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Memòria justificativa de recerca de les convocatòries BCC, BE, BP, CTP-AIRE, DEBEQ, FI, INEFC, NANOS i PIV

La memòria justificativa consta de les dues parts que venen a continuació:
1.- Dades bàsiques i resums
2.- Memòria del treball (informe científic)

Tots els camps són obligatoris

1.- Dades bàsiques i resums

Nom de la convocatòria

FI

Llegenda per a les convocatòries:

BCC	Convocatòria de beques per a joves membres de comunitats catalanes a l'exterior
BE	Beques per a estades per a la recerca fora de Catalunya
BP	Convocatòria d'ajuts postdoctorals dins del programa Beatriu de Pinós
CTP-AIRE	Ajuts per accions de cooperació en el marc de la comunitat de treball dels Pirineus. Ajuts de mobilitat de personal investigador.
DEBEQ (Modalitat A3)	Beques de Cooperació Internacional i Desenvolupament
FI	Beques predoctorals per a la formació de personal investigador
INEFC	Beques predoctorals i de col·laboració, dins de l'àmbit de l'educació física i l'esport i les ciències aplicades a l'esport
NANOS	Beques de recerca per a la formació en el camp de les nanotecnologies
PIV	Beques de recerca per a professors i investigadors visitants a Catalunya

Títol del projecte: ha de sintetitzar la temàtica científica del vostre document.
Estudi de les alteracions en la capacitat neuroprotectora dels astròcits reactius en l'envelliment

Dades de l'investigador o beneficiari

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Número d'expedient
2008FIC 00316

Paraules clau: cal que esmenteu cinc conceptes que defineixin el contingut de la vostra memòria.
Astròctis, envelliment ,estrés oxidatiu, neuroprotecció, factor neurotròfic derivat de la línia glial (GDNF)

Data de presentació de la justificació
22/04/2009

Nom i cognoms i signatura
del/de la investigador/a

Visttiplau del/de la responsable de la
sol·licitud

Resum del projecte: cal adjuntar dos resums del document, l'un en anglès i l'altre en la llengua del document, on s'esmenti la durada de l'acció

Resum en la llengua del projecte (màxim 300 paraules)

En el trabajo de estos cuatro años (2005-2008) hemos publicado tres artículos sobre las alteraciones de los astrocitos reactivos en el cerebro durante el envejecimiento. En el primer estudio, evaluamos la capacidad neuroprotectora de los astrocitos en un modelo experimental in vitro de envejecimiento. Los cambios en el estrés oxidativo, la captación del glutamato y la expresión proteica fueron evaluados en los astrocitos corticales de rata cultivados durante 10 y 90 días in vitro (DIV). Los astrocitos envejecidos tenían una capacidad reducida de mantener la supervivencia neuronal. Estos resultados indican que los astrocitos pueden perder parcialmente su capacidad neuroprotectora durante el envejecimiento. En el segundo estudio el factor neurotrófico derivado de la línea glial (GDNF) fue probado para observar sus efectos neurotróficos contra la atrofia neuronal que causa déficits cognitivos en la vejez. Las ratas envejecidas Fisher 344 con deficiencias en el laberinto de Morris recibieron inyecciones intrahippocampales de un vector lentiviral que codifica GDNF humano en los astrocitos o del mismo vector que codifica la proteína fluorescente verde humana como control. El GDNF secretado por los astrocitos mejoró la función de la neurona como se muestra por aumentos locales en la síntesis de los neurotransmisores acetilcolina, dopamina y serotonina. El aprendizaje espacial y la prueba de memoria demostraron un aumento significativo en las capacidades cognitivas debido a la exposición de GDNF, mientras que las ratas control mantuvieron sus resultados al nivel del azar. Estos resultados confirman el amplio espectro de la acción neurotrófica del GDNF y abre nuevas posibilidades de terapia génica para reducir la neurodegeneración asociada al envejecimiento. En el último estudio, examinamos cambios en la fosforilación de tau, el estrés oxidativo y la captación de glutamato en los cultivos primarios de astrocitos corticales de ratones neonatos de senescencia acelerada (SAMP8) y ratones resistentes a la senescencia (SAMR1). Nuestros resultados indican que las alteraciones en cultivos del astrocitos de los ratones SAMP8 son similares a las detectadas en cerebros enteros de los ratones SAMP8 de 1-5 meses de edad. Por otra parte, nuestros resultados sugieren que esta preparación in vitro es adecuada para estudiar en este modelo murino el envejecimiento temprano y sus procesos moleculares y celulares.

Resum en anglès (màxim 300 paraules)

In the work of these four years we have published three papers about the alterations of the reactive astrocytes in the brain aging. In the first study, we evaluate the neuroprotective capacity of astrocytes in an in vitro experimental model of aging. Changes in oxidative stress, glutamate uptake and protein expression were evaluated in rat cortical astrocytes cultured for 10 and 90 days in vitro (DIV). Aged astrocytes had a reduced ability to maintain neuronal survival. These findings indicate that astrocytes may partially loose their neuroprotective ability during aging. In the second study, glial cell line-derived neurotrophic factor (GDNF) was assayed for its neurotrophic effects against the neuronal atrophy that causes cognitive deficits in old age. Aged Fisher 344 rats with impairment in the Morris water maze received intrahippocampal injections of either a lentiviral vector encoding human GDNF in astrocytes or the same vector encoding human green fluorescent protein as a control. Astrocyte-secreted GDNF enhanced neuron function as shown by local increases in synthesis of the neurotransmitters acetylcholine, dopamine and serotonin. Spatial learning and memory testing showed a significant gain in cognitive abilities due to GDNF exposure, whereas control-transduced rats kept their performance at the chance level. These results confirm the broad spectrum of the neurotrophic action of GDNF and open new gene therapy possibilities for reducing age-related neurodegeneration. I

Resum en anglès (màxim 300 paraules) – continuació -.
In the last study, we examine changes in tau phosphorylation, oxidative stress and glutamate uptake in primary cultures of cortical astrocytes from neonatal senescence-accelerated prone mice (SAMP8) and senescence-accelerated resistant mice (SAMR1). Our results indicate that alterations in astrocyte cultures from SAMP8 mice are similar to those detected in whole brains of SAMP8 mice at 1–5 months. Moreover, our findings suggest that this in vitro preparation is suitable for studying the molecular and cellular processes underlying early aging in this murine model

2.- Memòria del treball (informe científic sense limitació de paraules). Pot incloure altres fitxers de qualsevol mena, no més grans de 10 MB cadascun d'ells.

Durante el periodo de esta beca de formación de personal investigador FI (2005-2008) he participado en la realización de tres artículos científicos donde se han estudiado las alteraciones de los astrocitos reactivos en el envejecimiento cerebral.

El primer artículo es "Astrocytes aged in vitro show a decreased neuroprotective capacity" Publicado en el Journal of Neurochemistry, 2007, 101, 794-805 por Pertusa M, García-Matas S, Rodríguez- Farré E, Sanfeliu C, Cristòfol R. (Ver anexo 1). En este primer trabajo se ha evaluado la capacidad neuroprotectora de los astrocitos en un modelo in vitro de astrocitos corticales de ratas Fisher 344, cultivados durante 10 y 90 DIV. El cerebro es particularmente vulnerable al estrés oxidativo debido a su alta tasa metabólica, a su alto contenido de ácidos grasos poli insaturados y a tener pocas defensas antioxidantes. Los radicales libres se generan constantemente en el cerebro in vivo. Los astrocitos tiene un papel fundamental en mantener la fisiología normal del cerebro y en protegerlo frente al estrés oxidativo. Sin embargo, los astrocitos son también vulnerables al estrés oxidativo. Su reacción a las concentraciones bajas de peróxido de hidrógeno implica la generación de especies reactivas de oxígeno (ROS), lipoperoxidación, y cambios en las defensas antioxidantes. Los astrocitos además son las principales células responsables de captar el glutamato extracelular y poseen receptores de glutamato de alta afinidad como el transportador glutamato/aspartato (GLAST). Cambios en la función de los astrocitos puede reducir su capacidad neuroprotectora y contribuir al envejecimiento neuronal. El objetivo de este estudio era establecer un modelo in vitro de envejecimiento de los astrocitos para estudiar su capacidad neuroprotectora. Primero,

determinamos parámetros de estrés oxidativo e inflamación, midiendo la generación de las ROS, la actividad mitocondrial y la expresión de proteína acídica fibrilar glial (GFAP), de S100β, de la enzima óxido nítrico sintasa inducible (iNOS), de la Cu/Zn superóxido dismutasa (SOD-1) y de la proteína hemo-oxigenasa 1 (HO-1) en los astrocitos cerebrales corticales de rata mantenidos en cultivo durante 90 DIV, frente a los cultivados en condiciones estándar de 10 DIV. En segundo lugar, evaluamos la función del transporte del glutamato en los astrocitos, determinando la captación del glutamato y la expresión de la proteína del transportador GLAST. Finalmente, la neuroprotección de los astrocitos fue probada midiendo la supervivencia neuronal en cocultivos de neuronas cerebrales corticales con astrocitos de 10 y de 90 DIV. Los métodos utilizados en este trabajo fueron los siguientes: cultivos primarios enriquecidos en astrocitos de corteza cerebral de ratas neonatales Fisher 344 de 2 días, se mantuvieron en cultivo durante 10 días (10 DIV) y 3 meses (90 DIV); cocultivos de neuronas de fetos de ratas Fisher 344 de 15 días y astrocitos, las neuronas se tiñeron con yoduro de propidio y el anticuerpo monoclonal neuronal NeuN (1:200) para ver su viabilidad; los astrocitos senescentes se identificaron por tinción con el kit de β-Galactosidasa; se hicieron Western blot de los astrocitos cultivados y se determinó la expresión de proteínas con los anticuerpos monoclonales anti-GFAP (1:40000) anti-S100β (1:500), anti-HO-1 (1:1000), y policlonales anti-SOD-1 (1:1000), anti-iNOS (1:200), y anti-GLAST (1:500); la captación de glutamato fue determinado con un método de radiométrico con glutamato tritiado; la actividad mitocondrial fue determinada por la prueba de MTT y la generación de ROS fue determinada usando 2,7 diclorofluorescein- diacetato (DCFH-DA) y la fluorescencia de DCF se midió en un fluorímetro. Los resultados de estos experimentos muestran que los astrocitos cultivados durante 3 meses muestran características de senescencia y activación glial. Esto se observa por una intensa tinción azul de β-Galactosidasa y un aumento en la expresión de proteínas reactivas gliales como GFAP y S100β con respecto a los astrocitos de 10 DIV. También se observa una reducción de la actividad mitocondrial a través del ensayo con MTT y un aumento de la producción de ROS a través de la prueba de diclorofluoresceina en los que los astrocitos de 90 DIV producen 6 veces más ROS que los astrocitos de 10 DIV. Además, en los astrocitos de 3 meses se observó a través de la técnica de Western Blot un aumento de proteínas nitrosiladas y un aumento de expresión de iNOS, SOD-1 y HO-1. La captación de glutamato es significativamente mayor en los astrocitos de 90 DIV comparados con los de 10 DIV y expresan 3 veces más receptores de glutamato GLAST. La exposición de los astrocitos a peróxido de hidrógeno inhibe la captación de glutamato de manera dosis dependiente y los antioxidantes propilgallato (50 µmol /L), trolox (100 µmol/L) y curcumina (10 µmol/L) revierten la inhibición del peróxido de hidrógeno. La capacidad neuroprotectora de los astrocitos fue determinada en cocultivos de astrocitos y neuronas y se comprobó la supervivencia neuronal a lo largo del tiempo. Las neuronas fueron cultivadas in vitro durante dos semanas ya sea solas, sobre una monocapa de astrocitos de 10 DIV o sobre una monocapa de astrocitos de 90 DIV. La supervivencia neuronal en el cultivo puro de neuronas fue menor al de los cocultivos, el cocultivo con mayor viabilidad neuronal fue el de neuronas con astrocitos de 10 DIV. Los

antioxidantes trolox (10 µmol/L) y curcumina (10 µmol/L) aumentaron la viabilidad neuronal en el cultivo puro de neuronas y en el cocultivo con astrocitos envejecidos durante 90 DIV. Los resultados de este estudio muestran que los astrocitos cultivados durante 3 meses (90DIV) adquieren características de senescencia y presentan una activación glial similar a la de los astrocitos de animales envejecidos. Estos astrocitos generan mayores niveles de ROS que los astrocitos de 10 DIV y esto conlleva una disminución de la actividad mitocondrial. La sobreexpresión de las proteínas HO-1 y SOD-1 puede ser una respuesta defensiva inicial contra la generación de ROS. En nuestros experimentos los astrocitos envejecidos (90DIV) presentan una reducción de su capacidad de mantener la viabilidad neuronal que se recupera en parte con antioxidantes. Todos estos resultados sugieren que los astrocitos tienen un papel importante en la progresión del envejecimiento cerebral. Además observamos un incremento de la captación de glutamato de los astrocitos envejecidos. Esto puede contradecir en principio la reducción en la captación de glutamato que se ha observado en los cerebros envejecidos. Sin embargo, la alta vulnerabilidad de la captación de glutamato al peróxido de hidrógeno observada en los astrocitos envejecidos sugiere que los astrocitos participan en la excitotoxicidad neuronal que se produce en los procesos neurodegenerativos. Los resultados de este trabajo sugieren que los astrocitos pierden parcialmente su capacidad neuroprotectora durante el envejecimiento cerebral y contribuyen al daño neuronal en los procesos neurodegenerativos.

El segundo artículo se titula "Expression of GDNF transgene in astrocytes improves cognitive déficits in aged rats"; publicado en Neurobiology of Aging 29 (2008) 1366-1379 por García-Matas S, Pertusa M, Mammeri H, Adell A, Rodrigo T, Mallet J, Cristòfol R, Sarkis C, Sanfeliu C (Ver anexo 2). En este segundo trabajo el factor neurotrófico derivado de la línea glial (GDNF) fue probado para observar si su expresión en astrocitos de hipocampo puede mejorar la atrofia neuronal que causa déficits cognitivos de ratas viejas. El GDNF es un factor neurotrófico miembro de la familia del factor transformante β del crecimiento. El GDNF tiene unos efectos regenerativos sobre neuronas dopaminérgicas nigrostriatales. Este factor neurotrófico tiene un poder neuroprotector sobre diversos tipos neuronales cuando sufren lesiones nerviosas. La administración terapéutica de GDNF se está estudiando como un tratamiento preventivo o paliativo de enfermedades como el Parkinson, la esclerosis lateral amiotrófica o la enfermedad de Huntington. El GDNF se expresa en neuronas y astrocitos. La liberación experimental de GDNF por parte de los astrocitos ha demostrado un papel neuroprotector in vivo en neuronas motoras y neuronas dopaminérgicas. El GDNF endógeno tiene un papel importante en el aprendizaje espacial en roedores. El aprendizaje espacial y la memoria se asocian con el hipocampo intacto y su correcta función. Déficits de memoria en el envejecimiento son similares a las que se producen cuando se lesiona bilateralmente el hipocampo. En este trabajo hemos estudiado los efectos regeneradores del GDNF en el aprendizaje espacial y la memoria después de una administración local en el hipocampo. En estos experimentos los astrocitos de la región dorsal del CA1 del hipocampo de ratas Fisher 344 envejecidas con déficits cognitivos fueron transducidas in vivo con un vector lentiviral que expresa el gen humano para el GDNF.

La eficacia de la liberación del GDNF o de la proteína fluorescente verde (GFP) usada como control y los cambios de comportamiento y de liberación de neurotransmisores fueron evaluados en este trabajo. Los objetivos de este estudio son la evaluación de la implicación de la región CA1 en el mantenimiento del aprendizaje espacial y la memoria durante el envejecimiento y el estudio del potencial papel neuroprotector de los astrocitos en los procesos neurodegenerativos. Para ello utilizamos ratas machos Fisher 344 jóvenes como controles y ratas machos Fisher 344 de 22 meses de edad como grupo experimental de ratas envejecidas. Los vectores lentivirales fueron proporcionados por el grupo del Dr C. Sarkis del Hospital Salpetrière de Paris. El vector lentiviral recombinante codifica el GDNF y se transduce en los astrocitos in vivo. La combinación de la cubierta de la glicoproteina de lisavirus Mokola con un promotor del citomegalovirus humano le da especificidad para expresarse en astrocitos in vivo. Otro vector que codificaba para GFP fue usado como vector control. El test de comportamiento para determinar el aprendizaje espacial y la memoria fue el test del laberinto acuático de Morris. En este test las ratas nadan en una piscina circular y tienen que hallar una plataforma de escape sumergida basándose en unas claves. Las ratas se sometieron a un preentrenamiento con la plataforma visible para descartar las que sufrían problemas morotes o visuales. Se pusieron cuatro claves para la orientación del animal en la búsqueda de la plataforma sumergida. Las ratas tuvieron cuatro ensayos por día durante doce días. El tiempo que tardaban en encontrar la plataforma fue medido y se hizo la media de los cuatro ensayos diarios. El último día se hizo una prueba de 60 segundo sin plataforma y se analizó el tiempo en que el animal nadaba en el cuadrante correcto donde debía haber estado la plataforma según las claves. Los animales con déficits de aprendizaje fueron designados como aquellos que nadaban en el cuadrante correcto un tiempo no superior al que correspondería por azar. Estos animales dos semanas después fueron divididos en dos grupos y fueron tratados con vectores lentivirales que expresaban GDNF o GFP. Se operaron un total de 23 ratas viejas: a un grupo de 7 animales se les inyectó GFP, a 8 animales se les inyectó GDNF y 8 animales no tenían déficits de aprendizaje se usaron como controles. La inyección de los vectores se produjo operando a los animales en un aparato estereotáxico y utilizando las coordenadas del área CA1 del hipocampo (AP -3.8mm, ML +/- 2mm, DV-2.4mm). Dos semanas después de la cirugía las ratas tenían cerca de 2 años de edad. Estos animales volvieron a realizar los tests del laberinto acuático de Morris realizando 4 ensayos por día y una prueba cada cuatro días hasta que completaron 48 ensayos. Después de completar los estudios de comportamiento se anestesiaron los animales y se les eutanasió extrayéndoles el cerebro que fue congelado. Cuatro hemicerebros del grupo inyectado con lenti-GFP fueron cortados en el criostato. Los cortes de 16 µm fueron teñidos con los anticuerpos GFP y GFAP y anticuerpos secundarios fluorescentes Alexa Fluor. El resto de los cerebros fueron diseccionados en 6 áreas hipocampales (dorsal y ventral CA1, dorsal y ventral CA2/3, dorsal y ventral del giro dentado), 5 áreas corticales (cingulado, frontal, temporal, entorrinal y parietal), septo y caudado. La determinación de los niveles en las diferentes regiones cerebrales de GDNF fueron determinados por ELISA. Se determinó la actividad de la colina acetil transferasa

(ChAT) midiendo la formación de ^{14}[C] acetilcolina a partir de ^{14}[C] acetilcoenzima-A. Los neurotransmisores dopamina, serotonina y los metabolitos de la dopamina: 3,4-dihidroxihenilacético (DOPAC) y el ácido homoválico (HVA) y el metabolito ácido de serotonina 5-hidroxiindoleacético (5-HIAA) fueron analizados por HPLC en los animales tratados con GFP, GDNF y un grupo de animales jóvenes. Se comprobó la expresión de los lentivirus, observamos que el GFP se expresó en la zona CA1 del hipocampo en sus capas oriens y el stratum radiatum. La proteína se expresaba selectivamente en los astrocitos, esto lo comprobamos por la coinmunolocalización en los astrocitos de GFAP y GFP mientras que la proteína GFP no se detectó en las neuronas de la capa molecular piramidal. Los niveles de GDNF detectados por ELISA fueron mayores en la zona CA1 del hipocampo del hemisferio inyectado. Las ratas envejecidas que tuvieron déficits de aprendizaje espacial y fueron tratadas con GDNF mejoraron en las pruebas del laberinto de Morris a niveles similares a las ratas sin déficits cognitivos mientras que las ratas tratadas con GFP no mejoraron. La velocidad de nado no fue afectada ni por la cirugía ni por la expresión transgénica. Los niveles de ChAT fueron mayores en el área CA1 dorsal y ventral del hipocampo de las ratas tratadas con GDNF que las ratas tratadas con GFP e incluso que las ratas jóvenes. También se midieron las concentraciones de dopamina y sus metabolitos DOPAC y HVA. La cantidad de dopamina se aumentó en las ratas tratadas con GDNF por encima de las ratas tratadas con GFP y las ratas jóvenes en el área CA1 dorsal y en todo el giro dentado. Una disminución en la cantidad de HVA se observó en las ratas tratadas con GFP y con GDNF en el área dorsal del CA1 y CA2/3, en las áreas corticales y el septo. En estas ratas también disminuyó la cantidad de DOPAC y HVA en el caudado. Los niveles de serotonina aumentaron en el grupo tratado con GDNF en todo el hipocampo dorsal y en giro dentado ventral más que en el grupo tratado con GFP. Ambos grupos de animales tuvieron un aumento de 5-HIAA en el giro dentado dorsal y en el área CA1 ventral. En conclusión una pequeña liberación crónica de GDNF por parte de los astrocitos en el área dorsal CA1 del hipocampo mejora la transmisión local colinérgica, dopaminérgica y serotoninérgica conduciendo a una mejora en el aprendizaje espacial y memoria de ratas envejecidas con déficits cognitivos. En este estudio se prueba que los astrocitos son un tipo celular apropiado para sobreexpresar GDNF en terapias génicas. Los déficits cognitivos no son del todo revertidos, esto sugiere que la acción paracrina del GDNF en la región CA1 del hipocampo no es suficiente para mejorar toda la actividad de aprendizaje. Los vectores lentivirales demuestran en este estudio una gran eficacia haciendo posible un tratamiento mayor y crónico que posiblemente produzca mejores resultados. Los resultados obtenidos en este trabajo demuestran el valor terapéutico de los vectores lentivirales que expresan GDNF transgénico en los déficits cognitivos de las ratas envejecidas. Esto abre la posibilidad a más estudios para explorar las posibilidades terapéuticas del GDNF en el envejecimiento humano y en la pérdida de memoria derivada de la enfermedad de Alzheimer.

El tercer artículo se titula "Dysfunction of astrocytes in senescence-accelerated mice SAMP8 reduces their neuroprotective capacity" publicado en Aging Cell 2008; 7 (5): 630-640 por García-Matas S, Gutierrez-Cuesta J, Coto-Montes A, Rubio-Acero R, Díez-Vives C, Camins A,

Pallàs M, Sanfeliu C, Cristòfol R. (Ver anexo 3). En este tercer trabajo estudiamos los cambios en la fosforilación de tau, el estrés oxidativo y la captación de glutamato en cultivos corticales primarios de astrocitos de ratones neonatos SAMP8 y de ratones resistentes a la senescencia SAMR1 intentando establecer un modelo in vitro para los estudios moleculares y celulares del envejecimiento en un modelo murino. Los ratones de senescencia acelerada (SAM) son un grupo de 12 cepas de ratones seleccionadas fenotípicamente a partir de la cepa AKR/J. Los de la cepa 8 de senescencia acelerada (SAMP8) manifiestan una avanzada senescencia irreversible con alteraciones patológicas, bioquímicas y de comportamiento mientras que los ratones SAMR1 presentan un patrón de envejecimiento normal. Los ratones SAMP8 tienen una media de vida de 10 meses mientras que los SAMR1 tienen una media de edad de 18.9 meses. Los ratones SAMP8 presentan déficits de memoria y aprendizaje, placas β-amiloideas, hiperfosforilación de la proteína tau y de la ciclina dependiente de kinasa 5 (Cdk5). Por estos motivos los ratones SAMP8 son un modelo adecuado para el estudio de los mecanismos moleculares que intervienen en los déficits cognitivos de los individuos envejecidos. La teoría de los radicales libres postula que las alteraciones oxidativas en las biomoléculas producidas por ROS contribuyen a la disfunción celular durante el envejecimiento. De hecho se han encontrado evidencias de un aumento del estrés oxidativo en los cerebros de los ratones SAMP8 envejecidos. Unos niveles superiores de lipoperoxidación, proteínas con enlaces carbonilo y ROS se han observado en los cerebros de los ratones SAMP8 envejecidos además de déficits de aprendizaje y memoria a la edad de 1-5 meses. También se ha observado en los ratones SAMP8 de 1-12 meses edad una disminución de la actividad de la superóxido dismutasa, catalasa, glutatión reductasa y glutatión peroxidasa con un incremento de la actividad acil-CoA oxidasa respecto a los ratones SAMR1 controles de la misma edad. Los astrocitos son importantes para la correcta función cerebral y defienden al cerebro frente al daño oxidativo. Los astrocitos presentan un gran número de sistemas antioxidantes como el glutatión, la glutatión transferasa, la catalasa y la superóxido dismutasa. Aunque son capaces de proteger a las neuronas frente a la toxicidad del peróxido de hidrógeno también son vulnerables frente al estrés oxidativo y reaccionan a bajas concentraciones de peróxido de hidrógeno generando ROS, lipoperoxidación y aumentando sus defensas antioxidantes. Además los astrocitos son los principales responsables de captar el glutamato extracelular previniendo así la excitotoxicidad neuronal. De este modo alteraciones en estos sistemas pueden reducir potencialmente la capacidad neuroprotectora de los astrocitos, contribuyendo a la neurodegeneración. Durante el envejecimiento se han observado elevados niveles de GFAP y S100β en regiones del cerebro de ratones y ratas. La producción de anión superóxido, la lipoperoxidación, la oxidación proteica y los depósitos de hierro están elevados en los cultivos de astrocitos envejecidos. El potencial de membrana mitocondrial de los astrocitos viejos está disminuido respecto al de los astrocitos jóvenes. Además se han observado cambios en la captación de glutamato glial durante el envejecimiento. Hasta ahora la disfunción neuronal se ha considerado como la principal causa de los déficits cognitivos en los ratones SAMP8. Sin embargo algunas evidencias sugieren que las alteraciones en los astrocitos contribuyen a la

senescencia acelerada observada en este modelo animal. Un aumento en la astrogliosis y microgliosis ha sido descrita en el hipocampo y corteza cerebral de los ratones SAMP8. El objetivo de este estudio ha sido estudiar si los astrocitos de los ratones SAMP8 presentan características relacionadas con su envejecimiento que nos permitan explicar su papel en las alteraciones bioquímicas y comportamentales observadas en los ratones SAMP8 envejecidos. Este estudio se llevó a cabo en astrocitos corticales debido a que muchos cambios bioquímicos estudiados en los ratones SAMP8 se han descrito en esta área cerebral. En los astrocitos SAMP8, encontramos hiperfosforilación de algunas formas de tau, activación de las proteínas quinasas Gsk3β y Cdk5 y un aumento del estrés oxidativo. La captación de glutamato en los cultivos primarios de astrocitos corticales de ratones SAMP8 esta disminuida comparada con la que hay en los astrocitos de los ratones SAMR1. También estudiamos la capacidad de neuroprotección de los astrocitos SAMR1 y SAMP8 en cocultivos con neuronas de ambas cepas. De ese modo demostramos que los astrocitos SAMP8 tienen reducida su capacidad de neuroprotección. Para este trabajo utilizamos diversos cultivos: a) cultivos primarios enriquecidos en astrocitos de corteza cerebral de ratones SAMP8 y SAMR1 neonatos de 2 días, estos cultivos contienen un 85-90% de astrocitos, 10-15% de microglía y 0.1-1% de oligodendroglía, los experimentos se llevaron a cabo cuando los astrocitos tenían 21 días in vitro; b) cocultivos de neuronas de fetos de ratones SAMP8 y SAMR1 de 15 días y astrocitos; y c) cultivos neuronales con medio condicionado durante 2 días por astrocitos de 21 DIV. Las neuronas se tiñeron con yoduro de propidio y el anticuerpo monoclonal neuronal NeuN (1:200) para ver su viabilidad. La generación de hidroperóxidos y el anión superóxido fue determinada mediante DCFH-DA y dihidroetidio. La lipoperoxidación lipídica fue determinada por medio de un kit de lipoperoxidación lipídica. Las proteínas con enlaces carbonilos fueron determinados a través del cromógeno 2,4 – dinitrofenilhidracina. Los cambios en el potencial de membrana mitocondrial se midieron usando rodamina 123 y su fluorescencia fue medida en el fluorímetro. Se hicieron Western Blot para determinar las siguientes proteínas con anticuerpos policlonales: anti-tau p[Ser^{199}] y anti-tau p[Ser^{396}] dilución (1:2000); anti-Gsk3β p[Tyr^{216}], anti- Gsk3β p[Ser^{9}], anti-Cdk y anti p35/p25 diluición 1:1000 y anti-GLAST dilución 1:500. La concentración de proteína se evaluó a través de la normalización con la β-actina (diluida 1:10000). Las bandas se midieron a través del software Quantity One. La captación de glutamato fue determinado con glutamato tritiado y líquido de centelleo en el aparato Wallac 1414 contador de líquido de centelleo. Los resultados que hemos obtenido en este trabajo indican cambios la expresión de la proteína tau, Gsk3β y Cdk5. Los análisis densitométricos del Western blot indican un aumento significativamente estadístico de la proteína tau fosforilada en Ser^{199} y Ser^{396} en los astrocitos de los ratones SAMP8 comparado con los astrocitos de los ratones SAMR1. También observamos un aumento significativo de Gsk3β fosforilada en Tyr^{216} (forma activa) y un descenso significativo de la proteína Gsk3β fosforilada Ser^{9} (forma inactiva) en los astrocitos de los ratones SAMP8 comparado con los astrocitos de los ratones SAMR1. Estos resultados indican una activación de la quinasa Gsk3β en los astrocitos de los ratones SAMP8. La expresión de la proteína Cdk5 no se diferencia entre los astrocitos de los ratones de ambas

cepas. La inducción de la actividad de Cdk5 se determinó por la proporción de p25/p35. La expresión de p35 se encontraba disminuida mientras que la expresión p25 se encontraba aumentada en los astrocitos de los ratones SAMP8 en relación con los astrocitos de los ratones SAMR1. La proporción p25/p35 se encontraba aumentada esto indica un aumento en la actividad de Cdk5 en los astrocitos de los ratones SAMP8. En los cultivos de astrocitos SAMP8 que fueron tratados con litio 10mM, un inhibidor específico de la actividad de Gsk3β, se observó un aumento de la expresión de Gsk3β fosforilada Ser^{9} y una disminución de la Tau fosforilada en Ser^{396}. El tratamiento con roscovitina 15 µm, un inhibidor no específico de la quinasa Cdk5, muestra una tendencia a disminuir la proporción de p25/p35 y de Tau fosforilada en Ser^{396} en los astrocitos SAMP8. Otros experimentos mostraron un aumento en el estrés oxidativo y un descenso del potencial de membrana mitocondrial en los astrocitos de los ratones SAMP8. Los cultivos de astrocitos de los ratones SAMP8 producen significativamente más anión superóxido que los astrocitos de los ratones SAMR1. También se encontraron mayores niveles de lipoperoxidación y formación de enlaces carbonilo en las proteínas de los astrocitos de los ratones SAMP8 respecto a los astrocitos SAMR1. El aumento de la producción de radicales libres puede estar relacionado con los cambios en el potencial de membrana mitocondrial, así que se midieron estos cambios de potencial y se observó una disminución en el potencial en los astrocitos de los ratones SAMP8. Además se hicieron experimentos de captación de glutamato y los resultados indican una disminución de la capacidad de captar glutamato de los astrocitos SAMP8 respecto a los SAMR1. Por último la capacidad neuroprotectora de los astrocitos fue evaluada en cocultivos de neuronas corticales determinando la supervivencia neuronal. Los resultados demuestran que los astrocitos SAMP8 tienen reducida su capacidad neuroprotectora. Los experimentos de neuronas con medio condicionado muestran que las neuronas viven más con medio condicionado por astrocitos, pero no llegan a los niveles de supervivencia que se obtienen si están en cocultivo. Esto indica que las diferencias en la capacidad neuroprotectora entre SAMR1 y SAMP8 no se deben a ningún componente secretado por los astrocitos al medio de cultivo. En conclusión hemos demostrado que los astrocitos en cultivo de los ratones neonatos SAMP8 presentan alteraciones similares a las descritas en los cerebros de los ratones SAMP8 de 1 a 5 meses de edad. También vemos alteraciones similares a las que ocurren en el cerebro envejecido, como el aumento de la generación de ROS que puede producir el aumento en la oxidación de sus lípidos y proteínas que a su vez pueden causar el cambio en la actividad mitocondrial, la fosforilación de tau y el transporte de glutamato. Todas estas alteraciones pueden comprometer el funcionamiento de los astrocitos. También hemos mostrado una disminución de la neuroprotección que proporcionan los astrocitos de los ratones SAMP8 a las neuronas en cultivo. Este trabajo demuestra que los astrocitos corticales de los ratones SAMP8 presentan disfunciones y respalda la hipótesis de que las alteraciones relacionadas con el envejecimiento en los astrocitos contribuyen a la neurodegeneración.

Estos trabajos forman parte de la tesis doctoral que se presentará en breve en la Facultad de Medicina de la Universidad de Barcelona.

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SUMARIO:

Journal of Neurochemistry, 2007, **101**, 794–805 doi:10.1111/j.1471-4159.2006.04369.x

Astrocytes aged *in vitro* show a decreased neuroprotective capacity

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Abstract

Alterations in astrocyte function that may affect neuronal viability occur with brain aging. In this study, we evaluate the neuroprotective capacity of astrocytes in an experimental model of *in vitro* aging. Changes in oxidative stress, glutamate uptake and protein expression were evaluated in rat cortical astrocytes cultured for 10 and 90 days *in vitro* (DIV). Levels of glial fibrillary acidic protein and S100β increased at 90 days when cells were positive for the senescence β-galactosidase marker. In long-term astrocyte cultures, the generation of reactive oxygen species was enhanced and mitochondrial activity decreased. Simultaneously, there was an increase in proteins that stained positively for nitrotyrosine. The expression of Cu/Zn-superoxide dismutase (SOD-1) and haeme oxygenase-1 (HO-1) proteins and inducible nitric oxide synthase (iNOS) increased in aged astrocytes. Glutamate uptake in 90-DIV astrocytes was higher than in 10 DIV ones, and was more vulnerable to inhibition by H_2O_2 exposure. Enhanced glutamate uptake was probably because of up-regulation of the glutamate/aspartate transporter protein. Aged astrocytes had a reduced ability to maintain neuronal survival. These findings indicate that astrocytes may partially loose their neuroprotective ability during aging. The results also suggest that aged astrocytes may contribute to exacerbating neuronal injury in age-related neurodegenerative processes.

Keywords: aging, astrocytes, astrocyte-neuron cocultures, glutamate uptake, neuroprotection, oxidative stress.

J. Neurochem. (2007) **101**, 794–805.

The brain is particularly vulnerable to oxidative stress because of its high metabolic rate, high level of polyunsaturated fatty acids and low antioxidant defences. Free radicals are constantly generated in the brain *in vivo*. Moreover, excessive production of the superoxide radical and hydrogen peroxide (H_2O_2) in the presence of iron or copper ions can result in the generation of the highly reactive hydroxyl radical (Halliwell 1992). Increased generation of reactive oxygen species (ROS) is physiologically controlled by the antioxidant defence system. Therefore, a decrease in antioxidant capacity will compromise cell function. Astrocytes play a fundamental role in maintaining normal brain physiology and in determining brain's susceptibility to oxidative damage (for a review see Cotrina and Nedergaard 2002). They modulate neuronal activity by releasing many modulatory molecules, such as ATP, glutamate, growth factors and cytokines. They also propagate calcium waves over long distances. In addition, astrocytes possess a high content of GSH, high GSH metabolism and elevated catalase and superoxide dismutase (SOD-1) enzyme activity. This enables them to protect neurons against oxidative injury (Makar *et al.* 1994; Desagher *et al.* 1996; Dringen *et al.* 2000). However, astrocytes are also vulnerable to oxidative stress. Their reaction to low concentrations of H_2O_2 involves generation of ROS, lipoperoxidation, and changes in antioxidant defences (Röhrdanz *et al.* 2001). Astrocytes are the cell type that is mainly responsible for clearing extracellular glutamate, as they have high-affinity glutamate transporters (for a review see: Andersson and Swanson 2000). Glutamate uptake into astrocytes can also be inhibited by the direct oxidation of glutamate transporters (Volterra *et al.* 1994; Trotti *et al.* 1997).

From a physiological point of view, brain aging has been proposed as a progressive loss of neuronal homeostatic reserve without causing neuronal death (Toescu and Verkhratsky 2003). Changes in the function of astrocytes

Received July 24, 2006; revised manuscript received October 18, 2006; accepted November 9, 2006.

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Abbreviations used: AD, Alzheimer's disease; DCF, dichlorofluorescein; DCFH-DA, 2′-7′-dichlorofluorescein diacetate; DIV, days *in vitro;* DMEM, Dulbecco's Modified Eagle Medium; FBS, foetal bovine serum; GFAP, glial fibrillary acidic protein; GLAST, glutamate/aspartate transporter; GPX, glutathione peroxidase; HBSS, HEPES-buffered saline solution; HO-1, haeme oxygenase-1; iNOS, inducible nitric oxide synthase; MCI, mild cognitive impairment; PBS, phosphate-buffered saline; PFA, paraformaldehyde; ROS, reactive oxygen species; SOD-1, superoxide dismutase; TCA, tricarboxylic acid.

may reduce their neuroprotective capacity, contributing to the neuronal aging development. Oxidative stress is believed to play a key role in brain aging progression, although the molecular mechanisms involved are not clear. Numerous evidences on alterations in astrocyte function during aging have been related to changes in redox homeostasis. Astrocyte activation increases progressively during aging in most brain regions, as estimated by an increased expression of glial fibrillary acidic protein (GFAP) and S100β protein; and GFAP and S100β mRNA (Amenta *et al.* 1988; O'Callaghan and Miller 1991; Kohama *et al.* 1995; Sheng *et al.* 1996; Morgan *et al.* 1997). Both proteins also increase with age in culture in mouse and rat cortical astrocytes (Andersson *et al.* 1994; Papadopoulos *et al.* 1998; Gottfried *et al.* 2002; Tramontina *et al.* 2002). S100β overexpression in astrocytes was considered as a neuroprotective response to neuronal damage. However, abundant evidence suggests that this response may be detrimental and accelerate age-related neurodegenerative processes (Sheng *et al.* 2000; Mrak and Griffin 2001). In addition, ROS production, lipoperoxidation and protein oxidation are elevated in several brain areas of aged rodents (for a review see: Poon *et al.* 2004) and in the brain of a senescence-accelerated animal model (Alvarez-García *et al.* 2006). It has been suggested that dysfunctional antioxidant defences contribute to cellular aging. However, opposing results in the expression or activity of the antioxidant enzymes have been reported (Benzi and Moretti 1995; Leutner *et al.* 2001; Balu *et al.* 2005; Comai *et al.* 2005; Siqueira *et al.* 2005; Keil *et al.* 2006). In aged astrocyte cultures, superoxide production, lipoperoxidation, protein oxidation and iron staining are also elevated, although antioxidant defences are maintained or increased (Papadopoulos *et al.* 1998; Gottfried *et al.* 2002; Klamt *et al.* 2002). However, astrocytes aged in culture show higher vulnerability to oxidative injury than young astrocytes (Papadopoulos *et al.* 1998; Gottfried *et al.* 2002). On the contrary, changes in glial glutamate uptake have been observed during brain aging (for a review see: Segovia *et al.* 2001). In a previous work, Gottfried *et al.* (2002) have shown that glutamate uptake is more sensitive to oxidative stress in aged astrocytes in culture.

Haeme oxygenase-1 (HO-1) is a stress protein involved in haeme catabolism, giving biliverdin, free iron and CO. Biliverdin and its metabolite bilirubin are physiological radical scavengers that protect neurons against oxidative stress (Llesuy and Tomaro 1994; Dore *et al.* 1999). HO-1 immunoreactivity is augmented in neurons and astrocytes of the aged brain and in age-related neurodegenerative diseases (Iijima *et al.* 1999; Schipper 2004; Schipper *et al.* 2006). Although HO-1 overexpression is believed to act as a neuroprotective mechanism, recent evidence suggests that glial HO-1 has a toxic role when high expression levels are induced (Shipper 2004; Song *et al.* 2006).

There is an increasing interest in finding out whether changes in the above-mentioned astrocyte functions accelerate aging processes. Such changes can alter synaptic efficacy and reduce neuronal survival. The aim of this study was to establish an *in vitro* model of astrocyte aging in order to study the neuroprotective ability of astrocytes. First, we assessed parameters of oxidative stress by measuring ROS generation, mitochondrial activity and the expression of GFAP, S100β, inducible nitric oxide synthase (iNOS) Cu/Zn SOD-1 and HO-1 proteins in rat cortical astrocytes maintained in culture for 10 and 90 days *in vitro* (DIV). Secondly, we evaluated the glutamate transport function of astrocytes by determining glutamate uptake and the expression of glutamate/aspartate transporter (GLAST) protein. Finally, astrocyte neuroprotection was tested by measuring cortical neuronal survival in cocultures with young and long-term astrocytes cultures.

Materials and methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), gentamycin and foetal bovine serum (FBS) were purchased from Gibco-BRL (Invitrogen, Paisley, UK). Culture plates, chamber slides and flasks were from NUNC (Roskilde, Denmark). L-[^{3}H]-Glutamate (specific activity, 51 Ci/mmol) was purchased from PerkinElmer Inc. (Wellesley, MA, USA). The fluorescent probe 2′-7′-dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Leiden, The Netherlands). L-Glutamic acid, H_2O_2, thiazolyl blue tetrazolium bromide (MTT), sodium orthovanadate, trolox, *n*-propyl gallate, curcumin, anti-GFAP and anti-S100β primary antibodies, anti-rabbit, anti-mouse and anti-sheep horseradish peroxidase-conjugated secondary antibodies were obtained from Sigma Chemical Co. (St Louis, MO, USA). Anti-NeuN and anti-iNOS antibodies were obtained from Chemicon International (Temecula, CA, USA); anti-SOD-1 antibody from Calbiochem (La Jolla, CA, USA); anti-2-nitrotyrosine antibody was from Abcam (Cambridge, UK); anti-α-actin and anti-HO-1 antibodies were from Stressgen Bioreagents (Victoria, BC, Canada); and anti-GLAST antibody was from Alpha Diagnostic International (San Antonio, TX, USA). The protease inhibitor cocktail Complete was from Roche Applied Science (Mannheim, Germany). The β-galactosidase staining kit was obtained from Cell Signalling Technology Inc. (Danvers, MA, USA). All other chemicals were of analytical grade.

Preparation of astrocyte cultures

Primary cultures enriched in astrocytes were established from cerebral cortical tissue of 2-day-old Fisher 344 rats (Iffa-Credo, l'Arbresle, France). Cultures were prepared as described elsewhere (Fauconneau *et al.* 2002). Briefly, brains were dissected free of the meninges, diced into small cubes and dissociated by pipetting for 2 min with a Pasteur pipette in DMEM containing 100 μg/mL gentamycin. The suspension was filtered through a 70-μm pore size nylon mesh cell strainer (Becton and Dickinson, Franklin Lakes, NJ, USA). Cells were then seeded in flasks and maintained in DMEM supplemented with 20% FBS at 37°C in a humidified atmosphere of 5% CO_2/95% air. The culture medium was changed twice a week. After 15 days, confluent cultures were mildly trypsinized in the presence of 0.02% EDTA. They were then reseeded at

0.25×10^6 cells/mL (0.75×10^5 cells/cm^2) in DMEM with 10% FBS in multi-well plates, chamber slides or flasks coated with poly-L-lysine. Experiments were performed at 7–10 days *in vitro* (10 DIV) and 3 months (90 DIV) after reseeding. The later cultures were treated with 10 μmol/L cytosine arabinoside to curtail astrocyte proliferation and to maintain the purity of the cultures. Fresh medium was added once a week. Selected experiments were undertaken either by adding cytosine arabinoside to 10 DIV cultures or by not adding it to 90 DIV cultures. No effect of this antimitotic agent on the assayed parameters was detected (data not shown).

Determination of neuronal survival in coculture with astrocytes

To study the neuronal protection response of 10- and 90-DIV astrocyte cultures, cocultures of neurons and astrocytes were prepared in eight chamber slides. Fresh neurons disaggregated from a fresh cerebral cortical tissue of 15-day foetuses were directly seeded at 1.5×10^6 cells/mL (4.5×10^5 cells/cm^2) on a confluent monolayer of 10- and 90-DIV astrocyte cultures. Sister pure neuron cultures were similarly processed. Neuronal survival was assessed at 14 DIV. Dead cells were detected by staining the cultures with 7.5 μmol/L propidium iodide (red fluorescence) for 30 min. After that, cultures were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA). Neuronal living cells were identified by immunostaining with NeuN antibody. Cells were permeabilized with 0.25% Triton in PBS for 30 min, washed with PBS, incubated with goat serum to block unspecific binding sites at 22–24 °C, and incubated with mouse NeuN antibody (diluted 1 : 200) overnight at 4°C. Cultures were then washed with PBS and incubated with anti-mouse Alexa Fluor 488 (green fluorescence; Molecular Probes, Leiden, The Netherlands) for 1 h at 22–24 °C. After washing with PBS, nuclei were stained by 5 μmol/L bisbenzimide. Finally, cultures were mounted under coverslips. Randomly chosen fields were examined using a fluorescence microscope (Nikon Eclipse E1000; Nikon, Tokyo, Japan) and digitally photographed with a ColorView camera (Soft Imaging Systems, Stuttgart, Germany). Neuronal survival was determined by counting immunoreactive neurons to NeuN, which were not propidium iodide stained, using the analysis software (Soft Imaging System).

β-Galactosidase staining

Astrocytes seeded on chamber slides were washed with PBS and fixed in 4% PFA in PBS for 30 min. This was followed by three washes with PBS. The degree of senescence in astrocyte cultures was evaluated using the senescence β-galactosidase staining kit following manufacture's instructions. This marker identifies senescent cells in culture (Dimri *et al.* 1995). Senescent astrocytes stained with a blue colour were observed using a microscope (Nikon Eclipse E1000) and digitally photographed with a ColorView camera.

Western blot analysis

After shaking the cultures for 4 h to eliminate microglial cells, the medium was removed and astrocytes were rinsed twice with PBS. Cells were lysed for 10 min on ice in ristocetin-induced platelet agglutination buffer [10 mmol/L PBS, 1% Igepal AC-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] containing a protease inhibitor cocktail (Complete) and 1 mmol/L orthovanadate. They were then collected and frozen at −20°C until assay. Protein extracts were quantified following the Bradford method. Then, 15 μg of the protein extracts were denatured at 100°C for 5 min, loaded onto a 15% SDS – polyacrylamide gel and electrophoresed. Proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA). This was incubated overnight at 4°C with the following primary antibodies: mouse anti-GFAP (diluted 1 : 40 000); mouse anti-S100β (diluted 1 : 500); sheep anti-SOD-1 (diluted 1 : 1000); mouse anti-nitrotyrosine (diluted 1 : 1000); rabbit anti-iNOS (diluted 1 : 200), mouse anti-HO-1 (diluted 1 : 1000); and rabbit anti-GLAST (diluted 1 : 500). Membranes were incubated for 1 h at 22–24 °C with horseradish peroxidase conjugated secondary antibodies. Proteins were detected with a chemiluminescence detection system based on the luminol reaction. Protein loading of the gels was controlled by staining blots with an antibody against α-actin (diluted 1 : 10 000). The immunoreactive bands were digitalized and a densitometry analysis was performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). The levels of protein immunoreactivity were normalized to that of α-actin.

Glutamate uptake

The culture medium was removed and astrocytes were washed with warm HEPES-buffered saline solution (HBSS) (136 mmol/L NaCl, 5.4 mmol/L KCl, 1.2 mmol/L $CaCl_2$, 1.4 mmol/L $MgCl_2$, 1.0 mmol/L NaH_2PO_4 and 10 mmol/L HEPES) containing 9 mmol/L glucose at pH 7.3. Astrocyte cultures were then incubated for 10 min at 37°C in HBSS with 9.8 nmol/L (500 nCi/mL) of [^{3}H]-glutamate and several concentrations of unlabeled glutamate in the range of 1–1000 μmol/L. Uptake was terminated by removing the medium and washing the cells three times with ice-cold HBSS. This was immediately followed by cell lysis in 0.2 N NaOH. Aliquots were taken for liquid scintillation counting (with Optiphase 'Hisafe' cocktail) and for Bradford's protein assay using bovine serum albumin as the protein standard. Radioactivity was analysed by scintillation counting in a Wallac 1414 Liquid Scintillator Counter (PerkinElmer, Boston, MA, USA). H_2O_2 effects on [^{3}H]-glutamate uptake were checked using 9.8 nmol/L of labeled glutamate plus 100 μmol/L unlabeled glutamate.

Mitochondrial activity

The mitochondrial activity of astrocyte cultures was determined by the MTT reduction assay (Hansen *et al.* 1989). At 10 and 90 DIV, MTT was added to the 96-well cultures to a final concentration of 0.5 mg/mL. At termination, formazan crystals were solubilized by adding 100 μL of extraction buffer [20% SDS in *N,N*-dimethylformamide/water (1 : 1), pH 7.4] [Correction added after online publication (19/03/07); pH 7.4 should read pH 4.7] and incubating overnight at 37°C. Absorbance was measured at 560 nm with a 620-nm reference wavelength in a plate reader (iEMS Reader MF; Labsystems, Vantaa, Finland).

Reactive oxygen species generation

Intracellular generation of ROS was determined using DCFH-DA as described previously (Sebastià *et al.* 2004). Briefly, cultures in 96-well plates were washed in HBSS and loaded with 10 μmol/L DCFH-DA for 20 min at 37°C. Wells were then washed with HBSS and 2′-7′-dichlorofluorescein (DCF) fluorescence was measured after 1 h incubation at 37°C in a fluorescence plate reader (Spectramax Gemini XS; Molecular Devices, Wokingham, UK) at

485 nm excitation/530 nm emission. The increase in intracellular ROS was quantified from a standard curve of DCF in methanol (0.5–100 nmol/L) and expressed as nmol of DCF/mg protein.

Statistical analysis

Experiments were performed with astrocytes from 3 to 6 primary cultures. Data were pooled and the results given as mean ± SEM. Statistical significance ($p < 0.05$) was determined by Student's *t*-test and by one- or two-way ANOVA followed by Dunnett's or Bonferroni's multiple comparison tests, respectively.

Results

Long-term astrocyte cultures presented characteristics of senescence and glial activation

Senescence characteristics in 10- and 90-DIV astrocyte cultures were evaluated using the β-galactosidase staining kit. Astrocytes with an intense blue stain were only clearly evident in 90 DIV cultures (Fig. 1). The expression of the glial proteins GFAP and S100β was determined by western blot in 10- and 90-DIV astrocytes. Long-term cultures showed an increase in GFAP and S100β bands. Densitometric analysis of the immunoblots showed that GFAP and S100β protein levels in 90 DIV cultures were 10- and 4-fold higher, respectively, than levels in the 10 DIV cultures (Figs 2a and b). The total protein content was maintained along the period of *in vitro* aging (27.78 ± 2.45 μg/well at 10 DIV vs. 29.59 ± 0.98 μg/well of 24-well plates at 90 DIV; lack of statistical significance).

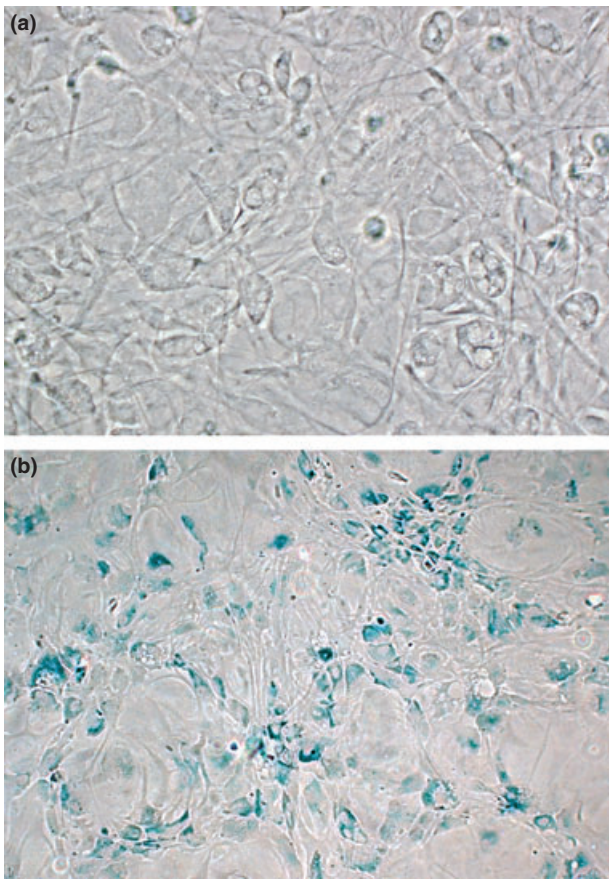

Fig. 1 A representative stain of β-galactosidase in astrocytes cultured for 10 (a) and 90 DIV (b). Blue-stained cells were only detected in 90 DIV cultures. A, B: 200×.

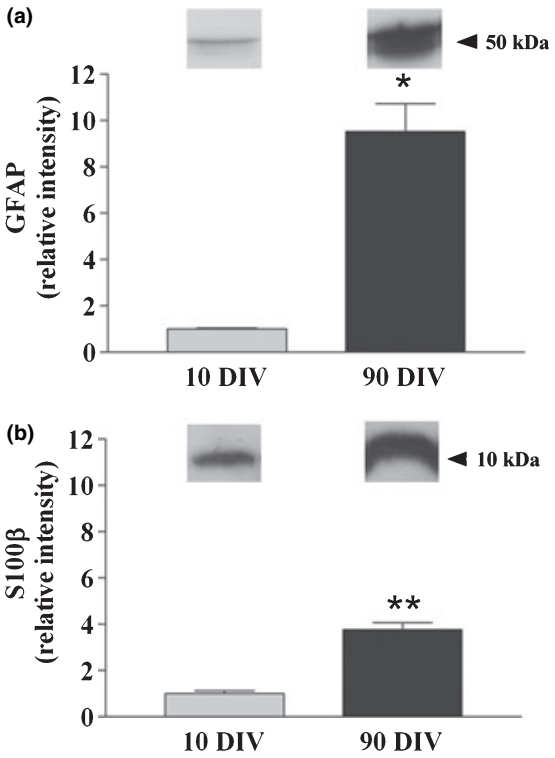

Fig. 2 Representative immunoblots and densitometry analysis of the expression of GFAP (a) and S100β (b) proteins in 10- and 90-DIV astrocyte cultures. Graphs are the summary of results from experiments repeated with at least three different cultures. The levels of GFAP and S100β immunoreactivity were normalized to that of α-actin and were expressed relative to the corresponding protein levels in 10-DIV cultures. Both protein levels were increased in 90-DIV astrocyte cultures. Data were compared by Student's *t*-test (*$p < 0.01$ and **$p < 0.001$).

Oxidative stress increased with age in the cultures

Mitochondrial activity was evaluated by MTT assay in astrocytes cultured for 10 and 90 DIV (Fig. 3a). Long-term cultures presented a significant reduction in mitochondrial activity when compared with young cultures. However, protein levels were not affected by a longer culture period (data not shown). To explore whether the reduction in mitochondrial activity was associated with ROS production, basal levels of hydrogen peroxide generated for 1 h were determined by the use of a DCFH probe (Fig. 3b). Ninety-DIV astrocyte cultures produced sixfold more ROS than 10 DIV cultures, as indicated by the significantly higher amount of DCF fluorescence detected in these cultures. One result of increased ROS production may be protein oxidation.

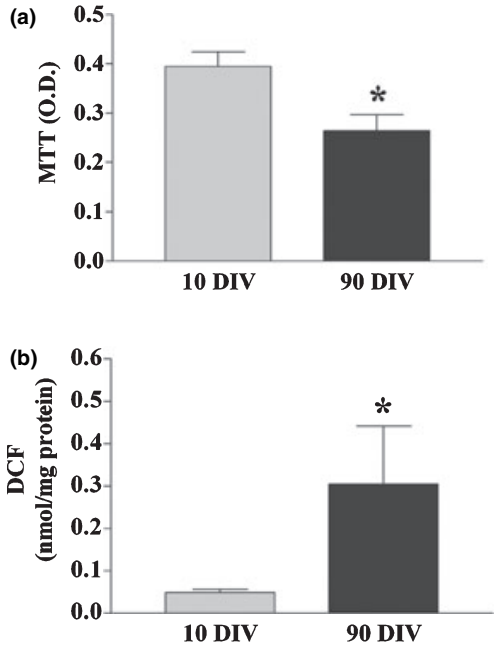

Fig. 3 Mitochondrial activity and ROS generation in 10- and 90-DIV astrocyte cultures. (a) Mitochondrial activity was measured by the MTT reduction assay. Data are expressed as absorbance (OD) of formazan and represent the mean ± SEM of 4–6 independent cultures. Mitochondrial activity was reduced in 90-DIV astrocyte cultures (*$p < 0.05$). (b) Intracellular ROS generation after 1 h, as measured by DCFH oxidation to DCF. Results are expressed as nmol of DCF/mg of protein and represent the mean ± SEM of 4–6 independent cultures. Ninety-DIV cultures generated significantly more ROS than 10-DIV cultures (*$p < 0.05$).

Therefore, we used western blot analysis to evaluate the presence of nitrotyrosine in proteins. Several proteins were observed, which stained positively for nitrotyrosine. We could quantify bands with an apparent molecular weight of approximately 120, 100, 75, 50, 45, 40 and 35 kDa. A significant increase in these proteins was detected in 90 DIV cultures, leading to a eightfold expression when compared with 10-DIV astrocyte cultures (Fig. 4a). Due that nitration of tyrosine residues in proteins is indicative of NO generation, we analysed the expression of iNOS protein by immunoblotting. Figure 4b shows that the iNOS expression level in 90-DIV astrocytes was twofold higher than that in 10-DIV astrocytes. The expression of SOD-1, the main superoxide radical scavenging enzyme, was determined by western blot. SOD-1 levels in 90 DIV cultures were 3.5-fold higher than that in the younger cultures (Fig. 4c). HO-1 protein is an enzyme that could be up-regulated by oxidative stress. Its expression level in short- and long-term cultures was also evaluated by immunoblotting. Astrocytes cultured for 90 DIV had a twofold expression level than those cultured for 10 DIV (Fig. 4d).

An increase in basal glutamate uptake was accompanied by up-regulation of GLAST expression in 90-DIV astrocytes

The ability of astrocytes to uptake glutamate was evaluated in 10 and 90 DIV cultures using different glutamate concentrations. Figure 5(a) shows that glutamate uptake in 90-DIV astrocyte cultures was significantly higher than that in 10 DIV cultures. Kinetic parameters obtained from the Eadie-Hofstee analysis revealed a statistically significant

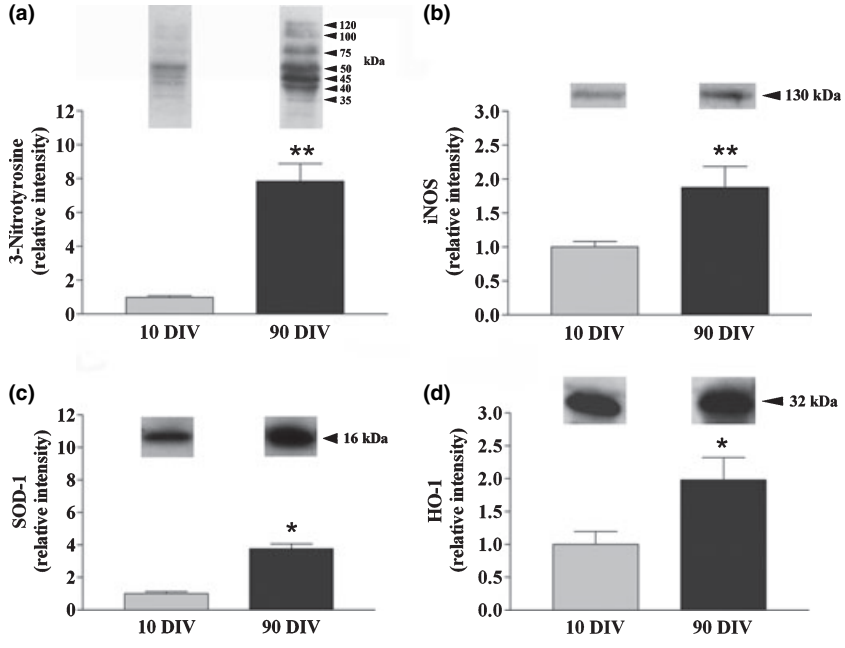

Fig. 4 Representative immunoblots and densitometry analysis of the expression of nitrotyrosine (a), iNOS (b), SOD-1 (c), and HO-1 (d) proteins in 10- and 90-DIV astrocyte cultures. Graphs are the summary of results from experiments repeated with at least three different cultures. The levels of nitrotyrosine, iNOS, SOD-1 and HO-1 immunoreactivity were normalized to that of α-actin and were expressed relative to the corresponding protein levels in 10-DIV cultures. All protein levels were higher in 90-DIV astrocyte cultures. Data were compared by Student's *t*-test (*$p < 0.01$ and **$p < 0.001$).

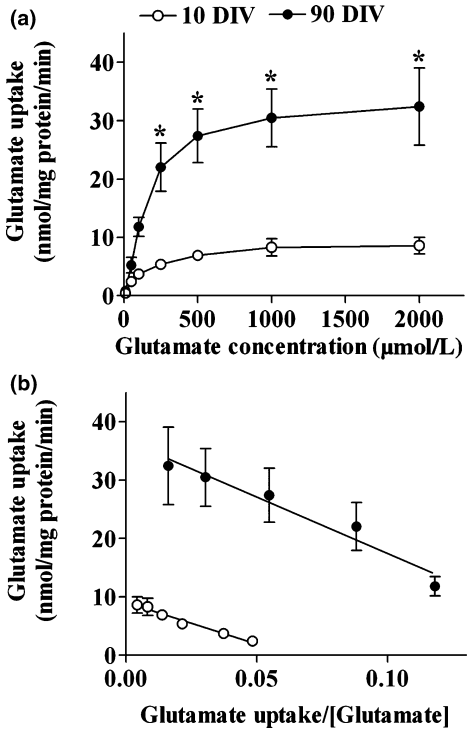

Fig. 5 (a) Glutamate uptake in astrocytes cultured at 10 and 90 DIV. Astrocytes were incubated with 9.8 nmol/L [^{3}H]-glutamate and several concentrations of unlabelled glutamate as described in Materials and methods section. Each point represents the mean ± SEM of five to six determinations. Two-way ANOVA followed by Bonferroni's test showed that culture age ($F_{1,65}$ = 83.2, p < 0.001) and glutamate concentration ($F_{6,65}$ = 16.67, p < 0.001) had a significant effect (*p < 0.001). (b) Eadie-Hofstee kinetic analysis of astrocyte glutamate uptake at 10 and 90 DIV. There was a significant increase in the V_{max} parameter of glutamate uptake in 90-DIV astrocyte cultures. However, there was no change in the K_m parameter.

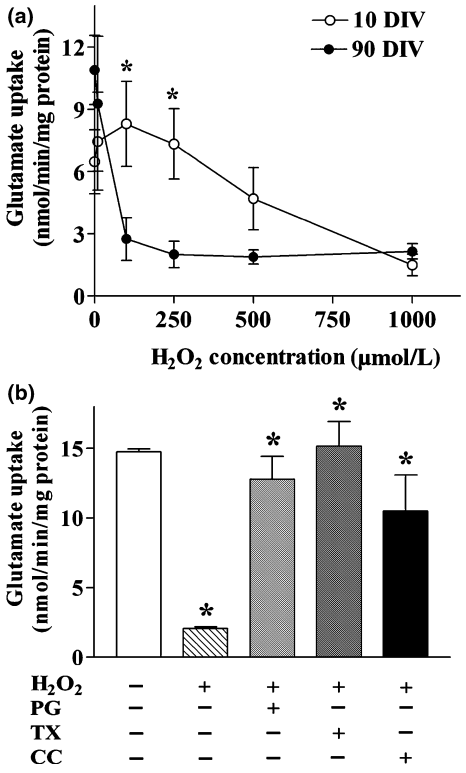

Fig. 6 Glutamate uptake inhibition by hydrogen peroxide (H_2O_2) in 10- and 90-DIV astrocyte cultures. Astrocytes were incubated with 9.8 nmol/L [^{3}H]-glutamate and 100 μmol/L of unlabelled glutamate. (a) Pre-incubation with different concentrations of H_2O_2 for 30 min before [^{3}H]-glutamate uptake measurement. (b) Effect of 50 μmol/L propyl gallate (PG), 100 μmol/L trolox (TX) and 10 μmol/L curcumin (CC) on 500 μmol/L H_2O_2-induced [^{3}H]-glutamate uptake inhibition in 90-DIV astrocyte cultures. Cultures were exposed to PG, TX and CC 30 min before and during the H_2O_2 treatment. Each point represents the mean ± SEM of five to six determinations. Statistical differences were determined by (a) Student's test (*p < 0.05); (b) One-way ANOVA followed by Dunnet's test (*p < 0.001 compared with all other groups).

increase in V_{max} in 90-DIV astrocytes when compared with 10-DIV astrocytes (V_{max} = 37 ± 2 and 9 ± 0.3 nmol/mg protein/min, p < 0.01, respectively) (Fig. 5b). There were no statistically significant differences between K_m values for 10- and 90-DIV astrocytes (143 ± 10 μmol/L and 193 ± 25 μmol/L, respectively). The exposure of astrocyte cultures to H_2O_2 resulted in concentration-dependent glutamate uptake inhibition (Fig. 6a). This inhibitory effect was potentiated in 90 DIV cultures. This indicates that there was an increase in susceptibility to oxidation injury with age in culture. The antioxidants propyl gallate (50 μmol/L), trolox (100 μmol/L) and curcumin (10 μmol/L) reversed the H_2O_2 inhibition in 90 DIV cultures (Fig. 6b), confirming the existence of a regulatory redox mechanism on the glutamate transporters similarly to that previously described in young astrocyte cultures (Volterra *et al.* 1994; Trotti *et al.* 1997; Sitar *et al.* 1999; Miralles *et al.* 2001).

In young and old cultures, the uptake was highly reduced by the inhibitor of glial glutamate transporter L-*trans*-pyrrolidine-2,4-dicarboxylate (to 85.75% and 79.09% for 15 and 90 DIV cultures, respectively). This suggests that the glial GLAST was mainly involved. Therefore, the expression of the GLAST transporter was examined in order to determine whether the increase in glutamate uptake observed in older astrocytes in culture was accompanied by an increase in GLAST protein levels. GLAST expression displayed an immunoreactive band with an apparent molecular mass of approximately 67 kDa (Fig. 7a). Western blot analysis revealed that GLAST expression in 90-DIV astrocytes was threefold higher than that in 10-DIV astrocytes (Fig. 7b).

Ninety-DIV astrocytes showed reduced neuroprotective capacity

The neuroprotective ability of astrocytes was tested in cocultures with cortical neurons by determining neuronal survival. Viable stained neurons were identified in pure

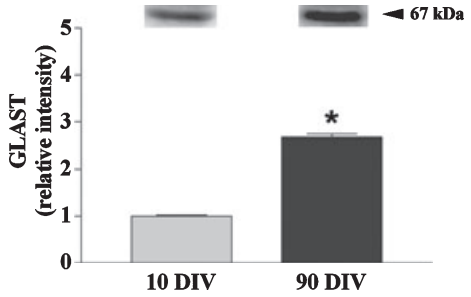

Fig. 7 A representative immunoblot of the expression of GLAST protein in 10- and 90-DIV astrocyte cultures. The anti-GLAST antibody recognized a transmembrane protein with a molecular mass of ~67 kDa. The graph is the summary of results from experiments repeated with at least three different cultures. The levels of GLAST immunoreactivity were quantified by normalization to α-actin and were expressed relative to the corresponding protein levels of 10-DIV cultures. GLAST protein levels were higher in 90-DIV astrocyte cultures. Data were compared by Student's *t*-test (*$p < 0.001$).

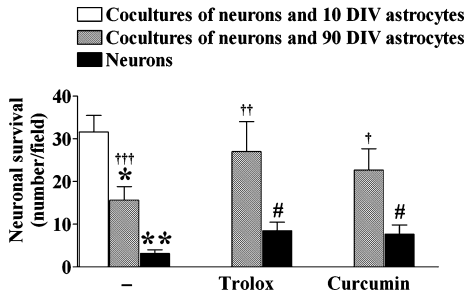

Fig. 8 Neuronal survival in the absence or presence of 10- and 90-DIV astrocytes and the effect of the antioxidants trolox and curcumin. Cortical neurons were cultured on a monolayer of 10- and 90-DIV astrocyte cultures for 2 weeks. Living neurons in cocultures with astrocytes or in pure neuron cultures were identified by cell staining with NeuN antibody and propidium iodide, as described in Materials and methods section. Neuron cultures and cocultures with 90-DIV astrocytes were grown in the absence or the presence of the antioxidants trolox (10 μmol/L) and curcumin (10 μmol/L). Results are expressed as the number of living neurons by field and are the mean ± SEM of five independent cocultures. Data were compared by Student's *t*-test (*$p < 0.01$ and **$p < 0.001$ relate to cocultures of neurons and 10-DIV astrocytes; $^{\#}p < 0.05$ relates to neuron cultures without antioxidant treatments; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ relate to the corresponding neuron cultures).

neuron cultures and in cocultures of neurons seeded on a monolayer of 10-DIV astrocytes or 90-DIV astrocytes for 2 weeks. After 2 weeks, the number of living cells in pure neuron cultures was notably reduced (Fig. 8). Cocultures of neurons with 10-DIV astrocyte cultures showed maximal neuroprotection, whereas 90-DIV astrocytes showed significantly reduced neuroprotection. Neuron cultures and cocultures of neurons with 90-DIV astrocytes were treated with the antioxidants trolox and curcumin. Both agents significantly increased the number of living cells in pure neuron cultures. In cocultures with aged astrocytes, the presence of antioxidants also enhanced neuronal survival. However, this survival rate was not statistically different from that either in cocultures with 10-DIV astrocytes or in untreated cocultures.

Discussion

The results of this study showed that astrocytes cultured 90 DIV acquired characteristics of senescence and generated higher levels of ROS than 10-DIV cultures. This was seen to have detrimental effects on neuronal protection. We observed an increase in GFAP and S100β proteins in older astrocytes in culture. Using a similar experimental approach, other authors also found increases in both of these proteins in mouse and rat cortical astrocytes cultured for 40–56 DIV compared with 7- to 10-DIV cultures (Papadopoulos *et al.* 1998; Gottfried *et al.* 2002; Tramontina *et al.* 2002). We found a higher level of GFAP at 90 DIV than that reported at 40 DIV (Papadopoulos *et al.* 1998; Gottfried *et al.* 2002). Our longer term astrocyte cultures could explain the major increase of GFAP expression. The expression of S100β at 90 DIV was slightly higher or in the same range than in the previous works (Gottfried *et al.* 2002; 40 DIV; Tramontina *et al.* 2002; 21–56 DIV). We have not determined the S100β secretion from the aged astrocytes *in vitro*, but the latter authors demonstrated a similar profile between cell accumulation and diminution of secretion from 7 to 56 DIV. In animal models of premature aging, such as senescence accelerated prone mice, increases in activated astrocytes overexpressing S100β and GFAP have been observed (Griffin *et al.* 1998; Wu *et al.* 2005). In the aging brain, astrocyte hyperplasia and hypertrophy occur in several areas, accompanied by an elevated content of GFAP and S100β (Amenta *et al.* 1988; Sheng *et al.* 1996; Nichols 1999). Furthermore, it is known that some activated phenotypes of aging glia are maintained *in vitro*, as overexpression of GFAP has been observed in cultures of astrocytes from aging cerebral cortex (Rozovsky *et al.* 1998). We also detected the presence of senescence β-galactosidase stain in 90-DIV cultured astrocytes. β-Galactosidase has been detected in cultured cells undergoing replicative senescence without proliferation (Dimri *et al.* 1995). It has also been found in aged brains after ischaemia (Takada *et al.* 2003). However, its cellular role is not known (Lee *et al.* 2006). The present article shows that astrocytes cultured for 90 DIV acquire aging and activation characteristics like those observed in the astrocytes of aged animals.

Glial fibrillary acidic protein and S100β overexpression has been related to enhanced oxidative stress. *In vitro*, Morgan *et al.* (1997) have shown up-regulation of the astrocyte GFAP gene by oxidative stress. Several authors have demonstrated that treatment of astrocyte cultures with

S100β results in activation of iNOS and NO release via the NFκB pathway (Hu *et al.* 1997; Lam *et al.* 2001). In this study, we observed an increase in ROS generation, accompanied by a remarkable enhancement in nitrated proteins in long-term astrocyte cultures. Nitration of tyrosine residues in cellular proteins is a good marker of oxidant generation derived from NO. Superoxide radicals react with NO to form the peroxynitrite radical, and this radical nitrates tyrosyl residues in proteins (Beckman 1996). We found an increase of iNOS protein expression in aged astrocytes indicative of NO production in these cultures. *In vivo* studies have reported controversial results on iNOS expression in cerebral cortex of aged rats (Uttenthal *et al.* 1998; Liu *et al.* 2004). NO-induced nitrosative stress can inhibit the mitochondrial respiratory chain in astroglial cell cultures. This leads to lower cellular energy (Calabrese *et al.* 2005). In this respect, we showed reduced mitochondrial activity in 90-DIV astrocyte cultures, probably derived from oxidative damage in mitochondria. Mitochondria are the major source of free radicals and may be also the major target for their damaging effects (Balaban *et al.* 2005). Several evidences suggest that mitochondrial oxidative stress is a cause of cell aging (Lee and Wei 2001; Sastre *et al.* 2003). For instance, it has been reported that decreased activities of mitochondrial complex enzymes were negatively associated to oxidative damage in brains from old mice (Kwong and Sohal 2000; Navarro 2004). Recently, lower membrane potential has been reported in astrocyte cultures from old mice (Lin *et al.* 2006). On the contrary, it has been suggested that chronic iNOS induction in astrocytes may lead to a decrease in GSH trafficking from astrocytes to neurones, thus preventing neuronal protection (Heales *et al.* 2004). In the present study, we examined the survival of cortical neurons at 2 weeks after coculturing with 10- and 90-DIV astrocytes. Long-term astrocytes were less able to protect cortical neurons than young astrocytes. This effect was partially counteracted by the addition of antioxidants to the cultures. This suggests that astrocyte's loss of antioxidant capacity may be one of the underlying factors behind the lack of neuroprotection in aging and age-related neurodegeneration. Accordingly, Lin *et al.* (2006) have demonstrated reduced neuroprotective ability of old astrocytes in cocultures with PC12 cells exposed to oxidative damage.

The immunoblotting studies revealed overexpression of HO-1 in 90-DIV astrocyte cultures. HO-1 expression in neurons and glia has been shown to increase against oxidative stress and traumatic injury (Fukuda *et al.* 1996; Dore *et al.* 1999; Llesuy and Tomaro 1994). HO-1 immunoreactivity is also augmented in brain aging and in age-related neurodegenerative diseases (Iijima *et al.* 1999; Schipper 2004). In general, this HO-1 overexpression has been considered as a neuroprotective response by generating the radical scavengers biliverdin and bilirubin. However, the chronic increase of HO-1 expression in astroglia in the temporal cortex and hippocampus has been associated with neurofibrillary pathology, specific cognitive deficits in Alzheimer's disease (AD), and mild cognitive impairment (MCI) (Schipper *et al.* 2006). These authors suggested that glial HO-1 up-regulation in MCI may produce mitochondrial damage. Thus, this would contribute to the early development of certain neuropathological characteristics of AD. This proposal is based on *in vitro* studies, in which overexpression of HO-1 in rat astrocytes promotes mitochondrial iron sequestration (Schipper *et al.* 1999); free radical-induced MnSOD expression (Frankel *et al.* 2000); and oxidative damage to mitochondrial lipids, proteins and, nucleic acids (Song *et al.* 2006). Accumulation of free ferrous iron generated from elevated HO-1 activity could result in a loss of cytoprotection (Suttner and Dennery 1999). Papadopoulos *et al.* (1998) detected iron staining and an increase in ferritin immunoreactivity in older astrocytes. In this article, HO-1 overexpression in astrocyte cultures may be early induced by increased generation of ROS. However, sustained HO-1 expression in long-term cultures would exacerbate alterations in mitochondrial activity and protein oxidation with detrimental consequences on astrocyte functions.

Antioxidant enzymes in astrocytes are considered to be a primary defence mechanism that protects neurons from oxidative damage. In our study, we have shown up-regulation of SOD-1 expression in astrocyte cultures with age. This result is in accordance with previous studies where increases in SOD and catalase activities were observed in cortical astrocytes cultured for 34–47 DIV compared with 7–13 DIV cultures (Papadopoulos *et al.* 1998; Klamt *et al.* 2002). In these studies, aged astrocytes had increased vulnerability to oxidative injury, and also elevated levels of lipoperoxidation and protein oxidation relative to young astrocyte cultures. As peroxidative stress has been shown to induce SOD activity (Benzi and Moretti 1995), the ROS generated in the initial periods of cultured astrocytes may induce SOD-1 overexpression as a protective mechanism. However, if this sustained response is not accompanied by elevated glutathione peroxidase (GPX) activity, the generation of hydrogen peroxide may increase with age, producing adverse effects. In fact, elevated levels of SOD-1 relative to GPX have been reported in aged murine brain (de Haan *et al.* 1992; Leutner *et al.* 2001). In addition, it has been shown that an altered antioxidant ratio SOD-1/GPX in cell lines leads to senescence-like changes (de Haan *et al.* 1996). On the contrary, in a recent paper, Son *et al.* (2005) demonstrated that a sequential induction of HO-1 and Mn-SOD could protect cultured astrocytes from a toxic dose of NO. In this sense, we cannot discard that simultaneous overexpression of HO-1 and SOD proteins may play a cytoprotective role under acute oxidative stress conditions. However, the data presented here suggest that the increase in HO-1 and SOD-1 expression in 90 DIV cultures may be detrimental, or at least is not enough to prevent the elevated levels of oxidative

stress shown in these cultures and to maintain a normal neuroprotective capacity.

Astrocytes have the ability to clear glutamate from the extracellular space, thus preventing neuronal excitatory damage. In the present article, the uptake of glutamate from the extracellular medium was evaluated for both of the astrocyte culture ages. Ninety-DIV cultures showed an increase in glutamate uptake. This was accompanied by overexpression of the glial GLAST. Gottfried *et al.* (2002) also demonstrated an increase in basal glutamate uptake in 40-DIV astrocyte cultures. We can consider that continuous increase in glutamate release in 90-DIV astrocytes may overexpress glutamate transporters and consequently increase glutamate uptake. In fact, Duan *et al.* (1999) revealed that glutamate could induce up-regulation of glutamate transport in murine cortical astrocyte cultures. *In vivo*, several studies have reported decreases in glutamate uptake in the cerebral cortex of aged rats when using neuronal synaptosomal preparations (Segovia *et al.* 2001). However, glutamate uptake was not modified in brain cortical slices of aged rats, with both neurons and astrocytes present (Dawson *et al.* 1989). Therefore, we can assume the presence of differential responses of neuronal and glial glutamate transport in brain aging. A reduced glutamate uptake in neurons together with an increased uptake in astrocytes may maintain normal synaptic glutamate concentration during aging. Indeed, extracellular glutamate brain concentrations have been reported unchanged during aging (Segovia *et al.* 2001).

Glutamate taken up by astrocytes is transformed into glutamine by the enzyme glutamine synthetase or is oxidized in the tricarboxylic acid (TCA) cycle (Bak *et al.* 2006). It has been reported that the metabolic fate of glutamate is concentration dependent, and the proportion of glutamate oxidized increases several times with higher extracellular concentration (Dienel and Cruz 2006). Therefore, glutamate derived from an increased uptake in aged astrocytes would have a higher entrance on the glutamate/TCA pathway than in the glutamate/GABA–glutamine cycle. However, an improvement on the metabolic status of the aged astrocyte is prevented by the decreased metabolic mitochondrial activity in these cells, as measured by MTT (mainly indicative of succinate-dehydrogenase activity, Slater *et al.* 1963).

Our results showed that glutamate uptake in young astrocytes was inhibited by acute H_2O_2 treatment, according to previous studies in cortical astrocyte cultures (Volterra *et al.* 1994; Trotti *et al.* 1997). However, Gottfried *et al.* (2002) have found higher efficacy of H_2O_2 in inhibiting glutamate uptake in astrocyte cultures. Differences in animal strain or culture preparation methods – primary or secondary (replated) astrocytes – may account for these effects. Furthermore, similarly to previous studies, we found higher vulnerability of aged astrocytes to glutamate uptake inhibition by H_2O_2 (Miralles *et al.* 2001; Gottfried *et al.* 2002). Although these cells presented a high vulnerability to oxidative stress, the free-radical inhibitory effect on their glutamate transporters could still be reverted by the antioxidant agents: propyl gallate, trolox and curcumin. It is tempting to hypothesize that in circumstances with elevated oxidative stress, as in age-related neurodegenerative processes, the inhibition of glutamate uptake into astrocytes may increase the extracellular accumulation of glutamate. This may enhance the vulnerability of neurons to excitotoxicity. A decline in glutamate uptake in the cerebral cortex has been reported in AD. However, no correlation was found with the levels of glial glutamate transporters (Beckstrom *et al.* 1999; Westphalen *et al.* 2003).

In conclusion, we have shown that astrocytes cultured for 90 DIV acquire characteristics of senescence and activation like those observed in astrocytes of aged animals. Increased ROS generation and nitrosative stress occur, which leads to decreased mitochondrial activity with age. Overexpression of HO-1 and SOD-1 proteins may be an initial defence response against increased ROS generation. However, sustained up-regulation of both proteins may have detrimental consequences on astrocyte functions. In our study, aged astrocytes had a reduced ability to maintain neuronal survival. This is probably at least partly because of a loss of their antioxidant capacity, as neuronal survival was partially recovered by antioxidant agents. All these results suggest that astrocytes have a major role in brain aging progression. In addition, we observed increased glutamate uptake in aged astrocytes. This may initially counteract the reduced neuronal glutamate uptake observed in brain aging. Nevertheless, the higher vulnerability of H_2O_2-induced glutamate uptake inhibition observed in aged astrocytes suggest that astrocytes are involved in the neuronal excitotoxicity that underlies age-related neurodegenerative processes. Altogether, these findings suggest that astrocytes partially lose their neuroprotective ability during brain aging and contribute to exacerbating neuronal injury in age-related neurodegenerative processes.

Acknowledgements

This study was supported by grants from FIS 03/0467 and Red CIEN V-2003-REDC10F-C from the Spanish Ministry of Health's Fondo de Investigaciones Sanitarias (FIS) and the Generalitat de Catalunya's DURSI 2005/SGR/00826. M. Pertusa and S. García-Matas received an IDIBAPS and a Generalitat de Catalunya fellowship, respectively. We thank A. Parull for his skilful technical assistance.

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Neurobiology of Aging 29 (2008) 1366–1379

NEUROBIOLOGY OF AGING

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Expression of GDNF transgene in astrocytes improves cognitive deficits in aged rats

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Received 26 September 2006; received in revised form 14 February 2007; accepted 20 February 2007
Available online 30 March 2007

Abstract

Glial cell line-derived neurotrophic factor (GDNF) was assayed for its neurotrophic effects against the neuronal atrophy that causes cognitive deficits in old age. Aged Fisher 344 rats with impairment in the Morris water maze received intrahippocampal injections at the dorsal CA1 area of either a lentiviral vector encoding human GDNF or the same vector encoding human green fluorescent protein as a control. Recombinant lentiviral vectors constructed with human cytomegalovirus promotor and pseudotyped with lyssavirus Mokola glycoprotein specifically transduced the astrocytes in vivo. Astrocyte-secreted GDNF enhanced neuron function as shown by local increases in synthesis of the neurotransmitters acetylcholine, dopamine and serotonin. This neurotrophic effect led to cognitive improvement of the rats as early as 2 weeks after gene transduction. Spatial learning and memory testing showed a significant gain in cognitive abilities due to GDNF exposure, whereas control-transduced rats kept their performance at the chance level. These results confirm the broad spectrum of the neurotrophic action of GDNF and open new gene therapy possibilities for reducing age-related neurodegeneration.

Keywords: Glial cell line-derived neurotrophic factor (GDNF); Lentiviral vector; Aging; Learning and memory; Gene therapy; Acetylcholine; Dopamine; Serotonin; Rat

1. Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor β superfamily. It was first isolated from culture medium of rat B49 glioblastoma cells in a search of trophic factors for midbrain dopaminergic neurons (Lin et al., 1993, 1994). GDNF has remarkable regenerative and restorative effects upon nigrostriatal dopaminergic neurons (Bowenkamp et al., 1997; Gash et al., 1996; Gill et al., 2003). Soon after its discovery, it was found that GDNF is a potent neurotrophic factor active on a broad spectrum of neuronal types and has neuroprotective effects in several experimental paradigms of nerve cell injury. For instance, GDNF reduces neuronal damage in axotomized neurons (Henderson et al., 1994; Oppenheim et al., 1995; Williams et al., 1996; Yan et al., 1995), in experimental brain trauma (Bakshi et al., 2006) and ischemia (Cheng et al., 2005; Kilic et al., 2003; Miyazaki et al., 1999; Wang et al., 1997), and in several excitotoxic (Martin et al., 1995; Nicole et al., 2001; Pérez-Navarro et al., 1996; Wong et al., 2005) and neurotoxic injuries modeling neurodegenerative diseases (Arenas et al., 1995; Ghribi et al., 2004; Ugarte et al.,

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0197-4580/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.neurobiolaging.2007.02.026

2003). Therapeutic GDNF administration has raised expectations that it could be a preventive or palliative treatment for Parkinson's disease (for review see: Eslamboli, 2005; Fernández-Espejo, 2004), amyotrophic lateral sclerosis (for review see: Bohn, 2004; McGeer and McGeer, 2005) and Huntington's disease (for review see: Alberch et al., 2004).

GDNF is expressed in both neurons and astrocytes (Lin et al., 1993; Marco et al., 2002; Nicole et al., 2001; Schaar et al., 1993). It signals through the two-component receptor complex GFRα-1/Ret (Treanor et al., 1996), also expressed in both cell types (Nicole et al., 2001). An increase in astrocyte GDNF synthesis and protein expression is believed to play an active role in neuron survival and plasticity after ischemia (Miyazaki et al., 2001; Tokumine et al., 2003) and excitotoxic damage (Ho et al., 1995; Marco et al., 2002). Accordingly, experimental strategies of GDNF delivery by astrocytes have shown to be neuroprotective in vivo for motor neurons (Parsadanian et al., 2006; Zhao et al., 2004) and dopaminergic neurons (Cunningham and Su, 2002; Do Thi et al., 2004; Ericson et al., 2005).

Endogenous GDNF plays an important role in cognitive abilities as demonstrated by the impaired spatial learning performance of mice that are heterozygous for a targeted deletion of the GDNF gene (Gerlai et al., 2001). Spatial learning and memory is associated with intact hippocampus function and it has long been known that memory deficits in old age are similar to those produced by bilateral hippocampal lesions (Broadbent et al., 2004; Good, 2002). Different categories of agents, such as neurotrophins (Fischer et al., 1991, 1994; Martínez-Serrano et al., 1996), antioxidant agents (Levin et al., 2002; Nishiyama et al., 1997) and cholinergic drugs (Bontempi et al., 2003; Hernández et al., 2006), have been assayed in aged rodents in a search for effective and harmless treatments that reverse the memory loss of normal aging and ameliorate memory loss in pathological aging caused by Alzheimer's disease. The potential of GDNF against age-related cognitive deterioration has not been fully explored. In a previous study, spatial learning and memory improved in aged rats after i.c.v. administration of GDNF but no brain regional correlates were analyzed (Pelleymounter et al., 1999). We studied the restorative effects of GDNF on spatial learning and memory after local delivery into the hippocampus. For this purpose, the astrocytes of the dorsal CA1 hippocampal area of cognitively impaired aged Fisher 344 rats were transduced in vivo with a lentiviral vector expressing the human gene for GDNF. The efficacy of transgene delivery, either GDNF or the human green fluorescent protein (GFP) used as a control, and the behavioral and neurotransmitter changes shown by the aged rats were determined throughout the study. Brain regional GDNF gene expression allowed the evaluation of the involvement of the hippocampal CA1 function in maintaining spatial learning and memory through aging. Vector specificity for the GDNF gene transfer to astrocytes enabled the study of the neuroprotective potential of these glial cells in neurodegenerative processes.

2. Methods

2.1. Animal groups

Thirty male 7-month-old Fisher 344 rats retired from breeding were purchased from Charles River (Lyon, France) and kept in the animal house of the University of Barcelona until they were old. They were maintained in standard conditions of temperature and humidity, with two animals per cage, a 12-h light:12-h dark cycle, and food and water ad libitum. Only animals showing a healthy general status at 22 months of age were included in the study. A group of 10 young male rats of the same origin were used to test the viral stock. A further group of 10 6-month-old male Fisher 344 rats were included in the study for comparative neurotransmitter determinations. All handling and experimental procedures were approved by the Animal Ethics and Health Committees of the University of Barcelona.

2.2. Lentiviral vectors

A recombinant lentiviral vector encoding human GDNF was constructed for the specific transduction of astrocytes in vivo. The combination of the lyssavirus Mokola glycoprotein (Mokola G) pseudotype with the human cytomegalovirus promoter (CMV) allowed efficient transgene expression under these experimental conditions. A vector encoding GFP instead of GDNF was used as a control vector. Vectors were constructed and produced as previously described (Bemelmans et al., 2005; Naldini et al., 1996). Briefly, the plasmids Flap-CMV-GDNF-WPRE and Flap-CMV-GFP-WPRE were derived from the lentiviral vector genome. These plasmids contain the backbone of the lentiviral genome including the central Flap sequence (Zennou et al., 2000). The GDNF and GFP transgenes were under control of the CMV promoter. The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was added 3′ to the GDNF or GFP gene to increase transgene expression (Zufferey et al., 1999). Lentiviral vector stocks were produced by transient co-transfection of 293 T cells with the vector genome plasmid, a packaging plasmid and a plasmid coding for the envelope that included the Mokola G gene. The cell supernatant for each transgene vector was harvested 48 h post-transfection, treated with DNase, filtered through 0.45 μm pore-sized filters, and subjected to ultra-centrifugation at 64,000 × *g* for 90 min. The virus pellet was resuspended in phosphate-buffered saline (PBS) and its physical titer was quantified by ELISA of the p24 capsid protein. Viral stocks were kept at −80 °C until use. At that moment, stocks were diluted with PBS to 30 ng/μl of p24 protein.

2.3. Behavioral testing

The behavioral test for spatial learning and memory was performed in a Morris water maze (Morris, 1981). The apparatus was modeled as described elsewhere (Chamizo et al.,

2006). Briefly, it measured 1.60 m in diameter and water was made opaque by the addition of 100 mL/L of latex. Water temperature was maintained at 22 ± 1 °C. Black curtains surrounded the pool. Inside the black enclosure four landmarks hanging from a false black ceiling defined the position of the hidden platform: (A) a 40-W fixed light; (B) a beach ball with horizontal color drawings; (C) a vertical white structure with three truncated cones of decreasing size; and (D) three silver bottles bound by their neck. Landmark objects were hung at 90° distance. The platform had a diameter of 0.11 m and was placed between landmarks A and B, 1 cm below the surface and 0.38 m from the side. The landmarks and the platform were semi-randomly rotated with respect to the room (90°, 180°, 270°, 360°) in a daily-equated way in order to ensure that the rats used these landmarks, rather than any inadvertently remaining static room cue, to locate the platform. A closed-circuit video camera recorded rat movements that were analyzed by computer. A pre-training test with the platform made visible, located 1 cm above the water surface, consisted of 8 trials during 2 days. The rat was given 90 s to find the platform, and then allowed to stay on for 30 s. When the rat did not reach the platform, it was picked up, placed on it and left there for 30 s. Rats that failed to find the visible platform were discarded from the study. For the acquisition test, the procedure was the same, but animals had to rely on the landmarks to find the hidden platform. Animals were given 4 escape trials per day for 12 days, a total of 48 trials. Latencies to reach the escape platform were recorded for each trial and averaged for the 4 daily trials. On the last day, a 60 s probe trial was performed with the platform removed. To analyze the rat's behavior, the surface of the pool was divided into four quadrants and the time that the rat spent in the quadrant where the platform was previously was calculated. Impaired aged rats were defined as those spending a time not different from chance performance (15 s out of 60 s) in the right quadrant during the spatial probe trial. Two weeks later, the impaired animals were divided in two groups that were treated for hippocampal astrocyte expression of GFP and GDNF, respectively (see surgical procedures below). Test assays were initiated 2 weeks after surgery, when rats were nearly 24 months old. Animals were given 4 escape trials per day and on the fourth day a probe trial was performed. This procedure continued, with a probe trial every 4 days until 48 escape trials had been conducted. Behavioral results for rats that did not survive until the end of the study were not included.

2.4. Surgical procedures

A total of 23 aged rats underwent surgery: 7 rats of the GFP group, 8 rats of the GDNF group and 8 unimpaired rats. Animals were anesthetized with 10 mg/kg xylacine (Rompun 2%, Bayer) and 80 mg/kg ketamine (Ketolar 50 mg/ml, Pfizer) i.m., and placed in a stereotactic apparatus. Bilateral infusions were performed into the CA1 area of the hippocampus at the coordinates: anterior–posterior −3.8 mm, medio-lateral +/−2 mm and dorso-ventral −2.4 mm, from bregma and dural surface. The right coordinates to reach were previously checked in 3 animals discarded from the study. One microlitre of the vector solution was delivered to the application point with a 25-gauge stainless steal cannula (Small Parts Inc., Miami, FL) connected to a Hamilton syringe through a Teflon tube. The syringe was attached to a micro-infusion pump (Bioanalytical systems Inc., West Lafayette, IN). The cannula was left in position for 5 min after delivery to prevent the solution from surging back.

2.5. Brain dissection and histology

After completion of the behavioral studies, animals were decapitated under light anesthesia with 40 mg/kg ketamine. Brains were quickly removed, immersed in cold saline solution and placed in a Kopf brain blocker. The cerebrum was cut into three blocks and then bisected sagittaly at the midline. A block containing the half dorsal hippocampal area of selected animals from the GFP group was fixed by immersion in 10% phosphate buffered formaldehyde, cryoprotected with 10% sucrose and frozen on a stainless steel plate over dry ice. Slices 16 μm thin were double-stained with the antibodies against GFP 1:1000 (polyclonal, Abcam, Cambridge, UK) and GFAP 1:400 (monoclonal, clone GA5, Sigma, St. Louis, MO), as previously described (Bemelmans et al., 2005). Secondary antibodies used were Alexa Fluor 488, 1:1000 and Alexa Fluor 546, 1:1000, respectively (Molecular Probes, Eugene, OR). Nuclei were counterstained with bisbenzimide (Sigma). For all other animals, both hemispheres were further dissected on a chilled glass plate, according to the procedure described in detail elsewhere (Baxter et al., 1999). Tissue samples for 6 hippocampal areas (dorsal and ventral CA1, dorsal and ventral CA2/3, dorsal and ventral dentate gyrus), 5 cerebral cortical areas (cingulate, frontal, temporal, entorhinal, parietal), septum and caudate (Paxinos and Watson, 1986), were weighed and frozen at −80 °C until analysis.

2.6. GDNF determination

The level of GDNF expression of the viral stock under the injection conditions used was tested in a group of 10 young rats of the same colony used for the experiment. A microinjection of the GDNF vector was performed in the dorsal CA1 area of the right hippocampus as described above. GFP vector was injected on the left side of half the animals and the other half were untreated. Rats were killed a week later, and the hippocampus dissected as described above. Regional tissue levels of GDNF were determined by ELISA with the GDNF E_{max} ImmunoAssay System kit (Promega), following the manufacturer's instructions. Long-term stability of this viral stock was previously checked and expression was stable for 1 year (unpublished data).

2.7. Choline acetyl transferase and monoamine neurotransmitter determinations

Choline acetyl transferase (ChAT) activity in the tissue samples was measured by the formation of ^{14}[C] acetylcholine from ^{14}[C]acetylcoenzyme-A (60 mCi/mmol; New England Nuclear, Perkin-Elmer, Wellesley, MA) and choline (Sigma). For this purpose, homogenates from left cerebral hemisphere regions were processed following the procedure described elsewhere (Araujo et al., 1990).

The monoamine neurotransmitters, dopamine and serotonin, the acid metabolites of dopamine 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) and the acid metabolite of serotonin 5-hydroxiindoleacetic (5-HIAA) were analyzed in the right hemisphere tissue samples of GFP, GDNF and young rat groups. Tissue samples were homogenized in a cold solution of 0.4 M perchloric acid, 0.01% EDTA, 0.1% sodium bisulfite and 0.01% cystine and centrifuged at 12,000 × *g* for 30 min. The concentrations of neurotransmitters and metabolites in supernatants were determined by HPLC using a 3-μm octadecylsilica (ODS) column (7.5 cm × 0.46 cm; Beckman) and detected amperometrically with a Hewlett-Packard 1049 detector set at oxidation potentials of 0.6 V (serotonin and 5-HIAA) and 0.7 V (dopamine, DOPAC and HVA). The mobile phase for 5-HT consisted of 0.15 M NaH_2PO_4, 1.8 mM octyl sodium sulfate, 0.2 mM EDTA (pH 2.8 adjusted with phosphoric acid) and 30% methanol. The mobile phase for dopamine consisted of 0.15 M NaH_2PO_4, 0.46 mM octyl sodium sulfate, 0.5 mM EDTA (pH 2.8 adjusted with phosphoric acid), and 18% methanol. Both mobile phases were pumped at 0.7 mL/min. Quantitative analyses were performed by the external standard method.

2.8. Statistics

Results are expressed as mean ± S.E.M. Statistical evaluation was performed with ANOVA procedures, followed by Bonferroni's or Newman–Keules's multi-range test for comparison of the mean group values.

3. Results

3.1. Astrocyte transduction with GFP and GDNF

The injection of lentiviral particles aimed at the dorsal CA1 hippocampal area yielded a wide GFP expression mainly in the stratum radiatum and also in the oriens layer of this area. No expression was detected outside the hippocampus. Transduced proteins were selectively expressed in astrocytes as demonstrated by overlapping of GFP and GFAP immunostaining whereas no GFP expression was detected in the neurons of the pyramidal molecular layer (GFP transduced rats, Fig. 1a–g). In a parallel study, we have confirmed the astrocyte-specific expression of the GFP reporter gene with this vector in several animal species (mouse, rat and non-human primate; Brizard et al., unpublished observations).

The ability of the GDNF-engineered lentiviral vector to drive a detectable production of GDNF was previously confirmed in a separate group of animals. GDNF levels determined by ELISA were higher in the dorsal CA1 trans-

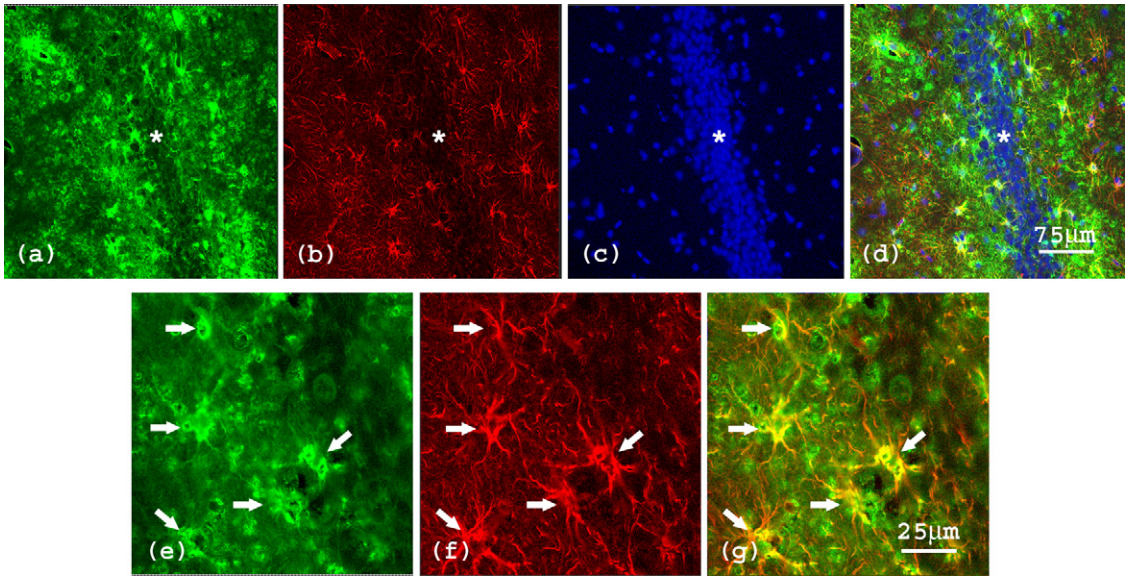

Fig. 1. Transgene expression of GFP driven by lentiviral vectors in the brain of aged rats. Confocal micrographs of a representative transduced CA1 region (a–d) show the immunostaining for GFP (green), for GFAP (red), the nuclei stained with bisbenzimide (blue) and triple staining, respectively. Asterisk indicates the pyramidal layer, with the oriens layer on the right and the stratum radiatum on the left of the images. Note that no GFP was detected in the neuron cell bodies of the pyramidal layer. At higher magnification, the cells secreting GFP (e) can be identified as GFAP immunostained astrocytes (f), showing in these cells double labeling (g) (see arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Levels of GDNF after lentiviral transduction with hGDNF gene

	GDNF (pg/mg tissue)		
	GDNF ($n=7$)	GFP ($n=3$)	Naïve ($n=3$)
Hippocampus: Dorsal			
CA1	288.1 ± 54.6[a,b]	5.6 ± 3.6	9.8 ± 3.9
CA2/3	173.3 ± 48.9[a,c]	4.1 ± 1.8	6.5 ± 0.8
Dentate gyrus	52.5 ± 20.1	4.0 ± 1.1	6.3 ± 1.8
Hippocampus: Ventral			
CA1	96.2 ± 44.7	7.0 ± 3.0	7.9 ± 1.2
CA2/3	58.5 ± 23.5	2.8 ± 0.7	4.9 ± 2.2
Dentate gyrus	15.8 ± 4.0	5.5 ± 1.3	3.3 ± 1.1

Experimental groups: GDNF, right hippocampus of young rats transduced with human glial cell line derived neurotrophic factor in the dorsal CA1 area; GFP, left hippocampus transduced with the control vector expressing human green fluorescent protein in the dorsal CA1; naïve, untransduced left hippocampus. Results are the mean ± S.E.M., $n=7$, 3 and 3, for GDNF, GFP and naïve group, respectively. Statistics: (a) significance of GDNF group as compared to GFP or naïve group, (b) significance of dorsal CA1 as compared to all the other regions, (c) significance of dorsal CA2/3 as compared to ventral dentate gyrus; ANOVA followed by Newman–Keules' test at the significance level of $p<0.05$.

duced with GDNF than in the GFP-transduced or untreated CA1 area of the contralateral side (Table 1). A lower GDNF over-expression was detected in the ipsilateral adjacent area CA2/3.

3.2. Improved performance of GDNF injected rats in the Morris water maze

Timing of the behavioral studies is indicated in Fig. 2a. The aged animals were clearly classified as non-impaired or learning-impaired, according to the results of the pre-test spatial probe trial (Fig. 2b, probe trial 0). Non-impaired rats spent 27.8 ± 3.6 s ($n=8$) and impaired rats spent 15.5 ± 4.3 s ($n=15$) out of the 60 s in the pool quadrant that previously contained the platform ($p<0.0001$ and $p=0.6746$ as against to chance value of 15 s, respectively). During prior training, latencies to find the hidden escape platform decreased steadily without significant group differences (two-way ANOVA, effect of day factor: $F(11,211)=4.915$, $p<0.0001$) (Fig. 2c).

In the spatial probe trials after surgery, the GDNF group showed steady learning improvement that was not present in the GFP group (Fig. 2b, probe trials 1–3). The latter rats kept their values of searching time in the correct quadrant at the chance level. By the end of the test-training period, GDNF rats showed values close to the unimpaired control group. The performance differences between GFP- and GDNF-injected rats were significant (ANOVA, $F(1,44)=9.869$, $p=0.0030$). No differences of latency to find the hidden platform during the 12 training days were detected between the two rat groups (Fig. 3d).

Swimming speed was not affected by either the surgery or the transgene expression. Pre-surgery values recorded

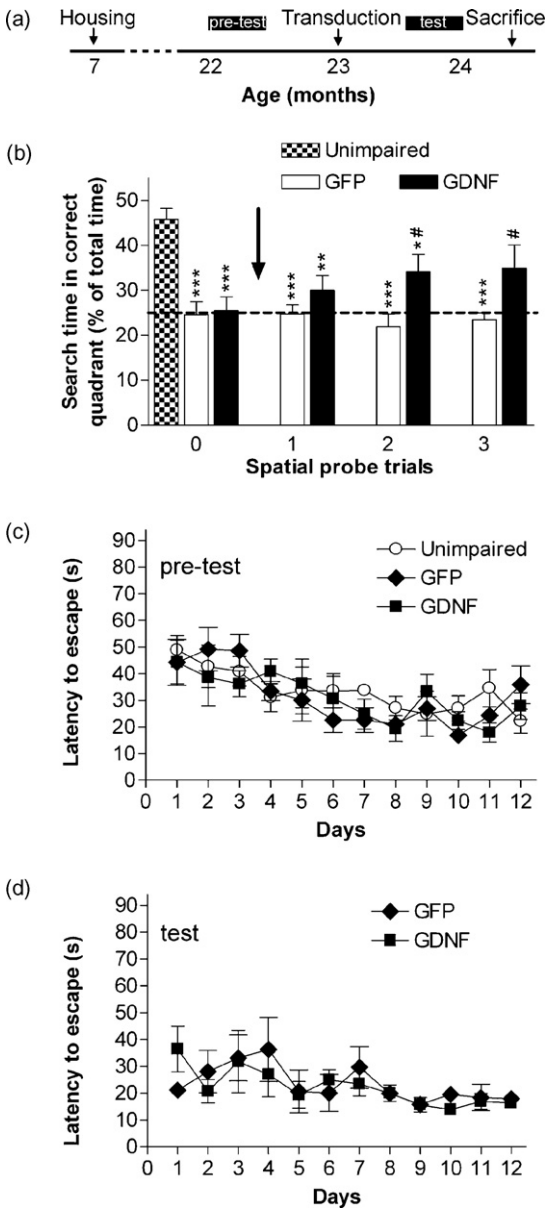

Fig. 2. Behavioral testing in the Morris water maze. (a) Experimental design scheduled for the aged rats, with pre-test and test indicating the behavioral studies performed before and after the surgical injection of the viral vectors, respectively. (b) Spatial probe trial results distinguished impaired and unimpaired rats in the pre-test (trial 0) and showed the steadily better performance of GDNF rats in the test (trials 1–3). Dotted line indicates chance performance. Arrow indicates surgery. Values are the mean ± S.E.M., $n=6$–8, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$, as compared with unimpaired rats; and $^{\#}p<0.05$, as compared to the GFP group; ANOVA followed by Newman–Keules' test. (c) The latencies to escape during the pre-test training did not differ between GFP and GDNF rat groups. Values are the mean ± S.E.M., $n=6$–7. (d) The latencies to escape during the test training did not differ, either, between GFP and GDNF rat groups, Values are the mean ± S.E.M., $n=6$–7.

in the pre-test probe trial were 21.58 ± 1.24 cm/s for the non-impaired rats, 21.61 ± 0.69 cm/s for the GFP group and 21.83 ± 0.81 cm/s for the GDNF group. Final swim speed measured in the last test probe trial was 22.73 ± 0.69 cm/s

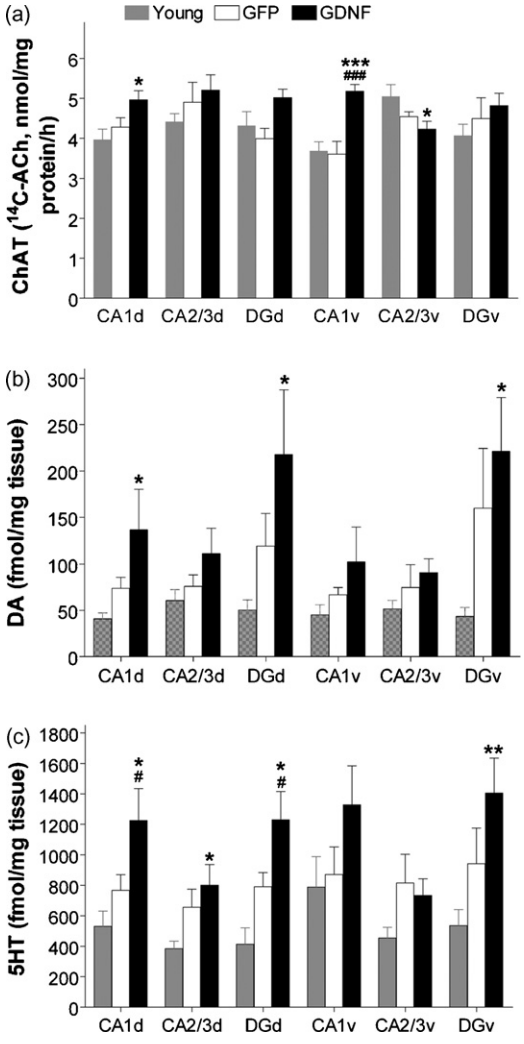

Fig. 3. Hippocampal regional concentration of neurotransmitters. (a) Choline acetyl transferase (ChAT) activity, indicative of acetylcholine levels, and (b) dopamine and (c) serotonin tissue levels. Young, 8-month-old rats; GFP, 24-month-old cognitively impaired rats transduced with human green fluorescent protein in the dorsal CA1 area; GDNF, 24-month-old cognitively impaired rats transduced with human glial cell line derived neurotrophic factor in the dorsal CA1. Numeric values are detailed in Tables 2–4, respectively. Values are the mean ± S.E.M., $n = 6$–8, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, as compared with young rats; and $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ as compared to the GFP group; ANOVA followed by Newman–Keules' test.

and 21.89 ± 1.07 cm/s for the GFP and the GDNF groups, respectively.

3.3. Increase of neurotransmitter levels in the hippocampus after GDNF transduction

Tissue concentration of ChAT, indicative of acetylcholine levels, in selected brain regions of young and aged impaired rats is shown in Fig. 3a (hippocampus areas) and Table 2 (all brain areas assayed). Two-way ANOVA indicated an effect of the cerebral brain region ($F(12,180) = 14.50$, $p < 0.0001$) and of the rat group ($F(2,180) = 7.56$, $p = 0.0007$). By paired ANOVA testing, GDNF rat group was significantly different

Table 2
Brain regional activity of choline acetyl transferase

	ChAT (^{14}C-ACh nmol/mg protein/h)		
	Young	GFP	GDNF
Hippocampus: Dorsal			
CA1	3.97 ± 0.26	4.28 ± 0.23	4.97 ± 0.23[a]
CA2/3	4.42 ± 0.20	4.90 ± 0.50	5.21 ± 0.38
Dentate gyrus	4.32 ± 0.35	3.99 ± 0.26	5.02 ± 0.21
Hippocampus: Ventral			
CA1	3.69 ± 0.23	3.61 ± 0.32	5.18 ± 0.17[a,b]
CA2/3	5.05 ± 0.29	4.54 ± 0.12	4.23 ± 0.19[a]
Dentate gyrus	4.07 ± 0.28	4.50 ± 0.52	4.82 ± 0.31
Cortex			
Cingulate	3.60 ± 0.25	4.22 ± 0.39	5.83 ± 0.58[a,b]
Frontal	3.36 ± 0.30	3.26 ± 0.14	4.06 ± 0.46
Parietal	3.00 ± 0.27	3.46 ± 0.28	4.09 ± 0.81
Temporal	3.03 ± 0.24	3.20 ± 0.39	3.24 ± 0.45
Entorhinal	3.64 ± 0.24	3.31 ± 0.08	3.38 ± 0.17
Septum	6.76 ± 0.76	5.10 ± 0.12	5.47 ± 0.38
Caudate	1.82 ± 0.32	3.22 ± 0.69	2.33 ± 0.27

Experimental groups: Young, 8-month-old rats; GFP, 24-month-old cognitively impaired rats transduced with human green fluorescent protein in the dorsal hippocampus CA1; GDNF, 24-month-old cognitively impaired rats transduced with human glial cell line derived neurotrophic factor in the dorsal hippocampus CA1. Values are the mean ± S.E.M. of 6–9 animals. Statistics: (a) significance of GFP or GDNF groups as compared to young rats and (b) significance of GDNF as compared to GFP group; ANOVA followed by Newman–Keules' test at the significance level of $p < 0.05$.

from GFP and from young rat groups (not shown). In GDNF transduced rats, an increase was detected in the whole CA1 area, as against GFP rats; whereas the ventral part increased also over young rat values. Cingulate cortex ChAT increased in GDNF rats over the other two groups. There was a decrease in the ventral CA2/3 area of GDNF rats, as against the group of young rats.

Regional brain concentration of dopamine is shown in Fig. 3b (hippocampus areas) and Table 3 (all brain areas assayed) and its metabolites DOPAC and HVA are shown in Table 3. Two-way ANOVA for brain region and rat group indicated an effect of the brain region factor for the concentration of dopamine and its metabolites (not shown), whereas the rat group factor was significant for both metabolites DOPAC ($F(2,321) = 12.98$, $p < 0.0001$) and HVA ($F(2,319) = 71.59$, $p < 0.0001$). The group effect was significant for dopamine levels when the brain regions were restricted to the hippocampus ($F(2,108) = 15.81$, $p < 0.0001$). In this area, the increase of dopamine shown by the GDNF group was significant in the dorsal CA1 area and in the whole dentate gyrus, as against the group of young rats. A decrease of HVA in both aged GFP and GDNF groups was observed in the dorsal CA1 and CA2/3, cerebral cortical areas and septum. In the caudate region of both aged rat groups there was a decrease in both metabolites, DOPAC and HVA.

Regional brain concentration of serotonin is shown in Fig. 3c (hippocampus areas) and Table 4 (all brain areas assayed) and its metabolite 5-HIAA is shown in Table 4. Two-

Table 3
Brain regional concentration of dopamine and its metabolites DOPAC and HVA

	Dopamine (fmol/mg tissue)			DOPAC (fmol/mg tissue)			HVA (fmol/mg tissue)		
	Young	GFP	GDNF	Young	GFP	GDNF	Young	GFP	GDNF
Hippocampus: Dorsal									
CA1	41 ± 6	74 ± 12	136 ± 44[a]	264 ± 87	180 ± 31	293 ± 79	287 ± 43	114 ± 35[a]	122 ± 28[a]
CA2/3	61 ± 12	76 ± 12	111 ± 27	260 ± 73	192 ± 53	229 ± 52	211 ± 16	117 ± 26[a]	123 ± 24[a]
Dentate gyrus	50 ± 11	119 ± 35	217 ± 70[a]	249 ± 91	237 ± 37	428 ± 115	257 ± 65	144 ± 29	173 ± 30
Hippocampus: Ventral									
CA1	45 ± 11	67 ± 8	102 ± 37	157 ± 24	179 ± 24	214 ± 60	201 ± 26	91 ± 12	159 ± 61
CA2/3	52 ± 9	75 ± 25	91 ± 15	264 ± 61	184 ± 75	228 ± 43	254 ± 37	251 ± 103	119 ± 22
Dentate gyrus	43 ± 10	150 ± 64	221 ± 58[a]	255 ± 80	313 ± 122	370 ± 74	259 ± 40	152 ± 41	196 ± 74
Cortex									
Cingulate	193 ± 46	279 ± 58	156 ± 33	533 ± 95	510 ± 128	364 ± 122	633 ± 47	338 ± 86[a]	233 ± 68[a]
Frontal	139 ± 58	132 ± 17	126 ± 40	354 ± 73	233 ± 43	203 ± 46	554 ± 98	164 ± 25[a]	115 ± 12[a]
Parietal	62 ± 9	93 ± 12	114 ± 21[a]	390 ± 113	311 ± 52	223 ± 49	776 ± 183	226 ± 27[a]	163 ± 40[a]
Temporal	701 ± 175	607 ± 149	633 ± 143	672 ± 146	501 ± 143	491 ± 47	590 ± 142	185 ± 39[a]	172 ± 18[a]
Entorhinal	299 ± 90	290 ± 64	436 ± 106	503 ± 89	323 ± 85	444 ± 47	455 ± 95	154 ± 31[a]	184 ± 12[a]
Septum	6702 ± 2156	7085 ± 1803	8081 ± 2165	9474 ± 2485	7844 ± 2300	9907 ± 2728	4437 ± 933	2476 ± 728[a]	2483 ± 562[a]
Caudate	22289 ± 3104	19901 ± 2339	19964 ± 3732	21965 ± 1829	10012 ± 2553[a]	11653 ± 1707[a]	10185 ± 850	3006 ± 613[a]	3040 ± 429[a]

Experimental groups: Young, 8-month-old untransduced rats; GFP, 24-month old cognitively impaired rats transduced with human green fluorescent protein in the dorsal hippocampus CA1; GDNF, 24-month-old cognitively impaired rats transduced with human glial cell line derived neurotrophic factor in the dorsal hippocampus CA1. Values are the mean ± SEM of 6–9 animals. Statistics: (a) significance of GFP or GDNF groups as compared to young rats; ANOVA followed by Newman–Keules' test at the significance level of $p < 0.05$.

Table 4
Brain regional concentration of serotonin and its metabolite 5HIAA

	5HT (fmol/mg tissue)			5HIAA (fmol/mg tissue)		
	Young	GFP	GDNF	Young	GFP	GDNF
Hippocampus: Dorsal						
CA1	531 ± 99	766 ± 104	1225 ± 210[a,b]	2259 ± 313	3097 ± 219	2787 ± 303
CA2/3	383 ± 50	656 ± 118	800 ± 136[a]	1992 ± 264	3045 ± 601	2881 ± 340
Dentate gyrus	413 ± 107	91 ± 93	1230 ± 186[a,b]	1729 ± 169	2888 ± 414[a]	3454 ± 263[a]
Hippocampus: Ventral						
CA1	789 ± 199	871 ± 181	1329 ± 256	2179 ± 236	3229 ± 262[a]	3052 ± 345[a]
CA2/3	454 ± 70	815 ± 188	733 ± 110	2210 ± 259	2799 ± 493	2210 ± 278
Dentate gyrus	535 ± 105	942 ± 235	1405 ± 229[a]	2458 ± 228	2660 ± 539	3268 ± 371
Cortex						
Cingulate	770 ± 152	1300 ± 94[a]	956 ± 143	1913 ± 232	3227 ± 1114	1669 ± 258
Frontal	1251 ± 158	1460 ± 226	1421 ± 203	1822 ± 158	1698 ± 244	1757 ± 221
Parietal	666 ± 136	1099 ± 187	1034 ± 181	1646 ± 203	1875 ± 304	1893 ± 237
Temporal	689 ± 104	1386 ± 313[a]	985 ± 137	1477 ± 208	1978 ± 156	1609 ± 112
Entorhinal	1005 ± 287	1314 ± 224	1614 ± 243	1897 ± 230	2012 ± 234	2344 ± 285
Septum	1384 ± 180	1972 ± 317	2084 ± 276	2963 ± 261	3590 ± 333	3713 ± 724
Caudate	736 ± 99	1352 ± 215[a]	1131 ± 152	2038 ± 153	1991 ± 240	2354 ± 300

Experimental groups: Young, 8-month-old rats; GFP, 24-month-old cognitively impaired rats transduced with human green fluorescent protein in the dorsal hippocampus CA1; GDNF, 24-month-old cognitively impaired rats transduced with human glial cell line derived neurotrophic factor in the dorsal hippocampus CA1. Values are the mean ± S.E.M. of 6–9 animals. Statistics: (a) significance of GFP or GDNF groups as compared to young rats and (b) significance of GDNF as compared to GFP group; ANOVA followed by Newman–Keules's test at the significance level of $p < 0.05$.

way ANOVA showed an effect of brain region (not shown) and rat group for serotonin and 5-HIAA ($F(2,319) = 25.45$, $p < 0.0001$ and $F(2,319) = 13.91$, $p < 0.0001$, respectively). The GDNF group increased serotonin levels in the whole dorsal hippocampus and in the ventral dentate gyrus more than the GFP group. Both groups of aged rats showed a hippocampal increase of 5-HIAA in the dorsal dentate gyrus and the ventral CA1 area. Serotonin also increased in cingulate and temporal cortical areas and in the caudate of control GFP aged rats, as against young rats.

4. Discussion

The over-expression of GDNF in hippocampal astrocytes induced a recovery of spatial cognitive abilities in aged impaired rats as demonstrated by an enhancement of memory retention of the platform location in the test probe trials. This better response in the Morris water maze cannot be attributed to a mere improvement of age-related motor impairment by GDNF. Several studies have shown that GDNF enhances motor function in aged rats and non-human primates, which has been related to dopaminergic induction and regeneration of the nigrostriatal pathway (Bowenkamp et al., 1996; Emerich et al., 1996; Grondin et al., 2003; Herbert and Gerhardt, 1997; Lapchak et al., 1997b). In the present study, GDNF was over-expressed only in a restricted area of the hippocampus and the swimming speed was not affected. In addition, we found no differences in the latency to escape between the rats expressing GDNF and control rats transduced with GFP during the test assays, where an increase in swimming ability would help the rats to find the platform in a shorter time. Latency to escape to the platform is routinely recorded as an additional spatial learning and memory measure, but its outcome can be affected by external factors. This is not the case with probe trial performance, which is accepted as the most accurate measurement because it requires memory of the precise location of the platform (Bucci et al., 1995; Frick et al., 1995). Accordingly, in previous studies in our behavioral facilities, the probe trial was established as a reliable result for learning acquisition and retention under our experimental conditions (Prados and Trobalon, 1998).

The present results confirm the previous report of a significant recovery of cognitively impaired 2-year old Fisher 344 rats in the Morris water maze 2 weeks after an i.c.v. injection of GDNF (Pelleymounter et al., 1999). The degree of spatial learning recovery after a GDNF transgene expression in dorsal CA1 astrocytes was similar to that obtained by earlier authors. Spatial learning and memory require the dorsal hippocampus to function (Compton, 2004; Porthuizen et al., 2004), with the CA1 area being particularly crucial for spatial discrimination tasks (Naghdi et al., 2003; Volpe et al., 1992). In old age, CA1 pyramidal neurons suffer a loss of functional synaptic contacts from the Schaffer collaterals and an alteration in Ca^{2+} regulation, both changes leading to plasticity and cognition deficits (Himeda et al., 2005; Rosenzweig and Barnes, 2003; Thibault et al., 2001). The absence of a generalized loss of cells and synapses in the hippocampus and the whole brain in normal aging (Hof and Morrison, 2004; Miller and O'Callaghan, 2005) facilitates amelioration of cognitive decline by neurotrophic action. Both GFRα1 and Ret receptor molecules are highly expressed in the hip-

pocampal neurons of the pyramidal layer of the Ammon's horn (Burazin and Gundlach, 1999; Sarabi et al., 2000; Serra et al., 2005). Therefore, GDNF secreted by CA1 transduced astrocytes was able to exert its trophic action on local neurons. Ectopic fiber sprouting is unlikely because GDNF secreted by transduced astrocytes is poorly transported by neurons to distal areas (Ericson et al., 2005) (Mammeri H. et al., unpublished observations). Nevertheless, a histological study should be performed when long-lasting GDNF gene expression in hippocampus is planned, to rule out unwanted effects in projection areas.

Neurochemical changes in cognitively impaired aged rats have been extensively studied in a search for the causes underlying frequent memory loss in older individuals. Atrophy of acetylcholine containing neurons is considered a hallmark of aging and dementia (Terry and Buccafusco, 2003), but this cholinergic dysfunction occurs within a wider context of neurotransmitter system changes (Decker and McGaugh, 1991; Stemmelin et al., 2000). Cholinergic and monoaminergic neurotransmitter systems are involved in the spatial learning and memory processes in the hippocampus (El-Ghundi et al., 1999; Everitt and Robbins, 1997; McNamara and Skelton, 1993; Richter-Levin and Segal, 1996; Seamans et al., 1998). Hippocampus is a target cholinergic area and no local decrease of ChAT activity was reported in aged rats, confirming our results, whereas ChAT decreases have been found in the cholinergic cell body containing areas of the basal forebrain and striatum in cognitively impaired rats (Gallagher et al., 1990). Hippocampus receives its dopaminergic innervation from the midbrain and is integrated in the mesolimbic system. Many studies have demonstrated an age-related decline in dopaminergic neurotransmission, mainly in the nigrostriatal system (Gallagher et al., 1990; Lee et al., 1994; Mínguez et al., 1999). In addition, decreased density of dopamine receptors D1–D5 have been reported in several brain areas, including the hippocampus of aged rat and human (Amenta et al., 2001; Hemby et al., 2003; Kaasinen et al., 2000). We found a widespread decrease of dopamine metabolite HVA in aged rats, indicative of a lower turnover rate. The effect was higher in the caudate, in which both metabolites, DOPAC and HVA, decreased. The role of the serotonergic system in aging impairment has raised controversy. Several authors have reported unchanged or enhanced serotonin levels and turnover in aged rats (Gallagher et al., 1990; Lee et al., 1994), while others reported decreases (Mínguez et al., 1999). Confirming the former we found a trend to higher serotonin and 5-HIAA concentrations in several brain regions of the aged rats. As regards serotonin receptors, no age-induced or cognitive-related changes have been reported in 5HT1A receptor (Borg et al., 2006; Lazaris et al., 2003).

GDNF over-expression in CA1 astrocytes induced a local neurotrophic effect. ChAT activity, indicative of acetylcholine levels, rose in hippocampus. In a study of motor activity enhancement, 2 weeks of i.c.v. infusion of GDNF induced an increase in ChAT activity in the septum, hippocampus, striatum and cortex of aged rats (Lapchak et al., 1997b). This study reported a similar enhancement of ChAT by GDNF or neural growth factor (NGF). The trophic effects of NGF on aged cholinergic neurons have been extensively studied (Rylett and Williams, 1994; Sofroniew et al., 2001). NGF binds with high affinity to the TrkA receptor, which is mainly located on cholinergic neurons in the basal forebrain nuclei and striatum (Holtzman et al., 1995). A partial recovery of the cognitive abilities in the Morris water maze, similar to that we obtained with GDNF, was reported initially (Fischer et al., 1987, 1991). In subsequent studies, an almost complete reversal was obtained either by a higher dosage through i.c.v. chronic infusion (Fischer et al., 1994) or by NGF-secreting cell implants at the two main cholinergic forebrain cell groups, nucleus basalis magnocellularis and medial septum/diagonal band of Broca (MS/DB) (Martínez-Serrano et al., 1996). Although hippocampus function resulted significantly enhanced by astrocyte-secreted GDNF, we did not obtain complete recovery of spatial learning. This could be due to a partial inability of locally expressed GDNF to recover the function of the circuitry entering the hippocampus from cholinergic cell bodies located in the MS/DB. A retrograde signaling of GDNF to these cell bodies (Coulpier and Ibáñez, 2004) may not be enough when functional terminals are already reduced. On the other hand, the complexity of the reciprocal cholinergic connections between CA1 and MS/DB (Gaykema et al., 1991; Nyakas et al., 1987) may modulate the functional response. In this regard, ventral CA1 that receives cholinergic afferents from a broader MS/DB area showed slightly higher ChAT enhancement than the greater GDNF-expressing dorsal CA1 area.

In addition to cholinergic enhancement, GDNF secreted by transduced astrocytes enhanced dopaminergic and serotonergic neurotransmission over control aged rats transduced with GFP. Dopamine and serotonin levels increased without the concentration of their metabolites rising, thus demonstrating a significant neurotransmitter synthesis increase. High variability of dopamine level led to a lack of statistical significance for its increase in the dorsal CA1 area, even though GDNF dopamine value nearly doubled the GFP group value. Given the reduction of dopamine receptor function in the hippocampus discussed above, the increased neurotransmitter levels would help to restore dopaminergic function. These tissue dopamine levels could hardly account for an increase in the risk of oxidative damage by dopamine auto-oxidation (Camp et al., 2000). However, the presence of hydroxylated adducts in hippocampus should be analyzed whenever a longer therapy is planned. While GDNF increased serotonin levels less than dopamine it similarly enhanced specific synaptic transmission, as long as there was no loss of serotonin receptors due to aging. As discussed above for the cholinergic neurotransmission, GDNF enhancement of the dopaminergic mesencephalic afferents (Gasbarri et al., 1997) and the serotonergic afferents from the median raphe (McKenna and Vertes, 2001) may not be enough to restore hippocampus functionality. Studies in Parkinson's disease models have demonstrated that GDNF induces higher func-

tional recovery when acting in the striatum terminals than in the substantia nigra dopaminergic bodies (Connor et al., 1999; Kirik et al., 2000). Therefore, an earlier and sustained therapy of GDNF in the hippocampus may be a better strategy for spatial memory recovery than its delivery to other targets. Nonetheless, the study of GDNF effects on other related brain areas should be considered.

GDNF showed a broad neurotrophic effect and probably also enhanced other neurotransmitters involved in learning and memory such as glutamate and GABA. GABAergic interneurons, present in the stratum pyramidale and stratum oriens express GFRα-1 (Sarabi et al., 2000), lose function with aging (Stanley and Shetty, 2004). Decrease of NMDA receptor density in the aged CA1 region correlates with spatial learning decline (Magnusson, 1998). GDNF has shown trophic effects on GABAergic neurons (García-Martínez et al., 2006) and neuroprotective effects against NMDA-mediated excitotoxicity (Nicole et al., 2001). Therefore GDNF may directly enhance these systems or/and counteract the alterations of the interactions between the different brain neurotransmitter systems that contribute to aging deterioration (Decker and McGaugh, 1991; Segovia et al., 2001).

The amelioration of senile neurons or damaged dysfunctional neurons in future human therapies may need a continuous supply of trophic factor (Niewiadomska et al., 2002). Chronic infusion of GDNF through a catheter implanted in the brain has been assayed in Parkinson's patients for periods of up to several years (Love et al., 2005), but may be accompanied by both practical and safety problems. Gene delivery ex vivo or in vivo allows selective local diffusion of the growth factor without undesirable side effects. Strategies in various experimental animal models of neural diseases include the implant of either astrocytes (Cunningham and Su, 2002; Do Thi et al., 2004; Ericson et al., 2005), neural progenitors (Akerud et al., 2001; Bakshi et al., 2006; Behrstock et al., 2006; Klein et al., 2005) or fibroblasts (Pérez-Navarro et al., 1996, 1999) engineered to secrete GDNF. However, the advances in the design of viral vectors convert in vivo gene therapy into an attractive strategy for long-term delivery of trophic factors to the nervous system. Neuroprotective effects in experimental models of neurodegenerative diseases have been obtained by direct in vivo transfer of GDNF gene with recombinant adenoviral (Acsadi et al., 2002; Bilang-Bleuel et al., 1997; Choi-Lundberg et al., 1998; Do Thi et al., 2004; Lapchak et al., 1997a), adeno-associated (Eslamboli et al., 2005; Kirik et al., 2000; McBride et al., 2003; Wang et al., 2002) and lentiviral vectors (Brizard et al., 2006; Georgievska et al., 2002; Guillot et al., 2004; Kordower et al., 2000; Wong et al., 2005). Replication-defective lentiviral vectors are safe and can sustain a strong expression of transgenes for long periods (Naldini et al., 1996). They can efficiently transduce neurons and glial cells and cause minimal inflammatory and immunological responses (Mazarakis et al., 2001; Wong et al., 2006). The combination of viral particle pseudotyping and gene promoter facilitates targeting of specific cell types (Bemelmans et al., 2005; Cronin et al., 2005). We constructed a lentiviral vector pseudotyped with Mokola G protein and its gene expression driven by the CMV promoter. This vector showed selectivity for transducing the astrocytes in the hippocampal area of injection.

In conclusion, a short chronic delivery of GDNF in the dorsal CA1 hippocampal astrocytes enhanced local cholinergic, dopaminergic and serotonergic transmission, leading to improved spatial learning and memory performance in cognitively impaired aged rats. Astrocytes proved to be an appropriated cell type for over-secreting GDNF in gene therapy strategies. Deficiencies were not totally reverted; suggesting that GDNF paracrine action in the CA1 area could be too restricted to obtain complete recovery of the complex behavioral learning task. Finally, lentiviral vectors showed high efficacy, making feasible longer chronic treatment which would probably have a better outcome. Therefore, the present results demonstrated the therapeutic value of lentiviral vectors expressing GDNF transgene in rat age-related cognitive decline. Further studies to explore the therapeutic possibilities of GDNF in human aging and Alzheimer's disease memory loss, are required.

Conflict of interest

The authors declare that there is no actual or potential conflict of interest.

Acknowledgements

This research was supported by grants FIS 03/0467 and Red CIEN V-2003-REDC10F-C from the Spanish Fondo de Investigaciones Sanitarias of the Ministry of Health and DURSI 2005/SGR/00826 of the Generalitat de Catalunya. M. Pertusa and S. García-Matas acknowledge a pre-doctoral fellowship from IDIBAPS and the Generalitat de Catalunya, respectively. We are grateful to Dr. H. Almirall and J.M. Marimón for their valuable advice and collaboration in the behavioral studies and to L. Campa and A. Parull for their skilful technical assistance. Confocal microscopic images were obtained by Dr. M. Calvo of the SCT of the University of Barcelona.

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Aging Cell (2008) **7**, pp630–640 Doi: 10.1111/j.1474-9726.2008.00410.x

Dysfunction of astrocytes in senescence-accelerated mice SAMP8 reduces their neuroprotective capacity

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Summary

Early onset increases in oxidative stress and tau pathology are present in the brain of senescence-accelerated mice prone (SAMP8). Astrocytes play an essential role, both in determining the brain's susceptibility to oxidative damage and in protecting neurons. In this study, we examine changes in tau phosphorylation, oxidative stress and glutamate uptake in primary cultures of cortical astrocytes from neonatal SAMP8 mice and senescence-accelerated-resistant mice (SAMR1). We demonstrated an enhancement of abnormally phosphorylated tau in Ser^{199} and Ser^{396} in SAMP8 astrocytes compared with that of SAMR1 control mice. Gsk3β and Cdk5 kinase activity, which regulate tau phosphorylation, was also increased in SAMP8 astrocytes. Inhibition of Gsk3β by lithium or Cdk5 by roscovitine reduced tau phosphorylation at Ser^{396}. Moreover, we detected an increase in radical superoxide generation, which may be responsible for the corresponding increase in lipoperoxidation and protein oxidation. We also observed a reduced mitochondrial membrane potential in SAMP8 mouse astrocytes. Glutamate uptake in astrocytes is a critical neuroprotective mechanism. SAMP8 astrocytes showed a decreased glutamate uptake compared with those of SAMR1 controls. Interestingly, survival of SAMP8 or SAMR1 neurons cocultured with SAMP8 astrocytes was significantly reduced. Our results indicate that alterations in astrocyte cultures from SAMP8 mice are similar to those detected in whole brains of SAMP8 mice at 1–5 months. Moreover, our findings suggest that this *in vitro* preparation is suitable for studying the molecular and cellular processes underlying early aging in this murine model. In addition, our study supports the contention that astrocytes play a key role in neurodegeneration during the aging process.

Key words: Astrocytes; glutamate uptake; neuroprotection; oxidative stress; senescence-accelerated mice; tau protein.

Introduction

The senescence-accelerated mouse (SAM) is comprised of a group of 12 strains developed from selective inbreeding of the AKR/J strain constructed by Takeda *et al*. (1981). The senescence-accelerated mouse prone-8 (SAMP8) strain manifests irreversible advancing senescence with pathological, biochemical and behavioural alterations, whereas the senescence-accelerated mice resistant-1 (SAMR1) strain presents a normal aging pattern (Takeda, 1999). For SAMP8 and SAMR1 strains, the median lifespans have been reported as 10 and 18.9 months of age, respectively (Takeda *et al*., 1994). Interestingly, the former exhibits age-related learning and memory deficits, as well as β-amyloid-like deposits in the brain (Flood & Morley, 1998; Chen *et al*., 2004). Furthermore, increases in hyperphosphorylated tau and cyclin-dependent kinase 5 (Cdk5) expressions and activation have also been detected in SAMP8 mice (Canudas *et al*., 2005). For these reasons, SAMP8 mice are regarded as a suitable rodent model for studying the molecular mechanisms underlying cognitive impairment in aged subjects.

The free radical theory of aging contends that oxidative alterations in biomolecules brought about by reactive oxygen species (ROS) contribute to cellular dysfunction during aging (Harman, 1956). Indeed, increasing evidence of elevated oxidative stress in the aged SAMP8 brain has been reported. Increased levels of lipoperoxidation, carbonyl proteins and ROS in the brain of SAMP8 mice have been observed, together with learning and memory deficits as early as 1–5 months of age (Liu & Mori, 1993; Sato *et al*., 1996a,b; Kurokawa *et al*., 2001; Yasui *et al*., 2002, 2003; Farr *et al*., 2003; Poon *et al*., 2004a; Alvarez-García *et al*., 2006). In addition, chronic administration of antioxidants, such as melatonin, α-lipoic acid, N-acetylcysteine and acetyl-L-carnitine, to SAMP8 mice not only reduced oxidative damage to neural lipids and proteins, but also lessened cognitive deficits (Okatani *et al*., 2002; Yasui *et al*., 2002; Farr *et al*., 2003; Poon *et al*., 2005). Decreases in superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase activities, as well as increases in acyl-CoA oxidase, which have been detected early in SAMP8 mice (1–12 months) compared with age-matched SAMR1 controls (Sato *et al*., 1996b; Kurokawa *et al*., 2001; Okatani *et al*., 2002; Alvarez-García

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Accepted for publication *29 May 2008*

et al., 2006; Sureda *et al*., 2006), may cause elevated generation of ROS. On the other hand, ROS generated by mitochondria or from other cell sites not only cause damage to mitochondrial components and DNA, but also trigger degradative processes that contribute to the aging process (Cadenas & Davies, 2000; Manczak *et al*., 2005). In fact, mitochondrial DNA deletions as well as inefficient mitochondrial hyperactivity were found in the brain of 4- to 8-week-old SAMP8 mice (Fujibayashi *et al*., 1998; Nishikawa *et al*., 1998). Differential gene expression profile in 12-month-old SAMP8 hippocampus revealed up-regulation of cytochrome *c* oxidase subunit I and III from the respiratory chain (Cheng *et al*., 2006).

Astrocytes play an essential role not only in maintaining normal brain physiology, but also in the brain's defence against oxidative damage (for a review, see Cotrina & Nedergaard, 2002). Astrocytes possess a great number of antioxidant systems, including glutathione, glutathione transferase, catalase and superoxide dismutase enzymes (Makar *et al*., 1994; Dringen *et al*., 2000). Although they are able to protect neurons from hydrogen peroxide (H_2O_2) toxicity (Deshager *et al*., 1996), astrocytes remain vulnerable to oxidative stress, since they react to low concentrations of H_2O_2 generating ROS and lipoperoxidation, and increasing antioxidant defences (Röhrdanz *et al*., 2001). Furthermore, astrocytes are primarily responsible for clearing extracellular glutamate, thereby preventing neuronal excitotoxiciy (for a review, see Anderson & Swanson, 2000). Thus, alterations in all these systems during aging may potentially reduce the neuroprotective capacity of astrocytes, further contributing to neurodegeneration. In fact, increasing evidence suggests that astrocytes play a significant role in neuronal aging. Heightened expression of glial fibrillary acidic protein (GFAP) and S100 β protein have been detected during aging in regions of the mouse and rat brain (O'Callaghan & Miller, 1991; Kohama *et al*., 1995; Amenta *et al*., 1998), as well as in mouse and rat cortical astrocytes aged in culture (Papadopoulos *et al*., 1998; Gottfried *et al*., 2002; Tramontina *et al*., 2002; Pertusa *et al*., 2007). Superoxide production, lipoperoxidation, protein oxidation, and iron staining remained elevated in aged astrocyte cultures, even though antioxidant defences had been maintained or increased (Papadopoulos *et al*., 1998; Gottfried *et al*., 2002; Klamt *et al*., 2002; Pertusa *et al*., 2007). Mitochondrial membrane potential in old astrocytes proved more depolarized than in young astrocytes (Lin *et al*., 2006). Furthermore, changes in glial glutamate uptake have been observed during brain aging (for a review, see Segovia *et al*., 2001). In aged astrocytes in culture, glutamate uptake was more vulnerable to inhibition by H_2O_2 exposition (Gottfried *et al*., 2002; Pertusa *et al*., 2007). Recent studies have shown that PC12 cells co-cultured with old astrocytes were more sensitive to the oxidant *tert*-buthyl H_2O_2 than those co-cultured with young astrocytes (Lin *et al*., 2006). In addition, we have shown that astrocytes aged *in vitro* have a reduced ability to maintain neuronal survival (Pertusa *et al*., 2007).

Until now, pathological neuronal dysfunction has been regarded as the main mechanism underlying the cognitive deficits in SAMP8 mice. However, some evidence suggests that altered astrocytes contribute to the accelerated senescence noted in this animal model. Increased astrogliosis and microgliosis have been reported in the hippocampus and cerebral cortex of SAMP8 mice (Nomura *et al*., 1996; Wu *et al*., 2005). In addition, D-serine, a glial modulator of nerve cells, is reportedly involved in the long term potentiation (LTP) dysfunction recorded in hippocampal slices from 12-month-old SAMP8 (Yang *et al*., 2005a). The aim of this study was to explore whether SAMP8 astrocytes exhibit aged-related characteristics consistent with a possible role in the biochemical and behavioural alterations observed in aged SAMP8 mice. The study was performed in cortical astrocytes since many senescence-related biochemical changes of SAMP8 mice have been described in this brain area, as mentioned above. In SAMP8 astrocytes, we found hyperphosphorylation in some forms of tau, protein kinase Gsk3β and Cdk5 activation, and increases in overall oxidative stress status. Glutamate uptake in primary cultures of cortical astrocytes from SAMP8 mice was decreased compared to that from SAMR1 mice. We also explored the neuroprotective capacity of SAMR1 and SAMP8 astrocytes in co-cultures with cortical neurons from both strains. Here, we have demonstrated that SAMP8 astrocytes suffer reduced neuroprotective abilities.

Results

Changes in tau, Gsk3β and Cdk5 protein expression in SAMP8 astrocytes

The hyperphosphorylation of tau was evaluated by Western blot using two phosphorylation-dependent and site-specific antibodies against tau in SAMR1 and SAMP8 astrocytes. Densitometric analysis of the immunoblots in SAMP8 astrocytes showed a statistically significant increase in phosphorylated tau at Ser^{199}/Ser^{396} sites compared with SAMR1 controls (Fig. 1). The expression of two Ser/Thr protein kinases involved in regulating tau phosphorylation, Gsk3β and Cdk5, was also determined by Western blot. We detected a significant increase in phosphorylated Gsk3β at Tyr^{216} (active form) and a significant decrease at Ser^{9} (inactive form) in SAMP8 astrocytes compared with SAMR1 (Fig. 2a). These results indicated activation of Gsk3β kinase in SAMP8 astrocytes in culture. Figure 3(a) shows that Cdk5 immunoblots in SAMP8 and SAMR1 astrocytes were not significantly different, indicating similar Cdk protein expression for both strains. The induction of Cdk5 kinase activity was determined using p25/p35 labeling. The expression of p35 was decreased while that of p25 increased in SAMP8 compared with SAMR1. The calculated p25/p35 ratio showed increased Cdk5 activity in SAMP8 astrocyte cultures. When SAMP8 astrocyte cultures were treated for 48 h with 10 mM lithium, a specific inhibitor of Gsk3β activity, a significant increase and decrease of phosphorylated Gsk3β at Ser^{9} and Tau at Ser^{396}, respectively, was detected (Fig. 2b). Roscovitine (15 μM), a non-specific Cdk5 kinase inhibitor, showed a tendency to decrease the p25/p35 ratio ($p = 0.2163$) and tau phosphorylation at Ser^{396} ($p = 0.1375$)

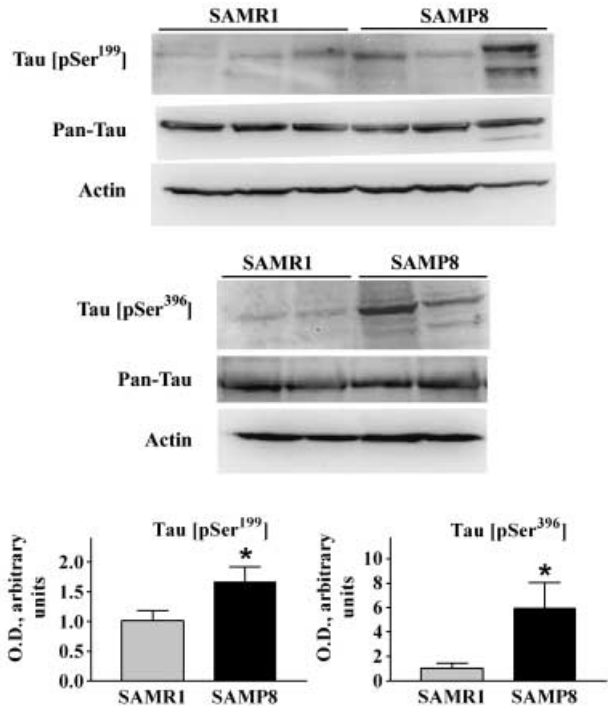

Fig. 1 Representative immunoblots and densitometry analysis of tau protein phosphorylation in SAMR1 and SAMP8 astrocyte cultures. Tau phosphorylation was assessed using phosphorylation-dependent antibodies anti-tau [pSer^{199}] and anti-tau [pSer^{396}]. Graphs summarize the results from five separate experiments. The immunoreactivity levels of each band in the SAMR1 and SAMP8 cultures were first normalized to that of the corresponding Pan-Tau and then to β-actin. Both phosphorylated protein levels were increased in SAMP8 astrocyte cultures. Data were compared using the Student's *t*-test (*$p < 0.05$).

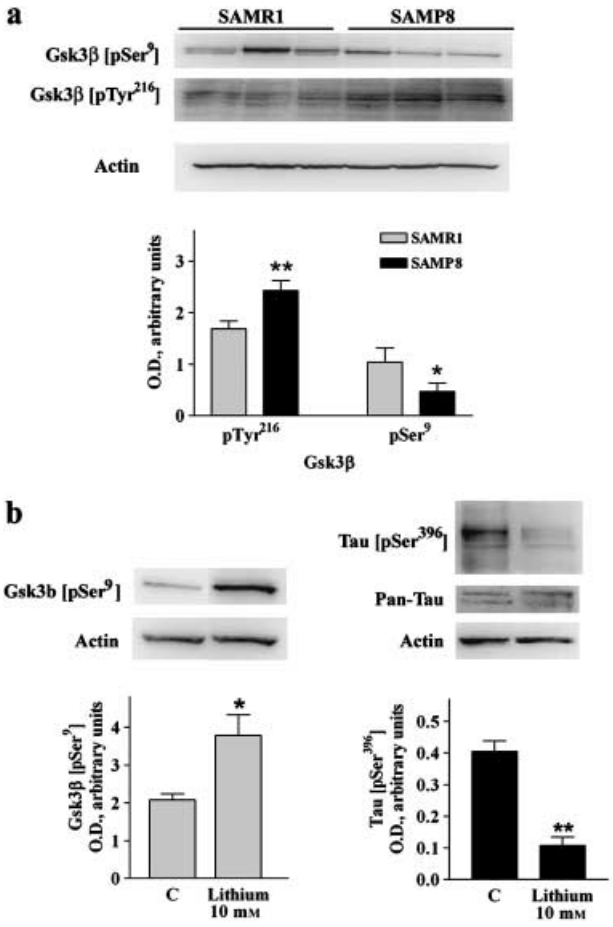

Fig. 2 (a) Representative immunoblots and densitometry analysis of Gsk3β protein phosphorylation in SAMR1 and SAMP8 astrocyte cultures. Gsk3β phosphorylation was assessed using phosphorylation-dependent antibodies anti-Gsk3β [pSer^{9}] and anti-Gsk3β [pTyr^{216}]. Graphs summarize the results from three to seven separate experiments. The immunoreactivity levels of each band in the SAMR1 and SAMP8 cultures were normalized to that of the corresponding β-actin. The protein levels of Gsk3β [pTyr^{216}] and Gsk3β [pSer^{9}] increased and decreased, respectively, in SAMP8 astrocyte cultures vs. SAMR1. (b) Effects of the Gsk3β inhibitor lithium (10 mM, 48 h) on Gsk3β phosphorylation at Ser^{9} and tau phosphorylation at Ser^{396} in the SAMP8 astrocyte cultures. Graphs summarize the results from three to four separate experiments. The immunoreactivity levels of tau pSer^{396} were first normalized to that of the corresponding Pan-Tau and then to β-actin. Lithium increased Gsk3β phosphorylation at Ser^{9}, indicating enzyme activity inhibition, and decreased tau phosphorylation at Ser^{396}. Data were compared using the Student's *t*-test (*$p < 0.05$, **$p < 0.01$).

in SAMP8 astrocytes (Fig. 3b). Roscovitine effects did not reach statistical significance due to the high variability found between samples from different cultures.

Oxidative stress increase and mitochondrial membrane potential decrease in SAMP8 astrocytes

Basal levels of superoxide anion radical generated for 1 h in SAMR1 and SAMP8 astrocytes were determined via a hydroethidium probe. As shown in Fig. 4, SAMP8 cultures produced significantly more superoxide radical than did those of SAMR1. No differences were observed between the two strains in terms of hydroperoxide generation (data not shown). The main source of superoxide radical production is the mitochondrial electron transport chain that occurs during normal respiration. Mn-superoxide dismutase transforms superoxide radical into H_2O_2, which may diffuse through the mitochondrial membrane to the cytoplasm. As H_2O_2 can be transformed into harmful radicals, increased generation of these oxidants may cause lipid and protein oxidation. Figure 5(a) shows significantly increased production of lipoperoxidation, as measured by malondialdehyde and 4-hydroxyalkenal accumulation, in SAMP8 astrocytes vs. SAMR1. Protein carbonyl formation, which is indicative of protein oxidation and protein damage, was significantly elevated in SAMP8 compared with those of SAMR1 (Fig. 5b). Enhanced free radical production may be related to changes in mitochondrial membrane potential. Therefore, we measured the accumulation of the fluorescent dye rhodamine 123, which is dependent on membrane potential, in SAMR1 and SAMP8 astrocyte cultures. SAMP8 astrocytes exhibited a significant reduction in the basal uptake of rhodamine 123 compared with SAMR1 astrocytes, indicative of lower mitochondrial membrane potential in those cultures (Fig. 6).

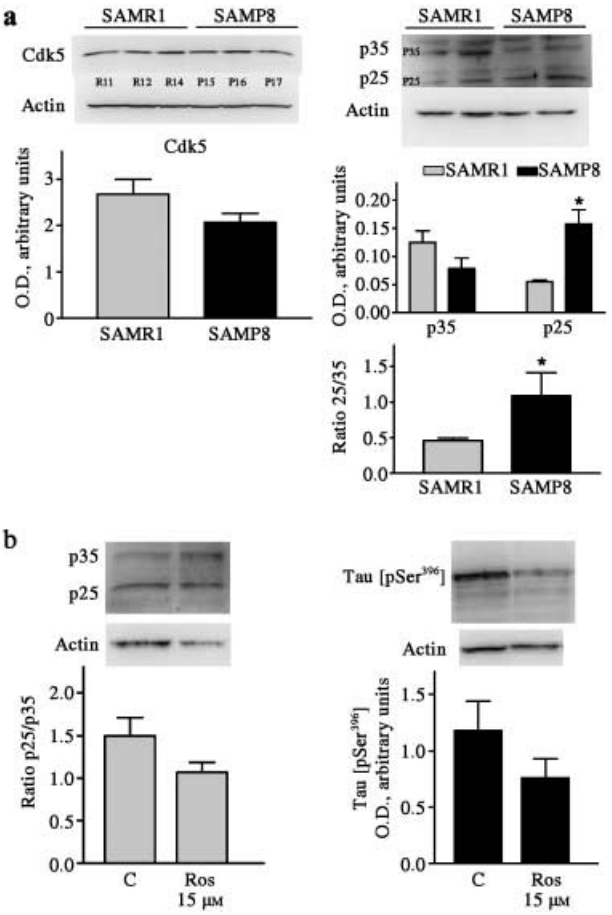

Fig. 3 (a) Representative immunoblots and densitometry analysis of Cdk5, p35 and p25 proteins in SAMR1 and SAMP8 astrocyte cultures. Graphs summarize the results from three to five separate experiments. The immunoreactivity levels of each band in the SAMR1 and SAMP8 cultures were normalized to that of the corresponding β-actin. SAMP8 and SAMR1 Cdk5 protein levels were not different. The p25/p35 ratio indicated a decrease in p35 band intensity, which resulted in an increase in p25 band intensity in SAMP8 astrocytes. (b) Effects of the Cdk5 inhibitor roscovitine (15 μM, 48 h) on p35 and p25 proteins and tau phosphorylation at Ser^{396} in the SAMP8 astrocyte cultures. Graphs summarize the results from 3–4 separate experiments. The immunoreactivity levels of tau $pSer^{396}$ were first normalized to that of the corresponding Pan-Tau and then to β-actin. Roscovitine slightly decreased the p25/p35 ratio ($p = 0.2163$) and tau phosphorylation at Ser^{396} ($p = 0.1375$). Data were compared using the Student's *t*-test (*$p < 0.05$).

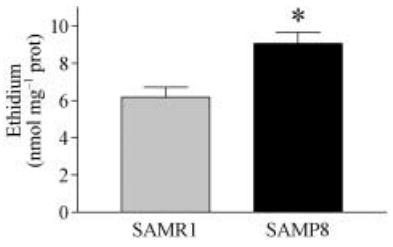

Fig. 4 Intracellular superoxide anion radical generation after 1 h in SAMR1 and SAMP8 astrocyte cultures, as measured by dihydroethidium oxidation to ethidium. Results are expressed as nmol of ethidium mg^{-1} of protein and represent the mean ± SEM of three independent cultures. SAMP8 cultures generated significantly more superoxide radicals than did SAMR1 cultures. *$p < 0.01$ compared with SAMR1 astrocytes by the Student's *t*-test.

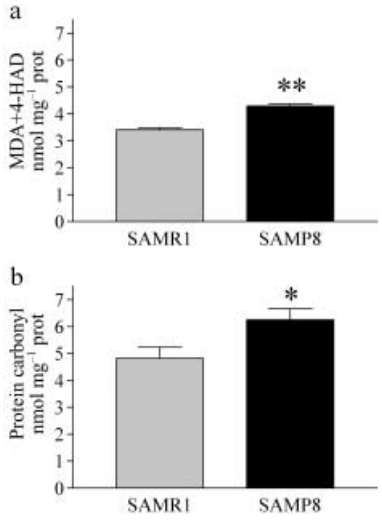

Fig. 5 Lipoperoxidation and carbonyl proteins in SAMR1 and SAMP8 astrocyte cultures. Lipoperoxidation (a) was expressed as nmol of malondialdehyde plus 4-hydroxyalkenal mg^{-1} of protein and carbonyl proteins (b) were expressed as nmol of protein carbonyl mg^{-1} of protein. Results represent the mean ± SEM of six independent cultures. We observed significantly more lipid and protein damage in SAMP8 cultures than in SAMR1 cultures. *$p < 0.05$ and **$p < 0.0001$ compared with SAMR1 astrocytes using the Student's *t*-test.

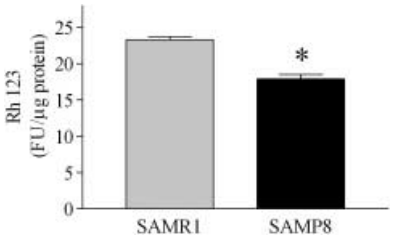

Fig. 6 Mitochondrial membrane potential in SAMR1 and SAMP8 astrocyte cultures, as measured by the fluorescent dye rhodamine 123 (Rh 123). Results are expressed as fluorescence units (FU) of Rh 123 per μg of protein and represent the mean ± SEM of three independent cultures. SAMP8 astrocytes showed a reduced basal rhodamine 123 uptake. *$p < 0.01$ compared with SAMR1 astrocytes using the Student's *t*-test.

Decreased glutamate uptake in SAMP8 astrocytes

The ability of astrocytes to uptake glutamate was evaluated in SAMR1 and SAMP8 cultures using an isotopic method with several glutamate concentrations. Figure 7(a) shows that glutamate uptake in SAMP8 astrocyte cultures was significantly lesser than in SAMR1 cultures. Kinetic parameters obtained from Eadie-Hofstee analysis revealed a statistically significant decrease in Km (92.78 ± 15.15 μM for SAMP8 and 222.2 ± 42.09 μM for SAMR1, $p < 0.05$) and Vmax (10.49 ± 0.67 nmol mg^{-1} protein min^{-1} for SAMP8 and 49.99 ± 5.09 nmol mg^{-1} protein min^{-1} for SAMR1, $p < 0.0001$) values in SAMP8 astrocytes (Fig. 7b). SAMP8 astrocytes treated with 100 μM trolox or 10 μM curcumin throughout the culture time period did not undergo a reversal of glutamate uptake inhibition (Fig. 7c). Expression of the glutamate-aspartate

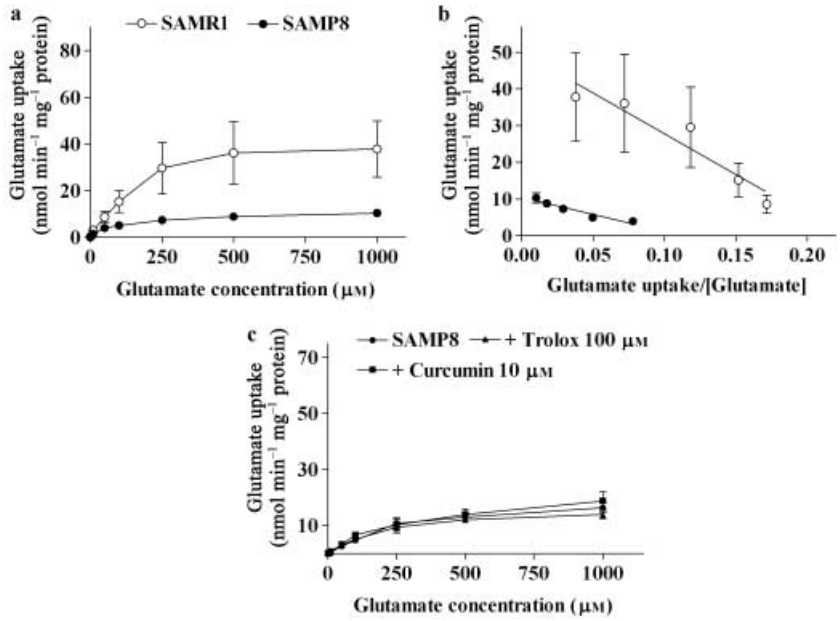

Fig. 7 (a) Glutamate uptake in SAMR1 and SAMP8 astrocyte cultures. Astrocytes were incubated with 9.8 nM [^{3}H]-glutamate and several concentrations of unlabelled glutamate as described in the Experimental procedures. Each point represents the mean ± SEM of five to eight determinations. Two-way analysis of variance followed by Bonferroni's test showed that strain ($F_{1,107} = 23.95$, $p < 0.0001$) and glutamate concentration ($F_{8,107} = 9.63$, $p < 0.0001$) had a significant effect. (b) Values from (a) (glutamate uptake and unlabelled glutamate concentrations from SAMR1 and SAMP8 astrocytes) were used to construct the Eadie-Hofstee representation to calculate Km and Vmax kinetic parameters. There was a significant decrease in the Km and Vmax values of glutamate uptake in SAMP8 astrocyte cultures (see Results). (c) Glutamate uptake in SAMP8 astrocytes treated during the culture period with 100 μM trolox or 10 μM curcumin. No significant effects were observed.

transporter (GLAST) was examined by Western blot and no changes were observed between SAMP8 and SAMR1 astrocytes (data not shown).

SAMP8 astrocytes show reduced neuroprotective capacity

The neuroprotective capacity of astrocytes was evaluated in co-cultures with cortical neurons by determining neuronal survival. Living neurons were identified by staining in co-cultures of SAMR1 or SAMP8 neurons seeded on a monolayer of either SAMR1 or SAMP8 astrocytes for 12 days. Thereafter, the number of living cells, either in SAMR1 or SAMP8 neurons, proved to be less in SAMP8 astrocyte co-cultures than in those of SAMR1 (Fig. 8a). These results revealed that SAMP8 astrocytes suffered a reduced neuroprotective capacity. No differences were found in the number of living neurons from SAMR1 or SAMP8 mice co-cultured with SAMP8 astrocytes. When SAMR1 and SAMP8 neurons were cultured in the presence of astrocyte-conditioned medium from SAMR1 or SAMP8 mice, their survival was higher than in the absence of astrocytes but lower than in co-cultures, and was similar for both types of astrocytes (Fig. 8b). These results suggested that differences in neuroprotective capacity are not due to a soluble compound secreted by astrocytes to the medium.

Taking into account the high astrocyte enrichment of the cultures, alterations observed in this work were attributed to the astrocyte population. However, some small contribution of contaminant microglial cells cannot be discarded.

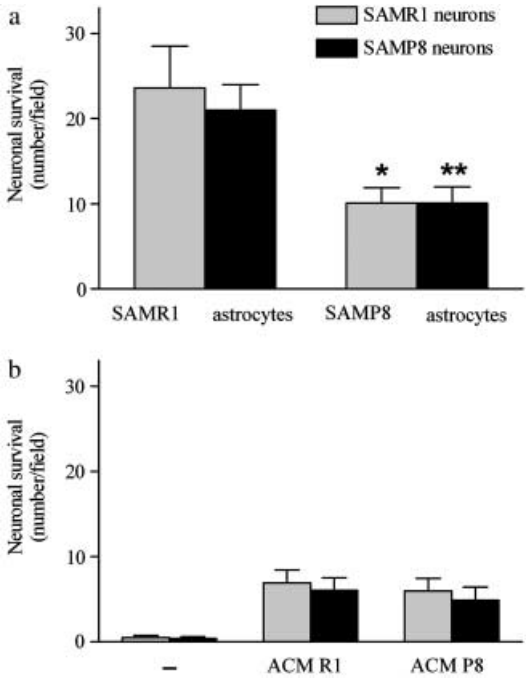

Fig. 8 Neuronal survival of SAMR1 and SAMP8 neurons in the presence of (a) astrocytes or (b) astrocyte-conditioned medium (ACM) from SAMR1 and SAMP8 mice. Cortical neurons were cultured on a monolayer of SAMR1 and SAMP8 astrocyte cultures or with 48 h ACM for 12 days. Living neurons in both conditions were identified by cell staining using NeuN or NF antibody and propidium iodide. Results are expressed as the number of living neurons by field and represent the mean ± SEM of seven independent co-cultures. Number of astrocytes by field is 230 ± 13 and 237 ± 16 for SAMR1 and SAMP8 astrocytes, respectively. Data were compared using the Student's *t*-test (*$p < 0.05$, **$p < 0.01$ related to co-cultures of neurons and SAMR1 astrocytes).

Discussion

Although mainly present in neuronal cells, tau protein has also been found in the astrocytes and oligodendrocytes of some neural diseases (for review, see Avila *et al.*, 2004). It has been largely demonstrated that the hyperphosphorylation of tau reduces its binding to microtubules, thereby resulting in neurofibrillary degeneration. This process occurs systematically in the neurons of human entorhinal and hippocampal regions during aging

(Delacourte *et al*., 2002, 2003). Recently, several authors reported an age-dependent pattern of glial tau aggregation in both human and baboon brains (Schultz *et al*., 2000; Yang *et al*., 2005b). Previous studies have shown that hyperphosphorylation of tau occurs in the brain of SAMP8 mice at 5 months of age (Canudas *et al*., 2005; Sureda *et al*., 2006). Consistent with these data, the present study has demonstrated increased tau phosphorylation at Ser^{199} and Ser^{396} in SAMP8 astrocyte cultures obtained from neonate brain, as compared with those of SAMR1 mice. Both sites have been shown to be phosphorylated *in vitro* by several protein kinases, such as Cdk5 and Gsk3β (see the review by Gong *et al*., 2005). Our results showed an increase and decrease of the active ($pTyr^{279}$) and inactive ($pSer^{9}$) form, respectively, of Gsk3β in SAMP8 vs. SAMR1, thus indicating activation of this enzyme. In addition, an increase in the p25/p35 ratio in SAMP8 astrocytes also denoted activation of the Cdk5 kinase. It is worth noting that the presence of functional Cdk5/p35 in murine cortical astrocyte cultures was recently reported by He *et al*. (2007). Therefore, both protein kinases may be responsible for the hyperphosphorylation of tau in SAMP8 astrocytes. The increase of Cdk activity was in agreement with the results in early senescent SAMP8 brains reported in the above-mentioned study of Canudas *et al*. (2005). On the other hand, no changes were reported in the phosphorylation at $pTyr^{279}$ and $pSer^{9}$ sites of Gsk3β between SAMP8 and SAMR1 brain. We cannot discard an increase of Gsk3β kinase activity in the absence of Gsk3β phosphorylation changes in senescent mice *in vivo*, according to the findings in old p25 transgenic mice with constant Cdk5 over-activation (Plattner *et al*., 2006). Therefore, in brains of early senescent SAMP8, Gsk3β kinase activity might be enhanced through Cdk5 activation. If this were the case, Gsk3β phosphorylation changes in astrocyte cultures would be indicative of inconsequential differences in the pathways of tau hyperphosphorylation as related to the brain tissue. The hyperphosphorylation of tau in cultures of SAMP8 neonate astrocytes may be considered as an early signal of an aging-related pathological process. The biological significance of astrocytic tau pathology in aging remains largely unknown. In certain diseases characterized by tau-based neurofibrillary pathology (tauopathies), tau aggregation in astrocytes appears to have no link with gliosis and, in fact, has been regarded as a degenerative process (Togo & Dickson, 2002). Moreover, a transgenic mouse model of human tau expression in astrocytes has shown an age-dependent pathology with abnormal tau phosphorylation leading to a focal neuron degeneration (Forman *et al*., 2005).

The regulation of tau hyperphosphorylation at different sites by protein kinases is a complex mechanism not well known, that probably requires the synergistic action of two or more kinases (Sengupta *et al*., 2006). Li *et al*. (2006) reported that Cdk5 phosphorylates tau at S^{404} and enhances Gsk3β-catalysed tau phosphorylation at S^{400} and S^{396}, suggesting that Cdk5 primes tau for Gsk3β in the brain. In our work, tau hyperphosphorylation at S^{396} was reduced by lithium and roscovitine, indicating a directly Gsk3β- and Cdk5-mediated tau hyperphosphorylation at this site in SAMP8 astrocytes. However, we cannot discard that Gsk3β-mediated tau hyperphosphorylation at Ser^{396} in SAMP8 astrocytes were a consequence of Cdk5-mediated phosphorylation at other sites in tau, as has been reported by Li *et al*. (2006) in rat brain. Further studies are necessary to identify the precise mechanisms underlying the hyperphosphorylation of tau in SAMP8 astrocytes.

There is wide consensus that free radicals are involved in the damaging processes associated with brain aging (Poon *et al*., 2004b). Interestingly, there is significant evidence that SAMP8 mice (age 1–5 months) possess a more elevated oxidative status compared with SAMR1 controls (Liu & Mori, 1993; Sato *et al*., 1996a,b; Kurokawa *et al*., 2001; Yasui *et al*., 2002, 2003; Farr *et al*., 2003; Poon *et al*., 2004a; Alvarez-García *et al*., 2006). In the current study, we demonstrated that SAMP8 astrocyte cultures present increased superoxide radical generation, lipoperoxidation and carbonyl proteins vs. SAMR1, which is consistent with *in vivo* studies. Elevated oxidative stress and abnormally phosphorylated tau in SAMP8 astrocytes suggest that neonatal astrocytes maturated in culture acquire characteristics similar to those observed *in vivo* in the brains of senescent SAMP8 mice. Thus, SAMP8 astrocyte cultures offer an effective model for studying the molecular and cellular processes underlying aging. Furthermore, these results support previous hypotheses contending that glial oxidative stress is involved in the aging process. In this context, several authors have demonstrated increased superoxide production, lipoperoxidation, protein oxidation and iron staining in astrocytes aged in culture (Papadopoulos *et al*., 1998; Gottfried *et al*., 2002; Klamt *et al*., 2002; Pertusa *et al*., 2007). It has been proposed that ROS generated by mitochondria accumulate during aging and are not only responsible for the damage present in mitochondrial components, but also cause degradative processes (Cadenas & Davis, 2000; Lenaz *et al*., 2006). Accordingly, we have detected lower mitochondrial membrane potential in SAMP8 astrocytes, which may stem from the elevated oxidative stress detected in these cultures. These results are in agreement with studies carried out by Lin *et al*. (2006) in which astrocytes cultured from older mice exhibited lower mitochondrial membrane potential than astrocytes cultured from younger mice. Age-related correlations between increases in ROS production and decreases in mitochondrial membrane potential have also been detected in hippocampal neurons cultured from old rats (Parihar & Brewer, 2007). On the other hand, our results are in agreement with those authors who observed altered mitochondrial dysfunction in SAMP8 mice. For instance, Nishikawa *et al*. (1998) detected an early stage mechanism underlying the age-associated mitochondrial dysfunction present in SAMP8 mouse brain, and Xu *et al*. (2007) reported that platelet mitochondrial membrane potential as well as hippocampal and platelet ATP content in SAMP8 mice decreased at early age compared with SAMR1. Thus, our results support the contention that mitochondrial alterations may play a key role in the aging process.

The consequences of tau pathology on astrocyte function have not been thoroughly investigated. Recently, a transgenic mouse model used to investigate tau pathology in astrocytes

revealed some loss of function as a consequence of increased tau expression and abnormally phosphorylated, ubiquitinated and filamentous tau (Forman *et al*., 2005; Dabir *et al*., 2006). These transgenic mice manifested neurodegeneration and reduced glutamate transport in those cerebral regions with robust astrocytic tau expression. In our own SAMP8 astrocyte cultures, we also noted decreased glutamate uptake in tandem with the above-mentioned increases in abnormally phosphorylated tau and oxidative stress. Alterations in glial glutamate transport may result from oxidative injury. Trotti *et al*. (1997) reported that glutamate transporters are vulnerable to oxidants due to the fact that they possess a redox regulatory mechanism. We were unable to reverse the inhibition of glutamate uptake in SAMP8 astrocytes using trolox and curcumin antioxidant treatments, even though we did reverse H_2O_2-induced glutamate uptake inhibition in astrocyte aged in culture (Pertusa *et al*., 2007). It is possible that a different antioxidant pattern treatment is required to reverse long-time oxidative effects. On the other hand, reduced glutamate uptake in the transgenic mouse of tau pathology has been associated with a decreased expression of glial glutamate transporters (Dabir *et al*., 2006). However, we could not detect any reduction in GLAST transporter expression in SAMP8 astrocytes. It has been suggested that alterations in the trafficking of glutamate transporters by tau pathology may lead to reduced glutamate uptake (Dabir *et al*., 2006). Additional studies are needed to evaluate the functional consequences of tau alterations in glial glutamate transport.

The role played by astrocytes in neuronal antioxidant defence is now widely understood (Deshager *et al*., 1996). Previous studies have demonstrated that functional alterations in astrocytes aged *in vitro* (Pertusa *et al*., 2007) or in astrocytes cultured from old mice (Lin *et al*., 2006) lead to reduced neuronal survival. We examined the survival of cortical neurons from SAMR1 and SAMP8 mice 12 days after co-culturing with SAMR1 and SAMP8 astrocytes. The latter were less able to protect cortical neurons from both strains than were SAMR1 astrocytes. When SAMR1 and SAMP8 neurons were cultured with astrocyte-conditioned medium from SAMR1 or SAMP8 astrocytes, similar neuronal survival was attained. Therefore, differences in neuroprotective capacity seem not to be mediated by soluble compounds released from astrocytes to medium. The attained neuroprotection was lower than that observed in co-cultures. Accordingly, it is well known that survival of neurons cultured in conditioned medium from astrocytes is significantly prolonged in relation to pure neuronal cultures, but is lower than that obtained when neurons and astrocytes are co-cultured (Bauer & Tontsch, 1990; Walsh *et al*., 1992). This suggests that several cellular mechanisms can underlie the neuroprotective role of astrocytes in addition to soluble factors. It can be suggested that alterations in oxidative stress, mitochondrial function, glutamate uptake and/or tau phosphorylation in SAMP8 astrocytes are underlying factors in the lost of neuroprotection. For instance, reduction in glial glutamate uptake capacity may elevate extracellular glutamate levels leading to neuronal excitotoxicity (Rohstein *et al*., 1996). Thus, changes in astrocyte function may play a key role in determining neuronal survival in the aged SAMP8 brain. Indeed, increases in GFAP expression and PK-11195 binding activity (a marker of gliosis), together with neurodegeneration, have been reported in the hippocampus and cerebral cortex of SAMP8 mice (Nomura *et al*., 1996; Wu *et al*., 2005; Sureda *et al*., 2006). Altered astrocytic function may contribute not only to brain neurodegeneration, but also to the premature learning and memory deficits observed in this murine model of early aging.

In summary, we have shown that astrocytes cultured from neonatal SAMP8 mice present similar alterations to those described in the whole brains of SAMP8 mice at 1–5 months of age. Similar to what occurs during brain aging, increased ROS generation in SAMP8 astrocytes can lead to lipid and protein oxidation, which in turn can cause changes in mitochondrial activity, tau phosphorylation and glutamate transport. All of these alterations can compromise astrocyte functionality. In this context, we have shown that SAMP8 astrocytes have reduced neuroprotective ability. This finding is in agreement with our previous studies showing decreased neuroprotection in astrocytes aged in culture (Pertusa *et al*., 2007). The present study demonstrates the dysfunction present in SAMP8 astrocytes and also supports the hypothesis that age-related alterations in astrocytes contribute to neurodegeneration.

Experimental procedures

Materials

SAMP8 and SAMR1 mice were provided by Harlan Interfauna Ibérica (Barcelona, Spain). Dulbecco's modified Eagle medium (DMEM), gentamycin and fetal bovine serum (FBS) were purchased from Gibco-BRL (Invitrogen, Paisley, UK). Culture plates, chamber slides and flasks were from NUNC (Roskilde, Denmark). L-[^{3}H]-glutamate (specific activity, 51 Ci mmol^{-1}) was purchased from PerkinElmer Inc. (Wellesley, MA, USA). The fluorescent probes 2′-7′-dichlorfluorescin diacetate (DCFH-DA), dihydroethidium and rhodamine-123 were obtained from Molecular Probes (Leiden, The Netherlands). L-glutamic acid, H_2O_2, thiazolyl blue tetrazolium bromide (MTT), trolox, curcumin, roscovitine and lithium chloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-tau [pSer^{199}], anti-tau [pSer^{396}] and anti-Pan-Tau antibodies were from Biosource International Inc. (Camarillo, CA, USA), anti-Cdk5, anti-p35 and anti-p25 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), anti-Gsk3β [pTyr^{216}] and anti-Gsk3β [pSer^9] from Bioreagents (Golden, CO, USA), anti-GLAST antibody from Alpha Diagnostic International (San Antonio, TX, USA), anti-NeuN from Chemicon International (Temecula, CA, USA) and anti-neurofilament (NF) from DAKO (Glostrup, Denmark). All chemicals were of analytical grade.

Astrocyte cultures

Primary cultures enriched in astrocytes were established using cerebral cortical tissue from 2-day-old SAMP8 and SAMR1 mice.

Brains were dissected free of the meninges, diced into small cubes and dissociated by incubation with a 0.5% trypsin-EDTA solution (Gibco) for 25 min. Cells were seeded at 0.25×10^6 cells mL^{-1} (0.75×10^5 cells cm^{-2}) in multiwell plates, chamber slides or flasks in DMEM supplemented with 2.5 mM glutamine, 100 μg mL^{-1} gentamycin and 20% FBS at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The culture medium was changed every 3–4 days and FBS was progressively lowered to 10% during the first 2 weeks. Experiments were routinely carried out at 21 days *in vitro*. Established astrocyte cultures of both SAMR1 and SAMP8 consisted of 85–90% astrocytes, 10–15% microglia and 0.1–1% oligodendroglia. Neither cell death nor morphological alterations were observed in SAMP8 cultures.

Neuronal survival in co-culture with astrocytes

To study the neuronal protective response of SAMR1 and SAMP8 astrocyte cultures, co-cultures of neurons and astrocytes were plated on 12-mm coverslips. Fresh neurons disaggregated from the cerebral cortical tissue of SAMR1 and SAMP8 15-day-old fetuses were directly seeded at 1.5×10^6 cells mL^{-1} (4.5×10^5 cells cm^{-2}) on a confluent monolayer of SAMR1 and SAMP8 astrocyte cultures. Cultured medium was DMEM supplemented with 0.2 mM glutamine, insulin B 100 mU L^{-1}, pABA 7 μM and 10% FBS. Neuronal survival was assessed at 12 DIV. Dead cells were detected by staining the cultures with 7.5 μM propidium iodide (red fluorescence) for 30 min. Cultures were subsequently washed with PBS and fixed with 4% paraformaldehyde. Living neurons were identified by immunostaining with NeuN or NF antibodies. Cells were permeabilized with 0.25% Triton in PBS for 30 min, washed with PBS, incubated with goat serum to block unspecific binding sites at room temperature, and incubated with mouse Neu-N or NF antibodies (diluted 1 : 200) overnight at 4 °C. Cultures were then washed with PBS and incubated with anti-mouse Alexa Fluor 488 (green fluorescence) for 1 h at room temperature. After washing with PBS, nuclei were stained with 5 μM bisbenzimide. Finally, cultures were mounted on microscope slides. Randomly chosen fields were examined using a fluorescence microscope (Nikon Eclipse E1000, Nikon, Tokyo, Japan) and digitally photographed with a ColorView camera (Soft Imaging Systems, Stuttgart, Germany). Neuronal survival was determined by counting immunoreactive neurons to NeuN or NF that were not propidium iodide stained, using the ANALYSIS software (Soft Imaging System). Astrocyte conditioned media were prepared from SAMR1 and SAMP8 astrocyte cultures at 21 DIV. For that purpose, astrocyte medium was changed to the neuronal medium used for co-cultures (see above). Conditioned media were collected after 48 h, centrifuged for 5 min at $300 \times g$ and used immediately. Neurons were seeded in astrocyte-conditioned media supplemented with 30% of fresh neuronal medium to provide a complete nutrient provision. Media were partially changed every 4 days with fresh conditioned media. At 12 DIV, neuronal survival was measured by the same procedure as in co-cultures.

Reactive oxygen species generation

Intracellular generation of hydroperoxides and superoxide anion radicals was determined using DCFH-DA and dihydroethidium, respectively, as previously described (Sebastià *et al.*, 2004). Briefly, cultures in 96-well plates were washed in HBSS and loaded with 10 μM DCFH-DA for 20 min at 37 °C. Wells were then washed with HBSS and basal 2′-7′-dichlorfluorescein (DCF) fluorescence was measured after 1 h incubation at 37 °C in a fluorescence plate reader (Spectramax Gemini XS, Molecular Devices, Wokingham, UK) at 485 nm excitation/530 nm emission. To measure superoxide anion generation, cultures in 96-well plates were loaded with 4.8 μM dihydroethidium and basal ethidium fluorescence was measured at 485 nm excitation/590 nm emission after 1 h at 37 °C.

Lipid and protein damage

Lipid peroxidation and protein damage were determined as described previously (Alvarez-García *et al.*, 2006). Lipid peroxidation was measured by determining malondialdehyde and 4-hydroxyalkenal using a Lipid Peroxidation Assay Kit from Calbiochem (EMD Biosciences Inc., Darmstadt, Germany). Protein damage was determined as carbonylated proteins. The chromogene 2,4-dinitrophenylhydrazine reacted with the carbonyl groups of the damage proteins. Protein carbonyls were determined at 366 nm.

Mitochondrial membrane potential

Changes in mitochondrial membrane potential were measured using rhodamine 123. Cultures in 96-well plates were incubated with 13 μM rhodamine 123 at 37 °C for 1 h. The cells were washed twice with HBSS, and fluorescence was determined at 507 nm excitation/529 nm emission.

Western blot analysis

After shaking the cultures for 4 h to eliminate microglial cells, the medium was removed and astrocytes were rinsed twice with PBS. Cells were lysed for 10 min on ice in RIPA buffer (10 mM PBS, 1% Igepal AC-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing a protease inhibitor cocktail (Complete) and 1 mM orthovanadate. They were then collected and frozen at –20 °C until assay. Protein extracts were quantified following the Bradford method. Subsequently, 15 μg of the protein extracts were denatured at 100 °C for 5 min, loaded onto a 15% sodium dodecyl sulfate–polyacrylamide gel and electrophoresed. Proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA). This was incubated overnight at 4 °C with the following primary polyclonal antibodies: anti-tau p[Ser^{199}] and anti-tau p[Ser^{396}] diluted to 1 : 2000; anti-Gsk3β p[Tyr^{216}], anti-Gsk3β p[Ser^9], anti-Cdk, and anti-p35/p25 diluted to 1 : 1000; anti-GLAST diluted to 1 : 500. Membranes were incubated for 1 h at room

temperature with horseradish peroxidase-conjugated secondary antibodies. Proteins were detected with a chemiluminiscence detection system based on the luminol reaction. Protein loading of the gels was controlled by staining blots with an antibody against β-actin (diluted 1 : 10 000). The immunoreactive bands were digitalized and a densitometry analysis was performed using Quantity One software (Bio-Rad). The levels of protein immunoreactivity were normalized to that of β-actin. In some experiments, SAMP8 astrocytes were treated with 10 mM lithium and 15 μM roscovitine for 48 h.

Glutamate uptake

Astrocyte glutamate uptake was determined as previously described (Pertusa *et al.*, 2007). Glutamate culture medium was removed from 96-well cultures and astrocytes were washed with warm HEPES-buffered saline solution (HBSS) (136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2PO_4 and 10 mM HEPES) containing 9 mM glucose at pH 7.3. Astrocyte cultures were then incubated for 10 min at 37 °C in HBSS with 9.8 nM (500 nCi mL^{-1}) of [^{3}H]-glutamate and several concentrations of unlabelled glutamate in the range of 1 μM to 1000 μM. Uptake was terminated by removing the medium and washing the cells three times with ice-cold HBSS. This was immediately followed by cell lysis in 0.2 N NaOH. Aliquots were taken for liquid scintillation counting (with Optiphase 'Hisafe' cocktail) and for Bradford's protein assay using bovine serum albumin as the protein standard. Radioactivity was analysed by scintillation counting in a Wallac 1414 Liquid Scintillation Counter (PerkinElmer).

Statistical analysis

Experiments were performed with astrocytes from three to eight primary cultures (*n* for each experiment is indicated in the corresponding figure legend). Data were pooled and the results given as mean ± SEM. Statistical significance ($p < 0.05$) was determined by the Student's *t*-test and two-way analysis of variance followed by Bonferroni's multiple comparison test.

Acknowledgments

This study was supported by grants from the Spanish Ministerio de Educación y Ciencia (SAF2006-13092-C02-02), Instituto de Salud Carlos III (RD06-0013-1004) and Generalitat de Catalunya (DURSI 2005/SGR/00826), and from Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED). S. García-Matas and R. Rubio-Acero received a Generalitat de Catalunya and IDIBAPS fellowship, respectively. We thank A. Parull for his skilful technical assistance.

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