Departamento de Farmacología y Terapéutica
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# *Nuevos abordajes terapéuticos basados en la melatonina y sus derivados para el tratamiento de la enfermedad de Alzheimer y el ictus.*

**TESIS DOCTORAL**

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# RESUMEN

El aumento de la longevidad de la población trae como consecuencia el aumento del riesgo de sufrir enfermedades neurodegenerativas como el Alzheimer (EA) o cerebrovasculares como el ictus. Estas enfermedades comparten mecanismos patológicos comunes, entre los que se encuentra el estrés oxidativo. Asimismo, los niveles de la neurohormona melatonina conocida por poseer propiedades antioxidantes, controlar el ciclo sueño-vigilia y mantener funciones inmunoreguladoras, disminuyen con la edad, y de forma más acusada en algunas enfermedades neurodegenerativas como la EA. Adicionalmente existen datos que muestran que la melatonina posee propiedades neuroprotectoras en modelos de EA y de ictus.

En base a estos antecedentes y a que tanto la EA como el ictus no poseen tratamientos capaces de detener o frenar la muerte neuronal, en esta tesis hemos evaluado si la melatonina en asociación con otros fármacos o nuevos derivados de la melatonina son capaces de mejorar la patología de la EA y de la isquemia cerebral.

En nuevos modelos *in vitro* de EA, en los que hemos combinado la patología βA y Tau en células SH-SY5Y y cultivos organotípicos de hipocampo, hemos demostrado que la asociación de melatonina con inhibidores de la acetilcolinesterasa utilizados en clínica para el tratamiento de la EA (galantamina y donepecilo), así como un nuevo híbrido melatonina-donepecilo, el ITH12291, son capaces de ofrecer neuroprotección y prevenir las distintas alteraciones relacionadas con la EA descritas en estos modelos. Adicionalmente, en modelos *in vitro* e *in vivo* relacionados con la isquemia cerebral, hemos puesto de manifiesto que dos nuevos derivados de melatonina, el ITH 12674 (híbrido melatonina-sulforafano) y el Neu-P11 (agonista melatoninérgico) pueden ofrecer neuroprotección y reducir el volumen de infarto cerebral en ratones.

En base a los resultados obtenidos en esta Tesis, proponemos que el uso de la melatonina como tratamiento coadyuvante o el uso de nuevos derivados de esta neurohormona pueden ser de utilidad para el tratamiento de enfermedades neurodegenerativas relacionadas con el envejecimiento, como son la enfermedad de Alzheimer y el ictus.

# ABSTRACT

The increased longevity of the population results in increased risk of neurodegenerative diseases like Alzheimer's disease (AD) or stroke. They also share a major common factor, oxidative stress. Likewise, for both, effective treatments are practically absent. On the other hand, the levels of the neurohormone melatonin (a potent antioxidant), capable of regulating different physiological functions, such as, the sleep-wake cycle and immunoregulatory functions are significantly reduced with aging. This decline is more pronounced in different neurodegenerative diseases, like AD. Furthermore, melatonin has been shown to be neuroprotective in different *in vitro* and *in vivo* models of AD and brain ischemia.

In new *in vitro* AD models in which we have combined βA and Tau pathology, we have shown that the combination of melatonin with acetylcholinesterase inhibitors used in clinic for Alzheimer's disease (galantamine and donepezil), and a new melatonin-donepezil hybrid, ITH12291 can protect and prevent several of the alterations described in the new *in vitro* models of AD. Similarly, two new melatonin derivatives, ITH 12674 (melatonin-sulforaphane derivative) and Neu-P11 (melatoninergic agonist) have shown to be effective in *in vitro* and *in vivo* cerebral ischemia models.

Therefore, we propose that the use of melatonin as adjuvant treatment, or the use of new derivatives of this neurohormone could be useful for treating neurodegenerative diseases associated with aging, such as AD and stroke.

## **ABREVIATURAS Y ACRÓNIMOS** (por orden de aparición)

- ✓ **EA:** enfermedad de Alzheimer
- ✓ **βA:** β-amiloide
- ✓ **AD:** Alzheimer's disease
- ✓ **EP:** enfermedad de Parkinson
- ✓ **t-PA:**activador de plasminógeno tisular recombinante
- ✓ **h:** horas
- ✓ **min:** minutos
- ✓ **PPA:** proteína precursora del β-amiloide
- ✓ **PS:** presenilina
- ✓ **$Ca^{2+}$:** calcio
- ✓ **FHP:** filamentos helicoidales pareados
- ✓ **ACh:** acetilcolina
- ✓ **AChE:** acetilcolinesterasa
- ✓ **ChAT:** acetilcolin transferasa
- ✓ **IAChE:** inhibidor de Acetilcolinesterasa
- ✓ **SNC:** sistema nervioso central
- ✓ **IL 1β:** interleucina 1β
- ✓ **TNF:** factor de necrosis tumoral
- ✓ **BDNF:** factor neurotrófico derivado de cerebro
- ✓ **NGF:** factor de crecimiento neuronal
- ✓ **ATP:** adenosín trifosfato
- ✓ **ERO:** especies reactivas de oxígeno
- ✓ **i.c.v:** intracerebroventricular
- ✓ **ml:** mililitro
- ✓ **g:** gramo
- ✓ **ACM:** arteria cerebral media
- ✓ **$Na^{+}$:** sodio
- ✓ **COX:** ciclooxigenasa
- ✓ **NO:** óxido nítrico
- ✓ **nNOS:** óxido nítrico sintasa neuronal
- ✓ **NFKB:** factor nuclear KB.
- ✓ **HIF1α:** factor inducible por hipoxia 1
- ✓ **iNOS:** óxido nítrico sintasa inducible
- ✓ **COX2:** ciclooxigenasa-2
- ✓ **BHE:** barrera hematoencefálica
- ✓ **ICAM-1:** molécula de adhesión intercelular-1
- ✓ **ET-1:** endotelina-1
- ✓ **ACI:** arteria carotidea interna
- ✓ **ACC:** arteria carotidea común
- ✓ **NAT:** N-acetil transferasa
- ✓ **HIOMT:** hidroxi-indol-O-metil transferasa
- ✓ **LCR:** líquido cefalorraquídeo
- ✓ **NQO2:** NAD(P)H quinona-óxido reductasa 2
- ✓ **HO-1:** hemo-oxigenasa 1
- ✓ **Nrf2:** factor 2 asociado con el factor nuclear eritroide 2
- ✓ **A.O:** ácido okadaico
- ✓ **GSH:** glutatión
- ✓ **cAMP:** adenosín monofosfato cíclico
- ✓ **EEUU:** Estados Unidos
- ✓ **EMEA:** asociación europea de medicamentos
- ✓ **PKC:** proteína cinasa C
- ✓ **SnPP:** protoporfirina de estaño
- ✓ **mg:** miligramo
- ✓ **POG:** privación de oxígeno y glucosa.

# 1. MARCO DE LA TESIS DOCTORAL

Este trabajo para la obtención de grado de Doctor, se ha realizado en el laboratorio L3 de la profesora Manuela García López que pertenece, al Instituto Teófilo Hernando de I+D del Medicamento y al Departamento de Farmacología y Terapéutica de la Facultad de Medicina, de la Universidad Autónoma de Madrid; bajo la tutela de la Doctora Manuela García López y el Doctor Fco Javier Egea Máiquez.

Entre las líneas de investigación del grupo se haya la que ha dado lugar a esta tesis Doctoral, concretamente el desarrollo de nuevos compuestos farmacológicos para el tratamiento de enfermedades neurodegenerativas como, la enfermedad de Alzheimer y el ictus.

# 2. JUSTIFICACIÓN DEL ESTUDIO

En las últimas décadas la población mundial ha sufrido importantes cambios en su estructura por edades, sobre todo en los países desarrollados. Así, debido al progresivo envejecimiento de la población mundial, ha aumentado la incidencia de enfermedades asociadas al envejecimiento como la enfermedad de Alzheimer (EA), la enfermedad de Parkinson (EP) o las enfermedades cerebrovasculares, como la demencia vascular o el ictus [1].

El término demencia deriva del latín "demens, dementatus" que significa sin mente. La demencia es definida por la Organización Mundial de la Salud en la Clasificación Internacional de las enfermedades como: “un síndrome debido a una enfermedad del cerebro, generalmente de naturaleza crónica o progresiva, en la que hay déficits de múltiples funciones corticales superiores.... que repercuten en la actividad cotidiana del enfermo". Desde un punto de vista cuantitativo y cualitativo, la EA es, dentro del grupo de las demencias degenerativas, la más relevante [2].

Datos de la Confederación Española de asociaciones de familiares de personas con EA y otras demencias (CEAFA) postulan que existen más de 600.000 pacientes diagnosticados, aunque se estima que el número real puede estar entorno a los 800.000 [3]. Asimismo, la sociedad Americana de Alzheimer estima que la prevalencia de la EA en Estados Unidos (EEUU) es de 5.1 millones y que este número podría casi triplicarse (13.8 millones) para el año 2050, si no se encuentra tratamiento paliativo o curativo para esta enfermedad [2]. Esto hace que se estime que el gasto económico que suponen las demencias sean unos 600.000 millones de dólares americanos anuales, superando el de otras patologías, como el cáncer [4].

Uno de los problemas añadidos a estas enfermedades -aparte del social, económico y sanitario- es que no existen tratamientos eficaces. En la actualidad, los tratamientos disponibles para tratar a los enfermos con EA consisten en mejorar la transmisión colinérgica mediante el uso de inhibidores de la acetilcolinesterasa (donepecilo, rivastigamina, galantamina) o inhibiendo la excitotoxicidad inducida por

glutamato con un antagonista no competitivo de los receptores NMDA (memantina). Sin embargo, estos tratamientos no consiguen frenar de forma eficaz la progresión de la enfermedad [5].

Por otro lado, el ictus es la segunda causa mundial de mortalidad y la primera de discapacidad en humanos [6]. Pese a que durante las dos últimas décadas se ha invertido mucho tiempo y dinero para descubrir nuevos tratamientos o compuestos neuroprotectores, a día de hoy, solo existe un tratamiento aprobado por las agencias reguladoras, el activador de plasminógeno tisular recombinante (t-PA) intravenoso. Sin embargo, este fribrinolítico muestra varios inconvenientes, como son una ventana terapéutica corta (3-4.5 h) y sus efectos secundarios adversos (hemorragia intracraneal) [7, 8], lo que limita su uso a menos del 20% de pacientes con ictus trombótico. Así, la búsqueda de nuevos tratamientos resulta de crucial interés.

Con el objeto de identificar nuevos abordajes terapéuticos para estas enfermedades, nos hemos centrado en la melatonina por las razones que se esgrimen a continuación. La melatonina, una neurohormona sintetizada en varios tejidos además de la glándula pineal, participa en diversos procesos biológicos como son la regulación del ritmo circadiano del sueño, la supresión del dolor y la inhibición de la inflamación, entre otros [9]. La melatonina y sus metabolitos poseen un marcado poder antioxidante, de ahí que sea potencialmente útil en patologías que presentan un componente de elevado estrés oxidativo en su patogénesis como son las EA y la isquemia/reperfusión cerebral. Desde un punto de vista terapéutico, la melatonina ha mostrado ser eficaz como uso en monoterapia o en combinación con otros fármacos, como con el donepecilo o la galantamina [10-12]. Además, es bien tolerado y muestra baja toxicidad, incluso a dosis altas, pero tiene el inconveniente de una vida media muy corta (30 min)[12, 13].

Con estas premisas en mente, en esta Tesis doctoral nos hemos interesado en investigar el uso de la melatonina, sola o en combinación con otros compuestos, así como la evaluación de nuevos derivados de la melatonina capaces de mejorar las propiedades farmacocinéticas y farmacodinámicas de la propia melatonina en modelos de EA e isquemia cerebral, con el objeto de identificar nuevos tratamientos para estas enfermedades que poseen una elevada prevalencia pero un escaso, y en muchos casos ineficaz, arsenal terapéutico.

# 3. INTRODUCCIÓN

## 3.1.ENFERMEDAD DE ALZHEIMER (EA)

### 3.1.1. PERSPECTIVA HISTÓRICA

En noviembre de 1901, una paciente de 51 años de edad llamada August D., ingresó en el hospital de Frankfurt a causa de un llamativo cuadro clínico, que continuó con una rápida y progresiva pérdida de la memoria, desorientación tiempo-espacial, paranoia, trastornos de la conducta y un grave trastorno del lenguaje. Fue estudiada por el Dr. Alois Alzheimer y otros médicos hasta su muerte, por septicemia y neumonía, en 1906.

El cerebro de la enferma fue remitido al Dr. Alzheimer, quien procedió a su estudio histológico. Su observación anatomoclínica describía la presencia de placas seniles, ovillos neurofibrilares y cambios arterioescleróticos cerebrales. Al año siguiente, publicó dichos resultados en la revista "Allgemeine Zeitschrift für Psychiatrie and Psychisch-Gerichtliche Medizin" con el título "Uber eine eigenartige Erkankung der Hirnrinde" (Una enfermedad grave característica de la corteza cerebral). Curiosamente, a pesar del gran impacto de esta enfermedad hoy en día, en aquel momento los hallazgos del Dr. Alzheimer tuvieron poco calado entre la comunidad científica de su época.

### 3.1.2. PATOFISIOLOGÍA DE LA EA

La EA puede tener un origen genético-hereditario o, como sucede en la mayoría de los casos, ser esporádica, debido a una causa aún desconocida. Las formas familiares de la EA suponen entre un 2-5% de todos los enfermos de EA y se caracterizan por poseer un inicio temprano (sobre los 40 años). Estos pacientes presentan mutaciones en la proteína precursora del beta amiloide (PPA) o de la presenilina-1 (PS1), enzima que participa en el procesamiento de la proteína βA. Por

otro lado, mutaciones en el gen Apolipoproteína E también se relacionan con la EA familiar, aunque de comienzo tardío [14].

Las formas no familiares son las más frecuentes (un 90-95%) y aparecen más tardíamente (a partir de los 65 años); en estos casos, el envejecimiento se considera un factor determinante. El mecanismo etiopatológico de la EA asociada al envejecimiento no está del todo dilucidado. Sin embargo, sí se conoce que los siguientes factores participan en su patología: (i) una acumulación aberrante de proteínas (placas de βA y ovillos neurofibrilares de proteína Tau hiperfosforilada), (ii) una disfunción colinérgica, (iii) neuroinflamación, (iv) disfunción mitocondrial, (v) estrés oxidativo y (vi) alteraciones en la homeostasis intracelular del calcio ($Ca^{2+}$). Curiosamente, la mayoría de estos procesos son comunes a muchas otras enfermedades neurodegenerativas, al ictus e incluso al propio envejecimiento [15].

A continuación se realizará una descripción de aquellas alteraciones más relevantes relacionadas con la fisiopatología de la EA (Figura 1).

#### *3.1.2.1* ***Placas de βA e hiperfoforilación de Tau***

Las dos principales características histopatológicas de la EA son las denominadas placas seniles y los ovillos neurofibrilares [16]. Las placas seniles están formadas por acúmulos del péptido βA. Existen diversas formas de procesamiento del péptido βA desde su precursor, la PPA. Por un lado, existe la ruta fisiológica denominada no amiloidógena, en la cual el dominio N-terminal es escindido por la α-secretasa, dando lugar a un péptido grande y extracelular llamado sPPAα. Éste contiene un fragmento de βA que incluye 28 aminoácidos del dominio extracelular y 12-15 residuos de la región de membrana del PPA [17]. Por último, el extremo C-terminal es escindido por la γ-secretasa. Sin embargo, existe otra ruta denominada amiloidógena, que es la ruta patológica, en la que la β-secretasa escinde el N-terminal de PPA y la γ-secretasa escinde el C-terminal. El péptido generado de 39-43 aminoácidos es secretado al espacio extracelular que, posteriormente, se agregará [18, 19] y dará lugar a las placas seniles encontradas en cerebros post-mortem de los

pacientes con EA [20, 21]. Por otro lado, los ovillos neurofibrilares son acumulaciones neurofibrilares intracelulares formadas por los filamentos helicoidales pareados (FHP) compuestos por la proteína asociada a microtúbulos Tau hiperfosforilada [22].

Como se ha comentado con anterioridad, las placas seniles y los ovillos neurofibrilares constituyen las características histopatológicas patognomónicas de esta enfermedad, que además se utilizan como marcadores diagnóstico [23]. En cambio, existe una gran controversia sobre si las placas de βA o la hiperfosforilación de la proteína Tau son causa o consecuencia de la enfermedad. Existen estudios que demuestran que la formación de FHP podría proteger del daño neuronal que se produce en esta enfermedad; así, se ha observado que ratones con mutaciones en la PPA y que muestran FHP, generan menos cantidad de βA y menor estrés oxidativo. Sin embargo, también existen datos que apoyan lo contrario; es decir, que las placas seniles y los FHP son los primeros responsables de la neurodegeneración y, que más tarde generarán estrés oxidativo y neuroinflamación [24, 25].

Desde que se describió la EA, se han desarrollado diversas hipótesis con el objetivo de explicar esta enfermedad; entre ellas, se encuentra **la hipótesis colinérgica**, **la hipótesis del βA**, **la hipótesis de Tau** o, por último, **la hipótesis de la neuroinflamación** [21, 22, 26]. Inicialmente, la comunidad científica se encontraba dividida en dos grandes corrientes: los “BAPtistas” y los “TAUistas”. Sin embargo, cada vez son más los datos que apoyan la interrelación entre βA y Tau [27]. Por ejemplo, en cultivo de neuronas y en cultivos organotípicos, los oligómeros de βA inducen la hiperfosforilación de Tau, hecho que se ha corroborado *in vivo* y también en un cultivo tridimensional de células neuronales humanas de un caso de Alzheimer familiar, en el que mutaciones que causan agregados de βA, producen también agregados de Tau hiperfosforilado [28-32]. En resumen, cabe destacar la importancia de entender la EA como una combinación de ambas teorías, y no como patologías independientes [27].

3.1.2.2 ***Degeneración colinérgica:***

La presencia de una neurodegeneración específica de las neuronas colinérgicas del prosencéfalo basal y del córtex en pacientes de EA permitió establecer las bases de la "**hipótesis colinérgica de la EA**" [33, 34]. Esta teoría se estableció en 1971 cuando se demostró que en el aprendizaje participaban las sinapsis colinérgicas y, que una pérdida de Acetilcolina (ACh) llevaba a pérdida de memoria [19]. Además, es sabido que tanto el aumento de βA como de estrés oxidativo, disminuyen la síntesis de ACh al disminuir la actividad de ACh transferasa (ChAT) [35].

La hipótesis colinérgica asentó las bases de la utilización de los inhibidores de la acetilcolinesterasa (IAChE) para el tratamiento de los pacientes de EA, cuyo objetivo es aumentar la biodisponibilidad de ACh en las sinapsis colinérgicas [36]; tratamientos que aún siguen vigentes.

3.1.2.3 ***La neuroinflamación:***

Otro de los fenómenos que está cobrando importancia en la patogénesis de las enfermedades neurodegenerativas como la EA, la EP o el ictus, es la neuroinflamación. Hasta ahora, se ha considerado que la neuroinflamación sólo se producía durante los estadios finales de la enfermedad y, que la activación glial acompañaba pero no contribuía a la patología amiloidea [37-40]. Sin embargo, estudios preclínicos, genéticos y bioinformáticos demuestran que la activación del sistema inmune acompaña a la patología de EA y contribuye a su patogénesis; de hecho, los pacientes con EA presentan un incremento en marcadores inflamatorios [37, 40, 41].

Las células residentes del sistema inmune en el sistema nervioso central (SNC), la microglía [42], son las mediadoras de la neuroinflamación asociada a la EA. Así, en autopsias de tejido cerebral de EA se ha detectado microglía rodeando a las placas de βA, ya que entre las funciones de estas células se encuentra la de eliminar/fagocitar agregados proteicos aberrantes u otros patógenos para mantener la homeostasia y plasticidad del SNC [42]. Además, las células gliales, no sólo producen citocinas pro-

inflamatorias interleucina 1β y factor de necrosis tumoral (IL1β y TNF, respectivamente), sino que su activación prolongada puede producir una pérdida de funciones tróficas (factor neurotrófico derivado del cerebro (BDNF), factor de crecimiento neuronal (NGF), entre otros) y la pérdida de propiedades protectoras de estas células, agravando así la patología de la EA [40, 43, 44].

#### *3.1.2.4 Alteraciones en la homeostasia del calcio intracelular*

Otra de las alteraciones descritas en la patología de la EA es la dishomeostasia del $Ca^{2+}$ intracelular. De hecho, existe una teoría desde los años 90 que apoya que esta dishomeostasia puede ser la causa de la EA [45, 46]. A favor de esta teoría está el hecho de que en las formas familiares de EA, los genes relacionados con la PPA y la presenilina participan en la señalización del $Ca^{2+}$ [47]. Sin embargo, en este caso también, sigue debatiéndose si la dishomeostasia del $Ca^{2+}$ es causa o consecuencia. Diferentes grupos de investigación han descrito que los acúmulos de βA causan alteraciones del $Ca^{2+}$, aumentando la concentración de este ión intracelular mediante la creación de poros en la membrana plasmática [48, 49] o, indirectamente, aumentando el flujo al interior o desde depósitos intracelulares [50]. Asimismo, se ha observado que la producción de estrés oxidativo afecta a la membrana, y por lo tanto, a la capacidad de la célula para extraer $Ca^{2+}$ de su interior [51-53]. Igualmente, existen evidencias que ponen de manifiesto que la dishomeostasia del $Ca^{2+}$ y los posteriores agregados de βA están estrechamente relacionados [54].

#### 3.1.2.5 *Disfunción mitocondrial*

La mitocondria es una organela que cuenta con la cadena transportadora de electrones para la producción de adenosina trifosfato (ATP) y es la mayor fuente de especies reactivas de oxígeno (ERO) [55, 56]. En relación a la función mitocondrial en la EA, se ha descrito que las neuronas de hipocampo de pacientes de EA muestran disfunción mitocondrial y disfunciones metabólicas. También se ha descrito que al disminuir la enzima citocromo C oxidasa (enzima de la cadena transportadora de

electrones) en estos pacientes, se produce un aumento de la producción de ERO y una disminución de las fuentes energéticas [23, 57]. Además, se ha demostrado que el propio βA es capaz de alterar la cadena transportadora, disminuyendo la actividad de enzimas clave en la dinámica mitocondrial [58]. Las alteraciones mitocondriales descritas anteriormente dan lugar a aumento del estrés oxidativo, alteraciones en el transporte axonal y mutaciones del ADN mitocondrial. Por último, las alteraciones en la dinámica mitocondrial (procesos de fisión y fusión) también contribuyen a incrementar la situación de estrés oxidativo, tal y como profundizaremos con mayor detalle a continuación [59, 60].

#### *3.1.2.6* ***Estrés oxidativo:***

La oxidación de lípidos, proteínas y ácidos nucleicos neuronales son fenómenos que aparecen en la EA [60, 61]. El cerebro humano, pese a que sólo ocupa el 2% del peso corporal total, consume el 20% del oxígeno total [62]. Este gran consumo de oxígeno aumenta la susceptibilidad al estrés oxidativo de este órgano, en comparación con otros. Más específicamente, la neurona es la célula con mayor metabolismo del cerebro, por lo que se trata de la célula más vulnerable al estrés oxidativo [63]. Se han descrito marcadores de estrés oxidativo, como la 3-nitrotirosina, 8-hidroxideoxiguanosina, malondialdehido y 4-hidroxinonenal, no sólo en cerebro, sino también en sangre de pacientes y/o en modelos animales de EA [64-68]. Además de acúmulos de ERO intracelulares, también se han observado alteraciones enzimáticas (superóxido dismutasa y catalasa) en el SNC y periférico de pacientes de EA [60, 69, 70].

En cuanto a cómo y cuándo se produce el estrés oxidativo en la EA, sigue sin estar dilucidado. Sin embargo, sí parece haber consenso en que tanto la disfunción mitocondrial [66], la acumulación aberrante de metales [66, 71], la hiperfosforilación de Tau [72] , la inflamación [73] y/o la acumulación de βA [66, 74] son los mecanismos que inducen al aumento de ERO [60]. Por el contrario, se ha descrito que el aumento de ERO contribuye a la acumulación de βA y a la hiperfosforilación de Tau, adquiriendo

mayor importancia de la que parecía en la patogénesis de la EA. Por todo ello, muchos investigadores proponen la utilización de marcadores de estrés oxidativo como biomarcadores diagnóstico/ pronóstico de la enfermedad, y también como diana para el desarrollo de nuevos medicamentos para su tratamiento [60, 66, 74, 75].

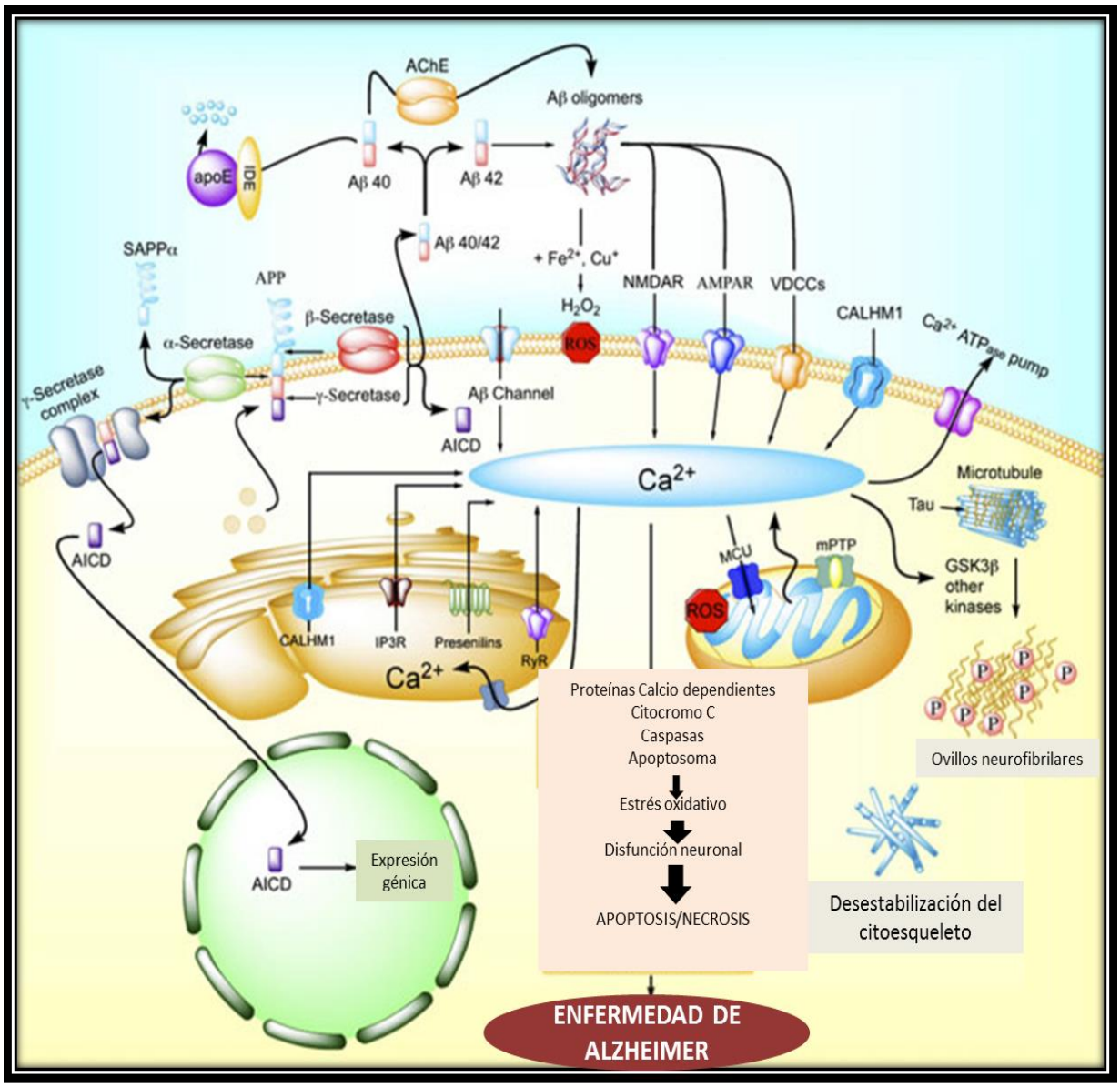


**Figura 1**: Alteraciones histopatológicas observadas en la EA. Figura adaptada de León *et*. al 2013 [5].

### 3.1.3. MODELOS ANIMALES PARA EL ESTUDIO DE LA EA

El desarrollo de modelos animales que reproduzcan las enfermedades humanas es una de las prioridades para entender la fisiopatología de cualquier enfermedad y para *a posteriori*, evaluar el potencial terapéutico de nuevos tratamientos antes de pasar a la fase clínica [76]. Sin embargo, en el caso de enfermedades con una fisiopatología compleja, como es el caso de la EA, estos modelos son difíciles de desarrollar y no reproducen la patología humana en su totalidad.

Dentro de los modelos *in vivo* de EA, actualmente se dispone de modelos de ratones modificados genéticamente y modelos en los que se inyecta intra-cerebralmente tóxicos, como por ejemplo, el péptido βA. A continuación, se resumirán algunos de los modelos *in vivo* de EA más destacados.

Los modelos genéticos son los más abundantes y los más consolidados. Esto se debe a que se conocen cuáles son las mutaciones humanas asociadas a las formas de EA familiar (como es el caso de las mutaciones en PPA y PS1) [77]. Entre otros, se han desarrollado ratones transgénicos para genes involucrados en el procesamiento y secreción de la proteína PPA, siendo el primer modelo descrito en 1995 el PDAPP (Platelet derived (PDGF) promoter) y distintas variedades posteriores como el TG2576, que contiene la mutación sueca, descrito en 1996. Estos ratones acumulan progresivamente βA y crean placas seniles, angiopatía cerebral amiloidea, astrocitosis, microgliosis, atrofia hipocampal, alteraciones en la neurotransmisión y déficits cognitivos y comportamentales [78-80].

A continuación, en 2001, mediante el cruce de ratones con mutaciones en PPA con ratones con mutaciones en la PS1 se obtuvo un doble mutante (APPswe/PS1) que mostraba un aumento en el ratio $\beta A_{42}/\beta A_{40}$, patología de βA acelerada, perdida neuronal y déficit cognitivo [81]. Este modelo presenta a su vez, ovillos pre-neurofibrilares que a pesar de no ser histopatológicamente compatibles con los que se ven en humanos con EA, por lo menos, muestra patología Tau [82]. Posteriormente, se desarrolló el ratón triple transgénico en el año 2003, mediante la inyección de constructos con las mutaciones de PPA y Tau en una única célula de un embrión

mutante homocigoto para PS1 (PS1M146V, APPswe, and TauP301L) [83]. Estos ratones desarrollan placas de βA y ovillos neurofibrilares con un perfil temporal y espacial parecido al observado en humanos, además de inflamación, disfunción sináptica y déficit cognitivo. Estos son sólo varios ejemplos de los numerosos transgénicos que se han desarrollado para la EA, que pueden consultarse en las revisiones de Webster et al. 2014 y en www.alzforum.org, para más detalle [80, 84].

Donde existe una mayor laguna es en la disponibilidad de modelos animales que reproduzcan la EA esporádica (que contribuyen más del 90% de los casos) [77]. Entre estos modelos se encuentra el de la inyección intra-cerebroventricular (i.c.v) del péptido βA en roedores [85, 86]. Esta inyección ocasiona déficits en aprendizaje y memoria y alteraciones neuropatológicas relacionadas con la enfermedad humana, como son la inflamación, activación glial y pérdida celular [85]. La ventaja de este modelo es que permite a los investigadores inyectar diferentes concentraciones y tipos del péptido βA y permite observar los cambios a mayor brevedad, sin tener que esperar meses para observar la patología, como es el caso de los transgénicos. No obstante, también cuenta con desventajas, ya que la concentración inyectada de βA es habitualmente muy superior a la encontrada en enfermos de EA [86].

Si bien es cierto que los modelos existentes han ayudado a entender la patología de la EA, existe una necesidad de generar nuevos modelos que mimeticen mejor la patología de la EA, para posteriormente explorar los nuevos tratamientos farmacológicos, tan necesarios para estos pacientes [87].

### 3.1.4 TRATAMIENTOS FARMACOLÓGICOS PARA LA EA

Los medicamentos que se utilizan en la actualidad para tratar a los enfermos de EA pueden englobarse en dos grandes grupos atendiendo a su mecanismo de acción. Los primeros en comercializarse fueron los **inhibidores de acetilcolinesterasa (IAChE),** entre los que se encuentran la tacrina (Cognex), el donepecilo (Aricept), la galantamina (Razadina, Reminyl) y la rivastigmina (Exelon). El primer IAChE comercializado, la tacrina, se encuentra actualmente en desuso por su corta vida media, pero sobre todo por sus efectos hepatotóxicos [94]. Después de los IAChE, se comercializó el

**antagonista no competitivo de los receptores NMDA,** la memantina (Namenda) [95]. En líneas generales, la memantina está indicada en las fases avanzadas de la enfermedad, mientras que los IAChE en los estadios leve-moderado [88] (**Figura 2**). Estos fármacos, en general, poseen una eficacia dudosa; pueden ralentizar modestamente la progresión de los síntomas cognitivos, pero finalmente, los pacientes acaban deteriorándose, y lo más preocupante, la mitad de los pacientes no responden al tratamiento [22]. A pesar de ello, el desarrollo de nuevos IAChE sigue siendo una estrategia vigente. En este sentido se sintetizó más recientemente un IAChE nuevo, memogain (GLN-1062), un pro-fármaco del éster benzóico de galantamina en administración intra-nasal, que se encuentra actualmente en investigación en fase clínica para la EA [89].

Pese a que durante los últimos 15-20 años se ha realizado un gran esfuerzo científico y económico por encontrar nuevos medicamentos para tratar esta devastadora enfermedad, lo cierto es que desde que se comercializó la memantina en el año 2007, aun no se ha aprobado ningún nuevo medicamento para la EA.

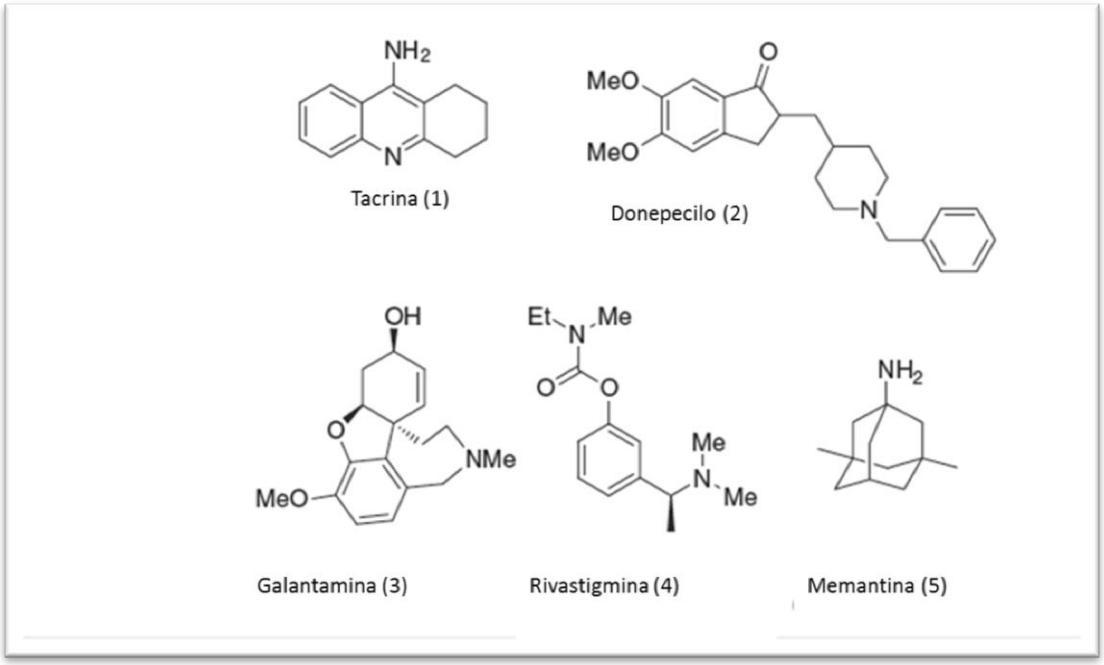


**Figura 2:** Fármacos utilizados en clínica para el tratamiento de la EA. Los inhibidores de Acetilcolinesterasa: Tacrina (1); Donepecilo (2); Galantamina (3) y Rivastigmina (4); y el antagonista no competitivo de los receptores NMDA, Memantina (5). Figura adaptada de León R. *et.* al 2013 [5].

Uno de los fenómenos más llamativos relacionados con el desarrollo de nuevos medicamentos para la EA, es que pese a que muchos compuestos han conseguido eficacia en los modelos preclínicos, cuando han llegado a la fase clínica, incluso en fase III, han fracasado. Esto puede ser debido a diversos factores como: (i) los modelos animales utilizados sólo se centran en una de las alteraciones descritas en la fisiopatología de la EA (por ejemplo la alteración del péptido βA), (ii) en los modelos animales no se encuentra la pérdida neuronal progresiva y severa como la observada en pacientes de EA, (iii) en los modelos animales no coexisten factores ambientales u otras comorbilidades como la hipertensión, la diabetes o la hipercolesterolemia, tal y como ocurre en los pacientes, (iv) los compuestos están dirigidos a una única diana, siendo la EA una enfermedad tan heterogénea, por lo que no sería suficiente y/o, (v) los pacientes que entran en los ensayos clínicos están en un estadio demasiado avanzado de la enfermedad, lo que dificulta frenar su progresión [22, 90, 91].

Las estrategias terapéuticas que están o han sido desarrolladas a los largo de estos últimos años se resumen en neurotrofinas, antioxidantes, estatinas, anti-inflamatorios no esteroideos, terapia de reemplazamiento hormonal, bloqueo de la excitotoxicidad, terapias frente a agregación de βA o la proteína Tau, inmunoterapias y efectores de secretasas, entre otros [22, 23, 90].

Por último, es interesante destacar el hecho de que cada vez esté tomando más importancia la necesidad de utilizar combinación de fármacos, así como fármacos multidiana, que no sólo están dirigidos a corregir una diana, sino que intentan abordar más de una alteración [5, 22, 91]. En ese sentido, cada vez son más los ensayos pre-clínicos y clínicos en los que se combinan fármacos para tratar la EA. Por ejemplo, la combinación de memantina con donepecilo para tratar la EA de moderada a severa (NCT00866060); la asociación de SB-742457 (un novedoso agonista de receptores 5-HT6) con donepecilo, que ha mostrado resultados interesantes en un ensayo en fase II [92] y la asociación de la vitamina E (como antioxidante) con memantina, que se encuentra en ensayo clínico en fase III [93].

## 3.2 LA ISQUEMIA CEREBRAL

### 3.2.1 INTRODUCCIÓN SOBRE LA HIPOXIA CEREBRAL

La hipoxia cerebral y la posterior reoxigenación es un componente central de diversas enfermedades como son el traumatismo cerebral, el síndrome de distrés respiratorio agudo, apnea de sueño obstructiva, enfermedad de la montaña, paro cardíaco y el ictus isquémico. El ictus es la segunda causa de mortalidad y primera de discapacidad [94]. De todos los casos de accidentes cerebrovasculares, el 87% son de tipo isquémico y el 13% son de tipo hemorrágico [6]. Por lo tanto, para el desarrollo de este trabajo nos hemos centrado en el ictus isquémico.

### 3.2.2 CONCEPTO DE LA PENUMBRA DEL INFARTO

El ictus se produce como consecuencia de una disminución brusca del flujo sanguíneo, alcanzando valores por debajo de 10-15 ml/100g/min, como consecuencia de un trombo o placa de ateroma en un vaso cerebral; la arteria más comúnmente afectada es la arteria cerebral media (ACM). La zona cerebral que sufre la mayor caída del flujo cerebral sufre un daño irreversible, las neuronas mueren por un proceso de necrosis y esa zona recibe el nombre de “core” o núcleo del infarto. Rodeando al núcleo del infarto, existe una zona subyacente denominada penumbra, donde el flujo desciende pero no de forma tan acusada (20 ml/100g/min); en esta zona las células están comprometidas energéticamente, pero pueden recuperarse si se consigue restaurar el flujo sanguíneo (**Figura 3**). Por lo tanto, la zona de penumbra es la susceptible de ser rescatada farmacológicamente.

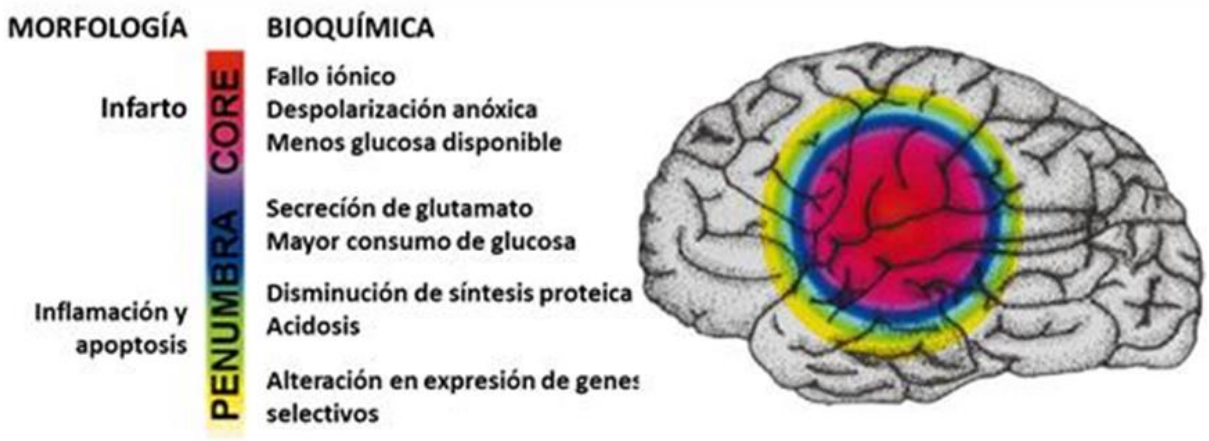


**Figura 3**: Esquema en el que se representa los procesos que tienen lugar en la penumbra isquémica y núcleo del infarto tras la obstrucción de un vaso cerebral. Figura adaptada de Dirnagl *et*. al 1999 [95].

### 3.2.3 PATOFISIOLOGÍA DEL ICTUS

#### *3.2.3.1* ***Déficit energético y la "cascada isquémica"***

El SNC requiere altas concentraciones de energía, la cual las obtiene mediante glucosa y oxígeno, que utilizará para generar ATP [96]. Al producirse la disminución de flujo sanguíneo, disminuye el aporte de glucosa y oxígeno, se inhibe la glucólisis y, por tanto, se desregula la cadena transportadora de electrones y se produce un fallo energético. El ATP es crucial para el mantenimiento iónico del cerebro y al faltar éste, distintas bombas, como la $Na^+/K^+$/ATPasa dependiente de ATP y la bomba de $Ca^{2+}$ ATPasa dependiente fallan. Como consecuencia, existe un aumento en el gradiente de sodio ($Na^+$) hacia el interior de la célula, que se acumula y, a su vez, aumenta el flujo de Cloro al interior. Por todo ello ocurre la despolarización de la membrana, se abren los canales de $Ca^{2+}$ dependientes de voltaje y la bomba $Na^+/Ca^{2+}$ funciona en reverso, produciéndose un incremento mayor de la concentración de $Ca^{+2}$ intracelular [97, 98]. Esta entrada masiva de $Ca^{2+}$ provoca la secreción de glutamato que resulta tóxico, produciendo muerte celular [99]. El daño se ve agravado ya que la liberación de glutamato activa receptores metabotrópicos y receptores de NMDA, AMPA y kainato,

que contribuirán a producir una sobrecarga de $Ca^{2+}$ en los terminales post-sinápticos [100]. Por último, este incremento de $Ca^{2+}$ intracelular ocasiona la activación de lipasas, proteasas y peroxidasas y producción de ERO, que finalmente conllevan a la muerte neuronal, también llamada excitotoxicidad [96]. Al conjunto de procesos descritos anteriormente que acontecen tras la caída del flujo sanguíneo cerebral, se conoce con el nombre de "cascada isquémica" (Figura 4).

#### *3.2.3.2 **Estrés oxidativo***

El estrés oxidativo es una alteración observada al inicio de la isquemia en el núcleo del infarto y está más que aceptada su contribución al daño neuronal [101]. El ión superóxido es el principal anión que se produce tras la reducción del aporte de oxígeno por alteración de la cadena transportadora de electrones en el núcleo del infarto [96]. Además, la NADPH oxidasa endotelial, de macrófagos y de la microglía y la ciclooxigenasa (COX) también producen ión superóxido [102, 103]. Por otro lado, se induce la óxido nítrico (NO) sintasa neuronal (nNOS) [104], la cual contribuye a la producción de NO y éste, junto con el superóxido, producen peroxinitritos, que son altamente reactivos. En condiciones fisiológicas, el aumento de ERO se contrarresta por el sistema antioxidante. En cambio, tras la isquemia éste se encuentra comprometido, por lo que no es capaz de cumplir eficientemente sus funciones [96]. Finalmente, durante la reperfusión, el aporte de oxígeno contribuye a una mayor generación de ERO [105]. El estrés oxidativo está estrechamente relacionado con la excitotoxicidad, pérdida energética y alteraciones en el equilibrio iónico, agravando de esta manera el daño del tejido y afectando también la zona de penumbra [106].

#### 3.2.3.3 ***Neuroinflamación***

Además del aumento de ERO, otro de los mayores contribuidores al daño producido durante la reperfusión es la respuesta inflamatoria. Ésta comienza con una activación de factores de transcripción sensibles a hipoxia como el factor nuclear kB (NFkB) o el factor inducible por hipoxia 1 (HIF1), que traen como consecuencia un aumento de producción de citocinas pro-inflamatorias como TNF, IL-1β e IL-6 o

interferón γ [107], que en última instancia producirán un aumento de la expresión de la óxido nítrico sintasa inducible (iNOS) y ciclooxigenasa-2 (COX-2) [108], que a su vez contribuyen a agravar la situación de estrés oxidativo [109]. Esta inflamación transcurre y aumenta durante la reperfusión afectando considerablemente a la viabilidad celular del tejido susceptible de recuperarse en zona de penumbra [96, 105].

#### 3.2.3.4 ***Ruptura de la barrera hematoencefálica (BHE)***

La isquemia cerebral está estrechamente relacionada no sólo con la falta de oxígeno y nutrientes sino con el aumento de la permeabilidad microvascular. La barrera hematoencefálica (BHE) constituye en el cerebro una barrera no sólo física, sino también metabólica y su buen funcionamiento resulta crucial para la supervivencia. Tras una situación de isquemia se produce la ruptura de la BHE, aumentando así la permeabilidad de ésta, produciendo la aparición de sustancias restrictivas en cerebro como aminoácidos excitatorios, quininas, prostaglandinas, metales u otras proteínas [96]. Una de las principales causas de esta ruptura es el aumento de estrés oxidativo que lleva a un aumento de ERO, que a su vez aumenta la producción de peroxinitrito, una sustancia pro-inflamatoria [110]. Además, a consecuencia de la neuroinflamación, aumenta la expresión de moléculas de adhesión de superficie de células endoteliales como, por ejemplo, la molécula de adhesión intercelular 1 (ICAM-1), P-selectina o E-selectina. Éstas, se unen a receptores de membrana de neutrófilos y cruzan así la membrana. De la misma manera, atraídos por los anteriores, macrófagos y monocitos también cruzan la BHE, activando la microglía y los astrocitos, que aumentarán la producción de tóxicos, empeorando así el daño secundario a la isquemia [95].

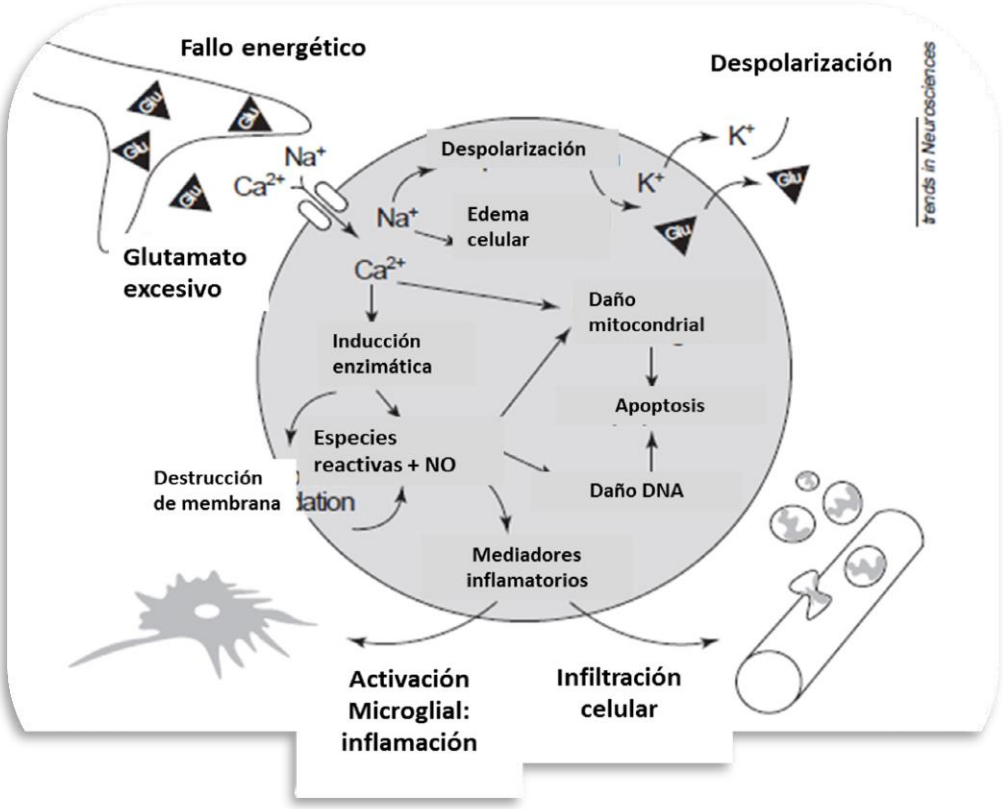


**Figura 4**: Resumen simplificado de las alteraciones que se producen tras una interrupción del flujo cerebral. Figura adaptada de Dirnagl *et*.al 1999 [95].

### 3.2.4 MODELOS EXPERIMENTALES DE ICTUS

La isquemia cerebral se ha estudiado en modelos animales durante más de 60 años. Los modelos animales de ictus pueden englobarse en dos grandes grupos: (i) los que mimetizan una isquemia global que simulan el paro cardíaco y (ii) los modelos de isquemia focal, que se centran, fundamentalmente, en ocluir la ACM, arteria ocluida con mayor frecuencia en humanos [111]. Los modelos de isquemia global se consiguen mediante la ligadura de dos arterias vertebrales y/o de las dos arterias carótidas comunes [112]. Por otro lado, para emular la isquemia focal son varios los modelos que se pueden utilizar, siendo los más comunes, el del filamento intraluminar, la inyección del vasoconstrictor endotelina-1, la oclusión directa de la arteria mediante su cauterización, la inyección de un trombo autólogo, la inyección de trombos artificiales y la fototrombosis [111, 112]. Cada uno de estos modelos tiene sus ventajas e inconvenientes tal y como se enumeran en la Tabla 1. Para la realización de este

trabajo de tesis doctoral, hemos empleado el modelo de ictus fototrombótico, que consiste en inyectar el agente fotoactivo rosa de Bengala, que al iluminarlo, produce coagulación microvascular. Las ventajas sobre la oclusión de la ACM son, entre otras, que se puede seleccionar la zona de la corteza cerebral que se quiere lesionar mediante coordenadas estereotáxicas, se puede regular el tamaño del infarto regulando el diámetro de la zona iluminada, es un método mínimamente invasivo y produce infartos menos variables [111, 113].

| Modelo | Procedimiento | Ventajas | Desventajas |
|---|---|---|---|
| Filamento intraluminar | • Insertar un filamento por la carotida interna tras la oclusión de la ACM. | • Poco invasiva<br>• Infartos reproducibles<br>• Produce isquemia transitoria y permanente | • Hipertermia tras el infarto<br>• Hemorragias subaracnoideas |
| Endotelina-1 (ET-1) | • Aplicación directa del vasoconstrictor ET-1 en la ACM. | • Poco invasiva | • La duración y magnitud de la isquemia muy variable |
| Trombo autólogo | • Inyectar un trombo autólogo en la ACI | • Reproduce mejor el infarto por embolismo humano<br>• Permite el estudio de trombolíticos | • Gran variabilidad en los infartos<br>• Reperfusión incontrolable |
| Embolismo sin trombo | • Inyección de "trombos artificiales (microsferas,etc) en ACI o ACC | • No riesgo de hipertermia<br>• Progresión lenta de la lesión, permite evaluar la ventana terapéutica | • Muchos infartos<br>• No hay reperfusión |
| Cirugía directa | • Ligación o sutura de la ACM | • Control del área donde suturas | • Invasiva<br>• No reperfusión<br>• Puede crear trauma |
| Fototrombosis | • Inyección de un agente fotosensible (Rosa de bengala), irradiación de la corteza con láser, produce coagulación microvascular | • Infartos en área y de tamaño definido<br>• Poco invasiva | • No produce el infarto en la ACM.<br>• Infartos menores |

**Tabla 1**: Ventajas e inconvenientes de los distintos modelos *in vivo* de isquemia cerebral focal disponibles. Figura adaptada de Jackman *et.* al 2011 [111].

### 3.2.5 TRATAMIENTO FARMACOLÓGICO PARA EL ICTUS

En la actualidad el único tratamiento aprobado por las agencias reguladoras para tratar los pacientes que han sufrido un ictus trombótico es el fibrinolítico activador de plasminógeno tisular recombinante (t-PA) [112, 114]. Su función consiste en restaurar el flujo sanguíneo cerebral. Sin embargo, su administración muestra ciertas particularidades que limita su uso a un número reducido de pacientes, el cual se estima que es inferior al 25 % [96]. Esta limitación de uso viene determinada porque la eficacia de la trombolisis con t-PA depende del tiempo que haya transcurrido desde que el paciente ha sufrido el infarto cerebral y recibe la medicación; el tiempo óptimo se establece entre 3-4.5 h. Sin embargo, en ese pequeño margen de tiempo, se tiene que haber establecido un diagnóstico diferencial entre ictus isquémico e ictus hemorrágico, lo que no siempre es posible. Adicionalmente, hay estudios que muestran que la trombolisis con t-PA incrementa el riesgo de sufrir hemorragias [115]. En cuanto a la prevención secundaria del ictus, en clínica se emplea la aspirina por sus propiedades antiagregantes [96].

Durante las últimas dos décadas se han realizado más de 40 ensayos clínicos fase III en los que se han evaluado compuestos con diversos mecanismos de acción (antagonistas del glutamato, antagonistas de los canales del $Ca^{2+}$, antioxidantes, estatinas, citicolina, albubina, magnesio, antagonistas de la serotonina, y antiinflamatorios, por ejemplo), pero desgraciadamente todos ellos han fracasado [116]. A pesar de este oscuro panorama, la búsqueda de nuevos tratamientos neuroprotectores/ neurorreparadores para el tratamiento del ictus sigue siendo una prioridad [96] .

## 3.3 MELATONINA

### 3.3.1 SÍNTESIS Y ACCIONES FISIOLÓGICAS DE LA MELATONINA

La melatonina (N-acetil-5 metoxitriptamina) es una neurohormona secretada por la glándula pineal de todos los mamíferos, incluidos los humanos, que fue aislada en 1958 por el grupo de Aaron Lerner [13, 117]. También está presente evolutivamente en varias plantas [118] y organismos unicelulares [119]. Recientemente, se ha descrito que existe una producción de melatonina extrapineal, concretamente en la retina, el tracto gastrointestinal, la médula ósea, los leucocitos, la cóclea, la glándula de Harderian, y la piel, el cerebro, el páncreas, la glándula tiroidea, la placenta, las células endoteliales y los leucocitos [120, 121]. Su amplio espectro de síntesis denota las diversas funciones fisiológicas que es capaz de regular [19]. El papel principal, y más reconocido de la melatonina es su implicación en la regulación del ciclo sueño-vigilia. Sin embargo, cada día se van descubriendo nuevas funciones para esta neurohormona, como son la inmunoreguladora, la anticancerígena o la secuestradora de radicales libres y antioxidante [13, 117, 122, 123].

La melatonina deriva de la serotonina, que se sintetiza a partir del triptófano [19]. Una vez sintetizada ésta, en dos pasos enzimáticos dará lugar a la melatonina [13]. El primer paso sintético está mediado por la N-acetil transferasa (NAT) que da lugar a la N-acetil serotonina [124], que posteriormente recibirá un grupo metilo, mediado por la enzima Hidroxiindol-O-metil transferasa (HIOMT) para formar la melatonina [125]. Una vez sintetizada, ésta no se almacena en la glándula pineal sino que difunde a capilares sanguíneos y al líquido cefalorraquídeo (LCR) (**Figura 5**) [126].

Muchos de los efectos anteriormente citados dependen de la acción de esta hormona sobre receptores de membrana acoplados a proteínas G, llamados MT-1 y MT-2 [117, 127], los cuales a su vez activan vías de protección que involucran a canales de $Ca^{2+}$ y las cinasas MAP, ERK1/2 y/o PI3K/AKT [128]. Existe también un tercer tipo de receptor, el MT-3, que se define como una quinona reductasa 2 (NQO2) [129]. Sin

embargo, su capacidad para secuestrar radicales libres parece ser independiente de receptor ya que requiere concentraciones superiores a las necesarias para mediar efectos receptor-dependientes [13]. En cuanto a la capacidad antioxidante de la melatonina, ésta actúa tanto como secuestradora de radicales libres como activando diferentes enzimas antioxidantes, como hemo-oxigenasa 1 (HO-1) vía Nrf2 (factor 2 asociado con el factor nuclear eritroide 2), entre otras [113, 130].

Debido al anillo indol aromático de su estructura, la melatonina puede donar electrones a las ERO y así, reducir radicales de hidroxilo, peróxido de hidrógeno, ácido hipocloroso o anión superóxido [19]. Además, tiene la ventaja adicional que sus metabolitos secundarios, terciarios y cuaternarios también son capaces de secuestrar especies reactivas, aumentando así su vida útil [131]. De este modo, se estima que una sola molécula de melatonina es capaz de secuestrar más de 10 especies reactivas de oxígeno y nitrógeno [115].

Por otra parte, la melatonina es capaz de prevenir el aumento aberrante de especies reactivas de nitrógeno al estimular sistemas antioxidantes como la superóxido dismutasa, la catalasa, la glutatión peroxidasa, la HO-1, aumentar los niveles de glutatión, así como de inhibir enzimas pro-oxidantes, como iNOS y COX. [19, 132, 133].

Por tanto, la melatonina posee una actividad antioxidante directa para secuestrar directamente radicales libres e indirecta mediante la activación de diferentes enzimas antioxidantes.

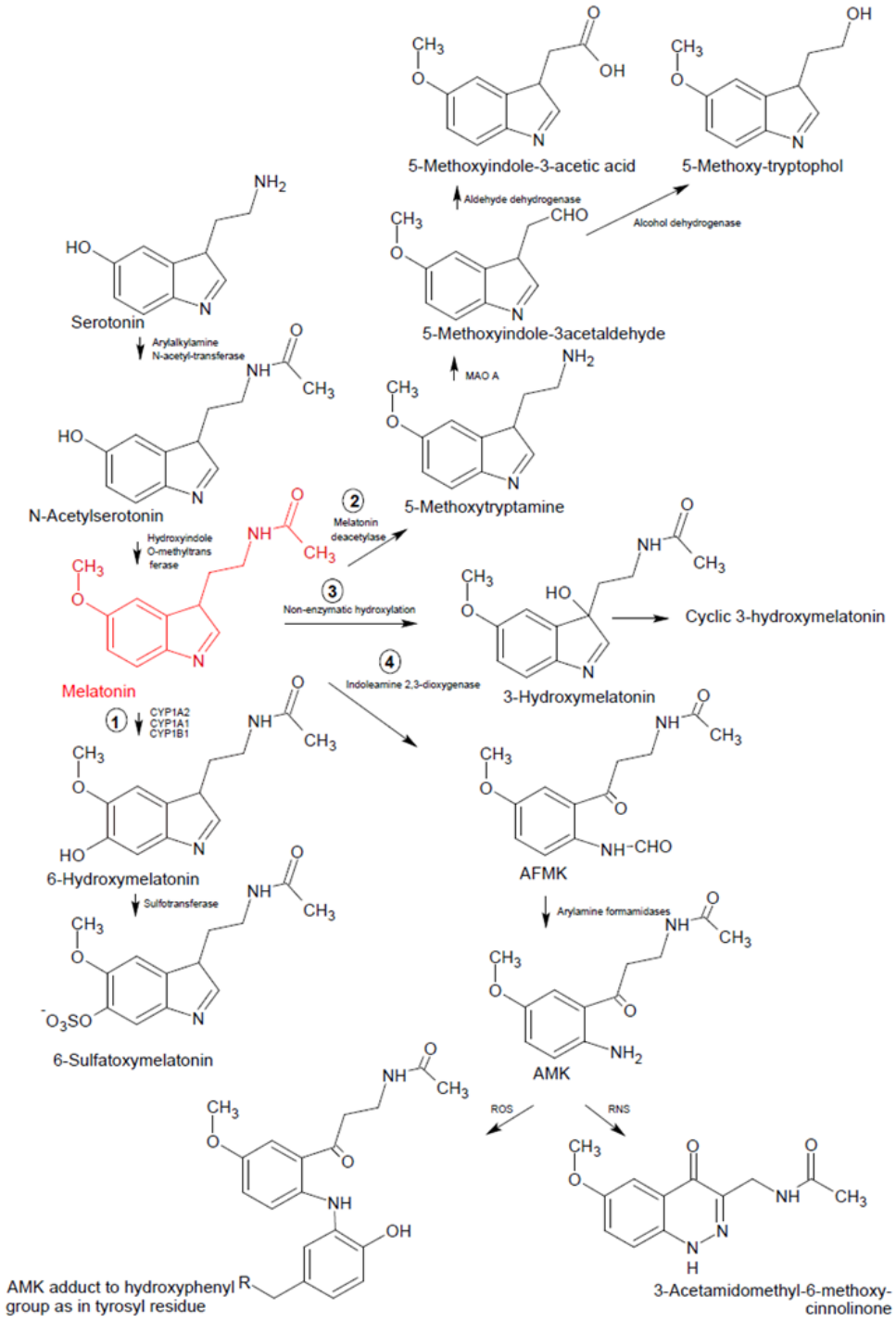


**Figura 5:** Ruta de síntesis endógena y metabolismo de la melatonina. Figura adaptada Escribano B. *et*. al, 2014 [117].

### 3.3.2 MELATONINA Y ENVEJECIMIENTO

Dadas las innumerables funciones que ejerce esta hormona, tal como se ha descrito anteriormente, la modificación de sus niveles en sangre y LCR, presumiblemente tendrán consecuencias a nivel fisiológico [134]. La producción de melatonina por la glándula pineal es superior en adultos jóvenes (18-54 años) que en personas mayores [135]; esta reducción tiene consecuencias sobre distintos sistemas como el inmunológico o el antioxidante [136, 137]. La reducción de los niveles de melatonina ocurre tanto en la glándula pineal, como en la sangre y el LCR, donde los niveles llegan a reducirse hasta la mitad [19, 138, 139]. La reducción de la producción de melatonina y la alteración de los ritmos circadianos pueden contribuir de forma significativa al aumento de estrés oxidativo y a las alteraciones degenerativas observadas en los ancianos [13]. Por otro lado, existe una amplia variabilidad interindividual de niveles de melatonina durante el envejecimiento y se ha postulado si estos niveles podrían utilizarse como marcadores de "buen envejecimiento" frente a niveles mucho menores, los cuales indicarían deterioros fisiológicos e incluso cognitivos [134].

### 3.3.3 MELATONINA Y EA

Los niveles de melatonina circulantes en sangre y en LCR en pacientes con EA son mucho menores (incluso una quinta parte) que en sujetos sanos de la misma edad [139]. Esta reducción se observa también en estadios preclínicos de la enfermedad; es decir, cuando aún no existen alteraciones cognitivas, por lo que se ha sugerido que la medida de los niveles de melatonina podría ser útil como marcador temprano de la enfermedad [13, 140]. Además, los pacientes con EA muestran alteraciones en la regulación del ritmo circadiano del sueño y presentan también falta de descanso durante la noche. Y por último, otra de las alteraciones descritas en estos pacientes es una disminución de los receptores MT2 y un aumento en los MT1 [141-144].

Por todo ello, un aporte exógeno de melatonina podría mejorar las alteraciones de sueño que sufren estos pacientes [19, 143]. Además de los datos aportados anteriormente, a nivel preclínico existe un gran número de evidencias que también apoyan el uso de melatonina como tratamiento de la EA. La melatonina ofrece neuroprotección frente a diversas alteraciones descritas en esta enfermedad [117] y ha demostrado efectos positivos en ratones transgénicos simples, dobles y triples de la EA [134]. En estos estudios, se ha evidenciado que la melatonina interactúa con el βA previniendo su agregación [145, 146]; puede regular la síntesis y la maduración de la PPA [19]; y es capaz de reducir la generación y deposición de βA *in vitro* e *in vivo* [145, 147-149].

Además de reducir la patología amiloide, la melatonina también es capaz de reducir la hiperfosforilación de Tau [13, 143]. Por ejemplo, al ser antioxidante, previene del estrés oxidativo y la hiperfosforilación de Tau causada por el inhibidor de fosfatasas ácido okadáico (A.O) [13, 150]. Por último, en el modelo triple transgénico de la EA, el cual desarrolla tanto la patología de βA como la de Tau [151, 152], la melatonina mejora la pérdida cognitiva que sufren estos ratones [153].

En cuanto a la hipótesis colinérgica, la melatonina es capaz de reducir eficazmente el aumento en la actividad de AChE inducida por βA [146]. También, en distintos modelos *in vivo* de la EA, se han visto mejorías debido al aumento de la actividad ChAT producida por la melatonina [154, 155].

También cabe destacar, la aplicabilidad de la melatonina para el tratamiento de la EA, debido a su gran capacidad antioxidante y la de sus derivados, los cuales son capaces de disminuir las ERO, disminuir la apoptosis y mitigar la producción de NO en corteza [19, 156, 157]. El aumento de ERO puede agravar la patología βA, pero también el βA puede aumentar los niveles de ERO. La melatonina, sin embargo, protege frente al estrés oxidativo en modelos *in vitro* e *in vivo,* convirtiéndose así en un buen candidato [19, 147, 154, 158].

Esta neurohormona también posee efectos anti-inflamatorios beneficiosos para la EA [159], ya que es capaz de reducir a la mitad la producción de citocinas pro-

inflamatorias IL-1β, IL-6, TNF [19], la expresión de NFkB, y la astrogliosis y microgliosis causada debido a la enfermedad [19, 117, 146].

En cuanto a las alteraciones mitocondriales, el tratamiento con melatonina mantiene los niveles de glutatión (GSH), disminuye la peroxidación lipídica observada en las membranas mitocondriales, impidiendo así también el daño en el ADN mitocondrial y la apoptosis [160-162].

Y por último, la melatonina reduce el aumento de $Ca^{2+}$ intracelular, inhibiendo la permeabilidad del poro de transición mitocondrial [163] y regula el metabolismo de la enzima adenosín monofosfato cíclico (cAMP), a través de los receptores MT2, que se encuentran disminuidos en la EA.

Por lo tanto, la melatonina es efectiva en prácticamente todas las alteraciones descritas en la EA, es decir: (i) previene la sobre-producción de βA, (ii) reduce la hiperfosforilación de Tau, (iii) disminuye las ERO por su actividad secuestradora y antioxidante, (iv) modula los procesos neuroinflamatorios, (v) muestra propiedades anticolinesterásicas, (vi) previene del daño mitocondrial y apoptótico y (vii) disminuye las alteraciones del $Ca^{2+}$ [19], hechos que justificarían su uso en esta enfermedad (**Figura 6**) [13, 19, 143].

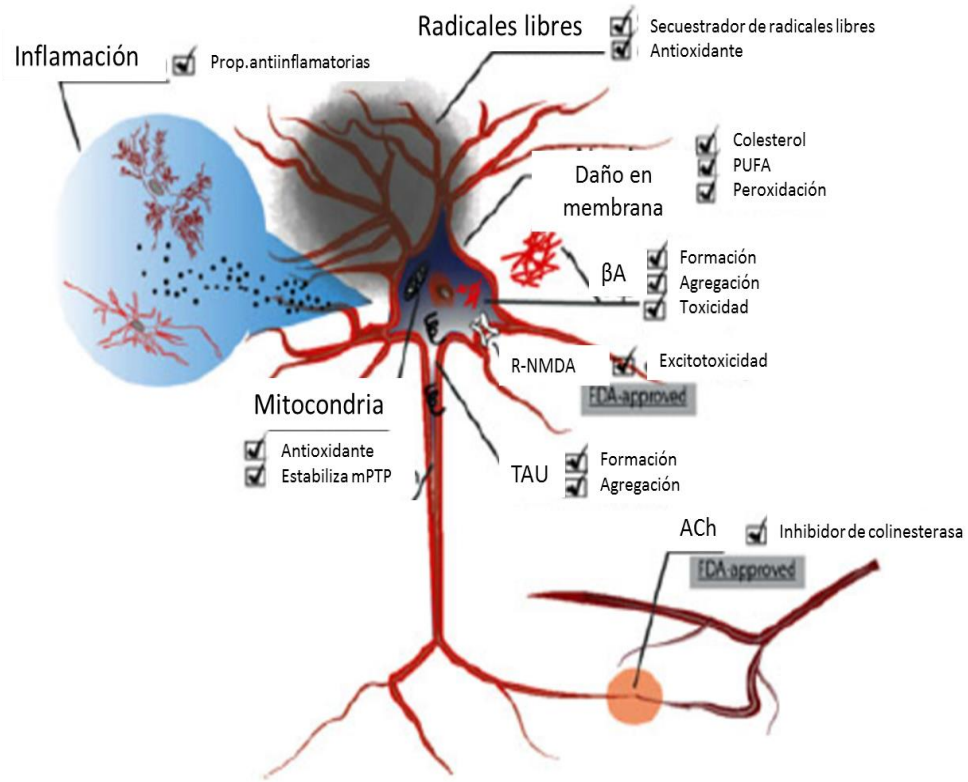


**Figura 6:** Ilustración que resume los efectos beneficiosos de la melatonina frente a la EA. Figura adaptada de Rosales-Corral *et*. al, 2012 [19].

### 3.3.4 MELATONINA E ISQUEMIA CEREBRAL

La disminución en la síntesis y secreción de melatonina durante el envejecimiento podría estar implicada en la fisiopatología de enfermedades con un componente de estrés oxidativo como las enfermedades neurodegenerativas y el ictus [117, 164]. El daño producido por la isquemia y posterior reoxigenación está muy relacionado con el aumento de ERO, tal y como se ha mencionado anteriormente [115, 117, 165].

Debido a su pequeño peso molecular, a su carácter lipofílico, a que no muestra prácticamente efectos secundarios, a su capacidad antioxidante, antiinflamatoria y antiapoptótica, la melatonina se ha empleado en varios estudios como tratamiento potencial de la isquemia cerebral. Así, en modelos *in vitro* e *in vivo* de isquemia, ha mostrado efecto neuroprotector [115, 165-167] y una disminución en la infiltración de neutrófilos, macrófagos/monocitos y microgliosis [117, 134, 168, 169] (Figura 7).

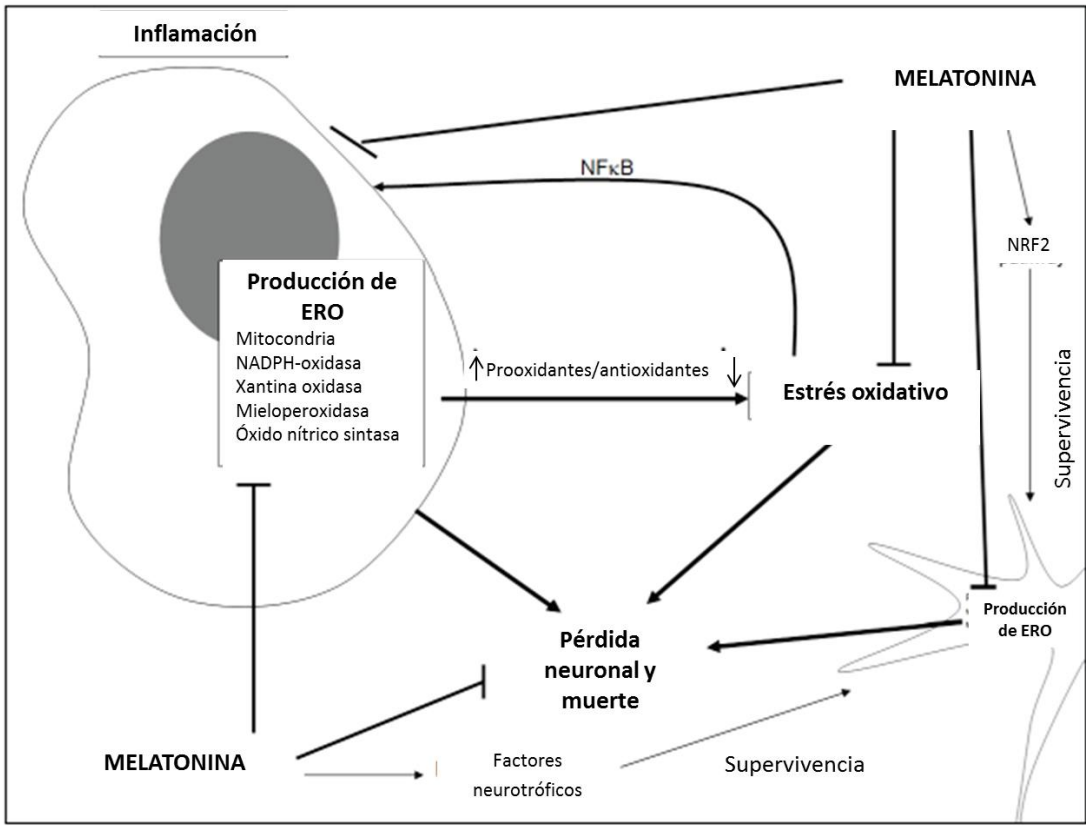


**Figura 7**: Acciones de la melatonina frente a isquemia cerebral a distintos niveles. Figura adaptada de Escribano *et*. al 2014 [117].

Por otro lado, se han descrito efectos positivos cuando es utilizada en combinación con t-PA en modelos animales de ictus [167, 170]. Por esta razón, se plantea la necesidad de desarrollar estudios clínicos en fase II y III para dilucidar si el uso de la melatonina en combinación con trombolíticos sería beneficioso o no [115, 171].

### 3.3.5 USO CLÍNICO DE LA MELATONINA: ENSAYOS CLÍNICOS

Después de todos los resultados positivos observados en investigación pre-clínica, durante los últimos 20 años son numerosos los ensayos clínicos realizados para probar el efecto de la melatonina en distintos campos de la medicina [172]. La melatonina ha mostrado ser efectiva en distintas condiciones patológicas como el glaucoma [173, 174]; la trombocitopenia [175]; enfermedades gástricas como la

enfermedad inflamatoria de intestinal [176]; enfermedades neurodegenerativas como la EA y la EP [177-179] y, para el control del sueño y el jet-lag [172]. También hay ensayos en los que la melatonina se ha administrado como coadyuvante para el tratamiento de enfermedades cardiovasculares, como la hipertensión arterial [180], la diabetes [181] o enfermedades infecciosas [182].

### 3.3.6 NUEVOS DERIVADOS DE MELATONINA

Posiblemente el mayor inconveniente que tiene la melatonina para extender su uso a la clínica es su corta vida media, de aproximadamente 30 min. De ahí que se esté haciendo un gran esfuerzo en sintetizar preparaciones de liberación prolongada, nuevos agonistas de receptores melatoninérgicos e incluso nuevos derivados de melatonina [13, 143, 183, 184] que permitan prolongar su vida media.

En este aspecto, existen compuestos (**Figura 8**) como el circadin (secreción prolongada de melatonina) o nuevos derivados de la melatonina como el ramelteon, la agomelatina, el tasimelteon y el TK-301 utilizados a día de hoy en clínica [13, 183]. El ramelteon (Rozerem, Takeda Pharmaceuticals, Estados Unidos) ha mostrado ser entre 3-16 veces más selectivo por los receptores de melatonina, que esta última. Además, tiene una vida media de 1-2.6 h y se aprobó en 2005 para el tratamiento del insomnio, siendo comercial en Estados Unidos (EEUU) y Japón [185]. El circadin (Neurim pharmaceuticals, Israel), es la primera preparación de melatonina con una formulación de liberación prolongada que se encuentra en el mercado desde el año 2007; se utiliza para el tratamiento de insomnio. Circadin libera melatonina manteniendo su perfil endógeno (8-10 h) y su vida media alcanza 3.5-4h. [186, 187]. La agomelatina (Valdoxan, Melitor, Thymanax, Servier pharmaceuticals, Neuilly-sur-Seine, France), actúa como agonista de los receptores melatoninérgicos y antagonista de los receptores 5HT2c; este fármaco fue aprobado en 2009 por la Agencia Europea de Medicamentos (EMEA) para el tratamiento de la depresión [188, 189]. El tasimelteon (Hetlioz, Vanda Pharmaceuticals, Washington, DC, USA) fue evaluado en un ensayo clínico en fase III en el año 2010, y desde el año 2014 se emplea como fármaco

huérfano para individuos ciegos con alteraciones en los ritmos de sueño [190-192]. Por último, TK-301 mostró en un ensayo clínico de fase II frente a insomnio en los EEUU, ser un fármaco tolerable, con buenas propiedades farmacocinéticas y farmacodinámicas [193]. Tiene actividad agonista sobre receptores MT1 y MT2, así como antagonista de los receptores serotoninérgicos 5HT2c y se propone como tratamiento para alteraciones del sueño en individuos ciegos y tratamiento de discinesias [13, 183, 194].

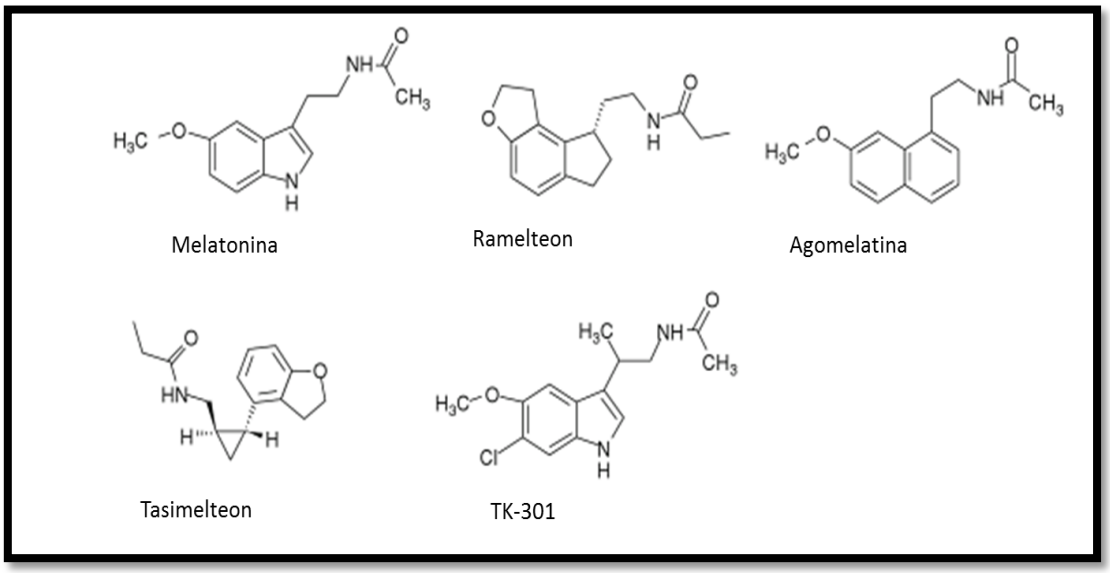


**Figura 8**: melatonina y sus derivados melatoninérgicos utilizados en clínica.

Además de los compuestos mencionados más arriba, tal y como hemos mencionado anteriormente, existen varios estudios que muestran el efecto positivo de la asociación de melatonina con otros compuestos utilizados en clínica, como el donepecilo o memantina para el tratamiento de la EA, o la asociación de melatonina con t-PA para el ictus [165, 195, 196]. Resultados previos de nuestro laboratorio demostraron cómo en un modelo de estrés oxidativo en las células SH-SY5Y, la asociación de concentraciones sub-efectivas de melatonina y galantamina producen una protección sinérgica interesante [11]. Por otro lado, ya son muchos los grupos de química médica que están sintetizando compuestos multidiana que incluyen melatonina en su estructura química [197]. Un ejemplo de ello, es uno de los derivados

estudiados en este trabajo, el ITH12291, el cual engloba la actividad de melatonina y un dominio del IAChE, donepecilo en la misma molécula [198]. Otro ejemplo de derivado también empleado en esta tesis es el ITH12674, que aúna las actividades de la melatonina y el sulforafano, un potente inductor del factor Nrf2, que ha mostrado propiedades neuroprotectoras en distintos modelos preclínicos relacionados con la neurodegeneración y la isquemia cerebral [199]. Por lo tanto, al igual que la melatonina, el potencial efecto de estos nuevos compuestos en tratamientos como la enfermedad de la EA e ictus parece justificado.

# 4 HIPÓTESIS DE TRABAJO

La hipótesis de esta tesis se sustenta en los siguientes hechos: (i) los niveles de melatonina se encuentran reducidos de forma progresiva con el envejecimiento y de forma más acusada en pacientes con enfermedades neurodegenerativas como la EA y (ii) la melatonina como terapia única o en combinación con otros fármacos ha mostrado propiedades antioxidantes, antiinflamatorias y neuroprotectoras frente a distintas enfermedades neurodegenerativas e ictus. Por ello, planteamos estudiar los efectos de diversos compuestos derivados de la melatonina, así como la melatonina en asociación con IAChE en modelos *in vitro* e *in vivo* relacionados con la EA y la isquemia cerebral con objetivo de evaluar su potencial terapéutico en estas patologías.

# 5 OBJETIVOS

**Los objetivos concretos de esta tesis han sido los siguientes:**

1) Desarrollar un nuevo modelo *in vitro* de la EA en células de neuroblastoma humano SH-SY5Y, en el que se combinen las patologías de βA e hiperfosforilación de Tau; una vez puesto en marcha el modelo, evaluar el efecto neuroprotector de la asociación de donepecilo y melatonina y un derivado que combina ambas propiedades, el ITH12291.

2) Desarrollar un modelo *in vitro* más crónico y complejo de la EA en el que se combinen las patologías de βA e hiperfosforilación de Tau en cultivo organotípico de hipocampo de rata; una vez puesto en marcha el modelo, evaluar el potencial efecto protector de la asociación de melatonina y galantamina en este modelo.

3) Evaluar farmacológicamente el efecto neuroprotector de un compuesto multidiana derivado de melatonina e inductor de Nrf2, el ITH12674, en distintos modelos *in vitro* de estrés oxidativo relacionados con la isquemia cerebral.

4) Evaluar el efecto y mecanismo neuroprotector del nuevo derivado melatoninérgico, Neu-P11, en modelos *in vitro* e *in vivo* de isquemia cerebral.

# 6 RESULTADOS

Los resultados de esta tesis se presentan como un compendio de los siguientes artículos científicos cuyas referencias son los siguientes.

- The melatonin-N,N-dibenzyl(N-methyl)amine hybrid ITH91/IQM157 affords neuroprotection in an in vitro Alzheimer's model via hemo-oxygenase-1 induction.
  Buendia I, Egea J, Parada E, Navarro E, León R, Rodríguez-Franco MI, López MG. ACS Chem Neurosci. 2015 Feb 18;6(2):288-96. doi: 10.1021/cn5002073. Epub 2014 Nov 25.
  PMID: 25393881

- Subthreshold Concentrations of Melatonin and Galantamine Improves Pathological AD-Hallmarks in Hippocampal Organotypic Cultures.
  Buendia I, Parada E, Navarro E, León R, Negredo P, Egea J, López MG.
  Mol Neurobiol. 2015 Jun 17. [Epub ahead of print]
  PMID: 26081146

- Melatonin-sulforaphane hybrid ITH12674 induces neuroprotection in oxidative stress conditions by a 'drug-prodrug' mechanism of action.
  Egea J, Buendia I, Parada E, Navarro E, Rada P, Cuadrado A, López MG, García AG, León R.
  Br J Pharmacol. 2015 Apr;172(7):1807-21. doi: 10.1111/bph.13025. Epub 2015 Feb 10.
  PMID:25425158

- Neuroprotective mechanism of the novel melatonin derivative Neu-P11 in brain ischemia related models.
  Buendia I, Gómez-Rangel V, González-Lafuente L, Parada E, León R, Gameiro I, Michalska P, Laudon M, Egea J, López MG.
  Neuropharmacology. 2015 Jul 15;99:187-195. doi: 10.1016/j.neuropharm.2015.07.014. [Epub ahead of print]
  PMID:26188145

Antes de presentar cada uno de los artículos originales en inglés, se adjunta el resumen de cada uno de ellos en español:

## 6.1. El nuevo híbrido melatonina-N,N-dibenzil(N-metilo)amina, ITH91/IQM157 ejerce neuroprotección en un modelo in vitro de Alzheimer mediante la inducción de Hemo oxigenasa-1.

En este trabajo, hemos investigado el efecto neuroprotector del nuevo derivado ITH91/IQM157, un híbrido de melatonina y N,N-dibenzil(N-metilo)amina, en un modelo *in vitro* de la EA en el que se combinan las patologías β-amiloide e hiperfosforilación de Tau (inducido por ácido okadáico, A.O) en las células de neuroblastoma humano SH-SY5Y.

La asociación de concentraciones subtóxicas de βA y A.O produjo una toxicidad significativa del 40%, muerte mayoritariamente apoptótica; esta muerte se asoció con una retracción de las prolongaciones celulares y acumulaciones proteicas aberrantes, teñidas mediante Tioflavina-S. En este modelo de toxicidad, el nuevo derivado, ITH91/IQM157, a concentraciones entre 1 y 1000 nM, redujo la muerte celular, medida por la técnica MTT; a 100 nM, previno la apoptosis, la retracción de las prolongaciones celulares y la formación de agregados de βA. El mecanismo protector ofrecido por el ITH91/IQM157 se revirtió con mecamilamina (antagonista de receptores nicotínicos), luzindol (antagonista de receptores de melatonina), celeritrina (inhibidor de proteína cinasa C (PKC)), PD98059 (inhibidor ERK 1/2), LY294002 (inhibidor PI3K/Akt), y protoporfirina de estaño (SnPP) (inhibidor de HO-1). Por lo tanto, demostramos que el nuevo derivado ITH91/IQM157, que aúna las actividades de la melatonina y de un IAChE en una única molécula, reduce la muerte neuronal inducida por βA y A.O, mediante un mecanismo de acción que implica los receptores nicotínicos y receptores de melatonina y las rutas pro-supervivencia PKC, Akt, ERK1/2, así como la inducción de HO-1.

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# The Melatonin–*N*,*N*-Dibenzyl(*N*-methyl)amine Hybrid ITH91/IQM157 Affords Neuroprotection in an in Vitro Alzheimer's Model via Hemoxygenase-1 Induction

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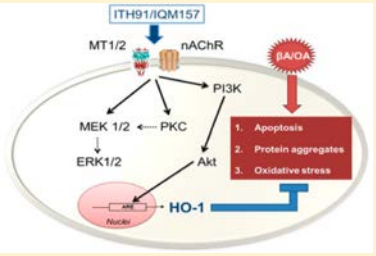
Supporting Information

**ABSTRACT:** We have investigated the protective effects of ITH91/IQM157, a hybrid of melatonin and *N*,*N*-dibenzyl(*N*-methyl)amine, in an in vitro model of Alzheimer's disease (AD)-like pathology that combines amyloid beta (A$\beta$) and tau hyperphosphorylation induced by okadaic acid (OA), in the human neuroblastoma cell line SH-SY5Y. Combination of subtoxic concentrations of A$\beta$ and OA caused a significant toxicity of 40% cell death, which mainly was apoptotic; this effect was accompanied by retraction of the cells' prolongations and accumulation of thioflavin-S stained protein aggregates. In this toxicity model, ITH91/IQM157 (1−1000 nM) reduced cell death measured as MTT reduction; at 100 nM, it prevented apoptosis, retraction of prolongations, and A$\beta$ aggregates. The protective actions of ITH91/IQM157 were blocked by mecamylamine, luzindol, chelerythrine, PD98059, LY294002, and SnPP. We show that the combination of melatonin with a fragment endowed with AChE inhibition in a unique chemical structure, ITH91/IQM157, can reduce neuronal cell death induced by A$\beta$ and OA by a signaling pathway that implicates both nicotinic and melatonin receptors, PKC, Akt, ERK1/2, and induction of hemoxygenase-1.




**KEYWORDS:** *SH-SY5Y, okadaic acid, beta-amyloid, melatonin, Alzheimer's disease, acetylcholinestaerase inhibitor, ITH91/IQM157, neuroprotection*

Alzheimer's disease (AD) is the most common form of dementia. There are about 27 millions of patients in the world, and this figure could increase to 107 million by the year 2050 if no treatment is found to delay the onset or the progression of the disease.[1] Therefore, the development of an effective treatment is a social, economic, and political global priority.

From a histopathological point of view, AD is characterized by two protein alterations, namely, tau hyperphosphorylation and excessive amyloid beta (A$\beta$) deposition, both related to neuronal degeneration.[2−4] This neurodegenerative process affects the cholinergic system, among others. Therefore, acetylcholinesterase inhibitors are the main drugs used today to treat these patients. For later stages of the disease, inhibition of NMDA receptors with memantine is also used. A meta-analysis for commercially available acetylcholinesterase inhibitors (AChEI) and memantine in combination for the treatment of patients with AD revealed only a modest trend favoring active treatment over placebo.[5] Therefore, the search for new compounds to treat this disease is still mandatory.

The use of multitarget compounds is emerging as an interesting strategy to treat different pathologies. These compounds combine, in a single molecule, complementary activities over different pathways of the pathophysiological cascade of AD. More specifically, our group has become interested in compounds that combine fragments derived from an inhibitor of acetylcholinesterase (AChEI) and melatonin for the following reasons: (i) AChEI are the drugs mainly used in clinic to treat AD patients; their mechanism of action is based on the improvement of cholinergic neurotransmission. (ii) The levels of the neurohormone melatonin, endowed with antioxidant properties,[6] are gradually reduced with age. In the cerebral spinal fluid (CSF), melatonin levels can be reduced by 50% when compared to young subjects; this reduction is even greater in AD patients (below 20%).[7−9] It is also worth mentioning that hippocampal CA1 and CA3 pyramidal neuronal loss can be reproduced in rats by removing their pineal gland, while replacement of melatonin in the drinking water recovers such loss.[10] Furthermore, melatonin has shown

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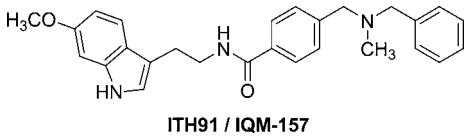
neuroprotective effect in several AD models,[8,11−14] and it has also shown beneficial effects in a double blind study on the sleep−wake rhythm and cognitive and noncognitive functions in Alzheimer's type dementia.[15] For all these reasons, melatonin could be beneficial in AD.[16] (iii) Previous results from our group have shown that the combination of subeffective concentrations of galantamine and melatonin offer a significant neuroprotective effect in SH-SY5Y cells against mitochondrial intoxication with rotenone and oligomycin A.[17] With these ideas in mind, we synthesized several melatonin−*N*,*N*-dibenzyl(*N*-methyl)amine hybrids;[18] the idea of keeping the AChEI activity, even if modest, was based on the fact that this target remains clinically valid for the majority of drugs (donepezil, rivastigmine, and galantamine) used today in AD patients. In this study, we have focused on ITH91/IQM157 (Figure 1) that shares chemical features of melatonin and the




ITH91 / IQM-157

**Figure 1.** Chemical structure of compound ITH91/IQM157, a melatonin−*N*,*N*-dibenzyl(*N*-methyl)amine hybrid.

AChEI AP2238, has low toxicity, is capable of crossing the blood-brain barrier in a predictive model, and has an interesting pharmacological profile with potential for the treatment of AD. It inhibits human AChE ($IC_{50}$ = 4.1 $\mu$M), displaces propidium from the peripheral anionic site of AChE (25% at 1.0 $\mu$M), presents antioxidant properties (ORAC = 1.5 trolox equiv), and protects neural cells against mitochondrial free radicals (26% at 1.0 $\mu$M).[18]

As mentioned earlier, there are two characteristic histopathological features in post-mortem brains of patients suffering from AD: senile plaques caused by accumulation of $\beta$A peptide and neurofibrillary tangles composed of hyperphosphorylated tau protein. It is also proposed that these alterations are not independent, but are interrelated.[19,20] Although there are several in vivo models that combine $\beta$A pathology with tau pathology, such as the double transgenic mice APPswe/TauVLW[21] or the triple transgenic PS1M146 V, APPswe, and TauP301L,[22] virtually no in vitro models combine these two alterations. Therefore, we have implemented an in vitro model that combines beta and tau pathology by combining $A\beta_{25-35}$ and okadaic acid in the human neuroblastoma cell line SH-SY5Y. We have used this model to evaluate the potential neuroprotective effects of the melatonin−*N*,*N*-dibenzyl(*N*-methyl)amine hybrid ITH91/IQM157.

## RESULTS AND DISCUSION

In order to set up the cytotoxicity model, we first performed concentration−response curves with A$\beta$ and okadaic acid in the human neuroblastoma cell line SH-SY5Y. Okadaic acid (OA), a phosphatase inhibitor that causes hyperphospholylation of tau protein,[23] was more effective to induce cell death than A$\beta$; in fact, maximum cell death achieved with A$\beta$ was near 40% (10 $\mu$M $\beta$A, Figure 2A), while with OA maximum cell death reach over 80% (30 nM OA, Figure 2B). Interestingly, when subeffective concentrations of both stimuli (1 $\mu$M of A$\beta$ and 3 nM of OA) were combined, we observed a significantly higher cytotoxic effect compared to each toxin alone (Figure 2C). This result was corroborated in a primary neuronal culture, in which a similar toxicity was observed (Supporting Information SI-1). Therefore, this result validates the use of a

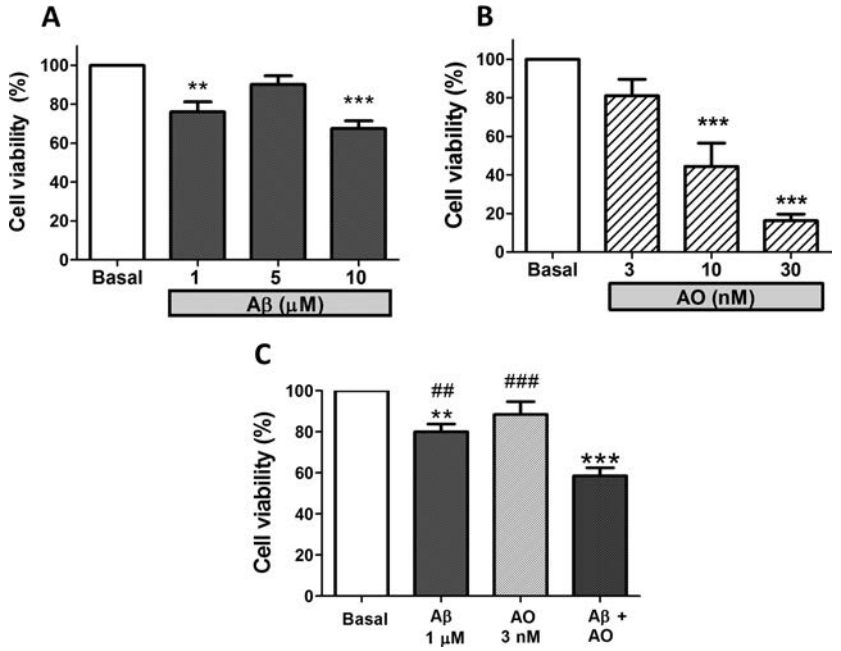



**Figure 2.** $A\beta_{25-35}$ ($\beta$A) and okadaic acid (OA) reduce cell viability of SH-SY5Y cells: combination of subeffective concentrations of A$\beta$ and OA cause significant cell death. Cells were incubated with the toxic stimuli for 20 h, and cell viability was assessed by the MTT technique. (A) Concentration−response curve with 1, 5, and 10 $\mu$M A$\beta$. For reasons that we do not completely understand, at 5 $\mu$M A$\beta$, we did not achieve a significant reduction in cell viability. (B) Concentration−response curve with 3, 10, and 30 nM OA. (C) Effect of 1 $\mu$M A$\beta$, 3 nM OA, and their association on SH-SY5Y cell viability. Values are expressed as means ± SEM of five different cultures, $^{***}P < 0.001$, $^{**}P < 0.01$ compared to basal; $^{###}P < 0.001$, $^{##}P < 0.01$ with respect to combination of both toxic stimuli.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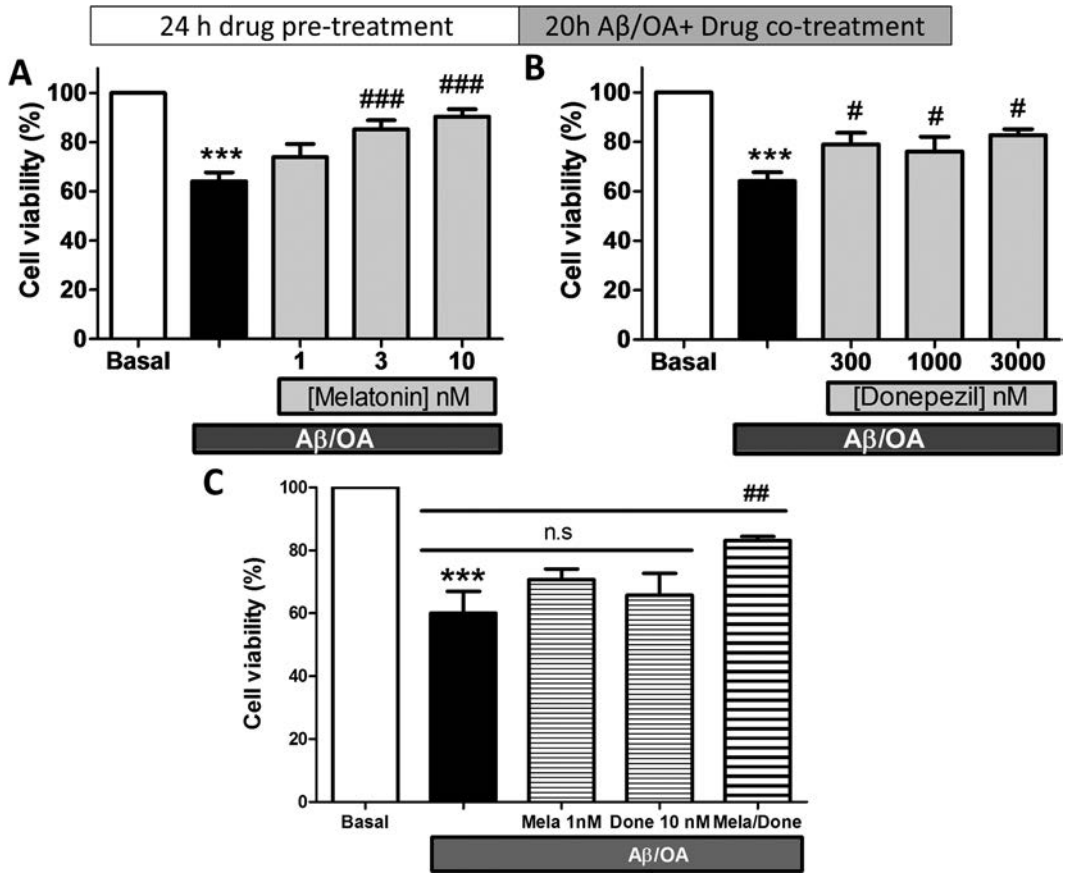


**Figure 3.** Combination of subeffective concentrations of melatonin and donepezil provide significant protection against A$\beta$/OA-induced toxicity. On the top part of the figure, a schematic illustration of the protocol used is represented. Cells were exposed for 20 h to 1 $\mu$M A$\beta$ plus 3 nM OA (A$\beta$/OA). When the neuroprotective compounds melatonin or donepezil were used, they were preincubated 24 h before adding the toxics and during the exposure to the toxics. Effect of increasing concentrations of melatonin (A) and donepezil (B) on the cell viability of SHSY5Y cells exposed to the combination of A$\beta$/OA, measured as MTT reduction. (C) Neuroprotective effect afforded by the association of subeffective concentrations of melatonin (1 nM) plus donepezil (10 nM). Data represent the mean ± SEM from seven different cultures, $^{***}P < 0.001$ compared to basal; $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$ compared with $\beta$A/OA group.

neuronal cell line instead of primary neuronal cultures which "replaces" the use of animals.

When we analyzed the apoptotic and necrotic populations in SH-SY5Y cells exposed to A$\beta$ (1 $\mu$M) in combination with OA (3 nM), from now on A$\beta$/OA, we found that cell death was mainly apoptotic (Figure 4B). These results are consistent with those described in animal models of AD where mutations associated with overexpression of A$\beta$ protein and mutations associated with hyperphosphorylation of tau are combined;[22,24] these animals show greater pathology and functional alterations in a more precocious way compared to monotransgenics. Besides the effects on cell death, subtoxic concentrations of A$\beta$/OA caused neurite retraction (Figure 5B), an effect related to tau hyperphosphorylation, which causes microtubule destabilization, cytoarchitecture loss, and, consequently, neurodegeneration.[3,23] This degeneration and cell death is also reflected in the emergence of more pyknotic nuclei in cells treated with A$\beta$/OA. We also found aggregates of thioflavin S staining as an indication of A$\beta$ aggregation (Figure 5E). Taken together, by combining subtoxic concentrations of A$\beta$ with OA, we have established a cytotoxicity model that displays several pathological markers of AD, such as neurite retraction, accumulation of protein aggregates, and apoptotic cell death. This model could, therefore, serve as a new cytotoxicity model to evaluate compounds with potential interest in the screening stage of AD-compounds, before moving into the in vivo studies that are more expensive and more time-consuming.

Having set the experimental conditions of toxicity induced A$\beta$/AO, we evaluated the potential cytoprotective effect of melatonin, the acetylcholinesterase inhibitor donepezil, and the association of subeffective concentrations of both. The experimental protocol consisted of preincubating SH-SY5Y cells for 24 h with increasing concentrations of the neuroprotective compounds prior to the addition of the toxic stimuli (A$\beta$ /OA), and maintaining the protective compounds for an additional 20 h period together with the toxins (see protocol on top of Figure 3). Melatonin showed a significant protective effect at the concentration of 3 nM (35.8% protection), and this protection increased in a concentration-dependent manner, being maximum at 10 nM (73% protection) (Figure 3A). We also evaluated the potential neuroprotective effect of donepezil; the range of concentrations was selected based on previous data from our group.[25] As represented in Figure 3B, donepezil was protective at concentrations ranging from 0.3 to 3 $\mu$M; however, a concentration-dependent effect was not observed.

To test the hypothesis that a significant neuroprotective effect could be achieved with the combination of subeffective concentrations of melatonin and an AChEI, we used 1 nM of melatonin plus 10 nM donepezil in the A$\beta$/OA toxicity model;


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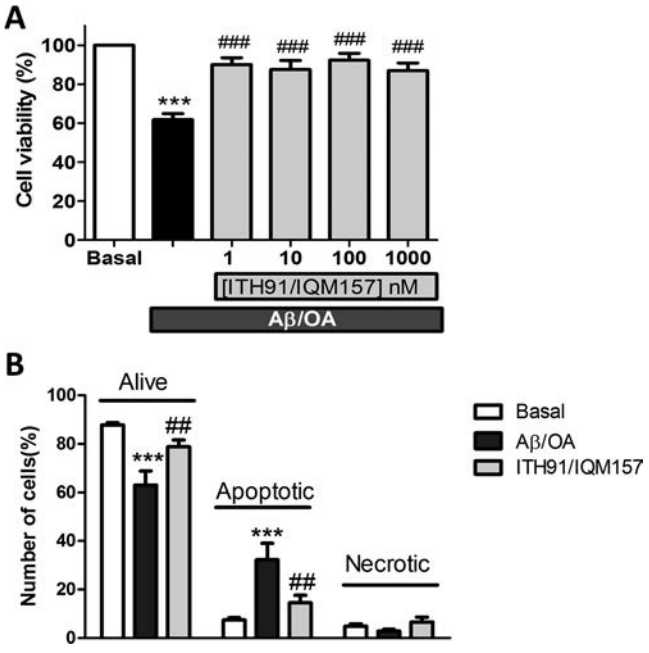


**Figure 4.** ITH91/IQM157 is neuroprotective against A$\beta$/OA toxicity by an antiapoptotic mechanism. (A) Effect of increasing concentrations of ITH91/IQM157 on the cell viability of cells exposed to A$\beta$/OA. (B) Percentage of alive, apoptotic, and necrotic cells, measured by flow cytometry in control cells or cells exposed to A$\beta$/OA alone or in the presence of ITH91/IQM157 at 100 nM. Data correspond to the mean ± SEM of four different cell batches; $^{***}P < 0.001$ significantly different from basal apoptotic cell death. $^{###}P < 0.001$, $^{##}P < 0.01$ significantly different from $\beta$A/OA-induced apoptotic cell death.

indeed, the drug combination afforded significant protection (57% protection) compared to the drugs alone (Figure 3C).

Next, we evaluated the potential neuroprotective effect of the melatonin−*N*,*N*-dibenzyl(*N*-methyl)amine hybrid ITH91/IQM157. Compared to melatonin or the acetylcholinestarase inhibitor donepezil, the neuroprotective actions found with ITH91/IQM157 were achieved at lower concentrations; at 1 nM, ITH91/IQM157 already offered maximum protection (Figure 4A). This hybrid improved the neuroprotective activity in comparison to the combination strategy of subeffective concentrations of melatonin (1 nM) and donepezil (10 nM); protection was 75% with 1 nM ITH91/IQM157 versus 57% with the combination strategy (Figure 3C). This finding agrees with our previous observation that combination of subeffective concentrations of melatonin and the AChEI galantamine offers significant neuroprotection.[17]

There are several potential advantages for a multifunctional molecule versus combination of different drugs covering the same mechanisms. First, association of several drugs may have different pharmacodynamics and pharmacokinetics; however, when a single molecule is developed, these properties can be optimized. Second, when two or more drugs are combined, frequently, there are complex pharmacological interactions that modify the effect of the other, giving increased secondary effects or reducing the effectiveness of one or more of the combined molecules. Finally, drugs directed to a single target might not always modify complex systems, even if they act in the way they are expected to precede. It is very common in the cell to have "backup" systems yielding the same effect such as gene expression, protein synthesis, receptors response, and protein degradation. Proteins and intermediates involved in these backup systems can be completely different and therefore, drugs targeting primary pathways will have no effect over this backup pathway, an effect known as redundancy. Multitarget therapeutics can be more efficacious making the biological system more sensitive to the action of a drug with two or more targets simultaneously, thereby, mitigating the redundancy

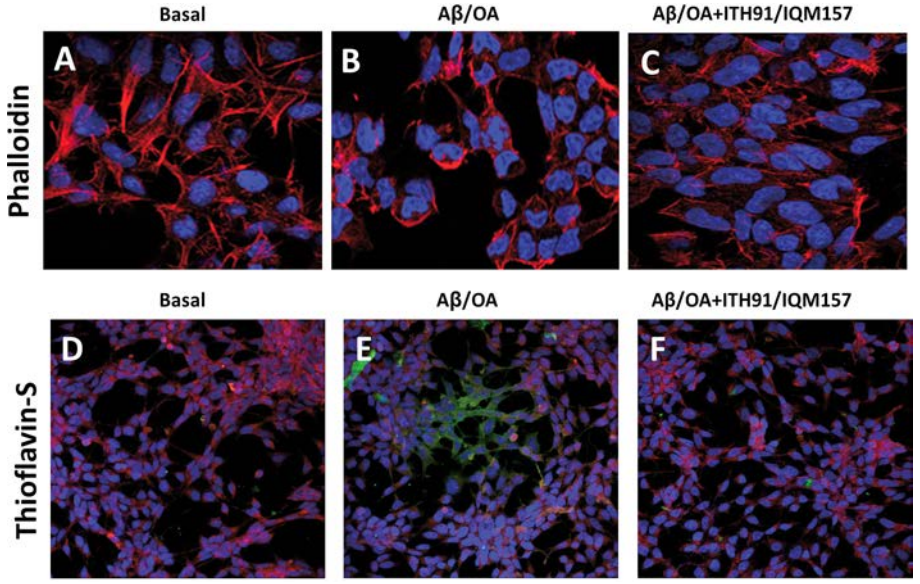


**Figure 5.** ITH91/IQM157 recovered cytoskeletal alterations and thioflavin-S aggregates induced by exposure of SH-SY5Y cells to A$\beta$/OA. Top part shows images of SH-SY5Y cells double stained with Hoechst 33342 (nuclei in blue) and phalloidin (cytoskeleton in red) under basal conditions (A), treated with A$\beta$/OA in the absence (B) or presence of 100 nM of ITH91/IQM157 (C). Bottom figures show images of SH-SY5Y cells stained with Hoechst 33342 (nuclei in blue), phalloidin (cytoskeleton in red), and Thioflavin-S (A$\beta$ aggregates in green) under basal conditions (D) or treated with A$\beta$/OA in the absence (E) or presence of 100 nM of ITH91/IQM157 (F). Images are representative of others obtained in three different cell batches.


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effect. Therefore, the complexity of interactions in the drug-combination approach has led to the hypothesis that one single molecule, acting on several targets at the same time, might be more effective for the drug development in complex diseases like AD.

Concerning the neuroprotective mechanism of action of ITH91/IQM157, both melatonin and nicotinic receptors seem to be implicated since both luzindole (a melatonin receptor antagonist) and mecamylamine (a nicotinic receptor antagonist) significantly reduced its protective effect (Figure 6A). The

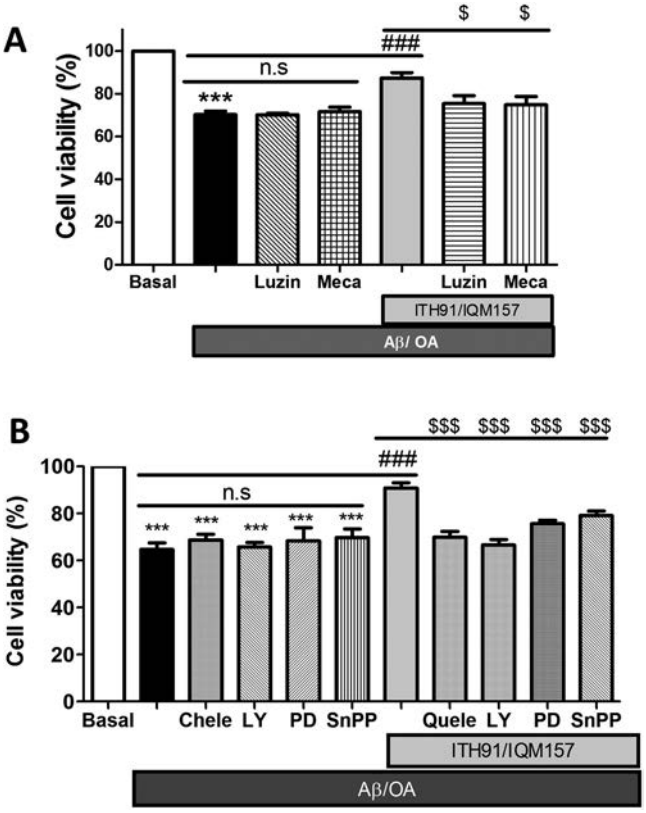


**Figure 6.** Neuroprotection elicited by ITH91/IQM157 involves melatonin receptors, nicotinic acetylcholine receptors, PI3K/Akt, ERK1/2, PKC, and induction of HO-1. (A) The melatonin receptor antagonist luzindole (3 $\mu$M) and the nAChR antagonist mecamylamine (10 $\mu$M) partially block the protective action of ITH91/IQM157. Both antagonists per se had no effect on cell death caused by A$\beta$/OA. (B) The protective effect of ITH91/IQM157 is prevented by the PKC inhibitor chelerythrine (1 $\mu$M), the PI3K/Akt antagonist LY294002 (10 $\mu$M), the ERK1/2 antagonist PD98059 (10 $\mu$M) and the HO-1 inhibitor Sn(IV) protoporphyrin IX dichloride (SnPP) (10 $\mu$M). The antagonists per se had no effect on cell death caused by $\beta$A/OA. Values are means ± SEM of seven experiments.***$P < 0.001$ significantly different from untreated cells; $^{###}P < 0.001$ in comparison to A$\beta$/OA; $^{\$\$\$}P < 0.001$, $^{\$}P < 0.05$ with respect to ITH91/IQM157 treated cells.

involvement of nAChRs has also been implicated in the protective effects of other AChE inhibitors like galantamine and donepezil.[26−28] The neuroprotective effect of ITH91/IQM157 was accompanied by the recovery of the cytoarchitecture and a reduction of thioflavin-S aggregates (Figure 5C and F). The reduction of protein aggregates can be related to actions of the melatonin substructure, since it is reported that melatonin can directly interact with A$\beta$ and prevent its aggregation[29,30] and it can also interfere with APP processing.[31−33] Furthermore, we previously reported that compound ITH91/IQM157 displaces propidium iodide from the peripheral acetylcholinesterase site, which is known to participate in $\beta$A aggregation.[34] Interaction with $MT_2$ receptors can stimulate phospholipase C and activate protein kinase C (PKC) via diacylglycerol, which in turn phosphorylates and inactivates GSK-3$\beta$, whose participation in APP synthesis[35,36] and tau hyperphosphorylation is well documented; this could be an additional mechanism for compound ITH91/IQM157. In fact, the protective mechanism of ITH91/IQM157 was partially inhibited by the PKC inhibitor chelerythrine (Figure 6B).

Our group and others have shown that activation of melatonin and nicotinic receptors can promote survival pathways such as those related to PI3K/Akt and ERK1/2[17,37,38]. Indeed, ITH91/IQM157 increased phosphorylation of ERK1/2 and Akt (Figure 7A and B) and its protective actions were prevented in the presence of inhibitors of these kinases (Figure 6B). Akt can phosphorylate GSK-3$\beta$ at position Ser-9, inactivating it[39−41] and can improve neuronal survival by (i) contributing to reduction of $\beta$A and tau pathology as mentioned above and/or (ii) promoting the nuclear translocation of Nrf2 (nuclear factor E2-related factor 2) to increase the cells defense mechanisms.[42]

Hemoxygenase-1 (HO-1) can be transcribed by Nrf2; it is an enzyme related to antioxidant, antineuroinflammatory, and neuroprotective actions. Compound ITH91/IQM157 was capable of inducing per se HO-1 (Figure 7C), and, most interesting, its protective actions were prevented when an inhibitor of this antioxidant enzyme (SnPP) was added to the cells (Figure 6B). These results indicate that part of its neuroprotective actions can be attributed to induction of HO-1 as already described for other neuroprotective drugs that interact with melatonin or nicotinic receptors.[37,43,44]

As a multifunctional drug, ITH91/IQM157 is endowed with different complementary mechanisms of action that could be useful to limit the complex physiopathological cascade of AD. One of those complementary actions, besides ACE inhibition and A$\beta$ aggregation, could be induction of HO-1 as part of its neuroprotective mechanism. In this study, we have focused on HO-1 because this enzyme seems to participate in the protective action of drugs that have a similar mechanism to compound ITH91/IQM157, for example, melatonin or nicotinic agonists.[37,45] Also induction of HO-1 by the ACEI galantamine has been related to protection of microvascular endothelial cells.[46] We see in this study that ITH91/IQM157 can induce HO-1 and that its protective actions are lost in the presence of the HO-1 inhibitor SnPP; this effect does not exclude the drug from having ACE inhibitory actions that could improve cognition or from reducing beta-amyloid aggregation that could contribute to reduce neuroinflammation and protecting neurons adjacent to the beta-amyloid plaques.

In conclusion, the melatonin−*N*,*N*-dibenzyl(*N*-methyl)-amine hybrid ITH91/IQM157 reduces cell vulnerability as well as A$\beta$ aggregates and disruption of the cytoskeleton in an in vitro AD-related model. The mechanism of action of ITH91/IQM157 involves melatonin and nicotinic receptors, activation of a signaling cascade that includes PKC, ERK1/2, and PI3K/Akt, and induction of the antioxidant and antineuroinflammatory enzyme HO-1; all of these actions can contribute to promote cell survival and thereby prevent neurodegeneration.


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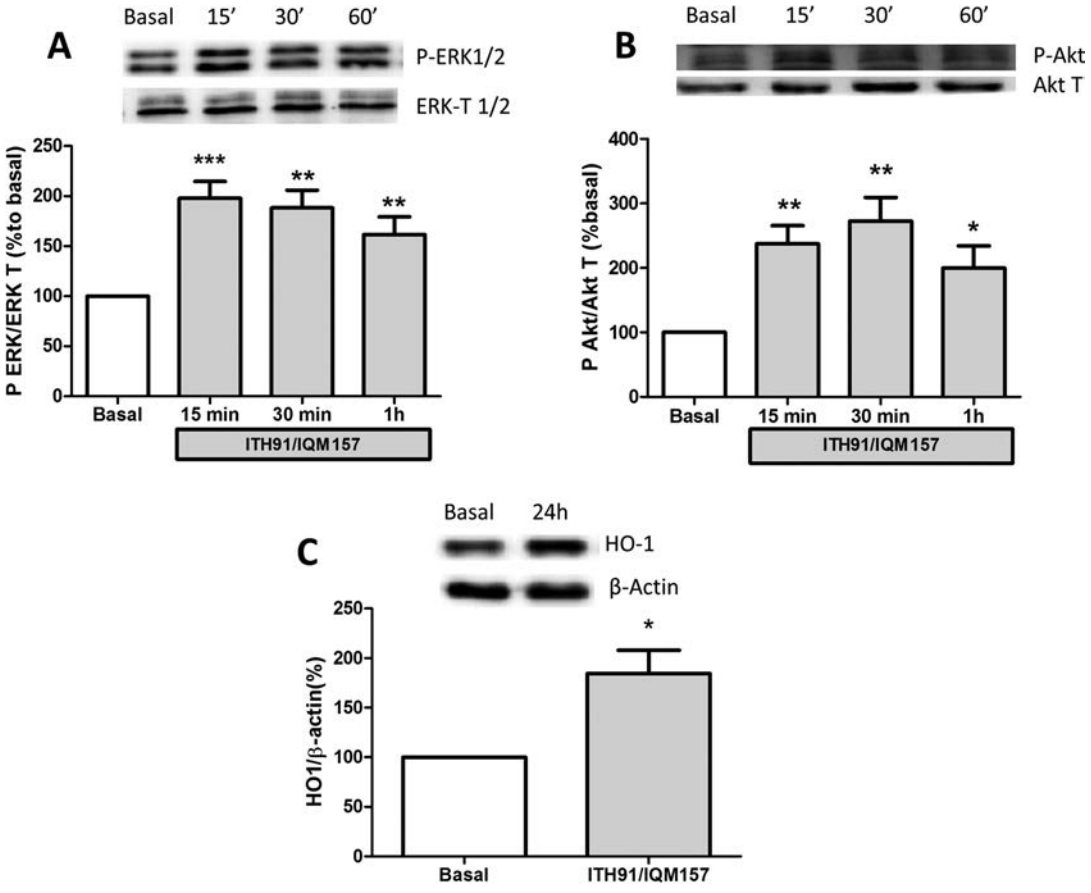


**Figure 7.** ITH91/IQM157 increases ERK1/2 and Akt phosphorylation and induces the antioxidant enzyme HO-1. ERK1/2 phosphorylation with respect to total-ERK1/2 (A) and Akt phosphorylation with respect to total-Akt (B) was analyzed, by Western blot, in SH-SY5Y cells treated for 60, 30, or 15 min with 100 nM ITH91/IQM157. The top part of the figures shows a representative immunoblot, and the histogram below shows the mean densitometric quantification of both kinases. (C) HO-1 induction in cells treated for 24 h with ITH91/IQM157 at 100 nM. The top part of the figure illustrates a representative immunoblot, and the bottom part a histogram with the densitometric quantification of HO-1 induction normalized with respect to $\beta$-Actin, under basal conditions or exposed to melatonin. Values correspond to the mean ± SEM of five experiments. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ significantly different from untreated cells.

## METHODS

**Materials.** Amyloid beta ($A\beta_{25-35}$), okadaic acid (AO), chelerythrine, PD98059 (2-(2-amino-3-methoxyphenyl)-(4*H*-1-benzopyran-4-one)), and LY294002 (morpholino-4-yl-8-phenylchromen-4-one), mecamylamine, were from Tocris scientific/Biogen, Madrid, Spain. Tin protoporphyrin (IV) from was Frontier Scientific Europe, Lancashire, U.K. Donepezil and melatonin were obtained from Sigma-Aldrich, Madrid, Spain, and ITH91/IQM157 was synthesized by the group of Dr. Rodríguez-Franco from the Instituto de Química Médica, Consejo Superior de Investigaciones Científicas (IQM-CSIC).

**Culture of the Human Neuroblastoma Cell Line SH-SY5Y.** SH-SY5Y cells were maintained in culture medium containing 10% inactivated fetal bovine serum, 15 nonessential amino acids, 1 mM sodium pyruvate (Invitrogen, Madrid, Spain), F12 nutrient medium (Ham12), MEM medium (Eagle's minimum essential medium) (Sigma-Aldrich, Madrid, Spain), $NaHCO_3$, 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin (Invitrogen, Madrid, Spain) in Milli-Q $H_2O$. Cells were grown initially in a flask and subcultured in 48-well plates at a density of $1 \times 10^5$ cells/well. Cells were maintained in an incubator in a humid atmosphere at 37 °C with 5% $CO_2$; they were used between 4 and 12 passages.

**Measurement of Cell Viability Using the MTT Method.** Cell viability was assessed by the detection of mitochondrial activity in living cells using the colorimetric analysis of blue tetrazolium bromide thiazolyl (MTT) (Sigma-Aldrich, Spain), previously described by Denizot and Lang.[47] Upon completion of the experiments, 50 $\mu$L of reagent MTT was added to each well to achieve a final concentration of 0.5 mg/mL; then, the cells were kept for 2 h in an incubator at 37 °C with 5% $CO_2$ and 95% air. Finally, 200 $\mu$L of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan salt and absorbance was measured in an ELISA reader at 540 nm. The absorbance obtained in basal conditions was taken as 100% cell viability.

**Measurement of Apoptosis and Necrosis with Annexin V−Phycoerythrin (PE) and 7-Aminoactinomycin D (7-AAD) by Flow Cytometry.** Apoptosis was determined by flow cytometry using an annexin V−PE (phycoerythrin) and 7-AAD double staining kit (BD Bioscience, Madrid, Spain) according to the manufacturer's instructions. Briefly, at the end of the experiment, cells were collected after centrifugation and resuspended in a solution containing 100 $\mu$L of 1× binding buffer, 5 $\mu$L of annexin V−PE, and 5 $\mu$L of 7-AAD. Cells were incubated at room temperature for 15 min in darkness, and then 100 $\mu$L of 1× binding buffer was added. Cells were then subjected to FACS analysis (Beckman Coulter, Madrid, Spain). Annexin V+/7-AAD− cells were considered as early apoptotic cells, annexin V+/7-AAD+ as late apoptotic cells, and annexin V−/7-AAD− as viable cells.

**Double Staining of SH-SY5Y Cells with Phalloidin and Hoechst.** We used phalloidin-rhodamine staining to detect the cellular cytoskeleton in our experimental conditions. Hoechst staining was concomitantly used to detect the nuclei. At the end of the experiment, SH-SY5Y cells were washed three times with PBS (NaCl 9 g/L, 10 mM $NaH_2PO_4$, 10 mM $K_2HPO_4$) and fixed with 2% paraformaldehyde dissolved in PBS for 15 min, permeabilized with 0.1% Triton in PBS for 1 min, and stained with phalloidin-rhodamine in PBS 1:1000 (Sigma-Aldrich, Madrid, Spain) for 20 min. Later, the


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cells were washed three times with PBS every 5 min; staining of the nuclei with Hoechst (5 $\mu$g/mL) was performed during the second wash (Invitrogen, Madrid, Spain). Finally, the slides were covered with coverslips adding glycerol-PBS (1:1 vol/vol) and imaged with a confocal microscope (TCS SPE, Leica, Wetzlar, Germany).

**Triple Staining of SH-SY5Y Cells with Thioflavin-S, Hoechst, and Phalloidin.** SH-SY5Y cells were fixed with 2% paraformaldehyde dissolved in PBS for 15 min and washed three times with PBS every 5 min. Later, they were permeabilized with 0.1% Triton for 1 min and washed three times with PBS before staining them with Thioflavin-S 0.5% for 10 min. Then, three consecutive washes with ethanol 80%, Milli-Q $H_2O$, and PBS were performed. Later, cells were stained with phalloidin-rhodamine in PBS 1:1000 (Sigma-Aldrich, Madrid, Spain) for 20 min, followed by three washes with PBS every 5 min; staining of the nuclei with Hoechst (5 $\mu$g/mL) was performed during the second wash (Invitrogen, Madrid, Spain). Finally, the slides were covered with coverslips adding glycerol-PBS (1:1 vol/vol) and imaged with a confocal microscope (TCS SPE, Leica, Wetzlar, Germany).

**Measurement of Protein Expression by Western Blot.** SH-SY5Y cells were lysed with 100 $\mu$L of cold lysis buffer containing 1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM $Na_3VO_4$. Once the amount of protein was quantified using the BCA Protein Assay Kit Reagent (Fisher Scientific, Madrid, Spain), electrophoresis was performed running 30 $\mu$g of proteins in polyacrylamide gels (PAGE) for 2 h at constant amperage. Proteins were transferred to PVDF membranes (Millipore Ibérica SA, Madrid, Spain) for 2 h at 70 mA. Later on, membranes were blocked for 2 h with TTBS + 4% albumin (Sigma-Aldrich, Madrid, Spain), incubated with anti-P-Akt, anti-total Akt (Santa Cruz Biotechnology, Santa Cruz, CA,), anti-P-ERK, anti-total ERK, anti-HO-1 (1:1000) (Chemicon, Temecula, CA), and anti-$\beta$ actin (1:10 000) (Sigma-Aldrich, Madrid, Spain) for 2 h. After washing several times with TTBS, the corresponding secondary antibodies (1:100 000) were added (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min. Finally, the membranes were revealed using ECL Advance Western Blotting Detection Kit (GE Healthcare, Barcelona, Spain) and quantified by using Scion-Image software.

**Statistical Analysis.** Data are presented as means ± SEM. Differences between groups were determined by applying a one-way ANOVA followed by a Newman−Keuls post hoc analysis. The level of statistical significance was taken at $p < 0.05$.

## ■ ASSOCIATED CONTENT

### Supporting Information

Figures showing (SI-1) effect of 1 $\mu$M A$\beta$, 3 nM OA, and their association on primary neuronal cell culture viability, and (SI-2) concentration−response curves of compounds ITH90/IQM156 and ITH91/IQM157 on the viability of SH-SY5Y cells exposed to A$\beta$/OA. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

⊥I.B. and J.E. have contributed equally to this work. I.B. has contributed to the concept/design, acquisition of data, data analysis/interpretation, and critical revision of the manuscript. J.E. has contributed to the concept/design, acquisition of data, data analysis/interpretation, drafting of the manuscript, critical revision of the manuscript, and approval of the manuscript. E.P. has contributed to acquisition of data and data analysis/interpretation. E.N. has contributed to acquisition of data and data analysis/interpretation. R.L. has contributed to acquisition of data, data analysis/interpretation, and critical revision of the manuscript. M.I.R.-F. has contributed to chemical synthesis of compounds ITH90/IQM156 and ITH91/IQM175 and critical revision of the manuscript. M.G.L. has contributed to the concept/design, drafting of the manuscript, critical revision of the manuscript, and approval of the manuscript.


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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

AD, Alzheimer's disease; A$\beta$, amyloid beta; AO, okadaic acid; AChEI, acetylcholine esterase inhibitors; $\alpha$7 nAChRs, $\alpha$7 nicotinic acetylcholine receptors; HO-1, heme oxygenase 1; MTT, blue tetrazolium bromide thiazolyl; DMSO, dimethyl sulfoxide; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; LY, LY294002 (morpholino-4-yl-8-phenylchromen-4-one); PD, PD98059 (2-(2-amino-3-methoxyphenyl)-(4*H*-1-benzopyran-4-one); SnPP, tin(IV) protoporphyrin IX dichloride

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## 6.2 La asociación de concentraciones subefectivas de melatonina y galantamina mejoran las alteraciones patológicas en un modelo de Alzheimer en cultivo organotípico de hipocampo.

La melatonina es una neurohormona que se encuentra considerablemente reducida o incluso ausente en pacientes de EA. En estos pacientes, los fármacos más utilizados en clínica son los IAChE. Sin embargo, su utilidad en clínica está limitada, debido entre otros factores, a sus efectos adversos. Además, como la asociación de fármacos es una estrategia farmacológica ampliamente utilizada en el tratamiento de diferentes enfermedades, como el cáncer o el síndrome de inmunodeficiencia adquirida, en este estudio nos propusimos evaluar si la combinación de melatonina y el IAChE, galantamina, podría ofrecer efectos beneficiosos en un nuevo modelo *in vitro* de la EA. Para ello, tratamos los cultivos organotípicos de hipocampo de rata con concentraciones subtóxicas de βA (0.5 μM) y de ácido okadaico (3nM) durante 4 días. Esta combinación de tóxicos produjo un 95% de muerte celular, siendo ésta mayoritariamente apoptótica. Además, esta combinación causó agregados de βA, hiperfosforilación de Tau, estrés oxidativo y neuroinflamación.

Bajo estas condiciones tóxicas, el co-tratamiento de las rodajas tanto con melatonina (1-1000 nM), como con galantamina (10-1000 nM), mostraron un efecto neuroprotector concentración dependiente. Además, la combinación de concentraciones subefectivas de ambos fármacos (1 nM de melatonina y 10 nM de galantamina) produjeron un efecto neuroprotector sinérgico y, además, previno la mayoría de las alteraciones relacionadas con la EA descritas en este modelo. Por lo tanto, podemos concluir que la suplementación de melatonina con menores dosis de IAChE podría ser una estrategia farmacológica interesante para los pacientes de Alzheimer.

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# Subthreshold Concentrations of Melatonin and Galantamine Improves Pathological AD-Hallmarks in Hippocampal Organotypic Cultures

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**Abstract** Melatonin is a neurohormone whose levels are significantly reduced or absent in Alzheimer's disease (AD) patients. In these patients, acetylcholinesterase inhibitors (AChEI) are the major drug class used for their treatment; however, they present unwanted cholinergic side effects and have provided limited efficacy in clinic. Because combination therapy is being extensively used to treat different pathological diseases such as cancer or acquired immune deficiency syndrome, we posed this study to evaluate if melatonin in combination with an AChEI, galantamine, could provide beneficial properties in a novel in vitro model of AD. Thus, we subjected organotypic hippocampal cultures (OHCs) to subtoxic concentrations of β-amyloid (0.5 μM βA) plus okadaic acid (1 nM OA), for 4 days. This treatment increased by 95 % cell death, which was mainly apoptotic as shown by positive TUNEL staining. In addition, the combination of βA/OA increased Thioflavin S aggregates, hyperphosphorylation of Tau, oxidative stress (increased DCFDA fluorescence), and neuroinflammation (increased IL-1β and TNFα). Under these experimental conditions, melatonin (1–1000 nM) and galantamine (10–1000 nM), co-incubated with the toxic stimuli, caused a concentration-dependent neuroprotection; maximal neuroprotective effect was achieved at 1 μM of melatonin and galantamine. Most effective was the finding that combination of sub-effective concentrations of melatonin (1 nM) and galantamine (10 nM) provided a synergic anti-apoptotic effect and reduction of most of the AD-related pathological hallmarks observed in the βA/OA model. Therefore, we suggest that supplementation of melatonin in combination with lower doses of AChEIs could be an interesting strategy for AD patients.

**Keywords** Alzheimer disease · Melatonin · Galantamine · Combined therapy · Inflammation · Oxidative stress

## Introduction

Among neurodegenerative diseases, Alzheimer's disease (AD) is the most common form of dementia. AD is an age-related neurodegenerative disease characterized by progressive loss of cognitive function, impaired memory, judgment and decision making, among others [1]. The brains of these patients show extracellular βA plaques and intraneuronal neurofibrillary tangles composed of hyperphosphorylated Tau protein which are considered major hallmarks for this disease and, are related to neurodegeneration [2–4]. However, glutamate excitotoxicity, free radical-mediated damage, and mitochondrial dysfunction are also found in AD and are considered common pathophysiological mechanisms with many other neurodegenerative diseases [5]. In spite of the evidences mentioned above, the exact pathogenesis of AD still remains to be fully elucidated and is thereby considered to be a multifactorial disease.

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In the treatment of complex diseases such as cancer, the combination of several one-target specific drugs has proven better results than a single one-target drug. This therapeutic improvement can be explained by the fact that diseases with a complex pathogenesis need to be approached with different drugs acting on the different pathways that lead to pathology. Therefore, combined therapy has opened a broad line of research for scientists in different fields [6]. In this respect, several studies have already been carried out showing the usefulness of combination of therapies for AD; for example, the use of memantine and acetylcholinesterase (AChE) inhibitors (AChEI) in patients with AD [7, 8].

Melatonin, originally discovered as a hormone synthesized mainly in the pineal gland, is also produced by cells of the immune system and many tissues, including brain, airway epithelium, bone marrow, gut, ovary, testes, skin, and likely other tissues [9]. Melatonin and its metabolites possess a potent antioxidant activity, and part of its therapeutic applications or preventive uses are based on this property [10, 11]. The beneficial effects of melatonin involve protection of nuclear DNA, membrane lipids and cytosolic proteins, observed in AD patients. Given that multiple lines of evidence have shown strong implications of oxidative stress in the pathogenesis of AD [12] and that melatonin levels are greatly reduced or absent [13] in AD patients, the development of approaches to reduce oxidative stress [14] and restore melatonin levels in AD patients may provide a new therapeutic opportunity.

AChEI are the major drug class used in the treatment of AD; however, their clinical usefulness is limited largely because of their adverse effects (confusion, hallucinations, extreme or sudden changes in behavior, nausea, or stomach pain) arising from excessive activation of the cholinergic system [6]. Galantamine is an alkaloid isolated from the Caucasian snow-drop (*Galanthus woronowii*) that was approved in February 2001 for AD treatment. It is a centrally acting, selective, reversible, and competitive AChEI, as well as from an allosteric modulator of the neuronal nicotinic receptor for acetylcholine, improving cholinergic transmission. Interestingly, it has shown to be protective in different models related to AD, ischemia, Huntington's disease, or amyotrophic lateral sclerosis (ALS) [12, 15–17].

There are molecular and physiological bases that support that melatonin may have an effective influence on several of the AD hypotheses to explain the cause of the disease [13]. Furthermore, melatonin has low toxicity and is able to reduce the side effects and increase the efficacy of other drugs [18], being proposed as an effective adjuvant in AD management [19, 20]. Additionally, previous data from our group have shown that sub-effective concentrations of melatonin combined with galantamine offer a synergic neuroprotective effect in an in vitro model of oxidative stress [12].

With this background in mind, the present study was carried out with the following objectives: (i) to develop a new in vitro model that reproduces AD-related pathological hallmarks by combining the toxic stimuli βA and okadaic acid (OA), which leads to hyperphosphorylation of Tau protein [21, 22] in organotypic hippocampal cultures and (ii) to determine if subthreshold concentrations of melatonin and galantamine reduce AD-like pathology and apoptosis in the toxicity model of βA/OA. The results of this study could provide new insights on the validity of this combination therapy in AD and also to develop new multitarget melatonin-cholinergic compounds.

## Materials and Methods

### Animals

All animal assays were carried out following the Guide for the Care and Use of Laboratory Animals and were previously approved by the Institutional Ethics Committee of the Autonomous University of Madrid, Spain, according to the European guidelines for the use and care of animals for research in accordance with the European Union Directive of 22 September 2010 (2010/63/UE) and with the Spanish Royal Decree of 1 February 2013 (53/2013). All efforts were made to minimize the number of animals used and their suffering.

### Preparation Organotypic Slice Cultures and Treatment

Organotypic hippocampal slice cultures (OHCs) were obtained from brains of 8- to 10-day-old Sprague Dawley rats. Cultures were prepared according to the method described by Stoppini et al. [23], slightly modified. Briefly, 300-μm-thick hippocampal slices were prepared and separated in ice-cold Hank's balanced salt solution (HBSS) composed of (in mM) glucose 15, $CaCl_2$ 1.3, KCl 5.36, NaCl 137.93, $KH_2PO_4$ 0.44, $Na_2HPO_4$ 0.34, $MgCl_2$ 0.49, $MgSO_4$ 0.44, $NaHCO_3$ 4.1, HEPES 25, 100 U/ml penicillin, and 0.100 mg/ml gentamicin. Four slices were placed on Millicell-0.4 μm culture inserts (Millipore, Madrid, Spain) within each well of a six-well culture plate with media, where they remained for 4 days before applying the different treatments. The culture media consisted of 50 % minimal essential medium (MEM), 25 % Hank's balanced salt solution, and 25 % heat-inactivated horse serum (Life Technologies, Madrid, Spain). After 4 days in culture, OHCs were treated with β-amiloyd$_{25\text{-}35}$ (βA, Sigma-Aldrich, Spain), okadaic acid (OA, Tocrisscientific/Biogen, Madrid, Spain), or the association of both toxic stimuli for 4 days, in the absence or presence of the protective drugs (melatonin, galantamine). OHCs were cultured in a humidified atmosphere at 37 °C and 5 % $CO_2$, and the medium was changed twice a week.

### Cell Death Measurement: Quantification of Propidium Iodide and Hoechst 33342 in OHCs

After finishing the experiment, OHCs were loaded with 1 μg/ml propidium iodide (PI) and Hoechst 33342 (Hoechst) during 30 min of incubation in the humidified atmosphere chamber.

PI and Hoechst fluorescence of CA1 region was measured in a fluorescence inverted NIKON eclipse T2000-U microscope. Wavelengths of excitation and emission for PI and Hoechst were 530 or 350, and 580 or 460 nm, respectively. Images were taken at magnifications of ×10. Fluorescence analysis was performed using the Metamorph programme version 7.0. Dividing the mean of PI fluorescence by the mean of Hoechst fluorescence, we normalized the amount of cell death. Data were normalized with respect to basal values that were considered as 100 %.

### TUNEL

The detection of DNA fragmentation and apoptotic bodies in OHCs were assessed with a terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling assay using a kit (TdT-mediated biotin-dUTP nick-end labeling; TdT-FragEL DNA fragmentation detection kit, Oncogene Research Products). At the end of experiment, OHCs were processed according to the manufacturer's protocol. Finally, the reaction was visualized by streptavidin-biotin-peroxidase complex and diaminobenzidine. Slices were counterstained with methyl green.

### Western Blot

OHCs were lysed in 100 μl ice-cold lysis buffer (1 % Nonidet P-40, 10 % glycerol, 137 mmol/l NaCl, 20 mmol/l Tris-HCl, pH 7.5, 1 μg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride, 20 mmol/l NaF, 1 mmol/l sodium pyrophosphate, and 1 mmol/l $Na_3VO_4$). Protein (30 μg) from the slice lysates was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Amersham). Membranes were incubated with the following antibodies: anti-PHF1 (generously gifted by Dr. Ávila) at 1:1000 and anti-β-actin at 1:100000 (Sigma, Madrid, Spain); Appropriate peroxidase-conjugated secondary antibody at 1:10000 was used to detect proteins by enhanced chemiluminescence.

### Thioflavin S Staining

Once the experiment was finished, OHCs were fixed with 4 % paraformaldehyde for 1 h and washed three times every 5 min with PBS. Then, slices were treated with 1:1 chloroform/ethanol for 1 h. Later, three washes in ethanol 100, 96, and 70 % followed by 5 min of water were made. Next, OHCs were incubated with Thioflavin S (1 mg/ml) for 1 h. Finally, they were washed three times (applying in the second wash 1 μg/ml Hoechst 33342) every 5 min with PBS and, mounted with Apathy on coverslips. Images were taken in a confocal microscope (TCS SPE; Leica, Wetzlar, Germany).

### ROS Measurement

To measure the cell's production of reactive oxygen species (ROS), we used the fluorescence probe CM-$H_2$DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) [24]. After the experiment, OHCs were loaded with 5 μM CM-$H_2$DCFDA and 1 μg/ml Hoechst 33342 (Hoechst) for 30 min at 37 °C. CM-$H_2$DCFDA crosses the cell membrane and is hydrolyzed by intracellular esterase to the non-fluorescent form, dichlorodihydrofluorescein; the latter reacts with intracellular ROS to form dichlorofluorescein, a green fluorescent dye. Fluorescence was measured in an inverted fluorescence microscope (inverted NIKON eclipse T2000-U microscope) and normalized to Hoechst fluorescence. Fluorescence analysis was performed using the Metamorph programme version 7.0. Data were normalized with respect to basal values that were considered as 100 %.

### Real-Time Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Sigma-Aldrich, Spain) and reverse transcribed. Quantitative real-time PCR analysis was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). Thermal cycling for the quantitative RT-PCR was carried out according to the manufacturer's recommendation, and the relative expression levels were calculated using the comparative ΔΔCt method. The primers were obtained from Signa-Aldrich, Madrid, Spain and primers sequences were as follows: IL-1β (FW: TCCTCTGTGACTCGTGGGAT; RV: GGGTGTGCCGTC TTTCATCA), TNFα (FW: CCACCACGCTCTTCTGTCTA ; RV: TGGAACTGATGAGAGGGAGC) and were normalize to β2-microglobulin as house-keeping gene (FW: ACCGTG ATCTTTCTGGTGCTTG; RV: TAGCAGTTGAGGAAGT TGGGCT).

### Glial Immunofluorescence

At the end of experiment, OHCs were processed for Iba-1 (microglia) and GFAP (astrocytes) immunofluorescence as follows: after fixation with 4 % paraformaldehyde for 1 h, the sections were washed three times for 5 min with PBS. They were then permeabilized with triton 0.1 % solution for 10 min and blocked them for an hour with normal goat serum

and BSA. To continue, OHCs were incubated with anti-Iba-1(Wako Chemicals, Rafer S.L) or with an anti-GFAP (Millipore, Spain) antibody over-night at 1:500. After washing with PBS, sections were incubated with anti-rabbit IgG or anti-mouse IgG secondary antibody, respectively, for 2 h and 30 min at 1:800. Then, slices were washed for three times for 5 min with PBS, applying in the second wash 1 μg/ml Hoechst 33342. Images were taken in a confocal microscope (TCS SPE; Leica, Wetzlar, Germany).

### Statistical Analysis

Statistically significant differences between groups were determined by a one-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc analysis. The level of statistical significance was taken at $p<0.05$.

## Results

### Cell Death Caused by β-amyloid and Okadaic Acid Alone or in Combination

We selected these two toxic stimuli as they are related to AD pathology: β amyloid (βA) to mimic amyloid pathology and okadaic acid (OA), a phosphatase inhibitor, to cause hyperphosphorylation of Tau protein. We first performed concentration-response curves with βA and OA in order to evaluate their toxicity in OHCs. For these experiments, after 4 days in culture, OHCs were exposed for another 4 days to increasing concentrations of the toxic stimuli; thereafter, cell viability was quantified as the ratio of PI /Hoechst fluorescence in CA1 hippocampal region. Representative images of PI uptake, as an indication of cell death, in OHCs treated with increasing concentrations of βA or OA can be visualized in Fig. 1a. βA increased the cell death of OHCs in a concentration-dependent manner (Fig. 1b); at the concentration of 5 μM, it almost doubled basal cell death. In the case of OA, significant cell death was achieved at the concentration of 3 nM and at 10 nM it almost tripled basal cell death (Fig. 1c).

In order to develop a new neurotoxicity model that combined both alterations, i.e., βA and hyperphosphorylation of Tau, OHCs were treated with subtoxic concentrations of βA and OA as we had previously described in the human neuroblastoma cell line SH-SY5Y [22]. The combination of 0.5 μM of βA with 1 nM of OA almost doubled (195±16 %) basal cell death measured as PI uptake (Fig. 2a); this cell death was significantly higher than that afforded by the toxic stimuli alone (108±12 % for βA and 147±14 % for OA) (Fig. 2a). As shown in Fig. 2c, e, most of the cells in CA1 treated with the combination of 0.5 μM of βA with 1 nM of OA (from now on βA/OA) were TUNEL-positive as compared to control (Fig. 2b, d); these results indicate that the βA/OA toxic stimuli caused mainly apoptotic cell death in the OHCs.

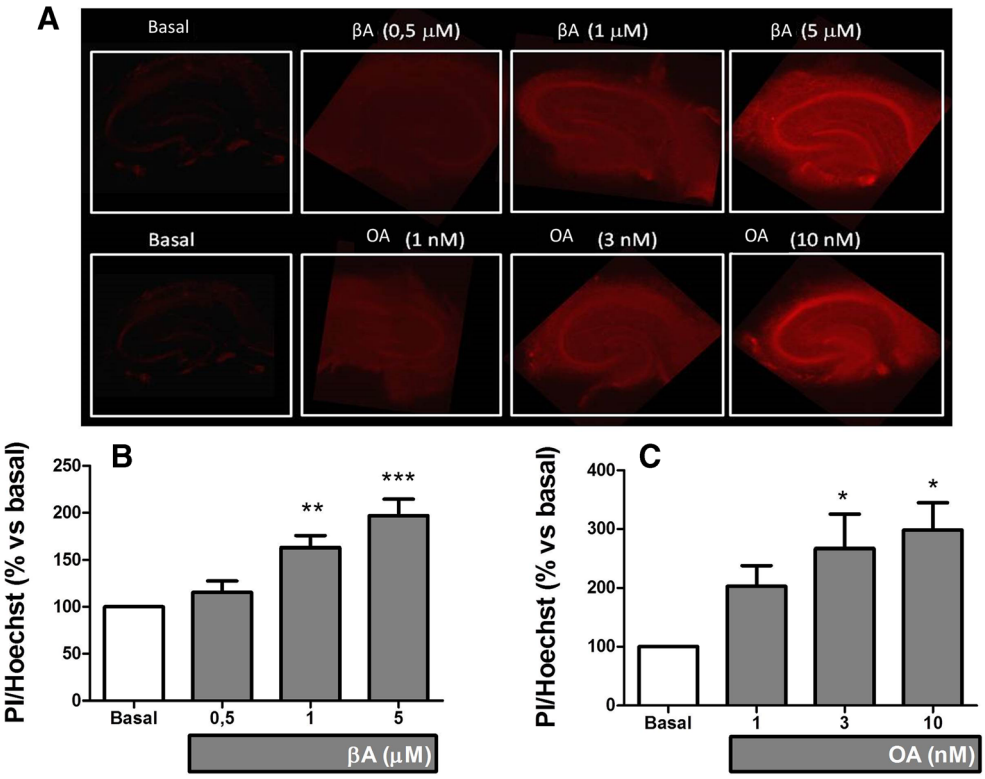


**Fig. 1** Cell damage increases in a concentration-dependent manner after exposure of organotypic hippocampal slices cultures (OHCs) to increasing concentrations of $\beta A_{25-35}$ (0.5, 1, and 5 μM) peptide and OA (1, 3, and 10 nM). **a** Representative photomicrographs of PI uptake in hippocampal slices after 4 days exposure to βA and OA. The graphs below show quantification of PI uptake normalized with respect to Hoechst fluorescence in CA1 in response to βA peptide **b** and OA **c**. Values are expressed as percentage of cell death in CA1 with respect to basal. *Bars* represent mean±SEM, $n=8$. ***$p<0.001$, **$p<0.01$, *$p<0.05$ significantly different from basal

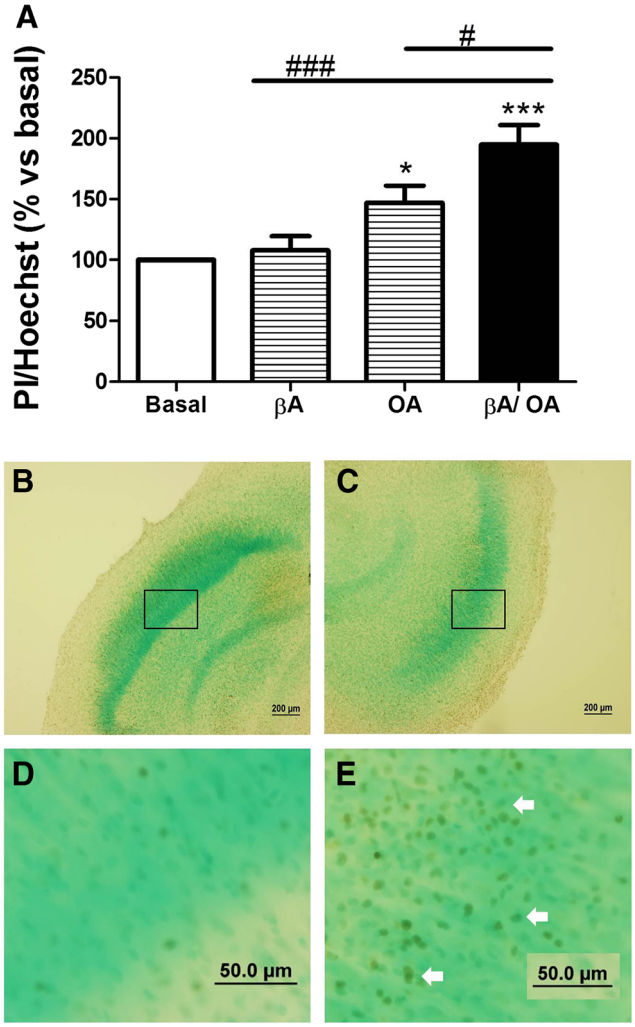


**Fig. 2** Combination of subtoxic concentrations of βA and OA offer a synergic toxic effect in OHCs. **a** Effect of 0.5 μM βA, 1 nM OA and their association (βA/OA), on the viability measured as PI uptake normalized with respect to Hoechst fluorescence in CA1. Values are expressed as means±SEM of eight different OHCs, ***$p<0.001$, *$p<0.05$ compared to basal; ###$p<0.001$, #$p<0.05$ with respect to the toxic stimuli on their own. **b** Shows TUNEL staining taken in CA1 in control OHCs; **d** is a magnification of the box shown in **b. c** Illustrates a greater number of TUNEL-positive cells in CA1 in an OHCc exposed for 4 days to the combination of 0.5 μM βA plus 1 nM OA; **e** a magnification of the area represented by the square in **c** can be seen. These photomicrographs are representative of four different cultures

### Effect of Melatonin and Galantamine in OHCs Exposed to βA/OA Toxicity

Once the βA/OA toxicity model was established, our aim was to determine if the combination of sub-effective concentrations of galantamine (an AChEI used in clinic for AD patients) and melatonin (a neurohormone with antioxidant properties known to be reduced with age and, particularly in AD patients [25]) could protect the OHCs under our toxic conditions. First, we performed concentration-response curves with galantamine and melatonin in order to select further on the sub-effective concentrations. As represented in Fig. 3a, co-incubation of increasing concentrations of melatonin with βA/OA, for 4 days, caused a concentration-dependent protection of the OHCs; significant protection was achieved at the concentration of 10 nM (56 % protection) while at 1 μM, PI uptake reached basal levels, indicating complete protection. In the case of galantamine, 10 nM did not afford significant protection, while complete protection was achieved at 100 nM (Fig. 3b).

According to the results described above, for the drug combination protocol, we selected the concentrations of 10 nM of

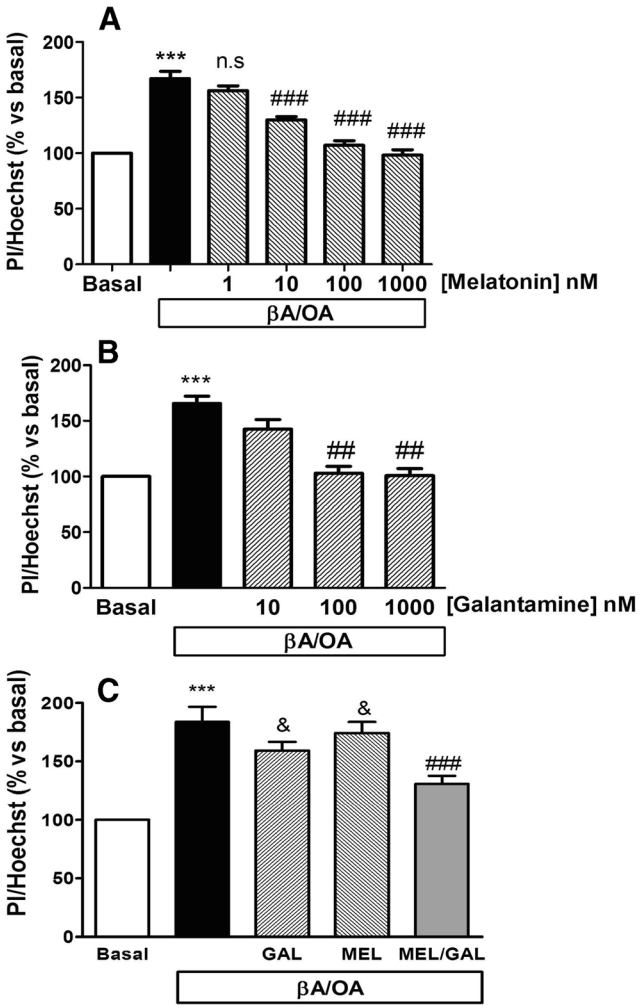


**Fig. 3** Concentration-dependent effect of melatonin, galantamine, and their combination, at sub-effective concentrations, in OHCs exposed to βA/OA. **a** Melatonin (1–1000 nM) causes concentration-dependent protection of OHCs when incubated for 4 days with βA/OA. **b** Galantamine (10–1000 nM) afforded significant protection at 100 and 1000 nM. **c** Combination of sub-effective concentrations of melatonin (1 nM) and galantamine (10 nM) (Mel/Gal) provided significant protection when compared to the drugs alone. Toxicity was measured as the ratio of PI/Hoechst fluorescence in CA1 and expressed as percentage of basal fluorescence (100 %). Values correspond to the means±SEM of 6–7 different animals, ***$p<0.001$, compared to basal; ###$p<0.001$ with respect to βA/OA; &$p<0.05$ compares protective drugs given alone or in combination

galantamine and 1 nM of melatonin (Mel/Gal) since they, per se, were not effective to provide protection; however, when associated, they afforded a highly significant protective effect against βA/OA-induced toxicity (Fig. 3c). These results indicate that the concentration of galantamine could be reduced up to ten times, maintaining a good protection, by adding 1 nM melatonin.

### Effect of Sub-effective Concentrations of Melatonin and Galantamine on βA Aggregates and Tau Hyperphosphorylation

Once we observed that sub-effective concentrations of melatonin plus galantamine could afford a significant protective effect, we were interested in knowing (i) if our toxicity model would reproduce two of the major pathological hallmarks associated to AD disease, i.e., βA aggregates and hyperphosphorylation of Tau, and (ii) if the combination of Mel/Gal could reduce these alterations. First, we analyzed βA aggregates in OHCs exposed to the toxic stimuli in the absence and the presence of the combination of melatonin plus galantamine. As shown in Fig. 4a, OHCs incubated with βA/OA increased Thioflavin S staining, the mean data showed a 2.2-fold increase in fluorescence. Co-treatment of the OHCs with the toxic stimuli, together with the protective drugs (Mel/Gal), reduced Thioflavin S aggregates to 1.5-fold (i.e., 63 % reduction). When we analyzed hyperphosphorylation of Tau by Western blot analysis, we observed that βA/OA increased Tau phosphorylation by 2.2-fold while Mel/Gal treatment reduced it by 73 % (Fig. 4b). Therefore, our toxic stimuli reproduced both, βA aggregates and hyperphosphorylation of Tau and, more interestingly, the association of sub-effective concentrations of melatonin and galantamine caused a marked reduction of these parameters.

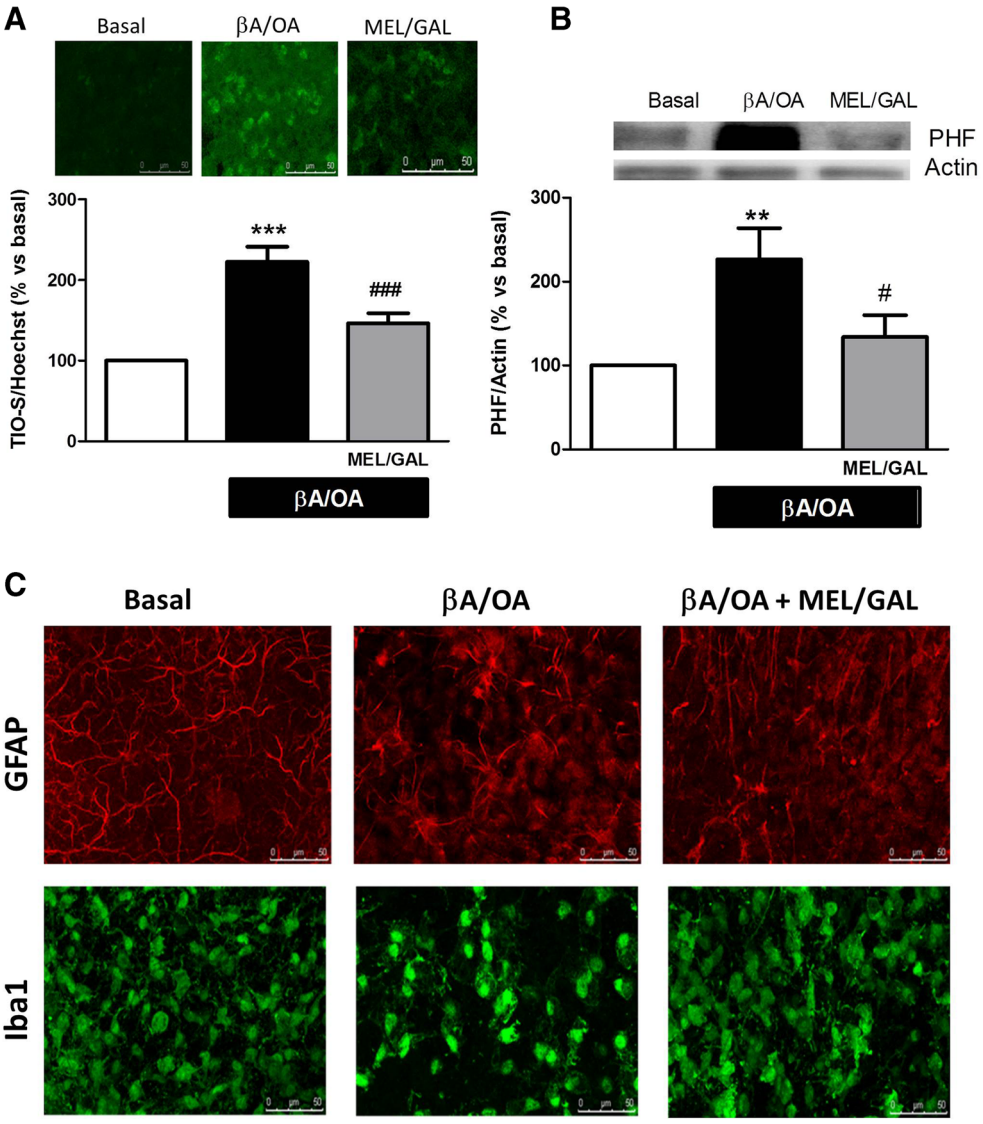


**Fig. 4** Combination of subthreshold concentrations melatonin and galantamine reduce Thioflavin S aggregates and Tau phosphorylation. **a** OHCs incubated for 4 days with βA/OA increased βA aggregates and, the combination of sub-effective concentrations of melatonin (1 nM) and galantamine (10 nM) (Mel/Gal) with the toxic stimuli, significantly reduced Thioflavin S fluorescence. **b** Similar results as in **a** were observed when hyperphosphorylation of Tau was measured by Western blot. **c** Representative images of Iba-1 labeled microglial cells and GFAP-labeled astrocytic cells in a non-treated OHCs (basal), treated with the toxic stimuli alone (βA/OA) or with the toxic stimuli and the combination of melatonin (1 nM) and galantamine (10 nM) (βA/OA + Mel/Gal). Values correspond to the means±SEM of six different animals, $^{***}p<0.001$, $^{**}p<0.01$, compared to basal; $^{###}p<0.001$, $^{\#}p<0.05$ with respect to βA/OA

### Neuroprotective Mechanism of the Combination of Melatonin with Galantamine

Neuroinflammation and oxidative stress are being increasingly related to different CNS pathologies like AD [12, 26]. Furthermore, neuroinflammation is closely related to microgliosis and astrogliosis found in AD [27]. Thus, we initially performed immunofluorescence staining of the OHCs against the microglial marker Iba1 and the astroglial marker GFAP, after the different treatments. Both microglia and astrocytes in OHCs exposed to βA/OA suffered morphological alterations, as can be seen in Fig. 4c.

In order to elucidate how βA/OA could be contributing to neuroinflammation, we measured the mRNA of the proinflammatory cytokines IL1β and TNFα extracted from the OCHs by qPCR. Exposure of OHCs to βA/OA caused 2.7-fold increase of IL1β and 2.5-fold increase of TNFα, indicating that neuroinflammation was taking place in the OHCs. The co-treatment of the slices with the toxic stimuli and the neuroprotectant combination of Mel/Gal reduced by over 50 % mRNA levels of IL1β (1.3-fold) and TNFα (1.2-fold), indicating that Mel/Gal were capable of controlling neuroinflammation in this model (Fig. 5a, b). Because we had previously shown that galantamine's neuroprotective actions were mediated by α7 nAChR [28, 29], we used α-bungarotoxin (a selective α7 nAChR antagonist) and luzindole (a melatonin receptor antagonist) to evaluate if these receptors were participating in the neuroprotective actions of Gal/Mel. Indeed, both α-bungarotoxin and luzindole (which did not afford any cell viability modification per se, data not shown) prevented the reduction of Il-1β and TNF-α mRNA levels caused by Gal/Mel in OHCs incubated with βA/OA (Fig. 5a, b).

Besides inflammation, we also looked at ROS production by using the fluorescent dye DCFDA. As represented in Fig. 5c, the toxic stimuli βA/OA almost doubled ROS production, and such effect was almost reduced to basal levels when Mel/Gal was present in the OHCs. As previously observed with IL1β and TNFα, the reduction of ROS production caused by the combination of Mel/Gal was blocked by luzindole and α-bungarotoxin.

Finally, to confirm that the protective effect of the neuroprotectant combination Mel/Gal was mediated by melatonin and α7 nAChRs, we measured cell viability by PI uptake; as illustrated in Fig. 5d, both luzindole and α-bungarotoxin prevented the protective effect of the

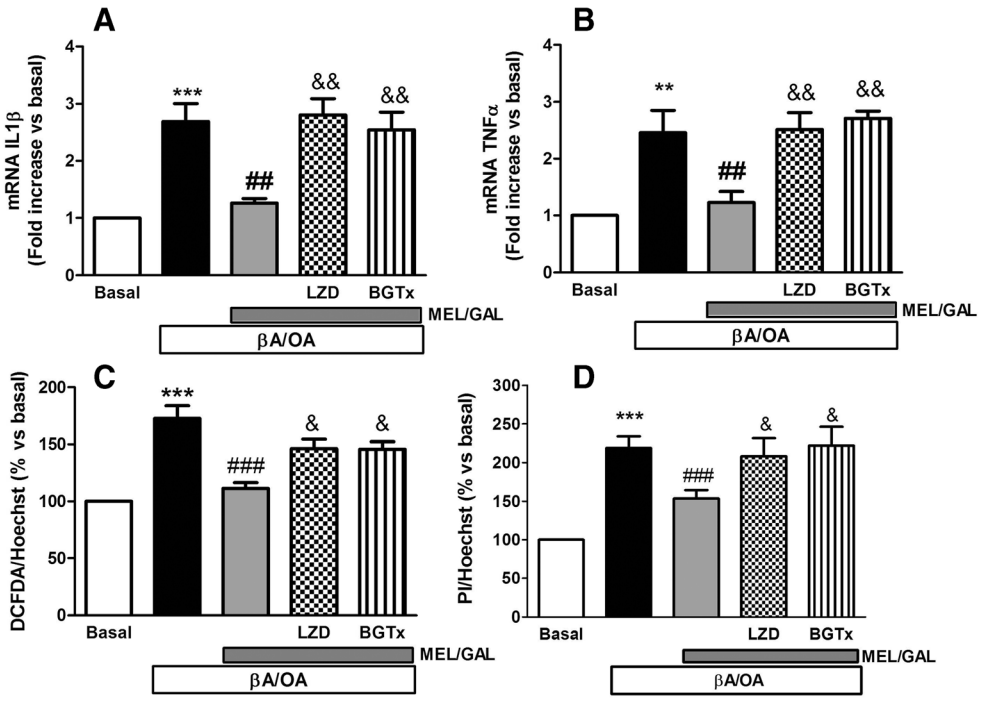


**Fig. 5** The antioxidant, anti-inflammatory, and neuroprotective effect of the combination of subthreshold concentrations of melatonin and galantamine is mediated by melatonin and nicotinic receptors. **a** Analysis of mRNA by qPCR of IL1β (**a**) and TNFα (**b**). **c** Illustrates ROS production measured as DCFDA fluorescence; this value was expressed as a ratio of Hoechst fluorescence in CA1 and **d** shows the viability measured as the ratio of PI/Hoechst fluorescence. In all the experiments, after 4 days in cultures, OHCs exposed for 4 days to the different treatments illustrated by the *horizontal bar* in the x axis; the melatonin antagonist luzindole (LZD) was used at 1 μM and the α7 nAChRs antagonist α-bungarotoxin (BGTx) was used at 100 nM. Data correspond to the mean and SEM of 9–4 animals. $^{**}p<0.01$, $^{***}p<0.001$ compared to basal; $^{###}p<0.001$ and $^{##}p<0.01$ compared to βA/AO alone; $\&\&p<0.01$, $\&p<0.05$ compared to the combination βA/OA + Mel/Gal

combination of Mel/Gal against βA/OA-induced toxicity. Thereby, confirming that melatonin and α7 nAChRs receptors are plasmalemmal targets for the protective actions of the combination Mel/Gal.

## Discussion

In the present study, we provide experimental evidence on the synergic neuroprotective effect of the combination of sub-effective concentrations of melatonin and galantamine in an AD-related pathology model. Association of nanomolar concentrations of melatonin and galantamine improves AD markers like (i) β-amyloid aggregation, (ii) Tau hyperphosphorylation, (iii) inflammation, and (iv) oxidative stress in organotypic hippocampal cultures (OHCs) subchronically exposed to βA and OA.

The organotypic hippocampal culture (OHCs) is a useful model for the study of neurodegeneration. This model allows us to incubate the slices for subchronic periods (4–7 days). Thus, we can reduce the concentrations of the toxic stimuli to more pathophysiological ones. In our work, we incubated the slices with βA or OA at concentrations 50-fold lower than concentrations used in the literature (Fig. 1) [30, 31] and we used $\beta A_{25\text{-}35}$ which has shown to be as toxic as other βA fragments [32]. Thus, we have developed a new model that combines βA and Tau pathologies using subtoxic concentrations of the toxic stimuli, $\beta A_{25\text{-}35}$ (0.5 μM) and OA (1 nM) for 4 days, which by themselves did not provide toxicity. Under these experimental conditions, subtoxic concentration of βA/OA produced an increment of cell death, which was confirmed as apoptotic by immunohistochemistry of OHCs (Fig. 2a–e). Postmortem analysis of AD brains shows that there is DNA fragmentation in neurons and glia of hippocampus and cortex as detected by TUNEL [33, 34]. Our results go in line with those obtained in the triple transgenic animal model of AD [35], where the functional alteration and pathological hallmarks appear earlier compared to double transgenic animals.

The most important finding was that the combination of low concentrations of melatonin (1 nM) and galantamine (10 nM), which did not afford protection by themselves, produced a potent neuroprotective, antioxidant and anti-inflammatory effect (Figs. 3, 4, and 5). Recently, several studies have reported that simultaneous administration of melatonin with other drugs enhances the protective effect of melatonin, as well as the effect of the second drug. In a previous work, we demonstrated that combination of sub-effective concentrations of melatonin and galantamine protects against oxidative stress in a human neuroblastoma cells [12]. In this previous work, the effective concentrations of melatonin and galantamine were higher probably because the toxic stimulus was more potent and it was applied during a shorter period. Here, we corroborated this data and expanded it to AD disease. By the use of the organotypic βA/OA model, some of the major pathological markers of AD are resembled, such as amyloid aggregates, Tau hyperphosphorylation, neuroinflammation, and oxidative stress (Figs. 4 and 5). We have proven that combined therapy of melatonin and galantamine is beneficial as it restored all these pathological alterations. The potential effect of melatonin in the different AD hypothesis has been recently reviewed, and the conclusion is similar to our results; the authors discuss that melatonin could prevent amyloid overproduction and reduce hyperphosphorylation of Tau [31]; it is an antioxidant and free radical scavenger and modulates proinflammatory processes and, curiously, it could work as an anticholinesterase agent. On the other hand, galantamine, which is one of the current treatments, has proven to be useful in multiple in vitro and in vivo AD models and in different clinical trials [36]. However, AChEI revealed only a modest trend favoring active treatment over placebo [37].

Glutamate excitotoxicity, mitochondrial dysfunction, and free radical-mediated damage are identified as pathophysiological mechanisms leading to neuronal death in neurodegenerative diseases [11]. Neuroprotective properties of melatonin against these three processes, added to its regulatory effects on circadian disturbances, point to melatonin as a therapeutic substance in the symptomatic treatment of neurodegenerative diseases. The multiple neuroprotective effects of melatonin, which include its anti-apoptotic, anti-inflammatory, and antioxidant actions, are mediated by both receptor and non-receptor pathways. On the other hand, it has been demonstrated that the neuroprotective effect of galantamine, a well-known AChEI used to treat AD, is associated to its ability to act on nicotinic receptors [28] and to its antioxidant effect [38]. Hence, both drugs could be good candidates to combat AD. In this study, we have observed that the neuroprotective effect of subthreshold concentrations of melatonin and galantamine is specifically mediated by both, melatonin and nicotinic receptors, as the blockade of those receptors with α-bungarotoxin and luzindole (Fig. 5) totally reversed their neuroprotective, antioxidant, and anti-inflammatory actions.

Melatonin exerts neuroprotective effects in different models of AD [13, 31, 39, 40]. Besides the pineal gland, melatonin is also produced in gastrointestinal tract, thymus, adrenal glands, thyroid gland, and other organs [41]. It must be noted that melatonin production declines with aging [13, 42, 43] due to dysfunction of the sympathetic regulation of pineal melatonin by the suprachiasmatic nucleus. The loss of melatonin during aging contributes to oxidative stress accumulation because its antioxidant effect is lost, and thereby it contributes to the progression of diseases with a free radical component like neurodegenerative diseases [13] or cardiovascular disease [44]. Supplementation of melatonin in aging animals reduced AD aberrant changes [45] due, in part, to the antioxidant effect of melatonin at mitochondrial level [46].

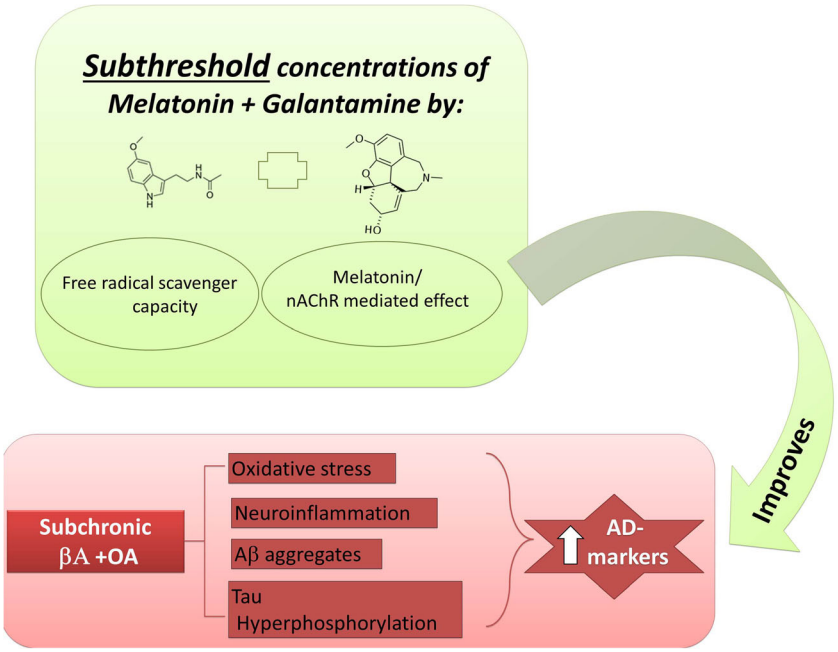


**Fig. 6** Main conclusions of this study. The combination of subtoxic concentrations of βA plus OA causes an increase of pathological markers related to AD-like βA aggregates, hyperphosphorylation of Tau, oxidative stress, neuroinflammation, and cell death in hippocampal slice cultures. These latter pathological markers, as well as neuronal apoptosis, can be reduced by combining subthreshold concentrations of melatonin and galantamine. We therefore assume that combination of subthreshold doses of melatonin and galantamine could be of potential interest in AD therapeutics not only to reduce pathology but also to reduce cholinergic side effects

Results from this study indicate that several advantages for melatonin supplementation with AChEI, as we have shown here for galantamine, can be withdrawn. From a clinical point of view, if melatonin supplementation is provided, the dose of the AChEI could be potentially reduced to as little as one tenth its effective dose; this would imply a significant reduction of cholinergic side effects, which could contribute to increase patient's therapeutic compliance and, indirectly, improve global efficacy of this group of drugs. On the other hand, since melatonin levels are significantly reduced or absent in AD patients [13, 42], the addition of melatonin to standard AD treatments could help restore internal melatonin levels and its associated homeostatic actions. In fact, a recent report by Wade et al. show that the addition of melatonin to an acetylcholinesterase inhibitor, with or without memantine treatment, has positive effects on cognitive function and sleep maintenance in mild to moderate AD patients compared to placebo [47]. There are also several studies that support the potential role of melatonin as an effective adjuvant in AD management [19, 20].

Taken all together, these results show that treatment of the OHCs with the combination of low concentrations of βA and OA for 4 days causes βA aggregates, hyperphosphorylation of Tau protein, oxidative stress, inflammation and glial alterations, resembling alterations shown in AD human brains. On the other hand, the combined therapy of subthreshold concentrations of melatonin and galantamine has proven to be powerful, as it prevented cell death, βA aggregates, hyperphosphorylation of Tau protein, oxidative stress, and glial alterations (Fig. 6). Thus, although more research is needed, melatonin could improve galantamine therapy for the treatment of AD, and also, these results could help to design new melatonin-acetylcholinesterase inhibitor derivatives as a new strategy for AD-therapy.

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**Compliance with Ethical Standards** All animal assays were carried out following the Guide for the Care and Use of Laboratory Animals and were previously approved by the Institutional Ethics Committee of the Autonomous University of Madrid, Spain, according to the European guidelines for the use and care of animals for research in accordance with the European Union Directive of 22 September 2010 (2010/63/UE) and

with the Spanish Royal Decree of 1 February 2013 (53/2013). All efforts were made to minimize the number of animals used and their suffering.

**Conflict of Interest** All authors have no conflict of interest.

**Author Contributions** Izaskun Buendia has contributed to acquisition of data, data analysis/interpretation, writing, and critical revision of the manuscript. Esther Parada has contributed to acquisition of data and data analysis/interpretation. Elisa Navarro has contributed to acquisition of data and data analysis/interpretation. Rafael León has contributed to critical revision of the manuscript. Pilar Negredo has contributed to critical revision of the manuscript. Javier Egea has contributed to concept/design, acquisition of data, data analysis/interpretation, drafting of the manuscript, critical revision of the manuscript, and approval of the article. Manuela García López has contributed to concept/design, drafting of the manuscript, critical revision of the manuscript, and approval of the article.

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## 6.3 El derivado de melatonina- sulforafano, el ITH12674 ofrece neuroprotección en modelos de estrés oxidativo mediante un mecanismo profármaco-fármaco

**ANTECEDENTES Y OBJETIVOS:** Las enfermedades neurodegenerativas son un grave problema que atañe a la población de edad avanzada. Sin embargo, no hay tratamientos efectivos que frenen su progresión. El estrés oxidativo y la neuroinflamación son dos alteraciones comunes en la patogenia de estas enfermedades. El factor de transcripción Nrf2 es el regulador más importante del estrés oxidativo, y la melatonina, una neurohormona endógena con propiedades antioxidantes cuyos niveles se ven reducidos con el envejecimiento. Con estos antecedentes, se sintetizó un nuevo derivado que combina los efectos de melatonina con la inducción de Nrf2, el ITH12674, con la intención de mejorar las propiedades neuroprotectoras.

**ABORDAJE EXPERIMENTAL:** Se diseñó y sintetizó este nuevo derivado y se evaluó su potencial efecto neuroprotector en distintos modelos de estrés oxidativo *in vitro* relacionados con enfermedades neurodegenerativas e isquemia cerebral.

**RESULTADOS**: Este nuevo derivado: (i) protegió en un modelo de estrés oxidativo en cultivo de neuronas corticales; (ii) disminuyó la producción de ERO; (iii) aumentó la concentración de GSH de las neuronas corticales; (iv) potenció la inducción de Nrf2 en células HEK293T transfectadas con ARE-luc; (v) protegió en cultivo organotípico de hipocampo frente a la privación de oxígeno y glucosa mediante la inducción de HO-1 y disminuyó la producción de ERO.

**CONCLUSIONES E IMPLICACIONES:** El nuevo derivado ITH12674 combina las rutas de señalización protectoras de los dos cabezas de serie (melatonina y sulforafano), mejorando así sus propias propiedades neuroprotectoras. Por tanto, estos compuestos abrirían una nueva línea de investigación en el tratamiento de las enfermedades neurodegenerativas e ictus.

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RESEARCH PAPER

# Melatonin–sulforaphane hybrid ITH12674 induces neuroprotection in oxidative stress conditions by a 'drug–prodrug' mechanism of action

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## BACKGROUND AND PURPOSE

Neurodegenerative diseases are a major problem afflicting ageing populations; however, there are no effective treatments to stop their progression. Oxidative stress and neuroinflammation are common factors in their pathogenesis. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is the master regulator of oxidative stress, and melatonin is an endogenous hormone with antioxidative properties that reduces its levels with ageing. We have designed a new compound that combines the effects of melatonin with Nrf2 induction properties, with the idea of achieving improved neuroprotective properties.

## EXPERIMENTAL APPROACH

Compound ITH12674 is a hybrid of melatonin and sulforaphane designed to exert a dual drug–prodrug mechanism of action. We obtained the proposed hybrid in a single step. To test its neuroprotective properties, we used different *in vitro* models of oxidative stress related to neurodegenerative diseases and brain ischaemia.

## KEY RESULTS

ITH12674 showed an improved neuroprotective profile compared to that of melatonin and sulforaphane. ITH12674 (i) mediated a concentration-dependent protective effect in cortical neurons subjected to oxidative stress; (ii) decreased reactive oxygen species production; (iii) augmented GSH concentrations in cortical neurons; (iv) enhanced the Nrf2–antioxidant response element transcriptional response in transfected HEK293T cells; and (v) protected organotypic cultures of hippocampal slices subjected to oxygen and glucose deprivation and re-oxygenation from stress by increasing the expression of haem oxygenase-1 and reducing free radical production.

## CONCLUSION AND IMPLICATIONS

ITH12674 combines the signalling pathways of the parent compounds to improve its neuroprotective properties. This opens a new line of research for such hybrid compounds to treat neurodegenerative diseases.

### Abbreviations

AD, Alzheimer's disease; ARE, antioxidant response element; GST, GSH S-transferase; $H_2$DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HO-1, haem oxygenase-1; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NDDs, neurodegenerative diseases; Nrf2, nuclear factor (erythroid-derived 2)-like 2; OGD/reox, oxygen and glucose deprivation plus re-oxygenation; OHCs, organotypic hippocampal cultures; PD, Parkinson's disease; PI, propidium iodide; Rot/olig, rotenone and oligomycin A combination; SnPP, tin-protoporphyrin IX; SULF, sulforaphane; TBH, tert-butyl hydroperoxide

## Tables of Links

| TARGETS |
| --- |
| Glutathione S-transferase (GST) |
| Haem oxygenase-1 (HO-1) |
| Papain |

| LIGANDS |
| --- |
| Glutathione (GSH) |
| Melatonin |
| Tin-protoporphyrin IX (SnPP) |

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

## Introduction

Due to an increased ageing population, neurodegenerative diseases (NDDs) such as Alzheimer's disease (AD), Parkinson's disease (PD) as well as ischaemic stroke are an increasing burden to society because of their cumulative morbidity and mortality. Common features of NDDs are cognitive and/or motor deficits, selective loss of neurons, the appearance of aberrant protein aggregates. In addition, there is abundant evidence of lipid peroxidation, protein nitration and nucleic acid oxidation (Di Carlo *et al.*, 2012).

Oxidative damage and mitochondrial dysfunction are thought to play a key role in the onset and development of NDD (Lin and Beal, 2006). For instance, the brains of AD patients are subjected to high oxidative stress, as demonstrated in *post-mortem* AD temporal cortex and hippocampus (Schipper *et al.*, 2006), and in astrocytes and neurons (Ramsey *et al.*, 2007). Similar results have been obtained for PD and other NDDs (Dasuri *et al.*, 2013). In normal conditions, cells respond to oxidative stress through an endogenous mechanism regulated mainly by the nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 is a member of the Cap 'n' Collar family of transcription factors that bind to the antioxidant response element (ARE) to regulate the antioxidant response (Nguyen *et al.*, 2010). It is sequestered in the cytosol by the Keap1 protein, which targets it for ubiquitination by CUL3–ROC1 ligase and subsequent degradation by the proteasome (Furukawa and Xiong, 2005). In the presence of oxidative stress, Nrf2 is released from Keap1, translocates into the nucleus and binds to the ARE sequences forming a complex with a small Maf protein to induce the 'phase II antioxidant response' (Zhang *et al.*, 2013). Phase II genes induced by Nrf2 include, among others, haem oxygenase-1 (HO-1) and GSH synthetic enzymes (Zhang *et al.*, 2013). Interestingly, despite abundant evidence for the occurrence of oxidative stress in NDDs, the Nrf2–ARE signalling pathway is attenuated or deleted in several neurodegenerative pathological conditions (Ramsey *et al.*, 2007). Therefore, there is increasing evidence supporting the use of the Nrf2–ARE transcriptional pathway as a key target for the treatment of NDDs. For example, in animal models of AD, induction of the Nrf2–ARE transcriptional pathway has been demonstrated to improve spatial learning (Kanninen *et al.*, 2009) and attenuate inflammation (Thimmulappa *et al.*, 2006) and oxidative stress. In PD, several inducers of Nrf2 have been shown to have the ability to protect cells and ameliorate symptoms *in vitro* (Jakel *et al.*, 2007) and *in vivo* (Chen *et al.*, 2009). There is also evidence of the therapeutic potential of the Nrf2–ARE pathway in Huntington disease (Calkins *et al.*, 2005; Ellrichmann *et al.*, 2011), amyotrophic lateral sclerosis (Vargas *et al.*, 2008) and brain ischaemic damage. Sulforaphane, a potent Nrf2 inducer isolated from sprouting broccoli, has been extensively studied in several NDD models. It has been found to have a wide neuroprotective profile (Tarozzi *et al.*, 2013) in numerous *in vitro* and *in vivo* oxidative stress models of AD (Kim *et al.*, 2013), PD (Jazwa *et al.*, 2011; Morroni *et al.*, 2013), cerebral ischaemia (Zhao *et al.*, 2006) and inflammation (Innamorato *et al.*, 2008); this effect is thought to be mediated by the induction of the Nrf2–ARE pathway.

Melatonin is a well-known multifunctional molecule that influences the circadian rhythms (Hardeland *et al.*, 2012), the immune response (Mauriz *et al.*, 2013), the cardiovascular (Dominguez-Rodriguez *et al.*, 2012) and digestive systems (Palileo and Kaunitz, 2011). Additionally, over the last decade, it has been extensively investigated because of its benefits in the CNS (Wang, 2009). The neuroprotective profile of melatonin is related, in part, to its potent antioxidant and scavenger effect (Tan *et al.*, 1993; Reiter *et al.*, 2009; Kilic *et al.*, 2012). As a scavenger, melatonin reacts with free radicals to form several metabolites that can also trap free radicals; this is known as the 'scavenger cascade of melatonin' (Tan *et al.*, 2007). A single molecule of melatonin is able to trap up to 10 free radicals (Tan *et al.*, 2001; 2007; Rosen *et al.*, 2006). Furthermore, melatonin increases the activity of

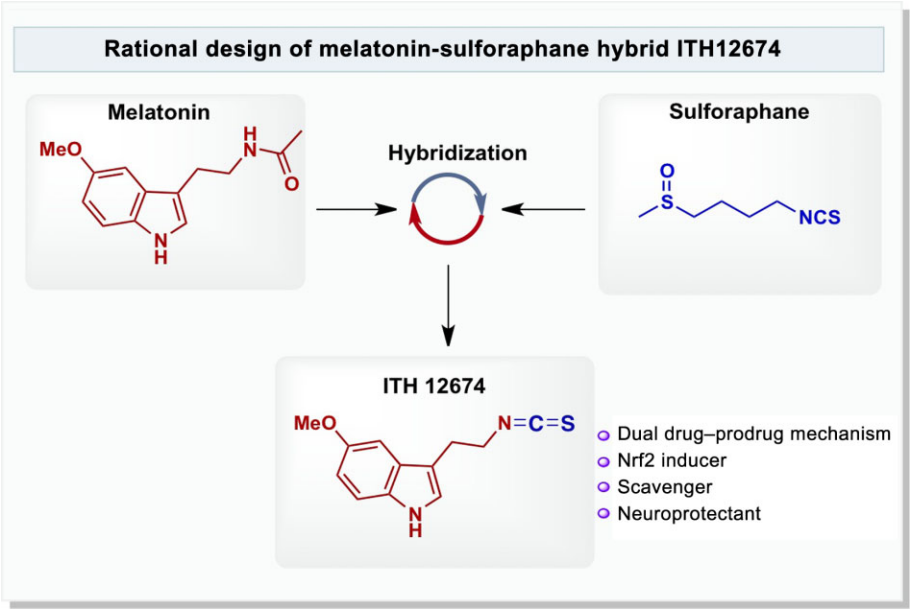



**Figure 1**
Rational design of melatonin–sulforaphane hybrid ITH12674.

several antioxidant enzymes (Luchetti *et al.*, 2010). Because of its antioxidant and neuroprotective activities, melatonin has been suggested as a possible treatment for oxidative stress-related disorders such as NDDs (Pandi-Perumal *et al.*, 2013).

In the light of the pleiotropic profile of melatonin and the interesting neuroprotective results obtained with sulforaphane, we have hypothesized that the combination of these compounds in one molecule could eventually result in complementary, additive neuroprotective effects with a potential therapeutic application for NDDs. Hence, we have generated a melatonin–sulforaphane hybrid, ITH12674 (Figure 1), designed to react with cysteines present in Keap1 to liberate Nrf2, which then acts as a drug and also to be conjugated with GSH inside the cell to generate a potent melatonin-like antioxidant compound, a prodrug of this conjugate. This drug–prodrug mechanism resulted in an improved pharmacological profile with therapeutic potential for the treatment of NDDs.

# Methods

## *Chemistry*

Compound ITH12674 was synthesized in our laboratories using an optimized synthetic protocol. A solution of N,N′-thiocarbonyldiimidazole (0.26 mmol, 46.8 mg) in THF (2 mL) was added to a solution of 2-(5-methoxy-1*H*-indol-3-yl)-ethanamine (0.26 mmol, 50 mg) in dry tetrahydrofuran (3 mL) at 0°C for 10 min. The resulting solution was allowed to warm-up to room temperature and stirred for 3 h until completion. Thereafter, the solvent was eliminated under reduced pressure and purified by flash chromatography on silica gel (hexane : $CH_2Cl_2$ 0–60%) to yield the product (ITH12674) as a pale yellow oil (54.4 mg, 90% yield); Rf 0.87 (dichloromethane, 100%); $^1$H NMR and $^{13}$C NMR data were in agreement with previously reported findings (Singh *et al.*, 2007); Rf 0.87 (DCM, 100%); $^1$H NMR (300 MHz, $CDCl_3$) $\delta_H$ 7.91 (1H, bs, NH), 7.20 (1H, d, $J$ = 8.6 Hz, $H^7$), 7.01 (1H, d, $J$ = 2.4 Hz, $H^4$), 6.91 (1H, d, $J$ = 2.4 Hz, $H^2$), 6.81 (1H, dd, $J$ = 2.4 Hz, $J$ = 8.6 Hz, $H^6$), 3.81 (1H, s, $OCH_3$), 3.69 (2H, t, $J$ = 6.8 Hz, $OCH_2CH_2NCS$ ), 3.06 (2H, t, $J$ = 6.8 Hz, $OCH_2CH_2NCS$); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$ 154.2, 131.4, 127.3, 123.8, 112.5, 112.2, 110.9, 56.1, 45.7, 26.5; HRMS ($ES^+$) mass calc'd. For $C_{12}H_{12}N_2SO$ 232.0670; found $[(M + H)^+]$ 233.0740, found $[(M + Na)^+]$ 255.0567; Anal. Calcd. For $C_{12}H_{12}N_2SO$: C, 62.04; H, 5.21; N, 12.06, S, 13.80. Found: C, 62.26; H, 5.38; N, 11.88; S, 13.56.

## *Animal experiments*

All experimental procedures were performed following the *Guide for the Care and Use of Laboratory Animals* and were previously approved by the institutional Ethics Committee of the Autonomous University of Madrid, Spain, according to the European Guidelines for the use and care of animals for research in accordance with the European Union Directive of 22 September 2010 (2010/63/UE) and with Spanish Royal Decree of 1 February 2013 (53/2013). All efforts were made to minimize animal suffering and to reduce the number of animals used. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

## *Isolation and culture of rat cortical neurons*

Cortical neuron culture was performed as previously described (Lorrio *et al.*, 2013). Briefly, pregnant rats (Sprague Dawley, SD) were decapitated and 18-day-old embryos were

quickly removed. The cortex was dissected under a stereomicroscope in PBS at 4°C. The tissue was digested with 0.5 mg·mL$^{-1}$ papain and 0.25 mg·mL$^{-1}$ DNAse dissolved in $Ca^{2+}$- and $Mg^{2+}$-free PBS containing 1 mg·mL$^{-1}$ BSA and 6 mM glucose at 37°C for 20 min. The papain solution was replaced by 5 mL of neurobasal medium supplemented with 10% FBS. Cells were resuspended in 5 mL neurobasal medium and plated at the desired density on plates coated with poly-D-lysine (0.1 mg·mL$^{-1}$). After 2 h of culture in the presence of FBS, the medium was replaced by fresh serum-free medium containing B27 supplement with antioxidants. Under these conditions, standard cell survival was 4 weeks; experiments were performed after 7–10 days in culture.

## Preparation of organotypic hippocampal slice cultures (OHCs)

Cultures were prepared according to the methods described by Stoppini *et al.* (1991) with some modifications. Briefly, 300 µM thick hippocampal slices were prepared from SD rats (8–10 days old) using a Mcilwain tissue chopper (Ted Pella, Inc., Redding, CA, USA) and separated in ice-cold HBSS. Four to six slices were placed per Millicell 0.4 µM culture insert and placed on a 6-well culture tray with media where they remained for 7 days. The culture media consisted of 50% MEM, 25% HBSS and 25% heat-inactivated horse serum. The medium was supplemented with 3.7 mg·mL$^{-1}$ D-glucose, 2 mmol·L$^{-1}$ L-glutamine and 2% of B27 supplement minus antioxidants and 100 U·mL$^{-1}$ penicillin. OHCs were cultured in a humidified atmosphere at 37°C and 5% $CO_2$, and the medium was changed twice a week.

## Cell treatment with compound solutions

Stock solutions were dissolved in DMSO at a concentration of $10^{-2}$ M. All solutions were stored in aliquots at –20°C. Once defrosted for a given experiment, the aliquot was discarded. The final concentrations of DMSO used (always <0.1%) did not cause neuronal toxicity.

## Neuroprotection experiments

*Toxicity elicited by rotenone/oligomycin A or TBH in cortical neurons.* Rat cortical neurons were plated at a density of 60 000 cells per well in 96-well plates. The cells were treated with neuroprotective compounds at the desired concentrations for 24 h. Thereafter, the compounds were withdrawn and the toxins added: (i) 30 µM rotenone plus 10 µM oligomycin A or (ii) 30 µM TBH for another 24 h. Toxins were incubated in 10% B27 minus antioxidant medium. At the end of the experiments, cell death was assessed by following the MTT reduction method.

*Quantification of cell viability by MTT reduction.* MTT was added (5 mg·mL$^{-1}$ per well) to the cell samples, which were then incubated in the dark at 37°C for 2 h. The formazan produced was dissolved by adding 100 µL of DMSO, resulting in a coloured compound; the optical density of this compound was measured in an ELISA reader at 540 nm. All MTT assays were performed in triplicate. Data are expressed as a percentage of MTT reduction, with the maximum control capability in each individual experiment taken as 100%.

*Oxygen and glucose deprivation in OHCs.* The inserts with OHCs were placed into 1 mL of the oxygen and glucose deprivation (OGD) solution composed of (in mM): NaCl 137.93, KCl 5.36, $CaCl_2$ 2, $MgSO_4$ 1.19, $NaHCO_3$ 26, $KH_2PO_4$ 1.18 and 2-deoxyglucose 11. The OHCs were then placed into an airtight chamber (Billups and Rothenberg, Del Mar, CA, USA) and were exposed to 95% $N_2$/5% $CO_2$ gas flow for 5 min to ensure oxygen deprivation. After that, the chamber was sealed for 15 min at 37°C. Control cultures were maintained for the same time under normoxic atmosphere in a solution with the same composition as that described above (OGD solution) but containing glucose (11 mM) instead of 2-deoxyglucose. After the OGD period, slice cultures were returned to their original culture conditions for 24 h (reoxygenation period).

*Quantification of cell death in OHCs by PI and Hoechst 33342 staining.* At the end of the experiment, the OHCs were loaded with 1 µg·mL$^{-1}$ PI and Hoechst 33342 (Hoechst) during the last 30 min of incubation. Mean PI and Hoechst fluorescence in the CA1 region in each slice, after a given treatment, were analysed. Fluorescence was measured in a fluorescence inverted NIKON eclipse T2000-U microscope (Nikon Instruments, Tokyo, Japan). Wavelengths of excitation and emission for PI and Hoechst were 530 or 350 and 580 or 460 nm respectively. Fluorescence was analysed using the Metamorph programme version 7.0 (Molecular Devices, Sunnyvale, CA, USA). Data were normalized with respect to control values that were considered as 1.

## Luciferase assays

To monitor the expression of Nrf2–ARE, HEK 293T cells were seeded on 24-well plates (100 000 cells per well), cultured for 16 h and transfected using calcium phosphate. Transient transfections of HEK293T cells were performed with the expression vectors pTK-Renilla and ARE-LUC (a gift from Dr J Alam, Department of Molecular Genetics, Ochsner Clinic Foundation, Baton Rouge, LA, USA). After transfection, cells were treated with ITH12674 at the indicated doses for 16 h. As a control, transfected cells were treated with sulforaphane (0.3 µM) for 16 h. Then, cells were lysed and assayed for luciferase activity with the dual luciferase assay system (Promega) according to the manufacturer's instructions. Relative light units were measured in a GloMax 96 microplate luminometer (Promega, Madrid, Spain) with dual injectors.

## Immunocytochemistry

Cortical neurons were seeded in 6 multiwell plates (50 000 cells per well) on poly-D-Lys-covered slides. Neurons were incubated with treatments for 2 h and then were fixed with 2% paraformaldehyde dissolved in PBS for 15 min and washed three times with PBS every 5 min. Later, they were permeabilized with 0.5% Triton X-100 for 1 min and washed three times with PBS. Cells were incubated with primary antibody (anti-Nrf2, H-300, SC-13032, Santa Cruz Biotechnology, Dallas, TX, USA) overnight. Then, three consecutive washes with PBS were performed before the samples were incubated with secondary antibody (45 min). To visualize the nuclei, cells were counterstained with Hoechst (5 µg·mL$^{-1}$) during the second wash (Invitrogen, Madrid, Spain). Finally, the slides were covered with coverslips, glycerol-PBS (1:1 v

$v^{-1}$) added and they were viewed with a confocal microscope (TCS SPE, Leica, Wetzlar, Germany).

### *Intracellular GSH measurement*

To quantify free GSH we used monochlorobimane (Kamencic *et al.*, 2000). Cells were incubated with monochlorobimane (100 μM) at a final volume of 50 μL of neurobasal media without B27 for 1 h. Then, cells were washed twice with Krebs–HEPES solution and fluorescence intensity was measured in a Fluostar optima microplate reader (BMG Labtech Offenburg, Germany) at excitation and emission wavelengths of 410 and 485 nm respectively. All measurements were performed in triplicate in five different cell cultures.

### In vitro *GSH conjugation and LC-IT-MS analysis*

Reactions were performed *in vitro* using a glutathione S-transferase (GST) enzyme. GSH (5 mM, final concentration), ITH12674 (1 mM, final concentration) and 10 U of GST were added to PBS (10 mM, pH 6.5) at a final volume of 500 μL. The reaction was maintained at 37°C for 1 h. As control, non-enzymatic reaction (mixture lacking GST) was incubated for 2 h at 37°C. Reactions were stopped by adding 100 μL of 20% trifluoroacetic acid. Samples were prepared for analysis by adding 400 μL of a 50:50 mixture of MeOH : $CH_3CN$. The reaction mixtures were analysed using an API QSTAR pulsar I LC-MS/MS system (Applied Biosystems, Madrid, Spain) equipped with an electrospray ionization source and connected to a LC system 1100 series (Agilent Technologies, Madrid, Spain). Sample components were separated in a 150 × 2.1 mm BetaBasic-18 $C_{18}$ column using a linear gradient mobile phase of 80% water with 0.1% formic acid and 20% acetonitrile. The LC-IT-MS was operated in positive ion mode.

### *Measurement of reactive oxygen species (ROS) production*

To measure the generation of ROS, we used the molecular probe $H_2$DCFDA (Ha *et al.*, 1997). $H_2$DCF reacts with intracellular ROS to form dichlorofluorescein, a green fluorescent dye. Neurons were seeded in 96-well clear bottom-black plates for 7–8 days. After the treatments, neurons were loaded with 10 μM $H_2$DCFDA for 45 min. Subsequently, neurons were washed twice with Krebs solution and kept for 15 min before the beginning of the experiment. Then, neurons were exposed to the ROS generator (rotenone and oligomycin A combination, rot/olig) for 4 h. Fluorescence was measured in a fluorescence microplate reader (Fluostar optima; BMG Labtech). Wavelengths of excitation and emission were 485 and 520 nm respectively. The fluorescence of control was normalized as 100% and the increase in fluorescence induced by a 4 h exposure to the ROS generator (rot/olig) was expressed as % control cells.

### *Western blot analysis*

Slices of each experimental group were lysed in 100 μL ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 $\mu g \cdot mL^{-1}$ leupeptin, 1 mM PMSF, 20 mM NaF, 1 mM sodium pyrophosphate and 1 mM $Na_3VO_4$). Proteins (30 μg) from these lysates were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corp.). Membranes were incubated with anti-HO-1 (1:1000) and anti-β-actin (1:50 000). Appropriate peroxidase-conjugated secondary antibodies (1:10 000) were used to detect proteins by enhanced chemiluminescence. Protein bands were scanned and density was analysed using the Scion Image analysis software (Scion Corporation, NIH, Bethesda, MD, USA). Control value was considered as 1.

### *Data analysis*

Data are presented as means ± SEM. Comparisons between experimental and control groups were performed by one-way ANOVA followed by Newman–Keuls *post hoc* test. Differences were considered to be statistically significant when $P \leq 0.05$. All statistical procedures were carried out using GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA, USA).

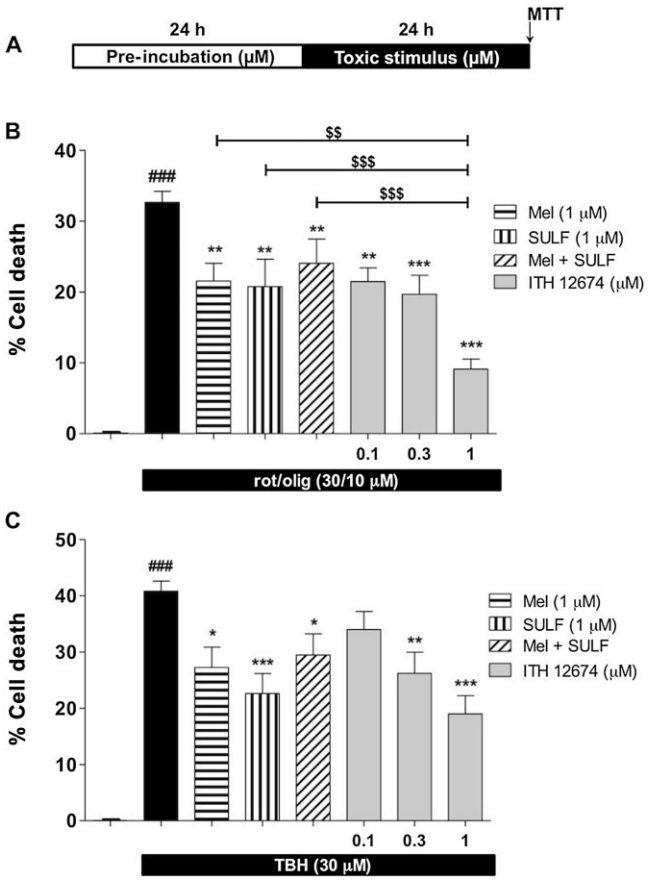
### *Chemicals*

The fluorescent dyes propidium iodide (PI), Hoechst 33342 and 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA), neurobasal, FBS, B27 supplement, B27 minus antioxidants (AO), minimal essential medium (MEM), HBSS and heat-inactivated horse serum were from Life Technologies (Madrid, Spain). Anti-HO-1 was purchased from Millipore (Madrid, Spain). Rotenone, oligomycin A, tert-butyl hydroperoxide (TBH), SULF, anti-β-actin, monochlorobimane, GSH S-transferase (GST), melatonin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), 2-deoxyglucose and poly-D-lysine were from Sigma-Aldrich (Madrid, Spain). Tin-protoporphyrin IX (SnPP) dichloride was from Tocris (Biogen, Madrid, Spain). pTK-Renilla was from Promega (Madison, WI, USA).

## Results

### *ITH12674 protects primary cortical neurons against oxidative stress*

To evaluate the neuroprotective profile of ITH12674 we first used two different *in vitro* models of oxidative stress, the combination of rot/olig (Egea *et al.*, 2007) and TBH (Kurz *et al.*, 2004). Rotenone and oligomycin A block complexes I and V, respectively, of the mitochondrial electron transport chain generating free radicals. In this study, we selected a pre-incubation protocol in order to evaluate the potential neuroprotective effect of ITH12674 derivative depending on its Nrf2 induction capability. Cortical neurons were pre-incubated for 24 h with increasing concentrations of ITH12674 and reference compounds [melatonin (1 μM) and sulforaphane (1 μM)] before the addition of toxic stimuli. Thereafter, treatments were removed and the correspondent toxic stimuli were added in fresh medium, without any compound and maintained for another 24 h. Neuronal viability was measured by the MTT reduction method (Figure 2A). Exposure of cortical neurons to rot/olig (30/10 μM) for 24 h significantly increased cell death to 33% with respect to untreated control (Figure 2B). Melatonin and sulforaphane significantly increased neuronal viability eliciting 40 and 44% protection, respectively, and their combination resulted in a similar protection of 35%. ITH12674 reduced the toxicity of rot/olig in a concentration-dependent manner, inducing 20% protection at a concentration of 100 nM; at 0.3 μM this protection increased to 41%. The highest neuroprotective




## Figure 2

ITH12674 exhibits concentration-dependent protection in cortical neurons against neuronal toxicity elicited by oxidative stress. (A) Experimental protocol: cortical neurons were pre-incubated with different treatments at defined concentrations for 24 h. Thereafter, neurons were subjected to toxic stimuli for another 24 h in the absence of the protecting compounds. (B) Cell death induced by the combination of rotenone (30 μM) and oligomycin A (10 μM) (rot/olig) and protection mediated by melatonin (1 μM), sulforaphane (SULF, 1 μM) and increasing concentrations of ITH12674 (0.1, 0.3 and 1 μM). (C) Cell death induced by TBH (30 μM) and its reduction induced by the previous compounds at the same concentrations. Data are expressed as cell death calculated as 100 minus % of viability assessed by the MTT technique and data were normalized as % basal. Data are mean ± SEM of triplicates of six independent experiments $^{###}P < 0.001$ comparing basal and toxic injured neurons; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ comparing toxic stimulus group in the absence of drugs; $\$\$P < 0.01$; $\$\$\$P < 0.001$ comparing ITH12674 group with reference compounds.

effect was reached at 1 μM (72%), which was more potent than reference compounds, either separately or combined.

TBH simulates the oxidative stress generated by complexed metals outside the cells (Kurz *et al.*, 2004). We used the same protocol described earlier (see protocol in Figure 2A). Incubation of cortical neurons with TBH for 24 h increased cell death to 40% (Figure 2C). ITH12674 significantly reduced TBH toxicity at concentrations of 0.3 and 1 μM, but not at 0.1 μM. In contrast, melatonin and sulforaphane elicited 33 and 47% protection, respectively. When given in combination, the protection decreased to 29%. In contrast, ITH12674 at 1 μM increased cell viability by 57%, being more potent than melatonin and sulforaphane at the same concentration.

### *Short-term decrease in GSH levels by ITH12674 due to GST-mediated conjugation with GSH*

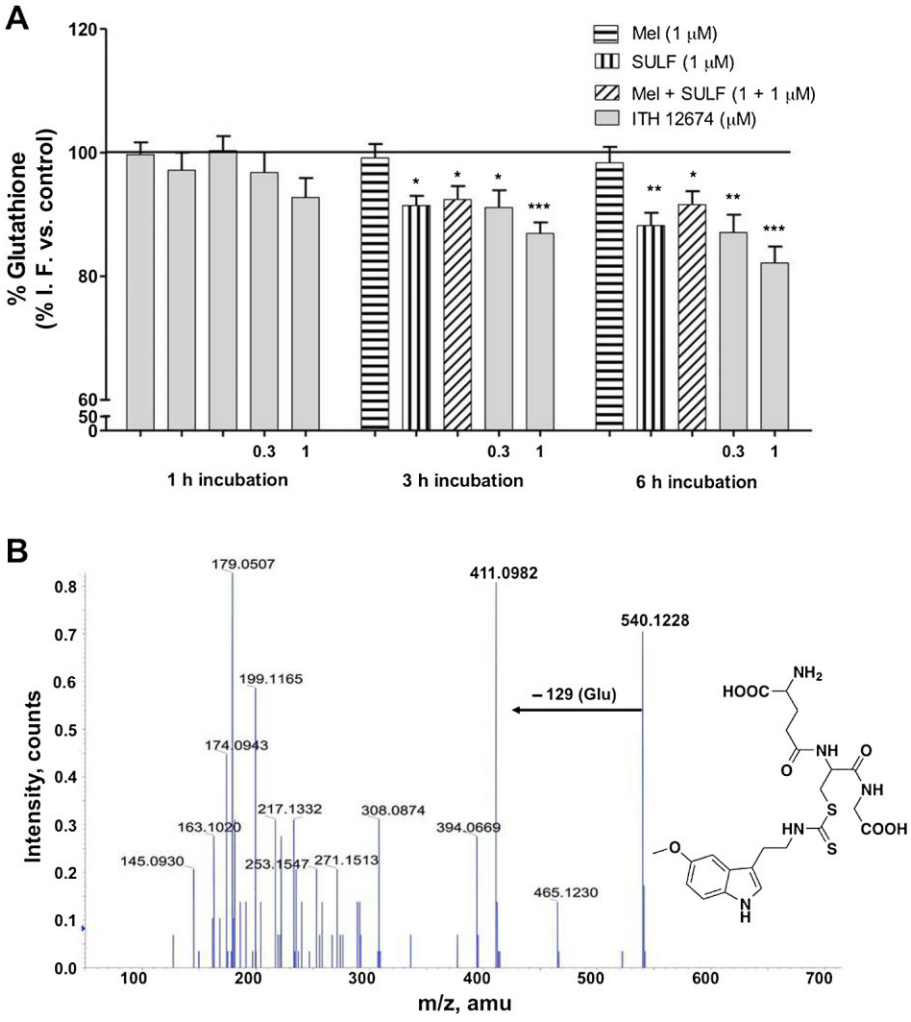
To evaluate the mechanism of action of ITH12674, we first focused on the GSH content of the treated neurons. GSH is the most important antioxidant inside the cells and its concentration is dependent on the expression of phase II enzyme. Electrophilic compounds decrease the levels of GSH by direct conjugation, which is catalysed by GST. Then, the redox system of the cell detects the GSH depletion, liberates Nrf2 that then translocates to the nucleus inducing the phase II antioxidant response. This mechanism is well documented for sulforaphane which, after conjugating with GSH, accumulates inside the cells (Ye and Zhang, 2001).

Thus, we were interested in measuring the time course for the changes in levels of GSH in response to, first, GSH depletion, and second, *de novo* synthesis of GSH due to phase II gene overexpression. Cortical neurons were incubated with increasing concentrations of ITH12674 (0.1, 0.3, 1.0 μM) and reference compounds melatonin or sulforaphane over three periods of time (1, 3 and 6 h). Following pre-incubation, treatments were removed and neurons were loaded with the dye monochlorobimane. As shown in Figure 3A, GSH concentrations after a 1 h pre-incubation with melatonin, sulforaphane or their combination were the same as those in untreated neurons. Incubation with each treatment over a period of 3 h resulted in a decreased GSH concentration in the presence of sulforaphane (8%) and more markedly in the presence of ITH12674, 15% decrease at 1 μM. Larger differences were observed with 6 h pre-incubation periods, where sulforaphane decreased the levels of GSH by 10%. ITH12674 decreased GSH levels in a concentration-dependent fashion showing a 20% decrease at 1 μM.

To demonstrate the conjugation of ITH12674 with GSH, we performed *in vitro* experiments in the presence of GST; the formation of the conjugate was analysed using LC-IT-MS. ITH12674 (1 mM) was mixed with reduced GSH (5 mM) in the presence of GST for 1 h at 37°C. The same conditions without GST were used as control reaction (data not shown). The expected molecular ion $[M + 1]^+$ of ITH12674–GSH conjugate ($m/z$ = 540) appears in the peak at 11.4 min (data not shown). The fragmentation pattern of this peak demonstrates its structure (Figure 3B) showing a characteristic peak of glutamate loss (–129) of the conjugate ($m/z$ = 411).

### *Long-term induction of GSH levels by ITH12674 is due to Nrf2–ARE transcriptional regulation*

The melatonin–sulforaphane hybrid ITH12674 was designed with the intention of combining the pharmacological properties of melatonin and the Nrf2 inducer abilities of sulforaphane. To confirm the Nrf2 inducer activity of ITH12674, we first studied the nuclear translocation of Nrf2 in the presence of ITH12674. Cortical neurons were treated with the hybrid compound (1 μM) or culture media for 2 h, then neurons were fixed and double stained with anti-Nrf2 and



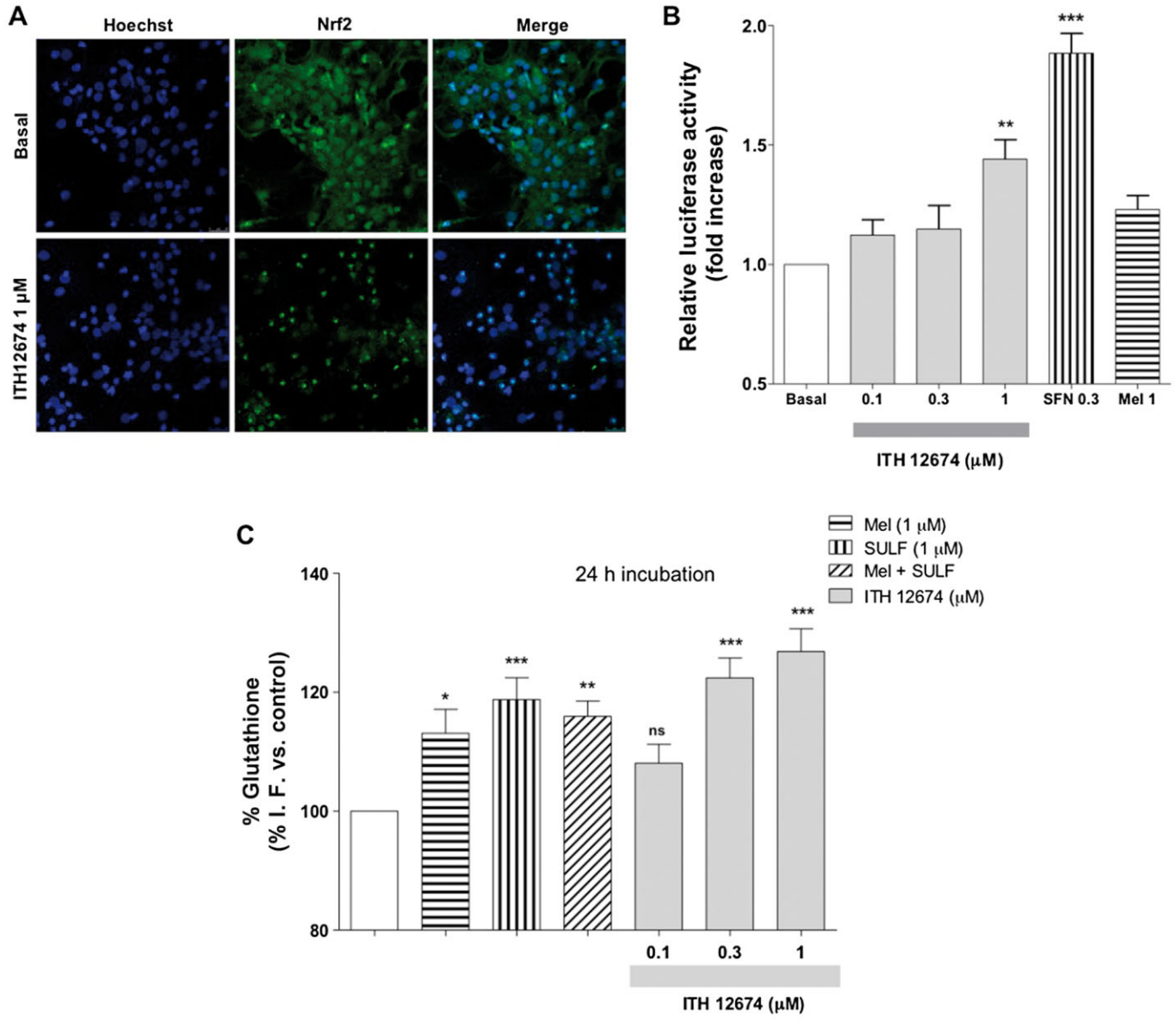

## Figure 3

ITH12674 decreased the levels of GSH in cortical neurons due to GST-mediated conjugation with GSH up to 6 h after incubation. (A) Cortical neurons were incubated with ITH12674 (0.3 and 1 μM) or reference compounds melatonin (1 μM), sulforaphane (SULF, 1 μM) or their mixture for 1, 3 and 6 h. Thereafter, treatments were removed and neurons were loaded with the fluorescent dye monochlorobimane for 1 h. Then, fluorescence intensity was measured. Data are expressed as % of fluorescence compared with vehicle control (DMSO). Data are mean ± SEM of triplicates of five independent experiments, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control conditions. (B) *In vitro* LC-MS/MS data for GST-mediated ITH12648–GSH conjugation, proposed chemical structure of the conjugate *m/z* 540 and its MS/MS fragmentation spectrum.

Hoechst. As shown in Figure 4A, in untreated neurons the Nrf2 was predominantly present in the cytosol; however, in the presence of 1 μM ITH12674 Nrf2 was predominantly located in the nucleus. Next, we tested the relative potency of ITH12674 with respect to melatonin and sulforaphane. HEK293T cells were transfected with ARE–LUC, and after overnight recovery, cells were stimulated for 16 h with increasing concentrations of ITH12674 (0.1, 0.3 and 1 μM), sulforaphane (0.3 μM) or melatonin (1 μM). ITH12674 increased reporter gene activity at the concentration of 1 μM, showing a 1.4-fold increase with respect to basal conditions (Figure 4B). sulforaphane, used as a control, almost doubled ARE expression at 0.3 μM and melatonin was not able to increase reporter gene activity in this model. As previously stated, *de novo* synthesis of GSH is regulated by phase II antioxidant genes as rate-limiting GSH synthetic enzymes are regulated by the ARE response. Thus, we measured GSH levels after 24 h. As shown in Figure 4C, a 24 h incubation of neurons with melatonin or sulforaphane increased the concentration of GSH by 17 and 20%, respectively, with respect to untreated controls. ITH12674, 0.3 and 1 μM, also increased the concentration of GSH after 24 h by 22 and 25%, respectively, with respect to control conditions (Figure 4C).

### *Antioxidant effect of ITH12674 in cortical neurons once conjugated with GSH*

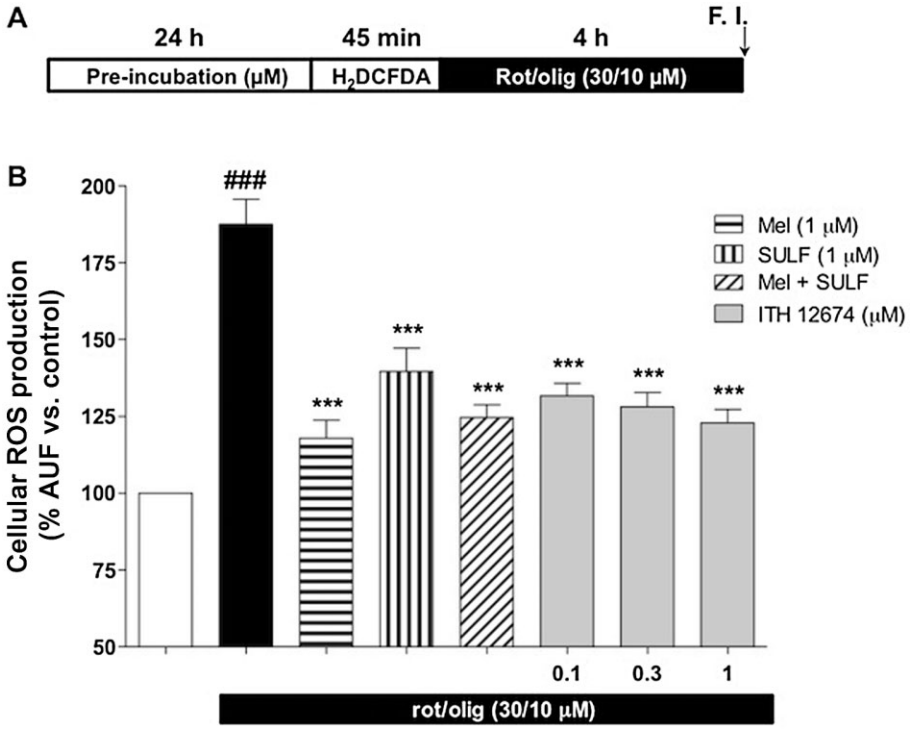
To correlate the neuroprotective effect of ITH12674 with its ability to induce Nrf2 and accumulation of the conjugate, we measured the mitochondrial ROS production induced by rot/olig, after 4 h, with the fluorescent dye $H_2$DCFDA. Cortical neurons were treated with increasing concentrations of ITH12674 (0.1, 0.3 and 1.0 μM) or reference compounds for




## Figure 4

ITH12674 induced Nrf2–ARE transcriptional response by nuclear translocation of Nrf2, measured as increased luciferase activity in HEK293T cells and increased GSH levels after a 24 h pre-incubation period. (A) Cortical neurons were treated with ITH12674 (1 μM) or culture medium (Basal) for 2 h, then they were processed for immunocytochemistry and stained with anti-Nrf2 (green) and Hoechst (blue). (B) HEK293T cells were transfected with the ARE–LUC reporter and Renilla control vectors. After transfection, cells were treated with either ITH12674 (0.1, 0.3 and 1 μM), sulforaphane (0.3 μM) or Mel (1 μM) for 16 h and luciferase activity was measured. Data are expressed as fold induction of luciferase activity compared with vehicle control (DMSO). Data are mean ± SEM of quadruplicates ($n = 4$), $^{**}P < 0.01$, $^{***}P < 0.001$ compared with basal conditions. (C) Cortical neurons were incubated with ITH12674 (0.1, 0.3 and 1 μM) or reference compounds melatonin (1 μM), sulforaphane (1 μM) or their mixture for 24 h. Thereafter, treatments were removed and neurons were loaded with the fluorescent dye monochlorobimane for 1 h. Then fluorescence intensity was measured. Data are expressed as % of fluorescence compared with vehicle control (DMSO). Data are mean ± SEM of triplicates of five independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control conditions.

24 h. Then, treatments were removed by washing and cells were loaded with the fluorescent dye for 45 min. After washing away the fluorescent dye, cells were incubated with rot/olig for 4 h (Figure 5A). Rot/olig incubation increased the production of ROS species by 87.5% with respect to untreated neurons. As shown in Figure 5B, ITH12674 and reference compounds reduced the production of ROS species. ITH12674 reduced ROS production to 31, 28 and 22% at the concentrations of 0.1, 0.3 and 1.0 μM respectively. Melatonin was the most potent, decreasing the production of ROS to only 17% over basal conditions. This reduction was similar to that produced by 1 μM ITH12674 and by the mixture of melatonin and sulforaphane. sulforaphane alone reduced ROS production to 39.7%.




## Figure 5

ITH12674 reduces ROS production in cortical neurons induced by the toxic combination of rot/olig after a 24 h pre-incubation. (A) Cortical neurons were pretreated with increasing concentrations of ITH12674 or reference compounds at 1 μM for 24 h. Then, treatments were removed and neurons were loaded with the fluorescent dye $H_2$DCFDA (45 min). Thereafter, the dye was eliminated and neurons were treated with ROS generator (rot/olig) over a 4 h period and fluorescence intensity was measured. Untreated cells without ROS generator were used as control. (B) Bar diagram of ROS production for basal conditions, ROS generator and treatments. Data are expressed as % of fluorescence compared with vehicle control (DMSO). Data are mean ± SEM of triplicates of five independent experiments, $^{###}P < 0.001$ comparing control and rot/olig-treated neurons; $^{***}P < 0.001$ compared with rot/olig conditions.

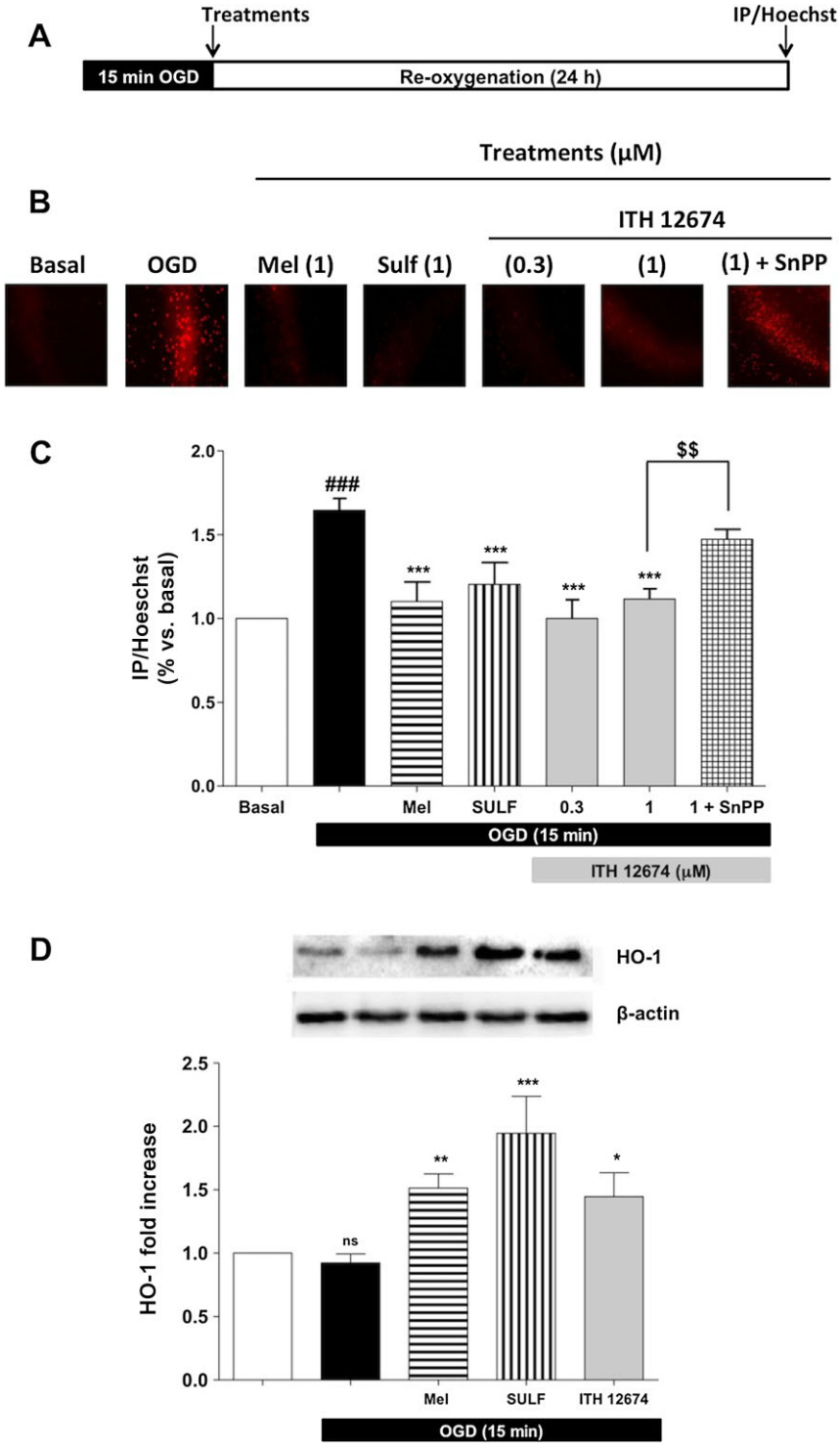
### *Participation of Nrf2/HO-1 in the neuroprotective effect of ITH12674 against OGD/reox*

To further investigate the neuroprotective effect evoked by ITH12674, we selected the oxygen and glucose deprivation plus reoxygenation (OGD/reox) model in OHCs as an *in vitro* model of brain ischaemia. This model has been demonstrated to reduce hippocampal neuron viability by ROS production and excitotoxicity (Parada *et al.*, 2013). After 15 min of OGD insult, OHCs were incubated with selected concentrations of ITH12674 (0.3 and 1 μM) and reference compounds, melatonin and sulforaphane (1 μM), for the 24 h re-oxygenation period (Figure 6A). OGD/reox increased cell death by 65% (1.65 ± 0.7) compared with control OHCs maintained in normoxia plus glucose conditions throughout the experiment (Figure 6C), as assessed by PI fluorescence in CA1 (Figure 6B). ITH12674 significantly reduced cell death at both concentrations, restoring OHCs to basal conditions at 0.3 μM (1.00 ± 0.13) and similarly at 1 μM (1.12 ± 0.06). ITH12674 was more potent than melatonin; it reduced toxicity to 10% (1.10 ± 0.13) and sulforaphane that reduced toxicity to 20% (1.20 ± 0.15) at the concentration of 0.3 μM where ITH12674 showed maximum protection.

As shown before (Figure 4), ITH12674 was able to induce Nrf2 and to increase GSH. To corroborate the participation of phase II enzymes in the neuroprotection mediated by ITH12674, we analysed the participation of the enzyme HO-1, one of the most important enzymes of the phase II antioxidant and anti-inflammatory response (Cuadrado and Rojo, 2008). After 15 min in OGD, OHCs were treated with ITH12674 and SnPP (HO-1 inhibitor, 3 μM); this resulted in a partial reversal of its neuroprotective effect (1.47 ± 0.07) (Figure 6C). To verify the expression of HO-1, at the end of the experiment, OHCs were collected and cell lysates were resolved on SDS-PAGE and analysed by immunoblot with anti-HO-1 (Figure 6D). Melatonin (1 μM) significantly increased, by 1.5-fold, the expression of HO-1 with post-OGD treatment. Sulforaphane was the most potent as, at 1 μM, it increased the expression of HO-1 by 1.9-fold. ITH12674, at 1 μM, also increased the expression of HO-1 in a statistically significant manner by 1.4-fold. Thus, ITH12674 exhibited similar potency to induce HO-1 as melatonin at the same concentration, but it was less potent than sulforaphane.

## Discussion

This study focuses on the neuroprotective profile of compound ITH12674 as well as on the signalling pathways involved in such protection. ITH12674 is a hybrid of




## Figure 6

Post-OGD treatment with ITH12674 elicits neuroprotection in organotypic hippocampal slice cultures and this effect is linked to HO-1 overexpression. (A) Experimental protocol: OHCs were exposed to 15 min of OGD followed by 24 h in control solution (Reox). ITH12674 or reference compounds were present during the 24 h reox period. (B) Microphotographs (original magnification 10×) of the CA1 subfield loaded with PI for each treatment. (C) Bar diagram of cell viability for basal conditions, OGD and treatments measured as the relationship of PI/Hoechst fluorescence in the CA1 subfield. (D) Top part of the figure illustrates the representative bands showing the expression of HO-1 obtained from hippocampal slices subjected to 15 min OGD and 24 h reox. Expression of HO-1 is presented as densitometric quantification using β-actin for normalization (bottom). Data are mean ± SEM of six independent experiments $^{###}P < 0.001$ comparing basal and OGD-injured slices; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ comparing (C) OGD group in the absence of treatments (D) with control slices. $^{\$\$}P < 0.01$ comparing OGD-injured slices treated with ITH12674 or ITH12674 plus SnPP (3 μM).

melatonin and sulforaphane designed to combine the broad pharmacological profile of melatonin and the Nrf2 inducer activity of sulforaphane in a single compound. We demonstrated that it has neuroprotective properties and characterized its mechanism of action, which was shown to involve (i) Nrf2 activation; (ii) reduction in ROS production; (iii) modulation of GSH levels; and, finally, (iv) overexpression of phase II enzymes including HO-1, a potent antioxidant and anti-inflammatory enzyme.

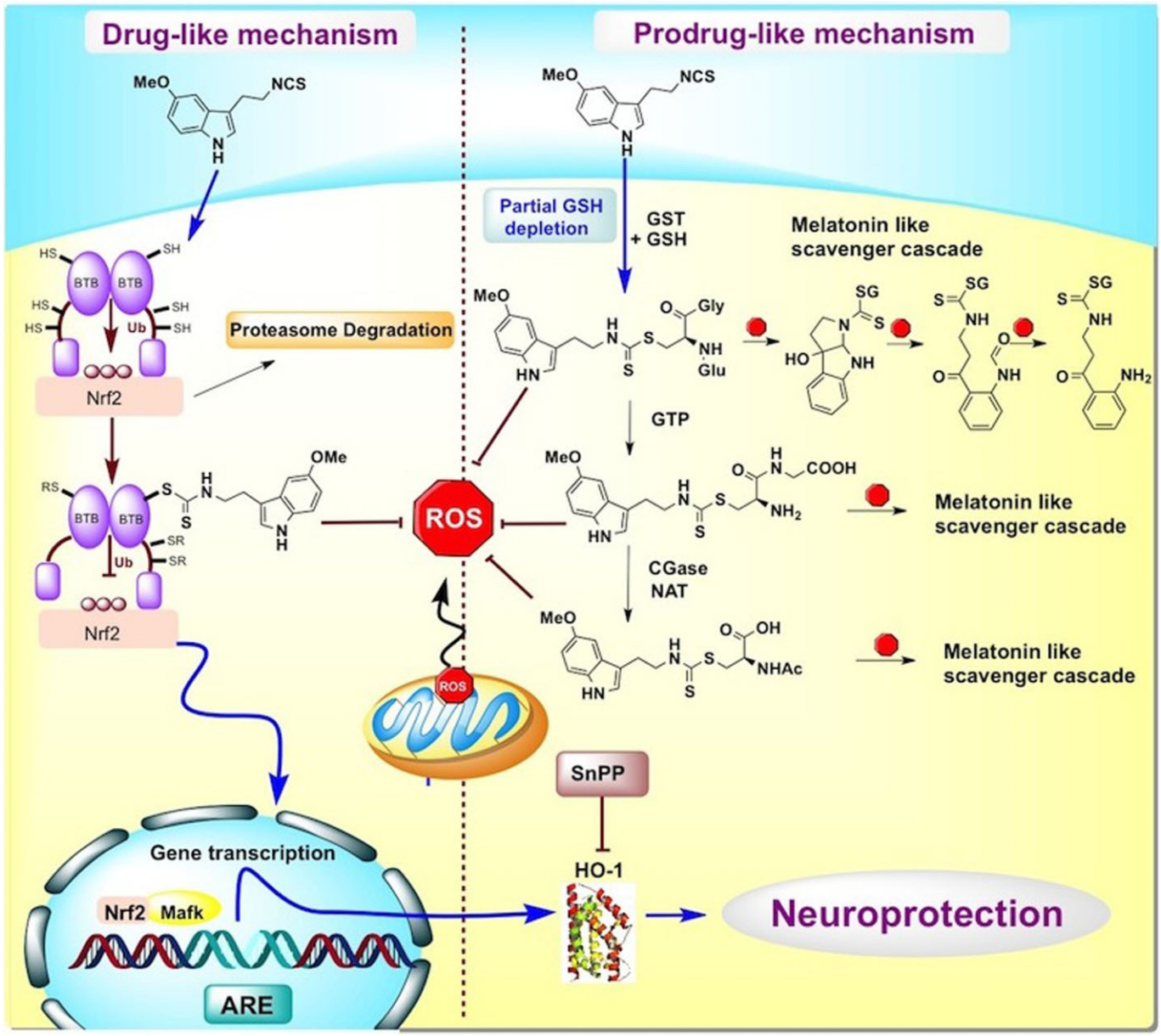
Mitochondrial dysfunction and oxidative stress are increasingly implicated in NDDs (Lin and Beal, 2006), and are thought to be the driving force of ageing. However, due to the participation of oxidative stress and neuroinflammation in the onset and development of NDDs and stroke, both hallmarks are increasingly used as key targets for the development of novel therapies for these pathologies. In this context, we focused our efforts to develop a new compound that is able to use both pathological hallmarks as targets. Thus, we first considered melatonin due to its neuroprotective profile in several models of oxidative stress related to neurodegeneration (Srinivasan *et al.*, 2011). Melatonin is able to protect from oxidative stress in different models by increasing the levels of Nrf2 protein (Wang *et al.*, 2012), and the subsequent activation of the Nrf2–ARE pathway and overexpression of phase II enzymes like HO-1 (Ding *et al.*, 2014; Parada *et al.*, 2014). Phase II enzyme induction by melatonin has been directly related to the nuclear translocation of Nrf2 rather than to an increase in its levels, but it depends on the model used (Ding *et al.*, 2014). The circulating levels of melatonin in aged patients have been found to be one-half of those of younger controls (Hardeland, 2013). Moreover, the production of melatonin decreases in aged individuals (Bubenik and Konturek, 2011), which suggests that decreased levels of this hormone are primary contributing factor for the development of NDDs (Reiter, 1994; Karasek, 2007).

Sulforaphane has also been demonstrated to reduce oxidative stress and this protective effect is mediated by the expression of phase II genes (Han *et al.*, 2007; Innamorato *et al.*, 2008). Sulforaphane is also able to increase the protein levels of Nrf2 (Fan *et al.*, 2013). Thus, ITH12674 might also increase Nrf2 protein levels. However, the induction of the Nrf2–ARE pathway has been directly related to the nuclear translocation of Nrf2 rather than to its levels in the cytosol. Sulforaphane is able to protect cells against oxidative stress by increasing the expression of phase II antioxidant enzymes (Danilov *et al.*, 2009). Kassahun *et al.* (1997) described the conjugation of the isothiocyanate moiety of sulforaphane and GSH to induce Nrf2 (Kassahun *et al.*, 1997). In that study, they demonstrated that the sulforaphane–GSH conjugate is metabolized by two peptidases that eliminate the γ-glutamyl (GTP) and the glycyl (C-glycyl peptidase) residues to generate a sulforaphane–Cys derivative. Finally, N-acetyl-transferase acetylates the sulforaphane–Cys derivative (Kassahun *et al.*, 1997). Furthermore, Zhang (2000) demonstrated that isothiocyanates are accumulated in several cell lines upon exposure to these compounds, causing the activation of phase II enzymes by the generation of GSH conjugates. It was also demonstrated that the accumulation of isothiocyanates and induction of Nrf2 were dependent on GSH (Ye and Zhang, 2001).

As described in the Introduction, the inclusion of the isothiocyanate moiety in ITH12674 was designed to implement a 'drug–prodrug' mechanism for the new molecule. The isothiocyanate moiety could react with key cysteine residues present in Keap1 to release Nrf2 (Figure 7). On the other hand, the GST enzyme catalyses its conjugation with GSH to generate an ITH12674–GSH conjugate (Figure 3B) that might be a potent-free radical scavenger (prodrug mechanism), showing a similar scavenger cascade as described for melatonin (Figure 7) (Tan *et al.*, 2007). The reaction of ITH12674 with GSH generates the conjugated compound with the dithiocarbamate functionality (Figure 7), which mimics the N-acetyl functionality present in melatonin. When the ITH12674–GSH conjugate is generated, it is accumulated inside the cell and it should be able to trap free radicals (Figure 7). This scavenger effect has been demonstrated for the dithiocarbamate analogue of melatonin (Pedras and Okanga, 1998). When this analogue was subjected to oxidative conditions, it was readily oxidized to the tricyclic analogue of cyclic 3-hydroxymelatonin (Pedras and Okanga, 1998). Therefore, the ITH12674–GSH conjugate might generate several intermediates able to react again with free radicals, as does cyclic 3-hydroxymelatonin, AFMK, AMK, AFM and melatonin (Tan *et al.*, 2007).

In support of this hypothesis (Figure 7), we demonstrated the improved neuroprotective effect of ITH12674 with respect to melatonin and sulforaphane. ITH12674 induced Nrf2 translocation to the nucleus at 1 μM (Figure 4A), and it was able to increase reporter gene activity at that concentration, being a better inducer than melatonin in this model (Figure 4B). However, sulforaphane is a better Nrf2 inducer than ITH12674 (Figure 4B), although sulforaphane and melatonin were less potent than ITH12674 at mediating neuroprotection against oxidative stress generated by rot/olig (Figure 2B). Similar results were obtained with the TBH model of oxidative stress (Figure 2C). In line with these results, ITH12674 reduced the production of intracellular ROS in a concentration-dependent manner to a larger extent than sulforaphane (Figure 3B), and this reduction might be mediated by the accumulation of ITH12674 inside the neurons as a GSH conjugate. This hypothesis explains the different protection evoked by ITH12674 against the rot/olig combination and TBH. The rot/olig combination generates free radicals inside the cell, and, therefore, the ITH12674–GSH conjugate would be in place to trap these radicals. We also demonstrated a decrease in the GSH content over a time period of 24 h (Figures 3A and 4C). For the first 6 h, the GSH content was partially depleted by the incubation with sulforaphane or ITH12674, but not with melatonin. Finally, the GSH content was increased after a 24 h pre-incubation period with ITH12674 in accordance with the results obtained by Zhang *et al.* and others (Gao *et al.*, 2001; Ye and Zhang, 2001).

There is compelling evidence to support the view that oxidative stress is enhanced after cerebral ischaemia and reperfusion, which in turn induces lipid peroxidation, protein and DNA oxidation, calcium overload, activation of intracellular signalling pathways, excitotoxicity and inflammation (Soane *et al.*, 2007). Taking into account the neuroprotective profile of melatonin and sulforaphane against oxidative damage in *in vitro* and *in vivo* models of cerebral ischaemia via induction of Nrf2 (Reiter *et al.*, 2005; Zhao *et al.*, 2006; Soane




## Figure 7

Schematic representation of the proposed 'drug–prodrug' mechanism of action of compound ITH12674.

*et al.*, 2010; Hall, 2011), we also studied the neuroprotective effect of ITH12674 in an *in vitro* model of brain ischaemia to elucidate the mechanism of action of this compound, that is, the free radical scavenger generated (prodrug mechanism, Figure 3B) and activation of Nrf2–ARE and phase II antioxidant enzymes (Figure 4A and B). Neuronal death was significantly reduced in OHC cultures treated with ITH12674 post-OGD (incubated during the 24 h reox period). Interestingly, ITH12674 evoked a slightly better neuroprotective effect than melatonin and sulforaphane at both concentrations tested. The protective effect of melatonin and sulforaphane in these models is, at least in part, associated with the expression of HO-1 (Parada *et al.*, 2014). Compound ITH12674 increased the expression of this antioxidant and anti-inflammatory enzyme in the post-OGD incubation protocol (Figure 6). Furthermore, the neuroprotective effect of ITH12674 against OGD/reox depends, partially, on the expression of HO-1 as co-incubation of ITH12674 with the HO-1 inhibitor, SnPP, reversed by 35% the neuroprotective effect of this hybrid. The partial reversal of the neuroprotective effect upon co-incubation with SnPP is also in line with the hypothesis that ITH12674 generates a GSH conjugate with antioxidant properties. Hence, the protection evoked by ITH12674 might be related, first, to the overexpression of HO-1 (Figure 6D) and, second, to the scavenger effect of the conjugate accumulated inside the neurons.

In conclusion, we have designed a melatonin–sulforaphane hybrid that possesses a dual drug–prodrug

mechanism of action (Figure 7), and has an improved neuroprotective profile compared to melatonin and sulforaphane in three different models of oxidative stress. Its neuroprotective action is dependent on its conjugation with GSH, the induction of Nrf2 and the overexpression of phase II enzymes as demonstrated by the overexpression of HO-1, a potent antioxidant and anti-inflammatory enzyme.

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## Author contributions

J. E. contributed to data acquisition, data analysis and interpretation, and critical revision of the manuscript. I. B. and E. N. contributed to data acquisition and data analysis/interpretation and critical revision of the manuscript. E. P. and P. R. contributed to data acquisition and data analysis/interpretation. A. C., M. G. L. and A. G. G. contributed to critical revision of the manuscript. R. L. contributed to concept/design, acquisition of data, data analysis/interpretation drafting of the manuscript, critical revision of the manuscript and approval of the article.

## Conflict of interest

The authors declare no conflict of interest.

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## 6.4 Mecanismo neuroprotector del nuevo derivado de melatonina, el Neu-P11, en modelos de isquemia cerebral.

Frenar la cascada isquémica alterando cualquiera de sus componentes es una estrategia interesante para tratar el ictus isquémico agudo. Durante la isquemia y, especialmente durante la reperfusión, uno de los mayores causantes de muerte celular es el estrés oxidativo. El efecto neuroprotector de la melatonina en modelos *in vivo* de isquemia cerebral se encuentra ampliamente descrito. En este sentido, nos propusimos probar el potencial efecto neuroprotector de un nuevo agonista de los receptores de melatonina MT1/MT2, el derivado Neu-P11, en modelos relacionados con la isquemia cerebral tanto *in vitro*, como *in vivo*. Y por último, determinar su mecanismo molecular.

El Neu-P11 mostró un buen perfil antioxidante, protegió frente a la excitotoxicidad inducida por glutamato y frente a la privación de oxígeno y glucosa en rodajas de hipocampo de rata y, redujo el volumen de infarto *in vivo*. En cuanto a su mecanismo de acción, su efecto neuroprotector fue revertido por el luzindol (antagonista de receptores de melatonina), por el AG490 (inhibidor de JAK2), el LY294002 (inhibidor de PI3/AKT) y el PD98059 (inhibidor de MEK/ERK1/2). Por tanto, podemos concluir que el nuevo derivado de la melatonina Neu-P11 protege frente a diferentes modelos de isquemia cerebral tanto *in vitro* como *in vivo*, mediante un mecanismo de acción que implica a los receptores de melatonina, así como las rutas de supervivencia, JAK/STAT, PI3K/Akt y MEK/ERK1/2.

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# Neuroprotective mechanism of the novel melatonin derivative Neu-P11 in brain ischemia related models

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ABSTRACT

Stopping the ischemic cascade by targeting its components is a potential strategy for acute ischemic stroke treatment. During ischemia and especially over reperfusion, oxidative stress plays a major role in causing neuronal cell death. Melatonin has been previously reported to provide neuroprotective effects in *in vivo* models of stroke by a mechanism that implicates melatonin receptors. In this context, this study was planned to test the potential neuroprotective effects of the novel melatonin MT1/MT2 receptor agonist, Neu-P11, against brain ischemia in *in vitro* and *in vivo* models, and to elucidate its underlying mechanism of action. Neu-P11 proved to be a good antioxidant, to protect against glutamate-induced excitotoxicity and oxygen and glucose deprivation in hippocampal slices, and to reduce infarct volume in an *in vivo* stroke model. Regarding its mechanism of action, the protective effect of Neu-P11 was reverted by luzindole (melatonin receptor antagonist), AG490 (JAK2 inhibitor), LY294002 (PI3/AKT inhibitor) and PD98059 (MEK/ERK1/2 inhibitor). In conclusion, Neu-P11 affords neuroprotection against brain ischemia in *in vitro* and *in vivo* models by activating a pro-survival signaling pathway that involves melatonin receptors, JAK/STAT, PI3K/Akt and MEK/ERK1/2.



## 1. Introduction

Stroke remains as one of the most devastating of neurological diseases, causing physical impairment or long term disability. Behind cardiovascular disease and cancer, stroke has become the third leading cause of death (Jahan and Vinuela, 2009). Despite the high medical need, only the recombinant thrombolytic tissue plasminogen activator (rtPA) is approved for these patients, but due to its limited therapeutic window and risk of bleeding, it is estimated that around 85% of all patients are excluded from treatment (Fisher, 2011). Therefore, it is crucial to find a new pharmacological approach for this disease (Jahan and Vinuela, 2009).

Oxygen and glucose deprivation during cerebral ischemia triggers a cascade of events that includes the disruption of membrane potential due to the reduction in ATP production and mitochondrial membrane damage, which leads to a release of excitatory amino acids such as glutamate (Bonde et al., 2003). Increased intracellular calcium due to activation of glutamate receptors leads to mitochondrial function impairment via activation of protein kinase, phospholipase, protease, nitric oxide synthase and release of free radicals (Schulz et al., 1995). Excessive reactive oxygen species (ROS) production can induce an inflammatory response (Gloire et al., 2006). Oxidative stress results in macromolecular damage, reducing cell viability and is also implicated in various disease states. NADPH oxidase (NOX) is one of the main contributors to such excessive ROS production. Another contributor to ROS generation is nitric oxide (NO), generated by inducible NO synthetase (iNOS) (Egea et al., 2012).

Stopping the ischemic cascade by targeting its components is one potential strategy for acute stroke treatment, known as the neuroprotection strategy. One appropriate target in stroke treatment is neutralizing these molecules by using free-radical

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scavengers (Katsura et al., 2012). Many different neuroprotection approaches, targeting different steps of the ischemic cascade, were previously tested in animal stroke models and clinical development programs (Hossmann, 2006). Despite the successful results in animal models regarding both infarct size reduction and improved functional outcome, no neuroprotective drug has yet demonstrated unequivocal efficacy in clinical trials that fulfilled regulatory requirements for approval (Fisher, 2011). The availability of a safe, even modestly, beneficial neuroprotective drug with a reasonable therapeutic time window for ischemic stroke would be of substantial benefit to a large number of patients.

Melatonin (N-acetyl-5-methoxytryptamine) is a neurohormone mainly produced by the pineal gland. It regulates the asleep–awake cycle, and it is used to control sleep and circadian rhythm disorders, although it has also be proven to have modulatory actions over the immune system and the antioxidant response (Tan et al., 2007). As a result of binding to high-affinity receptors (MT1 and MT2) expressed in immunocompetent cells, it promotes anti-inflammatory effects (Nair et al., 2011). Melatonin and its metabolites are involved in the modulation of oxidative stress (Ritzenthaler et al., 2013) in human acute stroke by preventing the increase of iNOS and eNOS, and also by decreasing excitototoxic glutamate release (Koh, 2008a); these two latter effects are mediated by the activation of MT2 melatonin receptors (Chern et al., 2012). In addition, due to its neuroprotective profile, melatonin is potentially useful for neurodegenerative diseases (Pandi-Perumal et al., 2013), although clinical trials are still not conclusive.

Neu-P11 (Piromelatine, N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-4-oxo-4H-pyran-2 carboxamide) is a novel melatonin (MT1/MT2) and 5-HT1A/1D receptor agonist (He et al., 2013). In two phase-I studies in healthy volunteers and in insomnia patients, piromelatine was found to be safe and well tolerated with no serious adverse events and showed a favorable, dose-proportional pharmacokinetic (PK) profile following oral intake. In a Phase II randomized placebo controlled sleep laboratory study in insomnia patients, piromelatine (20 and 50 mg daily, 1 month) significantly enhanced sleep maintenance, measured by polysomnography, in comparison to placebo. A recent study has tested its applicability in psychophysiological insomnia and Alzheimer's disease by improving neuronal viability and cognitive impairment (He et al., 2013). Neu-P11 also exerts antidepressant, anxiolytic (Tian et al., 2010), antidiabetic (She et al., 2009), antihypertensive (Huang et al., 2013) and antinociceptive effects (Liu et al., 2014). Besides its neurological actions, Neu-P11 has demonstrated usefulness in reducing intraocular pressure (She et al., 2009). More recently, it has proven to be protective against myocardial ischemia/reperfusion injury *in vitro* (Yu et al., 2014).

Our aim in this study is to elucidate the potential neuroprotective effect and mechanism of action of Neu-P11 against brain ischemia (*in vitro* and *in vivo* models), with the idea of postulating it as a new pharmacological approach in which melatonin's advantages are maintained and pharmacokinetics are improved.

## 2. Experimental procedures

### 2.1. Materials

Neu-P11 was provided by Neurim Pharmaceuticals LTD (Israel); Melatonin (from Sigma–Aldrich, Madrid, Spain); Luzindole (from Sigma–Aldrich, Madrid, Spain); LY294002 (morpholino-4-yl-8-phenylchromen-4-one) (PIP3K/Akt inhibitor), AG40090 (Jak2 inhibitor) and PD98059 (ERK1/2 inhibitor) were from Tocris scientific/Biogen, Madrid, Spain and; Chemicals to prepare different nutrient solutions were chemical grade from Panreac (Madrid, Spain). Chemicals were dissolved in DMSO; the same amount of DMSO was added to control cells or control brain slices.

### 2.2. Evaluation of the oxygen radical absorbance: ORAC test

The Oxygen Radical Antioxidant Capacity-Fluorescein (ORAC-FL) method, modified by Dávalos et al. (Davalos et al., 2004) was used to evaluate the ROS scavenging capacity of Neu-P11, compared to melatonin. Different concentrations of the compound were prepared in a final volume of 25 μl in 10 mM of PBS (pH 7.4) and 150 μl of FL reactive (94 nM of final concentration) were mixed in a black 96-well microplate at 37 °C. After 15 min, 25 μl of 2,2′-azobis(amidinopropane) dihydrochloride (AAPH) solution (12 mM final concentration) were added and the emitted fluorescence was measured in a FLUOStar optima microplate reader, (485 excitation and 520 emission filters) during 90 min. A FL + AAPH in PBS (blank) and a calibration curve of Trolox (1–8 μM) were included in each experiment. Results were normalized with respect to the blank curve, to later calculate the Area Under the Curves (AUC). Finally, regression equations between net AUC and antioxidant concentration were calculated for each concentration and represented as Trolox equivalents, where the ORAC-FL value of Trolox was taken as 1. Each sample was repeated 3–4 times and performed in duplicates.

### 2.3. SH-SY5Y cell culture and treatment

The neuroblastoma human cell line SH-SY5Y was used within passages 4–13. Cells were maintained in a 1:1 mixture of F-12 Nutrient Mixture (Ham12) (Sigma–Aldrich, Madrid, Spain) and MEM supplemented with 15 nonessential amino acids, 1 mM sodium pyruvate, 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (reagents from Invitrogen, Madrid, Spain) in flasks, and maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. For assays, SH-SY5Y cells were subcultured in 48-well plates (TPP, Zellkutur and Labortechnologie, Trasadingen, Switzerland) at a seeding density of $1 \times 10^5$ cells per well. Once the cells were grown at 75–80% of confluence, they were treated with the toxic stimuli Rotenone/Oligomycin (Rote/oligo) during 24 h or at 60% of confluence when the protective compounds were incubated 24 h prior to addition of the toxics (Rote/oligo). At the end of the experiment, cell viability was measured by MTT reduction.

### 2.4. Animal usage and care

Male rats (Sprague Dawley) and mice (C57BL/6J) were housed under controlled temperature and lighting conditions, and water and food were provided *ad libitum*. All animals were treated making all efforts to minimize animal suffering and reducing animal number. Guide for the Care and Use of Laboratory Animals was followed and all the procedures were pre-approved by the ethics committee for the care and use of animals in research of the Universidad Autónoma de Madrid and with the Spanish Royal Decree of 1 February 2013 (53/2013) and in accordance with the European Union Directive of 22 September 2010 (2010/63/UE).

### 2.5. Preparation of rat hippocampal slices

Experiments were performed in hippocampal slices from adult male Sprague Dawley rats (275–325 g) from a colony of our animal quarters. Rats were quickly decapitated under sodium pentobarbital anesthesia (60 mg/kg, i.p.). Forebrains were rapidly removed from the skull and placed into ice-cold Krebs bicarbonate dissection buffer (pH 7.4), containing: NaCl 120 mM, KCl 2 mM, $CaCl_2$ 0.5 mM, $NaHCO_3$ 26 mM, $MgSO_4$ 10 mM, $KH_2PO_4$ 1.18 mM, glucose 11 mM

and sucrose 200 mM. The hippocampi were quickly dissected and cut into transverse slices of 300 μm thick using a Tissue Chopper Mcllwain. After that, they were introduced into a chamber at 34 °C continuously bubbled with 95% $O_2$/5% $CO_2$ mixture for a stabilization period of 45 min.

### 2.6. Oxygen and glucose deprivation in rat hippocampal slices

Hippocampal slices corresponding to the control group were incubated 15 min in a Krebs control solution with the following composition: NaCl 120 mM, KCl 2 mM, $CaCl_2$ 2 mM, $NaHCO_3$ 26 mM, $MgSO_4$ 1.19 mM, $KH_2PO_4$ 1.18 mM and glucose 11 mM; this solution was equilibrated with 95% $O_2$/5% $CO_2$. On the other hand, oxygen and glucose deprivation (OGD) was induced by incubating the slices during 15 min in a glucose-free Krebs solution, equilibrated with a 95% $N_2$/5% $CO_2$ gas mixture; glucose was replaced by 2-deoxyglucose. The slices were returned back to an oxygenated normal Krebs solution containing glucose (reoxygenation period) for two hours. During the OGD and Reox period, melatonin 100 nM (as control drug) and Neu-P11 at different concentrations (1, 10, 100 and 1000 nM), were added to the different groups. Experiments were performed at 37 °C. To determine the signaling pathway in the neuroprotective effect of Neu-P11, the melatonin antagonist (Luzindole) and the JAK2, PI3K/AKT and ERK1/2 inhibitors (AG40090, LY294002 and PD98059, respectively) were added 30 min before and during the OGD and reoxygenation period.

### 2.7. Glutamate excitotoxicity in rat hippocampal slices

The relationship between glutamate receptor over activation, $Ca^{2+}$ overload and neuronal cell death has been extensively documented as being key elements in the ischemic cascade (Brassai et al., 2015). The protocol used to induce glutamate excitotoxicity in rat hippocampal slices was previously described by Molz and coworkers (Molz et al., 2008). Briefly, 300 μm thick rat hippocampal slices were stabilized for 45 min in a Krebs solution containing: NaCl 120 mM, KCl 2 mM, $CaCl_2$ 2 mM, $NaHCO_3$ 26 mM, $MgSO_4$ 1.19 mM, $KH_2PO_4$ 1.18 mM and glucose 11 mM, equilibrated with 95% $O_2$/5% $CO_2$. We then incubated the slices with glutamate (1 mmol/L) dissolved in a nutritive culture medium composed of 50% of KRB, 50% of Dulbecco's Modified Eagle's Medium (DMEM), 20 mM of HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a $CO_2$ atmosphere for 4 h. Neu-P11 (1, 10 or 100 nM) or melatonin (100 nM) was co-incubated with the glutamate solution, in randomly selected groups of slices, for 4 h.

### 2.8. Quantification of viability by MTT in hippocampal slices

Hippocampal cell viability was determined through the ability of the cells to reduce MTT as previously described (Denizot and Lang, 1986). Briefly, hippocampal slices were collected immediately after the treatment and incubated with MTT (0.5 mg/ml) in Krebs bicarbonate solution for 30 min at 37 °C. The tetrazolium ring of MTT can be cleaved by active dehydrogenases in order to produce a precipitated formazan. The formazan produced in the hippocampal slices was solubilized by adding 200 μl of dimethyl sulfoxide (DMSO), resulting in a colored compound whose optical density was measured in an ELISA microplate reader at 540 nm. Absorbance values obtained in control slices were taken as 100% viability.

### 2.9. Measurement of ROS production

To measure the generation of reactive oxygen species (ROS), we have used the molecular probe 2′,7′-Dichlorofluorescein diacetate ($H_2$DCFDA) purchased from Molecular Probes (Invitrogen, Madrid, Spain), which diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent form dichlorofluorescein (DCFH). DCFH reacts with intracellular $H_2O_2$ to form dichlorofluorescein (DCF), a green fluorescent dye (Ha et al., 1997).

At the end of the experimental protocol, hippocampal slices were loaded with 10 μM $H_2$DCFDA for 45 min in Krebs solution and Hoechst 33342 at 1 μg/ml was added during the last 5 min to stain nuclei. Subsequently, slices were washed twice with the preincubation Krebs solution. Fluorescence was measured in a fluorescence inverted NIKON eclipse T2000-U microscope. Wavelengths of excitation and emission were 485 and 520 nm, respectively. Fluorescence analysis was performed using the Metamorph programme version 7.0. Images were taken at CA1 at magnifications of 10×. ROS production was calculated dividing the mean DCFDA fluorescence by the mean Hoechst fluorescence. Data were normalized with respect to control values that were considered as 100%.

### 2.10. Western blot

Slices of each group were lysed in 100 μL ice-cold lysis buffer (1% Nonident P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris HCl pH 7.5, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium leupeptin, 1 mM sodium pyrophosphate, and 1 mM $Na_3VO_4$). The slices were then disaggregated using a Sonicator Ultrasound. The homogenates were centrifuged at 13,000 rpm for 5 min at 4 °C. 30 μg of protein fraction was resolved by PAGE-SDS and transferred to Immobilon-P membranes (Millipore Corp). Membranes were incubated with anti-total Erk1/2 and anti-P-Erk1/2 at 1:1000 (Santa Cruz Biotechnology, Heidelberg, Germany).

### 2.11. In vivo photothrombotic stroke model and drug administration

Three months old male C57 mice (30–35 g) were anesthetized with 1.5% isoflurane in oxygen under spontaneous respiration. Mice were then placed in a stereotaxic (David Kopf Instruments, Tujunga, CA, USA) and physiological temperature at 37 ± 0.5 °C was maintained by a servo-controlled rectal probe heating pad (Cibertec, Madrid, Spain). A small incision in the midline was made. Then, after removal of the periosteum, bregma and lambda points were identified. A cold-light (Zeiss KL 1500 LCD, Jena, Germany) was centered using a micromanipulator at 0.2 mm posterior and 1.5 mm lateral to bregma on the right side using a fiber optic bundle of 2 mm in diameter. One milligram (0.1 ml) dissolved in sterile saline of the photosensitive dye Rose Bengal (Sigma–Aldrich, St. Louis, MO, USA) was injected i.p and 5 min later the brains were illuminated during 20 min. After completion of the surgical procedures, the incision was sutured and the mice were allowed to recover for 24 h.

Animals were randomly distributed into 3 groups: (Jahan and Vinuela, 2009) Control: saline, (Fisher, 2011) Neu-P11: 1 mg/kg and (Bonde et al., 2003) Melatonin 1 mg/kg. Drugs were diluted in 0.9% NaCl sterile saline and were administered via i.p 5 min after photothrombotic stroke induction.

### 2.12. Infarct volume measurement

Twenty four hours after stroke induction, animals were anesthetized and sacrificed by decapitation and brains were quickly removed. Then, coronally sections of 1-mm-thick were cut and slices were incubated in 2% solution of triphenyltetrazolium chloride and then fixed in a buffered formalin solution. Thereafter, infracted tissue was defined by the unstained area and by the use of an unbiased stereological estimator of volume based on Cavalieri's principle (Avendano et al., 1995), the morphometric determination of cortical infarct volume was calculated.

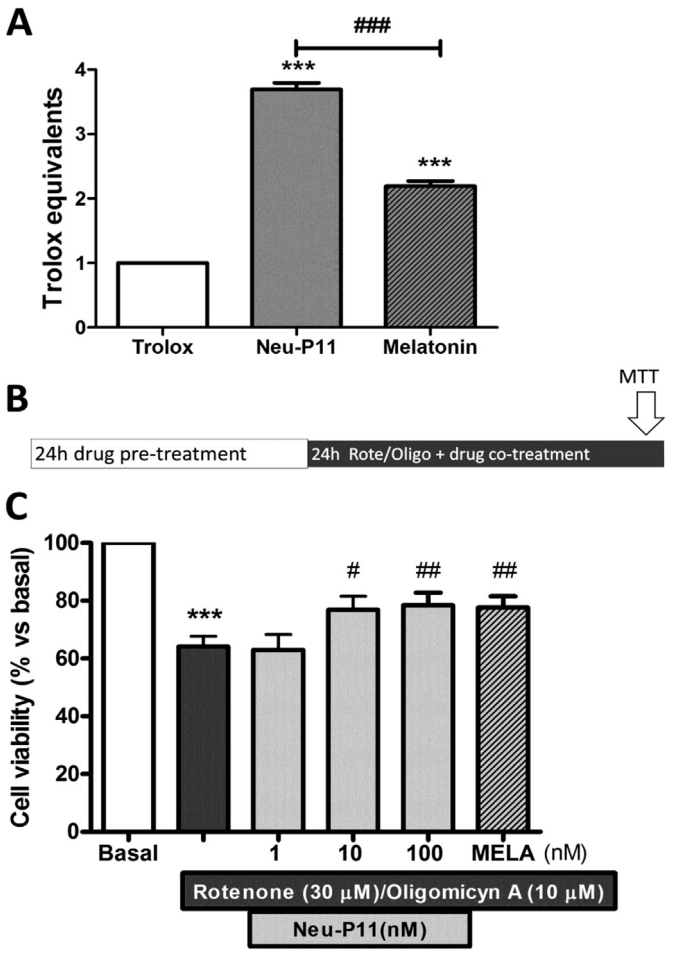


**Fig. 1.** Neu-P11 exerts antioxidant properties and neuroprotection against the combination of Rotenone (30 μM) and Oligomycin A (10 μM) (Rote/Oligo) induced toxicity in SH-SY5Y human neuroblastoma cells. (A) Antioxidant activity of increasing concentrations of Neu-P11 and melatonin was measured, normalized to trolox (1–8 μM). Data are expressed as means ± SEM of trolox equivalents of four different experiments. ***P < 0.001 compared to trolox; ###P < 0.001 compared to Neu-P11. (B) Illustrates the protocol used to test the neuroprotective effect of increasing concentrations of Neu-P11 against Rotenone (30 μM) and Oligomycin A (10 μM) (Rote/Oligo) induced toxicity. (C) Represents protection afforded by increasing concentrations of Neu-P11 (1–100 nM) and 100 nM of melatonin against Rote/Oligo toxicity following the protocol on B. Data represent the mean ± SEM from eight different cultures, ***P < 0.001 compared to basal; ##P < 0.01, #P < 0.05 compared to Rote/oligo group. One way anova followed by Newman–keuls was performed. Data were normalized to control values that were taken as 100% of cell viability.

### 2.13. Statistical analysis

Results were expressed as mean ± S.E.M. Statistical differences were determined with ANOVA test followed by Newman–Keuls post hoc using GraphPad Prism 5.00. Statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. Free radical scavenging and neuroprotective effects of Neu-P11 against rotenone/oligomycin A induced cell death in the human neuroblastoma cell line SH-SY5Y

Because melatonin is well known for its free radical scavenging properties (Galano et al., 2013), we first evaluated if Neu-P11 could scavenge free radicals in a similar fashion to melatonin. For this purpose we used the ORAC test (see Materials and Methods section) and trolox (a well-known vitamin E analog with potent antioxidative capacity) as reference compound (Davalos et al., 2004). Both Neu-P11 and melatonin showed capacity to scavenge free radicals. While melatonin showed 2.19 trolox equivalents, Neu-P11 showed 3.70 trolox equivalents; this value was significantly higher than that achieved with melatonin (Fig. 1A).

Once demonstrated Neu-P11ś capacity to scavenge free radicals, we next tested its ability to afford neuroprotection in an oxidative stress model that combines rotenone plus oligomycin A, blockers of the mitochondrial complexes I and V, respectively, in the human neuroblastoma cell line SH-SY5Y (Parada et al., 2010). As our group previously reported, exposure of SH-SY5Y cells to the combination of rotenone (30 μM) and oligomycin A (10 μM) during 24 h causes around 40% of cell death (Molz et al., 2008). Under these experimental conditions, we evaluated increasing concentrations of Neu-P11 (1, 10 and 100 nM) following the protocol described in Fig. 1B; melatonin was used as control. 1 nM of Neu-P11 did not afford neuroprotection against oxidative stress, while 10 nM and 100 nM caused 36% and 39% of protection. Melatonin at 100 nM served as positive control, and no differences between treatments were observed (Fig. 1C).

### 3.2. Neu-P11 affords neuroprotection against glutamate and oxygen and glucose deprivation followed by reoxygenation (OGD/Reox) in hippocampal slices

During cerebral ischemia, glutamate levels increase causing neuronal excitotoxicity as a result of excessive neurological stimulation (Kostandy, 2012). Glutamate receptor-mediated $Ca^{2+}$ overload appears to be the most relevant pathogenic pathway of $Ca^{2+}$ dysregulation related to various neurodegenerative diseases, as well as stroke (Choi, 1988). To evaluate the potential protective effect of Neu-P11 against glutamate-induced excitotoxicity, we used a more physiological preparation, the hippocampal slice model.

Following the protocol described on the top part of Fig. 2A, exposure of rat hippocampal slices for 4 h to 1 mM glutamate reduced cell viability of the hippocampal slices by 30%. Increasing concentrations of Neu-P11 (1, 10 and 100 nM) or melatonin at 100 nM were incubated during the 4 h exposure to glutamate. Under these experimental conditions, Neu-P11 afforded significant protection at 10 nM (52% protection) and 100 nM (67% protection); however, melatonin was not able to protect in this excitotoxicity model (Fig. 2A).

We next moved to the oxygen and glucose deprivation model in hippocampal slices as a more physiopathological model of brain ischemia. The protocol used consisted of exposing the hippocampal slices for 15 min to oxygen and glucose deprivation (OGD), followed by 2 h of reoxygenation (OGD/Reox) (see protocol on top part Fig. 2B); these experimental conditions caused 40% reduction of viability, measured as MTT reduction. When the slices were incubated during the OGD/Reox period with increasing concentrations (1–1000 nM) of Neu-P11, around 60% protection was achieved at the concentrations of 10 and 100 nM. In contrast to the results observed in the glutamate model, in this toxicity model, melatonin at 100 nM offered a similar protection to that of Neu-P11 (Fig. 2B). Therefore, the concentration of 100 nM of Neu-P11 was selected to continue with the following experiments. This protection was confirmed by measuring Propidium iodide uptake as cell death parameter. OGD/Reox caused a 65% of cell death, whereas the co-treatment with Neu-P11 (100 nM) reduced cell death to 35%, affording almost 50% of protection (Fig. 2C).

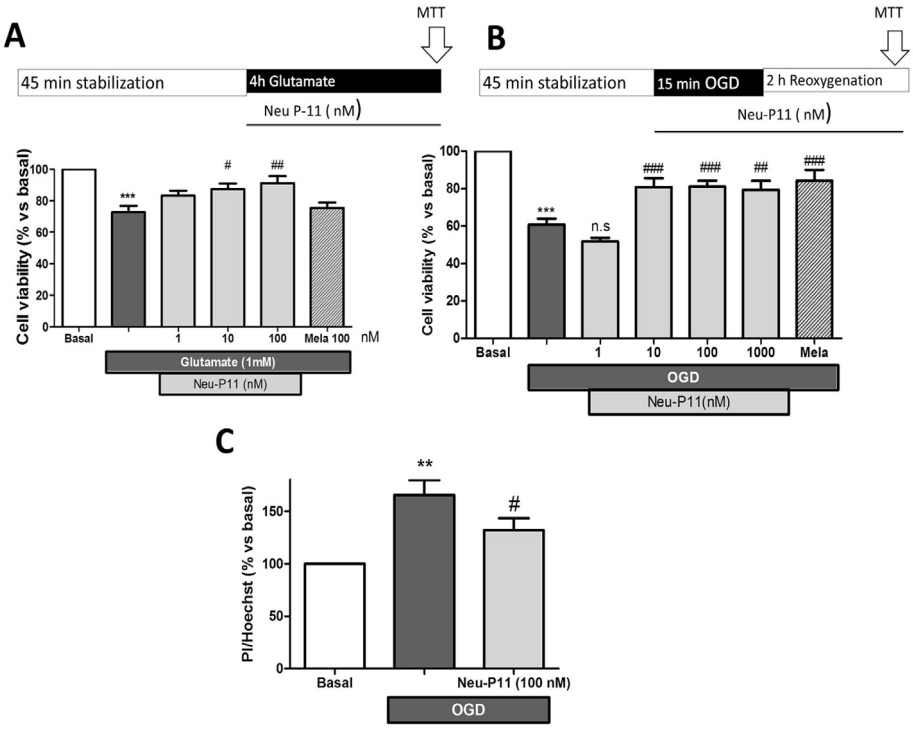


**Fig. 2.** Neu-P11 protects against glutamate and oxygen and glucose deprivation (OGD) induced neurotoxicity in brain hippocampal slices. Schematic illustration of the protocol used are represented on the top part of the figures A and B. (A) 4 h of glutamate induced cell death is prevented by increasing concentrations of Neu-P11 (10 and 100 nM). Neither Neu-P11 at 1 nM or melatonin at 100 nM exerted neuroprotection. Data are mean ± SEM of six independent experiments, ***$p < 0.001$ respect to basal; ##$p < 0.01$ and #$p < 0.05$ respect to glutamate group. (B) Quantitative analysis of cell viability measured by MTT reduction of hippocampal slices under basal conditions (basal) or subjected to Oxygen and glucose deprivation followed by reoxygenation (OGD/Reox) in the absence or presence of increasing concentrations of Neu-P11; the effect of 100 nM melatonin is also represented (Mela). Data are means ± S.E.M. of 7 different cultures. ***$p < 0.001$ comparing OGD respect to basal; ##$p < 0.01$, ###$p < 0.001$ comparing treatments to OGD/Reox (C) OGD/Reox increased cell death measured by propidium iodide uptake and Neu-P11 at 100 nM reduced the increased cell death. Data are means ± S.E.M. of 4 different experiments. **$p < 0.01$ comparing OGD respect to basal; #$p < 0.05$, comparing treatment to OGD/Reox. One way ANOVA followed by Newman–keuls was performed. Data were normalized to control values that were taken as 100% of cell viability.

### *3.3. Neu-P11 reduces the increase of reactive oxygen species (ROS) induced by OGD/Reox*

As stated in the Introduction, oxidative stress is one of the major factors that contribute to cell death during ischemia/reperfusion (Radak et al., 2014). Therefore, at the end of the experiments presented in Fig. 2B, ROS were measured as the fluorescence of $H_2$DCFDA in CA1 hippocampal region (see Fig. 3A for representative images under the different experimental conditions). As represented in Fig. 3B, the OGD/Reox almost doubled ROS production, whereas the treatment with Neu-P11 at 100 nM significantly reduced ROS production almost to basal levels.

### *3.4. Melatonin receptors (MT-1 and MT-2) are involved in the protective effects of Neu-P11*

In order to determine the participation of melatonin receptors in the neuroprotective effects of Neu-P11, we used the melatonin-receptors antagonist luzindole. As illustrated in Fig. 5, at 1 μM, luzindole antagonized the neuroprotective effect of Neu-P11. These data indicate that melatonin receptors are implicated in Neu-P11ś protective action.

### *3.5. Jak2/STAT3, Akt and MEK/Erk1/2 pro-survival signaling pathways are involved in the neuroprotective effect of Neu-P11*

To explore the intracellular signaling pathway induced by Neu-P11 to exert its protective effect, we used several kinase inhibitors related to kinases that are known to be activated to provide neuroprotection. First, we explored the Jak2/STAT3 pathway by using the Jak2 antagonist AG490 at 10 μM. As represented in Fig. 5A, AG490 blocked the protective effect afforded by Neu-P11 in hippocampal slices subjected to OGD/Reox. These results suggest that Neu-P11 protects the cells via activation of Jak2 survival pathway.

Further on, to evaluate if the PI3K/Akt intracellular pathway was implicated in Neu-P11ś neuroprotective effect, we used the PI3K/Akt family inhibitor LY294002 at 1 μM. As shown in Fig. 5A, this antagonist abolished the protective effect afforded by Neu-P11 in hippocampal slices subjected to OGD-Reox, reducing cell viability to almost OGD-group values. Thereby, the neuroprotective effect of Neu-P11 could be explained by the activation of PI3K/Akt signaling pathway.

Lastly, we used the MEK/ERK1/2 inhibitor PD98059, here we found that it reversed the neuroprotective effect of Neu-P11. Furthermore, at the end of the experiment, control, OGD/Reox and Neu-P11 treated slices were collected and phosphorylation of ERK1/2 was revealed by western blot analysis. As presented in Fig. 5B, exposure of brain slices to OGD/Reox reduced by 25% phosphorylation of ERK1/2; however, in slices co-incubated with Neu-P11, such reduction of phosphorylation was not observed. Taken together, these results support that Neu-P11 is promoting ERK1/2 phosphorylation to provide protection.

### *3.6. Neu-P11 reduces infarct volume in a photothrombotic stroke model*

After having demonstrated that Neu-P11 offered neuroprotection in different brain ischemia related *in vitro* models, we

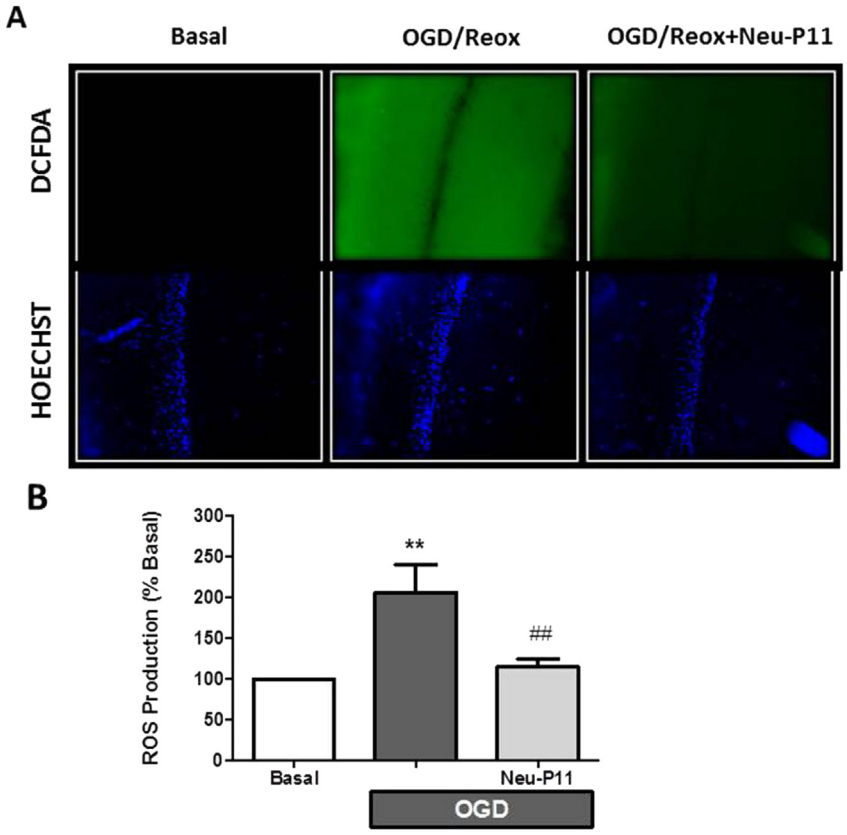


**Fig. 3.** Neu-P11 reduces OGD/Reox induced ROS production. (A) Top images show DCFDA fluorescence for ROS and bottom images show Hoechst fluorescence for nuclei of CA1 region of hippocampal slices under basal conditions, after exposure to OGD/Reox, or after exposure to OGD/Reox in the presence of 100 nM of Neu-P11; the protocol used was that described in Fig. 2B. (B) Average mean data ±S.E.M. of 5 independent experiments. **p < 0.01 with respect to basal and ##p < 0.01 in comparison to OGD/Reox. One way ANOVA followed by Newman–keuls was performed. Data were normalized to control values that were taken as 100%.

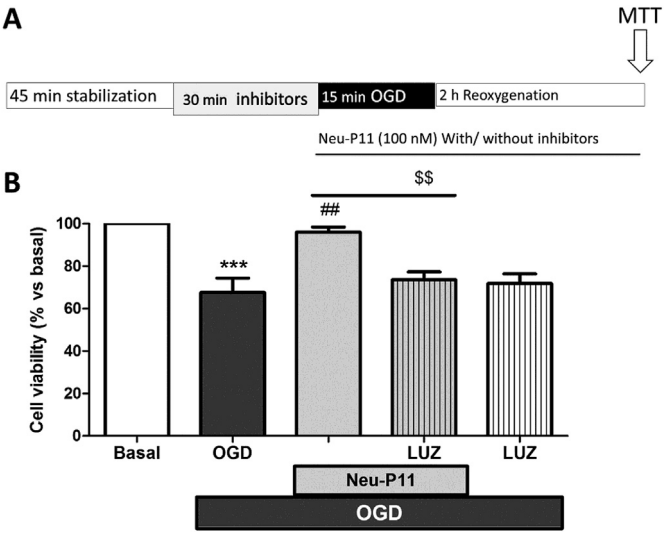


**Fig. 4.** Melatonin receptors are involved in the protective effect of Neu-P11. Slices were subjected to 30 min of the antagonists (Luzindole) before the period of 15 min OGD followed by 2 h reoxygenation, as shown in the protocol (A). Under these experimental conditions, 100 nM Neu-P11 showed a good neuroprotective effect, but in presence of 10 μM of Luzindole, this neuroprotection was lost. Antagonist *per se* did not affect cell viability (B). Data correspond to the mean ± S.E.M of 5 different animals; all variables were run in parallel. ***p < 0.001 respect to control, ##p < 0.01 respect to OGD/Reox and $$p < 0.01 with respect to Neu-P11. One way ANOVA followed by Newman–keuls was performed. Data were normalized to control values that were taken as 100% of cell viability.

were interested in evaluating its potential protective effect in an *in vivo* model. For this purpose, we used the photothrombotic model of brain ischemia in mice. Following the protocol shown in Fig. 6A, photothrombotic stroke caused a mean cortical infarct volume of 17.1%. Administration of Neu-P11 (1 mg/kg), 5 min after illumination, reduced the infarct volume by 35% (11.1%). For comparative purpose, we administered melatonin (1 mg/kg) and found that protection afforded by melatonin was not significantly different to that achieved with Neu-P11 (Fig. 6B and C). This data support the results obtained *in vitro* and postulate Neu-P11 as neuroprotectant for brain ischemia.

## 4. Discussion

In this study we have shown for the first time that the novel melatonin and serotonin 5-HT1A/1D receptor agonist Neu-P11 protects both in *in vitro* and *in vivo* brain ischemia models. Furthermore, we have elucidated, at least in part, its mechanism of action that implies the pro-survival signaling pathways of Jak2, Akt and MEK/ERK1/2.

In stroke as well as in neurodegenerative diseases, three major and frequently interrelated processes such as glutamate excitotoxicity, free radical-mediated damage and mitochondrial dysfunction have been identified as common pathophysiological mechanisms leading to neuronal death (Molz et al., 2008; Reiter, 1998; Iadecola, 2010). Here, we have explored Neu-P11ś capacity to protect in different models related to the common pathophysiological mechanisms related to brain ischemia mentioned above. First, we used the human neuroblastoma cell line SH-SY5Y exposed

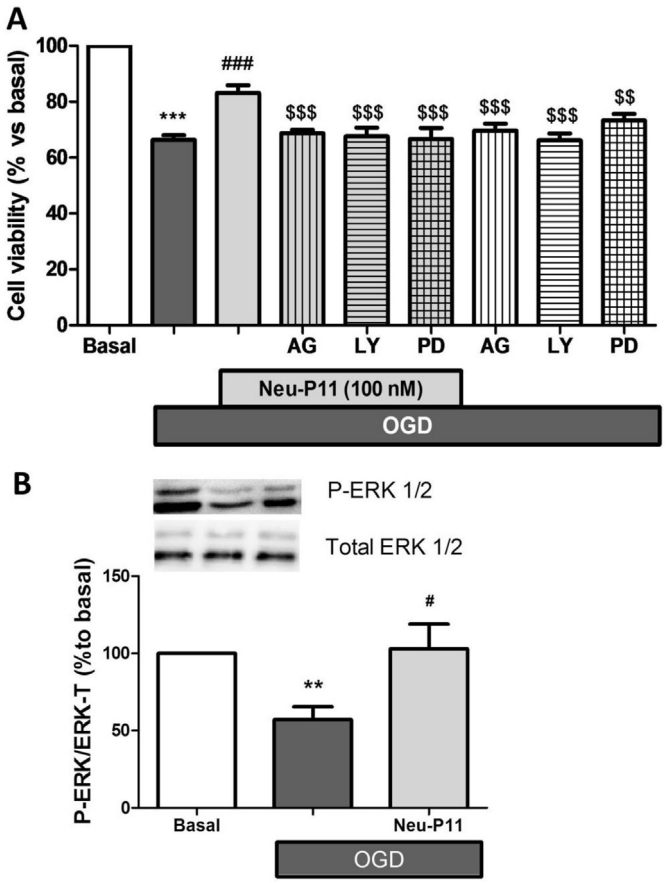


**Fig. 5.** Jak2/STAT3, PI3K/Akt and ERK1/2 survival pathways are involved in Neu-P11 neuroprotection against OGD/Reox. (A) The neuroprotective effect of Neu-P11 against OGD/Reox was reversed by Jak2/STAT3 antagonist AG490 (10 μM), PI3K/Akt antagonist LY294002 (10 μM), and the ERK1/2 antagonist PD98059 (10 μM). Cell viability was measured as MTT reduction at the end of the experiment. Data correspond to the mean ± S.E.M. of 5 animals. ***$p < 0.001$ in comparison to control; ###$p < 0.001$ comparing to OGD/Reox; \$\$\$$p < 0.001$ \$\$$p < 0.01$ in comparison to Neu-P11. (B) The decrease of ERK1/2 phosphorylation due to OGD/Reox is reversed by the use of Neu-P11, quantified by Western blot. Data correspond to the mean ± S.E.M. of 6 different cultures. **$p < 0.01$ in comparison to control; #$p < 0.05$ comparing to OGD/Reox. One way ANOVA followed by Newman–keuls was performed. Data were normalized to control values that were taken as 100%.

to a mixture of rotenone/oligomycin A, which cause mitochondrial disruption and secondary oxidative stress, and is considered a good *in vitro* model for ischemia (Galkin et al., 2009). In this model, Neu-P11 showed a good neuroprotective profile which related to its free radical scavenging properties. In fact, Neu-P11 was more potent than trolox, and even melatonin itself in the ORAC test (Fig. 1A and C).

Besides oxidative stress, glutamate excitotoxicity plays a major role in ischemic and traumatic brain insults (Choi, 1988; Trotti et al., 1998). By using a more complex model, the hippocampal slice instead of the SH-SY5Y cell line, we found that NeuP-11 was capable of reducing neuronal death secondary to glutamate excitotoxicity (Fig. 2A). Furthermore, Neu-P11 was protective and reduced ROS production in hippocampal slices exposed to OGD/Reox (Figs. 2B, C and 3).

Finally, we corroborated it protective effect in an *in vivo* model of brain ischemia. In concordance with the *in vitro* data, Neu-P11 was able to reduce infarct volume when administered at 1 mg/kg, 5 min post-stroke induction; its ability to reduce infarct volume at such a very low concentration avoids toxicity and other possible complications, making it an attractive candidate for further investigations directed to its usefulness in clinic (Fig. 6).

Of note is the observation that was while melatonin was protective in the OGD/Reox model it was ineffective in the glutamate excitotoxicity model. A possible interpretation for these results could lie on the fact that Neu-P11 also interacts with 5-HT1A/1D receptors (He et al., 2013). It has been reported that different 5HT1A agonists are able to attenuate excitotoxicity, proving neuroprotective in global and focal ischemia animal models (Marco et al., 2011). Furthermore, some 5HT1A agonists have demonstrated efficacy in phase IIb clinical trials for the treatment of ischemic stroke (Berends et al., 2005; Teal et al., 2009). In addition to brain ischemia, in Alzheimer's disease, Neu-P11 has shown neuroprotective properties by facilitating memory performance tasks in rats, requiring a more advantageous administration pattern compared to melatonin. Also, chronic treatment with Neu-P11, but not melatonin, has been reported to attenuate cellular loss induced by intra hippocampal Aβ injection (He et al., 2013). Neu-P11 has proved antidepressant and anxiolytic effects based on its interaction with 5HT1A/1D receptors; this effect correlates with the fact, that compared to melatonin, Neu-P11 presents better affinities for these receptors (Tian et al., 2010). In addition, Neu-P11 has demonstrated to be protective against myocardial ischemia/reperfusion injury *in vitro* (Yu et al., 2014).

Besides showing its neuroprotective component, we were interested in elucidating the neuroprotective mechanism of action of Neu-P11, as it is almost unknown. In this respect, we have shown that neuroprotection is mediated via melatonin receptors as previously reported (She et al., 2009). However, we provide new information as to its intracellular signaling pathway. Our results show that the blockers AG490, LY294002 and PD98059 reversed the neuroprotection afforded by Neu-P11 which indicates that Jak/Stat, Akt and Erk1/2, after melatonin receptor activation, is crucial for the neuroprotective effects of this compound (Figs. 4 and 5). This data support the pro-survival effect of these cellular pathways in the protection of different compounds such as melatonin (Kilic et al., 2005; Koh, 2008b; Nduhirabandi et al., 2011) and make Neu-P11 attractive for the treatment of different diseases.

Several authors propose antioxidants as good neuroprotective agents for brain ischemia, even though some of them like edaravone, sylimarin, minocycline or quercetin, among others, have not shown efficacy in clinic (Katsura et al., 2012; Wu et al., 2014; Lapchak, 2010; Borah et al., 2013; Chen et al., 2012; Pandey et al., 2012). In line with this, because melatonin is a direct and indirect antioxidant, it has been proposed as a neuroprotective agent (Reiter et al., 2010) and has demonstrated neuroprotection against oxidative stress implied in different neurodegenerative diseases such as AD (Dragicevic et al., 2011; Matsubara et al., 2003), PD (Acuna Castroviejo et al., 2002; Singhal et al., 2011), brain trauma (Tsai et al., 2011) and brain ischemia (Pandi-Perumal et al., 2013; Parada et al., 2014; Kilic et al., 2012).

As mentioned above, melatonin has therapeutic potential for many diseases but it has a poor pharmacokinetic profile (0.5 h half-life), which limits its use in clinic; therefore, there is an increasing need for developing prolonged-release preparations of melatonin or new melatonin agonists with a longer half-life (Pandi-Perumal et al., 2013; Turek and Gillette, 2004). In this respect, Neu-P11 shows a longer half live (1.5–3.0 h) (He et al., 2013) than melatonin, but there are also new melatonin derivatives being investigated such as ramelteon (it has 3–16 times higher affinity for MT receptors compared to melatonin), agomelatine, (melatonergic compound with antidepressant properties), tasimelteon or TIK-301 (Pandi-Perumal et al., 2013; Hardeland, 2010; Spadoni et al., 2011).

Taken together, our results support the idea that Neu-P11 protects against rotenone/oligomycin, glutamate and OGD *in vitro* and

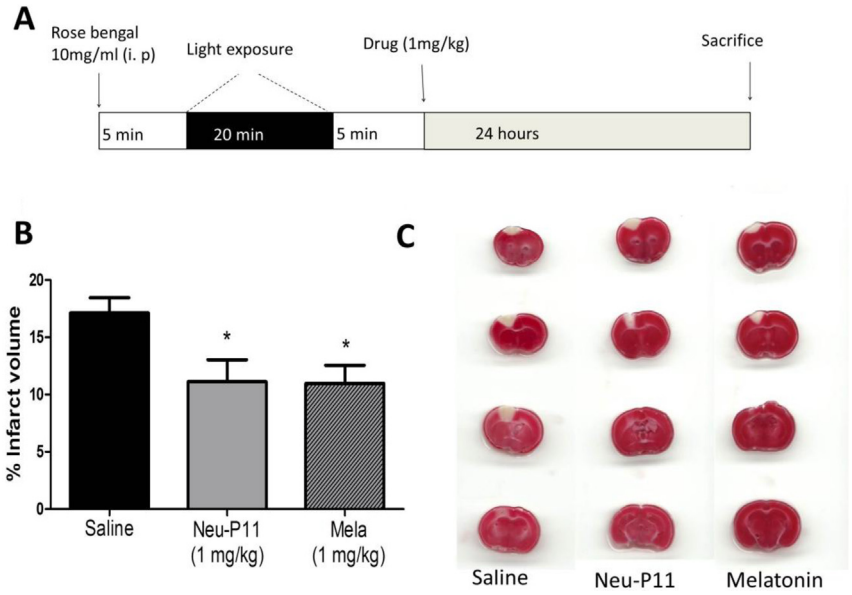


**Fig. 6.** Neu-P11 reduces the infarct volume in mice subjected to photothrombotic stroke. (A) Illustrates the protocol used, in which Neu-P11 or melatonin (intraperitoneal (i.p) at 1 mg/kg) were administered 5 min after the thrombotic stroke. (B) Data are expressed as a percentage (%) of the cortical infarct volume in mice. Note the reduction of infarct in mice receiving Neu-P11 and melatonin. Data are expressed as mean ± S.E.M. of eight animals per group. $^{*}p < 0.05$, compared to saline. (C) Representative photographs of the cortical infarcts of mice are presented. One way ANOVA followed by Newman–keuls was performed.

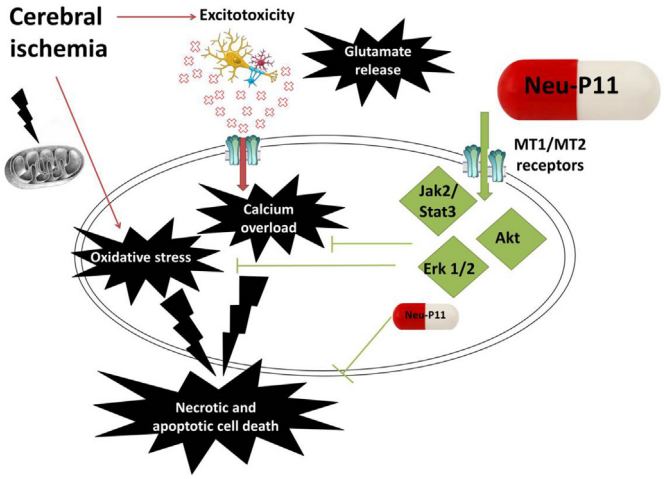


**Fig. 7.** Schematic illustration of the mechanism of action Neu-P11. Brain ischemia causes glutamate excitotoxicity, calcium overload and oxidative stress that, ultimately, lead to neuronal cell death. Neu-P11 was able to protect against brain ischemia induced cell death by acting on melatonin receptors and activating JAK2, AKT and ERK1/2 pro-survival signaling pathways.

reduces brain infarct volume *in vivo* by promoting Jak/Stat, MEK/Erk and Akt pro-survival signaling pathways (Fig. 7). Thus, it could be of a potential use against cerebral ischemia, although further investigations are required.

## Conflict of interest statement

We declare no conflict of interest.

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# 7 DISCUSIÓN

En esta Tesis hemos evaluado el potencial terapéutico de la melatonina en combinación con IAChE, así como de nuevos derivados de la melatonina para enfermedades tan prevalentes y sin tratamientos efectivos como son la EA y el ictus.

Nos hemos centrado en la melatonina porque, tal y como se ha mencionado en la Introducción, los niveles de esta neurohormona disminuyen con el envejecimiento y, de una forma más acusada, en las enfermedades que aquí nos ocupan. Adicionalmente, la melatonina muestra un perfil antioxidante, antiinflamatorio, inmunomodulador y neuroprotector que hace que sea potencialmente útil para el tratamiento de enfermedades neurodegenerativas y cerebrovasculares como el ictus [170-172] [13, 19, 143].

**En el primer y segundo estudio** hemos querido poner de manifiesto la prueba de concepto que la asociación de melatonina y un IAChE, así como un nuevo compuesto híbrido melatonina-IAChE (ITH91/IQM157) puede ser una estrategia útil para el tratamiento de la EA. El uso de los IAChE se sustenta en que son el grupo farmacológico mayoritariamente empleado en clinica para tratar a los enfermos con EA en la actualidad.

A pesar del gran esfuerzo científico y económico que se lleva realizando durante los ultimos veinte años para encontrar nuevos tratamientos para la EA, la realidad es que muchos de esos compuestos han dado resultados positivos a nivel preclínico, pero al llegar a la fase clínica han fracasado. Probablemente, tal como se mencionó en la Introducción, dicho fracaso pueda tener su base, a groso modo, en los siguientes dos motivos: (i) que los modelos disponibles de EA normalmente se centran en una de las causas de la enfermedad, fundamentalmente en la patología βA, y por tanto no están reproduciendo la patología de esta enfermedad y (ii) que los compuestos que se han desarrollado han ido dirigidos a una única diana, lo cual no sería suficiente para atajar una enfermedad con una fisiopatología tan compleja como la EA [21, 91].

Por tanto, dada la escasez de buenos modelos *in vitro* que permitan evaluar nuevos compuestos con potencial terapéutico en la EA, inicialmente desarrollamos un modelo de citotoxicidad en células de neuroblastoma humano SH-SY5Y, que combinaba concentraciones sub-efectivas de βA y ácido okadaico (A.O, un inhibidor de fosfatasas que produce hiperfosforilación de la proteína Tau) con la idea de reproducir ambas patologías. Los estímulos por sí solos, incubados durante 24 h, ocasionaron una muerte celular máxima del 40 % y del 80 % a las concentraciones de 10 µM de βA y 30 nM de A.O, respectivamente. Lo observación más interesante se obtuvo cuando se asociaron concentraciones sub-tóxicas de ambos estímulos (1 µM de βA y 3 nM de A.O), lo cual causó una muerte estadísticamente significativa respecto a la causada por cada estímulo tóxico por separado. Además, dicha muerte celular fue mayoritariamente de tipo apoptótica, resultado que concuerda con el tipo de muerte celular observada en modelos transgénicos de la EA [78, 85, 200]. Además, la asociación de βA/A.O causó una retracción de las prolongaciones celulares, un efecto estrechamente relacionado con la hiperfosforilación de Tau, ya que ésta lleva a una desestabilización de los microtúbulos, pérdida de la citoarquitectura celular y, consecuentemente, neurodegeneración. Adicionalmente, observamos que la asociación de βA/A.O incrementó la producción de agregados proteicos visualizados como un incremento de tinción de tioflavina-S. Por tanto, hemos implementado un nuevo y sencillo modelo de EA *in vitro* en el cual se articulaban muchas de las alteraciones histopatológicas encontradas en la EA, combinando las patologías de βA y Tau.

Una vez establecido el modelo de citotoxicidad, se evaluó el efecto neuroprotector de la melatonina, el donepecilo (un IAChE utilizado en clínica para la EA) y su asociación. Se seleccionaron concentraciones ya utilizadas por otros autores en la literatura [201] para realizar curvas concentración-respuesta para la melatonina (1, 3, 10, 100 nM) y para el donepecilo (1, 3, 100 y 300 nM). La melatonina protegió las células en un 36 % a la concentración de 3 nM y un 73% de neuroprotección máxima ya a 10 nM. El donepecilo, protegió desde la concentración de 0.3 µM (40 %).

Posteriormente, asociamos concentraciones sub-efectivas de ambos compuestos (1 nM de melatonina y 10 nM de donepecilo), obteniendo un marcado efecto sinérgico neuroprotector, validando nuestra hipótesis de actuar sobre varias dianas terapéuticas para incrementar el potencial protector.

En este primer estudio también se evaluó el compuesto ITH91/IQM157 (el híbrido melatonina−N,N‑Dibencil(N‑metil)amina) sintetizado por el grupo de la Dra. Rodriguez- Franco del Instituto de Química Médica del CSIC. Este compuesto fue seleccionado de un estudio previo en el que se describió que el ITH91/IQM157 poseía una baja citotoxicidad, era capaz de atravesar la BHE en un modelo predictivo, inhibía la AChE, desplazaba el ioduro de propidio del sitio aniónico periférico de la AChE y poseía un buen perfil antioxidante y protector frente al estrés oxidativo causado por rotenona/oligomicina A [198]. Cuando evaluamos el ITH91/IQM157 en el modelo de βA/A.O, observamos que ofrecía neuroprotección a concentraciones 10 veces menores (1 nM) que la propia melatonina. Posteriormente, estudiamos su mecanismo neuroprotector. Para ello, nos centramos en rutas de supervivencia previamente descritas en relación con la neuroprotección como son la activación de receptores nicotínicos α7, receptores de melatonina, PKC, PI3K/Akt y ERK1/2. Mediante el uso de antagonistas e inhibidores de cinasas, observamos que el efecto neuroprotector del ITH91/IQM157 dependía de su acción sobre receptores nicotínicos α7 y receptores de melatonina a nivel de membrana y, las vías de supervivencia ERK1/2, PI3K/Akt. Finalmente, determinamos que el ITH91/IQM157 era capaz de inducir la enzima antioxidante HO-1, la cual ha mostrado acciones neuroprotectoras en distintos enfermedades como la EA, EP, HD e ictus [113, 202-204].

El ITH91/IQM157 comparte las propiedades de la melatonina y de un IAChE; al tratarse de un compuesto único mostraría la ventaja de compartir una misma farmacocinética. Sin embargo, cuando se combinan distintos fármacos pueden existir interacciones farmacocinéticas que podrían modificar los efectos de cada uno de ellos,

aumentar potencialmente sus efectos secundarios o disminuir su efectividad. Además, un compuesto multidiana sería *a priori* más ventajoso que un compuesto "unidiana" para el tratamiento de enfermedades con una fisiopatología compleja como es la EA ya que sería capaz de actuar a distintos niveles de su cadena fisiopatológica [5].

**En el segundo trabajo**, decidimos seguir la misma idea de asociar patología βA y Tau pero en un modelo biológico más complejo y que permitiese tratamientos más crónicos. Para ello, empleamos cultivos organotípicos de hipocampo de rata. Una de las ventajas que ofrece el cultivo organotípico de cerebro es que las rodajas mantienen su viabilidad durante un tiempo mucho mayor, incluso hasta 15 días, conservando las estructuras fisiológicas y regiones de las rodajas de hipocampo de rata [205]. La posibilidad de ampliar el tiempo de incubación de los estímulos citotóxicos resulta sumamente interesante cuando queremos mimetizar, en modelos *in vitro*, modelos neurodegenerativos. Además, tal y como hemos comentado en la Introducción, además de las placas de βA y la patología Tau, la neuroinflamación y estrés oxidativo, son características patológicas relevantes en la EA. A este respecto, la presencia de glía, además de neuronas, en los modelos biológicos *in vitro* de enfermedad cobran especial interés para reproducir la patología neurodegenerativa [22, 23, 37]. Esta es otra ventaja añadida del cultivo organotípico, la presencia de distintos tipos celulares.

La incubación de los cultivos organotípicos de hipocampo de rata con la asociación de concentraciones sub-tóxicas de βA (0.5 µM) y A.O (3 nM) durante 4 días produjo una muerte celular sinérgica, estadísticamente significativa en comparación a la obtenida con cada estímulo tóxico por separado tal como sucedió en la línea celular SH-SY5Y en el primer estudio. Cabe mencionar que al tratarse de incubaciones subcrónicas, pudimos reducir la concentración de los tóxicos a concentraciones más patofisiológicas, alcanzando valores incluso 50 veces inferiores a los descritos en la literatura en este mismo modelo, el cultivo organotípico de hipocampo [206, 207]. El tipo de muerte celular obtenida fue mayoritariamente apoptótica, lo que concuerda con los análisis post-mortem de cerebros de pacientes de EA, los cuales muestran

fragmentación de DNA en neuronas y glía del hipocampo y corteza [208, 209], y el tipo de muerte celular descrita en los ratones transgénicos de EA, como el modelo de ratón triple transgénico [210] y en nuestro primer estudio en células SH-SY5Y. La incubación subcrónica de los cultivos organotípicos de hipocampo de rata con βA/A.O reprodujeron, además de la muerte celular, otras alteraciones patológicas asociadas con la EA como son la formación de agregados de βA , la hiperfosforilación de Tau, el incremento de marcadores de estrés oxidativo, astrogliosis y microgliosis y aumento de citocinas pro-inflamatorias (IL-1β y TNFα) [13, 19, 27, 83].

En este modelo *in vitro* de EA más complejo, también evaluamos el potencial efecto terapéutico de la asociación de concentraciones subefectivas de melatonina y un IAChE; en este caso empleamos la galantamina en vez del donepecilo como hicimos en el primer trabajo. A pesar de usar otro IAChE, también observamos que la asociación de concentraciones subefectivas de melatonina (1 nM) con galantamina (10 nM) ofrecía un efecto neuroprotector sinérgico. Adicionalmente, la combinación farmacológica resultó efectiva en (i) reducir los agregados de βA; (ii) reducir la hiperfosforilación de Tau; (iii) y prevenir el incremento de marcadores de estrés oxidativo y neuroinflamación ocasionados por la asociación subcrónica de βA/A.O.

El empleo de terapia combinada está cobrando un interés creciente en la comunidad científica para el tratamiento de enfermedades con etiopatogenia compleja [5]; de hecho, ya se utilizan con éxito frente al cáncer y en el síndrome de inmunodeficiencia adquirida, por ejemplo. Tanto en el estudio 1 como en el 2 hemos comprobado que la asociación de concentraciones sub-efectivas de un IAChE (fármacos mayoritariamente empleados en clínica para tratar los pacientes con EA) con melatonina puede resultar una terapia potencialmente interesante y efectiva. Los resultados positivos obtenidos con la combinación de melatonina con un IAChE obtenidos en el primer y en este segundo trabajo confirman y extienden los resultados previos del grupo en el que la asociación de la galantamina y melatonina protegió de forma sinérgica ante estímulos de estrés oxidativo en células SH-SY5Y [11]. También cabe mencionar que existen estudios de otros grupos que avalan el uso de un

antioxidante (en nuestro caso la melatonina) en combinación con otros fármacos, como la memantina con vitamina E, o el donepecilo con otros antioxidantes [11, 22, 92, 93].

La suplementación con melatonina durante el tratamiento con IAChE puede tener varias ventajas. En primer lugar, los niveles de melatonina que están considerablemente reducidos en la EA aumentarían y, por tanto, se favorecería una menor situación de estrés oxidativo [19]. Esta presunción se apoya en el hecho de que la administración de melatonina en animales viejos reduce las alteraciones descritas en la EA, en parte, debido a sus efectos antioxidantes [211, 212]. También, se han desarrollado diversos estudios en los que la melatonina como coadyuvante ha mostrado resultados positivos, como es el caso del co-tratamiento con memantina [195, 196]. Otra ventaja de suplementar con melatonina los pacientes tratados con un IAChE es que, según nuestros resultados, la dosis del IAChE podría reducirse potencialmente hasta 10 veces; ello significaría que al reducir la dosis se reducirían los efectos adversos secundarios [22, 201, 213]. Por lo tanto, se incrementaría el cumplimiento terapéutico y, posiblemente, la eficacia del tratamiento ya que muchos pacientes abandonan el tratamiento debido a los efectos secundarios.

Aunque se necesite más investigación para apoyar el tratamiento coadyuvante con melatonina e IAChE, pensamos que dada la baja toxicidad de la melatonina y, que ya se están usando los IAChE en clínica, deberían promocionarse nuevos ensayos clínicos para demostrar la eficacia de esta terapia combinada, que adicionalmente podría servir de prueba de concepto para desarrollar nuevos fármacos multidiana derivados de la melatonina con potencial terapéutico para la EA y otras demencias que se están tratando actualmente con IAChE tal como hemos demostrado con el ITH91/IQM157 en el primer estudio.

Como ya se comentó con anterioridad, la melatonina además de tener efecto antioxidante directo, es capaz de inducir el factor de transcripción Nrf2, que promueve una respuesta antioxidante e anti-inflamatoria. Adicionalmente, este factor de transcripción se ha relacionado con propiedades neuroprotectoras en distintos

modelos de enfermedades neurodegenerativas, como la EP, la EH y la EA, así como frente al ictus [113, 117, 199, 214]. Uno de los inductores clásicos de Nrf2, es el sulforafano. Este compuesto ha mostrado ser capaz de disminuir el estrés oxidativo mediante la inducción de genes de fase II. También es capaz de aumentar los niveles proteicos de Nrf2 y muestra efectos anti-inflamatorios [199, 215, 216]. Además, ha mostrado eficacia en modelos de EA e ictus [217-220].

Con estos antecedentes y con el objetivo de combinar las propiedades farmacológicas de la melatonina y el sulforafano en una única molécula, en el **tercer trabajo** se realizó un estudio monográfico sobre el híbrido melatonina-sulforafano, ITH12674 sintetizado por el Dr. Rafael León en el laboratorio de química médica del Instituto Teófilo Hernando en el departamento de Farmacología y Terapéutica de la Universidad Autónoma de Madrid.

El ITH12674 mostró las siguientes propiedades: (i) inducir Nrf2, (ii) reducir la producción de ERO, (iii) modular los niveles de GSH, (iv) e inducir la expresión de genes de fase II, como la enzima antioxidante y anti-inflamatoria, HO-1. Además, el hecho de incluir un isotiocianato en este nuevo compuesto, ofreció una ventaja adicional, dotar al compuesto con un mecanismo de acción profármaco-fármaco. Es decir, el isotiocianato reacciona con la cisteína presente en Keap1, inhibidor de Nrf2, liberándose así para su translocación al núcleo. Además, la enzima GST, catalizará su conjunción con GSH, generando un conjugado ITH12674-GSH (profármaco), que podría ser un potente secuestrador de radicales libres, mostrando una cascada de reducción de ERO parecida a la descrita para la melatonina [131]. Este conjugado, se mantendría en el interior celular, siendo capaz de secuestrar más radicales libres, tal y como se describió para un análogo ditiocarbonatado de melatonina [221]. Esta hipótesis se vio confirmada cuando demostramos que el ITH12674 mostró un perfil neuroprotector mejor que la propia melatonina y el propio sulforafano. Además, indujo la translocación de Nrf2 al núcleo a 1 µM y, pese a que no es tan potente inductor como el propio sulforafano, sí que mejoró esta propiedad de la melatonina. Tal y como demostramos, protegió mejor que los propios compuestos cabeza de serie frente a

estrés oxidativo generado tanto por rotenona/oligomicina A, como por terbutil-hidroperóxido, pudiéndose deber al conjugado con GSH que produce este compuesto. Por otro lado, este derivado aumentó la concentración de GSH tras 24 h de pre-incubación, tal y como está descrito en la literatura para la melatonina [222-224].

Dado que el aumento de ERO tras la isquemia cerebral, tiene como consecuencia la peroxidación lipídica, oxidación de proteínas y ADN, sobrecarga de $Ca^{2+}$, excitotoxicidad e inflamación [96, 114, 115, 225], y que tanto la melatonina como el sulforafano muestran un buen perfil protector en modelos *in vitro* como *in vivo* de isquemia cerebral [107, 113, 115, 167, 219, 226, 227] mediante la inducción de Nrf2, decidimos evaluar el ITH12674 en modelos de isquemia cerebral.

En este caso, el compuesto ITH12674 fue capaz de prevenir el daño causado por 15 min de privación de oxígeno y glucosa seguido de 24 h de reoxigenación (POG) en cultivos organotípicos de hipocampo de rata. Además, demostramos que esta protección, se debía, al menos parcialmente a la inducción de HO-1. Estos resultados concuerdan con resultados previos del grupo para la melatonina en este mismo modelo experimental de isquemia cerebral [113]. Más allá, indujo la expresión de enzimas antioxidantes y antiinflamatorias, como la HO-1, lo que lo convierte en un candidato interesante para el tratamiento de esta patología [7, 105].

Existen distintos autores que apoyan el uso de inductores de Nrf2 (como sulforafano, melatonina y curcumina) para la isquemia cerebral debido a su capacidad de inducir enzimas antioxidantes y anti-inflamatorias de fase II, como la HO-1. Además, estos compuestos ayudarían a la respuesta compensatoria neuronal endógena, mediante la cual, se ha observado que tras producir una isquemia cerebral, mediante la ligadura de la ACM y posterior reoxigenación, existe un pico a las 8 horas de aumento del factor de transcripción Nrf2, que se traduce en un aumento posterior (24 y 72 h) de la inducción de HO-1, así como de otras enzimas citoprotectoras: tioredoxina y GSH [227, 228]. Por lo tanto, mejorar esta función resultaría de especial interés. Esta característica sería interesante también para el tratamiento de distintas

enfermedades neurodegenerativas, como la EA, ya que en modelos animales de EA, como el ratón transgénico APP/PS1, se ha observado que existe una reducción de Nrf2, GSH y NQO-1 [229]. Estos resultados se han confirmado en humanos por otros autores, donde demostraron que existía una expresión mayoritariamente citoplasmática de Nrf2 en neuronas de hipocampo de estos pacientes, así como valores disminuidos de HO-1 en corteza e hipocampo [203, 230, 231].

A pesar de las grandes ventajas terapéuticas que muestra la melatonina, su uso en clínica se ve limitado por su farmacocinética, concretamente por su vida media tan corta (30 min). En este sentido, se han sintetizado nuevos derivados de melatonina o agonistas de los receptores de melatonina, cuyo objetivo, entre otros, ha sido incrementar su vida media [13, 183, 184]. Por ejemplo, el ramelteon tiene una vida media de entre 1 y 2.6 h y se utiliza en EEUU y Japón como tratamiento para el insomnio [185]; el circadin, es otro ejemplo, trabaja secretando melatonina y manteniendo su perfil endógeno (8-10h) y su vida media es 3.5-4 horas [186, 187]; la agomelatina fue aprobada en 2009 por la EMEA como tratamiento antidepresivo [188, 189] o, el tasimelteón y el TK-301, ambos agonistas de receptores de melatonina, utilizados para problemas de sueño [192, 193].

Continuando con los nuevos derivados de melatonina, en el **cuarto trabajo** hemos estudiado el Neu-P11 que posee una vida media más prolongada (1.5 a 3 h) que la melatonina (30 min) y, además de ser agonista de los receptores MT1/MT2, es agonista de los receptores 5-HT1A/1D [232], de ahí que pueda considerarse un compuesto multidiana con acciones melatoninérgicas. Este nuevo compuesto fue sintetizado por el grupo del Doctor Laudon, de los laboratorios Neurim Pharmaceuticals Ltd., de Israel.

Dado que el aumento de ERO y la disfunción mitocondrial son dos de los mecanismos moleculares comunes que llevan a la muerte neuronal tanto en la isquemia cerebral como en enfermedades neurodegenerativas, como la EA [233, 234], en el cuarto estudio exploramos las propiedades antioxidantes del Neu-P11 haciendo

hincapié en su potencial neuroprotector en modelos relacionados con la isquemia cerebral.

En la prueba del ORAC, que indica la capacidad de secuestrar ERO de un compuesto, el Neu-P11 fue más potente que la propia melatonina. Ello se correlacionó con un buen perfil neuroprotector en un modelo relacionado con la isquemia *in vitro* inducido por la combinación de rotenona y oligomicina A, los cuales inhiben el complejo I y V de la cadena transportadora de electrones, respectivamente, ocasionando estrés oxidativo y muerte celular [11, 235]. Además, siendo la excitotoxicidad otro de los eventos clave cuando se produce una caída del flujo cerebral, comprobamos que el Neu-P11 era capaz de reducir la muerte neuronal secundaria a la excitotoxicidad por glutamato en rodajas de hipocampo a concentraciones menores que las requeridas por la melatonina [236, 237]. El Neu-P11 también redujo el aumento de ERO y la muerte celular producida por la POG en rodajas de hipocampo de rata de una manera parecida a la propia melatonina, cuyo perfil neuroprotector en modelos de isquemia cerebral está ampliamente demostrado [120, 170-172]. Fue interesante observar que mientras la melatonina era capaz de proteger frente la POG, no fue así en el caso de la excitotoxicidad inducida por glutamato. Esto podría ser debido a la acción complementaria del Neu-P11 sobre los receptores 5-HT1A/1D, ya que existen antecedentes bibliográficos que muestran que los agonistas de receptores 5-HT1A reducen excitotoxicidad inducida por glutamato, protegiendo en distintos modelos de isquemia cerebral *in vivo* [238]. De hecho, compuestos con este mecanismo de acción han mostrado eficacia en ensayos clínicos en fase II para el tratamiento de infarto cerebral [239, 240].

Finalmente, corroboramos que la administración de Neu-P11 (1mg/kg), 5 min después de inducir la isquemia, era capaz de reducir el volumen de infarto cerebral en ratones expuestos a un ictus fototrombótico. Por tanto, el Neu-P11 mostró un buen perfil neuroprotector tanto en modelos *in vitro* como *in vivo* de isquemia cerebral.

En cuanto al mecanismo neuroprotector del Neu-P11, comprobamos que actúa sobre los receptores de melatonina, ya que el luzindol revirtió su efecto neuroprotector. Estos resultados concuerdan con lo publicado en la literatura, ya que este compuesto muestra una afinidad mayor por los receptores melatoninérgicos que la propia melatonina [241]. También indagamos en su mecanismo de acción intracelular. Nuestros resultados mostraron que las rutas de supervivencia Jak/Stat, Akt y Erk 1/2 participan en su mecanismo neuroprotector.

Otra de las ventajas del Neu-P11 en relación a su posible desarrollo clínico para el ictus, es que ya ha sido evaluado en humanos. Concretamente, en dos ensayos clínicos, en voluntarios sanos y pacientes con insomnio ha mostrado ser un fármaco bien tolerado y tener pocos o ningún efecto adverso. En un ensayo clínico de fase II, mostró ser eficaz (a 20 y 50 mg día/30 días) en mejorar la duración y calidad del sueño [232]. Además ha mostrado tener efecto antidepresivo, ansiolítico, antidiabético, anti-hipertensivo y antinociceptivo en modelos animales [241-244]. Cabe destacar que ha mostrado capacidad para mejorar los déficits cognitivos y la supervivencia neuronal en un modelo de EA [232]. Y por último, más recientemente mostró ser eficaz también en un modelo de infarto de miocardio en células [245]. Dado que el Neu-P11 ya se ha evaluado en humanos, pensamos que su estudio en pacientes con ictus podría ser factible en un corto plazo de tiempo, aunque tienen que ser desarrollados más estudios en otros modelos animales de ictus y en asociación o no con t-PA, antes de dar el paso a ensayo clínico.

Por tanto, el conjunto de resultados de esta Tesis ponen de manifiesto el interés que puede tener el uso de la melatonina como tratamiento coadyuvante de los inhibidores de la AChE, así como el desarrollo de nuevos derivados de melatonina multidiana para tratar enfermedades neurodegenerativas como la EA o el ictus.

# 8 CONCLUSIONES

De los resultados presentados en esta tesis doctoral se pueden extraer las siguientes conclusiones:

## Conclusiones parciales:

*Artículo 1: El nuevo híbrido melatonina-N,N-dibenzil(N-metilo)amina, ITH91/IQM157 ejerce neuroprotección en un modelo in vitro de Alzheimer mediante la inducción de Hemoxigenasa-1.*

- La asociación de concentraciones sub-tóxicas de βA y A.O causaron agregados de βA, retracción de las prolongaciones celulares y una muerte (mayoritariamente apoptótica) sinérgica en la línea celular de neuroblastoma humano SH-SY5Y;
- En éste modelo *in vitro* de EA, la asociación de melatonina con donepecilo, y el ITH12291, un híbrido melatonina-donepecilo derivado de ambos, protegieron a las células neuronales mediante un mecanismo de acción que implica a los receptores de melatonina, los receptores nicotínicos, PKC, AKT, ERK 1/2 y HO-1.

*Artículo 2: La asociación de concentraciones subefectivas de melatonina y galantamina mejoran las alteraciones patológicas en un modelo de Alzheimer en cultivo organotípico de hipocampo.*

- Concentraciones sub-tóxicas de βA y A.O en cultivo organotípico de hipocampo de rata, causaron agregados de βA e hiperfosforilación de Tau, estrés oxidativo, inflamación, alteraciones gliales y muerte neuronal.
- En este modelo citotóxico, la combinación de concentraciones sub-efectivas de melatonina y galantamina ofrecieron un efecto sinérgico, evitando la muerte celular y la aparición de las alteraciones histopatológicas descritas en este modelo *in vitro* de EA.

*<u>Artículo 3</u>: El derivado de melatonina- sulforafano, ITH12674 ofrece neuroprotección en modelos de estrés oxidativo mediante un mecanismo profármaco-fármaco.*

- El ITH12674, un derivado de melatonina-sulforafano, posee una actividad dual profármaco-fármaco que, supera las propiedades neuroprotectoras de sus cabezas de serie en distintos modelos de estrés oxidativo. Su mecanismo de acción implica la conjugación con GSH y la inducción del factor de transcripción Nrf2, y enzimas de fase II, como la HO-1.

*<u>Artículo 4:</u> Mecanismo neuroprotector de un nuevo derivado de melatonina, Neu-P11 en modelos de isquemia cerebral.*

- El Neu-P11, un nuevo derivado melatoninérgico, protege en distintos modelos *in vitro* e *in vivo* de isquemia cerebral. Su mecanismo protector se relaciona con su capacidad antioxidante y a la activación de las rutas de supervivencia Jak/Stat, Akt y Erk 1/2.

## Conclusión global:

Los resultados globales de esta tesis doctoral nos permiten **concluir** que la terapias basadas en el uso de melatonina, ya sea en combinación con otros fármacos o nuevos derivados multidiana que contengan melatonina en su estructura química, pueden ser un alternativa potencialmente útil para el tratamiento de enfermedades neurodegenerativas como la EA o las cerebrovasculares como el ictus; enfermedades que comparten un marcado componente de estrés oxidativo, neuroinflamación y muerte neuronal.

# 8 CONCLUSIONS

The conclusions that can be obtained from this PhD thesis are the following:

## Partial conclusions:

*Paper 1: The melatonin-N,N-dibenzyl(N-methyl)amine hybrid ITH91/IQM157 affords neuroprotection in an in vitro Alzheimer's model via hemo-oxygenase-1 induction.*

- The combination of sub -effective concentrations of βA and okadaic acid (O.A) in the human neuroblastoma cell line SH- SY5Y caused several of the pathological characteristics related to AD, i.e retraction of prolongations, aggregates of βA, increased oxidative stress markers and apoptotic cell death.
- In this model, the association of melatonin with donepezil, and the novel derivative, ITH12291 (a melatonin-donepezil hybrid), protected the neuroblastoma cells through a mechanism of action that involves melatonin receptors, nicotinic receptors, PKC , AKT , ERK 1/2 and HO-1, resulting in an interesting neuroprotective strategy for Alzheimer's disease.

*Paper 2: Subthreshold concentrations of melatonin and galantamine improves pathological AD-hallmarks in hippocampal organotypic cultures.*

- Hippocampal slices incubated for 4 days with low concentrations of βA and O.A showed βA aggregates, hyperphosphorylation of Tau, increased oxidative stress markers, neuroinflammation, glial alterations and cell death.
- The combination of sub- effective concentrations of melatonin and galantamine offered a synergistic effect, avoiding cell death, and preventing most of the histopathological alterations described in this model.

*Paper 3: Melatonin-sulforaphane hybrid ITH12674 induces neuroprotection in oxidative stress conditions by a 'drug-prodrug' mechanism of action.*

- The new melatonin - sulforaphane derivative, ITH12674 , through a dual "drug-predrug" mechanism improves the neuroprotective properties of melatonin and sulforaphane in different models of oxidative stress. The neuroprotective mechanism involves the conjugation with GSH and the induction of Nrf2 and, consequently, phase II enzymes, such as the HO-1 .

*Paper 4: Neuroprotective mechanism of the novel melatonin derivative Neu-P11 in brain ischemia related models.*

- The new melatoninergic derivative Neu- P11, protects in different *in vitro* (mitochondrial intoxication, glutamate exitotoxicity and oxygen and glucose deprivation) and *in vivo* (photothrombotic stroke) models related to brain ischemia. Its mechanism of action is related to its antioxidant capacity and activation of the survival pathways: Jak/Stat, Akt and Erk1/2.

## Overall conclusion:

The global results from this thesis lead to conclude that the use of melatonin, as combination therapy as well as its multi-target derivatives, could be a useful pharmacological strategy to treat diseases such as Alzheimer's disease and brain stroke, which share oxidative stress, neuroinflammation and cell death as common physiopathological factors.

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