neurofibrilares
PHF	Filamentos helicoidales pareados
PKA	Proteína cinasa A
PME-1	PP2A metilesterasa
PP1	Proteína fosfatasa 1
PP2A	Proteína fosfatasa 2A
PP2Ac	Subunidad catalítica de PP2A
PS-1	Presenilina 1
PS-2	Presenilina 2
6-BIO	(2′Z,3′E)-6-Bromoindirubin-3′ -oxime

INTRODUCCION

ENFERMEDAD DE ALZHEIMER

La enfermedad de Alzheimer (EA) es la forma mas común de demencia y, aunque su etiología aún se desconoce, el envejecimiento es el principal factor de riesgo. De hecho, el aumento de la expectativa de vida ha incrementado la incidencia de esta enfermedad (Kepe 2006). La EA se caracteriza por una pérdida progresiva de las funciones cognitivas, muerte neuronal, placas amiloideas (placas seniles) y marañas neurofibrilares (NFT), (por sus siglas en inglés). La EA muestra un daño gradual principalmente en el hipocampo y la neocorteza, las áreas del cerebro implicadas en procesos cognitivos y almacenamiento de la memoria (Mota et al., 2014). A pesar de los esfuerzos que se han hecho para encontrar un tratamiento adecuado, hasta el momento no existe cura.
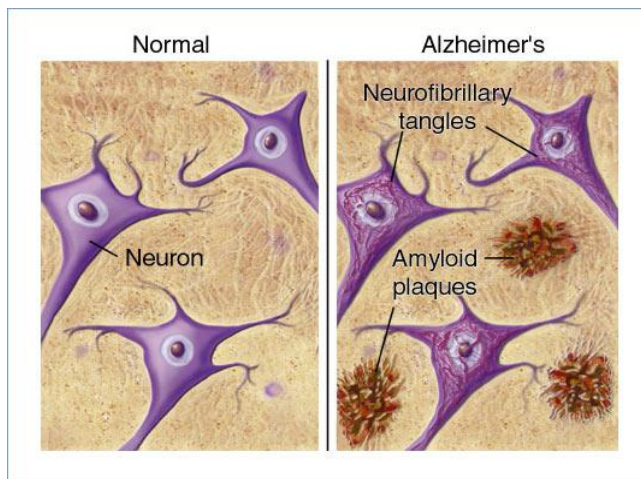
Figura 1. Esquema de las placas amiloideas y marañas neurofibrilares presentes en cerebros de pacientes con EA. (Tomado de Alzheimer's Disease Research, a program of the American Health Assistance Foundation.).

Los componentes principales de las placas amiloideas son agregados fibrilares del péptido β amiloide (Aβ) (Selkoe et al., 1994). El Aβ se forma a partir de cortes en la proteína precursora del amiloide (APP), que se sabe son llevados a cabo por enzimas llamadas β y γ secretasas, en la vía conocida como amiloidogénica.

Por otro lado, las marañas neurofibrilares provienen del ensamble anómalo de una proteína del citoesqueleto encargada de mantener polimerizados los microtúbulos axonales. Esta proteína se conoce como tau y en condiciones patológicas se agrega formando filamentos helicoidales pareados (PHF) y filamentos rectos. Estos filamentos

se acumulan en el interior de las neuronas (Iqbal y Grundke-Iqbal, 2008) y ambos tipos están compuestos predominantemente de proteína tau anormalmente hiperfosforilada (Goedert et al., 1988; Kondo et al., 1988; Lee et al., 1991). El patrón de localización de las marañas neurofibrilares y las placas amiloideas en la neocorteza depende de la severidad de la enfermedad. Ambos marcadores histopatológicos tienen un patrón de distribución y una temporalidad diferente. La progresión de la patología histopatológica se ha descrito ampliamente por Braak y Braak, la cual se caracteriza por seis etapas (Braak y Braak 1991). En la etapa I y II los cambios pueden no presentarse en manifestación clínica o puede existir un daño cognoscitivo leve, con presencia de marañas neurofibrilares limitadas a regiones de la corteza transentorrinal e hipocampo. El examen de tejidos de cerebro en las etapas I y II han sido utilizadas para intentar definir cambios patológicos tempranos en la proteína tau. Los casos en las etapas III y IV revelan poca destrucción en corteza cerebral, sin atrofia macroscópica detectable. En etapas avanzadas V y VI de la enfermedad, cuando se puede hacer un diagnostico más preciso de EA, hay un gran número de marañas neurofibrilares y hebras de neurópilo en prácticamente todas las subdivisones de la corteza cerebral. Una característica principal en estas etapas es la severa destrucción de las áreas de asociación neocorticales. Estas etapas corresponden con el criterio utilizado para la confirmación neuropatológica en la diagnosis clínica de la enfermedad (Braak y Braak 1991; Kepe et al., 2006). Estudios clínicos han demostrado una correlación entre el grado de demencia y la presencia de marañas neurofibrilares, pero no hay correlación clara con la presencia de depósitos amiloideos (Braak y Braak 1991; Giannakopoulos et al., 2003) pudiera ser que exista una correlación con los oligómeros de Aβ. La acumulación extracelular de placas amiloideas en ausencia de marañas neurofibrilares no produce la sintomatología completa de la EA. En algunos adultos mayores sin EA, se han detectado aproximadamente la misma cantidad de depósitos de Aβ en el cerebro, que pacientes con EA (Dickson et al., 1992). Es por eso que entender los mecanismos que producen los cambios bioquímicos de la proteína tau que conllevan a la formación de las marañas neurofibrilares, es un tema prioritario.

La EA es un padecimiento multifactorial y heterogéneo, se han descrito dos formas. La EA de inicio temprano (generalmete antes de los 65 años) se conoce como enfermedad de Alzheimer Familiar (EAF). La EAF se ha asociado a mutaciones en los genes del APP, presenilina 1 (PS-1) y presenilina 2 (PS-2). Las mutaciones en estos genes

afectan el procesamiento del APP, incrementando la producción de Aβ insoluble y su acumulación en placas (Hooper et al., 2008), sin embargo estas mutaciones se encuentran en menos del 1% de los casos de EA. En el tipo esporárido de EA (EAE), el envejecimiento es el principal factor de riesgo (Kepe et al., 2006), ya que se presenta posterior a los 65 años y da cuenta de más del 99% de los casos. El gen que codifica para la apolipoproteina E (ApoE) se ha asociado como factor de riesgo para la EAE, individuos con el genotipo ApoE4 tienen un riesgo mayor de padecer la EAE (Strittmatter et al., 1993).

TAU

Tau es una proteína asociada a microtúbulos, se expresa en todo el sistema nervioso central. En neuronas, tau se localiza predominantemente en axones, sin embargo también se ha encontrado en dendritas pero en niveles menores (Ittner et al., 2010). La función principal de tau es modular la estabilidad y el ensamblaje de los microtúbulos (Iqbal et al., 1994). También regula el transporte axonal de organelos celulares, incluyendo la mitocondria y el retículo endoplásmico (Ebneth et al., 1998).

La actividad de tau se regula principalmente por el grado de fosforilación. En su estado normal tiene entre 2 y 3 fosfatos por proteína, este es el grado de fosforilación para el funcionamiento correcto y su unión a microtúbulos. La hiperfosforilación baja la capacidad de ensamble y su unión a microtúbulos (Iqbal et al., 2005). En cerebros de pacientes de EA y adultos mayores de edad similar, existe la misma cantidad de tau, pero en los cerebros de los que padecen EA, tau se encuentra de 4 a 8 veces mas fosforilada (Khatoon et al., 1994).

La proteína tau esta codificada por un solo gen, el cual se localiza en 17q21 (Neve et al., 1986). En humanos existen 6 isoformas producidas por splicing alternativo (Goedert et al., 1989). Las isoformas de tau tienen entre 352 y 441 aminoácidos, difieren entre si por la presencia de 3 (3R) o 4 (4R) repeticiones en el extremo carboxilo terminal y por la presencia o ausencia de uno o dos insertos ácidos (en el extremo amino terminal). El peso molecular se encuentra entre 48 y 68 kDa (Hasegawa 2006). Las repeticiones 3R y 4R localizadas en la región C-terminal, son las regiones de unión a microtúbulos (Evans et al., 2000). Las isoformas 3R se unen a los microtúbulos con menor afinidad que las isoformas 4R, ya que estas tienen un sitio mas de unión (Dayanandan et al., 1999).

Las isoformas de tau están reguladas durante el desarrollo. En mamíferos, en etapas fetales solo se expresa la isoforma mas pequeña, en organismos adultos se expresan todas las isoformas (Hasegawa 2006).
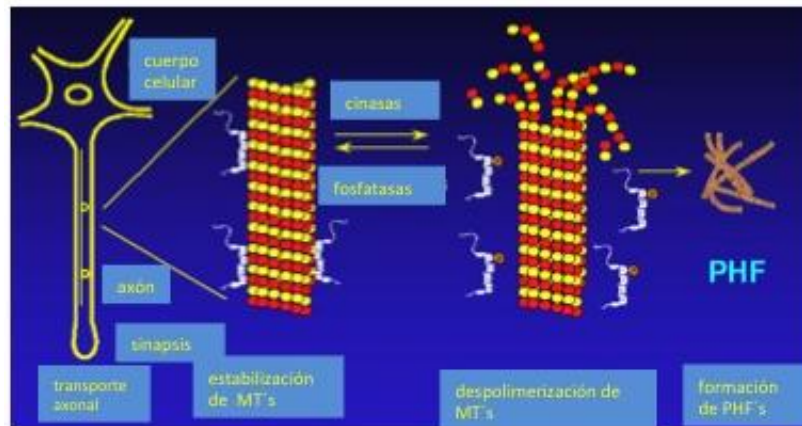
Figura 2. Esquema del funcionamiento de la proteína asociada a microtúbulos tau. En estado desfosforilado tau se une a los microtúbulos y promueve su ensamblaje. Cuando las proteínas cinasas fosforilan a tau, esta se desensambla de los microtúbulos. La hiperfosforilación provoca la agregación de tau en el citosol y la formación de PHF.

Además de ser regulada por fosforilación, la proteína tau puede sufrir modificaciones post-traduccionales como glicosilación, glicación, truncación, nitración, poliaminación, ubiquitinación, sumoilación, oxidación, etc. (Martin et al., 2011).

La patología de tau, no solo se presenta en la EA, también se observa en otra familia de enfermedades neurodegenerativas, conocidas como taupatías. Entre ellas se encuentran la parálisis supranuclear progresiva, degeneración corticobasal, enfermedad de Pick, demencia frontotemporal con parkinsonismo asociado al cromosoma 17 (FTDP-17) (Lee y Leugers 2012). Todas estas enfermedades presentan la agregación de la proteína tau hiperfosforilada, cambios en la conducta, demencia, anormalidades de lenguaje y disfunción motora (Ittner et al., 2011). En la FTDP-17 existen mutaciones en el gen de tau, lo cual muestra el papel crucial de esta proteína en el desarrollo de ciertas enfermedades neurodegenerativas (Hutton et al., 1998).
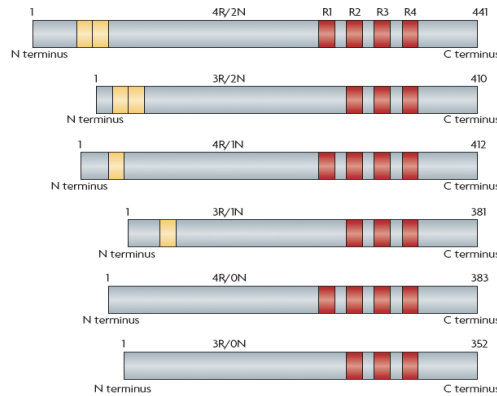
Figura 3. Las 6 isoformas de la proteína tau en humanos. En amarillo se muestras los insertos ácidos en el extremo N-terminal y en rojo se observan los sitios de unión a microtúbulos (Ballatore et al., 2007).

En la EA, parece ser que la hiperfosforilación de tau precede a su acumulación en las neuronas afectadas. Aproximadamente el 40% de la proteína tau hiperfosforilada se encuentra en el citosol no polimerizada en marañas neurofibrilares (Iqbal et al., 2005). La proteína tau polimerizada en marañas neurofibrilares no se une a la tubulina ni promueve el ensamblaje de microtúbulos, lo que finalmente afecta la morfología del axón y el transporte axonal. Por otro lado la proteína tau hiperfosforilada que se encuentra libre en el citosol, tampoco es capaz de unirse a la tubulina ni promover el ensamblaje de microtúbulos, también inhibe el ensamblaje y rompe las red de microtúbulos (Iqbal y Grundke-Iqbal 2008). La proteína tau hiperfosforilada secuestra a la tau que se encuentra en estado normal, también secuestra a otras proteínas neuronales de unión a microtúbulos como MAP1A/B y MAP2 (Alonso et al., 1994). El efecto tóxico de tau parece deberse en gran parte a la hiperfosforilación, ya que la desfosforilación la regresa a su estado no patológico (Alonso et al., 1994). Por esta razón la hiperfosforilación ha recibido especial atención. Tau es fosforilada en más de 80 residuos de Ser/Thr (Morishima-Kawashima et al., 1995; Hanger et al., 1998) y es sustrato de diferentes cinasas (Wang et al., 2007). Algunas de las cinasas que participan en la hiperfosforilación anormal de la proteína son: la GSK3 (cinasa glucógeno sintasa 3), cdk5 (cinasa dependiente de ciclina 5), CK-1 (caseina cinasa 1), PKA (proteína cinasa A), CAMKII (calcio calmodulina cinasa II), ERK 1/2 entre otras (Iqbal y Grundke-Iqbal 2008).

El mecanismo molecular por el cual se lleva a cabo la fosforilación de tau no se conoce completamente. La fosforilación de tau es catalizada por proteínas cinasas y revertida

por proteínas fosfatasas. De hecho, se ha propuesto que un desequilibrio entre la acción de diversas cinasas y fosfatasas subyace a los cambios bioquímicos en tau.

La cinasa GSK3β es una de las más estudiadas ya que fosforila un gran número de residuos en el proceso de hiperfosforilación de tau. De igual manera la proteína fosfatasa 2A (PP2A) es una de las principales en la desfosforilación de tau.

GSK3

La cinasa glucógeno sintasa 3 (GSK3) es una cinasa de serina/treonina, que fue descrita inicialmente como la enzima capaz de fosforilar e inactivar a la glucógeno sintasa. Actualmente se ha descrito su participación en diferentes procesos celulares como proliferación, diferenciación, dinámica de microtúbulos, ciclo celular, función neuronal, señalización de insulina, apoptosis, y desarrollo embrionario (Frame y Cohen 2001; Rayasam et al., 2009).
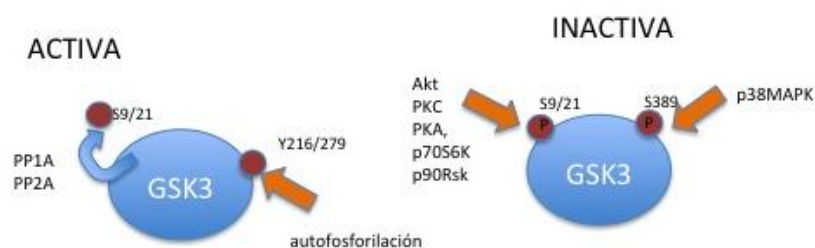
Figura 4. Esquema de GSK3α/β , sitios donde se modula y algunas de las proteínas involucradas .

La GSK3 está presente en todo el reino animal (Plyte et al., 1992), se expresa en todos los tejidos, y particularmente en el cerebro existen cantidades elevadas de ambas isoformas (Woodgett, 1990). GSK3 está constitutivamente activa y se inactiva en respuesta a señales celulares (Doble y Woodgett 2003). Existen dos isoformas codificadas por genes diferentes: GSK3α (51kDa) y GSK3β (47kDa) (Woodgett 1990). GSK3α y GSK3β son idénticas en un 98% en el sitio de unión del ATP, pero difieren en los dominios amino y carboxilo terminal (Woodgett 1990).

En 2002 se identificó una variante de GSK3β, -GSK3β2-, la cual se expresa exclusivamente en el sistema nervioso (Mukai et al., 2002). El mecanismo de acción de esta variante aun no está bien caracterizado. La actividad de GSK3 es regulada por diversos estímulos y vías de señalización. Los mecanismos de regulación pueden ser clasificados en:

- Regulación por fosforilación:

La actividad de GSK3 se reduce significativamente por la fosforilación en el extremo N-terminal de la serina 9 en GSK3β y serina 21 en GSK3α. Estas fosforilaciones en serina inhiben a GSK3 ya que generan una conformación de pseudosustrato en el sitio de unión al sustrato. Se han descrito múltiples cinasas capaces de fosforilar la Ser9/21 de GSK3, como Akt, PKC, PKA, p70S6K, p90Rsk entre otras (Jope y Johnson 2004). Una de las vías que regula de esta manera a GSK3 es la de insulina, mediante la activación de la cinasa Akt, esta fosforila directamente la Ser9/21 (Cross et al., 1995). También se han descrito otros 2 sitios regulatorios en GSK3β. La Thr43 que probablemente sea modificada por la cinasa ERK (Ding et al., 2005), esta fosforilación correlaciona con la modulación a la baja de GSK3β. La Ser389 y la Thr390 de GSK3β son fosforiladas por p38MAPK (Thornton et al., 2008), se sugiere que la fosforilación en ambos residuos hace mas suceptible a la Ser9 de ser fosforilada pero no promueve una inhibición directa (Medina et al., 2011).

Por otro lado la fosforilación en las tirosinas 279 de GSK3α y 216 de GSK3β parecen correlacionar con el incremento en la actividad de cinasa (Hughes et al., 1993). Posiblemente esta fosforilación la lleven a cabo otras cinasas de tirosina (Hartigan et al., 2001) y/o mediante autofosforilación (Cole et al., 2004). La proteína fosfatasa 1 (PP1) y la proteína fosfatasa 2A (PP2A), pueden desfosforilar la Ser9/21 y provocar la activación de GSK3.

- Regulación por asociación con complejos proteícos

Uno de los mecanismos de regulación de GSK3 es su interacción con proteínas. Un ejemplo es que GSK3 forma parte de las proteínas de la vía Wnt, descrita a detalle mas adelante.

Otro ejemplo es la interacción de GSK3 con la proteína FRAT, por un mecanismo que no se conoce por completo. Parece ser que la asociación de GSK3 con FRAT1 actua de manera inhibitoria (Yost et al., 1998), mientras que la asociación con FRAT2 incrementa la fosforilación mediada por GSK3 en algunos residuos (Stoothoff et al., 2005).

- Regulación por pre-fosforilación/especificidad de sustrato

Algunos de los sustratos de GSK3 no requieren una secuencia de aminoácidos muy específica, pero necesitan una fosforilación previa llevada a cabo por otra cinasa en un residuo de Ser/Thr ubicado 4 aminoacidos C-terminal a la Ser o Thr que será

modificada por GSK3 (Jope y Johnson 2004). Esta pre-fosforilación facilita la unión del grupo fosfato a la proteína blanco.

- Regulación por localización subcelular

GSK3 se encuentra principalmente en el citosol, pero también está presente en núcleo y mitocondria (Bijur y Jope 2003).

- Regulación por cortes proteolíticos

Recientemente se ha propuesto un nuevo mecanismo de regulación de GSK3. Esta consiste en el corte de un fragmento de la región N-terminal de GSK3 (incluyendo la Ser9/21) , este corte es llevado a cabo por la calpaina. La eliminación de este fragmento, provoca la activación de GSK3 (Goñi-Oliver et al., 2007).

Lo anterior muestra que GSK3 es una cinasa compleja y con diferentes mecanismos de regulación. La desregulación de GSK3 se ha asociado a su vez a patologías como trastornos afectivos, diabetes, algunos tipos de cáncer y enfermedades neurodegenerativas como la EA (Rayasam et al., 2009).

La proteína tau tiene 85 sitios fosforilables (45 serinas, 35 treoninas y 5 tirosinas). El cerebro de pacientes con EA presenta 40 sitios de fosforilación y GSK3 puede fosforilar 23 de ellos (Hanger et al., 2009).

Se ha demostrado en modelos de ratón que la sobreexpresión de GSK3β induce neurodegeneración. En este modelo también se ha demostrado que GSK3β fosforila a tau en sitios relacionados a la EA (Lucas et al., 2001; Engel et al., 2006; Plattner et al., 2006).

En modelos transgénicos de *Drosophila*, la sobre-expresión de tau y GSK3 incrementan la hiperfosforilación de tau e induce neurodegeneración (Jackson et al., 2002).

Estudios en ratones transgénicos que sobre expresan tau humana, muestran que la inhibición de GSK3β reduce la fosforilación y la agregación de tau (Pérez et al., 2003; Noble at al., 2005). De igual manera la hiperfosforilación de tau y la neurodegeneración producida por la sobre-expresión de GSK3β incrementa si se sobreexpresa a su vez tau con mutaciones propias de la FTDP-17. Este modelo sugiere que se podría prevenir la progresión de la taupatía inhibiendo GSK3β en etapas tempranas (Engel et al., 2006).

Se ha demostrado que tau contribuye a la neurodegeneración hipocampal inducida por la sobre-expresión de GSK3β, en un modelo de ratón knock-out para tau (Gómez de Barreda et al., 2010). También en modelos transgénicos se ha observado que inhibidores de GSK3β decrementan la fosforilación de tau y los depósitos de amiloide (Serenó et

al., 2009).

En cerebros de pacientes se ha demostrado que GSK3β colocaliza con las NFT (Yamaguchi et al., 1996). De igual manera Pei y colaboradores demostraron que la GSK3β activa está presente en el citoplasma cuando la proteína tau hiperfosforilada se empieza a acumular (Pei et al., 1999).

También estudios recientes han reportado que polimorfismos en el promotor y el gen de GSK3β son factores de riesgo para la EAE (Mateo et al., 2006; Schaffer et al., 2008).

La inhibición de GSK3β por sales de litio reduce la fosforilación de tau y afecta la estabilidad de los microtúbulos (Mercado-Gómez et al., 2008). Aún cuando existe amplia evidencia de la participación de esta cinasa en la hiperfosforilación de tau, el mecanismo (posibles alteraciones en vías de señalización como Wnt, ver más adelante), por el cual esta cinasa se encuentra desregulada, está aún por elucidarse.

Además de estar involucrada en la hiperfosforilación de tau en la EA, la actividad de GSK3 se ha encontrado asociada en procesos como el déficit cognitivo, la producción de Aβ, la respuesta inflamatoria y la reducción en la síntesis de acetilcolina (Hooper et al., 2008).

PP2A

La proteína fosfatasa 2A constituye una familia de fosfatasas de serina/treonina con diversas funciones en señalización celular. Las holoenzimas PP2A son heterotrímeros (fig. 5) compuestos de una subunidad C catalítica (Cα y Cβ), una subunidad estructural (Aα y Aβ), y una subunidad regulatoria B, la cual pertenece a una de las 4 diferentes familias: B, B', B'' o B''' (Sents et al., 2013; Sontag y Sontag 2014). Parece ser que la variabilidad de las subunidades B influye en la especificidad del sustrato y en la localización subcelular. Se estima que la combinación de subunidades puede generar mas de 100 diferentes holoenzimas triméricas (Kowluru y Matti 2012). Las subunidades A y C son ubicuas, pero algunas subunidades B son tejido específicas (Kowluru y Matti 2012).

La subunidad catalítica de PP2A (PP2Ac) sufre diversas modificaciones post-traduccionales que regulan su activación. Estas modificaciones incluyen la metilación, la fosforilación y la nitración.

PP2Ac puede ser regulada por carboximetilación en la leucina 309, esta metilación es reversible y es catalizada por la leucina carboxil metiltransferasa 1 (LCMT-1) (De Baere et al., 1999) y la PP2A metilesterasa (PME-1) (Lee et al., 1996). PME-1 se une

directamente al sitio activo de PP2Ac, removiendo el grupo metilo e inactivando a la PP2A. La metilación de la Leu-309 incrementa la afinidad de PP2A por algunas subunidades regulatorias, por lo tanto puede regular la actividad y la especificidad de PP2A (Shi 2009).
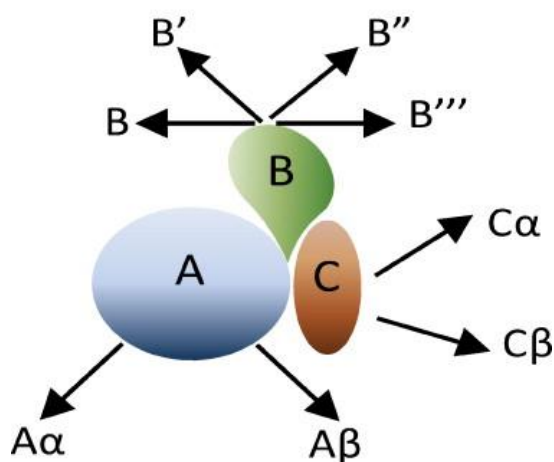
Figura 5. PP2A. Composición de subunidades y las variantes que existen en cada una.

PP2Ac también puede fosforilarse en la tirosina 307. A diferencia de la metilación en la Leu-309 que parece facilitar el ensamblaje de la holoenzima y la activación de PP2A, la fosforilación en la Tyr-307 inhibe la función catalítica de PP2A (Chen et al., 1992).

La fosforilación en la Tyr-307 se cree que puede interferir en la interacción con algunas subunidades B´ (Sents et al., 2013). La activación de GSK3 estimula la la fosforilación inhibitoria de la Tyr-307, mientras que la inhibición de GSK3 decrementa dicha fosforilación (Yao et al., 2011)

PP2A a su vez también es regulada por la unión a inhibidores endógenos como I_1^{PP2A} e I_2^{PP2A} (Li y Damuni 1998).

PP2A es una de las principales fosfatasas en el cerebro, en un cerebro sano la actividad de PP2A es aproximadamente 71% comparada con otras fosfatasas como PP2B ($\approx$7) y PP5 ($\approx$11). En la EA la actividad de PP2A se reduce en un 50% (Liu et al., 2005).

En pacientes con EA se ha encontrado un incremento del 20% en los niveles de los inhibidores I_1^{PP2A} e I_2^{PP2A} (Martin et al., 2013). También se ha reportado incremento en los niveles de mRNA de ambos inhibidores en la neocorteza y su colocalización con PP2A y con tau hiperfosforilada (Tanimukai et al., 2005).

Se ha reportado que en cerebros de pacientes con EA hay un decremento en los niveles

de mRNA de PP2A (Vogelsberg-Ragaglia et al., 2001). También se ha visto un incremento en la fosforilación en la Tyr-307 (Liu et al., 2008) y un decremento de la metilación en la Leu-309 en cerebros de pacientes de EA (Songtag et al., 2004). Se ha sugerido que la modulación negativa de las fosfatasa contribuye a la hiperfosforilación de la proteína tau y su agregación (Martin et al., 2013). PP2A contribuye aproximadamente en un 70% del total de la actividad de desfosforilación de tau (Liu et al., 2005).

VIA WNT/ β-catenina

Las Wnts son una familia de glicoproteínas palmitoiladas secretadas (Logan y Nusse 2004), son parte fundamental de procesos como proliferación celular, mantenimiento de células troncales, determinación el destino celular en la embriogénesis y la homeostasis tisular (Clevers y Nusse 2012). En humanos son similares en tamaño y van desde los 39kDa a los 46kDa (Miller 2002).

Se han descrito dos vías de señalización activadas por ligandos Wnt: la vía canónica también conocida como vía Wnt/β-catenina y la vía no canónica o independiente de β-catenina. La vía canónica de Wnt es la mejor caracterizada. Los ligandos Wnt1, Wnt3a y Wnt8 frecuentemente transducen a través de esta vía.

En ausencia de ligando Wnt, la proteína β-catenina es degradada en el citoplasma por la acción de un complejo multiproteíco compuesto por la Axina, APC, CK1 y GSK3 (MacDonald et al., 2009). CK1 fosforila en la Ser45, y posteriormente GSK3 fosforila en Ser33/37/41 de β-catenina (fig. 6). La fosfo- β-catenina es reconocida por la E3 ubiquitin ligasa, β-Trcp y degradada vía proteasoma. (Barker 2008; MacDonald et al., 2009).

Para encender la vía de señalización canónica, es necesario que los ligandos Wnt se unan a los receptores Frizzled (FZ) de 7 dominios transmembranales y a su co-receptor LRP5/6 (fig. 6). El complejo Wnt-Fz-LRP5/6 induce la fosforilación y el reclutamiento en la membrana de la proteína adaptadora Dishevelled (Dvl), lo cual a su vez recluta a la axina y como consecuencia se desensambla el complejo de degradación de β-catenina, lo que permite su acumulación en el citoplasma y su entrada al núcleo, donde se une a los factores de transcripción de la familia LEF/TCF y de esta manera se activa la expresión de genes Wnt (Logan y Nusse 2004). A pesar de que el mecanismo por el cual la vía Wnt inhibe a GSK3 no se conoce totalmente, se ha reportado que el

encendido de la señalización Wnt hace que GSK3 sea secuestrada en vesículas, previniendo así su interacción con sustratos citoplásmicos (Taelman et al., 2010).
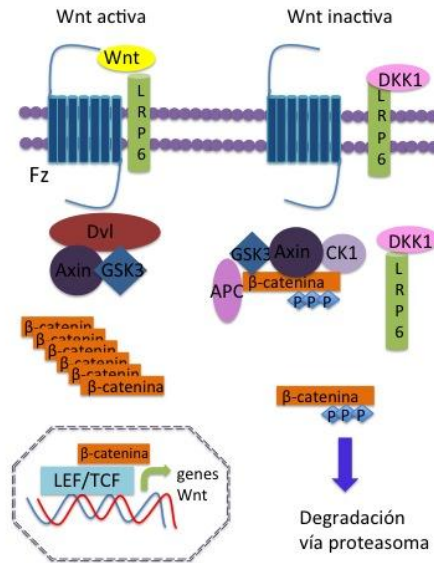
Figura 6. Vía WNT/β catenina. En presencia del ligando Wnt hay una inhibicón de GSK3 y esto permite acumulación de β-catenina en el citoplasma, su translocación al núcleo y transcripción de genes Wnt. En ausencia de ligando Wnt, CK1 y GSK3 fosforilan a β-catenina y la marcan para degradación vía proteasoma.

Existen varios moduladores de la vía Wnt canónica, uno de los mejor caracterizados es Dickkopf-1 (Dkk-1). Dkk-1 es una glicoproteína, con un peso molecular de entre 24 y 29kDa, que inhibe específicamente la vía de señalización Wnt/β-catenina (Bafico et al., 2001). Dkk-1 se une al co-receptor LRP5/6 y lo internaliza (fig. 6), impidiendo el encendido de la vía canónica (Niehrs 2006). En desarrollo es indispensable para la formación de la cabeza y extremidades en ratón.

La señalización Wnt juega un papel importante es procesos como diferenciación celular, migración y actividad sináptica. Los ligandos Wnt participan en el desarrollo del sistema nervioso, se han relacionado al proceso de sinaptogénesis, neurogénesis (Varela-Nallar e Inestrosa 2013), y en la modulación de la plasticidad sináptica (Inestrosa y Arenas 2010). También las vías Wnt se relacionan con padecimientos como cáncer, enfermedades cardiovasculares, enfermedad de Parkinson, EA, entre otras (Inestrosa y Varela-Nallar 2014).

La expresión de Wnts en el cerebro adulto, su papel en la modulación de la neurogénesis y la plasticidad sináptica sugieren que la señalización Wnt juega un papel importante en el mantenimiento y la protección de conexiones neurales a lo largo de la

vida (revisado por Ortiz-Matamoros et al., 2013).

Estudios recientes sugieren que la vía Wnt/β-catenina participa en la formación de la memoria en adultos (Maguschak y Ressler 2008, 2011), mientras que la desregulación de esta vía se ha relacionado con la EA, la cual se asocia con la pérdida de la memoria.

Se ha demostrado que la activación de la vía Wnt inhibe GSK3β y tiene un efecto neuroprotector en cultivos de neuronas hipocampales y en modelos transgénicos de la EA (Oliva et al., 2013). En cerebros de pacientes con la EA familiar se ha reportado un decremento en los niveles de β-catenina (Zhang et al., 1998). Otra evidencia de la participación de la vía canónica de Wnt en la EA es que se ha identificado al co-receptor LRP6 como un gen de riesgo (De Ferrari et al., 2007; Alarcón et al. 2013). El modulador negativo de la vía Wnt/β-catenina, Dkk-1 incrementa sus niveles en cerebros de pacientes con EA y se ha encontrado colocalizando con NFTs y con neuritas distróficas (Caricasole et al., 2004). También se ha reportado un incremento en los niveles de Dkk-1 en modelos transgénicos de EA y FTD (Rosi et al., 2010).

PLANTEAMIENTO DEL PROBLEMA

Recientemente la vía WNT se ha asociado con algunas enfermedades neurodegenerativas, entre ellas la EA. A pesar de que ya se han descrito modificaciones en la vía de señalización WNT asociadas al envejecimiento, se desconoce cómo estas modificaciones se asocian a la hiperfosforilación de tau y si es a partir de la activación de GSK3β. La mayoría de los estudios se han llevado a cabo en cerebros post-mortem, en modelos de cultivos celulares y en modelos transgénicos donde no se ha evaluado el impacto del envejecimiento en la activación de GSK3β mediada por cambios en la señalización WNT. En el presente proyecto, empleando un modelo *ex vivo* de rebanadas hipocampales metabólicamente activas, estudiamos la fosforilación de tau en residuos que se encuentran fosforilados en la EA, activando o inhibiendo la vía Wnt en ratas jóvenes y viejas y analizamos el mecanismo involucrado.

HIPÓTESIS

Durante el envejecimiento ocurren modificaciones en la vía de señalización WNT que pueden modular positivamente a la cinasa GSK3β e inducir la fosforilación de tau en residuos relacionados con la EA.

OBJETIVO

Estudiar la modulación de la vía Wnt y los cambios en la fosforilación de tau en el hipocampo de ratas jóvenes y viejas.

OBJETIVOS PARTICULARES

1.-Estudiar el efecto del inhibidor de GSK3β (6-BIO) en la fosforilación de la proteína tau (PHF-1, p-Ser199/202, p-Ser214) en rebanadas hipocampales de ratas jóvenes y viejas.

2.-Estudiar el efecto de 6-BIO en componentes de la vía Wnt/β-catenina (GSK3β p-Ser9, GSK3 α/β p-Tyr279/216, β-catenina: Ser33/37/Thr41 y p-Ser33/37/Thr41).

3.-Estudiar el efecto del inhibidor de la vía Wnt (Dkk-1) en la fosforilación de tau (PHF-1, p-Ser199/202, p-Ser214) en rebanadas de hipocampo en diferentes edades de la rata (3 y 24 meses).

4.- Estudiar el efecto de Dkk-1 sobre la fosforilación de GSK3β: (GSK3β p-Ser9, GSK3 α/β p-Tyr279/216, y β-catenina: Ser33/37/Thr41 y p-Ser33/37/Thr41).

5.- Estudiar mediante un ensayo de actividad el efecto de Dkk-1 sobre el nivel de actividad de la fosfatasa PP2A.

6.- Evaluar si la inhibición de la vía canónica de Wnt modifica la cantidad total de la subunidad catalítica de PP2A, la metilación en la leucina 309 y la fosforilación en tirosina 307 (análisis por Western-blot del nivel de PP2A total, PP2A pY307, metil-PP2A leu309).

MATERIALES Y MÉTODOS

- Obtención de rebanadas

Se utilizaron ratas Wistar machos (3 y 18-24 meses). Se anestesia a las ratas con pentobarbital sódico. Se decapita y se saca el cerebro, se deja sumergido en líquido cefalorraquídeo artificial (ACSF) [126mM NaCl, 3.5mM KCl, 1.2mM NaH$_2$PO$_4$, 1.3mM MgCl$_2$, 2mM CaCl$_2$, 11mM glucosa, 2mM NaHCO$_3$, pH 7.4] por 5 minutos a 4°C y se oxigena con una mezcla 95% O$_2$ y 5% CO$_2$ durante todo el procedimiento. Se fija el cerebro en la placa del vibratomo y se obtienen rebanadas de hipocampo, a partir de cortes coronales de 400µm. Las rebanadas se dejan equilibrar 1 h en ACSF a temperatura ambiente. Posteriormente se aplica el tratamiento (PBS o Dkk-1 200ng/ml; DMSO 0.07% o 6-BIO 20µM). Se incuba con el tratamiento por 3 horas, a 37°C.

Después las rebanadas se sonican en 200µl buffer de lisis [Tris-HCl 50mM pH 7.5, NaCl 150mM, Nonidet P40 1%, deoxicolato 0.5%, COMPLETE cocktail inhibidor de proteasas,y Halt cocktail inhibidor de fosfatasas (Thermo Scientific, Inc., USA)] para realizar electroforesis y Western-blot. Los homogenados se centrifugaron a 9500 xg durante 30 minutos y posteriormente almacenados a -70°C hasta su uso.

- Western Blot

De las muestras obtenidas se cargaron 25-40µg de proteína en un gel SDS-PAGE al 12%. Luego de la electroforesis, las proteínas se transfirieron a una membrana de nitrocelulosa. La membrana se bloquea en leche 5% en TBS-tween 0.1%, y suero de caballo (0.3-0.5%) por 3h a temperatura ambiente. Posteriormente se incubó la membrana con el anticuerpo primario (tabla 1) toda la noche a 4°C. Después se lavan 3 veces con TBS-T 0.1%, finalmente se incuba durante 1h con el anticuerpo secundario (anti mouse IgG acoplado a peroxidasa; anti rabbit IgG acoplado a peroxidasa, 1:7000) a temperatura ambiente, se incubó la membrana con sustrato quimioluminiscente HRP y se detectó por quimioluminiscencia en placas fotográficas Kodak XOmat films. Se utilizó actina como control de carga.

Anticuerpo	Sitio	Dilución
Tau phospho Ser199/202	p-Ser199/202	1:1000
PHF-1	p-Ser396/404	1:50
Tau phospho Ser214	p-Ser214	1:500
Anti-GSK3β (phospho-Ser9)	p-Ser9	1:1000
Anti-GSK3 (α+β) (p-Y216/279)	Tyr216/279	1:1000
Phospho-β-Catenin	p-Ser33/37/Thr41	1:250
PP2Ac	Subunidad catalítica de PP2A	1:2000
Anti-Active-β-catenin	Ser33/37/Thr41 (desfosforilada)	1:1000
Anti-PP2A alpha (phospho Y307)	p-Tyr 307	1:3000
Anti –PP2A alpha (methyl L309)	Methyl Leu-309	1:1000

Tabla 1. Anticuerpos utilizados.

- Inmunoprecipitación y ensayo de actividad de PP2A

Se utilizaron rebanadas hipocampales controles o incubadas con Dkk-1 (200ng/ml) para medir la actividad de PP2A utilizando el kit para ensayo de actividad de PP2A (Millipore). Brevemente, se homogenizaron las rebanadas hipocampales en buffer de lisis [20mM imidazole-HCl; 2mM EDTA; 2mM EGTA; pH 7.0; 10 μg/ml de aprotinina, leupeptina, pepstatina; 1mM benzamidine y 1mM PMSF], se centrifugaron a 2000 xg por 5 min a 4°C y los sobrenadantes se colectaron y se incubaron con anti-PP2A subunidad C y perlas de proteína A agarosa durante 2h a 4°C . Se lava 3 veces con TBS y una vez con buffer de ensayo Ser/Thr. Las proteínas inmunoprecipitadas se incuban con 60μl de fosfopéptido (K-R-pT-I-R-R) a 30°C en agitación lenta. Las muestras obtenidas se utilizan para en ensayo de actividad de PP2A, utilizando el buffer de verde malaquita para detección de fosfatos. Después de 15 minutos de incubación las muestras se analizan en el espectrofotómetro a 630 nm.

- Análisis de datos

Los resultados se analizaron utilizando una t de Student pareada. El análisis de las bandas obtenidas en el Western Blot se hizo utilizando el programa NIH ImageJ. Los valores de la densidad óptica de las proteínas se normalizó con el valor de la actina.

En el ensayo de actividad de PP2A, los resultados se presentan como el porcentaje de actividad relativa a la rebanada control. $p < 0.05$ se consideró estadísticamente significativo

RESULTADOS

Primero examinamos si el nivel de fosforilación de tau en los homogenados de hipocampo de ratas jóvenes y viejas era dependiente de la actividad constitutiva de GSK3β y si era sitio dependiente. Utilizamos el inhibidor específico de GSK3β, 6-BIO 20μM (fig. 7). Tanto las rebanadas hipocampales de ratas jóvenes como las de ratas viejas mostraron que la inhibición de GSK3β decrementa aproximadamente en un 50% la fosforilación respecto a la fosforilación basal en los epitopes Ser199/202 y Ser396/404 (fig. 7a, b, d, e). Sin embargo el nivel de fosforilación de la Ser214 no cambió en presencia de 6-BIO en ninguna de las edades evaluadas (fig. 7c, f). Estos resultados indican que los residuos Ser199/202 y Ser396/404 dependen de la actividad constitutiva de GSK3.
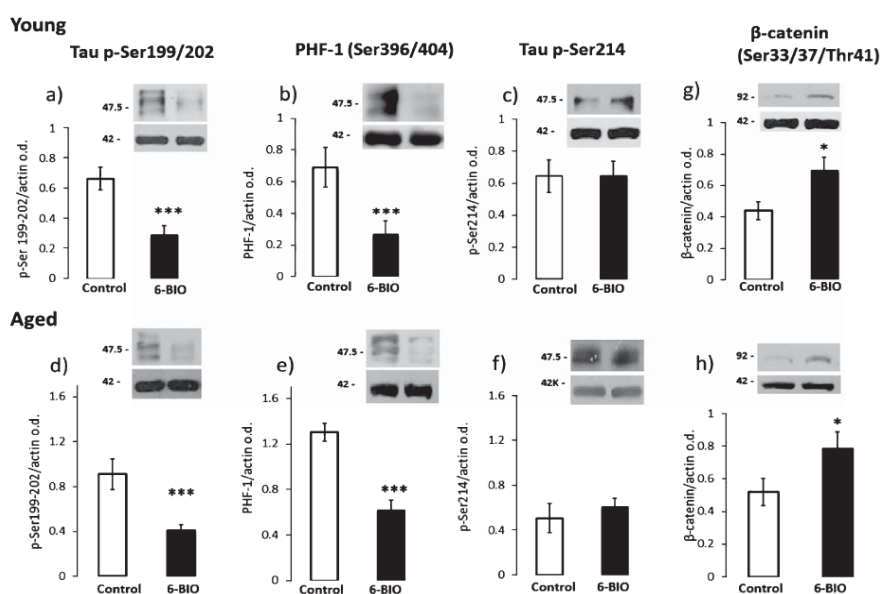
Figura 7. La inhibición de GSK3β decrementa la fosforilación de tau en residuos específicos en ratas jóvenes y viejas. Rebanadas hipocampales de ratas jóvenes (3meses) y viejas (24 meses) se incubaron con 6-BIO o vehiculo (0.07% DMSO) durante 3 horas. La inhibición de GSK3β baja los niveles de tau fosfo-Ser199/202 (a,d) y fosfo-Ser396/404 (PHF-1) (b,e). Por otro lado la fosfo Ser-214 permanece sin cambio en presencia del inhibidor (c,f). La cantidad de β-catenina activa (Ser33/37/41 defosforilada) incrementa con la inhibición de GSK3β (g,h). Las barras representan el análisis densitométrico y son la media ± error estándar de 7-10 experimentos independientes. *p ≤ 0.05 ***p ≤ 0.001.

También analizamos si el inhibidor de GSK3β, 6-BIO modificaba los niveles de β-catenina, ya que dicha proteína forma parte de la cascada de señalización Wnt, y su degradación está mediada por la actividad de GSK3β. De acuerdo con lo anterior, encontramos un incremento significativo en los niveles de β-catenina activa

(Ser33/37/Thr41), tanto en rebanadas de hipocampo de ratas jóvenes como en ratas viejas (fig. 7g, h). Ya que GSK3β es regulada negativamente por la vía Wnt, analizamos si el inhibidor específico de la vía canónica de Wnt, Dkk-1 modula la fosforilación de tau de manera diferencial respecto a la edad. Encontramos que la exposición de las rebanadas hipocampales de ratas jóvenes a Dkk-1 no tiene efecto significativo en los niveles de fosforilación en ninguno de los sitios analizados (fig. 8a, b, c). Sin embargo en las rebanadas de ratas viejas, se observó un incremento significativo en los niveles de fosforilación en Ser199/202 y Ser396/404 (fig. 8d, e). De manera interesante encontramos también un incremento significativo en la fosforilación de tau Ser214 (fig. 8f). No se encontró incremento en los niveles totales de proteína tau (Tau46), en ninguno de los tratamientos.
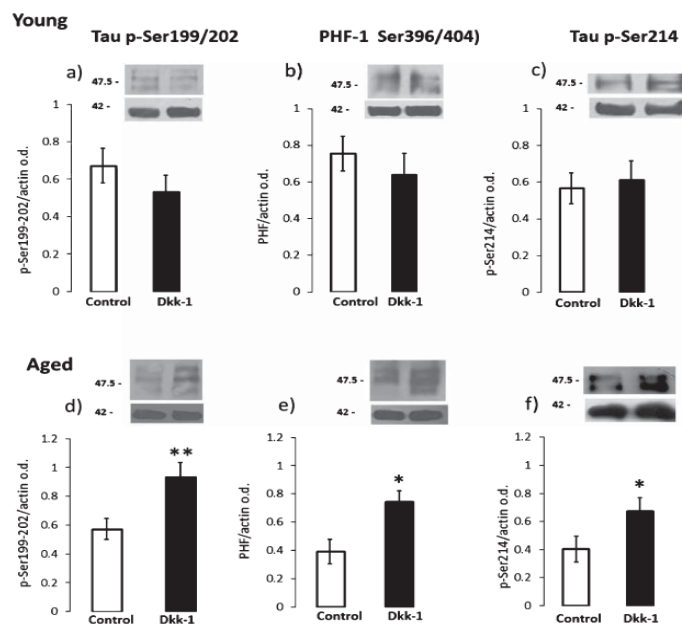
Figura 8. Efecto de Dkk-1 en la fosforilación de tau en rebanadas hipocampales de ratas jóvenes y viejas. Las rebanadas hipocampales se incubaron por 3h con el inhibidor de la vía Wnt. Dkk-1 no modificó significativamente la fosforilación de tau en ninguno de los epitopes analizados en ratas jóvenes (a,b,c). Sin embargo en hipocampos de ratas viejas, Dkk-1 incrementa la fosforilación en todos los epitopes evaluados (d,e,f). Las barras representan el análisis densitométrico y son la media ± error estándar de 7-16 experimentos independientes. $**p \leq 0.01$ $*p \leq 0.05$.

A pesar de que la regulación de GSK3β por la vía Wnt no es a través de la Ser9, evaluamos en nuestro modelo los niveles de la fosforilación inhibitoria de GSK3β en este epitope (fig. 9). Encontramos que en las rebanadas hipocampales de ratas jóvenes los niveles de GSK3β fosfo-Ser9 disminuyeron ligeramente, mientras que en las

rebanadas de ratas viejas, no encontramos una diferencia significativa (fig. 9). Para corroborar la activación de GSK3β, evaluamos la fosforilación en los residuos de β-catenina dependientes de dicha cinasa. Encontramos que el tratamiento con Dkk-1 incrementó la fosforilación de β-catenina en los residuos GSK3β dependientes (p-Ser33/37/Thr41) y consistentemente disminuyeron los niveles de β-catenina desfosforilada en estos residuos (activa) en el hipocampo de ambos grupos (fig. 9).
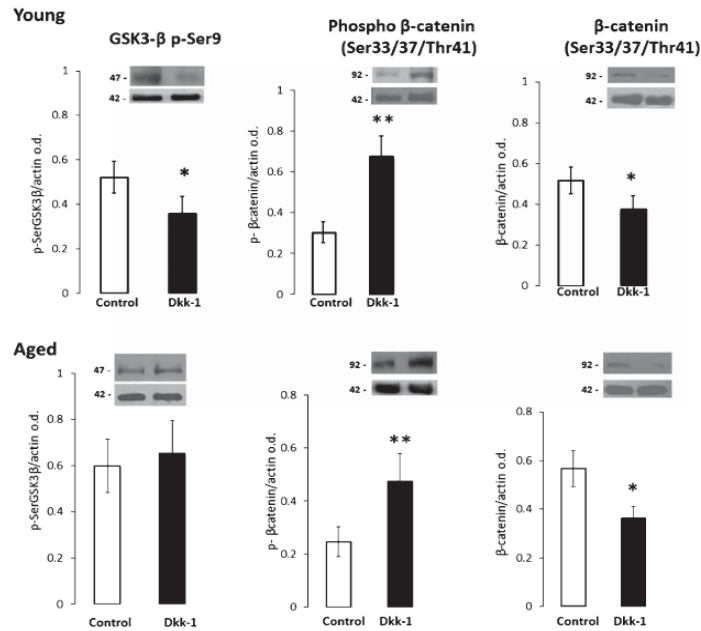
Figura 9. Efecto de Dkk-1 en la fosforilación de GSK3β y β-catenina en rebanadas hipocampales de ratas jóvenes y viejas. En el hipocampo jóven, la exposición al Dkk-1 redujo ligeramente los niveles de GSK3β fosfo-Ser9. En el hipocampo de ratas viejas no se observó una diferencia significativa en GSK3β fosfo-Ser9. Después de la incubación con Dkk-1, los niveles de fosfo β-catenina se elevan, y se reducen los niveles de β-catenina no fosforilada en el hipocampo en ambas edades. Western blots representativos de 7-12 experimentos independientes. Las barras representan el análisis densitométrico y son la media ± error estándar. **p ≤ 0.01 *p ≤ 0.05.

Ya que la proteína fosfatasa PP2A puede regular la actividad de GSK3β (Hernández et al., 2010) y también participa en la desfosforilación de tau (Gong et al., 2000), analizamos la participación de dicha fosfatasa en el incremento de la fosforilación de tau inducida por Dkk-1 en el hipocampo de ratas viejas (fig. 10).
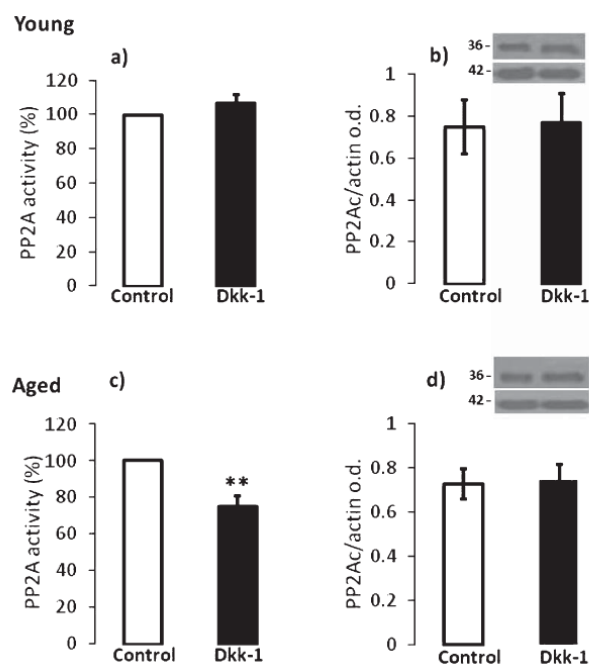
Figura 10. La inhibición de la vía canónica de Wnt decrementa la actividad de PP2A en hipocampo de ratas viejas.. Rebanadas hipocampales de ratas jóvenes y viejas tratadas con Dkk-1 o vehiculo (PBS) se usaron para medir la actividad de PP2A. En el hipocampo joven, no se observó cambio en la actividad (a) o el contenido de la subunidad catalítica PP2Ac (b). En el hipocampo de ratas viejas, se observó un decremento significativo en la actividad de PP2A (c), sin cambios en el contenido total de PP2Ac (d). Las barras representan el análisis densitométrico de los western blots (b,d) y son la media ± error estándar. Los resultados de ensayo de actividad se presentan como el porcentaje de actividad relativa a la rebanada control (a,c).**$p \leq 0.01$

Encontramos una disminución de aproximadamente 30% en la actividad de PP2A en las rebanadas hipocampales de ratas viejas incubadas con Dkk-1 (fig. 10c), pero no se observó cambio en los niveles totales de la subunidad catalítica de PP2Ac (fig. 10d). En las rebanadas de animales jóvenes no encontramos diferencia en la actividad de la PP2A ni en los niveles totales de PP2Ac (fig. 10 a, b). Posteriormente quisimos investigar el mecanismo asociado a la reducción en la actividad de PP2A, analizamos la metilación de la subunidad catalítica en la leucina 309, que como se mencionó anteriormente indica un incremento en la actividad de PP2A y la fosforilación en la tirosina 307, que sugiere un decremento en la actividad.
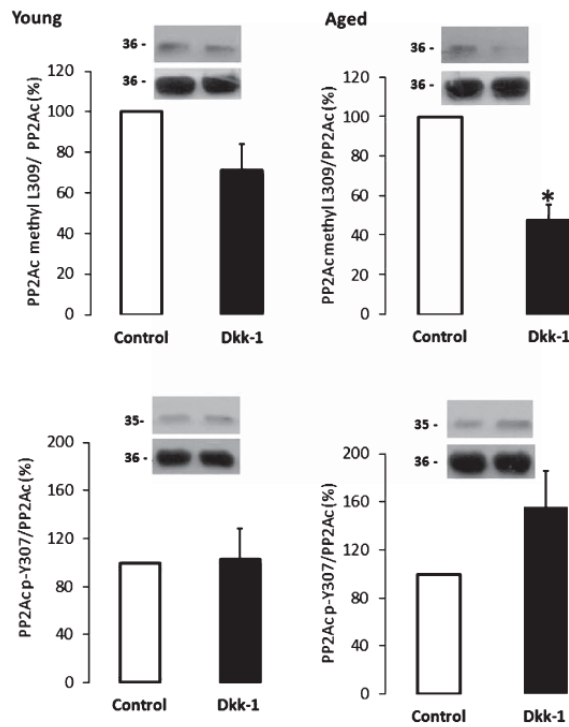
Figura 11. Efecto de Dkk-1 en la fosforilación y metilación PP2A a diferentes edades. Las rebanadas hipocampales se incubaron por 3h con el inhibidor de Wnt, Dkk-1. La metilación en la leucina 309 disminuye con la exposición a Dkk-1 en hipocampo de ratas viejas. Las barras representan el análisis densitométrico de los western blots 3-4 experimentos independientes y son la media ± error estándar. *p ≤ 0.02

Encontramos una reducción significativa en la metilación en Leu309 en las rebanadas hipocampales de ratas viejas mientras que en el hipocampo de ratas jóvenes no se observaron diferencias (fig. 11). Con respecto a la fosforilación en la tirosina 307 encontramos un ligero incremento en el hipocampo de las ratas viejas que correlaciona con el decremento en la metilación en la Leu309. En las ratas jóvenes no se observó diferencia alguna.

DISCUSION

El envejecimiento es la manifestación de una serie de cambios que ocurren a lo largo del tiempo en todos los niveles de organización biológica como molecular, celular, sistémico y del organismo. Estos cambios se expresan frecuentemente como una disminución de la capacidad adaptativa celular, un deterioro progresivo de las funciones fisiológicas y en el humano se asocia con una variedad de enfermedades como la isquemia, la aterosclerosis, el cáncer, la sarcopenia, la osteoporosis, la EA, la enfermedad de Parkinson y finalmente, con el aumento del riesgo de muerte.

Varias vías de señalización celular pueden verse alteradas en el proceso de envejecimiento (Carlson et al., 2008), así como alteraciones en la expresión de algunos genes (Kenyon et al., 2010). Los cambios dependientes del envejecimiento parecen ser perjudiciales para la homeostasis celular y por lo tanto contribuyen a la patología tisular (Carlson et al., 2008). El principal factor de riesgo para la EA es el envejecimiento mismo. Los cambios propios de la edad en el cerebro, como la inflamación y el incremento de radicales libres se ha sugerido pudieran agravar la patología.

Una de las interrogantes respecto a la EA, es cuáles cambios metabólicos participan en la alteración del balance proteínas cinasas/fosfatasas que contribuye a la hiperfosforilación de tau. Sin embargo las vías de señalización que están alteradas en el cerebro envejecido y que probablemente modifiquen este balance no se conocen completamente. La vía Wnt juega un papel importante en el mantenimiento de células troncales y de la homeostasis del sistema nervioso central, su desregulación se ha relacionado con enfermedades neurodegenerativas como la EA. A través de esta vía se modulan múltiples procesos relacionados con crecimiento, desarrollo y senescencia celular.

En virtud de que esta vía modula la actividad de una de las cinasas que se han implicado en el desarrollo de un estado anormalmente fosforilado de la proteína tau, en este estudio describimos su participación durante el envejecimiento. El principal hallazgo de este trabajo fue encontrar que efectivamente, la desregulación de la vía de señalización Wnt parece ser crucial para aumentar la actividad de GSK3β, particularmente en el tejido envejecido lo que conlleva a un incremento en la fosforilación de tau en sitios relacionados con la EA.

Aquí mostramos que al inhibir la actividad constitutiva de GSK3β en rebanadas hipocampales metabólicamente activas, se disminuye considerablemente el nivel de fosforilación de tau en ciertos residuos específicos como la Ser199/202 y Ser396/404 de manera similar en ratas jóvenes y viejas (fig. 7a, b, d, e). Esto sugiere una asociación directa entre la actividad constitutiva de GSK3β y la fosforilación de tau *ex vivo* en estos residuos. Por otro lado, encontramos que los niveles de fosforilación de tau en la Ser214 no se modifican en presencia del inhibidor de GSK3β en ninguna de las edades analizadas (7c, f). Esto muestra que este residuo no es directamente dependiente de la actividad constitutiva de GSK3β como los otros residuos evaluados. Aunque se ha demostrado que GSK3β es capaz de fosforilar la Ser214 de tau, se requiere una fosforilación previa de otra cinasa, como la proteína PKA (Leroy et al., 2010; Zhu et al., 2010).

La inhibición de la GSK3β por el compuesto 6-BIO, que compite por el sitio del ATP de la cinasa, es mucho más específica que la analizada por otros grupos utilizando un inhibidor más general como el LiCl. En virtud de que la inhibición de la GSK3β se asocia con un incremento en el contenido de las proteína efectora de la vía, la β-catenina, analizamos la defosforilación de ésta en residuos dependientes de GSK3β (Ser33/37/Thr41) en presencia de 6-BIO (7g, h). Encontramos que efectivamente la inhibición selectiva de la GSK3β incrementa los niveles de β-catenina activa, además de disminuir la fosforilación en residuos específicos de tau en rebanadas de hipocampo de ratas jóvenes y viejas.

En células no estimuladas, generalmente GSK3β se encuentra constitutivamente activa y su regulación se lleva a cabo principalmente mediante un mecanismo de inhibición (Doble y Woodgett 2003). La vía canónica de Wnt regula negativamente la actividad de GSK3β, y para incrementar su actividad utilizamos el inhibidor endógeno de esta vía, Dkk1. El Dkk-1 se expresa en muy pequeñas cantidades en el cerebro adulto pero se ha reportado un incremento en su expresión bajo ciertas condiciones patológicas. La expresión de Dkk-1 está regulada por la proteína p53, la cual es el sensor más importante de daño al DNA en células eucarióticas. Se ha sugerido que Dkk-1 puede estar involucrado en procesos de daño al DNA y muerte neuronal en eventos de neurodegeneración (Caraci et al., 2008).

Existe evidencia previa del efecto que ejerce Dkk-1 sobre la fosforilación de tau, y de hecho, se ha reportado que Dkk-1 co-localiza en neuronas positivas para tau

hiperfosforilada en varios modelos transgénicos de la EA (Rosi et al., 2010). Otros trabajos además han reportado que la exposición del hipocampo a Dkk-1 induce muerte neuronal, fosforilación de tau y astrocitosis reactiva (Caricasole et al., 2004; Scali et al., 2006; Esposito et al., 2008). Además también está reportado que en ratones, los niveles de Dkk-1 incrementan con la edad, y la pérdida de este antagonista aumenta la neurogénesis hipocampal lo que podría contrarrestar cierto deterioro cognitivo asociado con el envejecimiento (Seib et al., 2013).

En nuestro modelo, encontramos que la regulación positiva de GSK3β en presencia de Dkk-1 incrementa la fosforilación de tau (Ser199/202, Ser396/404, Ser214) en las rebanadas hipocampales únicamente de ratas viejas (fig. 8d, e, f). En las rebanadas hipocampales de ratas jóvenes no encontramos una diferencia significativa en ninguno de los epítopes analizados (fig. 8a, b, c). Es importante resaltar que incluso la fosfo-Ser214 que en nuestro modelo no mostró depender directamente de GSK3β, incrementó su inmunoreactividad después de la incubación con Dkk-1. Como se mencionó anteriormente este epitope puede ser fosforilado por PKA, si previo a esto GSK3β fosforila la Thr212 (Zheng-Fischhöfer et al., 1998). La Ser214 es un sitio interesante además ya que su fosforilación inhibe la interacción de tau con los microtúbulos y es uno de los sitios específicos reportados en la EA (Augustinack et al., 2002). Respecto al envejecimiento, estudios realizados en monos reportan que hay un incremento en la fosforilación de tau Ser214 en la corteza dorsolateral de asociación y este incremento es dependiente de la edad (Carlyle et al., 2014).

Aunque generalmente GSK3β requiere sustratos previamente fosforilados por otra cinasa (Fiol et al., 1987), se ha demostrado que puede fosforilar a tau en Ser396/400/404 directamente (Leroy et al., 2010). Pero se sabe que las fosforilaciones previas incrementan la eficacia catalítica de GSK3β. Se han reportado muchas cinasas capaces de fosforilar previamente los sitios de GSK3β, entre ellas PKA, CAMKII (Wang et al., 2007) y Cdk5 (Sengupta et al., 1997; Noble et al., 2003; Landrieu et al., 2010). Pudiera ser que el incremento en la sensibilidad a la fosforilación en tau observada en ratas viejas con la inhibición de la vía Wnt, refleje una desregulación generalizada en varias proteínas cinasas. En apoyo a esto, se ha encontrado un ligero incremento en las subunidades regulatorias de Cdk5 y PKA, p25 y, R1β respectivamente en cerebros de ratas viejas (Yu et al., 2009). Otros estudios en cerebros post-mortem con EA sugieren que algunos sitios de tau son fosforilados en etapas

tempranas de le enfermedad y se han asociado a la actividad de cinasas como las MARK y las MAPK, pero en etapas más avanzadas la inmunoreactividad de anticuerpos como el AT8 (Ser199/202,Thr205), AT100 (Thr212, Ser214), y PHF-1 (Ser396/404) apuntan a un incremento en la actividad de GSK3β y Cdk5 (Augustinack et al., 2002). Cdk5 incluso se ha visto incrementada en pacientes con deterioro cognitivo leve (Sultana y Butterfield 2007).

A pesar de que la Ser9 de GSK3β indica un decremento en la actividad de la cinasa, en nuestro modelo no observamos un efecto significativo del Dkk-1 en la fosforilación de la Ser9 en el hipocampo de ratas viejas (fig. 9). Esto es consistente con trabajos previos que muestran que la vía Wnt no regula la fosforilación en la Ser9, aunque si disminuye la actividad de la enzima (Ding et al., 2000), también se ha reportado que una mutación en Ser9 no afecta la señalización Wnt (McManus et al., 2005). El mecanismo molecular por el cual es GSK3β es regulada parece ser complejo y no se conoce en su totalidad. Se ha reportado que en la cascada Wnt/β-catenina, GSK3β es secuestrada desde el citosol a complejos multivesiculares previniendo así su interacción con sus sustratos citoplásmicos (Taelman et al., 2010). Parece ser que la inhibición de la vía canónica de Wnt no altera la fosforilación en la Ser9 de GSK3β, la cual depende directamente de otras cinasas entre ellas Akt (Cross et al., 1995). También se ha reportado que la fosfo-Ser9 puede ser desfosforilada eficientemente por PP2A (Hernández et al., 2010; Qian et al., 2010). Sin embargo otros trabajos muestran que la inactivación de GSK3 con litio genera un incremento en la fosforilación de la Ser9, posiblemente por un efecto inhibitorio sobre PP2A (Planel et al., 2001).

En nuestro modelo, corroboramos la inhibición de la vía canónica de Wnt mostrando la disminución de la β-catenina activa, desfosforilada en los sitios dependientes de GSK3β (Ser33/37/Thr41). Y consistentemente encontramos un incremento en los niveles de β-catenina fosforilada en dichos sitios en rebanadas hipocampales de ratas jóvenes y viejas (fig. 9).

El incremento de la fosforilación de tau en varios de los sitios analizados, que ocurrió solamente en el hipocampo de ratas viejas, puede deberse a un cambio en el equilibrio entre cinasas y fosfatasas. Esto es, en el tejido joven, aunque el Dkk-1 incremente la actividad de GSK3β, es posible que las fosfatasas encargadas de mantener el equilibrio apropiado, funcionen correctamente y que en el tejido de ratas viejas, este equilibrio se rompa. Es por esto que decidimos analizar la actividad y contenido de una de las fosfatasas involucradas es la defosforilación de tau.

La PP2A es responsable de más del 70% de la actividad total de las fosfatasas celulares (Liu et al., 2005) y es una de las principales enzimas implicadas en la desfosforilación de tau (Goedert et al., 1995; Arias et al., 1993). Por dicha razón decidimos medir la actividad de PP2A, así como el contenido total de la subunidad catalítica PP2Ac posterior a la incubación con Dkk-1 en rebanadas hipocampales de ratas jóvenes y viejas. Aunque no encontramos diferencias en contenidos totales de PP2Ac en ninguna de las edades analizadas (fig. 10b, d), la actividad de PP2A se encontró significativamente disminuida posterior al tratamiento con Dkk-1 en el hipocampos de ratas viejas (fig.10 a,c). Estos resultados muestran que la edad es un factor importante en la regulación de la actividad de las fosfatasas, aunque el mecanismo que subyace se desconoce. Queda demostrado que la PP2A baja su actividad en presencia de Dkk-1 en hipocampo de ratas viejas, y que posiblemente participe un mecanismo en respuesta a la inhibición de la vía Wnt. Se ha reportado que en cerebros de ratón hay un decaimiento en la actividad de PP2A en el envejecimiento (Veeranna et., 2011) y también se ha reportado que en EA la actividad esta reducida en un 50% (Liu et al., 2005). Consistente con nuestros resultados, no se han reportado cambios en el contenido de PP2Ac entre ratas de 3 y 24 meses (Yu et al., 2009). Entonces, este estudio sugiere que posterior a la inhibición de la vía Wnt, la reducción de la actividad de PP2A exacerba la fosforilación de tau en el hipocampo de ratas viejas. Pudiera ser entonces que exista un equilibrio entre la actividad de PP2A/GSK3β y que condiciones moleculares asociadas con el envejecimiento alteren este equilibrio y se aumente la susceptibilidad a la hiperfosforilación de tau.

La actividad de PP2A puede ser inhibida por fosforilación en tirosina 307 y por la reducción en la metilación en leucina 309 en la subunidad catalítica (Chen et., 1992; Lambrecht et., 2013). En este trabajo encontramos que la reducción en la actividad de PP2A correlaciona con una disminución significativa en la metilación en las rebanadas hipocampales de ratas viejas después de la incubación con Dkk-1. En las regiones cerebrales afectadas en los pacientes con EA, también se ha observado un decremento en los niveles de metilación de PP2A concomitante al incremento en la fosforilación de tau (Sontag et al., 2004). A pesar de no poder demostrar cuál es el mecanismo involucrado en que Dkk-1 favorece la reducción en la metilación de la fosfatasa, nuestros resultados muestran una clara correlación entre la reducción de la actividad de PP2A y el incremento en la fosforilación de tau durante el envejecimiento.

En resumen, el presente trabajo demuestra en un modelo de rebanadas de hipocampo una respuesta diferencial a la fosforilación de tau posterior a la activación de GSK3β modulada por la vía Wnt en tejido joven y viejo y apoya el papel de ciertos cambios asociados con el envejecimiento como promotores de una mayor vulnerabilidad a la fosforilación de tau semejante a lo que puede ocurrir en el cerebro de pacientes con EA.
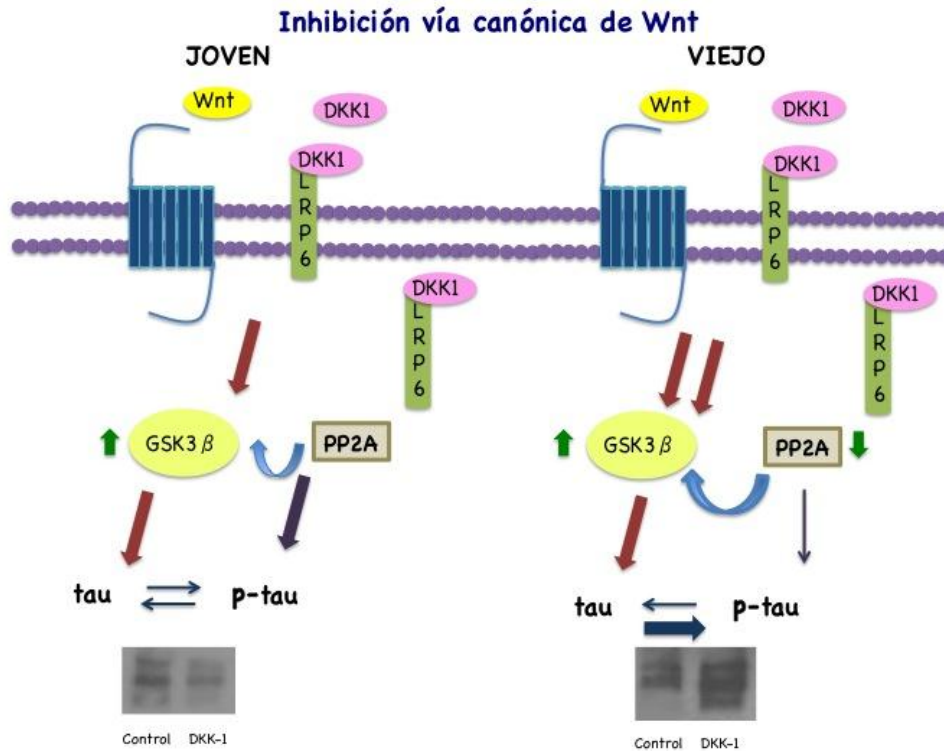
Figura 12. Modelo de la inhibición de la vía canónica de Wnt en rata joven y vieja. En ambos casos el Dkk-1 se une al co-receptor LRP6 y lo internaliza. Esto evita que se active la vía y se incrementa la actividad de GSK3β. En rata joven, GSK3β fosforila la cinasa tau y la PP2A la desfosforila manteniendo un estado de equilibrio entre fosforilación/desfosforilación. En rata vieja la inhibición de Wnt disminuye la actividad de PP2A. GSK3β fosforila a tau pero la baja de la actividad de PP2A impide revertir este estado de fosforilación.

HALLAZGOS

1.- La actividad basal de GSK3β está involucrada con la fosforilación de sitios de tau implicados en la hiperfosforilación de esta proteína en la Enfermedad de Alzheimer en el hipocampo de ratas jóvenes y viejas.

2.- La inhibición de la vía canónica Wnt con Dkk-1 induce la activación de GSK3β en forma similar en el hipocampo de ratas jóvenes y viejas pero solo resulta en la hiperfosforilación de tau en las rebanadas de hipocampo de ratas viejas.

3.- El incremento en la fosforilación de tau en presencia de Dkk-1 en hipocampo de ratas viejas va acompañado de una disminución en la actividad de la PP2A, que es la mayormente involucrada con el mantenimiento del equilibrio entre fosforilación/defosforilación de tau.

CONCLUSION:

Estos resultados sugieren que en individuos jóvenes existe un balance óptimo entre la actividad de cinasas/fosfatasas capaz de contrarrestar los efectos de la activación de GSK3β sobre la fosforilación de tau.

En el envejecimiento, este balance se encuentra afectado y la disminución de la actividad de la PP2A parece ser un factor crítico para incrementar la fosforilación de tau en una situación de inhibición de la vía canónica Wnt.

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PAPEL CENTRAL DE LAS VÍAS DE SEÑALIZACIÓN QUE INDUCEN CRECIMIENTO CELULAR (INSULINA Y Wnt) EN ENVEJECIMIENTO Y LONGEVIDAD

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VÍA DE SEÑALIZACIÓN INSULINA/PI3K Y SU RELACIÓN CON PROCESOS DE ENVEJECIMIENTO

La búsqueda de genes relacionados con la longevidad ha llevado a caracterizar algunas vías de señalización dentro de las células cuyo estado de actividad (a la alta o a la baja) determinan incrementos en la esperanza de vida de los organismos estudiados. Desde hace más de una década se conoce que en vertebrados –y posiblemente en mamíferos–, la vía de señalización activada por insulina o el factor de crecimiento semejante a insulina (IGF-1) tiene efectos muy importantes sobre la longevidad (Kimura et al., 1997; Brown-Borg et al., 2001).

Tanto la insulina como el IGF-1 se unen a su receptor (RI), el cual es una proteína transmembranal tetramérica con actividad de tirosina cinasa. La unión de la insulina induce la fosforilación en residuos de tirosina del RI. Una vez fosforilado, el RI atrae proteínas adaptadoras como el sustrato del receptor de insulina (IRS). Las proteínas adaptadoras activan la vía de señalización de la cinasa de fosfatidil inositol (PI3K), que a su vez activa a cinasas como

Akt, que actúa sobre varias proteínas, jugando un papel muy importante en procesos como la síntesis de proteínas, el crecimiento celular y la apoptosis o muerte celular programada. Por su parte, la cinasa Akt regula a la cinasa glucógeno sintetasa (GSK3), que desempeña un papel central en el crecimiento y desarrollo de los organismos y controla la división celular, además de regular a una enzima blanco del metabolismo, la cinasa mTOR (figura 1).

Los estudios sobre el control genético del envejecimiento y la longevidad se han desarrollado de manera muy importante tanto en el nemátodo *Caenorhabditis Elegans* (C. Elegans) como en la mosca de la fruta, *Drosophila melanogaster*. Entre otras, dos mutaciones en genes de *C. Elegans* resultan en un aumento significativo en la longevidad: Age-1, un hómologo del gen en mamíferos que codifica para la subunidad catalítica de la enzima PI3K (Morris et al., 1996) y Daf-2, que es dependiente de temperatura y que produce casi el doble de longevidad, pero requiere la activación de un segundo gen, el Daf-16, homólogo de la familia de los factores de transcripción FOXO (*forkhead transcription factor*). Aparentemente, las mutaciones en Daf-2 generan
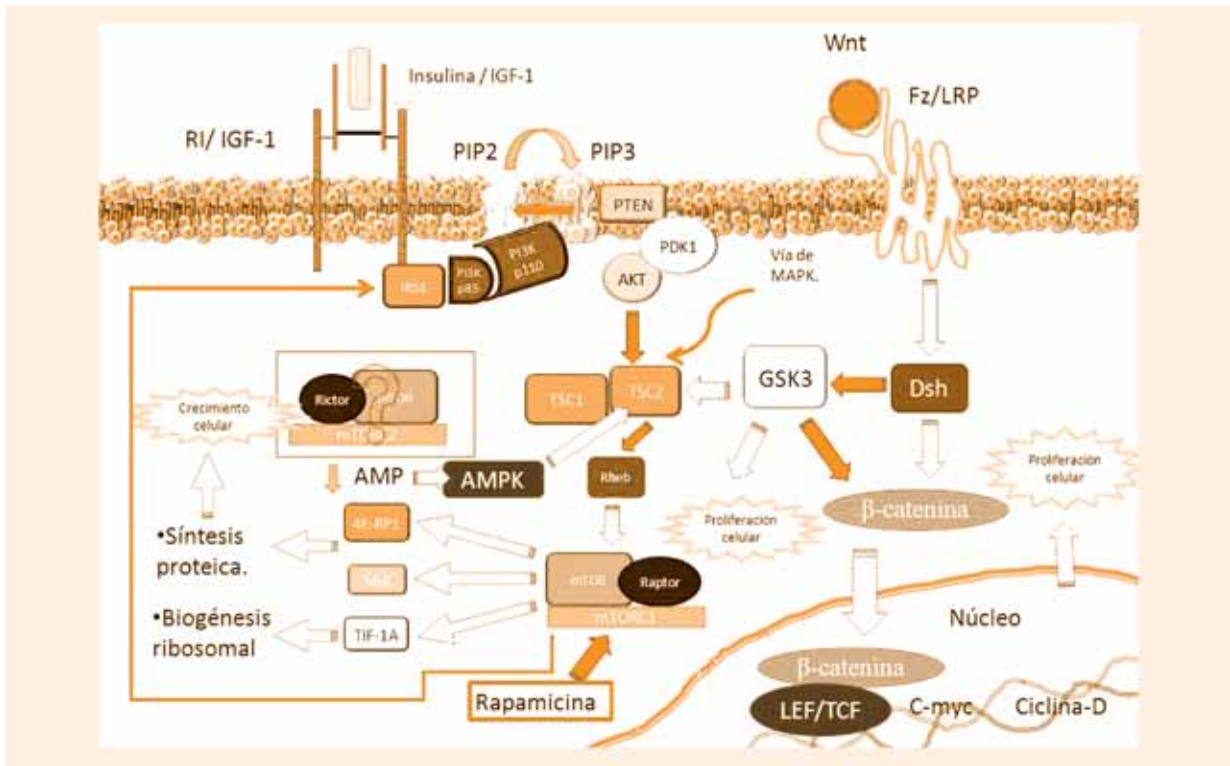

Figura 1. Vías de señalización PI3K y Wnt, involucradas en la regulación de la proliferación y crecimiento celular durante el desarrollo y el envejecimiento. Las flechas naranjas significan activación y las rojas inactivación. La función del complejo mTORC2 es poco conocida.

señales internas que también se pueden producir durante periodos de ayuno prolongado y equivaldrían al efecto de disminución de la vía metabólica activada por insulina. Daf-2 es un gen homólogo del gen en mamíferos que codifica para una proteína semejante al RI y regula la actividad de varias enzimas con actividad de cinasas promoviendo su fosforilación y manteniendo en el citoplasma al factor de transcripción Daf-16. Cuando Daf-2 se inactiva, Daf-16 se mueve al núcleo y promueve resistencia al estrés y aumento de la longevidad. En Drosophila, algunas mutaciones que producen longevidad también se relacionan con el sustrato del receptor de insulina Chico (Clancy et al., 2001). En mamíferos, el panorama parece ser mucho más complejo.

Por ejemplo, ratones con mutaciones en el RI presentan disminución del tejido adiposo y longevidad, aunque en mamíferos estos efectos parecen también depender de un sistema hormonal que no está presente en gusanos o moscas: la hormona de crecimiento (GH) (Bartke, 2005). La restricción calórica también tiene efectos importantes en longevidad; entre otros, se sabe que el ayuno reduce la intensidad y duración de la secreción de insulina requerida para la homeostasis de la glucosa, reduce las señales intracelulares generadas por la insulina y promueve la expresión de enzimas antioxidantes que se asocian con longevidad (Bonkowski et al., 2006).

De manera muy interesante, basta mutar el IRS2 en el cerebro para que varias de estas características se expresen y se observe aumento de longevidad en ratones, lo que sugiere que una inactivación selectiva de este receptor en el cerebro puede impactar la longevidad en humanos (Tagushi et al., 2007).

En el humano, el papel en la longevidad mediada por inactivación de la señalización por insulina todavía es controversial, aunque se ha demostrado que individuos centenarios muestran un incremento en la sensibilidad periférica a la insulina y niveles reducidos de insulina circulante (Baribieri et al., 2008). En estudios más recientes en nonagenarios, la longevidad parece asociarse con diferencias en la sensibilidad a la insulina (Rozing et al., 2009). De hecho, a medida que los mamíferos envejecen, se desarrolla una hiperinsulinemia compensatoria para poder mantener la homeostasis de la glucosa y prevenir la progresión a la diabetes de tipo 2. Sin embargo, el aumento de la concentración de insulina circulante tiene efectos negativos en el cerebro y puede disminuir la esperanza de vida. Se ha propuesto que atenuando la señalización de insulina a través de la proteína IRS2 en el cerebro de los ancianos se puede evitar el efecto nocivo de la hiperinsulinemia que se desarrolla con el sobrepeso y la edad (Taguchi et al., 2007). Otras estrategias que llevarían al mismo efecto protector, disminuyendo la insulina circulante y atenuando la vía de señalización de insulina cerebrales, son el ejercicio físico, la restricción calórica y la pérdida de peso, además de la reducción de la señalización a través de la GH (Bonkowski et al., 2006).

LA CINASA MTOR COMO BLANCO TERAPÉUTICO EN ENVEJECIMIENTO

La reducción de la vía de insulina/IGF-1 regula la longevidad de varios organismos eucariontes aparentemente a través de la inhibición de la actividad de mTOR. Un mecanismo propuesto que media la longevidad en organismos con baja actividad de mTOR es la autofagia celular, probablemente necesaria para la remoción de proteínas y organelos dañados, manteniendo el correcto funcionamiento celular. También se ha visto que la señalización celular mediada a través de la enzima mTOR regula el comportamiento de alimentación en varios modelos animales de mamíferos (Tsang et al., 2007; Arsham y Neufeldet, 2006; Blagosklonny, 2007). A su vez, la hiperactividad de mTOR está relacionada con varias enfermedades crónico-degenerativas y con cáncer (figura 2).

En el sistema inmune, mTOR regula la proliferación de células inmunitarias, así como la expresión de moléculas involucradas en procesos autoinmunes (MHCII), pudiendo ser un blanco terapéutico para enfermedades autoinmunes y terapia inmunosupresora en transplantes de órganos. Muchos de los diferentes tipos de cáncer poseen mutaciones en diferentes genes que intervienen en la vía de señalización de mTOR, como la fosfatasa PTEN y la cinasa PI3K, que producen mayor actividad de mTOR, por lo que pueden ser controlados con inhibidores de mTOR. En diabetes mellitus tipo 2 se ha asociado la resistencia a insulina con la fosforilación del sustrato del receptor de insulina 1(IRS1) en residuos de serina mediada por mTOR. En la obesidad se ha visto que la mayor actividad de mTOR favorece la lipogénesis y la proliferación de adipocitos. En enfermedades cardiovasculares como la hipertrofia cardiaca, la inhibición de la actividad de mTOR también puede tener uso terapéutico.

La mTOR es miembro de las cinasas de lípidos fosfoinosítidos (PIKK); es una cinasa de las llamadas de serina/treonina. Esta enzima puede formar dos complejos principales conformados dependiendo del tipo de proteínas con las cuales interactúe: mLST8/GβL más Raptor, en el caso del complejo mTORC1, y mLST8/GβL más Rictor/mAvo3 en el caso de mTORC2 (Wang y Proud, 2006; Tsang et al., 2007).

La señalización a través de la cinasa mTOR se activa por hormonas y factores de crecimiento como la propia insulina y el IGF-1. Otros factores que regulan la actividad de mTOR son señales de daño celular como bajas concentraciones de oxígeno, estado energético celular, estrés oxidante y daño al ADN (a través de p53), los cuales inhiben la actividad de mTOR (Tsang et al., 2007; Arsham y Neufeldet, 2006). Tanto la vía celular que se activa a través de PI3K/Akt como la vía de MAPK participan en la activación de mTOR y, de manera inversa, la cinasa dependiente de AMP (AMPK), activada durante estrés energético, la inhibe (figura 1).

Una de las funciones celulares de mTOR en eucariontes es la importación de nutrientes, la traducción de RNA mensajeros y la biogénesis de ribosomas que conlleva el crecimiento del tamaño y masa celulares. La cinasa mTOR tiene como blanco varias cinasas, como eEF2K, RSK y S6K, las cuales regulan la actividad de factores iniciadores de la síntesis de proteínas, así como algunos reguladores de la transcripción de genes (figura 2).

Existe evidencia de que la disminución de la señalización mediada por PI3K/Akt/mTOR aumenta la longevidad de manera muy significativa en ratones (Sharp et al., 2005). Los individuos centenarios son muy sensibles a la insulina y la sensibilidad a la insulina parece ser un muy buen indicador de una actividad de mTOR reducida. De esta manera, la longevidad parece asociarse de manera muy significativa con la reducción de la actividad de esta cinasa, lo que apunta al desarrollo de estrategias farmacológicas para inhibirla (Bagloskonny, 2006). Es también notable que la deficiencia de sirtuina-1 (SIRT1) –molécula implicada en los efectos moleculares sobre longevidad en mamíferos que produce la restricción calórica– resulta en un incremento en la señalización a través de mTOR. En este sentido, se ha encontrado que también el resveratrol que activa SIRT1 reduce la actividad de mTOR (Ghosh et al., 2010).

El principal inhibidor de mTOR es la rapamicina producida por el hongo *Streptomyces hygroscopicu*. La rapamicina se une a FKBP12 y este complejo a mTORC1, probablemente interfiriendo con la unión del dominio PIKK de mTOR con sus sustratos y evitando así su activación (Tsang et al., 2007). La rapamicina bloquea la fosforilación de varias cinasas blanco de mTOR como las cinasas de la proteína ribosomal S6 de la subunidad 40s ribosomal (S6K). También se han desarrollado varios análogos de mTOR cuya efectividad para inhibirla se está probando. Sin embargo, es importante también el análisis estructural de la interacción de mTOR y sus análogos para poder producir un fármaco ideal para la terapia deseada, teniendo en cuenta el balance
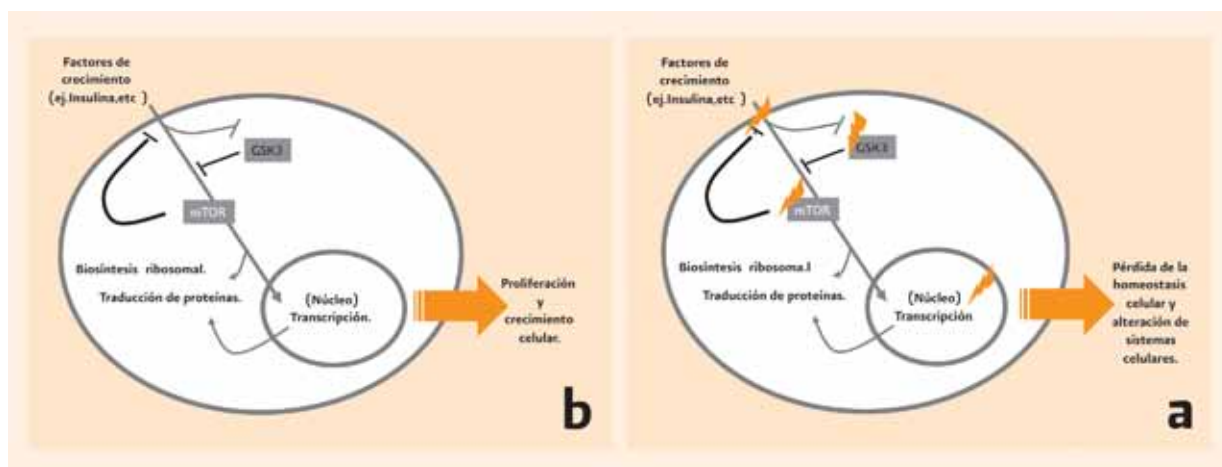

Figura 2. Vía de mTOR en el desarrollo normal (a) y el envejecimiento patológico (b).

entre crecimiento celular normal y anormal. La rapamicina y sus análogos son bien tolerados por humanos y representan una alternativa interesante como droga "anti-edad" (Wang y Proud, 2006; Tsang et al., 2007). Recientemente se ha demostrado que el tratamiento con rapamicina durante la vida ya adulta extiende significativamente la vida en ratones machos y hembras a través de su mecanismo de acción que inhiben TOR (Harrison et al., 2009).

La rapamicina ya se usa en clínica en humanos, particularmente para el tratamiento de cáncer. Al menos tres análogos de rapamicina están siendo utilizados para algunos tipos específicos de cáncer que responden de manera favorable, como el cáncer de células renales. Se ha propuesto también el uso de la rapamicina en enfermedades neurodegenerativas debido a su hidrofobicidad, lo que permite su paso a través de la barrera hematoencefálica y debido a que en el cerebro promueve la autofagia y remoción de agregados proteicos, como los de huntingtina (enfermedad de Huntington) y la tau (enfermedad de Alzheimer) (Tsang et al., 2007).

WNT, SUS RECEPTORES Y CO-RECEPTORES

Recientemente se han implicado otras vías de señalización que modulan procesos relacionados con crecimiento, desarrollo y senescencia celular como la vía conocida como Wnt/β-catenina.

Las proteínas Wnt son una familia de glicoproteínas secretadas ricas en cisteína que participan en diferentes procesos celulares. Durante el desarrollo embrionario juegan un papel importante en la proliferación celular, diferenciación, orientación, adhesión, supervivencia y apoptosis (Li et al., 2006; Patapoutian y Reichardt, 2000). En el adulto, esta vía Wnt ha sido asociada a procesos de homeostasis o equilibrio tisular (MacDonald et al., 2009). La señalización Wnt aberrante está implicada en diferentes patologías, como enfermedades cardiovasculares, envejecimiento, cáncer, diabetes, neurodegeneración y procesos inflamatorios (Maiese et al., 2008).

Hasta el momento se han descrito tres vías principales de activación por ligandos Wnt. La primera –y principal– es la vía Wnt/β-catenina, conocida como vía canónica; involucra a ligandos como Wnt1, Wnt3a y Wnt8 (Maiese et al., 2008) y cuenta con dos receptores, el receptor Frizzled

(FZ) de 7 dominios transmembranales, y el co-receptor LRP5/6. Para la activación de esta vía de señalización es necesario que Wnt se una a ambos receptores. En ausencia de Wnt, la proteína β-catenina es capturada por la proteína adaptadora axina, la cual también recluta a la proteína APC (*adenomatous polyposis coli*), a la caseína cinasa 1 (CK1) y a la glicógeno sintetasa (GSK3). A todo este complejo se le conoce como complejo de destrucción, ya que su formación genera la degradación de la β-catenina. La CK1 y la GSK3 fosforilan de manera secuencial a β-catenina en la región aminoterminal y la marca para su degradación vía proteasoma (Barker, 2008; MacDonald et al., 2009). La eliminación continua de β-catenina evita su acumulación citoplásmica y su traslocación al núcleo; por lo tanto, se inhibe la transcripción de los genes blanco procrecimiento como ciclina-D y c-myc. Por otro lado, si Wnt se une a su receptor y co-receptor, se forma un complejo trimolecular (Fz-Wnt-LRP5/6); esto provoca el reclutamiento de la proteína adaptadora Dishevelled (Dvl). El receptor LRP5/6 se activa y se recluta el complejo de destrucción; de esta manera se inhibe la fosforilación y degradación de β-catenina que se acumula en el citoplasma y entra al núcleo para interactuar con factores de transcripción de la familia Lef/Tcf y, así, activar a los genes blanco de Wnt (MacDonald, 2009).

La vía Wnt/calcio sólo tiene un receptor principal, el FZ, y se activa por un incremento en los niveles de calcio intracelular; esto activa a las proteínas dependientes de calcio como la cinasa C(PKC). Así como hay diferentes Wnts, también hay diferentes receptores FZ; en esta vía participan Wnt-4, Wnt5a, Wnt11 y el receptor FZ2 (Miller, 2002; Maiese et al., 2008). Al activarse la vía Wnt/calcio, se activan proteínas Gtriméricas y así se da el aumento del calcio y activación de CamKIIy PKC, entre otras moléculas de señalización.

La tercera vía es la Wnt/PCP, denominada de polaridad planar celular, ya que regula la polaridad celular mediante la reorganización del citoesqueleto (Miller, 2002). Esta vía también incluye al receptor FZ y por medio de proteínas G monoméricas activa a la cinasa JNK y genera cambios en el citoesqueleto.

WNT Y ENVEJECIMIENTO

Las proteínas Wnt en mamíferos adultos se han asociado con procesos de renovación de células troncales. Conforme avanza la edad, las células salen de manera permanente del ciclo celular, evitando su proliferación y entrando a un proceso que se conoce como senescencia celular (Sharpless et al., 2007). Evidencias experimentales sugieren que la vía canónica de Wnt regula positivamente la renovación de células troncales y progenitoras, retardando así el inicio de cambios relacionados con el envejecimiento. Por ejemplo, la activación de la vía canónica Wnt regula positivamente el mantenimiento de células troncales adultas, la neurogénesis en adultos (Lie et al.,2005), la regeneración del pelo (Ito et al.,2009) y del hueso en ratones adultos (Chen et al.,2007). La sobreexpresión de la cinasa GSK3 promueve el proceso de senescencia en células humanas y de ratón; esto sugiere que la inhibición de la vía canónica podría ser responsable del declive de la proliferación celular dependiente del envejecimiento (Zmijewski y Jope, 2004). Otro miembro de la vía de señalización que está involucrado en el proceso patológicos es el receptor LRP5/6, el cual parece estar mutado en pacientes con enfermedad de arteria coronaria (Mani et al., 2007).

La enfermedad de Alzheimer (EA) es una enfermedad neurodegenerativa en la cual el principal factor de riesgo es el envejecimiento. La EA se caracteriza por la presencia de marañas neurofibrilares compuestas de la proteína tau hiperfosforilada, depósitos extracelulares de la proteína β-amiloide (Aβ), muerte neuronal progresiva y demencia. Se ha sugerido que componentes de las vías Wnt pueden participar en este padecimiento. La proteína cinasa GSK3 ha recibido especial atención en el estudio de esta enfermedad, ya que es la principal enzima que fosforila tau; además se ha encontrado que un incremento en los niveles de la GSK activa en neuronas precede la agregación de tau en las marañas neurofibrilares (Inestrosa et al., 2007). En neuronas hipocampales de rata, la inhibición de GSK3 con LiCl protege a las células del daño inducido por Aβ (Inestrosa et al., 2007). Esto sugiere que la vía canónica de Wnt puede estar involucrada en la neurodegeneración dependiente de Aβ. En tejido cerebral de autopsias de pacientes de EA, existe una inducción del inhibidor de la vía canónica Wnt, Dkk1 (Caricasole et al., 2004).

Por otro lado, se sabe que la sobreactivación de la vía Wnt promueve la división celular y la formación de tumores. Lo anterior se puede ver como un evento contrario al proceso de senescencia celular, por lo que se puede sugerir que la vía Wnt canónica activa, retarda el proceso de envejecimiento (DeCarolis et al., 2008).

Si bien existe evidencia que señala a las proteínas Wnt como proteínas que retardan el envejecimiento, recientes publicaciones aseguran que bajo ciertas condiciones las Wnts pueden acelerar dicho proceso en cultivos celulares (Schelleret al., 2006).

GSK3 ENLACE ENTRE LA VÍA MTOR Y LA VÍA WNT

La vía Wnt es importante en el desarrollo de eucariontes y en procesos de proliferación, supervivencia y determinación del destino celular. En ausencia de señal de Wnt, la β-catenina es degradada en el complejo de destrucción que incluye a la cinasa GSK3β y axina entre sus componentes. La estabilización sostenida de β-catenina es común en diversos tipos de cáncer, implicando la señalización constitutiva de Wnt en varios neoplasmas. Los miembros del complejo de destrucción se asocian con el complejo TSC1/2 de la vía de mTOR. Mientras la axina y la GSK3 se disocian del complejo TSC1/2 por estimulación de Wnt, Dsh se asocia con TSC2 y estimula la actividad de S6K1. En condiciones de estrés energético,TSC2 se fosforila por AMPK y por GSK3β, promoviendo la actividad de TSC2 e inhibiendo la acción de mTORC1. Este proceso es inhibido por la señalización de Wnt, ya que a través de ella se estimula la actividad de TSC2 e inhibe la cinasa mTOR. Por otro lado, la estimulación con factores de crecimiento promueve la fosforilación de TSC2 promoviendo la activación de mTOR. La inhibición de GSK3β es esencial para la activación de mTORC1. Sin embargo, aún falta entender cómo se regula la actividad de TSC2 por GSK3 y axina en diferentes estados energéticos y de señalización (Inoki et al., 2006; Huang et al., 2009) (figura 1). De esta manera, existe una comunicación cruzada entre diferentes vías de señalización que debe ser tomada en cuenta al momento de profundizar sobre las bases moleculares de la longevidad y el envejecimiento.

CRECIMIENTO, DIFERENCIACIÓN Y ENVEJECIMIENTO: ¿CARAS DE LA MISMA MONEDA?

La senescencia celular parece estar asociada con una activación sostenida de la enzima mTOR, que a su vez forma parte de vías metabólicas que controlan el ingreso de nutrientes, el crecimiento celular y las señales que inducen división celular. Muchas de las mutaciones en genes que producen aumento en la longevidad se asocian con inactivación de vías de señalización que controlan la actividad de la mTOR. De hecho, en humanos algunas de las características asociadas al envejecimiento como la hipertrofia celular, la aterosclerosis, la hipercoagulación y la tumorogénesis, la diabetes, la osteoporosis y la obesidad se asocian típicamente con una hiperactividad de mTOR (Blagosklonny, 2006). El envejecimiento no puede ser genéticamente programado porque no ha sido seleccionado evolutivamente; así, más bien aquellas vías metabólicas seleccionadas evolutivamente para favorecer un programa de crecimiento en respuesta a factores de crecimiento y nutrientes necesarios para garantizar la exitosa reproducción de la especie, y que son útiles en una etapa de la vida, pueden no serlo una vez terminada la etapa reproductiva. Por tanto, su activación sostenida puede estar involucrada en diversas manifestaciones del envejecimiento (Blagosklonny, 2008). De esta manera, diversas alternativas farmacológicas (rapamicina, resveratrol) y no farmacológicas (restricción calórica, ejercicio físico) se perfilan como herramientas útiles para lograr un envejecimiento exitoso, libre de las patologías asociadas a él.

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Review Article

GSK3 Function in the Brain during Development, Neuronal Plasticity, and Neurodegeneration

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GSK3 has diverse functions, including an important role in brain pathology. In this paper, we address the primary functions of GSK3 in development and neuroplasticity, which appear to be interrelated and to mediate age-associated neurological diseases. Specifically, GSK3 plays a pivotal role in controlling neuronal progenitor proliferation and establishment of neuronal polarity during development, and the upstream and downstream signals modulating neuronal GSK3 function affect cytoskeletal reorganization and neuroplasticity throughout the lifespan. Modulation of GSK3 in brain areas subserving cognitive function has become a major focus for treating neuropsychiatric and neurodegenerative diseases. As a crucial node that mediates a variety of neuronal processes, GSK3 is proposed to be a therapeutic target for restoration of synaptic functioning and cognition, particularly in Alzheimer's disease.

1. GSK3 Signaling Pathway

Many diseases of the central nervous system are characterized by changes in the structural organization of neuronal networks, developmental abnormalities, or dysregulation of signaling pathways, leading to altered brain plasticity and, ultimately, neurodegeneration. The proline-directed serine/threonine kinase, glycogen synthase kinase 3 (GSK3), has been suspected to be a contributing factor in psychiatric illness and age-associated neurodegenerative diseases for some time [1]. The involvement of GSK3 misregulation in a variety of brain abnormalities strongly supports its pivotal role as a metabolic crossroads for controlling basic mechanisms of neuronal function from brain bioenergetics to establishment of neuronal circuits, modulation of neuronal polarity, migration, neuronal proliferation, and survival [2]. In particular, the role of GSK3 in phosphorylation of cytoskeletal proteins impacts neuronal plasticity, as cytoskeletal constituents are involved in the development and maintenance of neurites, and changes in the rate of stabilization/destabilization of microtubules (MT) could influence major cellular compartments of neurons, such as dendrites, spines, axons, and synapses.

The metabolic function of GSK3 was first described in glycogen metabolism, as GSK3 phosphorylates glycogen synthase in response to insulin [3]. Since then, research has identified a multitude of substrates and functions for this enzyme. GSK3 exists in cells as two distinct gene products, α and β, which exhibit high homology in the catalytic domain but differ in the N- and C-terminal sequences [4]. GSK3 is ubiquitous throughout the animal kingdom [5] and is widely expressed in all tissues with particularly abundant levels in the brain [4], where the neuron-specific isoform GSK3β2 is found [6].

GSK3 is unique because it is constitutively active, and upstream signals downregulate its activity by phosphorylation at specific residues. The most important phosphoresidues are serine (Ser) 21 for GSK3α and Ser9 for GSK3β, which inhibit its kinase activity [2, 7–10], while phosphorylation on tyrosine (Tyr) residues (Tyr 216/279 for GSK3β and GSK3α, resp.), is required for its activation [11–13]. The latter kind of phosphorylation is mediated by
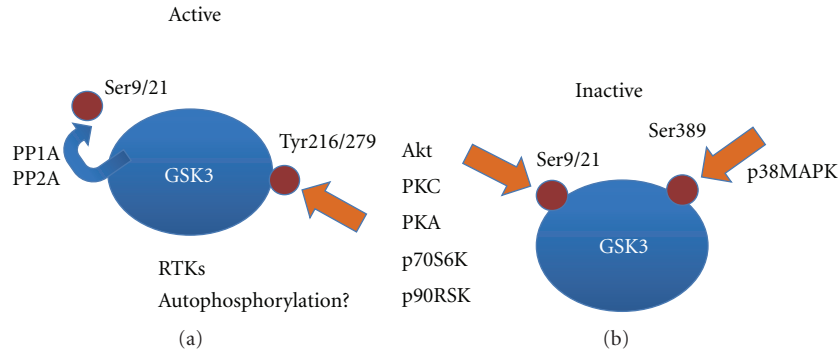
Figure 1: Modulation of GSK3 activity by phosphorylation. Protein phosphatases 1 and 2A activate GSK3 by removing Ser9/21 phosphorylation. It has also been reported that phosphorylation in tyrosine residues by members of the receptor tyrosine kinase family of cell surface receptors (RTKs) or by autophosphorylation may activate GSK3. On the other hand, signaling networks activate several protein kinases, which may bring about phosphorylation of different residues and inhibition of GSK3.

diverse tyrosine kinases [14] or by autophosphorylation [15] (Figure 1).

Multiple kinases can phosphorylate Ser21/9, including Akt, protein kinases A and C, p70S6K, and p90RSK [16]. In contrast, protein phosphatases 1 (PP1) and 2A (PP2A) dephosphorylate the inhibitory site of GSK3, resulting in activation of the enzyme. In addition to the inhibitory phosphorylation of GSK3β described above, an additional inhibitory site at Ser389 has been detected in the brain, which is phosphorylated by p38 mitogen-activated protein kinase (MAPK) [17].

In addition to its phosphorylation state, GSK3 activity may be regulated by proteolysis through disruption of the axin-β-catenin complex [18] or N-terminal cleavage by the calcium-dependent protease calpain [19]. GSK3 activity also depends on its cellular localization. Although GSK3 is predominantly located in the cytosol, it is also present in nuclei and mitochondria, where it is highly activated compared with the cytosolic pool [20]. Nuclear GSK3 regulates the expression of diverse genes via various transcription factors, such as Ap-1, β-catenin, c-myc, and p53 [16]. Subtle control of GSK3-mediated activation and inhibition is required to ensure a proper balance among cell morphoregulation, proliferation, and growth. Thus, prolonged inhibition of GSK3 is associated with hypertrophic cell growth [21], while sustained activation is associated with neurodegeneration [22]. Unlike other kinases, the majority of GSK3 substrates require a "priming" phosphorylation on Ser/Thr residues, which is catalyzed by a protein kinase other than GSK3 [2, 10, 16].

2. Implications of GSK3 Activity in Brain

In adulthood, both GSK3α and GSK3β are expressed in mice adult brain and are particularly enriched in hippocampus, neocortex, and cerebellum [23]. In rodent adult hippocampus GSK3β is more abundant than GSK3α [24], and in aged hippocampus GSK3β is elevated, but not GSK3α [25]. Two splice variants of the GSK3β gene are found in neurons from mouse, rat, and human: GSK3β1 and GSK3β2, the

latter being highly expressed during brain development and specific to neurons [6, 26–28]. The two isoforms are differentially involved in phosphorylation of different substrates [29] and establishment of neuronal polarity and axon guidance [2, 30–32].

The importance of GSK3 in brain function has been established by several studies in transgenic mice, which have shown different neurological defects depending of the specific GSK3 isoform involved. While deletion of GSK3β is lethal, heterozygote mice survive and present increased anxiety and reduced exploration [33–35]. Conversely, knockout GSK3α mice are quite normal [36], although neuron-specific knockout of GSK3α results in reduced anxiety, locomotor activity, and aggression [37]. Overexpression of an inhibitory phosphorylation-resistant form of GSK3 results in increased locomotor activity and has been proposed as a model of manic illness [38]. Moreover, overexpressed GSK3β in dentate gyrus results in tau-dependent neurodegeneration of this region [39]. In the brain, GSK3 regulates developmental processes, including neurogenesis, migration, axon growth and guidance, and synaptic plasticity [40], and its activity is controlled through several signaling pathways activated by growth factors, wingless (Wnt) proteins, G-protein-coupled receptors (GPCR), β-arrestin, among other proteins [41].

Abnormal activation of GSK3 has been associated with several neurological and psychiatric disorders that share developmental abnormalities and altered neurocircuitry maintenance, such as schizophrenia, bipolar disorder, autism, and Alzheimer's disease (AD) [42–46]. GSK3 is indeed a common therapeutic target for neuropsychiatric drugs [41, 47].

3. Signaling Pathways Involved in GSK3 Activity in Brain

GSK3 is a downstream component of several signaling pathways in the brain. One of the most studied is the phosphoinositide-3-OH kinase (PI3K)/Akt pathway, which plays a crucial role in differentiation and survival of neuronal and glial cells [48]. Growth signals, Ras proteins [49],
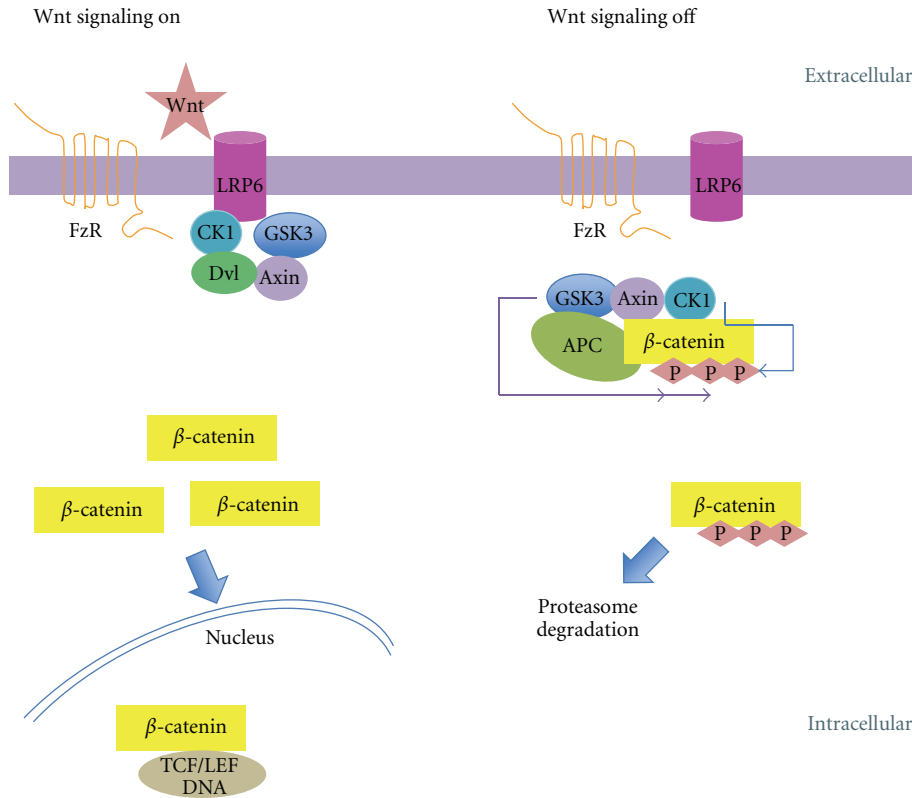
FIGURE 2: Canonical Wnt signaling and GSK3 regulation. Wnt activation through Frizzled receptor (FzR) induces destabilization of the protein complex composed of axin, adenomatous polyposis coli (APC) protein, β-catenin, casein kinase (Ck1), and GSK3, which results in GSK3 inhibition leading to the induction of β-catenin/TCF target gene expression. When Wnt signalling is off the GSK3/axin complex is not inhibited and β-catenin phosphorylated and is degraded by the proteasome machinery.

or diminished phosphatase and tensin homolog (PTEN) all activate the catalytic subunit of PI3K, which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) and activates phosphoinositide-dependent protein kinase-1 (PDK-1). Meanwhile, signaling proteins with pleckstrin homology (PH) domains accumulate at sites of PI3K activation on the inner surface of the plasma membrane through interactions between their PH domains and the phospholipid products of PI3K. Next, the serine-threonine kinase Akt/protein kinase B is recruited and phosphorylated by PDK-1, which stimulates the catalytic activity of Akt, in turn phosphorylating GSK3 to downregulate its activity.

The canonical Wnt pathway is also classically involved in negative regulation of GSK3. Although the role of Wnt proteins in mature neurons remains largely unexplored, recent data indicate that Wnts are important mediators of neuronal function, neuronal morphology, neurogenesis, and synaptic plasticity [50–52]. Interestingly, Wnt signaling has also been implicated in neurological disorders associated with developmental abnormalities, such as schizophrenia [53], as well as in chronic neurodegenerative diseases, such as AD [54]. Extracellular secreted Wnt proteins activate Frizzled receptor and/or the low-density lipoprotein-related protein 5 and 6 (LRP5/6) receptors, leading to the characteristic activation of the Wnt canonical pathway [55].

Due to Frizzled activation, the Dishevelled mammalian homolog Dvl1 is recruited, inducing destabilization of the protein complex composed of axin, adenomatous polyposis coli (APC) protein, β-catenin, and GSK3β, which results in GSK3β inactivation [56]. Inhibition of GSK3β favors an increase in unphosphorylated β-catenin levels, allowing interaction with members of the lymphoid enhancer factor/ T-cell factor (LEF/TCF) family of transcription factors and, as a consequence, promoting the expression of cell survival genes [57]. Although the molecular mechanism of GSK3 inhibition is not completely understood, Wnt signaling has recently been reported to trigger the sequestration of GSK3 from the cytosol to multivesicular organelles, preventing its interaction with cytoplasmic substrates [58] (Figure 2).

The outcome is different in the absence of the Wnt stimulation, which may occur due to lack of Wnt ligands or the presence of Wnt negative modulators, such as the extracellular protein Dickkopf-1 (DKK1), which regulates the canonical Wnt signaling, or the secreted Frizzled-related protein, which modulates both canonical and noncanonical Wnt signaling [59]. Under these circumstances, GSK3β is activated and able to phosphorylate its target proteins. Several regulators also target β-catenin/GSK3β signaling. For example, the product of disrupted in schizophrenia 1 (DISC1) gene inhibits GSK3β activity through a direct physical interaction, causing stabilization of β-catenins.

DISC1 loss-of-function in the dentate gyrus has been shown to result in reduced neural progenitor proliferation and to elicit hyperactive and depressive behaviors in mice [60], suggesting the involvement of GSK3β overactivation in mental illnesses, such as depression and schizophrenia. Moreover, DISC1 function seems to be essential for neural progenitor proliferation in embryonic brains and in the dentate gyrus of adult brains through its ability to control GSK3 activity and to maintain β-catenin levels, which ultimately impacts the neural circuitry [60].

GSK3β is also a downstream mediator of dopamine signaling via the dopamine D2 receptor/β-arrestin 2/PP2A complex. In this signaling pathway, Akt activates neuregulin-1 signaling leading to inhibition of GSK3β activity [61]. Interestingly, neuregulin-1 has been also implicated as schizophrenia risk factor [62].

In addition to the described role of GSK3β in neurodevelopment, it has been recently found the potentiation of Notch signalling by PI3K through GSK3β inhibition [63]. The Notch pathway has been implicated in controlling cell fate, differentiation, development as well as synaptic plasticity, learning and memory [64].

4. GSK3: A Switch for Cytoskeletal Reorganization and Synaptic Plasticity

Changes in neuronal morphology and plasticity are affected by GSK3-induced phosphorylation of proteins involved in the modulation of MT and neurofilament stabilization, which affect the cytoskeleton [65]. Among these proteins are tau, microtubule-associated protein 2 (MAP2), microtubule-associated protein 1B (MAP1B), collapsin response mediator protein 2, APC, axin, neurofilaments, kinesin light chain, and cytoplasmic linker protein [9, 16, 30, 31, 40, 53, 66–70].

The induction of polarity during neuronal development is essential for the establishment of circuits that support complex functioning [71, 72]. Subcellular location of the inactive form of GSK3β varies depending on the state of neuronal polarization, as it moves from nonpolarized neurites to the neurite tip that will form the axon at the beginning of the differentiation process. Local inactivation of GSK3 is important to allow axonal growth concurrent with its activation in dendrites [73–76]. These mechanisms support the establishment of neuronal polarity, which is dependent on the stability and dynamism of the MT in each neuronal compartment [40, 53]. The relationship between GSK3β and the microtubule stabilizing protein complex APC-mPar3, which are both present at the tip of the actively growing nascent axon, is important for the establishment of neuronal polarity. Shi and colleagues [74] have demonstrated that spatially regulated GSK3 activity in hippocampal neurons during development leads to axonal generation [74]. The inactivation of GSK3 at the nascent axon is required for mPar3 targeting through APC and kinesin-mediated transport at the plus end of the axonal MT [74].

Two further studies showed that GSK3β inhibition in hippocampal neurons induces formation of multiple axons [75, 76]. However, the role of GSK3 in neurodevelopment remains only partially understood due to contradictory data;

other studies have found that GSK3 inhibition induces axonal spreading, reduces axonal elongation, and increases growth cone size, but it does not induce the formation of multiple axons [66, 68, 77–79].

One mechanism related to both synaptic reorganization and MT dynamics is Wnt signaling [80–82], which directs the growing axon towards the synaptic terminal. This process involves the reduction of axonal growth speed and the extension of axonal distal portions at the growth cone [83] until arborization forms functional synaptic endings where the presynaptic apparatus can be assembled. Transmembrane proteins, such as neuroligin/neurexin and cadherins, are also involved in this process and serve to regulate assembly on both sides of the synapse [52, 84]. Wnt proteins have a fundamental role in synapse formation, acting as retrograde signals that regulate assembly of the presynaptic apparatus [84]. Specifically, Wnt7a has a dual function in synaptic differentiation, promoting axon remodeling and increasing incorporation of synaptic proteins [66, 84]. These effects are linked to changes in the reorganization and dynamics (stabilization-destabilization) of MT, which are achieved through the canonical Wnt signaling, independent of the transcription pathway, in which GSK3β activity is inhibited, and, consequently, the phosphorylation state of the axonal MAP1B is reduced [84–86]. The addition of Wnt7a to neuronal cultures reduces MAP1B phosphorylation and induces MT depolymerization from growing areas of the axon, promoting axonal growth cone enlargement and axonal spread [51, 66, 87]. The classical inhibition of GSK3β by lithium chloride (LiCl) reproduces the effects of Wnt7a, inducing axonal arborization and widening and enlargement of the growth cone through remodeling of axonal MT during postnatal development of cerebellar cells [52, 87, 88]. On the other hand, it has been shown that Wnt7a increases the level of Synapsin I (SynI), which is known to be involved in synapse formation, as well as in the maturation and transport of synaptic vesicles in areas of growth [87, 89, 90]. Accumulation of SynI promotes both axonal remodeling and synaptogenesis during cerebellar development [87] and is mimicked by LiCl treatment [66, 88, 91].

GSK3 is also present in mature synapses [92], where its activity, along with that of cyclin-dependent kinase (Cdk5), participates in the recovery of synaptic vesicles during high neuronal activity. During this process, Cdk5 phosphorylates the GTPase dynamin I, and then GSK3β phosphorylates the same dynamin I [93]. Both phosphorylation events are necessary and sufficient to trigger and maintain activity-dependent bulk endocytosis of vesicles [94].

As a result of controlling different morphofunctional aspects of adult brain plasticity, GSK3 also plays a role in long-term potentiation (LTP) [95, 96] and long-term depression (LTD). LTP might be considered the electrophysiological correlate of learning based on its synaptic mechanisms and long-lasting experience-dependent cortical circuits [97–99]. On the other hand, LTD has been suggested as a mechanism to enhance the signal-to-noise ratio of sensory input from stored memories [97]. Some studies have shown that GSK3β inhibition upregulates and maintains LTP [24, 50, 91, 100–102], while GSK3β remains active during

LTD [101]. In rat hippocampus, GSK3β overactivation has been shown to impede LTP and affect synapses by decreasing both synaptic transmission and release of the presynaptic neurotransmitter glutamate [91]. This is regulated by proteins associated with synaptic vesicles, such as SynI [103–108], which is considered to be a synaptic plasticity marker [109, 110]. GSK3β activation inhibits SynI expression after LTP induction and simultaneously disrupts SynI clustering, which results from elevated neuronal activity [91].

An other evidence that underscores the importance of GSK3 in brain plasticity is derived from experiments conducted in rat hippocampus by Gómez de Barreda and colleagues. The authors found that inhibitory phosphorylation of GSK3 at Ser9 increased at the time of LTP induction was maintained for up to one hour *in vivo* and was significantly higher in the hippocampal CA1 and dentate gyrus subregions, which are involved in learning and memory acquisition [39]. Transgenic mice overexpressing GSK3 showed reduced LTP induction [100]. These data confirm the significant participation of GSK3 in LTP regulation by enabling LTP when its catalytic activity is inhibited and preventing LTP when it is overactive. The inhibition of the two main signaling pathways (insulin/PI3K and Wnt) which induced an activation of GSK3 also prevents the induction of LTP [50, 64, 111–113].

GSK3 has been shown to induce LTD through presynaptic and postsynaptic mechanisms. In the presynaptic neuron, upregulation of GSK3 decreases the expression of SynI [91], which has been linked to a decrease in glutamate release [103]. In the postsynaptic neuron, GSK3 activation causes changes in levels of synapse-associated proteins [114, 115], evident as downregulation of the NR2A/B subunits of NMDA receptors and of the scaffolding protein postsynaptic density 93 (PSD93) [24, 91]. In addition, a transient activation of NMDA receptors and endocytosis of AMPA receptors occurs [116, 117], leading to the loss of GSK3 inhibition due to insufficient Ca^{2+} entry. This GSK3 inhibition is mediated by NMDA-PI3K-Akt signaling [112, 118]. Over-activity of GSK3 may also induce MT destabilization in dendrites and axons [80, 86, 119] (Figure 3).

Overexpression of GSK3β in mice prevents the induction of LTP [100] and causes spatial memory deficits [120]. These data suggest that GSK3β plays an essential role in memory formation through three general processes: (i) phosphorylation of substrates involved in synaptic remodeling, necessary for the establishment of new connections, (ii) turnover of cytoskeletal proteins such as MAPs, actin, and tubulin, promoting disassembly, a condition required for a proper synaptic reorganization, and (iii) involvement in the two major forms of synaptic plasticity in the brain, LTP, and LTD [121].

In summary, the functional consequence of GSK3 overactivation in mature neurons is inhibition of LTP and induction of LTD [101, 121], which could be linked to deficiencies of memory and learning characteristic of some neurological diseases, such as AD.

5. GSK3 and Alzheimer's Disease

AD represents a serious epidemiological problem, as it is now recognized as the most common age-related neurodegenerative disease. Evidence supports a role for GSK3 in producing some of the characteristic hallmarks of AD: extracellular accumulation of amyloid-β protein (Aβ) and intraneuronal neurofibrillary tangles (NFTs) composed of hyperphosphorylated forms of tau and inflammatory markers [122]. All of these effects contribute to synaptic and neuronal loss and memory decline [123, 124].

It has been proposed that overactivation of GSK3 in AD leads to inhibition of LTP and may partially explain the learning and memory deficits present early in this neurodegenerative disorder. On the other hand, changes in GSK3 activity may be a molecular link between the two main histopathological markers: Aβ overproduction and tau hyperphosphorylation [39, 46, 125, 126].

The NFTs that accumulate in AD are anomalous filamentous structures composed mainly of abnormal, hyperphosphorylated forms of tau protein [127]. Hence, numerous studies have focused on identification of the protein kinases and phosphatases regulating tau phosphorylation *in vivo*. GSK3β was recognized as a primary kinase involved in tau phosphorylation, as was apparent from the first studies that termed it tau protein kinase-I [128]. Thus, GSK3β has been identified as one of the major enzymes mediating tau hyperphosphorylation at the residues implicated in neurodegenerative tauopathies, including AD [129].

Normally, tau protein contains a total of 85 phosphorylable sites: 45 Ser, 35 Thr, and 5 Tyr. Of these, 40 have been identified as phosphorylated in insoluble tau in AD brain: 28 Ser, 10 Thr, and 2 Tyr, and GSK3β can phosphorylate 23 of these sites [130]. Although GSK3β commonly needs priming phosphorylation of tau, three sites were recently found that can be phosphorylated by GSK3β alone, without priming: Ser396, Ser400, and Ser404 [131]. Furthermore, initial phosphorylation of the Ser214 by cAMP-dependent protein kinase was shown to lead to the rapid modification of four additional sites by GSK3β [131]. Studies in transgenic mouse models have shown that overexpression of GSK3β results in neurodegeneration and have unequivocally demonstrated that GSK3β phosphorylates tau in AD-related phosphoepitopes *in vivo* [93, 132, 133]. Moreover, co-overexpression of tau and GSK3β synergistically increased tau phosphorylation and induced neuronal death in a transgenic model in *Drosophila* [134] while GSK3 inhibition reduces the phosphorylation and aggregation of tau [135, 136]. Similarly, tau hyperphosphorylation and neurodegeneration after GSK3β overexpression are exacerbated by co-overexpression of tau with mutations characteristic of frontotemporal dementia with parkinsonism, associated with chromosome 17 (FDTP-17). This study also showed that tauopathy progression could be prevented by administration of a GSK3β inhibitor at the first signs of pathology [133]. Tau knockout mice overexpressing GSK3β show reduced hippocampal degeneration, indicating that tau partially contributes to the pathology observed in mouse brain [39]. Finally, GSK3β inhibitors decrease tau phosphorylation and amyloid deposition in
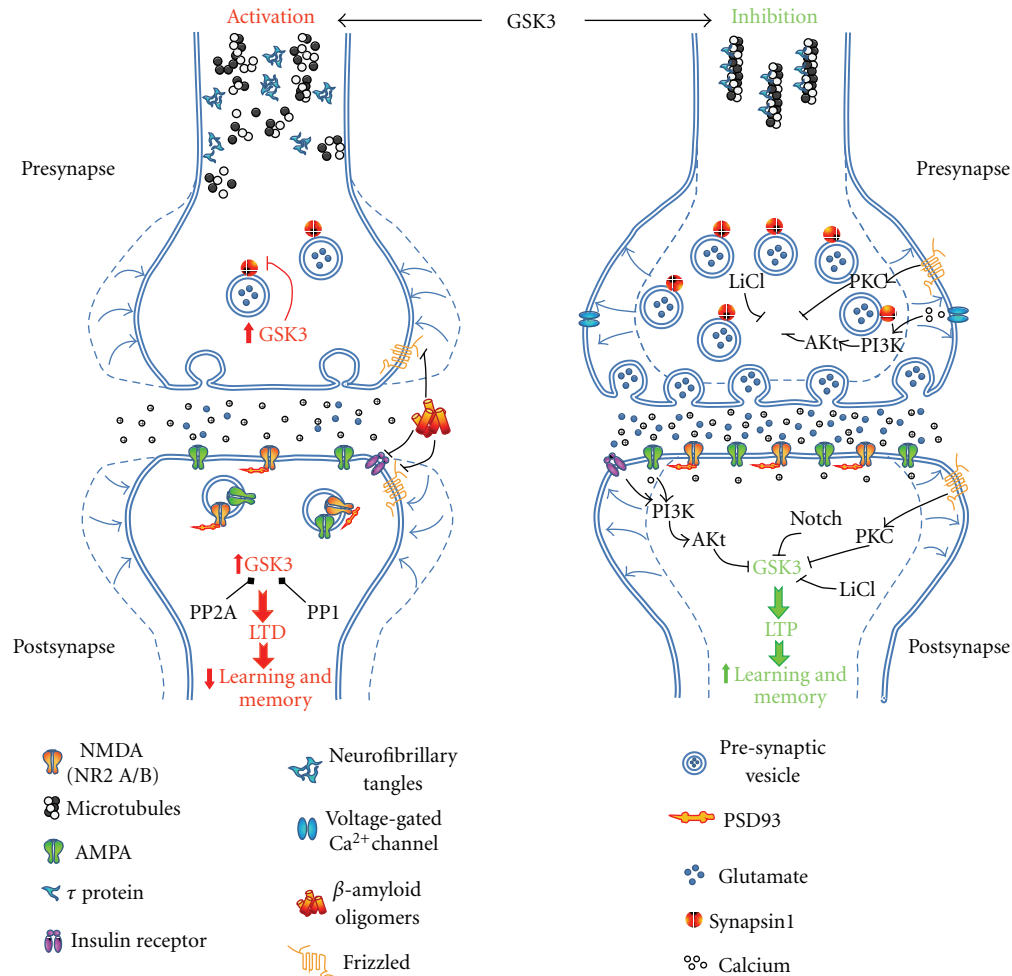
FIGURE 3: Schematic representation of pre- and postsynaptic mechanisms involved in neuronal plasticity and the role of GSK3. In the presynapse GSK3 activity decreases the expression of SynI reducing the release of glutamate while in postsynapses GSK3 transiently activates NMDA receptors leading to endocytosis of AMPA receptors and reduces the levels of PSD93 protein, favoring LTD. In contrast, Wnt and PI3K signaling pathways or pharmacological inhibition of GSK3 by LiCl supports the induction of LTP, facilitating learning and memory. GSK3 inhibition is also involved in axon and dendritic widening in both pre- and postsynaptic sites. Serine/threonine phosphatases PP1 and PP2A can activate GSK3 regulating phosphor-GSK3 levels through its dephosphorylation. GSK3 is important in the modulation of multiple signaling pathways including Notch pathway that plays an important role in different developmental processes. In AD, amyloid-β oligomers inhibit Wnt and insulin signaling pathways leading to activation of GSK3. In addition, GSK3 overactivation mediates τ hyperphosphorylation and microtubule destabilization.

a double transgenic mouse model coexpressing human mutant amyloid precursor protein (APP) and tau [137]. In brains of AD patients, GSK3β colocalizes with NFT [138], and active GSK3β is present in neuronal cytoplasm of neurons with tangle-like inclusions when abnormal tau hyperphosphorylation begins [139]. In fact, polymorphisms in GSK3 were recently reported to be risk factors for late-onset AD [140, 141].

Evidence suggests that GSK3β regulates APP processing [126, 142], leading to increased production of Aβ. Neuronal exposure to Aβ increases GSK3β activity through PI3K inhibition [143], causing a positive feedback loop. Aβ peptide can regulate GSK3 activity, acting as an insulin receptor antagonist and preventing activation of PI3K and Akt. In turn, the absence of activated Akt prevents the inhibitory

phosphorylation of GSK3, increasing its activity [144]. Aβ seems to interfere with the Wnt canonical pathway as well, leading to increased GSK3 activity [145]. Thus, deregulation of GSK3 in AD might be due, in part, to alterations in insulin and Wnt signaling. In the canonical Wnt signaling pathway, the gene for LRP6 coreceptor has been identified as a risk factor for late-onset AD in ApoE4-negative individuals [146]. Interestingly, it has been suggested that the Wnt pathway might be inhibited by ApoE protein, which likely binds to the coreceptor LRP5/6 [147]. Moreover, the ApoE4, implicated in sporadic AD [148], may activate GSK3 [46, 149].

Wnt dysregulation has also been implicated in AD. For example, protein Dickkopf-1 negatively modulates the canonical Wnt signaling pathway and thus activates GSK3. DKK1 colocalizes with NFT and dystrophic neurites in
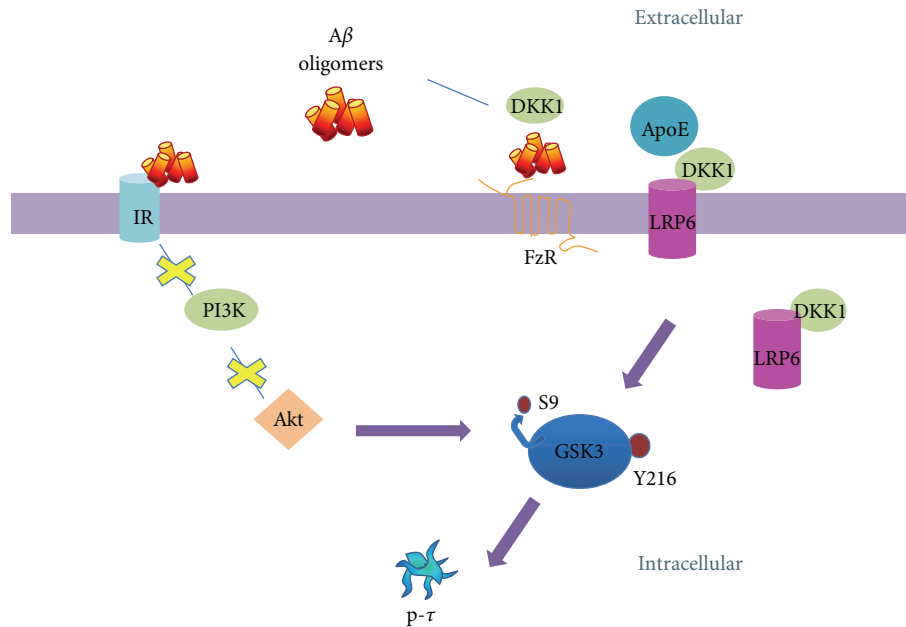
FIGURE 4: Proposed model of GSK3 activation by amyloid-β protein in AD. Amyloid-β oligomers bind to the insulin receptor and inhibit PI3K/Akt pathway, and Akt is unable to phosphorylate and inactivate GSK3. Aβ also induces the expression of DKK1, which internalizes LRP6 receptor and inhibits Wnt signaling leading to GSK3 activation. Aβ can bind to Frizzled receptor (FzR) and inactivate Wnt signaling as well. ApoE also inhibits this signaling pathway and activates GSK3. Tau hyperphosphorylation and NFT formation may result from GSK3 overactivation.

degenerating neurons of AD brains [150]. Moreover, using Wnt and PI3K signaling inhibitors, cultured cortical neurons have shown increased tau phosphorylation and morphological changes mediated by GSK3β [151]. Taken together, this evidence suggests an important role for GSK3 in AD and supports the notion that GSK3 could be the link between amyloid and tau pathology [46] (Figure 4).

6. Concluding Remarks

GSK3 has attracted a great deal of interest due to the myriad of processes it controls. GSK3 is implicated in many fundamental functions, ranging from bioenergetics to developmental and plasticity events, particularly in the brain. Altered GSK3 activity in the brain negatively influences neuronal structure, which in turn may affect maintenance of neuronal circuits that support cognitive function. The use of therapeutic drugs to control GSK3 activity has been hampered by the variety of substrates targeted by this enzyme and the long-term ramifications of its downstream signaling. Future studies could focus on identifying spatiotemporal expression patterns of specific GSK3 isoforms in the brain with the goal of developing specific inhibitors for clinical use in devastating neurological diseases, such as AD.

Acknowledgments

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Role of Wnt Signaling in the Control of Adult Hippocampal Functioning in Health and Disease: Therapeutic Implications

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Abstract: It is well recognized the role of the Wnt pathway in many developmental processes such as neuronal maturation, migration, neuronal connectivity and synaptic formation. Growing evidence is also demonstrating its function in the mature brain where is associated with modulation of axonal remodeling, dendrite outgrowth, synaptic activity, neurogenesis and behavioral plasticity. Proteins involved in Wnt signaling have been found expressed in the adult hippocampus suggesting that Wnt pathway plays a role in the hippocampal function through life. Indeed, Wnt ligands act locally to regulate neurogenesis, neuronal cell shape and pre- and postsynaptic assembly, events that are thought to underlie changes in synaptic function associated with long-term potentiation and with cognitive tasks such as learning and memory. Recent data have demonstrated the increased expression of the Wnt antagonist Dickkopf-1 (DKK1) in brains of Alzheimer´s disease (AD) patients suggesting that dysfunction of Wnt signaling could also contribute to AD pathology. We review here evidence of Wnt-associated molecules expression linked to physiological and pathological hippocampal functioning in the adult brain. The basic aspects of Wnt-related mechanisms underlying hippocampal plasticity as well as evidence of how hippocampal dysfunction may rely on Wnt dysregulation is analyzed. This information would provide some clues about the possible therapeutic targets for developing treatments for neurodegenerative diseases associated with aberrant brain plasticity.

Keywords: Alzheimer´s disease, Hippocampal plasticity, neurodegeneration, neurogenesis, neurorepair, Wnt signaling.

INTRODUCTION

The mature brain undergoes continuous morphological changes in response to external and internal stimuli through the turnover and reorganization of neuronal networks and synapses [1-3]. These morphological adjustments determine the ability of neurons to incorporate new information from the internal and external environment and largely depend on the proper functioning of a variety of signaling pathways that control neuronal circuitry activity and neuronal shape. The secreted Wnt signaling proteins activate a variety of signaling pathways that modulate neuronal connectivity through downstream molecules involved in a number of physiological processes ranging from cellular morphology to gene expression [4]. All signaling events implicated in the Wnt pathway must occur in a coordinate fashion so synaptic contacts remain dynamic in the adult brain thus allowing a continuous fine balance between synaptic formation and synapse disassembly [5]. The hippocampus is a critical plastic brain region where significant network changes occur underlying a lifelong synaptic remodeling according to experience [3,6,7]. The hippocampus is particularly enriched in signaling molecules that influence many aspects of structural plasticity and network dynamics [8-11] besides being one of the two active neurogenic regions in the adult brain. The hippocampus is one of the most affected structures in pathological aging and emerging evidence has revealed a significant role of disrupted Wnt signaling in the mechanisms of neuronal death and dysfunctional plasticity subserving neurodegenerative conditions such as AD [12] and frontotemporal dementia (FTD) [13].

Wnt Signaling Pathways

Wnts are secreted cysteine-rich glycosylated and lipid modified proteins [14]. Palmitoylation at a conserved cysteine seems to be essential for Wnt signaling since removing the palmitate group or mutations in the cysteine residues result in loss of Wnt activity [15]. There are 19 Wnt genes identified in mammals, including human [16] with molecular sizes ranging from 39 kDa to 46 kDa [17]. Wnt signaling starts mainly by interaction of Wnt ligands to one of the 19 types of Frizzled (Fz) receptors. Wnt transduction pathways are complex and even when they have been studied as linear signaling cascades, it has been recently suggested that all Wnt pathways should be considered as part of an integrated cellular signaling network where different intra and extracellular stimuli converge [18]. The best characterized Wnt signaling is the canonical Wnt/β-catenin. In the absence of Wnt ligand, cytoplasmic β-catenin protein is degraded by the action of a complex composed of scaffolding axin

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protein, tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3) [19]. CK1 phosphorylates Ser45 of β-catenin priming the subsequent phosphorylation of GSK3 on Ser33/37/41. Phosphorylated β-catenins are recognized by the E3 ubiquitin ligase β-Trcp, which targets β-catenins for proteasomal degradation. Extracellular Wnt ligands bind to the seven-pass transmembrane receptor Fz and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor. Wnt-Fz-LRP5/6 complex recruits the scaffolding protein dishevelled (Dvl) and axin on the plasma membrane leading to GSK3 inactivation. Without GSK3 phosphorylation, β-catenin accumulates in the cytoplasm and enters the nucleus, where it binds members of the lymphoid enhancer-binding factor/T cell-specific transcription factor (LEF/TCF) family and activates Wnt target genes expression [14]. Although the molecular mechanism of GSK3β inhibition is not completely understood, Wnt signaling has recently been reported to trigger the sequestration of GSK3β from the cytosol to multivesicular organelles, preventing its interaction with cytoplasmic substrates [20]. Another Wnt signaling pathway, the Wnt/PCP depends of Fz and Dvl to activate Rho GTPases and the Jun N-Terminal kinase (JNK). PCP components include Fz receptors, and the four pass trans-membrane protein Van Gogh like1 and 2 (Vangl1/2), Celsr1, 2 and 3, prickle and Dvl. Downstream these molecular elements, the PCP pathway acts through Rho and Rac small GTPases to control cytoskeleton remodeling and Jun N-Terminal

kinase (JNK) to regulate gene expression [21]. Wnt ligands can also induce the release of calcium from intracellular stores, probably *via* heterotrimeric G-proteins in the so-call Wnt/Ca^{2+} pathway [22]. Wnt signaling through Fz receptor and Dvl mediates the activation of phospholipase C (PLC). PLC cleaves phosphatidylinositol 4,5-biphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,2,5-triphosphate (IP3). IP3 diffuses through the cytosol and interacts with endoplasmic reticulum calcium channels, resulting in the release of calcium ions and activation of the calcium calmodulin-dependent protein kinase II (CAMKII). DAG and calcium ions activate protein kinase C (PKC) [23]. PKC has several downstream intracellular targets including the nuclear transcription factors NFkB and cAMP responsive element-binding (CREB). CAMKII promotes the nuclear import of cytoplasmic protein nuclear factor associated with T cells (NFAT), which induces the expression of several genes.

In addition to Fz receptors and LRP5/6, Wnt ligands can also bind to single pass transmembrane receptor tyrosine kinase (RTKs) of the Ryk and Ror families [24]. Wnt signaling through Ryk leads to the activation of Src proteins, and Wnt binding to Ror2 can inhibit β-catenin/TCF complexes and activate JNK [18]. Wnt4, Wnt5a and Wnt11 have been identified as activators of the non canonical Wnt pathways (Fig. **1**).

In the hippocampus several Wnt and Fz receptors have been found to be expressed throughout life. In the rat, Wnt4,
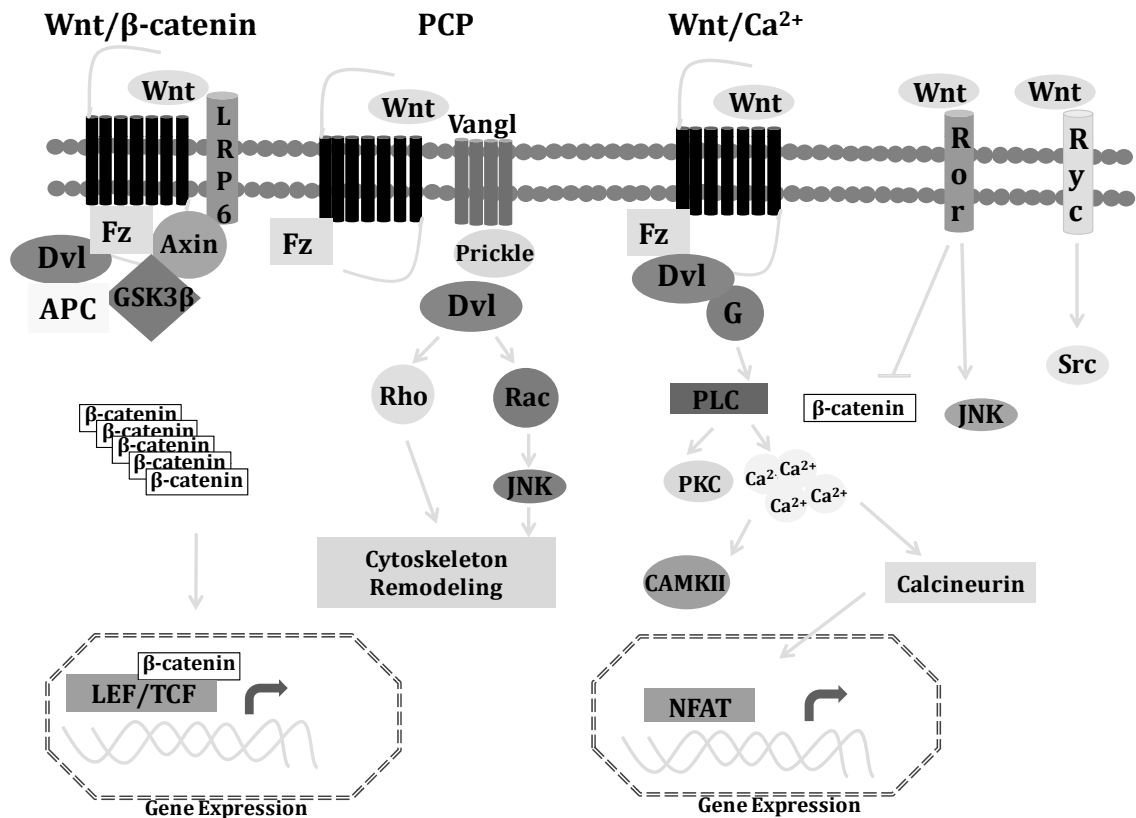
Fig. (1). Schematic representation of Wnt signaling pathways. Wnt proteins activate several signaling pathways. APC, adenomatous polyposis coli; Dvl, Dishevelled; Fz, Frizzled; GSK-3β, glycogen synthase kinase-3β; LRP5/6, low density lipoprotein receptor related protein 5/6; CaMK, Ca2$^+$/calmodulin dependent kinase; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; Ror, receptor tyrosine kinase-like orphan receptor 1/2; Tcf/Lef, T-cell factor/lymphoid enhancer factor.

Wnt5a, Wnt7a, Wnt8b and Wnt11 are present in hippocampal neurons from the embryonic stages to the adult stage [25,26]. Particularly Wnt7b expression remains in the DG blades and also outlines the pyramidal cell layer of CA3 in the adulthood [25]. Also, Fz3, Fz5 and Fz8 are expressed during hippocampal development [27] while Wnt3, Wnt5, and Wnt7a/b, Fz1, Fz2, Fz5, Fz8 and Fz9 increase during hippocampal synaptogenesis [27,28]. Fz9 has a selective expression pattern in the hippocampus and was found in both, neurons and astrocytes throughout life [29,30]. The specific pattern of genes encoding Wnt ligands, receptors, and inhibitory proteins reported in the adult hippocampus evidences the potential role for Wnt signaling in broad hippocampal functions.

Wnt Inhibitors

Wnt signaling is regulated by several types of secreted regulatory proteins, among the most well characterized are secreted Frizzled related protein (sFRP1 to sFRP5) [31,32], Dickkopf (DKK1 to DKK4) [33], and the Wnt-inhibitory factor 1 (WIF1) [34]. However, a variety of endogenous inhibitors such as Wise/SOST, Cerberus, IGFBP, Shisa, Waif1, APCDD1, and Tiki1 have also been described [reviewed in 35]. DKK1 interferes with Wnt canonical signaling preventing the binding of Wnt ligands to LRP5/6 co-receptors. WIF1 and sFRPs were initially found as Wnt scavengers that bind to Wnts and prevent Wnt-Fz activation [36]. Recently, sFRPs have been shown to act as counterparts of Wnts in gain-of- and loss-of-function experiments [reviewed in 37]. It should be noted that other functions of some sFRPs have been reported, such as the regulation of axon guidance by binding to Fz receptors [38]. It has been demonstrated that DKK1 induction is dependent upon induction by c-Jun [39] and p53, which is a sensor of DNA damage in cells [40].

Wnt in the Hippocampus: From Development to Adult Plasticity

Various studies have shown a crucial participation of Wnt pathways at early stage of hippocampal development [41] and in fact, the expression pattern of Lef1 (a gene of the LEF1/TCF family of transcription factors) as well as other LEF1/TCF proteins are critical for the regulation of dentate gyrus granule cells generation and the entire hippocampal maturation [42,43]. Actually, the conditional inactivation of β-catenin in mice, results in an impairment of hippocampus development [44] and the inhibition of canonical Wnt signaling by DKK1 induces severe defects in the hippocampal structure [45]. Wnt proteins also exert influence on various features of neuronal circuit assembly through modifications of the neuroskeleton organization and synaptic assembly [4,5,46-53]. Studies in cultured hippocampal neurons have found that β-catenin are mediators of dendritic morphogenesis since the overexpression of a stabilized form of β-catenin leads to the development of a more complex dendritic arborization. In contrast, sequestration of β-catenin by overexpressing the intracellular domain of N-cadherin, decreases dendritic arborization and dendritic branch length. Moreover, the increased dendritic growth and arborization after high K^+ depolarization depends on the intracellular contents of β-catenin and on the increase of Wnt activity

since the number of dendritic branches decreases in DKK1-expressing neurons [54]. Interestingly, hippocampal dendritic arborization after depolarization depends on Wnt2 expression, which indeed belongs to the group of genes responsive to the transcription factor CREB, involved in plasticity events [55]. *In vitro* experiments in hippocampal neurons isolated from rats at embryonic day 18 have also shown a role for the non-canonical Wnt pathway function in dendritic arborization, in view that Wnt7b acting through Dvl1 increases dendritic branching by downstream activation of the Rac GTPase and the c-Jun N-terminal kinase (JNK) pathway. This effect is mimicked by Dvl1 overexpression and blocked by the Wnt antagonist sFRP, which is in line with the results from hippocampal neurons derived from a Dvl1 mutant mice [56]. Dvl1 is largely accumulated in developing axons where it directly regulates the function of the molecular complex PAR3-PAR6-aPKC (atypical protein kinase C) involved in axonal and dendritic differentiation in the hippocampus. The interaction of Dvl1 with aPKC resulted in its stabilization and activation of this atypical kinase. Additionally, treatment with conditioned media form cultured neurons expressing Wnt5a activates aPKC and promotes axonal differentiation. Together these results show that the effect of Wnt5a in the establishment of neuronal polarity depends on Dvl1-aPKC interaction [57] and demonstrates the critical role of Wnt during neuritic development.

Wnt signaling is also involved in presynaptic assembly and function. In cultured hippocampal neurons Wnt7a enhances the number of clusters of the presynaptic vesicle markers, synaptophysin, synaptotagmin and SV-2 through a mechanism independent of GSK3β activity and β-catenin stabilization in view that it does not require changes in Wnt-dependent gene expression. Moreover, administration of Wnt7a to hippocampal neurons induces spontaneous synaptic vesicle recycling and modulates the efficacy of synaptic vesicles exocytosis. These results point out the role of Wnt7a in the formation of new active sites for vesicle recycling and neurotransmitter release [26]. Other additional effect of Wnt7a on controlling neurotransmitter release seems to depend on its ability to relocalize nicotine acetylcholine receptors (α7-nAChRs) in presynaptic terminals. In hippocampal neurons Wnt7a induces the dissociation of APC from the β-catenin complex allowing the interaction between APC and α7-nAChRs [58,59]. As mentioned, Cerpa *et al.* [26] showed that Wnt7a decreases the paired pulse facilitation and increases the miniature excitatory post-synaptic currents (mEPSC) frequency enhancing neurotransmitter release at the CA3-CA1 synapses in hippocampal slices from adult rat. Similarly, Wnt3a rapidly increases mEPSC in embryonic hippocampal neurons depending on a fast influx of calcium ions from the extracellular space. Further, in this work the authors reported that the Wnt3a effects were also dependent on the presence of the LRP6 co-receptor suggesting a crosstalk between canonical and non-canonical Wnt signaling pathways [60]. In agreement with these findings the increased number of excitatory presynaptic sites elicited by Wnt3a was dependent on Fz1 activation in cultured neurons [61] (Fig. **2**).

There is evidence that emphasizes the involvement of Wnt receptors and ligands in the modulation of neuronal
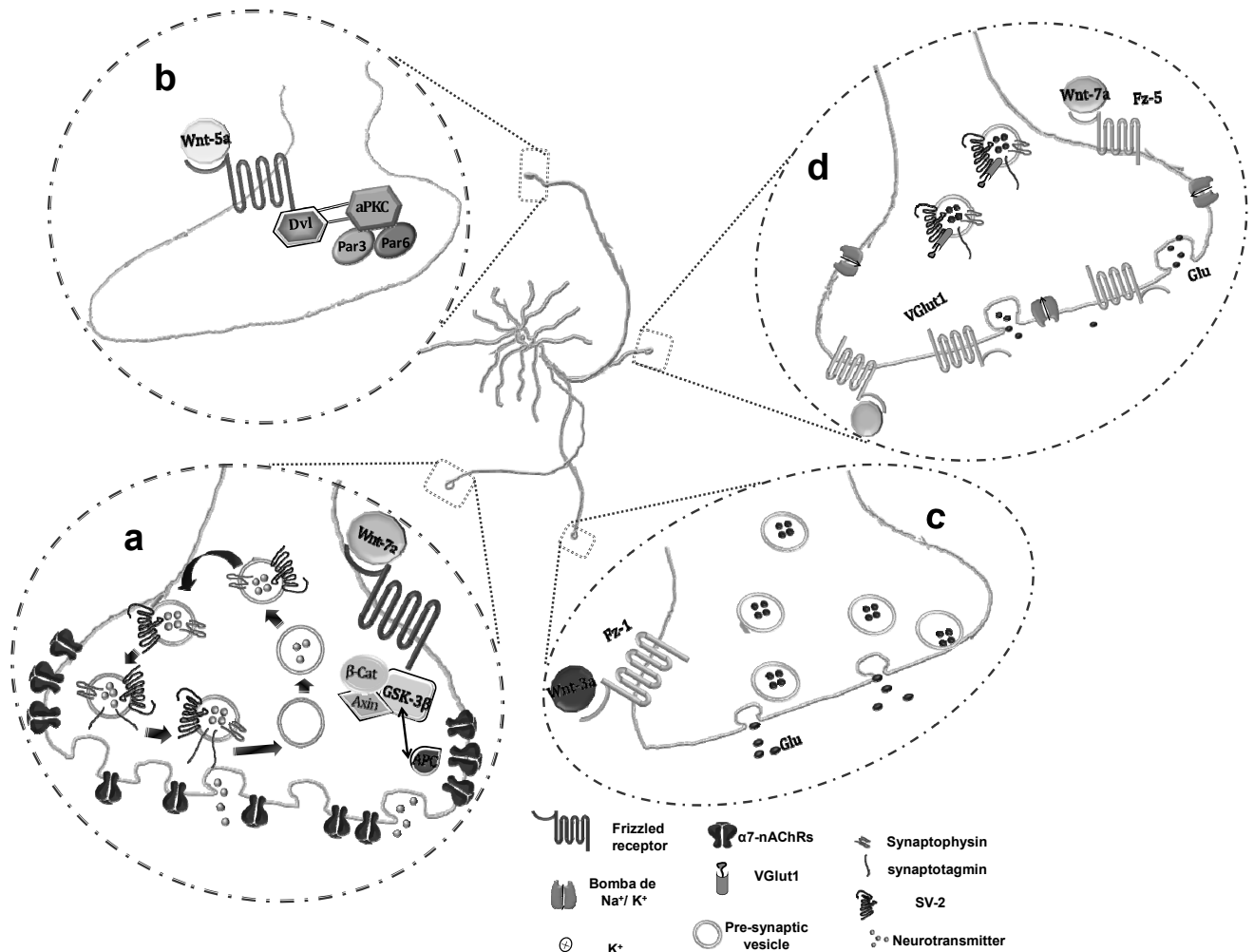
Fig. (2). Role of Wnt signaling in the presynapsis. (**a**) Wnt-7a increases the clusters of presynaptic proteins (synaptophysin, synaptotagmin and SV-2), enhances spontaneous synaptic vesicles recycling and relocalizes α7-nAChRs. This last effect depends on APC dissociation from the β-catenin complex enabling the APC and α7-nAChRs interaction. (**b**) Wnt-5a promotes axon differentiation acting on Dvl which accumulates in developing axons and modulates the complex PAR3-PAR6-aPKC involved in neuronal polarization. (**c**) Wnt-3a binds to Fz-1 and increases excitatory presynaptic sites. (**d**) During depolarization, Wnt-7a modulates presynaptic components, inducing the clustering of Fz-5 with VGlut1.

circuit assembly at pre- and post-synaptic levels. Depolarization of hippocampal neurons by high K^+ induces the colocalization of Fz5 receptors with the pre- and post-synaptic markers, VGlut1 and PSD-95, respectively and mediates the effects of Wnt7a on synapse formation [28]. Recently, Wnt7a has been found to act also at a postsynaptic level promoting specifically the formation of excitatory synapses in hippocampal neurons. Even more, Wnt7a enhances dendritic spine density and maturity while Wnt7a-Dvl1 deficient mice show defects in spine morphogenesis and in mossy fiber-CA3 synaptic transmission [62].

Interestingly, in hippocampal slices, Wnt5a enhances a calcium-dependent increase in the amplitude of field excitatory postsynaptic potentials (fEPSP). Besides, Wnt5a leads to short term changes in postsynaptic density protein-95 (PSD-95) distribution promoting its recruitment from a diffuse membrane pool to clusters in dendritic spines of

mature hippocampal neurons. This effect has been attributed to a JNK1-dependent phosphorylation of PSD-95 on Ser295 [63]. Wnt5a modulates the activity of glutamatergic synaptic transmission increasing fEPSP amplitude at CA3-CA1 synapses dependent of both, AMPA and NMDA components of the excitatory postsynaptic currents (EPSCs) [64-67]. The ligand Wnt5a also affects inhibitory synapses in hippocampal neurons through its ability to induce GABA$_A$ receptors surface expression by promoting their insertion and clustering in the neuronal membrane. This effect enhances the efficacy of GABA synapses at the postsynaptic level as evidenced by a raise in the amplitude of GABA-currents and increasing GABA$_A$ receptors, effects mediated by CAMKII activation [68] (Fig. **3**).

Long term exposure of cultured rat hippocampal neurons to Foxy-5 (formylated hexapeptide derived from the sequence of Wnt5a) that mimics the full action of the Wnt5a molecule,
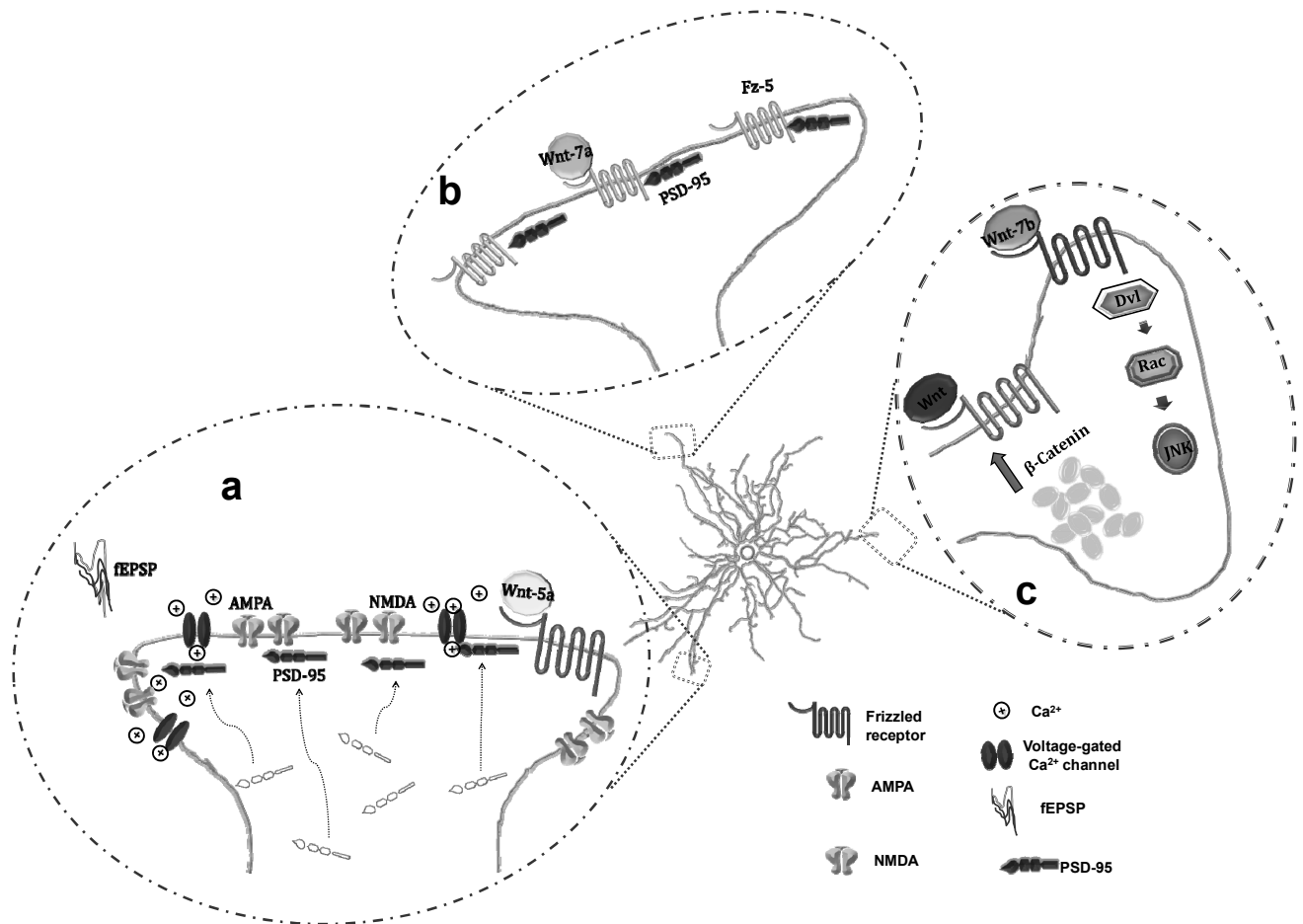
Fig. (3). Role of Wnt signaling in the postsynapsis. (**a**) Wnt-5a augments fEPSP amplitude, promotes the recruitment of PSD-95 to dendritic spines, and modulates AMPA and NMDA responses. (**b**) Wnt-7a modulates postsynaptic components, inducing the clustering of Fz-5 with PSD-95. (**c**) The increase in β-catenin contents mediate dendritic morphogenesis. Wnt-7b augments dendritic branching acting through Dvl1 and downstream by Rac and JNK activation.

show the importance of this ligand in the mechanisms involved in neurite length promoting the membrane cluster of the SV2 (synaptic vesicle protein 2) and PSD-95 proteins. In addition, Foxy-5 increased mEPSC amplitude and frequency [69]. Therefore, Wnt5a appears to be a postsynaptic mediator of synaptic differentiation and plasticity in the hippocampus stimulating dendrite spine morphogenesis, inducing de novo dendritic spines formation, and increasing the size of the preexisting ones [70].

Besides the described Wnt effect on structural plasticity new evidence also suggests a role for the Wnt signaling in functional hippocampal events. It has been shown that in hippocampal brain slices inhibition of Wnt signaling impairs long-term potentiation (LTP) while activation of Wnt/β-catenin pathway facilitates LTP expression [71]. Additional evidence supports the idea that GSK3β inhibition is essential for LTP, since phosphorylation of the kinase at the inhibitory residue Ser9 is enhanced upon LTP induction in CA1 and dentate gyrus *in vivo*. Moreover, LTP is impaired in transgenic mice conditionally overexpressing GSK3β and this deficit is reversed by lithium treatment [72]. As it has been proposed that LTP might be the electrophysiological correlate of learning and memory, these results suggest that

GSK3β is a key participant in these cognitive processes. The complexity of LTP expression may rely in the activity of several transduction pathways that act in concert to modulate diverse aspects of synaptic plasticity. In agreement, recent evidence shows that during the process of memory consolidation Notch signaling is transiently attenuated concomitantly with a transient increase in soluble β-catenin levels and GSK3β phosphorylation, indicating Wnt signaling activation in this event [73]. Another study reported that the Wnt-mediated suppression of GSK3β activity allows the activation of the mammalian target of rapamycin (mTOR) by strong synaptic activity, which is crucial for the induction of late phase of LTP and involves protein synthesis [74]. On the other hand previous studies revealed that GSK3β activation is required for memory reconsolidation in adult brain, as observed in Morris water maze performance of heterozygous GSK3β knockout mice impaired in their ability to form long-term memories [75].

Spatial learning has been associated with a selective increase of Wnt7 and Wnt5a levels in the hippocampus. The increase in Wnt7 levels was site and temporally specific since it was observed only in the granule cells of the dentate gyrus and expressed until 7 and 30 days after water maze

[76]. Recently, studies on the relationships between Wnt/ β-catenin pathway and spatial memory have described that the expression of the calcium/calmodulin-dependent protein kinase type IV (CaMKIV) is modulated by Wnt3a and that the administration of lithium restores the levels of CaMKIV and improves the spatial memory deficits in a transgenic model of AD [77]. CaMKIV participates in the regulation of CREB-dependent genes involved in memory and neuronal survival [78, 79].

Recent evidence suggests that the Wnt pathway is not only an inductor of plastic events, but that it can be activated by synapse-dependent experience. Studies in animals exposed to an enriched environment (EE) have shown an enhancement of Wnt7a/b levels in postsynaptic CA3 pyramidal neurons. On the contrary, Wnt signaling inhibitor sFRP1 suppresses the increase on synapse numbers elicited by EE and reduces synapse numbers in control mice. Interestingly, Wnt7a/b application to CA3 neurons mimicks the effects of EE of synapse numbers, while eliciting excitatory activity in CA3 neurons elevates Wnt7a/b levels [3].

Altogether, the evidence highlights the importance of Wnt function in the regulation of hippocampal synaptic plasticity along life in events ranging from the appropriate neuronal circuit assembly, and the modulation of pre and postsynaptic terminals remodeling to the cognitive performance in experimental models.

Wnt and Hippocampal Neurogenesis

Hippocampal neurogenesis takes place in the subgranular zone (SGZ) of the adult hippocampus, which constitutes a niche of stem and progenitor cell types that are continuously dividing and generating neurons that affect learning and memory [80]. The functional impact of new neurons on the existing neural circuitry and their contribution in hippocampal physiology under both healthy and pathological states has been demonstrated [reviewed in 81]. Each of the steps in neurogenesis is mediated by different signaling pathways, extracellular cues and cell-intrinsic mechanisms [10]. Wnt signaling and Wnt proteins play a critical role in stem cell self-renewal [82] as well as in the proliferation of the neural progenitor pool [83] and neuronal differentiation from neuronal precursor cells (NPCs) [84] in the developing central nervous system. Additionally, the canonical Wnt pathway has been shown to be involved in all different stages of adult neurogenesis in the SGZ [for review, see 85]. Evidence that Wnt signaling is a main pathway regulating neurogenesis comes from *in vivo* studies showing that in presence of the Wnt inhibitor sFRP2/3, there is a decrease in the percentage of adult hippocampal progenitors that differentiate into neurons. Furthermore, it has been shown that the orphan nuclear receptor Tlx activates Wnt/β-catenin signaling thus stimulating neural stem cell proliferation and self-renewal [86]. A recent work showed that Tlx can activate the expression of Wnt7a and the canonical Wnt/β-catenin pathway, suggesting that NSCs control their self-renewal in an autocrine manner [86]. In culture Wnt3 not only stimulates neuroblast proliferation but also instructs adult hippocampal progenitors to differentiate into neurons [87]. In particular, Wnt3a signaling has been shown to be

essential for the normal growth of the hippocampus during development [41] whereas in adult neural stem cells, β-catenin that accumulates in response to Wnt3a induces the transcription of Neurod1 [88] a transcriptional factor that is essential for neuronal differentiation, maturation and survival [89]. Interestingly, Wnt3 protein levels and NeuroD1 mRNA levels decrease with aging along with a reduction in neurogenic differentiation of NPCs in the aged brain. However, *in vitro* the expression of receptors involved in Wnt signaling does not seem to be altered in the aged NSC [90].

Adult hippocampal astrocytes express Wnt family members like Wnt3 [87,90] and adult hippocampal progenitors express receptors for Wnts and other components of the Wnt/β-catenin signalling pathway [87], thus accumulating evidence suggests that a muticellular niche is needed for providing the required molecular signaling [87,91-93] necessary for neurogenesis to take place. Astrocytes have been shown to instruct differentiation of neural progenitor cells (NPCs) [90,94,95] and Wnts released by astrocytes have been shown to promote NPCs proliferation by inducing the expression of the mitotic regulator survivin [93].

Neurogenesis (in particular neuronal progenitor proliferation) has been shown to diminish during aging [96,97] along with the functional decline of hippocampal mediated learning and memory. In line with these observations, the experimentally induced decrease in neurogenesis has been positively correlated with impairment on long-term retention in different memory tasks [80]. Until lately, the target genes of Wnt/β-catenin signaling responsible for the different stages involved in adult neurogenesis had been scarcely identified. However, in a recent work Miranda *et al.* show that Wnt mediated signaling in the aged brain of mice led to a decrease of survivin expression in NPCs and to a diminished proliferation, while survivin protein levels increased after the activation of the canonical Wnt pathway in NPCs. Interestingly, the authors showed that the decrease in the neurogenesis rate in the aged brain relies on a deficit of NPCs in cell cycle progression dependent on the reduced levels of chromosomal pass aging protein survivin. Furthermore, it was suggested that a decrease in the TCF/LEF promoter activity occurs during aging and is dependent on the down regulation of Wnt genes [93].

So far, experimental evidence suggests that fine regulations in Wnt signaling, in particular in the canonical pathway differentially regulate aspects of neurogenesis thus promoting proliferation, differentiation an even survival of neurons [98, 99] and that during aging, Wnt signaling acting though genes such as NeuroD1 and survivin is modified thus altering different stages of the neurogenesis process. In line, it has been shown that the deletion of the tumor suppressor APC from a subset of NPCs leads to a modest decrease in cell proliferation in the DG of young adult mice, and to a reduction of neuroblasts. Thus suggesting that APC depleted NSC fails to pursue their normal differentiation pathway [100].

Involvement of the Wnt Pathway in Neurodegenerative Diseases

Neurodegenerative diseases are generally associated to multiple neuronal abnormalities linked with changes at different levels of the structural and functional organization of neuronal networks, developmental defects or dysregulation of cellular signaling pathways that lead to synaptic atrophy and finally neuronal death. Altered Wnt signaling has been implicated in acute and chronic neuronal dysfunction associated to psychiatric conditions [101,102], ischemia [103,104], temporal lobe epilepsy [105], Alzheimer´s disease (AD) [12,106-110] and with some forms of frontotemporal dementia (FTD) [13].

The proposed relationship of altered Wnt signaling function with several psychiatric diseases is based on the evidence which shows that lithium has a positive effect on the treatment of the bipolar disorder symptom [111]. More recently various components of the Wnt pathway (e.g. Wnt2, Wnt7b and Fz9) have been found upregulated by chronic administration of antidepressant treatments. Particularly Wnt2 expression was reported to be highly elevated in the rat hippocampus, and the viral overexpression of Wnt2 was sufficient to produce antidepressant-like behavioral actions in an animal model of depression [112]. In this same line it has also been reported that chronic electroconvulsive sessions are accompanied by a CREB dependent increase of Fz6 mRNA levels in the granule cell layer of the dentate gyrus and in the CA3 region. Also, vector-mediated inhibition of the Fz6 gene results in depressive-like behavior in response to chronic unpredictable stress [113]. Thus, it is feasible to postulate that Wnt has a significant role in the modulation of highly complex emotional behaviors and its malfunctioning is implicated in the expression of some psychiatric symptoms.

Wnt antagonism seems also to take part in the induction of neuronal death as has been suggested by the fact that DKK1 levels are increased in the brain in different models of ischemia, excitotoxicity and exposure to amyloid-β peptide (Aβ) [reviewed in 114]. DKK1 is hardly detectable in the healthy brain, but it is strongly induced in brain tissue from AD patients or from patients with temporal lobe epilepsy and hippocampal sclerosis [109].

One of the neurodegenerative diseases in which Wnt signaling has been extensively documented is AD. More than a decade ago, it was suggested that sustained loss of function of Wnt signaling would determine the onset and development of AD [12]. Presenilin (PS) mutations that appeared in familiar AD were associated with altered intracellular trafficking and turnover of β-catenins as well as with down-regulated Wnt signaling [115,116].

Many other components of the Wnt signaling pathway have been implicated in the molecular pathology of AD. In Drosophila models of neurodegeneration, Wnt dysregulation can lead to neurofibrillary pathology [117] and the overexpression of Dvl1 increases the non-amyloidogenic α-secretase cleavage of APP [118] while DKK1-neutralizing antibodies are protective against synaptic loss in mouse models of AD [119]. Many of the pathological neuronal

responses attributed to Wnt dysregulation in AD come from multiple evidences about the Wnt-dependent GSK3β activity regulation [reviewed in 120]. Experimental data suggest that GSK3β regulates APP processing [121,122] and prevents Aβ toxicity [123]. On the other hand, Aβ seems to interfere with the Wnt canonical pathway as well, leading to increased GSK3β function [109, 124], generating a vicious cycle that might exacerbate neuronal injury. Regarding to the canonical Wnt signaling pathway, the gene for LRP6 co-receptor has been identified as a risk factor for late-onset AD in ApoE4-negative individuals [125]. Interestingly, it has been reported that the Wnt pathway might be inhibited by ApoE protein, which likely binds to the coreceptor LRP5/6 [126]. Moreover, the ApoE4, implicated in sporadic AD [127] may activate GSK3β [128,129]. The canonical Wnt signaling inhibitor, DKK1 induces GSK3β-mediated tau phosphorylation in the hippocampus [130] as well as in cultured neurons [131]. Interestingly, DKK1 has been found elevated and colocalizing with neurofibrillary tangles and dystrophic neurites in degenerating neurons of AD brains [109]. Taken together, these evidences suggest that Wnt signaling might be a crucial pathological pathway that contributes to AD-related neurodegeneration and a link between amyloid and tau pathology.

Recent studies have found that the Wnt5a ligand and its receptor Fz5 were up-regulated in the brain of a mouse model of AD and in cultured cortical neurons by Aβ exposure [132]. Interestingly in this report the authors also found that Wnt5a signaling elicited the expression of proinflammatory cytokines such as interleukin 1β (IL-1β).

Microglia plays an important role in inflammation and the hippocampus is densely populated with this type of cell. During aging and pathological conditions, the activated form of microglia has been found to be increased in the hippocampus [133,134] and the canonical Wnt signaling seems to have a role in microglia activation. Halleskog *et al.*, [135] reported an increase in β-catenin expression in microglial cells that undergo proinflammatory morphogenic transformation in pathogenic neuroinflammation such as occurs in AD. In addition, in cultured microglia expressing both receptors, Fz and LRP5/6, Wnt3a can stabilize β-catenin and specifically enhance the expression of proinflammatory immune response genes that exacerbate the production and release of IL-6, IL-12 and tumor necrosis factor α (TNF-α). Given the significant role of neuro-inflammation in AD, the participation of Wnt in this process deserves to be analyzed in depth.

Recently, an elegant functional genomic analysis has shown a major role of Wnt dysregulation in brain samples from FTD associated with mutations in the progranulin gene and an important increase of the Fzd2 in a knockout progranulin gene mouse model [13].

Together, the above revised studies suggest that excessive and reduced Wnt function acting through the three signaling pathways may be involved at different steps of the neurodegenerative process in the adult brain. The specific disease outcome of such Wnt uncontrolled function may depend on the neuronal metabolic context and the cellular

type (neurons, astrocytes or microglia) where Wnt signaling is exacerbated or inhibited.

Therapeutic Implications

An ideal compound with neuroprotective potential against hippocampal dysfunction should decrease or delay neuronal death, enhance neurogenesis, improve cognitive function, and control neuroinflammation. In view of the role played by Wnt pathways in maintaining neuronal homeostasis in the healthy hippocampus and their potential participation in brain disease it is feasible to suggest the targeting of Wnt signaling pathways to modify the progression of synaptic impairment, inflammation and ultimately neuronal death. In addition, the fact that Wnt signaling is involved in different steps of neural stem cell renewal suggests that purified Wnt proteins can be used as tools for cell replacement therapies in the treatment of neurodegenerative diseases and brain injury [86]. Adult neural stem cells are promising as therapeutic strategies given their potential to proliferate and differentiate into neurons and glia. On one hand, new neurons may incorporate into preexisting circuits, thus providing functionality to an altered circuit, but also, glial cells are part of the cellular niche needed for neuronal proliferation and survival. Among the different molecular signaling pathways for neurogenesis to occur, Wnt has shown to be a crucial one. It has been shown that following exercise in aged animals astrocytic Wnt3 levels increase thus re-stimulating different stages of neurogenesis [90]. Therapeutic strategies to increase Wnt positive effects may include the use of lithium which has been shown to enhance proliferation of adult hippocampal progenitors *in vitro* inducing them to become neurons at particular concentrations. Moreover, experiments in which lithium is administered to a mouse strain encoding a double-mutant form of APP, have shown to stimulate adult hippocampal progenitor cells proliferation and neuronal differentiation along with the improvement in cognitive functions through the inhibition of GSK3β and subsequent activation of Wnt/β-catenin signaling [136]. In a recent work, Matrisciano *et al.* [137] show that chronic mild restrain stress, which associates to hippocampal damage leads to an over expression of DKK1 levels and that inducing stress in a mice strain where DKK1 levels are reduced, not only diminishes neuronal loss, but increases neurogenesis and dendritic arborization [137].

Wnt signaling also mediates a positive neuron-astrocyte crosstalk for neuroprotection as has been found in mesencephalic neurons where Wnt1 effects depends on the astroglial response to oxidative stress and inflammation after neuronal injury, and requires Fz1 receptor and β-catenin stabilization to transmit pro-survival signals into the neuronal nucleus [138].

However to deep into the design of a molecule with Wnt positive effect, it should be considered that a fine balance between different Wnt ligands exists in the brain. For example, experimental models of behavior suggest that the inability of rats to tolerate stressful environments and the resulting anxiety may be associated with the enhanced expression of the Wnt2 gene and the resistance to the increase of Wnt7b levels in the ventral tegmental area [139] thus stressing the possibility that these ligands act in concert

to promote brain adaptation when facing harmful situations. The functional reestablishment of a proper balance between different Wnt agonists and antagonist of the canonical Wnt pathway in anxiety as well as in major depression disorders may represent the biggest challenge to restore neuronal homeostasis in malfunctioning cells.

Previous work has proposed that different canonical and non-canonical Wnt agonists, acting at different levels can be protective in AD [140]. Particularly the non-canonical Wnt activator, Wnt5a has a defending role against synaptic failure evoked by Aβ oligomers making this molecule a possible therapeutic target for AD therapy [65,67]. Likewise the search for compounds directed to neutralize the action of the Wnt inhibitors, e.g. DKK, may be a promising avenue for developing neuroprotective drugs for this devastating disease.

Given that Fz2 was found selectively elevated in the brain of patients with FTD and Fz2 activates a non-canonical Wnt pathway it is suggested that modulation of this pathway could be therapeutically beneficial for this demential illness.

However pharmacotherapy aimed at modulating Wnt pathways should consider the timing for application as well as the targeted cell type and brain region in order to provide specificity to the molecular processes underlying a specific neurodegenerative disease. For example, in terms of brain inflammation enhanced β-catenin signaling in microglia could be either beneficial or detrimental for the disease outcome. Also, Wnt activates proliferative pathways that may lead to uncontrolled cellular proliferation and tumor growth but as previously discussed may also represent a powerful therapeutic strategy for neuronal protection.

CONCLUSIONS

Wnt signaling regulates many aspects of hippocampal development and continues critically involved in the adult regulating plasticity mechanisms. However the complexity of events that are under the control of the Wnt pathways poses the need to perform further studies that unravel the biological roles of the highly dynamic Wnt signaling through different stages of brain maturation as well as in disease. The expression of different genes and proteins of the Wnt signaling pathway in regions of the central nervous system that control learning and memory opens the possibility to understand how they can influence the processes of neuronal plasticity that are altered in dementias and neuropsychiatric disorders. Thus, new pharmacological developments can be specifically targeted at a particular disease entity to enhance treatment efficacy.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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Susceptibility to GSK3β-Induced Tau Phosphorylation Differs Between the Young and Aged Hippocampus after Wnt Signaling Inhibition

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Abstract. The abnormal phosphorylation of the microtubule-associated protein tau is a prominent aspect of Alzheimer's disease (AD). Considerable evidence suggests that glycogen synthase kinase 3β (GSK3β) and the protein phosphatase 2A (PP2A) are involved in normal and pathological tau phosphorylation. However, the mechanisms underlying a shift of the phosphorylation/dephosphorylation balance that leads to abnormal tau phosphorylation remains unknown. The canonical Wnt pathway negatively regulates GSK3β activity, and this signaling pathway has also been found to be dysregulated in the AD brain. Here, we report that the Wnt antagonist Dkk-1 selectively increases tau phosphorylation in the hippocampus of aged rats at Ser199/202, Ser396/404, and Ser214 sites. In the aged hippocampus, the inhibition of Wnt signaling is also accompanied by reduced PP2A activity. This study suggests that aging promotes tau hyperphosphorylation after Wnt inhibition, due to an imbalance between GSK3β and PP2A activities.

Keywords: Aging, GSK3β, hippocampal slices, PP2A, tau phosphorylation, Wnt signaling

INTRODUCTION

The microtubule-associated protein tau is expressed throughout the central nervous system, where it is predominantly associated with axonal microtubules (MT) and is expressed at lower levels in dendrites, where it is involved in signaling functions [1]. Tau contains sequence motifs that promote its association with tubulin, leading to MT stabilization. It is believed that in a hyperphosphorylated state, tau proteins detach from axonal MT, which favors their aggregation into the paired helical filaments (PHF) [2, 3]. The accumulation of hyperphosphorylated tau proteins in neurons is the hallmark lesion in many age-related neurodegenerative diseases, including Alzheimer's disease (AD), Pick's disease, corticobasal degeneration, progressive supranuclear palsy, and frontotemporal dementia with Parkinsonism linked to chromosome 17, which are collectively referred to as tauopathies (reviewed in [4]). In AD, conformational changes and the truncation of tau have been reported [5–9]. However, the most established cause of dysfunctional tau in neurons is the abnormal accumulation of hyperphosphorylated forms [10–13]. Hence, numerous studies have focused on identifying the protein kinases and phosphatases that regulate the balance of the tau phosphorylation/dephosphorylation rate and the signaling

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pathways implicated in this process. Considerable evidence suggests that glycogen synthase kinase 3β (GSK3β) and the protein phosphatase 2A (PP2A) are involved in the tau pathology underlying AD [14]. GSK3β was recognized as a primary kinase involved in tau phosphorylation, as was apparent from the first studies that termed it tau protein kinase-I [15], and is one of the major enzymes mediating tau hyperphosphorylation at the residues implicated in AD [16]. Furthermore, GSK3β overexpression results in neurodegeneration and increased tau hyperphosphorylation in transgenic models [17], and the inhibition of GSK3 reduces the phosphorylation and aggregation of tau [18]. Importantly, the overexpression of GSK3β in the dentate gyrus results in tau-dependent neurodegeneration of this hippocampal region [19]. In the brains of AD patients, GSK3β co-localizes with neurofibrillary tangles [20], and active GSK3β is present in the neuronal cytoplasm of neurons with tangle-like inclusions when abnormal tau phosphorylation starts [16]. GSK3β is unique because it is constitutively active, and upstream signals downregulate its activity via phosphorylation at specific residues (reviewed by [21]). The canonical Wnt pathway is one of the major signaling pathways that negatively regulate GSK3β activity. Although the role of Wnt proteins in mature neurons remains largely unexplored, recent data indicate that Wnts are important mediators of neuronal function, neuronal morphology, neurogenesis, and synaptic plasticity [22]. Additionally, dysregulation of the Wnt pathway has been found to be involved in the physiopathology of AD [23]. Expression of the Wnt antagonist Dickkopf-1 (Dkk-1) is induced during neurodegenerative processes associated with AD [24] and brain ischemia [25]. Dkk-1 is expressed by degenerating neurons in the brain from AD patients, where it colocalizes with neurofibrillary tangles and dystrophic neurites [24]. In cultured neurons, Dkk-1 causes GSK3β activation and tau phosphorylation

[26]. Active GSK3β was found to co-localize with Dkk-1 and phospho-tau in a transgenic mouse model expressing familial AD [27]. Taken together, these data suggest that inhibiting the canonical Wnt pathway may result in tau hyperphosphorylation via increased GSK3β activity. So far, no studies have addressed whether aging contributes to increased vulnerability to tau phosphorylation through a GSK3β-dependent mechanism. Indeed, this issue is relevant given that the most recognized risk factor for sporadic AD is aging. Therefore, we investigated the effects of GSK3β inhibition and activation by the Wnt inhibitor Dkk-1 on tau phosphorylation at AD-related phosphoepitopes in hippocampal slices from young and aged rats as well as the underlying mechanisms.

MATERIALS AND METHODS

Animals

Male Wistar rats (3 or 18–20 months old) were used throughout the study and handled with all precautions necessary to diminish their suffering according to the Regulations for Research in Health Matters (México) and with the approval of the local Animal Care Committee.

Metabolically competent hippocampal brain slices

Metabolically active brains slices from rat hippocampus were obtained as described by Gong et al. [28], with slight modifications. Briefly, male Wistar rats (3 or 18–20 months old) were deeply anesthetized with sodium pentobarbital anesthesia, and the brains were removed. The brains were immersed in ice-cold artificial cerebrospinal fluid (aCSF) (126 mM NaCl, 3.5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.3 mM MgCl$_2$, 2 mM CaCl$_2$, 11 mM glucose, 2 mM NaHCO$_3$, pH 7.4) for 5 min and oxygenated with a mixture of

Table 1
Identities and characteristics of the primary antibodies employed in this study

Antibody	Site	Dilution	Source
Tau phospho- Ser199/202	p-Ser199/202	1 : 1000	Chemicon, USA
PHF-1	p-Ser396/404	1 : 50	Kindly provided by Dr. Peter Davies
Tau phospho- Ser214	p-Ser214	1 : 500	Abcam, UK
Tau46	Total tau	1 : 500	Cell Signaling Technology, USA
Anti-GSK3β (phospho-Ser9)	p-Ser9	1 : 1000	Cell Signaling Technology, USA
Phospho-β-catenin	p-Ser33/37/Thr41	1 : 250	Cell Signaling Technology, USA
PP2Ac	Catalytic subunit	1 : 2000	Millipore, USA
Anti-active-β-catenin	Ser37/Thr41 (unphosphorylated)	1 : 1000	Millipore, USA
Anti-PP2A α (phospho Y307)	p-Tyr 307	1 : 3000	Abcam, UK
Anti-PP2A α (methyl L309)	Methyl Leu-309	1 : 1000	Abcam, UK

95% O_2 and 5% CO_2 during the entire procedure. The brains were fixed in a plate and placed in a vibroslice chamber (Campden Instruments, Ltd., IN, USA) immersed in ice-cold aCSF. Hippocampal coronal slices (400 μM thick) were obtained and were equilibrated for 1 h at room temperature. After the recovery period, the slices were placed in a 37°C water bath and incubated in aCSF in the presence or absence of 6-bromo-indirubin-3′-oxime (6-BIO, 20 μM, Calbiochem, Merck-Millipore, Germany), a selective and potent GSK3β inhibitor, or with the canonical Wnt signaling inhibitor (Dkk-1 200 ng/ml, R&D Systems, MN, USA) for 3 h. After incubation, the hippocampus was sonicated in 200 μl lysis buffer containing Tris HCl pH 7.5, NaCl 50 mM, Nonidet P40 1%, deoxycholate 0.5%, COMPLETE protease inhibitor cocktail table (Roche, UK), and Halt phosphatase inhibitor cocktail (Thermo Scientific, Inc., USA). The homogenates were centrifuged at 9500 × g for 30 min,

and the supernatants were collected and stored at −80°C until use.

Western blotting

The homogenates of the hippocampal slices were used for protein blotting, and the protein (25–40 μg) was subjected to 12% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were blocked with a solution composed of TBS with 0.1% tween 20, 5% non-fat dry milk, and 0.3% horse serum for 3 h at room temperature. After blocking, the membranes were incubated with primary antibodies (Table 1) overnight at 4°C. The membranes were washed 3 times with TBS 0.1% tween 20, incubated with either goat anti-mouse IgG or goat anti-rabbit horseradish peroxidase-coupled secondary antibodies (1 : 7000, Santa Cruz Biotechnology, Inc., USA) for 1 h at room temperature and detected by
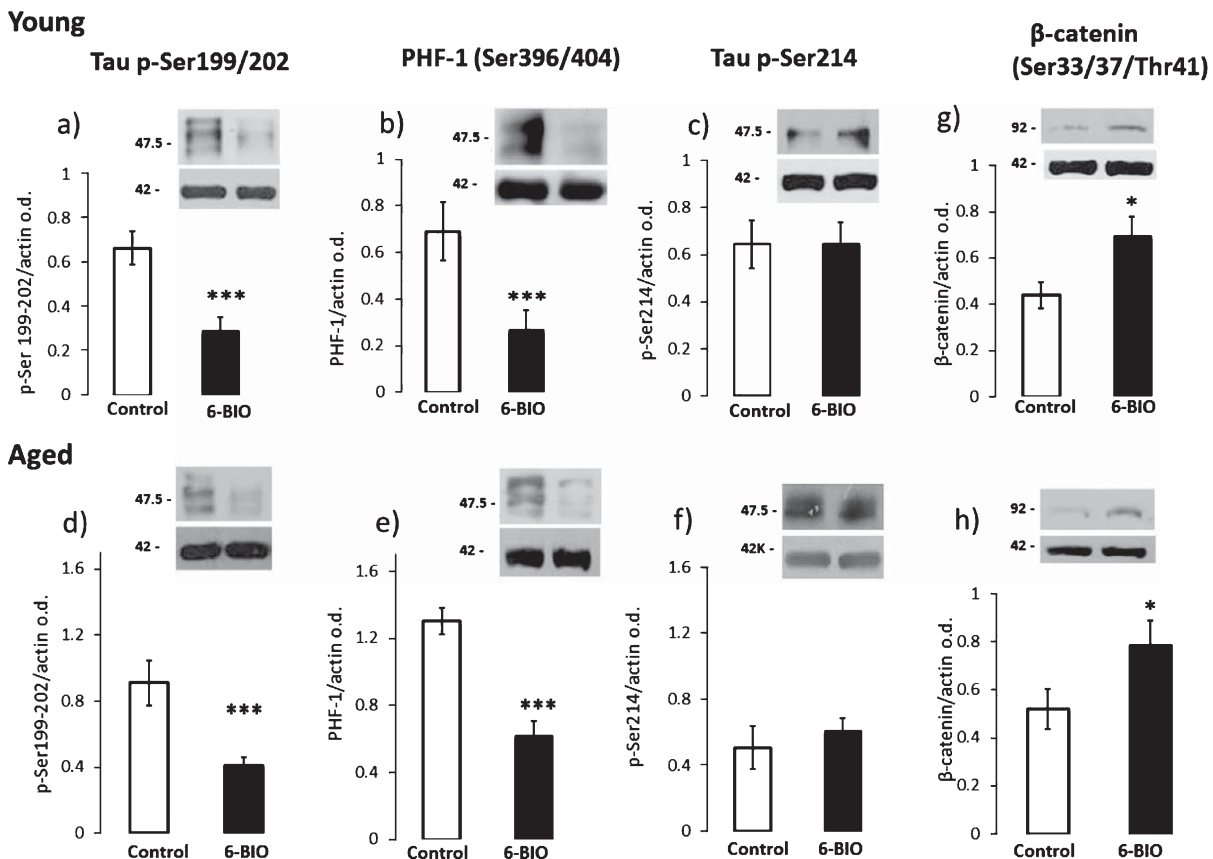

Fig. 1. Decreased tau phosphorylation at specific residues after selective inhibition of GSK3β is accompanied with increased active β-catenin levels. Hippocampal slices of young (3 months old) and aged (18–20 months old) rats were incubated with 6-BIO (20 μM) or vehicle (0.07% DMSO) for 3 h. GSK3β inhibition decreased the expression of phospho-Ser199/202 (a, d) and phospho-Ser396/404 (PHF-1 site) (b, e), while phospho-Ser214 remained unchanged (c, f). The active β-catenin (unphosphorylated Ser33/37/Thr41) pool is increased after GSK3β inhibition (g, h). Bars represent the densitometric analysis and are the mean ± SEM from 7 to 10 independent experiments. *$p \leq 0.05$, ***$p \leq 0.001$.

chemiluminescence (Millipore, USA) on Kodak X-Omat films. A monoclonal antibody against β-actin was used as an internal loading control. The negative controls were incubated without primary antibody.

Immunoprecipitation for protein phosphatase 2A (PP2A) activity assay

Metabolically active brain slices treated with Dkk-1 (200 ng/mL) or aCSF were used to measure PP2A activity using the PP2A activity assay kit (Millipore, USA) according to the manufacturer's instructions. Briefly, the hippocampal slices were homogenized in a buffer containing 20 mM imidazole-HCl; 2 mM EDTA; 2 mM EGTA; pH 7.0; 10 μg/ml of aprotinin, leupeptin, pepstatin; 1 mM benzamidine; and 1 mM PMSF. The homogenates were centrifuged at 2000 × g for 5 min at 4°C, and the supernatants were collected and incubated with anti-PP2A C subunit and protein A agarose beads for 2 h at 4°C. The beads were washed 3 times with TBS and once with the Ser/Thr Assay Buffer. The immunoprecipitated proteins were incubated with 60 μl of threonine phosphopeptide (K-R-pT-I-R-R) at 30°C. The samples were assessed for PP2A activity using Malachite Green phosphate detection buffer. After 15 min of incubation, the samples were analyzed in a spectrophotometer at 630 nm. The results are presented as the percent of the activity relative to control hippocampal slices.

Data analysis

Group differences were analyzed using a paired Student's *t*-test. The densitometric analysis of the western blot bands was conducted using NIH ImageJ software. The normalized protein values were obtained from actin bands from each experimental condition, and the results are expressed as the optical density of the normalized values (o.d.). $p < 0.05$ was considered to represent statistically significant differences.

RESULTS

We first examined whether the level and site-specific phosphorylation of tau in the hippocampal homogenates of rats is affected by the constitutive activity of GSK3β at different ages. We used the compound 6-BIO because it is a specific inhibitor of GSK3β [29]. The hippocampal slices obtained from young (3 months old) and aged (18–20 months old) rats showed similar sensitivities to GSK3β inhibition with regard to the basal expression of phospho-Ser199/202
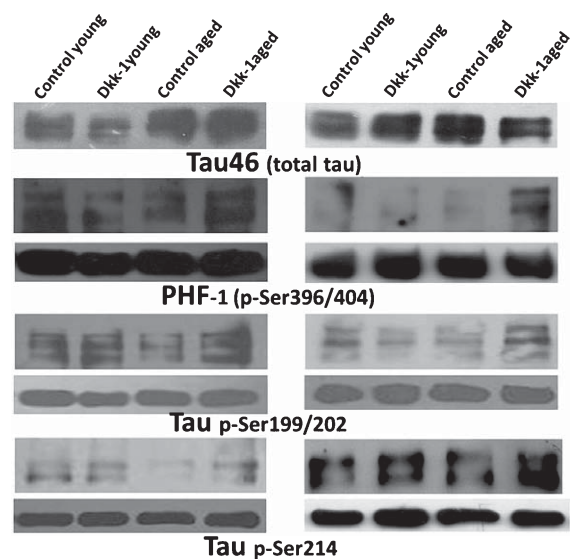

Fig. 2. Effect of Dkk-1 on tau phosphorylation at different ages. Hippocampal slices were exposed for 3 h to the Wnt inhibitor, Dkk-1. Western-blot of four samples running in parallel obtained from different young and aged rats showing changes on total tau (tau46) and phosphorylation at the Ser199/202, Ser396/404, and Ser214 epitopes.

and phospho-Ser396/404 (PHF-1 site) (up to 50% reduction, $p < 0.001$) (Fig. 1a, b and d, e). However, the expression of phospho-Ser214 was not affected by 6-BIO at any age (Fig. 1c, f). These results indicate that tau phosphorylation at the specific sites, phospho-Ser199/202 and PHF-1 strongly depends on the constitutive activity of GSK3β in both the young and aged hippocampus and that GSK3β inhibition did not modify the total tau contents because the phosphorylation levels of the Ser214 residue did not change. We next examined whether the inhibitor 6-BIO alters β-catenin, which are involved in the canonical Wnt signaling and have a degradation pathway that is mediated by GSK3β activity. As expected, we found a significant increase ($p < 0.05$) in the active (unphosphorylated) β-catenin contents in hippocampal slices from young and aged animals (Fig. 1g, h) indicating that inhibition of basal GSK3β activity for a short, 3-h period appears to exert an effect on active β-catenin at both ages.

Because GSK3β activity is negatively modulated by Wnt signaling, we next examined whether the canonical Wnt inhibitor Dkk-1 modulates tau phosphorylation differentially by age. We found that the exposure of young hippocampal slices to Dkk-1 did not influence significantly the levels of any of the tau phosphorylation sites. However, in hippocampal slices from aged rats, increased phosphorylation
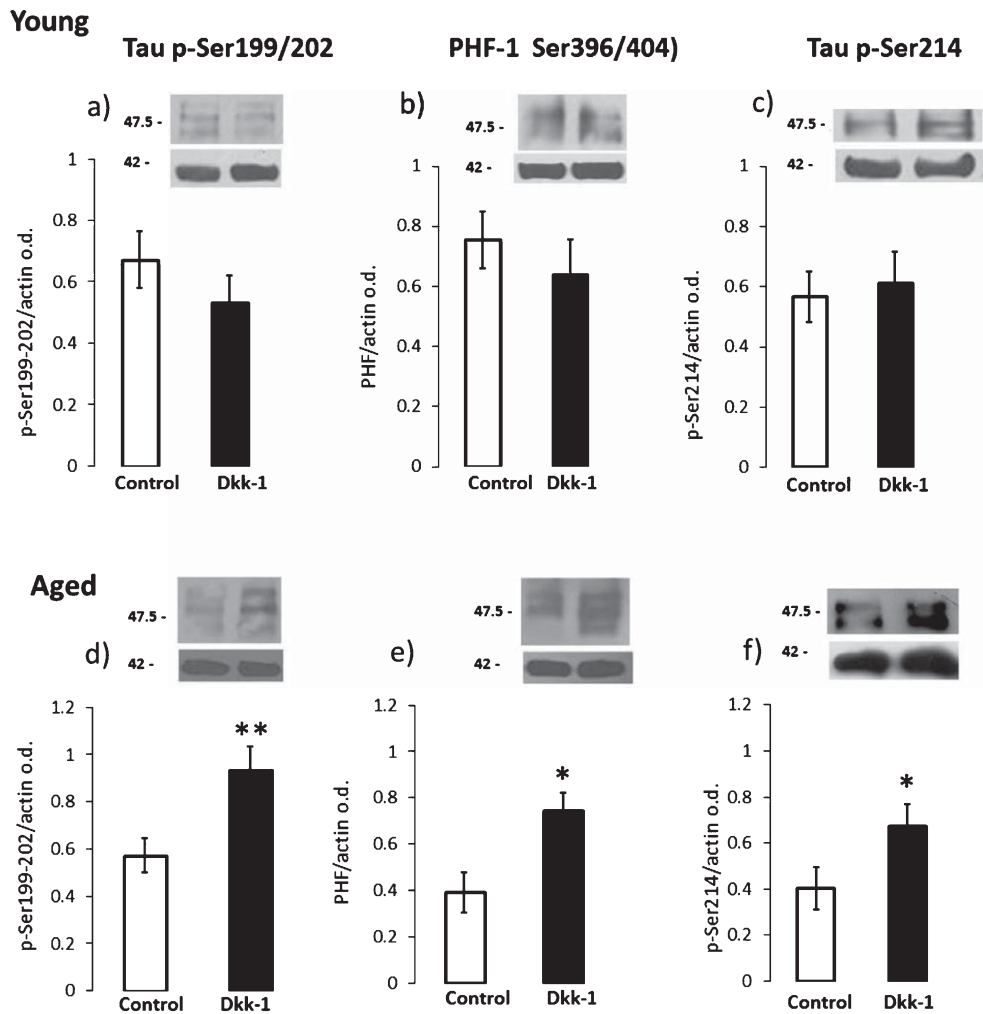
Fig. 3. Effect of Dkk-1 on tau phosphorylation at different ages. Hippocampal slices were exposed for 3h to the Wnt inhibitor. Dkk-1 did not affect significantly tau phosphorylation in young rats (a, b, c). However, in the aged hippocampus, Dkk-1 increased tau phosphorylation in all studied epitopes (d, e, f). Representative western blots are shown. Bars represent the densitometric analysis and are the mean ± SEM from 7 to 16 independent experiments. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

levels of Ser199/202 (160%, $p < 0.01$) and Ser396/404 (190%, $p < 0.05$) were observed (Figs. 2 and 3a, b compared with d, e). Remarkably, after incubation with Dkk-1, a significant increase in the expression of phospho-Ser214 (170%, $p < 0.05$) was also found without increase levels of tau analyzed by an independent phosphorylation epitope, Tau46 (Figs. 2 and 3c compared with f). Because Dkk-1 inhibits the endogenous Wnt/β-catenin pathway, we examined the levels of GSK3β phosphorylated at Ser9 and as well as β-catenin contents (Fig. 4). The expression levels of phospho-Ser9 were significantly decreased ($70 \pm 12\%$ $p < 0.04$) in hippocampal slices from young rats and remained unchanged in old age. However after Dkk-1, an increased expression of phospho-β-catenin

was found in both young and aged rat hippocampus (230% and 200%, respectively, $p < 0.01$) concomitant with decreased levels of unphosphorylated (active) β-catenin pool in the young and aged hippocampus (67% and 65%, respectively, $p < 0.05$). These results indicate a β-catenin destabilization pattern after GSK3β activation.

Given that PP2A may regulate the activity of GSK3β [30] and is also involved in the phosphorylation state of tau [31, 32], we tested the possible involvement of this phosphatase in the increased sensitivity of the aged hippocampus to Dkk-1 effects. The PP2A activity and the protein contents of the PP2A catalytic subunit are shown in Fig. 5. We found a marked reduction in PP2A activity of nearly 30% ($p < 0.01$) in the aged
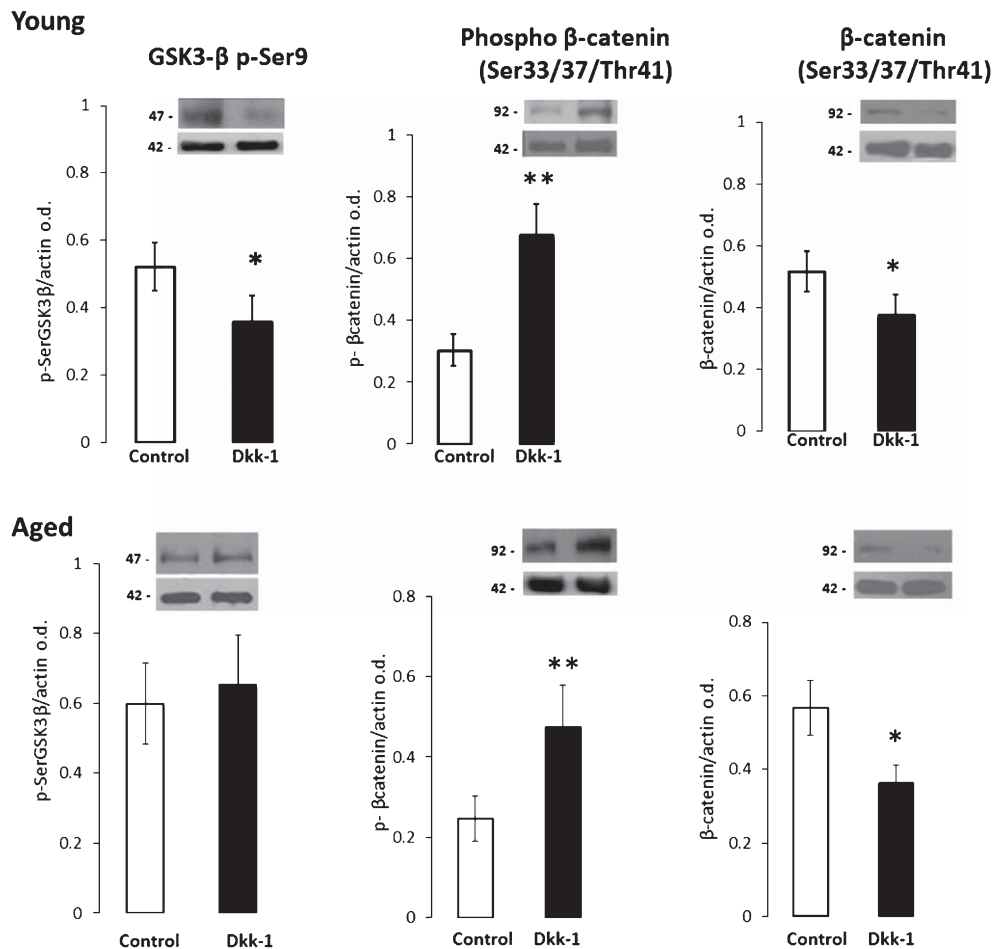
Fig. 4. Effect of Dkk-1 on GSK3β phosphorylation and β-catenin levels in young and aged hippocampal slices. In the young hippocampus, exposure to Dkk-1 reduced the phospho-Ser9 content, indicating an increased activation of GSK3β. After incubation with Dkk-1, the expression of phospho-β-catenin is achieved, resulting in a decrease in the total active β-catenin pool in the young and aged hippocampus. Representative Western blots from 7 to 12 independent experiments. Bars represent the densitometric analysis and are the mean $\pm$ SEM. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

hippocampus after treatment with Dkk-1 (Fig. 5a, c) that was not accompanied by changes in the expression of the PP2A catalytic subunit (Fig. 5b, d). To get insight on the mechanism associated with the reduction of PP2A activity, we analyzed two posttranslational modifications of the PP2A catalytic subunit. As observed in Fig. 6, the methylation of PP2A on the Leu309 residue was significantly reduced after Dkk-1 in the hippocampus from aged rats (45%, $p < 0.02$) while the content of the phospho-Tyr307 was found slightly increased correlating with a down regulation of PP2A activity.

DISCUSSION

In the search for age-related changes that are risk factors for developing AD, one important question that

remains unanswered is what metabolic alterations participate in shifting the balance between protein kinase and phosphatase activities that may contribute to tau hyperphosphorylation. However, the underlying key signaling pathways in the aged brain that might modify the balance between protein kinases and phosphatases are not completely known. In this regard, dysregulation of the Wnt pathway seems to be crucial in increasing GSK3β activity during aging and might have a pivotal role in the induction of AD-related phospho-epitopes, as we show in the present study. Herein, we have reported that the selective inhibition of constitutively active GSK3β by 6-BIO strongly downregulates tau phosphorylation at two main phosphorylation sites, Ser199/202 and Ser396/404, similarly in young and aged hippocampus. These results suggest a significant association between constitutive GSK3β activity
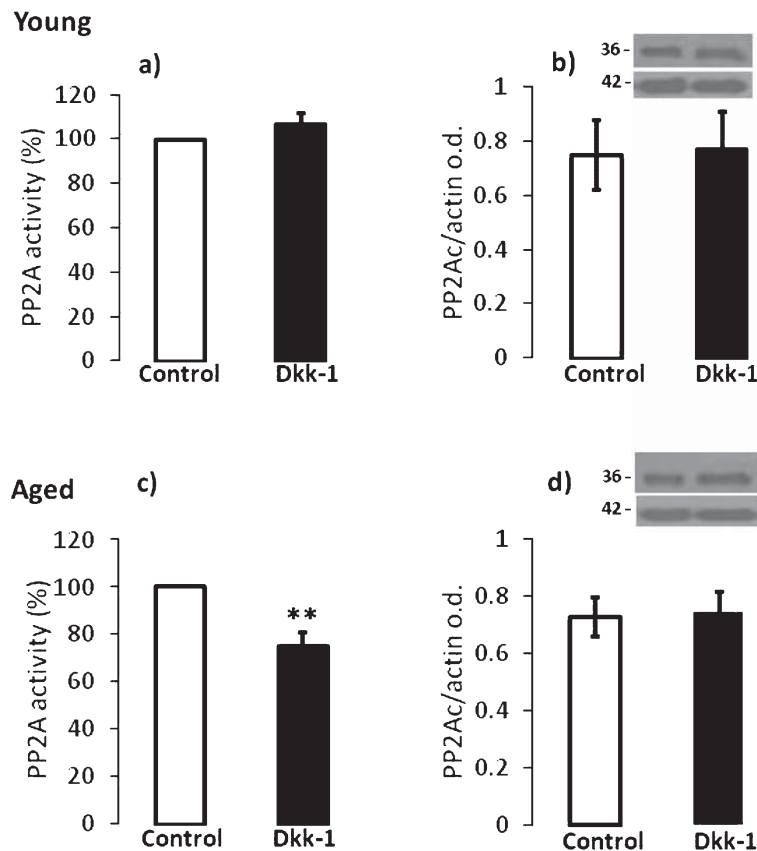
Fig. 5. Wnt signaling inhibition decreases PP2A activity in aged rats. Hippocampal slices of young and aged rats treated with Dkk-1 or vehicle (PBS) were used to measure PP2A activity. In the young hippocampus, no changes in activity (a) or PP2A catalytic subunit content (b) are observed. In aged hippocampus, a significant decrease in PP2A activity is observed (c), without changes in the catalytic subunit (d). Bars represent the densitometric analysis of the western blot results (b, d) and are the mean $\pm$ SEM. $**p \leq 0.01$.

and the expression of these two phosphorylated sites *in vivo*. In contrast, the Ser214 site was not affected at any age by GSK3β inhibition. However, the upregulation of GSK3β activity by Dkk-1 selectively increased tau phosphorylation in the aged hippocampus at the three sites studied: Ser199/202 (which is part of the AT8 site), Ser396/404 (PHF-1), and Ser214. It is noteworthy that p-Ser214 immunoreactivity was increased in the hippocampus of aged rats after Dkk-1 exposure. The expression of this phosphoepitope is particularly interesting, as it can be phosphorylated by protein kinase A (PKA) if a previous phosphorylation of the Thr212 is achieved by GSK3β; furthermore, it is one of the most specific sites for Alzheimer's tau (AT100) that strongly inhibits tau-microtubule interactions [33–37].

Although the majority of GSK3β substrates usually require prior phosphorylation by another kinase at a priming residue for subsequent phosphorylation by GSK3β [38], it has been demonstrated, at least

in vitro, that GSK3β is able to phosphorylate tau at the PHF-1 site directly and efficiently [39]. However, taking into account that several protein kinases have been shown to act as priming enzymes for GSK3β phosphorylation, including the cyclin-dependent kinase, Cdk-5 [40–42], it is possible that the increased sensitivity to tau phosphorylation by Wnt inhibition in the aged brain might reflect widespread protein kinase dysregulation. In fact, the regulatory subunit of Cdk5, p25, and the PKA regulatory subunit, R1β, have been found to be slightly increased in brains from old rats [43]. Studies in human AD brains have suggested that some tau sites are phosphorylated early in the pathological process and have implicated MARK and MAPK kinases, but a later stages of disease progression, the appearance of immunoreactivity to the antibodies AT8 (Ser199/202, Thr205), AT100 (Ser214, Thr212), and PHF-1 points to a pronounced activity of GSK3β and Cdk5 [36]. The latter kinase has also been
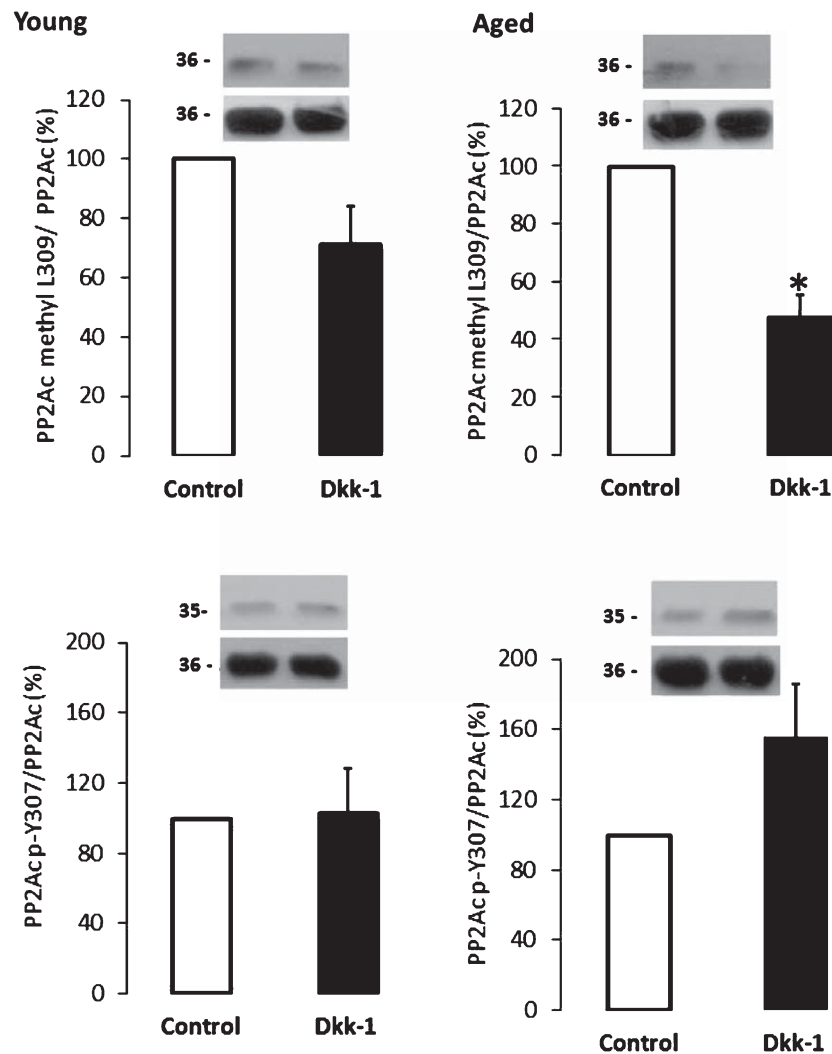
Fig. 6. Effect of Dkk-1 on PP2A phosphorylation and methylation at different ages. Hippocampal slices were exposed for 3 h to the Wnt inhibitor, Dkk-1. Western-blot of 3–4 samples running in parallel obtained from different young and aged rats showing changes on PP2A phosphorylation at the Tyr307 and methylation at Leu309. Bars represent the densitometric analysis of the western blot results and are the mean ± SEM. *$p \leq 0.02$.

found to be increased in patients with mild cognitive impairment [44].

GSK3β is partially active in unstimulated cells and is regulated in a predominantly inhibitory manner by signaling mechanisms [45]. In the present work, we used the Wnt canonical signaling inhibitor Dkk-1, which has been shown to be expressed in AD brains [24] and to co-localize with hyperphosphorylated tau-bearing neurons in several transgenic mouse models [27]. Other works have also reported that hippocampal exposure to Dkk-1 directly induces neuronal death, tau phosphorylation, and reactive astrocytosis [24, 26, 46].

Although phosphorylation at Ser9 is a major means of inactivating GSK3β, we did not observe a significant effect of Dkk-1 on Ser9 phosphorylation in the aged hippocampus. This finding is in line with previous reports that show that mutation at Ser9 does necessarily affect Wnt signaling [47, 48]. However, inhibition of the canonical Wnt signaling was corroborated by a strong reduction in the active β-catenin and an increase in the phosphorylated β-catenin pool in both young and aged hippocampal slices.

The molecular mechanism of GSK3β modulation seems to be complex and is not completely understood. Wnt signaling has recently been reported to trigger the sequestration of GSK3β from the cytosol to multivesicular organelles, preventing its interaction with cytoplasmic substrates [49]. In fact, Ser9 of axin-bound

GSK-3 can remain insulated to further phosphorylation [47]. Thus, Wnt inhibition does not necessarily affect the phosphorylation state of this particular Ser9 epitope, which mostly depends on Akt phosphorylation [50]. In addition, phospho-Ser9 is efficiently removed by PP2A [30, 51], which we found to be significantly reduced in the presence of Dkk-1 in the aged tissue. Curiously, other works have found that inactivation of GSK3β by lithium results in an increase in phosphorylation at Ser9, possibly via an inhibitory effect on PP2A activity [52]. Hence, it is possible that the level of phospho-Ser9 observed during Wnt inhibition by Dkk-1 in the aged hippocampus did not result in the complete inhibition of GSK3β toward tau but that residual activity contributed to tau phosphorylation at the sites we analyzed and was enhanced by decreasing PP2A activity. How PP2A activity is decreased in the presence of Dkk-1 is not clear, but it is possible that a regulatory mechanism may be operating in the aging tissue to balance Wnt/β-catenin signaling inhibition.

PP2A accounts for more than 70% of cellular phosphatase activity [53] and is one of the major enzymes implicated in tau dephosphorylation [34, 54]. A decline in PP2A activity has been reported during mouse aging [55], and PP2A activity has also been shown to be decreased by almost 50% in AD brains [53]. However, in support of our findings, no changes in the PP2A catalytic subunit expression have been reported between 3- and 24-month-old rats [43]. Thus, the current study suggests that under Wnt signaling impairment, reduced PP2A activity potentiates tau phosphorylation in the aged hippocampus. In accord with this conclusion, it is possible that a feedback loop between PP2A and GSK3β dysregulation is associated with aging leading to an increased susceptibility for tau hyperphosphorylation. PP2A activity can be inhibited by tyrosine phosphorylation and/or by reduced methylation of its catalytic subunit [56, 57]. Here, we found that the diminished PP2A activity correlates with a significant reduction of the enzyme methylation in the aged hippocampus after Dkk-1 exposure. Interestingly, in AD-affected brain regions a reduced PP2A methylation concomitant with increased tau phosphorylation levels has been reported [58]. Although we cannot provide evidence for the mechanism by which Dkk-1 favors the reduced methylation of the enzyme, our results show that there is a clear correlation between the reduced activity of PP2A and the increase in tau phosphorylation during aging.

In general, the present results suggest that aging might be affecting the interplay between protein kinase and phosphatase activities associated with some defects in signal-transduction pathways, such as the canonical Wnt signaling, which increase the risk for tau hyperphosphorylation.

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