Universidade de Santiago de Compostela
Facultade de Medicina
Departamento de Ciencias Morfolóxicas

Sistema Renina-Angiotensina y degeneración dopaminérgica: Papel de la activación microglial, el complejo NADPH-oxidasa y canales de potasio mitocondriales dependientes de ATP. Implicaciones en neuroprotección.

Tesis doctoral

Belén Joglar Santos

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Fotografía de portada: Inmunofluorescencia doble para OX-42, GFAP ó TH (verde) y para los receptores AT1 ó AT2, ó para las subunidades del complejo NADPH-oxidasa gp91 o p47 (rojo) en cultivos primarios mesencefálicos (a-l) o sustancia negra de ratón (m-q). AII, angiotensina II; AT1, receptor de AII de tipo 1; AT2, receptor de angiotensina de tipo 2; OX-42 (marcador de microglía); GFAP, proteína acídica fibrilar glial (marcador de astroglía); TH, tirosina hidroxilasa (marcador de neuronas dopaminérgicas). Escala: 30 µm (p), 50 µm (a–d, l, o y q), 75 µm (e–k) y 100 µm (m, n).

D. José Luis Labandeira García , Catedrático de Anatomía de la Facultad de Medicina de la Universidad de Santiago de Compostela y ***Dña. Jannette Rodríguez Pallares*** , Profesora Titular de la misma Universidad,

HACEN CONSTAR QUE:

Dña. Belén Joglar Santos ha realizado bajo nuestra dirección el trabajo de su Tesis Doctoral *Sistema Renina-Angiotensina y degeneración dopaminérgica: Papel de la activación microglial, el complejo NADPH-oxidasa y canales de potasio mitocondriales dependientes de ATP. Implicaciones en neuroprotección*, y que dicho trabajo reúne todas las condiciones necesarias para ser presentado para su valoración por la comisión correspondiente.

Y para que así conste y surta los efectos oportunos,

lo firmamos en Santiago de Compostela, a 22 de Mayo de 2012.

José Luis Labandeira García
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Belén Joglar Santos

A mis padres, Alfonso y Tere,

a mis hermanos, Felipe y Judit,

a Manu.

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Sistema Renina-Angiotensina y degeneración dopaminérgica: Papel de la activación microglial, el complejo NADPH-oxidasa y canales de potasio mitocondriales dependientes de ATP. Implicaciones en neuroprotección.

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3. Abreviaturas

- **6-OHDA** 6-hidroxidopamina
- **A(1-7)** Angiotensina (1-7)
- **AI** Angiotensina I
- **AII** Angiotensina II
- **AIV** Angiotensina (3-8)
- **AT1** Receptor de angiotensina de tipo 1
- **AT2** Receptor de angiotensina de tipo 2
- **BDNF** Factor neurotrófico derivado del cerebro
- **CNTF** Factor neurotrófico ciliar
- **COMT** Catecol-orto-metil-transferasa
- **EORs** Especies oxigenadas reactivas
- **EP** Enfermedad de Parkinson
- **EPPR** Elemento de respuesta del proliferador de peroxisomas
- **GAP** Proteína activadora de GTPasas
- **GDI** Inhibidor de disociación de GDP
- **GDNF** Factor neurotrófico derivado de la glía
- **GEF** Factor intercambiador de nucleótidos de guanina
- **GPe** Globo pálido externo
- **GPi** Globo pálido interno
- **iNOS** Sintasa de óxido nítrico inducible
- **iPS** Células pluripotentes inducidas
- **K(ATP)** Canales de potasio dependientes de ATP
- **L-DOPA** Levodopa
- **LRRK2** Quinasa 2 rica en repeticiones de leucina
- **MAO-B** Monoamino oxidasa-B
- **MAP-2** Proteína de unión a microtúbulos 2
- **mitoK(ATP)** Canales de potasio mitocondriales dependientes de ATP
- **$MPDP^+$** 1-metil-4fenil-2,3-dihidropiridina
- **MPP^+** 1-metil-4-fenilpiridina
- **MPTP** 1-metil-4-fenil.1,2,3,6-tetrahidropiridina
- **NMDA** N-metil D-aspartato
- **NST** Núcleo subtalámico
- **PARK-1** A-sinucleína
- **PARK-2** Ubiquitina E3 ligasa
- **PARK-7** DJ-1
- **PINK-1** Quinasa 1 p-ten inducida
- **PLZF** Factor de transcripción promielocítico de dedos de Zinc
- **PPAR-γ** Receptor gamma activado por peroxisomas
- **PRR** Receptor de (pro)renina
- **RXR** Receptor X de retinoides
- **sK(ATP)** Canales de potasio de membrana dependientes de ATP
- **SNC** Sistema nervioso central
- **SNpc** Sustancia negra pars compacta
- **SRA** Sistema renina-angiotensina
- **SNpr** Sustancia negra pars reticulata
- **SUR** Receptor sulfonilurea
- **UHCL-1** Ubiquitina carboxil-hidroxilasa 1

- VMAT-2 Transportador vesicular monoamino de tipo 2
- CRD Dominio de repetición rico en cisteínas
- RBD Dominio de unión a Rho
- ROCK Rho-quinasa

INTRODUCCIÓN

1.- INTRODUCCIÓN

1.1- ENFERMEDAD DE PARKINSON

La enfermedad de Parkinson (EP) es la segunda enfermedad neurodegenerativa más común después de la enfermedad de Alzheimer. Fue descrita por primera vez en 1817 por James Parkinson con el nombre de "Parálisis Temblorosa" (Kempster y col., 2007). Se estima que la incidencia de la EP es de 8-18 personas por cada 100000 al año y afecta a más del 1% de la población mayor de 60 años llegando hasta el 4% en la población mayor de 80 años (de Lau y Breteler, 2006). La EP es una enfermedad neurodegenerativa caracterizada por la degeneración progresiva de las neuronas dopaminérgicas de la sustancia negra pars compacta (SNpc) y por la presencia de inclusiones proteicas citoplasmáticas intraneuronales conocidas como cuerpos de Lewy. Esta pérdida de neuronas dopaminérgicas tiene como consecuencia un marcado déficit de dopamina a nivel del estriado. La dopamina es un neurotransmisor que es sintetizado por las neuronas mesencefálicas de la SNpc y del área tegmental ventral, así como también por otros grupos de neuronas como las neuronas hipotalámicas de los núcleos arcuato y periventricular (Carlsson y col., 1962). Las neuronas de la SNpc inervan el estriado a través de la vía nigroestriatal. La dopamina actúa como neuromodulador controlando importantes funciones fisiológicas tales como los movimientos voluntarios, aprendizaje y producción de hormonas. El déficit de dopamina a nivel del estriado da lugar a los principales síntomas clínicos de la EP: acinesia, rigidez y temblor (Hughes y col., 1992; Bergman y Deuschl, 2002). Se sabe que además de la SNpc, otras áreas del cerebro se ven afectadas, y que la degeneración también tiene lugar fuera del sistema nervioso central (SNC) como es el caso del sistema nervioso entérico (Lebouvier y col., 2009).

1.1.1- CARACTERÍSTICAS CLÍNICAS

Se caracteriza por una serie de alteraciones tanto motoras como no motoras. Las principales alteraciones motoras son del tipo de la bradicinesia (lentitud en la realización de movimientos), que se manifiesta en dificultad para caminar y en la ausencia de mímica o expresión facial. En los casos más extremos puede derivar en acinesia, temblor, rigidez e inestabilidad postural (Obeso y col., 2010). Estas alteraciones son consecuencia de la pérdida progresiva de las neuronas dopaminérgicas de la SNpc y de sus proyecciones hacia el estriado (vía nigroestriatal). Entre las

manifestaciones no motoras caben destacar los problemas disautonómicos (incontinencia urinaria, disfunción sexual…), la depresión y los trastornos cognitivos; debidos a que el proceso degenerativo afecta también a distintas estructuras extranígricas tales como el locus coeruleus, núcleo dorsal del rafe y el núcleo basal de Meynert (Ikeda y col., 1978; Mínguez-Castellanos y Escamilla-Sevilla, 2005; Lim y Lang, 2010).

1.1.2- NEUROPATOLOGÍA

Aunque clínica y patológicamente, ambas formas de la EP (esporádica y familiar) difieren en varios aspectos significativos, ambas muestran las mismas anormalidades bioquímicas a nivel cerebral.

La principal característica neuropatológica de la EP es la pérdida de neuronas dopaminérgicas de la SNpc. Sin embargo, es importante enfatizar el hecho de que la neuropatología no está totalmente restringida a esta vía nigroestriatal, sino que se pueden encontrar anormalidades histológicas en otros grupos celulares dopaminérgicos y no dopaminérgicos (Przedborski, 2005). Además de esta disminución de dopamina en el estriado también se detectan déficits de neuropéptidos tales como la encefalina y la sustancia P en la SNpc y en el estriado; y de somatostatina en el hipocampo, el tronco cerebral, bulbo olfatorio y en la corteza frontal (Agid y Blin, 1987; Fearnley y Less, 1991; Mc Geer y col., 1988). Los síntomas típicos de la enfermedad no aparecen hasta que al menos un 50% de las neuronas dopaminérgicas de la SNpc ha degenerado, lo que se corresponde a su vez con una pérdida del 70-80% de la dopamina estriatal (Braak y col., 2004; Samii y col., 2004).

La segunda característica neuropatológica más importante es la presencia de inclusiones intraneuronales conocidas como cuerpos de Lewy, descritas por Lewy en 1912, que suelen aparecer en las neuronas dopaminérgicas supervivientes de la SNpc y en todas las regiones afectadas del cerebro. Son agregados esféricos eosinófilos que se localizan a nivel citoplasmático compuestos por una variedad de proteínas tales como α-sinucleína, parkina, ubiquitina y neurofilamentos (Dickson y col., 2009; Wolters, 2009). La presencia de estas estructuras se considera un indicativo neuropatológico de la enfermedad, aunque no exclusivo, ya que existen casos de individuos en los que presentando toda la sintomatología asociada a la EP, no se han detectado cuerpos de Lewy en exámenes post-morten. La actividad que los cuerpos de Lewy desempeñan es controvertida, ya que no está claro si causan la degeneración de la célula en la que se

hayan o la protegen acumulando proteínas que de otra forma podrían ser dañinas (Jellinger, 2009).

1.1.3- FISIOPATOLOGÍA: alteración de los circuitos de los ganglios basales

Algunos trastornos del movimiento como es el caso de la EP se producen como consecuencia de la alteración de los circuitos de los ganglios basales.

Los ganglios basales son un grupo de núcleos subcorticales localizados en el telencéfalo e interconectados entre sí con otras estructuras cerebrales (diencéfalo y mesencéfalo). Constituyen un gran sistema de recepción y procesamiento de aferencias corticales, talámicas y de núcleos del tronco del encéfalo. Las principales estructuras que lo conforman se encuentran clasificadas en núcleos de entrada, de salida e intrínsecos. El núcleo de entrada es el cuerpo estriado y los núcleos de salida son el globo pálido interno (GPi) y la sustancia negra pars reticulada (SNpr). Finalmente, se consideran núcleos intrínsecos el globo pálido externo (GPe), el núcleo subtalámico (NST) y la SNpc. Estos núcleos son responsables en gran parte de los programas motores corticales (Obeso y col., 2008).

Como hemos mencionado anteriormente, independientemente de la causa, la EP se debe fundamentalmente a una degeneración de la SNpc, lo que significa una pérdida de neuronas dopaminérgicas nigroestriatales. La dopamina ejerce un papel modulador, de forma que activa a las neuronas estriatales GABAérgicas que contienen el neuropéptido sustancia P (vía directa) e inhibe a las neuronas que contienen encefalina (vía indirecta). Por ello, tras la degeneración del sistema dopaminérgico, se produce una disminución del impulso excitador de la vía directa y un aumento del impulso inhibidor de la vía indirecta. El aumento de la inhibición de la vía indirecta conlleva a una sobreinhibición del GPe, una desinhibición del NST y un incremento de la excitación del GPi y de la SNpc; mientras que la menor activación de las neuronas estriatales de la vía directa produce una reducción en su influencia inhibitoria sobre el GPi y la SNr. Este incremento en la actividad neuronal de estos dos núcleos producirá una sobreinhibición del tálamo y en consecuencia, una hipoestimulación del córtex, lo que favorecerá una disminución de la producción de los movimientos (Albin y col., 1990; De Long y Wichmann, 2007; Obeso y col., 2008; Vitek, 2008).

1.1.4- ETIOLOGÍA

Aunque su etiología continúa siendo incierta, hoy en día se considera que el origen de la enfermedad se debe a la interacción de múltiples factores. Estudios epidemiológicos han revelado que la mayoría de los casos de EP (90%) son esporádicos y de aparición tardía (Tanner, 2003). Y que el 10% restante se relaciona con una aparición temprana de los síntomas y con antecedentes familiares (Mizuno y col., 2001). El desarrollo del síndrome parkinsoniano o familiar se atribuye a la aparición de mutaciones en determinados genes entre los que se incluyen α-sinucleína (PARK-1), ubiquitina carboxil-hidroxilasa-1 (UHCL-1), ubiquitina E3 ligasa (PARK-2), DJ-1 (PARK-7), quinasa 1 p-ten-inducida (PINK-1, PARK-6) y LRRK2 (Qian y col., 2010). Sin embargo, el caso de la EP idiopática parece ser el resultado final de un conjunto de interacciones entre múltiples factores, entre los que caben destacar la propia vulnerabilidad de las neuronas dopaminérgicas, una predisposición genética, y la exposición a toxinas ambientales. Entre las toxinas ambientales tanto agentes infecciosos como pesticidas y metales pesados se han asociado con el desarrollo y progresión de la EP (Qian y col., 2010).

Otra hipótesis que se baraja es que toxinas endógenas puedan ser las responsables de la neurodegeneración en la EP. Esto significa que alteraciones en el metabolismo puedan crear sustancias tóxicas debido a exposiciones ambientales o a diferencias en las vías metabólicas heredadas (Dauer y Przedborski, 2003).

1.1.5- PATOGÉNESIS

En base a los datos obtenidos de numerosos estudios llevados a cabo con modelos neurotóxicos de la EP parece que la degeneración de neuronas dopaminérgicas de la vía nigroestriatal no resulta de la acción de un único factor deletéreo, sino de la convergencia de múltiples factores patogénicos. Muchos de estos factores nocivos son sintetizados en el interior de las propias neuronas dopaminérgicas, mientras que otros se originan fuera de éstas como por ejemplo de la población glial. Algunos de estos factores nocivos son: estrés oxidativo, disfunción mitocondrial, carencia de factores neurotróficos, neuroinflamación y excitotoxicidad.

Así mismo, teniendo en cuenta las investigaciones realizadas en casos genéticos de la EP, se ha visto la gran importancia de la agregación del exceso de proteínas con respecto a los mecanismos de muerte neuronal, perturbaciones en los sistemas de

degradación proteica, y la acumulación de proteínas no deseadas (Przedborski e Ischiropoulos, 2005).

Estrés oxidativo:

Las especies oxigenadas reactivas (EORs) son moléculas derivadas del metabolismo del oxígeno muy reactivas debido a que poseen electrones desapareados, lo que hace que actúen como radicales libres que interaccionan y alteran diversos componentes celulares. Las células eucariotas han desarrollado mecanismos para protegerse del daño producido por estas especies reactivas y repararlo. Cuando la generación de EORs excede la capacidad reparativa de la célula ésta sufre muerte necrótica o apoptótica. Las principales fuentes de EORs en la célula son la cadena respiratoria mitocondrial y el complejo NADPH-oxidasa (Babior, 1999; Babior, 2004; Szeto, 2006; Rodriguez-Pallares y col., 2007, 2008). Aunque los niveles de estrés oxidativo en individuos de edad avanzada están elevados en ausencia de enfermedad, en los individuos afectados se ha visto la existencia de peroxidación lipídica y nitración proteica, con una clara disminución de moléculas antioxidantes como el glutatión (Gandhi y Wood, 2005; Yuan y col, 2007; Zhou y col., 2008). Se ha sugerido que la proteína DJ-1 puede actuar como molécula antioxidante y que una mutación en el gen que la codifica que causa una forma de EP está relacionada con el estrés oxidativo (Dodson y Guo; 2007).

Disfunción mitocondrial:

La mitocondria es la principal fuente de EORs en la célula y se originan en los complejos I y III de la cadena respiratoria mitocondrial. El hecho de que algunas EORs no atraviesen con facilidad la membrana mitocondrial interna hace que estos orgánulos sean especialmente vulnerables. El estrés oxidativo generado en el interior de la mitocondria tiene un efecto deletéreo sobre muchas moléculas: sobre proteínas relacionadas con el metabolismo energético generando una caída en los niveles de ATP que finalmente afectará a la viabilidad celular, y sobre lípidos de membrana que sufren peroxidación lipídica desencadenando el inicio del proceso apoptótico. Numerosas neurotoxinas se han relacionado con la disfunción mitocondrial y el aumento en los niveles de EORs. Se ha visto que PINK-1 tiene propiedades neuroprotectoras y se localiza en la mitocondria, y que mutaciones en dicho gen provocan una forma recesiva de la EP (Gu y col., 2010; Xie y col., 2010; Keane y col., 2011).

Carencia de factores neurotróficos:

Los factores neurotróficos son proteínas capaces de unirse a receptores de determinadas células para estimular su supervivencia, crecimiento o diferenciación. Algunos tipos celulares del sistema nervioso central (SNC) pueden sintetizar una variedad de moléculas neurotróficas tales como el factor neurotrófico ciliar (CNTF), el factor neurotrófico derivado del cerebro (BDNF) y el factor neurotrófico derivado de la glía (GDNF), que tienen la capacidad de asegurar la supervivencia de las células nerviosas vecinas. En el SNC adulto normal estos factores se expresan de forma constitutiva a bajas dosis, pero tras un daño o lesión sus niveles aumentan por una sobreexpresión de los mismos. Por lo tanto, una reducción en esta capacidad de respuesta supondría la alteración de un importante mecanismo de defensa y contribuiría a la degeneración celular (Olanow y Tatton, 1999). Estudios en el líquido cefalorraquídeo y en regiones de la vía nigroestriatal de pacientes con EP muestran que los niveles de algunos factores neurotróficos están alterados en comparación con individuos de la misma edad no afectados (Mogi y col, 1999; Nagatsu y col., 2000; Nagatsu y Sawada, 2007).

Inflamación:

La inflamación es el principal mecanismo de defensa frente a eventos patogénicos y toxinas ambientales. La respuesta inflamatoria del cerebro es llevada a cabo por las células gliales, en especial por la microglía. De modo que ante cualquier deterioro del tejido nervioso, ya sea en situaciones de lesión neuronal, enfermedad e incluso envejecimiento, la glía posee la capacidad de responder experimentando de manera gradual cambios morfológicos y funcionales, acompañados de la producción de citocinas pro-inflamatorias y la adquisición de funciones fagocíticas, lo que se conoce como “gliosis reactiva o glía activada” (Wu y col., 2002; Depino y col., 2003; Gao y col., 2003; Ouchi y col, 2005; Zhang y col., 2005). Se trata de una respuesta inflamatoria multifactorial que proporciona mecanismos de defensa frente a diversidad de agresiones y que va dirigida a eliminar los agentes nocivos e inhibir sus efectos perjudiciales. Sin embargo, una activación excesiva o descontrolada puede resultar extremadamente nociva e inducir o potenciar la progresión del proceso degenerativo (Lull y Block, 2010). Diversos estudios han revelado la presencia de microglía activada en varias regiones del cerebro de pacientes en estadíos tempranos de la EP (Ouchi y col., 2005; Gerhard y col., 2006). Así mismo, en análisis post-morten de la sustancia

negra de pacientes afectados por la enfermedad se ha visto un incremento en la activación microglial (Ghosh y col., 2007), así como en la expresión de citoquinas pro-inflamatorias, tales como interleucina-1α e interleucina-1β (Mogi y col., 1994; Hunot y col., 1999; Nagatsu y col., 2000). Además, hay estudios genéticos que describen la existencia de una asociación entre la existencia de polimorfismos del gen de la interleucina-1 con un incremento en el riesgo de padecer EP (Nishimura y col., 2000; Dodel y col., 2001; Mcgeeret y col., 2002; Schulte y col., 2002). Todo esto sugiere que la activación microglial y la consecuente liberación de citocinas juegan un papel importante en el proceso neuropatológico de la EP.

Excitotoxicidad:

El glutamato es un neurotransmisor común en numerosas sinapsis de tipo excitatorio. Una activación excesiva de los receptores del glutamato se asocia con el daño neuronal en muchas enfermedades neurológicas. Concretamente, en los afectados de EP la vía glutamatérgica está hiperactivada (Przedborski, 2005). John Olney denominó este fenómeno como "excitotoxicidad" (Olney, 1990). Esta forma de toxicidad es mediada al menos en parte por una activación excesiva de los receptores N-metil D-aspartato (NMDA) cuya consecuencia inmediata es la entrada de calcio al interior celular (Pivovarova y Andrews, 2010). Este aumento en los niveles de calcio intracelular junto con la unión del calcio a determinadas proteínas como la calmodulina conlleva a la activación de la sintasa de óxido nítrico neuronal que a su vez genera un aumento en la producción de óxido nítrico, el cual reaccionaría con radicales superóxido para la formación de peróxido nítrico y radicales libres, ambos potentes agentes oxidantes (Beckman y col., 1990). Además, el óxido nítrico puede producir una alteración irreversible de la cadena respiratoria mitocondrial (Bolanos y col., 1996) provocando disfunción sináptica, pérdida de plasticidad y degeneración neuronal (Mattson, 2007).

Alteración del sistema proteolítico:

Hay evidencias que sugieren que fallos del sistema ubiquitina-proteosoma y la formación de proteínas mal plegadas también están implicados en la patogénesis molecular de ambas formas de la EP, familiar y esporádica (Moore y col., 2005; Gu y col., 2010). De hecho, se han observado déficits tanto funcionales como estructurales del proteosoma en la SNpc de pacientes con EP (McNaught y col., 2002; 2003;

McNaught y Olanow, 2003). Así mismo, se han hecho estudios con ratas exponiéndolas a inhibidores sintéticos del proteosoma y se sugirió que éstas reproducían las principales características de la EP (McNaught y col., 2004). Esto sugiere que quizás una disfunción proteosomal puede ser el punto final común que precipita la degeneración de neuronas dopaminérgicas en la EP (Moore y col., 2005). La acumulación y agregación de proteínas potencialmente citotóxicas, incluyendo la α-sinucleína, en los cuerpos de Lewy podría generar el desplegamiento generalizado de proteínas y el consiguiente estrés proteolítico, lo cual desembocaría finalmente en el fallo de la función del sistema ubiquitina-proteosoma.

1.1.6 -TRATAMIENTO

Las terapias actuales para la EP están dirigidas a mejorar la capacidad funcional del paciente durante el mayor tiempo posible, pero no modifican la progresión del proceso neurodegenerativo. Se basan fundamentalmente en el tratamiento farmacológico enfocado a suplir el déficit de dopamina y/o mimetizar sus efectos.

i) Tratamiento farmacológico

El tratamiento clásico para EP se basa en el uso del precursor de la dopamina, la levodopa (L-dopa) que reduce muchos de los síntomas motores de la enfermedad pero no afecta tanto a los síntomas no motores (Yuan y col., 2010). Actualmente, se usa en combinación con carbidopa que evita la conversión periférica de L-dopa a dopamina evitando así los efectos adversos de la dopamina a nivel sistémico y aumenta la biodisponibilidad de L-dopa en el cerebro (Chaná, 2010).

Hay mucha variabilidad en cuanto a la repuesta del tratamiento entre los distintos pacientes, pero en la mayoría de los casos los efectos beneficiosos son a corto plazo (Olanow y col., 2004). El tratamiento con L-dopa a largo plazo está asociado con numerosos efectos adversos a nivel motor (fenómenos “on-off”, discinesias…) limitando así su efectividad (Olanow y col., 2004; Jankovic, 2005; Rezak, 2007). De hecho algunos estudios sugieren que la L-dopa puede ser neurotóxica y que promueve aún más la degeneración neuronal (Jenner y Brin, 1998; Mytilineou y col., 2003; Muller y col., 2004; Weiner, 2006).

Otro tipo de fármacos utilizados son los inhibidores de la monoamino oxidasa-B (seleginina, rasagilina) (MAO-B) que inhiben el metabolismo *in vivo* de la dopamina y usados en combinación con la L-dopa potencian sus efectos antiparkinsonianos. Se

piensa que prolongan la acción sináptica de la dopamina al reducir su metabolización (Perez-Lloret y Rascol; 2011).

Por otro lado, están los fármacos anticolinérgicos que resultan útiles en pacientes con síntomas leves, especialmente cuando predomina el temblor y la rigidez, así como también distintos agonistas dopaminérgicos como la apomorfina, que pueden ser administrados de forma única para retrasar la necesidad de L-dopa, o en combinación con ésta para aumentar su efectividad, ya que potencian sus efectos y permiten disminuir la dosis total utilizada (Pondal y col., 1996; Frucht y col., 2002).

Otro tipo de fármacos empleados como terapia son los inhibidores de otro enzima que metaboliza la dopamina, la catecol-orto-metil-transferasa (COMT). Se suelen administrar de forma conjunta con la L-dopa (Schapira y col., 2009).

La eficacia limitada de estos tratamientos así como sus efectos colaterales hacen que se investiguen continuamente tratamientos alternativos. Una alternativa la representan los agentes neuroprotectores que buscan preservar las células y circuitos neuronales con el fin de retrasar, ralentizar o incluso detener la aparición y progresión de la EP una vez iniciado el tratamiento. Estos fármacos neuroprotectores tienen como diana los mecanismos patogénicos descritos anteriormente.

Se ha visto que el tratamiento con distintas moléculas antioxidantes disminuyen el estrés oxidativo generado en las neuronas dopaminérgicas (Singh y col., 2007a). Ejemplo de estos antioxidantes son las vitaminas A, C y E que consiguen reducir los niveles de peroxidación lipídica. Los inhibidores de la MAO-B como la rasagilina, además de incrementar la disponibilidad de dopamina en el estriado tienen propiedades antioxidantes. El selenio posee una actividad neuroprotectora por su relación con el metabolismo del glutatión. Otras moléculas capaces de reducir el estrés oxidativo son la N-acetil-cisteína, inhibidores de la enzima conversora de angiotensina, nicotina (Lopez-Real y col., 2005; Muñoz y col., 2004; Soto-Otero y col., 2002), melatonina, agentes quelantes de hierro, cafeína o estrógenos (Rodríguez-Perez y col., 2010).

Moléculas neuroprotectoras relacionadas con la función mitocondrial son la coenzima Q10 y la creatina. La coenzima Q10 es un componente clave de la cadena respiratoria mitocondrial implicada en la generación de ATP y tiene propiedades antioxidantes. La creatina es una molécula capaz de almacenar energía química y permite la formación de ATP en la mitocondria (Bonucelli y Del Dotto, 2006).

Para reducir la excitotoxicidad que ocurre en pacientes de EP se han probado inhibidores de la liberación de glutamato como el riluzol, antagonistas de receptores

NMDA y bloqueantes de canales de calcio (Rodriguez y col., 1998; Bonuccelli y Del Dotto, 2006; Pivovarova, 2010).

La carencia de factores neurotróficos se puede compensar mediante el uso de neuropéptidos como el GDNF (Yasuhara y col., 2007; Evans y Barker 2008; Diógenes y Outeiro, 2010). Otros fármacos tienen propiedades neuroprotectoras relacionadas con la expresión in situ de factores neurotróficos, favoreciendo la expresión de estos factores por parte de células presentes en el propio sistema nervioso (Sanchez y col., 2002). El ejercicio físico también favorece la supervivencia al inducir la expresión y aumentar la disponibilidad de diversos factores neurotróficos dentro y fuera del sistema nervioso (Villar-Cheda y col., 2009).

Debido al papel que la inflamación tiene en la patogénesis de la EP, el uso de agentes que reduzcan la reacción microglial o moléculas anti-inflamatorias como inhibidores de la ciclooxigenasa (aspirina, meloxicam) o anti-inflamatorios no esteroideos (ibuprofeno, naproxeno) han mostrado tener efectos beneficiosos (Bonuccelli y Del Dotto, 2006; Wilms y col., 2007; Gao y col., 2011).

Para evitar los daños producidos por alteraciones en el procesado de las proteínas se ha propuesto el uso de inhibidores de la función del proteosoma (Yamamoto y col., 2007). Por otro lado, se sabe que el hierro acelera la agregación de la α-sinucleína de modo que agentes quelantes del hierro (desferoxamine, agente V-28) también resultan beneficiosos al reducir la formación de agregados proteicos (Singh y col., 2007b).

ii) Tratamiento quirúrgico

Con anterioridad a la comercialización de la L-dopa ya se empleaban técnicas quirúrgicas como tratamiento de la EP. En la actualidad, se suelen utilizar en los casos de pacientes que se encuentran en fases tardías de la enfermedad, en las que las complicaciones derivadas del uso de la L-dopa son inhabilitantes.

La primera estrategia neuroquirúrgica que se realizó fue la talamotomía con la que se conseguía reducir el temblor contralateral (Singh y col., 2007a). Posteriormente, este tratamiento fue sustituido por la palidotomía, lesión del GPi, con la que se obtenía una mejora del temblor contralateral y sobre todo de las discinesias. En la actualidad, el principal tratamiento quirúrgico frente a la EP es la estimulación cerebral profunda del NST ó del GPi. Con esta técnica se consigue una mejora de los síntomas motores de la EP y una reducción de las complicaciones motoras inducidas por el tratamiento con

L-dopa. Ambos núcleos, el NST y el GPi, están hiperactivos en la EP y su inhibición con la estimulación a alta frecuencia produce un claro beneficio motor (Vitek, 2008).

Cabe destacar que sólo del 5 al 10% de los pacientes con EP son candidatos adecuados para este tipo de intervenciones quirúrgicas. Por este motivo se está investigando en el campo de la nanotecnología el desarrollo de sistemas basados en implantes de nanotubos de carbono o nanochips, sistemas que permitirían una mayor seguridad y precisión en la liberación de los impulsos y reducir los posibles efectos colaterales (Singh y col., 2007a).

iii) Terapia celular

La terapia celular en la EP se basa generalmente en el transplante de neuronas productoras de dopamina para reemplazar aquellas que han degenerado durante la patogénesis de la enfermedad. Es una estrategia prometedora ya que potencialmente se podría conseguir la completa restauración de la capacidad funcional (Hedlund y Perlmann, 2009).

Los transplantes celulares pueden actuar de distintos modos para lograr la recuperación del paciente: mediante la liberación de dopamina directamente por parte de las células transplantadas, por medio de la liberación de factores neurotróficos que favorezcan la supervivencia y la funcionalidad de las neuronas situadas en la SNpc, o por la acción local que permita la reinervación y el rebrote de terminales estriatales para la liberación de dopamina (Takahashi, 2007).

En la mayoría de los estudios las células proceden de tejido mesencefálico ventral de fetos humanos (Subramanian, 2001; Levy y col., 2004), pero presenta varios problemas, por un lado, las implicaciones éticas y por otro, que la fuente de obtención es muy limitada y se requieren cantidades sustanciales para que sea eficaz. Por estos motivos, en los últimos años la búsqueda de una fuente celular alternativa como por ejemplo el uso de células madre o células pluripotenciales inducidas (iPS) ha recibido especial atención (Arenas, 2010; Meyer y col., 2010; Kim, 2011).

iv) Terapia génica

Los avances en medicina molecular han permitido desarrollar estrategias de terapia génica para el posible tratamiento de la EP. Una de las principales ventajas de esta técnica es que permite la manipulación de la liberación del producto génico en localizaciones concretas, en las cantidades necesarias y de forma regulada mediante el

uso de vectores víricos (adenovirus o lentivirus) o no víricos (lisosomas) (Singh y col., 2007a).

En la actualidad, los ensayos clínicos de terapia génica se basan fundamentalmente en tres estrategias terapéuticas distintas. En primer lugar, aumentando los niveles de dopamina (terapia dirigida a aumentar la actividad de los genes implicados en la síntesis de dopamina). En segundo lugar, modulando el fenotipo neuronal (consiste en silenciar la hiperactivación del NST, se trata de convertir las neuronas excitatorias a un fenotipo inhibitorio) y por último, estrategias de neuroprotección (basadas en la expresión de factores neurotróficos como GDNF o neurturina) (Bjorklund y Kordower, 2010; Feng y Maguire-Zeiss, 2010).

1.1.7- MODELOS EXPERIMENTALES

Disponer de modelos experimentales de la EP es fundamental para poder seguir avanzando en la investigación. Un modelo experimental debería reproducir la mayoría, sino todas las características de la enfermedad en humanos. Los modelos nos permiten no solo investigar acerca de la etiología de la enfermedad, sino que también facilitan el desarrollo de nuevos agentes neuroprotectores o estrategias terapéuticas (von Bohlen y Halbach, 2005; Duty y Jenner, 2011).

Existen dos tipos de modelos experimentales, modelos *in vitro* y modelos *in vivo.* Los modelos *in vitro* proporcionan una importante herramienta para investigar los mecanismos moleculares y bioquímicos implicados en los procesos tóxicos. Son muy útiles para llevar a cabo estudios que son difíciles de realizar en los modelos *in vivo*. La principal limitación es que deben representar lo más exactamente posible el sistema *in vivo* que es objeto de estudio (Zeng y col., 2006). El uso de modelos animales permite no solo realizar investigación básica sino también investigar diferentes estrategias terapéuticas como requisito previo a ser testadas en pacientes. Un modelo animal de la EP adecuado debería reproducir las características principales de la misma, tales como, lesión selectiva de neuronas dopaminérgicas que evolucione con el tiempo, depleción de dopamina en el estriado y presencia de los cuerpos de Lewy. Así mismo desde el punto de vista del comportamiento, lo idóneo sería que presentase acinesia, rigidez y temblor.

Generalmente los modelos experimentales de la EP se basan en el uso de una única toxina o un único gen, diferenciando así modelos basados en el uso de toxinas y modelos genéticos (von Bohlen y Halbach, 2005; Meredith y col., 2008).

i) Modelos basados en el uso de toxinas

Entre las toxinas más usadas que inducen la degeneración de neuronas dopaminérgicas destacan la reserpina, 6-hidroxidopamina (6-OHDA), 1-metil-4-fenil-1,2,3,6-terahidropiridina/ 1-metil-4-fenil-piridina (MPTP/MPP^+), paracuat y rotenona.

* Modelos farmacológicos de depleción de dopamina: son los primeros modelos que se desarrollaron y se basan en la utilización de determinadas drogas que producen depleción de dopamina, tales como la α-metil-p-tirosina o la reserpina (inhibidor del transportador vesicular monoaminérgico) (Betarbet y col., 2002).

* Lesiones con 6-OHDA: es uno de los modelos más usados. Se trata de un compuesto neurotóxico, análogo noradrenérgico que presenta algunas similitudes estructurales con la dopamina y la norepinefrina, teniendo así alta afinidad por los transportadores catecolaminérgicos, transportadores de dopamina y transportadores de norepinefrina. Puede entrar por tanto en las neuronas dopaminérgicas y en las neuronas noradrenérgicas produciendo daño en las vías catecolaminérgicas de ambos sistemas nerviosos, central y periférico, se cree que por un efecto combinado de las EORs y quinonas (Blandini y col., 2008). Diversos estudios sugieren que además de las EORs fomadas por la autooxidación de la propia toxina (Soto-Otero y col., 2000) y la posible inhibición del complejo I de la cadena respiratoria mitocondrial (Glinka y col.,1997), una activación microglial temprana y EORs derivadas de la activación del complejo NADPH-oxidasa juegan un papel importante en la neurodegeneración dopaminérgica inducida por la 6-OHDA (Rodriguez-Pallares y col., 2007).

Es un modelo imperfecto ya que no manifiesta todos los síntomas, pero sin embargo resulta muy útil para testar terapias sintomáticas, estrategias neuroprotectoras y transplantes celulares.

Concretamente en esta tesis la 6-OHDA ha sido utilizada para llevar a cabo diferentes estudios *in vitro.*

* Lesiones con pesticidas: paracuat, rotenona y maneb son algunos de los pesticidas que permiten obtener modelos de EP. Sus mecanismos de acción están relacionados fundamentalmente con la generación de estrés oxidativo y la inhibición del complejo I mitocondrial (Allain y col., 2008).

* Lesiones con inhibidores del proteosoma: el hecho de que anomalías en el procesado de proteínas sea una característica tanto de la forma familiar como esporádica de la EP hacen del sistema proteolítico ubiquitin-proteosomal una diana para el desarrollo y utilización de modelos de EP (Mcnaught y Olanow, 2006).

* Lesiones con MPTP: El uso de MPTP es uno de los pocos casos en los que sus efectos como neurotoxina fueron descubiertos primero en humanos y después, en base a esto, se desarrolló un modelo animal. Su uso como herramienta en investigación comenzó a adquirir importancia a principios de la década de los ochenta tras la identificación de cierto número de heroinómanos del norte de California que presentaban síntomas idénticos a los de la EP (Burns y col., 1985; Langston, 1985). Aunque fue identificado inicialmente como agente parkinsoniano en humanos, se ha demostrado que ejerce efectos similares en otros primates (Jenner, 2003), así como en gatos y en varios tipos de roedores (solo cepas específicas de ratón son sensibles a la administración de MPTP) (Hamre y col., 1999). Estructuralmente es muy similar a varios agentes ambientales conocidos, como el herbicida paracuat y el insecticida rotenona, de los cuales se ha demostrado que inducen degeneración de células dopaminérgicas mediante mecanismos de acción ligeramente diferentes (Betarbet y col., 2000).

Se trata por tanto de una neurotoxina que cuando es inyectada de manera sistémica en primates no humanos y algunos roedores replica los signos característicos de la EP, incluyendo temblor, rigidez, inestabilidad postural y acinesia. Su mecanismo de acción consiste principalmente en la inhibición del complejo I mitocondrial. En la actualidad, el modelo con MPTP en ratón es útil para estudiar los cambios neuropatológicos y neuroquímicos, mientras que para estudiar el comportamiento el modelo en monos resulta más adecuado.

Dado que en esta tesis se utilizaron modelos *in vitro* e *in vivo* basados en el uso de esta neurotoxina, se explica a continuación su mecanismo de acción en más detalle:

El MPTP actúa como neurotoxina afectando preferentemente a las neuronas dopaminérgicas de la SNpc. Es altamente lipofílico por lo que puede atravesar la barrera hematoencefálica con facilidad. Una vez que atraviesa la barrera el primer paso en la toxicidad del MPTP ocurre a nivel de los astrocitos donde es metabolizado por acción del enzima MAO-B a 1-metil-4-fenil-2,3-dihidropiridina ($MPDP^+$) y a continuación, por un mecanismo aún no muy claro se desprotona originando el metabolito activo MPP^+

(Smeyne y Jackson-Lewis, 2005). El MPP^+ es un componente polar y no puede salir libremente de su ambiente glial. Se ha sugerido que puede haber un transportador específico que mueve activamente esta molécula polar al exterior, aunque hasta el momento este mecanismo se desconoce (Russ y col., 1996; Inazu y col., 2003). Una vez en el espacio extracelular, el MPP^+ puede entrar en el interior de las neuronas monoaminérgicas ya que tiene alta afinidad por los transportadores de dopamina, así como también por los transportadores de norepinefrina y serotonina, produciendo en todas ellas alteraciones bioquímicas y degeneración neuronal. La degeneración es mucho más prominente en las neuronas dopaminérgicas donde una vez dentro puede seguir tres vías o rutas (Dauer y Przedborski, 2003):

1) Puede entrar en el interior de la mitocondria donde interfiere con el complejo I de la cadena transportadora de electrones (Ramsay y Singer, 1986):

El MPP^+ entra en la mitocondria por difusión a través de la membrana interna mitocondrial, transporte activo mediado por un gradiente de membrana. Una vez en la mitocondria, inhibe la respiración celular mediante el bloqueo del enzima transportador de electrones NADH ubiquinona óxido-reductasa (complejo I) (Nicklas y col., 1985; Suzuki y col., 1990). Este bloqueo produce una reducción en la síntesis de ATP celular. Aunque este proceso parece ser el principal paso en el bloqueo de la función mitocondrial, diversos estudios han mostrado que el MPP^+ puede actuar también inhibiendo directamente los complejos III (ubiquinol ferrocitocromo C óxido reductasa) y IV (ferrocitocromo C óxido reductasa o citocromo C oxidasa) de la cadena transportadora de electrones (Mizuno y col., 1988). La pérdida de energía a través de cada una de estas vías tiene múltiples consecuencias, incluyendo la formación de radicales libres (que reaccionan para formar peróxido de hidrógeno), y la formación de radicales hidroxilo. El fallo energético que tiene lugar en el interior de las neuronas dopaminérgicas como consecuencia del bloqueo del complejo I mitocondrial no parece ser la causa inmediata de la muerte neuronal en la SNpc, sino que es debida a la acción de los componentes generados en la célula de manera secundaria (Cleeter y col., 1992). Así mismo, el óxido nítrico producido y liberado por las células gliales puede entrar en las neuronas a través de la membrana por difusión simple. En este punto, los radicales superóxido y el óxido nitríco, que no son particularmente dañinos por sí mismos pueden interaccionar y formar peroxinitrito, una de las moléculas oxidantes más dañinas (Ischiropoulos y al-Mehdi, 1995; Przedborski y col., 2000; Przedborski y Vila, 2003).

2) Puede permanecer en el citosol e interaccionar con enzimas citosólicos, especialmente con aquellos que poseen carga negativa (Klaidman y col., 1993).

3) Puede ser secuestrado en el interior de vesículas citoplasmáticas a través de los transportadores vesiculares monoamino de tipo 2 (VMAT-2) (Liu y col., 1992). Se trata de transportadores dependientes de protones que secuestran neurotransmisores monoamino en el interior de las vesículas sinápticas. Estructuralmente, el MPP^+ es muy similar a estos neurotransmisores monoamino de modo que puede ser transportado al interior de las vesículas a través de este tipo de transportadores, evitando así la entrada en la mitocondria donde podría inhibir el complejo I de la cadena transportadora de electrones. Por este motivo, se ha planteado la hipótesis de que puede tratarse de un potencial mecanismo protector por reducción de los efectos deletéreos de diferentes toxinas monoaminérgicas.

ii) Modelos genéticos

Se han descubierto varios genes asociados a la EP, lo cual es muy útil para desarrollar nuevos modelos animales. En la actualidad, se pueden distinguir tres tipos de modelos genéticos. El primero de ellos consiste en la deleción de genes importantes para el desarrollo o supervivencia de las neuronas dopaminérgicas o su fenotipo. En segundo lugar, modelos basados en la expresión o deleción de genes conocidos asociados con el desarrollo de la forma familiar de la EP, y por último modelos genéticos basados en el uso de vectores virales para controlar la expresión de genes o mutaciones conocidas y relacionadas con la EP familiar a nivel de las neuronas dopaminérgicas de la SNpc (Meredith y col., 2008).

De todos los genes identificados los más estudiados son: PARK-1, PARK-2, UCHL-1, DJ-1, PINK-1 (Dauer y Przedborski, 2003; Dawson y col., 2010). Los animales más usados son ratones y moscas (*Drosophila melanogaster*). Concretamente, las moscas transgénicas son excelentes modelos genéticos de la EP, sin embargo, la principal desventaja es que presentan un sistema nervioso mucho más simple que el de roedores y primates, así como un rango limitado de comportamientos locomotores que podamos testar (Maries y col., 2003). Por otro lado, trabajar con roedores y primates es mucho más caro de ahí que se estén desarrollando nuevos modelos genéticos en otros organismos como peces, anfibios y lombrices (Pienaar y col., 2010).

1.2- SISTEMA RENINA-ANGIOTENSINA

El sistema renina-angiotensina (SRA) representa uno de los sistemas filogenéticamente más antiguos y fue descrito por Tigerstedt y Bergman en 1898. Está formado por una familia de péptidos bioactivos con distintas actividades biológicas: angiotensina-(1-8) (AII), angiotensina-(3-8) (AIV) y angiotensina-(1-7) (A(1-7)). El péptido efector es la AII que ejerce una variedad de acciones a través de dos receptores específicos, el receptor de angiotensina tipo I (AT1) y el receptor de angiotensina tipo II (AT2). La función clásica de este sistema es la regulación de la presión sanguínea y de la homeostasis de sodio y agua (von Bohlen y Halbach, 2006).

En la actualidad, se sabe que además del SRA clásico existen SRA locales en diferentes tejidos entre los que se incluyen el corazón, riñón y páncreas, así como también en el aparato reproductor, sistema linfático, en tejido adiposo y cerebro (Benigni y col., 2010). Los SRA locales ejercen diferentes funciones en cada uno de los órganos y pueden operar de manera independiente, como es el caso de las glándulas adrenales y el cerebro, o interaccionando con el SRA periférico como el corazón y riñón. Por otro lado, también se ha descrito la existencia de un SRA intracelular (De Mello, 2003; Re y Cook, 2006). El estudio de estos SRA locales e intracelular ha puesto de manifiesto la existencia de otros efectos de la AII completamente diferentes a los del sistema clásico, tales como efectos pro-inflamatorios, proliferativos y pro-fibróticos. También se ha visto que la AII promueve la producción de EORs, el crecimiento celular, apoptosis, migración y diferenciación celular, la remodelación de la matriz extracelular y regulación de la expresión génica y que además puede activar múltiples vías de señalización intracelular conduciendo finalmente al daño tisular (Ruster y Wolf, 2006). Numerosos estudios relacionan el SRA con el inicio y progresión de enfermedades cardiovasculares y otras enfermedades relacionadas con la edad (Ruiz-Ortega y col., 2001), mientras que el SRA del cerebro se ha vinculado con procesos neurodegenerativos (Kehoe y col., 2009; Mertens, 2010; Labandeira-Garcia y col., 2011) y a su principal efector, la AII, como uno de los principales inductores de inflamación y estrés oxidativo (Phillips y Kagiyama, 2002; Touyz y col., 2002).

Componentes y biosíntesis:

La AII es generada a partir del angiotensinógeno a través de una cascada enzimática donde intervienen en un primer momento la renina originando angiotensina I (AI) y a continuación, el enzima convertidor de angiotensina originando así la propia AII. Por último, la activación de varias aminopeptidasas conlleva a la degradación de la AII y a la generación de metabolitos biológicamente activos tales como la AIII, AIV y A(1-7), y posteriormente de metabolitos inactivos (Jones y col., 2008).

1.2.1- SISTEMA RENINA-ANGIOTENSINA EN EL CEREBRO:

Tal y como hemos mencionado, el cerebro incluyendo los ganglios basales y el sistema dopaminérgico tiene su propio SRA con todos sus componentes, (Allen y col., 1992; Mendelsohn y col., 1993; Saavedra, 2005; Phillips y de Oliveira, 2008). Los primeros indicios surgieron cuando se vio que el angiotensinógeno, AI y AII no pueden atravesar la barrera hematoencefálica (Martin y col., 2006). Algunos de los componentes están ampliamente distribuidos por todo el cerebro mientras que otros tienen una distribución más restringida (Davisson, 2003), lo que sugiere que la formación de los distintos péptidos requiere de múltiples interacciones celulares. Existe una hipótesis que sugiere que el cerebro posee mecanismos enzimáticos alternativos, distintos de los del sistema clásico (Saavedra, 2005). Por otro lado, se ha visto que la renina no solo está implicada en la formación de estos componentes sino que podría desempeñar otros papeles en el cerebro. Existen datos que indican que la renina y su precursor pro-renina, que también posee actividad biológica, son capaces de unirse a un receptor común, receptor de (pro)renina (PRR) generando una cascada de señalización intracelular (Nguyen y col., 2002; apartado 1.2.3).

Cabe destacar que el SRA del cerebro no es totalmente independiente del sistema periférico, ya que la AII periférica puede interactuar en regiones específicas del mismo, concretamente, a nivel de los órganos circunventriculares (zonas específicas del SNC que carecen de una barrera hematoencefálica normal) (Pan, 2004).

1.2.2- RECEPTORES DE AII

Las acciones fisiológicas de la AII en el cerebro, mediada por los receptores AT1 y AT2 tal y como hemos comentado anteriormente, implican modulaciones en la actividad neuronal que son iniciadas por cambios en la actividad de los canales y en las corrientes iónicas de membrana (von Bohlen y Albrecht, 2006).

Los receptores AT2 se expresan predominantemente en tejidos fetales, lo cual es indicativo de su papel en el desarrollo y en los procesos de diferenciación, función completamente diferente a la función cardiovascular del control de la presión sanguínea mediada a través de los receptores AT1. Tras el nacimiento, la expresión de los receptores AT1 y AT2 se revierte, es decir, la expresión de los receptores AT1 predomina sobre la de AT2; llegando incluso a desaparecer por completo la expresión de AT2 en algunos tejidos (Unger y col., 1996). Por otro lado, en el caso de incidentes patológicos la expresión de AT2 se ve incrementada.

i) Receptor AT1:

El gen humano AT1 se localiza en el cromosoma 3q y codifica para una proteína de 40-42 KDa (359 aminoácidos). La mayoría de las especies expresan un único gen autosómico AT1, pero en roedores hay dos genes relacionados que son denominados $AT1_A$ y $AT1_B$. Estos dos receptores son idénticos en el 95% de sus secuencias aminoacídicas. Ambos son similares en cuanto a la unión al ligando y activación pero difieren en su distribución, localización cromosómica y regulación transcripcional (de Gasparo y col., 2000; Inagami y col., 1994; Iwai e Inagami, 1992).

Las vías de señalización de los receptores AT1 incluyen las cascadas clásicas activadas por proteínas G, que generan incrementos de calcio intracelular y activación de la quinasa C. Estas vías son las responsables de generar y mediar respuestas inmediatas, tales como la liberación de vasopresina y oxitocina. Otras vías de señalización iniciadas por la unión de la AII a los receptores AT1 son reacciones dependientes de fosforilación, algunas de las cuales implican el incremento en la expresión de factores de transcripción tales como c-Fos y c-Jun. Estas cascadas de señalización se cree que están implicadas en el crecimiento celular mediado por los receptores AT1 (Culman y col., 2002). Los receptores AT1 neuronales median las acciones estimuladoras de la AII en la presión sanguínea, en el balance de agua y electrolitos, en la liberación de vasopresina y en el sistema nervioso simpático. En los últimos años se ha visto una clara implicación de la AII a través de AT1 en la activación

de procesos inflamatorios y generación de estrés oxidativo (Cassis y col., 2010; Saavedra y col., 2011).

ii) Receptor AT2:

Los receptores AT2 muestran una homología del 32-34% con respecto a los AT1 y representan una proteína de 363 aminoácidos con siete dominios transmembrana hidrofóbicos (Kambayashi y col., 1993; Nakajima y col., 1993). El gen humano AT2 se localiza en el cromosoma X (Gard, 2002).

Se sabe que los receptores AT2 están implicados en el desarrollo embrionario del cerebro, en la regeneración y protección neuronal y en el inicio de procesos apoptóticos. Adicionalmente, también pueden modular algunas de las acciones clásicas tales como la proliferación celular, el control de la sed y la presión sanguínea (Culman y col., 2002; Hoiruchi y col., 2010).

Una variedad de mediadores de señalización han sido descritos para los receptores AT2. Están acoplados a una proteína G_i, y los mecanismos de señalización implican la inhibición de quinasas activadas por mitógenos, la alteración en los niveles intracelulares de GMP_c y la inhibición de la fosforilación. Diversos estudios indican que la AII a través de los receptores AT2 también está implicada en eventos pro y/o antiapoptóticos (Culman y col., 2002).

1.2.3- OTROS RECEPTORES DEL SRA

Uno de los aspectos emergentes del SRA es el descubrimiento de un receptor transmembrana que une tanto renina como su proenzima, la pro-renina, y que por ello se denominó receptor de pro-renina/ renina (PRR). Este receptor actúa como cofactor para renina y pro-renina incrementando su actividad enzimática (Nguyen y col., 2002).

i) Estructura del receptor PRR

PRR es un receptor de 350 aminoácidos, sin homología con ninguna proteína conocida y está compuesto por un péptido señal (indicativo de proteínas secretadas), un dominio extracelular (responsable de la unión a renina y pro-renina), un dominio transmembrana único y un dominio citoplasmático (implicado en la señalización intracelular).

Aunque este receptor posee capacidad para unir tanto renina como pro-renina, posee mayor afinidad por la pro-renina. El gen que lo codifica, ATP6ap2, se localiza en el cromosoma X y recibió este nombre porque en 1998 se caracterizó una proteína de 8,9 KDa que co-precipitaba con una ATPasa vacuolar. Esta proteína resultó ser un fragmento del PRR, llamado M8-9, que se corresponde con el dominio citoplasmático, transmembrana y una parte del dominio extracelular. Como posteriormente se vio que sólo había un gen para ambas proteínas, se concluyó que ambas procedían del mismo mensajero.

ii) Renina/Pro-renina y PRR

La renina es un componente clave del SRA ya que cataliza el paso limitante del sistema, la conversión del angiotensinógeno a AI, precursora del resto de péptidos bioactivos. La formación de la renina madura o renina activa tiene lugar a través del corte proteolítico del prosegmento adicional de 43 aminoácidos localizado en el extremo N-terminal de su precursor, la pro-renina (Nguyen y Muller, 2010). Este prosegmento se localiza bloqueando el sitio catalítico del enzima (“handle region”) (Suzuki y col., 2003).

La unión de la renina madura al receptor PRR se traduce en un aumento de su actividad catalítica hasta 4 o 5 veces más. Y la unión de la pro-renina tiene como consecuencia su activación mediada por un cambio conformacional, sin ruptura del prosegmento (activación no proteolítica). La activación no proteolítica es reversible, y

también puede ser inducida por pH bajo (3,3) o baja temperatura (4ºC) (Batenburg y Danser, 2008).

Varios estudios han establecido que el receptor PRR juega un papel doble en la regulación del SRA. Por un lado, facilitar la formación de AI y de AII; y por otro desencadenar el inicio de una cascada de señalización intracelular en la que intervienen las MAP quinasas (proteínas activadas por mitógenos) y que tiene como consecuencia el incremento en la síntesis de moléculas pro-fibróticas (Shan y col., 2008).

Todos estos efectos del PRR se han observado en presencia de los antagonistas de los receptores AT1 y AT2, indicando de este modo que se trata de un efecto independiente de la generación de AII (Nguyen y Contepras., 2008). Así mismo, se vio que utilizando ARN de interferencia contra PRR se bloqueaban dichos efectos (Huang y col., 2006; 2007).

Finalmente, Schefe y col. en 2006 identificaron el factor de transcripción promielocítico de dedos de Zinc (PLZF) que interacciona directamente con el dominio C-terminal de PRR. Cuando se activa el PRR, PLZF se transloca al núcleo y reprime la transcripción del propio PRR, creando un bucle de retroalimentación negativo. De este modo, cuando aumentan los niveles de renina, como ocurriría en el caso de un bloqueo del SRA, se suprime la expresión de PRR para prevenir así una activación excesiva del receptor. Por ahora, se desconoce si el aumento de los niveles de pro-renina ejercería el mismo efecto.

1.2.4- SISTEMA RENINA-ANGIOTENSINA Y ENFERMEDAD DE PARKINSON

Numerosos estudios han puesto de manifiesto la existencia de una relación entre el SRA y enfermedades neurodegenerativas, entre ellas la EP. Varios de ellos han mostrado que la AII y sus receptores juegan un papel importante en funciones estriatales y dopaminérgicas (Banks y col., 1994; Jenkins y col., 1997; Rodríguez-Pallares y col., 2004). Además, en el estriado de pacientes con EP se ha observado una marcada reducción en la expresión de los receptores AT1. Por otro lado, se ha publicado que los niveles de los receptores AT2 están disminuidos en el núcleo caudado, pero no en el putamen, ni en la SNpc (Ge y Barnes, 1996). También se ha visto que el enzima convertidor de angiotensina está ampliamente distribuido en el cerebro, incluyendo la vía nigroestriatal y los ganglios basales (Strittmatter y col., 1985; Chai y col., 1990; Jenkins y col., 1997) y que pacientes con EP presentan un incremento en la actividad

del enzima convertidor de angiotensina a nivel del fluido cerebroespinal (Konings y col., 1994). También se ha observado una asociación entre polimorfismos genéticos del gen que codifica el enzima convertidor de angiotensina y la enfermedad (Lin y col., 2002).

En los últimos años, se ha relacionado el SRA con procesos inflamatorios y estrés oxidativo, dos de los principales mecanismos patogénicos en la EP. Se ha visto que la AII tiene acciones pro-inflamatorias y que es capaz de producir EORs a través de la activación del complejo NADPH-oxidasa, lo cual podría contribuir al menos en la progresión de la enfermedad (revisado en Mertens y col., 2010; Labandeira-Garcia y col., 2011).

1.3- COMPLEJO NADPH-OXIDASA

Estudios recientes han demostrado que en varios tipos celulares, incluyendo las neuronas, las EORs juegan un papel crucial en la vía de señalización de la AII mediada por los receptores AT1 y que la AII activa al complejo NADPH-oxidasa (Griendling y col., 2000; Wang y col., 2004; Garrido y Griendling, 2009).

El complejo NADPH oxidasa es la segunda fuente de EORs intracelular después de la mitocondria (Babior 1999, 2004). Es un complejo enzimático formado por varias subunidades. Por un lado, tiene un componente de membrana, el citocromo b558 (formado por las subunidades p22 y gp91) y por otro, varias subunidades citosólicas (p47, p67 y p40) y la proteína-G Rac. Cuando estas subunidades están separadas en el espacio el complejo está inactivo y tras un estímulo las subunidades citosólicas se fosforilan, se activa Rac y a continuación se translocan a la membrana y se unen al citocromo b558 constituyendo así el complejo activo. El producto final de la activación es la formación de EORs (Block, 2008).

El complejo NADPH-oxidasa es el responsable de la producción de radicales libres en gran variedad de tipos celulares, entre los que destacan la microglía, macrófagos, neutrófilos y células epiteliales. Los radicales libres tales como el anión superóxido son deletéreos para las neuronas, especialmente para las neuronas dopaminérgicas, las cuales son especialmente vulnerables al daño oxidativo. Además, este superóxido puede reaccionar con el óxido nítrico formando peroxinitrito que es aún más tóxico que los anteriores (Liu, 2006). Por otro lado, las EORs pueden actuar sobre las vías de señalización intracelulares que inducen la activación microglial e incrementar de este modo la producción de moléculas pro-inflamatorias (Qin y col., 2004).

Se ha sugerido que las EORs originadas por acción de la NADPH-oxidasa desarrollan inicialmente un mecanismo de señalización intracelular común a todos los tipos celulares, y posteriormente un sistema defensivo especializado en macrófagos (Babior, 1999, 2004). La respuesta inflamatoria en torno a las neuronas y terminales muertas puede inducir un daño no específico en otras neuronas y contribuir así a la progresión a largo plazo de la muerte de las neuronas dopaminérgicas, tal y como se ha observado en las enfermedades autoinmunes (Vominckel y col., 1997).

Por último, se ha observado que la inhibición de la actividad NADPH-oxidasa disminuye la pérdida de neuronas dopaminérgicas inducida por diferentes neurotoxinas en modelos experimentales de la EP (Wu y col., 2003; Rey y col., 2007; Rodríguez-

Pallares y col., 2007, 2008), y que la microglía es la principal fuente de EORs derivadas de la activación de la NADPH-oxidasa (Block, 2008). Por otra parte, estudios recientes de nuestro laboratorio han mostrado que el bloqueo de la actividad NADPH-oxidasa en neuroesferas de células progenitoras mesencefálicas induce un aumento en la generación de células dopaminérgicas (Parga y col., 2010).

1.4- RECEPTOR GAMMA ACTIVADO POR PEROXISOMAS (PPAR-γ)

Los receptores PPARs son factores de transcripción dependientes de ligando que pertenecen a la superfamilia de receptores hormonales nucleares. Se han identificado 3 tipos: alfa, delta/beta y gamma. Juegan un papel importante en el metabolismo de la glucosa y de lípidos, y en los procesos de proliferación y diferenciación celular (Schoonjans y col., 1996; Berger y Moller, 2002). En estudios recientes se ha visto un importante papel anti-inflamatorio tanto en órganos periféricos como a nivel del SNC (Kapadia y col., 2008).

1.4.1- ESTRUCTURA, MECANISMO DE ACCIÓN Y FUNCIÓN

i) Estructura:

El gen de PPAR-γ humano se localiza en el cromosoma 3 y genera al menos tres transcritos de ARNm, PPAR-γ1, PPAR-γ2 y PPAR-γ3. PPAR-γ1 y PPAR-γ3 codifican para la misma proteína mientras que PPAR-γ3 codifica una proteína que contiene 28 aminoácidos adicionales en el extremo N-terminal (Kapadia y col., 2008).

A nivel proteíco todos los PPARs presentan una organización estructural similar que consiste en cuatro dominios funcionales. El dominio A/B en el extremo N-terminal, que a su vez contiene una región de activación funcional independiente de ligando (AF1), responsable de la fosforilación de PPAR; el dominio C o dominio de unión al ADN, promueve la unión de PPAR al elemento de respuesta del proliferador de peroxisomas (EPPR) presente en la región promotora de los genes diana; el dominio D, sitio de acoplamiento de cofactores (co-activadores/co-represores) y el dominio E/F o dominio de unión del ligando que contiene una región de activación funcional dependiente de ligando (AF2) que promueve el reclutamiento de los cofactores requeridos para la transcripción génica.

ii) Mecanismo de acción:

PPAR-γ es un factor de transcripción que se activa tras la unión a un ligando endógeno o exógeno. Forma heterodímeros con el receptor X de retinoide (RXR) y se une específicamente al EPPRs en la región promotora de los genes diana (Cunard y col., 2002; Lehrke y Lazar, 2005). Tras la unión a la región promotora se produce el reclutamiento de co-activadores, que se incorporan a la maquinaria transcripcional basal y activan así la transcripción génica (Argmann y col., 2005). En ausencia de ligando, el

heterodímero se asocia a un co-represor que suprime el proceso de transcripción (Ehrmann y col., 2002). Varias proteínas actúan como co-activadores o co-represores mediando así la capacidad de estos receptores nucleares para iniciar o suprimir el proceso de transcripción. Los principales coactivadores son la proteína de unión a PPAR y el co-activador del receptor esteroideo 1 (Zhu y col., 1996, 1997), mientras que entre los co-represores se encuentran el mediador del silenciamiento del receptor de la hormona tiroidea y retinoides y el co-represor del receptor nuclear (Chen y Evans, 1995; Horlein y col., 1995).

iii) Función:

La isoforma PPAR-γ se comporta como "sensor molecular" siendo capaz de unirse a un amplio abanico de moléculas relacionadas con el metabolismo, y ha sido objeto de estudio fundamentalmente en diabetes y obesidad debido a su papel regulador en el metabolismo de la glucosa (Desvergne y col., 2004; Lehrke y Lazar, 2005). PPAR-γ muestra un patrón de expresión restringido y se localiza principalmente en el tejido adiposo donde regula la diferenciación de adipocitos y el metabolismo lipídico (Cunard y col., 2002). También se expresa en las células del sistema inmune como monocitos y macrófagos, y células B y T. En el cerebro adulto normal los niveles de expresión son relativamente bajos, se localizan principalmente en las células granulares del giro dentado (Braissant y col., 1996), también a nivel de los núcleos caudado y putamen y globo pálido de los ganglios basales así como en el tálamo y el córtex piriforme (Moreno y col., 2004). Estudios recientes indican que la expresión de PPAR-γ en el cerebro se localiza mayoritariamente en las células microgliales y astrocitos, células responsables de la respuesta inflamatoria. En este sentido, hay estudios que muestran que PPAR-γ puede modular la producción de citocinas inflamatorias y radicales libres, de manera que su activación se correlaciona con una disminución en la activación microglial y en la activación de los macrófagos lo que se traduce finalmente en un descenso de la liberación de mediadores inflamatorios (Schintu y col., 2009).

1.4.2- PPAR-γ Y ENFERMEDAD DE PARKINSON

Numerosos estudios han puesto de manifiesto que los procesos inflamatorios juegan un papel clave en el desarrollo de la EP así como en la toxicidad inducida por neurotoxinas como MPTP. Tras ver que la activación de PPAR-γ inhibía la expresión de la sintasa de óxido nítrico inducible (iNOS) en macrófagos periféricos, así como en

modelos de neuroinflamación, ratones tratados con MPTP fueron a su vez tratados con ligandos sintéticos de PPAR-γ y vieron que efectivamente el efecto anti-inflamatorio de PPAR-γ ejercía un efecto neuroprotector (Heneka y Landreth, 2007). Breider y col. vieron que el tratamiento con pioglitazona, agonista de PPAR-γ, protegía la degeneración dopaminérgica a nivel de la SNpc inducida por MPTP (Breider y col., 2002). Esto mismo fue corroborado posteriormente por Dehmer y col. (2004). En ambos trabajos se vió que este compuesto inhibía la activación microglial y astrocitaria, y disminuía el número de células iNOS-positivas tanto en el estriado como en la SNpc. Resultados similares fueron obtenidos usando como modelo de neurodegeneración dopaminérgica ratas tratadas con un lipopolisacárido. En este caso, también se vió que el tratamiento con pioglitazona reducía la activación microglial y el estrés oxidativo y restauraba la función mitocondrial (Hunter y col., 2007). Sin embargo, estudios posteriores demostraron que el efecto neuroprotector de pioglitazona en los ratones tratados con MPTP se debía a que esta sustancia actúa inhibiendo el enzima MAO-B lo que supone una reducción de MPP^+ en el cerebro (Quinn y col., 2008). Más recientemente, Schintu y col. (2009) han demostrado que otro agonista de PPAR-γ, la rosiglitazona, también previene la pérdida neuronal dopaminérgica inducida por la administración de MPTP a ratones. Y comprobaron mediante la administración de un inhibidor de PPAR-γ, el GW9662, que efectivamente este efecto de la rosiglitazona era específico de la activación de PPAR-γ.

En los últimos años, se ha sugerido que los agonistas de PPAR-γ podrían ejercer su papel neuroprotector mediante la inhibición de la respuesta inmune, inhibiendo la activación microglial y astrocitaria así como la liberación de mediadores pro-inflamatorios (Storer y col., 2005; Gurley y col., 2008). Y más concretamente, en modelos de isquemia cerebral y hemorragia intracerebral, el tratamiento con rosiglitazona tiene un efecto anti-inflamatorio mediado por la inhibición de la vía del factor de transcripción NFkB, lo que conlleva una atenuación en la expresión de genes proinflamatorios y en la producción de citocinas (Chen y col., 2006; Luo y col., 2006; Tureyen y col., 2007). Del mismo modo, se ha visto que en modelos de MPTP en ratones, la rosiglitazona es capaz de reducir la respuesta glial que tiene lugar en la SNpc (Schintu y col., 2009).

Por otro lado, se sabe que existe una relación entre PPAR-γ y el SRA. Sunagawa y col. observaron que la activación de PPAR-γ suprimía la expresión de los receptores AT1 en las células de la musculatura lisa vascular (Sugawara y col., 2001) y

posteriormente, Zhao y col. (2005) que la AII inducía la activación de PPAR-γ a través de los receptores AT2. Hoy en día existen compuestos que tienen un efecto doble, de manera que por un lado bloquean los receptores AT1 y por otro, actúan como agonistas parciales de PPAR-γ.

1.5 – CANALES DE POTASIO DEPENDIENTES DE ATP

Los canales de potasio dependientes de ATP (K(ATP)) fueron descubiertos por Akinori Noma en miocitos cardiacos (Noma, 1983) y posteriormente, se vio que también se expresaban en muchos otros tipos celulares, tales como, músculo cardíaco, células beta pancreáticas y en varias regiones del cerebro. Estos canales se localizan en diferentes partes de la célula incluyendo la membrana plasmática (sK(ATP)) y la membrana interna mitocondrial (mitoK(ATP)) (Busija y col., 2004).

1.5.1- ESTRUCTURA

La estructura de los canales sK(ATP) ha sido ampliamente estudiada y se trata de proteínas octaméricas formadas por dos tipos de subunidades: por un lado, Kir6.x o subunidades formadoras del poro, y por otro, SUR (receptor sulfonilurea) o subunidades reguladoras también llamadas sensores metabólicos. En los canales funcionales cuatro subunidades Kir se unen a cuatro subunidades SUR. Hasta el momento se han identificado dos tipos de subunidades Kir6.x, Kir6.1 y Kir6.2, y dos isoformas de la subunidad SUR, SUR1 y SUR2. A su vez se han descrito dos variantes de la subunidad SUR2, SUR2A y SUR2B siendo estas últimas las más importantes. Se cree que los canales mitoK(ATP) tienen una estructura similar a los sK(ATP) con los dos tipos de subunidades aunque la composición exacta se desconoce (Bednarczyk, 2009; Avshalumov y col., 2005).

1.5.2- FUNCIÓN

En general, los canales K(ATP) se consideran sensores metabólicos ya que su apertura depende del estado metabólico de la célula (Liss y Roeper, 2001). En función de las concentraciones relativas de potasio en el líquido intersticial, citosol y mitocondria las consecuencias de la apertura de estos canales dependerán de su localización, de este modo, la activación de los canales sK(ATP) produce la hiperpolarización de la célula, mientras que la apertura de los canales mitoK(ATP) produce la despolarización de la organela. A través de diferentes estudios se ha puesto de manifiesto que manipulaciones a nivel de estos canales K(ATP) pueden ser una importante estrategia en el tratamiento de diferentes enfermedades entre las que se incluyen la diabetes, hipertensión arterial, fallo cardíaco e isquemia (Oldenburg y col., 2002; Yellon y Downey, 2003; Busija y col., 2004; Hanley y Daut, 2005; Costa y Garlid, 2008). Inicialmente, los estudios sugerían que los canales sK(ATP) eran los que

dirigían o mediaban los efectos protectores, pero investigaciones más recientes apuntan a que realmente son los canales mitocondriales los que juegan un papel más importante. Numerosos estudios han mostrado que la apertura de los canales mitoK(ATP) produce un incremento en la producción de EORs a partir de la mitocondria (Obata y Yamanaka, 2000; Oldenburg y col., 2002; Reinhardr y col., 2003; Costa y Garlid, 2008). Así mismo, hay estudios recientes sugieren que la AII no solo estimula la formación de EORs a nivel citosólico a través de la activación del complejo NADPH-oxidasa sino también a partir de la mitocondria en diferentes tejidos (de Cavanagh y col., 2007; Zhang y col., 2007), así como que la mitocondria es en gran medida la responsable de la generación de estrés oxidativo y juega un papel relevante en los procesos de neurodegeneración y envejecimiento (de Cavanagh y col., 2007; Schapira, 2008). Numerosos estudios apoyan la idea de que los canales mitoK(ATP) son los responsables de modular los niveles intracelulares de EORs (Andrukhiv y col., 2006; Costa y Garlid, 2008; Mattson y Liu, 2003) a través de la regulación en la formación y liberación de EORs a nivel mitocondrial, así como la integración de señales procedentes de diversas fuentes (Oldenburg y col., 2002; Facundo y col., 2007; Fornazari y col., 2008). Aunque varios estudios corroboran que la apertura de los canales mitoK(ATP) conlleva a un aumento en los niveles de EORs hay controversias al respecto ya que algunos sugieren que no es necesaria la apertura de estos canales para observar dicho aumento (Minners y col., 2007). La mayoría de estas discrepancias pueden ser debidas a diferencias en las condiciones experimentales.

1.5.3- CANALES MITOK(ATP) Y ENFERMEDAD DE PARKINSON

Estos canales fueron descritos originalmente en corazón, aunque son particularmente abundantes a nivel del SNC, y más concretamente a nivel de la SN y del estriado (Zini y col., 1993; Busija y col., 2004; Avshalumov y col., 2005). De hecho, las mitocondrias a nivel del tejido nervioso contienen siete veces más canales mitoK(ATP) por miligramo de proteína que el hígado o corazón (Bajgar y col., 2001; Bednarczyk, 2009).

Se ha visto que estos canales en las neuronas dopaminérgicas se abren en respuesta a una inhibición parcial del complejo I mitocondrial, así como en respuesta a una disminución de ATP y a un incremento del estrés oxidativo (Liss y col., 1999; Avshalumov, 2005; Liss y col., 2005). Por otro lado, se ha visto que la AII a su vez estimula la producción de EORs a través de la apertura de estos canales (Kimura y col.,

2005a). Por otro lado, en un estudio reciente de nuestro laboratorio se ha visto que el bloqueo de los canales sK(ATP) y mitoK(ATP) en neuroesferas de células progenitoras mesencefálicas durante los procesos de proliferación y diferenciación produce un aumento en la generación de células DA (Parga y col., 2010).

1.6- LA PROTEÍNA RHO Y SU BLANCO RHO-QUINASA

1.6.1- VÍA DE SEÑALIZACIÓN Rho/ROCK

i) La superfamilia de las GTPasas pequeñas:

La proteína Rho pertenece a la superfamilia de las GTPasas pequeñas que comprende más de 100 proteínas estructuralmente relacionadas. Estas GTPasas experimentan cambios en su conformación espacial y su localización subcelular dependiendo del nucléotido de guanina. Las GTPasas son activas cuando tienen unido GTP e inactivas cuando contienen GDP. En su estado activo se unen a sus efectores que a su vez regulan un gran número de funciones biológicas (Van Aelst y D´Souza-Schorey, 1997; Bishop y Hall, 2000; Mueller y col., 2005). Son controladas por distintas clases de proteínas reguladoras (Boguski y McCormick, 1993) y salvo raras excepciones, su activación está mediada por la acción de factores intercambiadores de nucléotidos de guanina denominados GEF (*guanine nucleotide exchange factors*). Estos factores desplazan al inhibidor de disociación de GDP (GDI) y liberan el residuo isoprénico unido a la GTPasa que sirve de anclaje a la membrana plasmática y catalizan el intercambio de GDP a GTP. La unión del GTP induce un cambio conformacional en la GTPasa, activándola y permitiendo así el acoplamiento a sus efectores. Las proteínas activadoras de las GTPasas, conocidas como GAP (*GTPase activating proteins*), estimulan la hidrólisis intrínseca del GTP y producen la conversión de las GTPasas a su estado inactivo ligado a GDP y GDI.

La superfamilia de las GTPasas pequeñas según sus relaciones estructurales y funcionales se ha subdividido en 5 subfamilias: Ras, Rho, Rab, Arf y Ran. Los miembros de la subfamilia Rho (RhoA, RhoB, Rac1 y Cdc42) son de especial interés, ya que controlan la formación de las distintas estructuras que conforman el citoesqueleto de la actina (Jalil y col., 2005; Ding y col., 2011). Además, regulan muchas otras funciones, entre las que se incluyen la transcripción génica y el metabolismo lipídico. RhoB tiene acciones opuestas a RhoA e inhibe la expresión génica mediada por RhoA. Los efectos de RhoA en la arquitectura celular están mediados por las proteínas serina/ treoninquinasas dependientes de Rho (Aspenstro, 1999) y se dividen en dos subgrupos: las proteinquinasas PKC/ PKN y las Rho-quinasas (ROCK1 y ROCK2) (Mueller y col., 2005).

ii) Isoformas de ROCK y su distribución en el tejido:

Rock es una serina/ treoninquinasa, que fue identificada hace aproximadamente 16 años como una proteinquinasa dependiente de Rho y con una masa molecular de unos 160 kDa (Mukai y col., 2001; Clerk y Sugden, 2000). Se han descrito dos genes distintos que codifican dos isoformas de ROCK: ROCKI (también conocida como ROCKβ o p160ROCK) y ROCKII (conocida como ROCKα) (Nagawa y col., 1996). Las dos isoformas tienen una coincidencia del 65% en su secuencia aminoacídica y del 92% en sus dominios quinasa (Amano y col., 2000; Riento y Ridley, 2003; Kang y col., 2011). También presentan homología con otros miembros del grupo de las ACG quinasas tales como la quinasa de distrofia miotónica, la quinasa de distrofia dependiente de CDC-42 y la citron quinasa. En términos generales, el dominio catalítico de todas estas quinasas se localiza en el extremo amino-terminal, a continuación hay una región enrollada que contiene el dominio de unión a Rho (RBD) y por último, en su dominio carboxi-terminal contiene un dominio de repetición rico en cisteínas (CRD).

A pesar de la gran similitud en las secuencias aminoacídicas de ambas isoformas se ha visto una gran diferencia en cuanto a su distribución en los tejidos, lo que nos indica la existencia de distintas funciones para cada isoforma *in vivo*. ROCKII se expresa principalmente en cerebro, mientras que ROCKI muestra los niveles más altos de expresión en tejidos no neuronales, entre los que se incluyen corazón, pulmón y músculo esquelético (Mueller y col., 2005). La expresión de ROCKII en cerebro bovino se ha observado principalmente en las neuronas piramidales del hipocampo y en el córtex cerebral, aunque también hay expresión en las células de Purkinje del cerebelo (Hashimoto y col., 1999). Resulta interesante que en cerebro de ratón, durante el desarrollo post-natal los niveles de expresión de ROCKII se incrementan gradualmente (Komagome y col., 2000).

iii) Regulación de la actividad ROCK

En 1999 se describió que el extremo C-terminal de ROCK regula negativamente su actividad quinasa (Amano y col., 1999). Concretamente, el dominio C-terminal de ROCK se pliega hacia atrás sobre su dominio quinasa formando de este modo un bucle auto-inhibitorio que mantiene a ROCK en su estado inactivo. Por otro lado, se cree que la unión de Rho al dominio RBD interrumpe esta interacción entre el dominio catalítico y la región C-terminal autoinhibitoria, lo que resultaría en la activación del enzima ante una señal extracelular.

iv) Actividad ROCK

Uno de los principales sustratos de ROCKII es el enzima LIM-quinasa, que fosforila e inactiva la cofilina (Maekawa y col., 1999; Ohashi y col., 2000). La cofilina estimula la despolimerización de la actina por lo que su efecto neto sería la estimulación de la formación de fibrillas. Otro de los sustratos de ROCK es la fosfatasa de la cadena ligera de miosina. Cuando ROCK está activa fosforila esta fosfatasa inhibiéndola y de este modo favorece la contracción de las células musculares lisas vasculares, la formación de fibras de estrés y la migración celular (Loirand y col., 2006; Joshi y col., 2008; Narumiya y col., 2009; Zhou y Liao, 2009). Entre otros sustratos de ROCK también se encuentran proteínas de los filamentos intermedios tales como vimentina, proteína acídica fibrilar glial y neurofilamentos que tras su fosforilación por ROCK pueden producir despolimerización (Mueller y col., 2005). Entre otros candidatos como sustratos de ROCK están la proteína asociada a microtúbulos 2 (MAP2) y tau (Amano y col., 2003). La fosforilación de tau disminuye su actividad para promover el ensamblaje de los microtúbulos *in vitro.* Además, ROCKII fosforila una proteína neuronal (proteína mediadora de respuesta a colapsina 2) involucrada en el desarrollo del sistema nervioso (Arimura y col., 2000). Por lo tanto, ROCKII parece ser un punto donde se integran varias vías de señalización intracelular, especialmente aquellas que regulan la contractilidad de las fibras de actina-miosina. Por último, cabe destacar que cada vez existen más evidencias que sugieren que anomalías en la regulación de la actividad ROCK juegan un papel importante en la patogénesis de muchas enfermedades neurológicas como es el caso del infarto cerebral, enfermedad de Alzheimer y esclerosis múltiple entre otras (Kubo y Yamashita, 2007; Shin y col., 2008).

1.6.2- ROCK Y ENFERMEDAD DE PARKINSON

Se ha descrito que ROCK participa en diferentes procesos fisiológicos modulando la migración, proliferación y supervivencia celular. Por otro lado, la inhibición de ROCK mediante el uso de inhibidores específicos del enzima como Fasudil o Y-27632, ha mostrado que limita la activación leucocitaria e infiltración y disminuye la invasión y metástasis de células tumorales (Ding y col., 2009). Además, se ha observado una activación anómala de la vía Rho/ROCK en varios desórdenes del SNC, que puede ser importante en la patogénesis de varias enfermedades neurológicas (Mueller y col., 2005). También se ha descrito que la activación de RhoA promueve la migración transendotelial de monocitos vía ROCK, lo cual podría llevar a desencadenar

procesos pro-inflamatorios. Hay estudios que afirman que ROCK puede estar implicada en inflamación y daño tisular (Büyükafsar y col., 2006; Ruiz-Ortega y col., 2006; Sheikh y col., 2009). Además, se ha publicado que la vía Rho/ ROCK está implicada en la regulación de la presión sanguínea ya que el bloqueo de esta vía a nivel del nucleo del tracto solitario disminuye la presión sanguínea, el pulso cardíaco y la actividad simpática renal (Ito y col., 2003). Esto podría indicar que ROCK podría regular también la transmisión neuronal (Sasaki, 2003). Así mismo, se ha visto que el inhibidor de ROCK Y-27632 inhibe la liberación de acetilcolina a nivel de los nervios colinérgicos periféricos (Büyükafsar y Levent, 2003). Desde este punto de vista la cascada de señalización Rho/ ROCK podría participar en la liberación de neurotransmisores a partir de los nervios sensoriales, y la inhibición de esta vía podría reducir la transmisión neuronal. Por otro lado, Sheikh y col. en 2009 han descrito que el fosfolípido lisofosfatidilcolina es capaz de inducir a través de la activación de ROCK una respuesta proinflamatoria en la que están implicados la activación microglial y astrocitaria, lo cual podría contribuir a procesos neuroinflamatorios y neurodegenerativos que tienen lugar en la patogénesis de las enfermedades neurodegenerativas como es el caso de la EP. También se ha descrito que la AII a través de sus receptores AT1 puede activar la vía Rho/ ROCK (Ruiz-Ortega y col., 2006). En un estudio renal en ratas tratadas con AII se ha visto que el tratamiento con el inhibidor Y-27632 disminuye el daño tubular, el número de células inflamatorias y la sobreexpresión renal del factor de crecimiento tisular y los parámetros proinflamatorios (Rupérez y col., 2005).

JUSTIFICACIÓN Y OBJETIVOS

2.- JUSTIFICACIÓN Y OBJETIVOS

La enfermedad de Parkinson (EP) es una enfermedad neurodegenerativa frecuente caracterizada por la pérdida progresiva de neuronas dopaminérgicas (Parkinson, 2002; Meyer y col., 2010). Numerosos estudios han puesto de manifiesto que tanto el estrés oxidativo (Gandhi y Wood, 2005; Yuan y col, 2007; Zhou y col., 2008), como los procesos inflamatorios (Ouchi y col., 2005; Gerhard y col., 2006) y la disfunción mitocondrial (Gu y col., 2010; Xie y col., 2010; Keane y col., 2011) juegan un papel clave en el inicio y/o al menos, en la progresión de la EP.

En la actualidad, se sabe que además del SRA clásico existen SRA locales en diferentes tejidos, entre ellos el cerebro. Además existen datos que relacionan al SRA cerebral con procesos inflamatorios y estrés oxidativo (Kehoe y col., 2009; Mertens, 2010). Se ha visto que la AII, principal efector del sistema, tiene acciones pro-inflamatorias (Benigni y col., 2010) y que es capaz de producir EORs a través de la activación del complejo NADPH-oxidasa (Phillips y Kagiyama, 2002; Touyz y col., 2002). Sin embargo, no se conoce el papel que los distintos componentes del SRA pueden tener en el desarrollo de la EP. Por este motivo nos hemos planteado los siguientes objetivos:

- 1) Estudiar el papel de AII y de sus receptores (receptores AT1 y AT2) en la degeneración dopaminérgica inducida por neurotoxinas tanto *in vitro* como *in vivo*.
- 2) Estudiar la posible implicación del receptor PRR en la degeneración dopaminérgica inducida por neurotoxinas *in vitro*.

Por otro lado, se ha visto que el receptor PPAR-γ juega un importante papel anti-inflamatorio tanto a nivel periférico como a nivel del sistema nervioso central (Kapadia y col., 2009), y que además existe una relación entre PPAR-γ y el SRA (Sunagawa y col., 2001; Zhao y col., 2005). Por ello nos hemos planteado como tercer objetivo:

- 3) Estudiar el posible papel neuroprotector del receptor PPAR-gamma en la degeneración dopaminérgica que se produce en modelos animales de EP.

Además, numerosos estudios han demostrado que la apertura de los canales mitoK(ATP) produce un incremento en la producción de EORs derivados de la

mitocondria (Obata y Yamanaka, 2000; Oldenburg y col., 2002; Reinhardr y col., 2003; Costa y Garlid, 2008) y se ha propuesto una posible interacción entre el SRA y la mitocondria cuyo fundamento aún no ha sido clarificado (de Cavanagh y col., 2007; Zhang y col., 2007; Schapira, 2008). Basándonos en estos datos nuestro siguente objetivo ha sido:

- 4) Estudiar la posible implicación de los canales mitoK(ATP) en la neurodegeneración dopaminérgica inducida por dosis bajas de neurotoxinas.
- 5) Conocer el papel de los canales mitoK(ATP) en el efecto de la AII sobre la degeneración de neuronas dopaminérgicas inducida por dosis subletales de neurotoxinas.

Por último, se sabe que el enzima ROCK forma parte de la cascada de señalización intracelular tras la activación de los receptores AT1 (Ruiz-Ortega y col., 2006; Lorenzo y col., 2007; Higuchi y col., 2007). Sin embargo se desconoce la implicación de esta vía de señalización en la degeneración dopaminérgica inducida experimentalmente. Por ello nos hemos planteado como objetivo de esta tesis:

- 6) Estudiar el papel del enzima ROCK en los mecanismos neurodegenerativos inducidos por la neurotoxina MPTP y potenciados por AII, tanto *in vitro* como *in vivo*.

MATERIAL Y MÉTODOS

3.- MATERIAL Y MÉTODOS

3.1- DISEÑO EXPERIMENTAL

3.1.1- Papel de la AII cerebral en la respuesta inflamatoria en animales tratados con MPTP como modelo de enfermedad de Parkinson: implicación en la progresión de la degeneración dopaminérgica

Para estudiar el efecto de la AII en la respuesta microglial inducida por la neurotoxina MPTP se realizaron estudios tanto *in vitro* como *in vivo*. *In vitro* se emplearon cultivos primarios mesencefálicos, excluyendo así los posibles efectos debidos a cambios inducidos por la AII en la función vascular cerebral. *In vivo* usamos ratones que no fueron tratados ni intracerebral ni intraventricularmente para preservar de este modo las condiciones fisiológicas del animal. En los cultivos, se estudiaron los efectos de la AII en la neurodegeneración dopaminérgica y la activación glial inducidas por el metabolito activo del MPTP, el MPP^+, y la posible vía de señalización intracelular implicada. Para ello se trataron con antagonistas de los receptores AT1 y AT2, inhibidores de la proteína quinasa C (PKC) o inhibidores del complejo NADPH. In vivo, se usaron ratones de la cepa C57BL/6J tratados con candesartán, antagonista de los receptores AT1, para estudiar el papel de estos receptores en la degeneración dopaminérgica inducida por el MPTP y su efecto sobre la activación glial y del complejo NADPH-oxidasa tanto a nivel del estriado como de la SNpc. Los análisis *in vivo* se realizaron tanto a tiempos cortos (modelo agudo) como a tiempos largos (modelo crónico).

3.1.2- Efecto del bloqueo de los receptores de pro-renina/renina *in vitro* sobre la muerte neuronal dopaminérgica

Tras describir en nuestro laboratorio la presencia y localización del receptor de pro-renina/renina (PPR) con técnicas de inmunofluorescencia tanto en tejido de mono “Macaca fascicularis” como en cultivos primarios mesencefálicos, nos propusimos estudiar el papel del receptor PPR en la neurodegeneración dopaminérgica inducida por la neurotoxina 6-OHDA. Para ello cultivos primarios mesencefálicos fueron tratados con el antagonista del receptor PRR, “handle region peptide” (HRP), y con renina, agonista del receptor PRR, 30 min antes del tratamiento con dosis bajas de la neurotoxina 6-OHDA.

3.1.3- Implicación de PPAR-γ en el efecto neuroprotector y antiinflamatorio del bloqueo del receptor de AII de tipo 1. Efecto del antagonista de los receptores AT1, telmisartán y de la deleción del receptor AT1 en un modelo en ratón de enfermedad de Parkinson

Para evaluar "*in vivo*" la implicación de PPAR-γ en la neurodegeneración dopaminérgica se utilizaron ratones tratados con MPTP como modelo de EP. Ratones de la cepa C57BL/6J fueron divididos en 4 grupos experimentales. El grupo control no recibió ningún tratamiento, el grupo MPTP recibió 5 inyecciones intraperitoneales de MPTP y fue sacrificado 7 días después de la última inyección. Otro grupo de animales fue tratado con MPTP y Telmisartán, antagonista de los receptores AT1 y agonista parcial del receptor PPAR-γ, y un cuarto grupo que fue tratado con MPTP, Telmisartán y con un inhibidor de PPAR-γ, el GW9662. Del mismo modo, se utilizaron ratones knockout para el receptor AT1a y se dividieron en 3 grupos experimentales: un grupo de animales que recibieron inyecciones con salino se emplearon como grupo control, un segundo grupo de animales fue tratado con MPTP y el último grupo recibió inyecciones con MPTP y el inhibidor GW9662 para estudiar el papel de los receptores AT1 y de PPAR-γ en la degeneración dopaminérgica inducida por esta neurotoxina.

3.1.4- Papel de los canales de potasio mitocondriales dependientes de ATP en la toxicidad de la 6-hidroxidopamina sobre las neuronas dopaminérgicas

Para estudiar la posible implicación de los canales mitoK(ATP) en la toxicidad de la 6-OHDA sobre las células dopaminérgicas se llevaron a cabo estudios *in vitro*. Por un lado, se utilizaron cultivos mesencefálicos tratados con el antagonista de los canales mitoK(ATP), 5-Hidroxidecanoato (5-HD), para estudiar el efecto de la inhibición de estos canales sobre la neurodegeneración dopaminérgica inducida por distintas dosis de 6-OHDA, así como sobre los niveles de superóxido en las neuronas dopaminérgicas. También se analizaron los posibles cambios inducidos por la 6-OHDA en el potencial de la membrana mitocondrial interna en las neuronas dopaminérgicas y el efecto de la inhibición de los canales mitoK(ATP) sobre estos cambios. Por otro lado, se obtuvieron cultivos enriquecidos en neuronas para estudiar el papel de los canales mitoK(ATP) de las células gliales sobre la degeneración inducida por la 6-OHDA.

3.1.5- Determinación de la implicación del enzima Rho-quinasa en la neurodegeneración dopaminérgica inducida por AII

Para estudiar la posible implicación del enzima Rho-quinasa (ROCK) en los mecanismos neurodegenerativos de las células dopaminérgicas se llevaron a cabo estudios tanto "*in vitro*" como "*in vivo*". Para los estudios "*in vitro*" cultivos primarios mesencefálicos fueron tratados con el inhibidor de ROCK, Y-27632, 30 minutos antes de la correspondiente dosis de MPP^+ o MPP^+ y AII. Para evaluar "*in vivo*" la implicación de ROCK en la neurodegeneración dopaminérgica se utilizaron ratones tratados con MPTP como modelo de EP. Ratones de la cepa C57BL/6J fueron divididos en 3 grupos experimentales. El grupo control no recibió ningún tratamiento, el grupo MPTP recibió 5 inyecciones intraperitoneales de MPTP y fue sacrificado 7 días después de la última inyección. Y el tercer grupo de animales fue tratado con MPTP e Y-27632.

3.2- CULTIVOS CELULARES

3.2.1- Cultivos primarios mesencefálicos

Las células mesencefálicas fueron obtenidas de tejido mesencefálico ventral a partir de embriones de rata de Sprague-Dawley de 14 días de gestación. Todos los experimentos fueron llevados a cabo de acuerdo con los "Principios de Cuidado de Animales de Laboratorio" de la Comisión Europea del 24 de Noviembre de 1986 (86/609/EEC), sobre protección y experimentación animal, reduciendo en lo posible el sufrimiento del animal y utilizando el mínimo de animales necesarios para conseguir resultados fiables.

Los embriones fueron extraídos por cesárea de la madre profundamente anestesiada. La suspensión celular fue preparada según Nikkhah y colaboradores (1994), que realizaron modificaciones de la técnica previamente descrita por Björklund y colaboradores (1983). Para la preparación de la suspensión celular se procedió a la disección de la porción mesencefálica ventral del cerebro. Dicho tejido mesencefálico fue rápidamente incubado en 0,05% de DNAsa (Sigma) disuelta en Medio Esencial Mínimo de Dulbecco (DMEM, Invitrogen) con 0,1% de tripsina (Sigma), durante 20 minutos en un baño termostático a 37ºC. A continuación, se hicieron varios lavados y se disoció el tejido mecánicamente. La suspensión celular resultante fue centrifugada a 50g durante 5 minutos, tras la cual se eliminó el sobrenadante cuidadosamente y el pellet fue

resuspendido en el volumen final requerido. Posteriormente, se procedió a la estimación del número de células viables usando una tinción con naranja de acridina (Sigma) y bromuro de etidio (Sigma) observando la muestra al microscopio de fluorescencia.

Finalmente, las células fueron sembradas en placas de cultivo de 35 mm previamente tratadas con poli-L-lisina (100 µg/ml; Sigma) y laminina (4 µg/ml; Sigma) a una densidad de $5x10^5$ células/ml de medio de cultivo suplementado con un 10% de suero bovino fetal (FBS). Dos días después, el medio fue reemplazado por medio fresco. Los distintos tratamientos se realizaron entre los días 4 y 6 DIV en función del diseño experimental (Tabla 1, apartado 3.2.3). Las células tratadas fueron comparadas siempre con sus respectivos controles. Los cultivos fueron mantenidos durante 7 ó 8 días *in vitro* (DIV) tras los cuales las células fueron teñidas para distintos marcadores y/o procesadas inmunohistoquímicamente (Tabla 3) o utilizadas para estudios de RT-PCR a tiempo real (apartado 3.6).

3.2.2- Cultivos primarios mesencefálicos enriquecidos en neuronas

Para la obtención de cultivos enriquecidos en neuronas, las células fueron tratadas 48 horas después de la siembra con citosina-β-D-arabino-furanosido (Ara-C), que actúa como inhibidor de la replicación de la población glial. Pasados 2 días el medio de cultivo fue reemplazado por medio fresco para realizar posteriormente los correspondientes tratamientos. Este método nos permite enriquecer el cultivo en neuronas hasta en un 85% de pureza.

3.2.3- Tratamientos empleados *in vitro* (Tabla 1)

Tratamiento	**Actividad**
5-HD	Antagonista de los canales mitocondriales de potasio dependientes de ATP
6-OHDA	Neurotoxina dopaminérgica
AII	Agonista de los receptores de AII
Apocinina	Inhibidor del complejo NADPH-oxidasa
Ara-C	Inhibidor de la progresión del ciclo celular, antimitótico
CCCP	Protonóforo
Chelerytrine chloride	Inhibidor selectivo de la PKC
Diazóxido	Agonista de los canales de potasio dependientes de ATP
GBR 12935	Inhibidor específico de los transportadores de dopamina
Glibenclamida	Antagonista de los canales de potasio dependientes de ATP
HRP	Antagonista del receptor PRR
MPP^{+}	Neurotoxina dopaminérgica
PD 123319	Antagonista de los receptores AT2
Renina	Agonista del receptor PRR
Y-27632	Inhibidor del enzima ROCK
ZD 7155	Antagonista de los receptores AT1

3.3- MODELOS ANIMALES

3.3.1- Para la obtención de **modelos animales de EP** se utilizaron dos estrategias experimentales:

- ratones de la cepa C57BL/6J (Charles River Laboratories) tratados con la neurotoxina MPTP. Para los estudios en modelos crónicos, los ratones recibieron 5 inyecciones intraperitoneales (i.p.) de MPTP (30 mg/kg/día) y fueron sacrificados 7 días después de la última inyección. Y para los estudios en modelos agudos, los ratones fueron expuestos a una única inyección i.p. de MPTP (40 mg/kg/día) y sacrificados 24 horas después de la misma.

- ratones transgénicos knockout para Agtr1a (The Jackson Laboratory) tratados con MPTP siguiendo el modelo crónico descrito previamente.

3.3.2- Tratamientos empleados *in vivo* (Tabla 2)

Tratamiento	**Actividad**
Candesartán	Antagonista de los receptores AT1
Telmisartán	Antagonista de los receptores AT1 y agonista parcial de PPAR-γ
GW9662	Inhibidor de PPAR-γ
Y-27632	Inhibidor de ROCK

3.4- ESTUDIOS BIOQUÍMICOS

3.4.1- Estudios de producción de O^- intracelular y de EORs derivadas de O^-

Para estudiar la producción de O^- intracelular y las EORs derivadas de O^- se emplearon cultivos primarios mesencefálicos crecidos sobre cubreobjetos que fueron tratados con dihidroetidio (DHE) diluido en tampón fosfato salino durante 30 minutos a 37ºC. Posteriormente, los cultivos fueron lavados, fijados y procesados para inmunofluorescencia anti-TH para la detección de niveles de DHE en células dopaminérgicas, o anti-OX-42 para la detección en células microgliales.

3.4.2- Estudios del potencial de membrana interna mitocondrial

Para estudiar el potencial de membrana interna mitocondrial se emplearon dos ensayos: por un lado utilizando como marcador clorometil-tetrametilrosamina metil éster (CMTMR) y por otro, 5,5´,6,6´-tetracloro-1,1´,3,3´-tetraetilbencimidazolilcarbocianina iodide (JC-1). Se utilizaron cultivos primarios mesencefálicos crecidos sobre cubreobjetos (para CMTMR) y cultivos crecidos en placas de 96 pocillos (para JC-1). En el primer caso, 3 horas después de los correspondientes tratamientos el medio de cultivo fue suplementado con CMTMR e incubado a 37ºC y 5% de CO_2 durante 15 minutos. A continuación, el medio fue retirado y las células lavadas con tampón DPBS frío y fijadas con paraformaldehido al 4% durante 10 minutos. Por último, fueron procesadas para inmunofluorescencia anti-TH o anti-OX-42. La fluorescencia fue visualizada mediante microscopia confocal y medida con un procesador de imagen digital. En el segundo caso, tras el correspondiente tratamiento, se añadió JC-1 a cada uno de los pocillos que fueron incubados durante 30 minutos a 37ºC y en oscuridad. A continuación, el medio fue retirado y las células lavadas con tampón DPBS. La intensidad de fluorescencia fue

medida en un lector de placas multifuncional. El potencial de membrana fue expresado como la relación entre la fluorescencia de los agregados y de los monómeros de JC-1. Como control positivo las células fueron tratadas con el protonóforo carbonil cianida m-clorofenilhidrazono (CCCP).

3.4.3- Estudio de la masa mitocondrial

Para llevar a cabo estudios de la masa mitocondrial total se emplearon cultivos primarios mesencefálicos crecidos sobre cubreobjetos. Tres horas después del tratamiento correspondiente, el medio fue suplementado con Mitotracker Green FM (MTGFM) e incubado durante 30 minutos a 37ºC y con un 5% de CO_2. A continuación, las células fueron lavadas y fijadas con paraformaldehido al 4% durante 10 minutos. Tras la fijación, las células fueron procesadas para inmunofluorescencia anti-TH (ver apartado 3.7). La fluorescencia fue visualizada mediante microscopia confocal y medida con un procesador de imagen digital (Image J).

3.4.4- Determinación de los niveles de MPP^+

Para estimar los niveles de MPP^+ en el cerebro de ratones control y ratones tratados con MPTP, los estriados fueron pesados, sonicados y centrifugados. A continuación, el sobrenadante fue recogido para su posterior análisis en un espectrómetro de masas.

3.4.5- Determinación de la actividad NADPH-oxidasa

La actividad NADPH-oxidasa, tanto en cultivos primarios como en tejido de cerebro, fue medida por quimioluminiscencia con lucigenina en un lector de placas. Los resultados fueron expresados como unidades relativas de luz por minuto y por µg de proteína.

3.4.6- Determinación de la actividad del enzima convertidor de AII

La actividad del enzima convertidor de AII fue medida por fluorimetría (en cultivos y en tejido de cerebro). Para ello se utilizó como sustrato un análogo del enzima convertidor de angiotensina, hippuryl-L-histidil-L-leucine (Hip-His-Leu). Finalmente, el producto His-Leu fue cuantificado en un lector de placas y la actividad fue expresada como nmoles de His-Leu producidos por mg de proteína por minuto.

3.4.7- Determinación de la actividad Rho-quinasa

La actividad Rho-quinasa (ROCK) fue medida con un kit de ensayo (Cell Biolabs, Inc, San Diego, CA, USA) siguiendo las instrucciones de uso. El kit consiste en un inmunoensayo enzimático desarrollado para la detección específica de la fosforilación llevada a cabo por ROCK a nivel de la subunidad 1 de la fosfata de miosina en el residuo Thr696. Para ello, el tejido fue homogeneizado en un tampón de lisis (50 mM Tris-HCl, pH 7,5, 150 mM NaCl, 1 mM 2-glicerofosfato, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 1 mM Na_3VO_4) que incluye un cóctel de inhibidores de proteasas (P8340, Sigma). La concentración de proteína fue medida usando el kit de ensayo de proteína *Pierce BCA* (Thermo Scientific, Fremont, CA, USA). Y la actividad fue medida en un lector multiplaca (Infinite M200, TECAN) a una absorbancia de 450nm.

3.4.8- Ensayos de recaptación de dopamina

Para determinar la recaptación de dopamina, los cultivos con sus correspondientes tratamientos fueron incubados con [^{3}H]dopamina durante 30 minutos a 37ºC y fue medida en un espectrómetro de centelleo. Así mismo, la recaptación inespecífica fue determinada en presencia de GRB 12935 (inhibidor específico de los transportadores de dopamina). Los resultados se expresaron como porcentaje respecto al grupo control.

3.4.9- Cromatografía líquida de alta resolución

Para confirmar los efectos funcionales de la lesión de MPTP, los niveles de dopamina y sus metabolitos fueron medidos a nivel del estriado en cerebros de ratones control y ratones tratados con MPTP. Para ello, siete días después de la última inyección de MPTP los ratones fueron sacrificados y los cerebros extraídos. El estriado fue diseccionado, homogeneizado y centrifugado a 14000g durante 20 minutos a 4ºC. El sobrenadante fue recogido, filtrado e inyectado en el sistema de HPLC (Shimadzu LC Prominence). La dopamina y sus metabolitos, ácido 3,4-dihidroxifenilacético (DOPAC) y ácido homovanílico (HVA), fueron separados mediante una columna analítica de fase reversa (Waters Symmetry 300 C18; 150 x 3.9 mm, tamaño de partícula 5µm, Waters). El flujo empleado para la fase móvil (70 mM KH_2PO_4, 1 mM ácido octanosulfónico, 1 mM EDTA, 10% MeOH, pH 4) fue de 1 ml/min. La cuantificación fue llevada a cabo mediante un detector electroquímico colorimétrico (ESA Coulochem III). Los datos

fueron adquiridos y procesados con el software de *Shimazu LC solution* y los resultados fueron expresados como nanogramos por microgramo de tejido en peso húmedo.

3.5- EXTRACCIÓN DE ARNm y RT-PCR CUANTITATIVA

La técnica de PCR a tiempo real fue empleada para estimar por un lado, los niveles relativos de las subunidades p47 y gp91 del complejo NADPH-oxidasa tanto en cultivos primarios mesencefálicos como en estriado y sustancia negra de tejido de animales de experimentación. Y por otro, los niveles relativos de ARNm de RhoA y ROCK II en la sustancia negra de cerebros de ratón. El paso previo a este análisis fue la extracción del ARN total mediante el uso de trizol y la posterior retrotranscripción del ARN para la obtención del ADNc.

3.6- INMUNOMARCAJE

De manera previa al inmunomarcaje los cultivos celulares fueron fijados con paraformaldehido al 4%, mientras que los animales fueron sacrificados por sobredosis de anestesia, perfundidos transcardíacamente con paraformaldehido al 4% y los cerebros extraídos, crioprotegidos y seccionados en un criostato a 20 μm.

En función del objetivo del experimento se llevaron a cabo técnicas de inmunohistoquímica colorimétrica (simple), inmunofluorescencia (simple y doble) o histoquímica.

En caso de inmunomarcaje colorimétrico tanto las células en cultivo como el tejido fueron tratados con una solución de agua oxigenada para inactivar la peroxidasa endógena. Posteriormente fueron incubados con suero normal (de la misma especie que el anticuerpo secundario) para bloquear los antígenos inespecíficos presentes en las células, junto con seroalbúmina bovina y Triton X-100 para permeabilizar la membrana celular. A continuación, fueron incubados con el/los anticuerpo/s primario/s correspondiente/s (Tabla 3) junto con el suero, la seroalbúmina y Triton; y seguidamente incubados con el/los anticuerpo/s secundario/s marcados bien con biotina (inmunohistoquímica) o con un fluoróforo ((fluoresceína isotionicianato) FITC o (cianina 3) Cy3, inmunofluorescencia). Para inmunohistoquímica después del anticuerpo secundario fueron incubados con un preparado comercial del complejo avidina-biotina-peroxidasa (ABC-Kit) y revelados empleando peróxido de hidrógeno y

diaminobenzidina (DAB) con la que se obtiene un precipitado marrón, o DAB junto con sulfato de níquel que permite un marcaje más intenso de color negro. En el caso del inmunomarcaje fluorescente, los núcleos fueron contrastados con Hoechst 33342.

Para el marcaje de microglía activada en tejido el primer paso fue la eliminación de la actividad peroxidasa endógena mediante la incubación con peróxido de hidrógeno. A continuación, el tejido fue incubado con una solución de tampón fosfato y Triton y posteriormente, con la misma solución junto con el marcador GSI-B4. Por último, fue incubado con el kit ABC y revelado con peróxido de hidrógeno y DAB más sulfato de níquel.

Para la tinción con violeta de cresilo el tejido fue incubado durante 10 min a 57ºC con la solución de violeta y a continuación fue deshidratado en una estufa a 57ºC.

Tabla 3: anticuerpos primarios/ marcadores empleados

Antígeno	Especie	Tipo	Marcaje
Angiotensinogeno	Cabra	Policlonal	Marcador de angiotensinógeno
AT1	Conejo	Policlonal	Marcador de receptores de AII de tipo I
AT2	Conejo	Policlonal	Marcador de receptores de AII de tipo II
Calbindina	Conejo	Policlonal	Marcador de neuronas dopaminérgicas A10
CD11b	Ratón	Monoclonal	Marcador de microglia activada y en reposo/ macrófagos
GFAP	Ratón	Monoclonal	Marcador de astrocitos
GFAP	Conejo	Policlonal	
gp91^{phox}	Cabra	Policlonal	Marcador de la subunidad gp91 del complejo NADPH-oxidasa
Integrin αM	Cabra	Policlonal	Marcador de microglía activada/ macrófagos
NeuN	Ratón	Monoclonal	Marcador neuronal
OX-42	Ratón	monoclonal	Marcador de microglía activada y en reposo/ macrófagos
OX-6	Ratón	Monoclonal	Marcador de microglía activada
p47^{phox}	Cabra	Policlonal	Marcador de la subunidad p47 del complejo NADPH-oxidasa
PRR	Cabra	Policlonal	Marcador del receptor de pro-renina
TH	Ratón	Monoclonal	Marcador de neuronas dopaminérgicas
TH	Conejo	Policlonal	
CMTMR			Marcador para estimar el potencial de membrana interna mitocondrial
DHE			Marcador para detectar superóxido
GSI-B4	Griffonia simplicifolia		Marcador de microglía activada
Hoechst 33342			Marcador nuclear
JC-1			Marcador de hiperpolarización de la membrana mitocondrial
MTGFM			Marcador para estimar la masa mitocondrial
Violeta de cresilo			Marcador de los grumos de Nissl

3.7- CUANTIFICACIÓN

3.7.1- Cuantificación de células en cultivo

Las células en cultivo marcadas inmunohistoquímicamente para TH, OX-6 o GFAP fueron cuantificadas usando un microscopio invertido de contraste de fases (Eclipse, Nikon) a 100 aumentos. Se estimó el número de células en cinco campos microscópicos (0,5 x 0,5cm) elegidos aleatoriamente a lo largo de la placa de cultivo. Las células marcadas por inmunofluorescencia fueron cuantificadas empleando un microscopio acoplado a una fuente de epi-fluorescencia.

Los resultados fueron obtenidos de al menos 3 experimentos independientes (cultivos obtenidos por separado) con un tamaño mínimo de muestra de 4 placas por grupo. Estos resultados fueron normalizados comparando los contajes de los grupos experimentales con los del grupo control de la misma tanda, expresando así los resultados como porcentaje de células positivas respecto al grupo control, para contrarrestar la posible variabilidad entre tandas.

3.7.2- Cuantificación de células en tejido

La cuantificación del número de células en la SNpc (neuronas dopaminérgicas) y en el estriado (células microgliales y astrocitos) se llevó a cabo empleando un método estereológico no sesgado. Dicho análisis se realizó con la ayuda del sistema CAST-Grid Olympus (Computer Assisted Stereologicla Toolbox). El número total de células se estimó empleando la metodología del fraccionador óptico (West y col., 1991) a 100 aumentos y el coeficiente de error asociado fue calculado según Gundersen y Jensen (1987). Los resultados fueron expresados en valores absolutos. Por un lado, el número total de neuronas dopaminérgicas y por otro, la densidad (número total de células/mm^3) de células microgliales y astrocitos.

3.7.3- Densidad óptica

La densidad de terminales dopaminérgicos en el estriado se estimó midiendo la densidad óptica (D.O.) de la inmunoreactividad para TH en al menos cuatro secciones por animal a nivel del estriado central. Para ello se empleó el programa de análisis de imagen NIH-Image 1.55 (Wayne Rasband, MIMH) en un ordenador personal Macintosh. Las imágenes fueron tomadas con una videocámara (CCD-72, MTI) conectada a una fuente de iluminación constante y fría (Norther Light). Para cada

sección, el valor de D.O. se obtuvo delimitando el área del estriado, y dicho valor fue corregido substrayendo el correspondiente fondo tomado a nivel del cuerpo calloso. Los resultados se expresaron como porcentaje respecto al grupo control (100%).

3.8- ANÁLISIS ESTADÍSTICO

Los resultados obtenidos fueron expresados como media ± error estándar. Las comparaciones entre dos grupos fueron realizadas con el test de t-Student, mientras que para las comparaciones múltiples se utilizó un análisis de la varianza (ANOVA) de una vía seguido del test de Bonferroni o del test de Holm-Sidak dependiendo del estudio. Se consideró la existencia de diferencias estadísticamente significativas cuando $p<0,05$, y la normalidad de las poblaciones y la homogeneidad de las varianzas fue testada antes de cada ANOVA. Los programas utilizados para todos los análisis estadísticos y la elaboración de los histogramas fueron SigmaStat 3.0.1 y SigmaPlot 8.0.2 (Jandel Scientific).

RESULTADOS

4.- RESULTADOS

4.1- Papel de la AII cerebral en la respuesta inflamatoria en animales tratados con MPTP como modelo de enfermedad de Parkinson: implicación en la progresión de la degeneración dopaminérgica

Belen Joglar, Jannette Rodríguez-Pallares, Ana Isabel Rodriguez-Perez, Pablo Rey, Maria Jose Guerra y Jose Luis Labandeira-Garcia (2009) The inflammatory response in the MPTP model of Parkinson´s disease is mediated by brain angiotensin: relevance to progresion of the disease. J Neurochem 109: 656-669.

La neurotoxina MPTP reproduce la mayoría de las características bioquímicas y patológicas de la EP, de ahí su uso para la obtención de modelos animales de la enfermedad. Por otro lado, sabemos que las EORs generadas como consecuencia de la inhibición del complejo I mitocondrial así como de la activación del complejo NADPH-oxidasa microglial juegan un papel importante en la toxicidad del MPTP. Sin embargo, el mecanismo exacto a través del cual se regula la respuesta microglial aún no está claro. Además del sistema Renina-angiotensina (SRA) clásico existen SRA locales en diferentes tejidos entre los que se incluye el cerebro. El principal efector del SRA es el péptido AII que a través de los receptores AT1 desencadena la respuesta inflamatoria y estrés oxidativo, y mediante la activación del complejo NADPH-oxidasa induce sobre las células inflamatorias la liberación de altos niveles de EORs. El SRA cerebral modula la liberación de dopamina a nivel del estriado pero se desconoce si tiene un papel importante en el estrés oxidativo generado como consecuencia de la activación microglial y en la degeneración dopaminérgica. En este estudio se ha demostrado que en cultivos primarios mesencefálicos la degeneración dopaminérgica inducida por la neurotoxina MPTP/MPP^+ es amplificada por acción de AII e inhibida mediante el uso de antagonistas de los receptores AT1, y que la PKC, la activación microglial y la activación del complejo NADPH-oxidasa median este efecto. Del mismo modo, en ratones se ha visto que el uso de antagonistas de los receptores AT1 inhibe tanto la degeneración dopaminérgica como la activación microglial temprana y la activación del complejo NADPH-oxidasa. Por tanto, el SRA cerebral juega un papel importante en la activación microglial temprana y generación de EORs derivadas de la activación del complejo NADPH-oxidasa, que contribuyen al inicio y/o progresión de la EP. Así, manipulaciones del SRA podrían resultar efectivas como estrategia neuroprotectora, tal y como se ha observado en estudios previos en enfermedades cardiovasculares.

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The inflammatory response in the MPTP model of Parkinson's disease is mediated by brain angiotensin: relevance to progression of the disease

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Abstract

The neurotoxin MPTP reproduces most of the biochemical and pathological hallmarks of Parkinson's disease. In addition to reactive oxygen species (ROS) generated as a consequence of mitochondrial complex I inhibition, microglial NADPH-derived ROS play major roles in the toxicity of MPTP. However, the exact mechanism regulating this microglial response remains to be clarified. The peptide angiotensin II (AII), via type 1 receptors (AT1), is one of the most important inflammation and oxidative stress inducers, and produces ROS by activation of the NADPH-oxidase complex. Brain possesses a local angiotensin system, which modulates striatal dopamine (DA) release. However, it is not known if AII plays a major role in microglia-derived oxidative stress and DA degeneration. The present study indicates that in primary mesencephalic cultures, DA degeneration induced by the neurotoxin MPTP/MPP^+ is amplified by AII and inhibited by AT1 receptor antagonists, and that protein kinase C, NADPH-complex activation and microglial activation are involved in this effect. In mice, AT1 receptor antagonists inhibited both DA degeneration and early microglial and NADPH activation. The brain angiotensin system may play a key role in the self-propelling mechanism of Parkinson's disease and constitutes an unexplored target for neuroprotection, as previously reported for vascular diseases.

Keywords: angiotensin, microglia, NADPH-oxidase, neurodegeneration, oxidative stress, Parkinson.

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Parkinson's disease (PD) is a common neurodegenerative disorder characterized by a progressive loss of dopaminergic (DA) neurons. A number of studies have shown that oxidative stress (OS), neuroinflammation, and microglial activation play a pivotal role, at least in the progression of PD (Block and Hong 2005). The neurotoxin MPTP reproduces most of the biochemical and pathological hallmarks of PD. MPTP produces a parkinsonian syndrome after its conversion to MPP^+ ion by type B monoamine-oxidase. MPP^+ is then accumulated in DA neurons by the DA reuptake system, and impairs mitochondrial respiration by inhibiting complex I (Przedborski *et al.* 2000). Recent studies, however, have shown that microglial activation and microglial NADPH-derived free radicals play major roles in the toxicity of MPTP, and that DA neurons may be under the dual effect of reactive oxygen species (ROS) generated as consequence of mitochondrial complex I inhibition and activated microglia (Gao *et al.* 2003a,b; Wu *et al.* 2003). However, the exact mechanism regulating the microglial response remains to be clarified.

Several recent studies have suggested a major role for neuropeptides in neurodegeneration and microglial signaling, which constitutes an unexplored area with potential

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Abbreviations used:ACE, angiotensin-converting enzyme; AII, angiotensin II; AT1, angiotensin type 1 receptor; DA, dopamine; GFAP, glial fibrillary acidic protein; OS, oxidative stress; PD, Parkinson's disease; PKC, protein kinase C; RAS, renin–angiotensin system; RLU, relative light units; ROS, reactive oxygen species; SNc, substantia nigra; TH, tyrosine hydroxylase; TH-ir, TH immunoreactivity; VM, ventral mesencephalon.

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therapeutic applications (Block *et al.* 2006; Noda *et al.* 2007). The peptide angiotensin II (AII), via type 1 (AT_1) receptors, is one of the most important known inflammation and OS inducers, and produces ROS by activation of the NADPH-oxidase complex (Seshiah *et al.* 2002; Touyz *et al.* 2002; Cai *et al.* 2003), which is the most important intracellular source of ROS apart from mitochondria (Babior 1999, 2004). The effects of local renin–angiotensin systems (RAS) have been extensively studied in renal and vascular tissues, as OS and chronic inflammation are the hallmarks of atherosclerosis (Zalba *et al.* 2001; Suzuki *et al.* 2003). In vascular degenerative disease and others mediated by OS and chronic inflammation, AII acts on resident cells and inflammatory cells, and inhibition of AII is much more effective than antioxidants or anti-inflammatory drugs (Münzel and Keany 2001). Brain (McKinley *et al.* 2003; Saavedra 2005), including the basal ganglia and the DA system (Allen *et al.* 1992; Mendelsohn *et al.* 1993; Brown *et al.* 1996; Rodriguez-Pallares *et al.* 2004), possesses a local RAS. It is well known that there is interaction between DA and AII in tissues in which AII induces OS, inflammation and degenerative diseases (Hussain *et al.* 1998; Zeng *et al.* 2006). Interestingly, AII also modulates striatal DA release (Allen *et al.* 1992; Mendelsohn *et al.* 1993; Brown *et al.* 1996). However, it is not known if AII plays a major role in microglial activation and DA degeneration in PD. In the present study, we focused on the main animal model of PD, which involves intoxication with MPTP/MPP^+, a neurotoxin that induces DA neuron degeneration by mechanisms possibly similar to those of environmental neurotoxins potentially responsible for the disease.

Materials and methods

Experimental design

The effect of AII on the microglial response induced by MPTP has been studied *in vitro* in primary mesencephalic cultures, which excluded possible indirect effects due to AII-induced changes in the brain vessel function, and *in vivo* in mice that were not subjected to intracerebral or intraventricular injections in order to preserve physiological conditions and provide evidence that the observations have *in vivo* implications. In cultures, we studied the effects of AII and AII-receptor antagonists on glial activation and DA neuron degeneration, and the possible cell signalling pathway involved in the observed effects. Given that it has been reported that AII enhances the inflammatory response in peripheral cells through the pathway AII/AT1 receptors/protein kinase C (PKC)/NADPH complex activation/ROS generation pathway (Seshiah *et al.* 2002; Cai *et al.* 2003), we treated cultures with AT1 antagonists, or PKC inhibitors, or inhibitors of the NADPH activation in order to study the possible involvement of this pathway in the observed effects.

In mice, given that glial reaction may be a consequence of neuronal DA death, we used two different groups of MPTP-treated animals. A first group of animals were subjected to complete MPTP intoxication following a subacute/chronic MPTP model, and killed 7 days after the last MPTP injection to study the effect of MPTP and AT1 antagonists on DA cell death. A second group of mice were injected with a single injection of MPTP and killed 24 h later, to study the glial activation at stages prior to occurrence of significant loss of DA neurons (Jackson-Lewis *et al.* 1995; Hébert *et al.* 2003), and the effects of AT1 antagonists on this early glial activation. All experiments were carried out in accordance with the 'Principles of laboratory animal care' (NIH publication No. 86–23, revised 1985) and approved by the corresponding committee at the University of Santiago de Compostela (see Supporting information for further details on methodological procedures).

Primary mesencephalic cultures

Cell suspensions were obtained from ventral mesencephalon (VM) of rat embryos 14 days of gestation (E14). Control cultures contained about 40% neurons (i.e. Neun-positive cells; see immunohistochemistry below), 43% astrocytes and 12% microglial cells. After 4 days *in vitro*, cultures were exposed to MPP^+ (01–0.5 μM; Sigma, St Louis, MO, USA) alone or MPP^+ and AII (100 nM; Sigma) for a further 4 days. To determine the AII receptor subtype effects, cultures were treated with the AT1 receptor antagonist ZD 7155 (1 μM; Sigma) or the AT2 receptor antagonist PD 123319 (1 μM; Sigma) 16 h before adding MPP^+ alone or MPP^+ and AII. To study the possible effects of the NADPH-oxidase complex activation, some cultures were treated with apocynin (an inhibitor of NADPH-oxidase; 0.25 mM; Fluka, Buchs, Switzerland) 30 min before treatment with the MPP^+ alone or MPP^+ and AII. To study the possible activation of the NADPH-oxidase complex via PKC, some cultures were treated with chelerythrine chloride (a selective PKC inhibitor; 10 μM; Sigma). To obtain neuron-enriched cultures, cytosine-β-D-arabino-furanoside (Ara-C; 1 μM; Sigma) was added 48 h after seeding the cells. After 4 days *in vitro*, the cultures were then treated with MPP^+ (01–0.5 μM; Sigma) alone or MPP^+ and AII (100 nM; Sigma) for a further 4 days. This method can enrich neurons to > 85% purity (about 9% astrocytes and < 1% microglia). Cultures were then processed for histology, angiotensin-converting enzyme (ACE) and NADPH activity, or RT-PCR studies (see below).

Cultures processed for histology were fixed with 4% paraformaldehyde and then processed for immunohistochemistry with a mouse monoclonal anti-tyrosine hydroxylase (TH; Sigma; 1 : 30 000) as DA marker, a mouse monoclonal anti-NeuN (Chemicon, Temecula, CA, USA; 1 : 2000) as a neuronal marker, a mouse monoclonal anti-glial fibrillary acidic protein (GFAP; Chemicon, Kidlington, Oxford, UK; 1 : 1000) as a marker of astrocytes, a mouse monoclonal anti-CD11b (anti-complement receptor-3, clone MRC OX-42; Serotec, Kidlington, Oxford, UK; 1 : 1000) as a marker of resting and reactive microglial cells/ macrophages, OX-6 (a mouse monoclonal antibody directed against a monomorphic determinant of the rat major histocompatibility complex class II antigens, expressed by activated microglia but not by resting cells; 1 : 200; Serotec) as a marker of reactive microglia/ macrophages, a rabbit polyclonal anti-AT1- or anti-AT2-receptor antibody (Abcam, Cambridge, UK; 1 : 400), or a goat polyclonal anti-$gp91^{phox}$ or anti-$p47^{phox}$ NADPH subunits (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 100 and 1 : 200, respectively. Cells were counted with phase contrast microscopy (Eclipse, Nikon, Tokyo, Japan; 100× magnification). In addition, cultures grown on glass coverslips were processed for double immunofluorescence against AT1- or AT2-receptors, or $gp91^{phox}$ or $p47^{phox}$,

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and TH, GFAP, or OX-42 to study the co-localization of these markers. Co-localization of markers was confirmed by confocal laser microscopy (TCS-SP2; Leica, Heidelberg, Germany).

In vivo treatment with MPTP and the AT1 receptor antagonists

Male C57BL-6 mice (Charles River, l'Arbresle, France) weighing 20–25 g (i.e. 7 weeks old) at the beginning of the experiments were used for both *in vivo* experiments. The mice were divided into four groups (A–E). Mice in group A (n = 18) were used as normal (i.e. non-lesioned) controls, and received intraperitoneal and subcutaneous injections of saline (see group C below). Mice in group B (n = 30) were injected with MPTP (Free base, Sigma; 30 mg/kg/day, intraperitoneally; in saline) with a single injection (group B1) or with five injections (every second day for 9 days; group B2), and with subcutaneous injections of saline. Mice in group C (n = 20) were injected with MPTP as with group-B mice, but received subcutaneous injection of candesartan (2 × 0.25 mg/kg/day; AstraZeneca, Mölndal, Sweden) from 2 weeks before MPTP treatment until they were killed. Mice in groups D and E were used as controls. Mice in group D (n = 10) received subcutaneous injection of candesartan as above and saline injection instead of MPTP, and were used to study possible effects of candesartan alone. Mice in group E (n = 10) were treated with candesartan as above or saline, and finally injected with a single doses of MPTP (30 mg/kg; 30 min after the last candesartan injection) to quantify striatal levels of MPP^+ (Przedborski *et al.* 1996; see below). Mice were killed 90 min (group E), 24 h (groups B1, C1, and D1) or 1 week (groups B2, C2, and D2) after the MPTP or saline treatment, and were then processed for histology, mass spectrometry, ACE and NADPH activity, or RT-PCR studies (see below).

For histology, the animals were killed and perfused with 4% paraformaldehyde, and the brains cut on a freezing microtome. Sections were processed with rabbit polyclonal antibodies to TH (Pel-Freez, Rogers, AR, USA; 1 : 500; as a marker of DA terminals), rabbit polyclonal antibodies to GFAP (Chemicon, 1 : 2000; as a marker of astrocytes), rabbit polyclonal antibodies to AT1 and AT2 receptors (Abcam, 1 : 400), goat polyclonal antibodies to angiotensinogen (R&D Systems, Minneapolis, MN, USA; 1 : 100). Activated microglial cells were stained histochemically with *Griffonia simplicifolia* isolectin B4. Selected sections were processed for double immunofluorescence, and co-localization of markers was confirmed by confocal laser microscopy as above.

The total numbers of TH immunoreactivity (TH-ir) neurons, microglial cells and astrocytes were estimated by an unbiased stereology method (i.e. the optical fractionator). Stereological analysis was carried out with the Olympus CAST-Grid system (Computer Assisted Stereological Toolbox; Olympus, Albertslund, Denmark). In order to confirm that MPTP induces cell death the total number of Nissl-stained (i.e. Cresyl violet-stained) neurons in the substantia nigra (SNc) was estimated by the same unbiased stereology method. The density of striatal DA terminals was estimated as the optical density of the striatal TH-ir with the aid of NIH-Image 1.55 image analysis software (Wayne Rasband, MIMH, Bethesda, MD, USA).

Estimation of MPP^+ levels, NADPH-oxidase subunits mRNA expression, NADPH activity, ACE activity, and [^{3}H]DA-uptake

For estimation of MPP^+ levels, striata were weighed, sonicated and centrifuged, and the supernatant was used for determinating MPP^+ levels (Hows *et al.* 2004). HPLC separation was accomplished in a Waters Alliance 2795 system (Waters, Milford, MA, USA), with an Atlantis dC18 column (2.1 × 50 mm, 3 μm). Eluates were detected with a Quattro MicroTM API ESCI triple-quadrupole mass spectrometer fitted with Z-spray.

Total RNA from the striatum or VM or mesencephalic cultures was extracted with Trizol (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Real-time PCR was used to examine relative levels of the NADPH-oxidase subunits $gp91^{phox}$ and $p47^{phox}$ mRNA as an index of NADPH activity (Jones *et al.* 1995; Rueckschloss *et al.* 2002; Touyz *et al.* 2002).

NADPH oxidase activity in cultures and brain tissue was measured by lucigenin-enhanced chemiluminescence with a Infinite M200 multiwell plate reader (TECAN, Salzburg, Austria), as described by Griendling *et al.* (1994) and Hong *et al.* (2006), respectively. Chemiluminescence was measured as relative light units (RLU/min/μg protein).

Angiotensin-converting enzyme activity was assayed with hippuryl-L-histidyl-L-leucine (Hip-His-Leu; Sigma) as substrate, as described previously by Hemming *et al.* (2007). Fluorescence was assayed in a 96-well plate format with an Infinite M200 multiwell plate reader (TECAN; excitation, 355 nm; emission, 535 nm), and was expressed as nmol of His-Leu produced per milligram of protein per minute.

Uptake of [^{3}H]DA was determined by incubation of cultures for 30 min at 37°C with 20 nM [^{3}H]DA ([2,5,6-^{3}H] DA; 1 μCi, 12 Ci/mmol; Amersham Biosciences, GE Healthcare, Buckinghamshire, UK) in the absence (untreated cultures) or presence of different drugs (100 nM AII or 1 μM ZD 7155 or 10^{-4} M candesartan or 0.25 mM apocynin). Non-specific uptake values were defined in the presence of 10 μM GRB 12935 (Sigma), a specific inhibitor of the DA transporter. Results are expressed as percentages of untreated control culture responses.

Statistical analysis

All data were obtained from at least three independent experiments and were expressed as mean ± SEM. Multiple comparisons were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. The normality of populations and homogeneity of variances were tested before each ANOVA. Differences at $p < 0.05$ were considered as statistically significant. Statistical analyses were carried out with SigmaStat 3.0 from Jandel Scientific (San Rafael, CA, USA).

Results

Location of AII receptors and NADPH-oxidase subunits in DA neurons and glial cells.

The cultures showed cells immunoreactive for TH, as marker of DA neurons, astroglial markers (i.e. GFAP) and microglial markers (i.e. OX-42, OX-6). The average number of TH-positive cells in a control culture dish was 1846 ± 67. Confocal microscopy and double immunolabeling for AT1 or AT2 and TH or GFAP or OX-42 revealed that angiotensin receptors (i.e. AT1 and AT2 receptors) were located in DA neurons, but also in astrocytes and microglia. Double immunofluorescence revealed that major membrane ($gp91^{phox}$) and cytosolic ($p47^{phox}$) subunits of the NADPH-

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oxidase complex were present in microglia (i.e. OX-42-positive cells), cells immunoreactive for GFAP and TH-ir neurons (Fig. 1a–l; Fig. S1). Finally, the activity of endogenous RAS in cultures was confirmed by determination of ACE activity (23.7 ± 1.4 nmol His-Leu/min/mg protein). The presence of angiotensin receptors and angiotensinogen in mice SNc was confirmed by immunohistochemistry. Double immunofluorescence revealed that DA neurons in the SNc express AT1 receptors and less intense labeling for AT2 receptors, and confirmed astrocytes as the major source of angiotensinogen in the SNc (Fig. 1m–q). Endogenous RAS activity was confirmed by determination of ACE activity in the VM (17.44 ± 0.3 nmol His-Leu/min/mg protein) and striatum (25.2 ± 0.6 nmol His-Leu/min/mg protein) of control mice.

Effect of AII and AII receptor antagonists on MPTP/MPP$^+$-induced degeneration of TH-ir cells

Cultures were treated with different doses of MPP$^+$ (0.1–0.5 μM for 4 days; Fig. 2 and Fig. S2). No significant difference in the number of TH-ir neurons was observed after 4-day treatment with 0.1 μM MPP$^+$ or 0.1 μM MPP$^+$ and AII. However, cultures treated with doses of 0.25 or 0.5 μM contained significantly less TH-ir cells than the control cultures. Treatment with 0.25 μM MPP$^+$ induced the loss of a low number of TH-ir cells (of about 25% decrease), which was significantly enhanced by simultaneous treatment with AII (Fig. 2a). The effect of 0.25 μM MPP$^+$ alone or 0.25 μM MPP$^+$ + AII was blocked (i.e. no significant difference from control cultures) by simultaneous treatment with the AT1 receptor antagonist ZD 7155, but was not significantly affected by simultaneous treatment with the AT2 receptor antagonist PD 123319. It is particularly interesting to note that the AT1 antagonist ZD 7155 blocked the effect of low doses (0.25 μM) of MPP$^+$ alone (i.e. without addition of exogenous angiotensin; Fig. 2c). This suggests that endogenous AII amplified the MPP$^+$ toxicity in cultures. This was also confirmed in mice treated with MPTP and candesartan alone (see below). Furthermore, cultures subjected to simultaneous

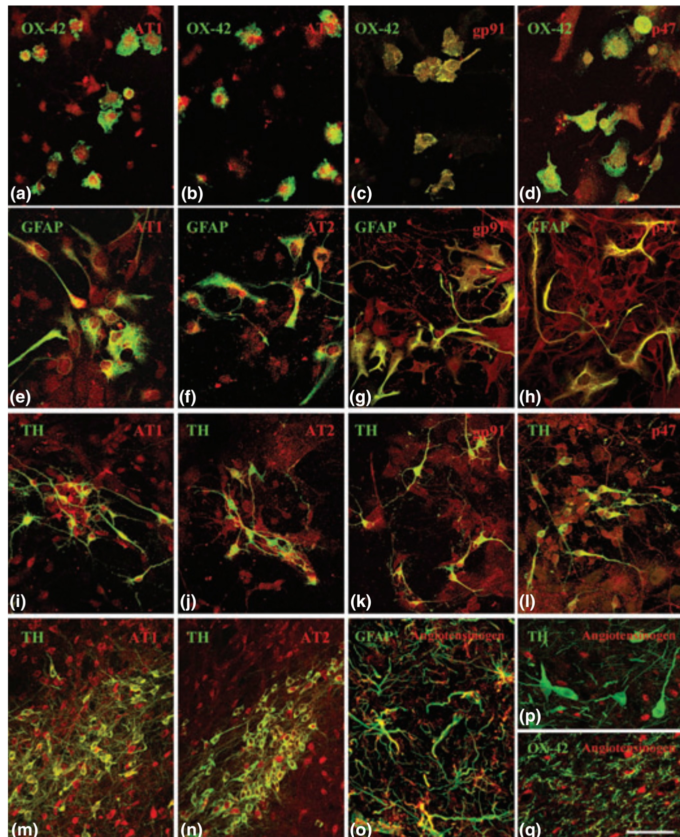

Fig. 1 Double immunofluorescence for OX-42, GFAP or TH (green) and AT1 or AT2 receptors, or gp91^{phox} or p47^{phox} NADPH subunits (red) in primary mesencephalic cultures (a–l) or mouse substantia nigra (m–q). AT1 and AT2 receptors and gp91^{phox} and p47^{phox} subunits show co-localization (yellow) with the microglial marker OX-42 (a–d), the astroglial marker GFAP (e–h), and the dopaminergic marker TH (i–l). In the substantia nigra, AT1 and AT2 receptors were observed in dopaminergic (yellow) and non-dopaminergic (red) cells (m, n), and numerous astrocytes (green) contained (yellow) angiotensinogen (o; red). No significant co-localization of angiotensinogen (red) and TH or the microglial marker OX-42 was observed (green; p, q). AII, angiotensin II; AT1, angiotensin type 1 receptor; AT2, angiotensin type 2 receptor; GFAP, glial fibrillary acidic protein; TH, tyrosine hydroxylase. Scale bar: 30 μm (p), 50 μm (a–d, l, o, and q), 75 μm (e–k).and 100 μm (m, n).

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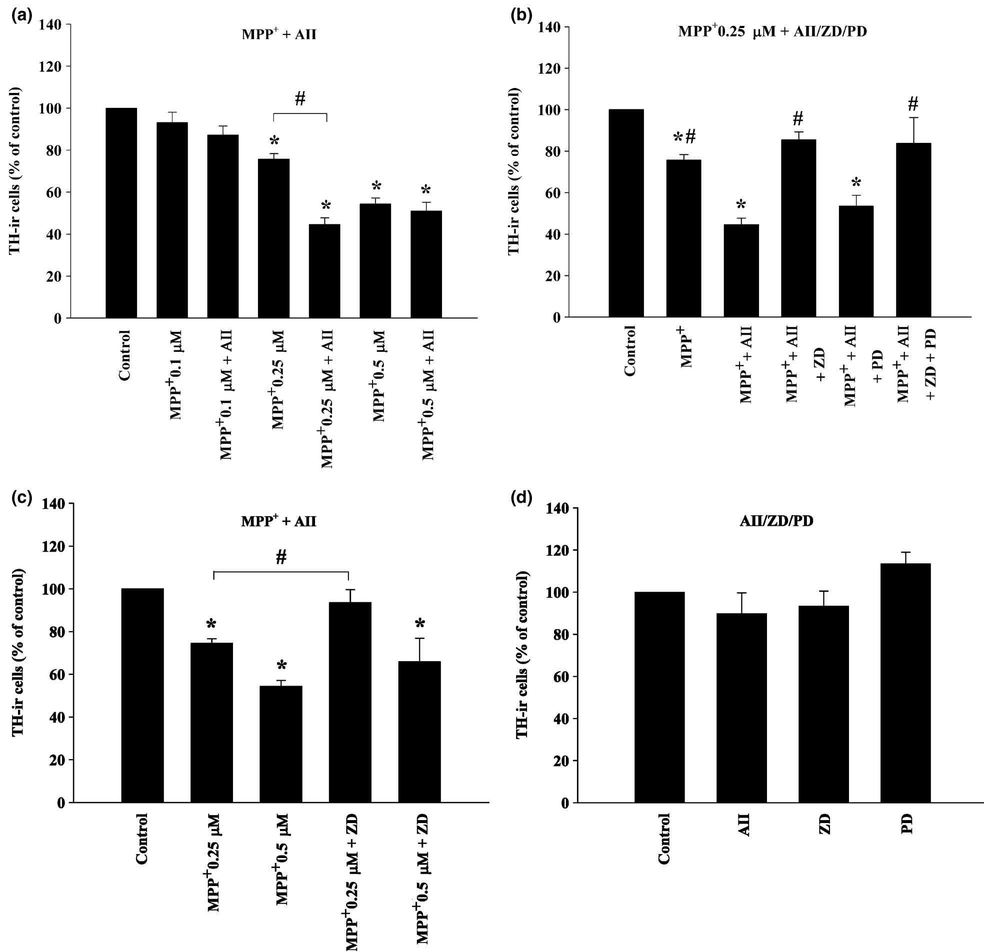

Fig. 2 Effects of different treatments on the number of TH-ir cells. (a) Treatment with 0.25 μM MPP^+ induced a significant loss of TH-ir neurons, which was significantly increased by AII (100 nM); however, the effect of higher doses of MPP^+ (0.5 μM) was not increased by AII. (b) The amplifying effect of AII was inhibited by ZD 7155 but not by PD 123319. (c) The decrease in the number of TH-ir cells induced by 0.25 μM MPP^+ (i.e. without addition of exogenous AII) was blocked by treatment with the AT1 receptor antagonist ZD 7155, which presumably inhibited the effect of endogenous angiotensin. The loss of TH-ir cells induced by 0.5 μM MPP^+ was not significantly affected by ZD 7155. (d) No significant effect was observed after treatment with AII or antagonists alone. The data are expressed as percentages of the number of TH-ir cells obtained in the respective control cultures (100%). Data represent mean ± SEM. $^*p < 0.05$ compared with control group (untreated cells); $^\#p < 0.05$ compared with MPP^+ + AII in B (one-way ANOVA and Bonferroni *post hoc* test). AII, angiotensin II; PD, PD 123319; TH, tyrosine hydroxylase; ZD, ZD 7155.

treatment with AT1 + AT2 receptor antagonists (i.e. 0.25 μM MPP^+ + AII + ZD 7155 + PD 123319) did not contain significantly lower numbers of TH-ir cells than the controls, suggesting that the deleterious effects of AII on TH-ir cells are prevented by blockage of AT1 receptors and that simultaneous stimulation of AT2 receptors is not a significant factor in this effect (Fig. 2b). Treatment of cultures with higher doses of MPP^+ alone (0.5 μM, 4 days; or 1 μM 48 h, not shown) induced a greater decrease in the number of TH-ir neurons (of about 50% decrease), which was not significantly modified by simultaneous treatment with AII or the AT1 antagonist ZD 7155 (Fig. 2a

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and c). No significant difference in the number of TH-ir neurons was observed after treatment with AII (100 nM) alone. Similarly, no significant changes in the number of TH-ir cells were observed after treatment with ZD 7155 or PD 123319 alone (Fig. 2d).

In control mice (i.e. not injected with MPTP) the DA neurons in the SNc compacta were intensely immunoreactive to TH, and a dense evenly distributed TH-ir was observed throughout the striatum, which indicated the presence of a dense network of nigrostriatal DA terminals (Fig. 3). As expected, the number of Nissl-stained neurons counted in Cresyl-violet stained sections (13205 ± 770) was slightly higher than that of TH-immunoreactive (TH-ir) neurons since some non-DA neurons located in the area of the SNc were

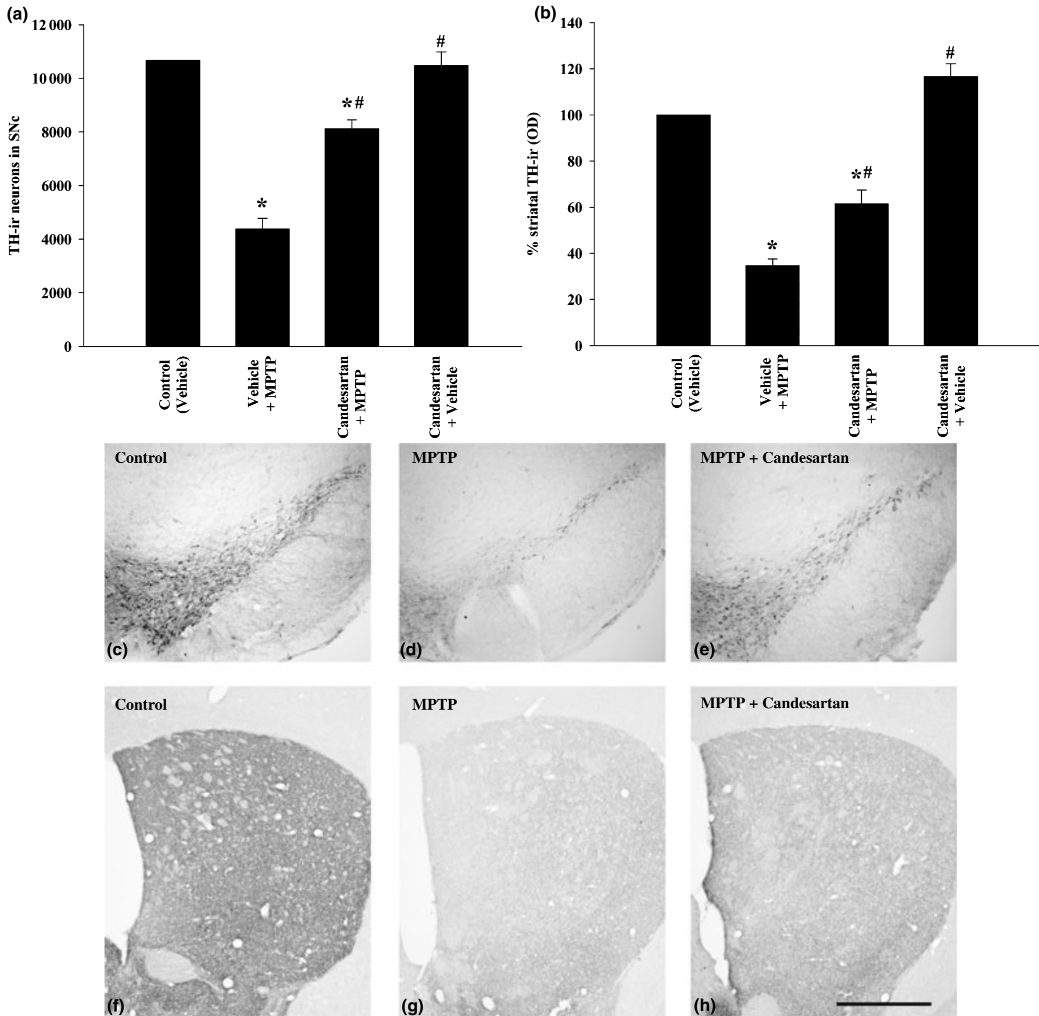

Fig. 3 Dopaminergic (TH-ir) neurons (a and c–e) or terminals (b and f–h), in the substantia nigra compacta (SNc; a, c–e) or striatum (ST; b, f–h) 1 week after a five-injection treatment with vehicle, vehicle + MPTP (5 × 30 mg/kg), candesartan + MPTP (5 × 30 mg/kg), or candesartan + vehicle. The DA neurons were quantified as the total number of TH-ir neurons in the SNc (a). Density of striatal DA terminals was estimated as optical density and expressed as a percentage of the value obtained in the control group (b). Data represent mean ± SEM. *$p < 0.05$ compared with control group, #$p < 0.05$ compared with group treated with MPTP + vehicle (one-way ANOVA and Bonferroni *post hoc* test). Scale bar: 500 μm.

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also included. Mice subjected to subcutaneous injection of saline and intraperitoneal injection of MPTP, showed a bilateral reduction in the number of TH-ir neurons in the SNc and a marked reduction in the TH-ir in both striata (Fig. 3). Furthermore, sections from group-B mice showed a significant reduction in the number of Cresyl-violet stained neurons in the SNc (7651 ± 876). Mice subjected to treatment with candesartan and intraperitoneal injection of MPTP showed a bilateral reduction in the number of TH-ir neurons in the SNc, although this reduction was significantly less than that observed in group-B mice not treated with candesartan. Similarly, the mice treated with MPTP and candesartan showed a significant reduction in the striatal TH-ir relative to the control mice as a consequence of the loss of striatal DA terminals. However, this reduction was significantly less than that observed in mice not treated with candesartan. This suggests that brain endogenous AII increases the neurotoxic effect of MPTP on the DA system, as previously observed in mesencephalic cultures. No significant changes were observed in mice treated with candesartan alone (Fig. 3).

[^{3}H] Dopamine uptake assay and striatal levels of MPP^+

MPP^+ is accumulated by the DA uptake system. Therefore, it is possible that changes in MPTP or MPP^+-induced neurotoxicity were caused by increased or decreased MPP^+ uptake due to changes in the DA transport activity. We measured [^{3}H] DA uptake in the absence or presence of those compounds that modified the effect of MPP^+. No significant changes ($p > 0.05$) in the uptake of [^{3}H] DA were observed after treatment with 100 nM AII (103 ± 12%) or 10^{-6} M ZD 7155 (115 ± 16%) or 0.25 M apocynin (95 ± 12%) or 10^{-4} candesartan (113 ± 14%) in comparison with non-treated controls (100%), which is consistent with the results of previous studies on effect of AII on DA uptake (Mendelsohn *et al.* 1993; Brown *et al.* 1996).

In order to determine if treatment with candesartan could act by modifying MPTP pharmacokinetics such as penetrance into the brain, biotransformation of MPTP to MPP^+ or MPP^+ removal from the brain, we measured striatal levels of MPP^+ in mice. We observed no significant differences in striatal levels of MPP^+ between mice treated with candesartan and MPTP (4.08 ± 0.59 ng/mg striatal tissue) and mice treated with saline and MPTP (4.19 ± 1.0.68 ng/mg striatal tissue).

Effect of AII and AII receptor antagonists on MPTP/ MPP^+-induced glial response

Treatment with 0.25 μM MPP^+ induced a significantly higher number of OX-6-ir cells (i.e. activated microglial cells) than in control cultures. Treatment with MPP^+ + AII induced further increase in the number of OX-6-ir cells, and this increase was blocked by simultaneous treatment with the AT1 antagonist ZD 7155 (Fig. 4a and b). However, we did not observe any significant change in the number of astrocytes (i.e. GFAP-ir cells) in cultures treated with 0.25 μM MPP^+, or MPP^+ + AII, or MPP^+ + AII + ZD 7155 (Fig. 4c). Cultures were treated with the antimitotic agent Ara-C to eliminate glial cells and to study the possible role of glial cells in the increasing effect of AII on DA neuron degeneration. Cultures treated with Ara-C and low doses of MPP^+ (0.25 μM) + AII did not contain significantly lower numbers of cells than control cultures or those treated with low doses of MPP^+ (0.25 μM) alone. This indicates that glial cells play a major role in the effects of AII on DA neuron degeneration (Fig. 4d). Additional treatments (see above) did not induce any significant change in the glial population of cultures treated with Ara-C.

In order to study whether neuroprotection by AT1 antagonists in mice is associated with inhibition of MPTP-induced microglial response, we analyzed the expression of isolectin B4, a marker for activated microglia, and GFAP, a marker for astrocytes in the striatum and VM. The glial response was studied 1 day after a single injection of MPTP (i.e. prior to significant DA neuron death), and 1 week after five-injection MPTP treatment (i.e. when DA neuronal loss is practically complete). Control mice (i.e. saline-injected mice) showed minimal and non-significant glial activation. Twenty-four h after a single MPTP injection, there was intense microglial activation as well as an increased ACE activity in the VM (29.2 ± 1.2 nmol His-Leu/min/mg protein) and striatum (38.9 ± 1.3 nmol His-Leu/min/mg protein). However, only a slight increase in the number of GFAP-ir cells was observed, which was statistically significant in the striatum but not in the nigra (Figs 5 and 6; Fig. S3). MPTP-induced microglial activation was, however, significantly lower in mice treated with candesartan + MPTP (Figs 5a,b, and 6a-f). One week after the five-injection MPTP treatment (i.e. 2 weeks after the first injection), the microglial activation had notably decreased but it was still higher than in controls or candesartan-treated mice (Fig. 5a,b; Fig. S3). However, 1 week after the MPTP treatment there was a marked increase in reactive astroglia (i.e. GFAP-ir) in animals treated with MPTP or MPTP and candesartan, although significantly less in the candesartan-treated group (Figs 5c, d and 6g–l).

Role of PKC and NADPH complex activation in the AII-induced effects

NADPH-oxidase complex is composed of membrane-bound subunits and cytosolic subunits, and the translocation of cytosolic subunits to the membrane leads to NADPH activation and ROS generation. In mesencephalic cultures, intense NADPH activity was detected by luminescence (103.3 ± 3.4 RLU/min/μg protein). Furthermore, immunohistochemical studies revealed immunoreactivity for major membrane ($gp91^{phox}$) and cytosolic ($p47^{phox}$) NADPH-oxidase subunits. The expression of $gp91^{phox}$ and $p47^{phox}$

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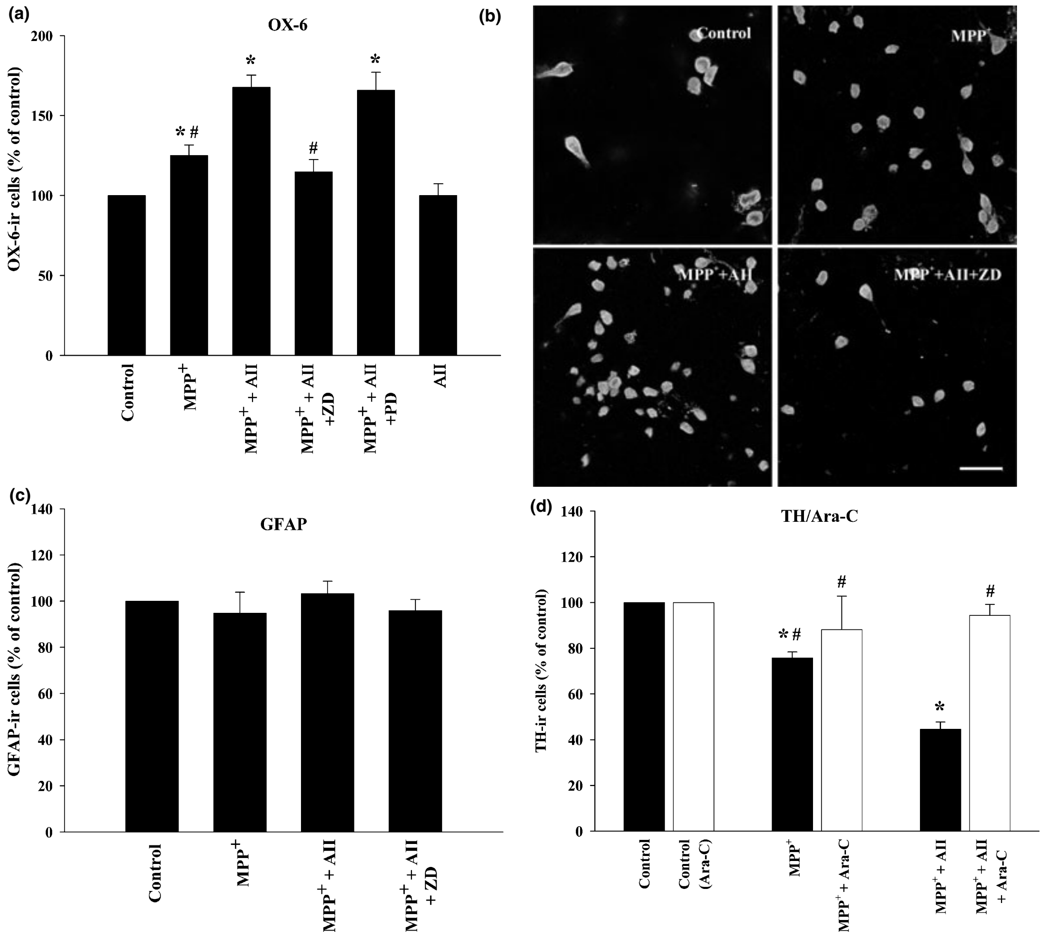

Fig. 4 Role of AII in MPP⁺-induced microglial (OX-6-ir; a, b) and astroglial (c) activation in cultures, and role of glial cells in the AII-induced decrease in the number of TH-ir surviving neurons (d). (a, b) 0.25 μM MPP⁺ induced a significant increase in microglial activation, which was increased by AII and inhibited by ZD 7155 but was not affected by PD 123319. No significant increase in microglial activation was observed after treatment with AII alone. (c) No significant change in GFAP-ir cells was induced by the same treatments. (d) Cultures treated with Ara-C and MPP⁺ + AII did not contain significantly lower numbers of cells than those treated with MPP⁺ alone, or control cultures. The data are expressed as percentages of the number of cells obtained in the respective control cultures (100%). Data represent mean ± SEM. $^{*}p < 0.05$ compared with control group (untreated cells); $^{\#}p < 0.05$ compared with group treated with MPP⁺ + AII (one-way ANOVA and Bonferroni *post hoc* test). AII, angiotensin II; TH, tyrosine hydroxylase. Scale bar: 50 μm.

mRNA in the mesencephalic cultures was confirmed by real time RT-PCR. Real time RT-PCR and luminescence analysis also revealed that treatment with AII significantly increased mRNA levels of gp91^{phox} and p47^{phox} and NADPH activity, respectively, in comparison with controls (Fig. 7a). The increasing effect of AII on mRNA levels of NADPH subunits was blocked by treatment with the AT1 receptor antagonist ZD 7155, which indicates that AII induces activation of the NADPH complex via AT1 receptors in the cultures. AII-induced increase in NADPH subunits and NADPH activity was also blocked by treatment with the selective PKC inhibitor chelerythrine, which indicates that AT1 receptors act via PKC activation in the induction and translocation of cytosolic subunits to the membrane and activation of NADPH in the present model (Fig. 7a).

The possible involvement of NADPH complex activation in AII-induced increase in MPP$^+$ toxicity was also studied by treatment of cultures with the NADPH inhibitor apocynin.

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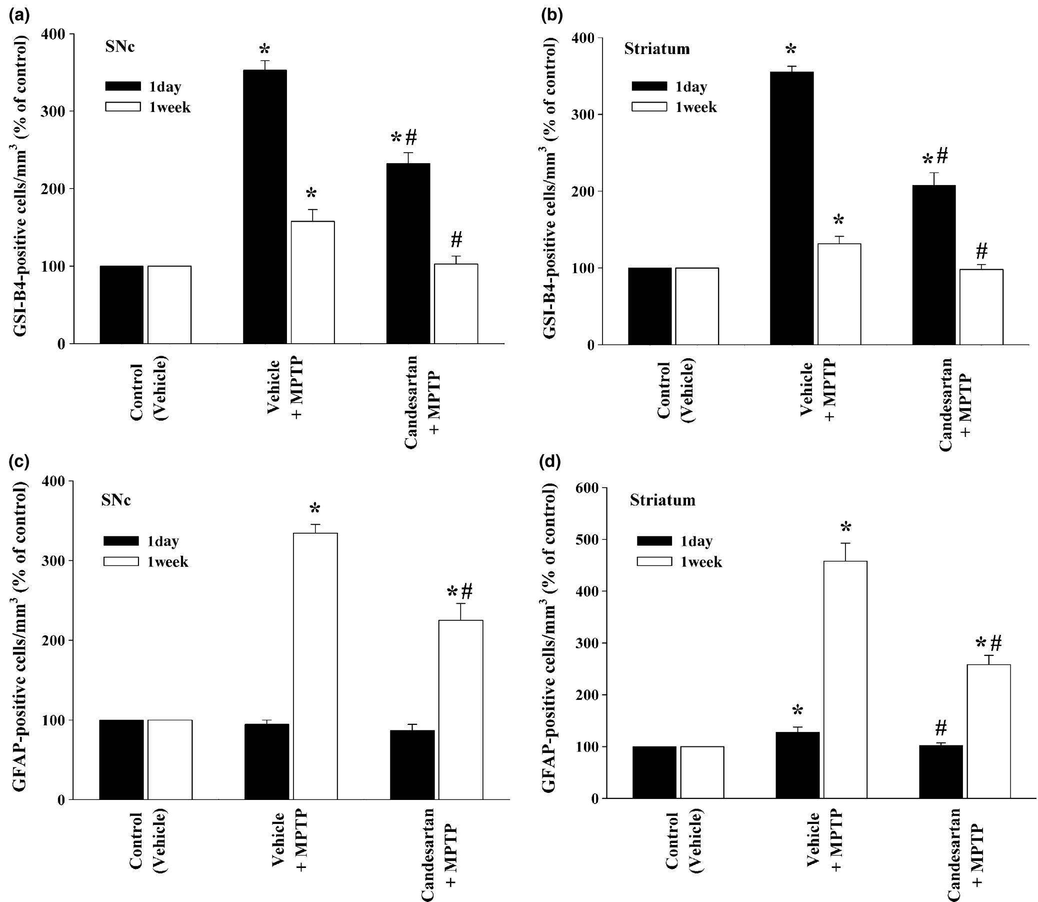

Fig. 5 Activated microglial (a and b) or astroglial (c and d) cells in the substantia nigra compacta (SNc; a and c) or striatum (ST; b and d) 1 day or 1 week after treatment with vehicle, MPTP + vehicle, or MPTP + candesartan. The microglial and astroglial cells were quantified as the number cells per mm^3, and the data are expressed as percentages of the number of cells obtained in the respective controls (100%). Data represent mean ± SEM. *$p < 0.05$ compared with control group, #$p < 0.05$ compared with group treated with MPTP + vehicle (one-way ANOVA and Bonferroni *post hoc* test).

The loss of TH-ir neurons induced by low doses of MPP^+ (0.25 μM) increased significantly after simultaneous treatment with AII. However, treatment with apocynin (0.25 mM) blocked the loss of TH-ir neurons induced by AII (Fig. 7b).

In mice, the expression of $gp91^{phox}$ and $p47^{phox}$ mRNA in the VM and the striatum was confirmed by real time RT-PCR, and NADPH activity determined in VM (34.4 ± 2.1 RLU/min/μg protein) and striatum (45.5 ± 1.5 RLU/min/μg protein) by luminescence. There was a significant increase in NADPH activity and mRNA levels of $gp91^{phox}$ and $p47^{phox}$ in mice treated with MPTP relative to control mice treated with saline. However, mice treated with candesartan + MPTP showed a significant decrease in NADPH activity and mRNA levels of $gp91^{phox}$ and $p47^{phox}$ relative to mice treated with MPTP alone (Fig. 7c and d).

Discussion

Brain RAS has been usually involved in the regulation of cardiovascular and fluid-electrolyte homeostasis. AII is involved in modulation of neurotransmitters such as norepinephrine, serotonin and DA in the basal ganglia, and interactions between RAS and DA are particularly interesting. (Mendelsohn *et al.* 1993; Brown *et al.* 1996). However, AII, via AT1 receptors, has also been shown to be one of the most important inflammation and OS inducers in several

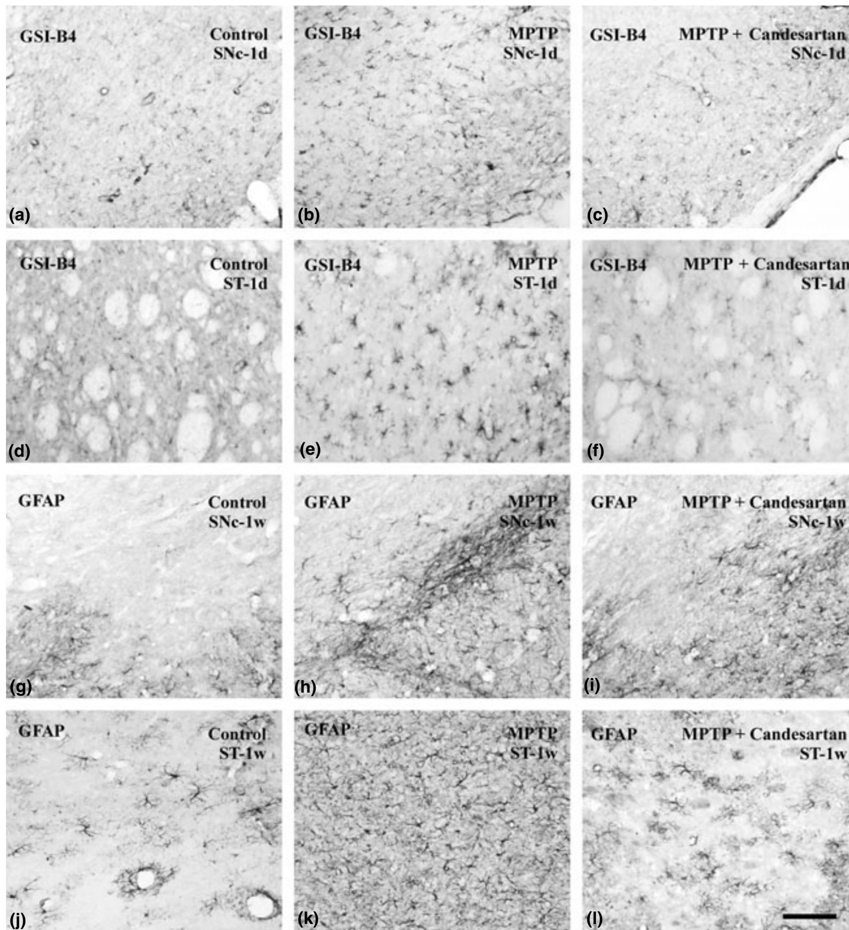

Fig. 6. Photomicrographs showing changes in microglial (a–f) and astroglial (g–l) activation 1 day (a–f) and 1 week (g–l) after treatment at central levels of the substantia nigra (SNc; a–c, g–i) and striatum (d–f, j–l) in mice treated with vehicle (i.e. controls; a, d, g, and j), with MPTP alone (b, e, h, and k), or with MPTP + candesartan (c, f, i, and l). One day post-treatment, the microglial activation was lower in the treated group (c, f) than in the mice that did not receive candesartan (b, e). One week after treatment, the astroglial activation was lower in the treated group (i, l) than in the mice that did not receive candesartan (h, k) (see also Fig. S3). Scale bar: 100 μm.

tissues (Seshiah *et al.* 2002; Touyz *et al.* 2002; Cai *et al.* 2003), and produces ROS by activation of the NADPH-oxidase complex. AII may normally be involved in the modulation of DA neurotransmission, and enhance the microglial/inflammatory response after an initial DA lesion induced by neurotoxins such as MPTP in the present experiments or by other factors in PD. It is possible that impaired interaction between DA and AII plays a role in the progression of degenerative processes, as recently reported in other tissues (Bek *et al.* 2006). In accordance with this, we have previously shown that ACE inhibitors decrease MPTP-induced DA cell death and OS (Muñoz *et al.* 2006), and Grammatopoulos *et al.* (2007) have observed that the AT1 antagonist losartan protects against MPTP toxicity. However, the mechanism involved in this effect had not previously been studied in the MPTP model of PD. Furthermore, losartan only reduced MPP^+ toxicity in the presence of exogenous AII, and a protective role for AII was suggested. These discrepancies may be related to methodological differences, since Grammatopoulos *et al.* (2007) used much higher doses of MPP^+ (5–10 μM) than in the present study.

The results of the present study indicate that the DA cell loss induced by the neurotoxin MPTP/MPP^+ is amplified by AII and inhibited *in vivo* and *in vitro* by AT1 receptor

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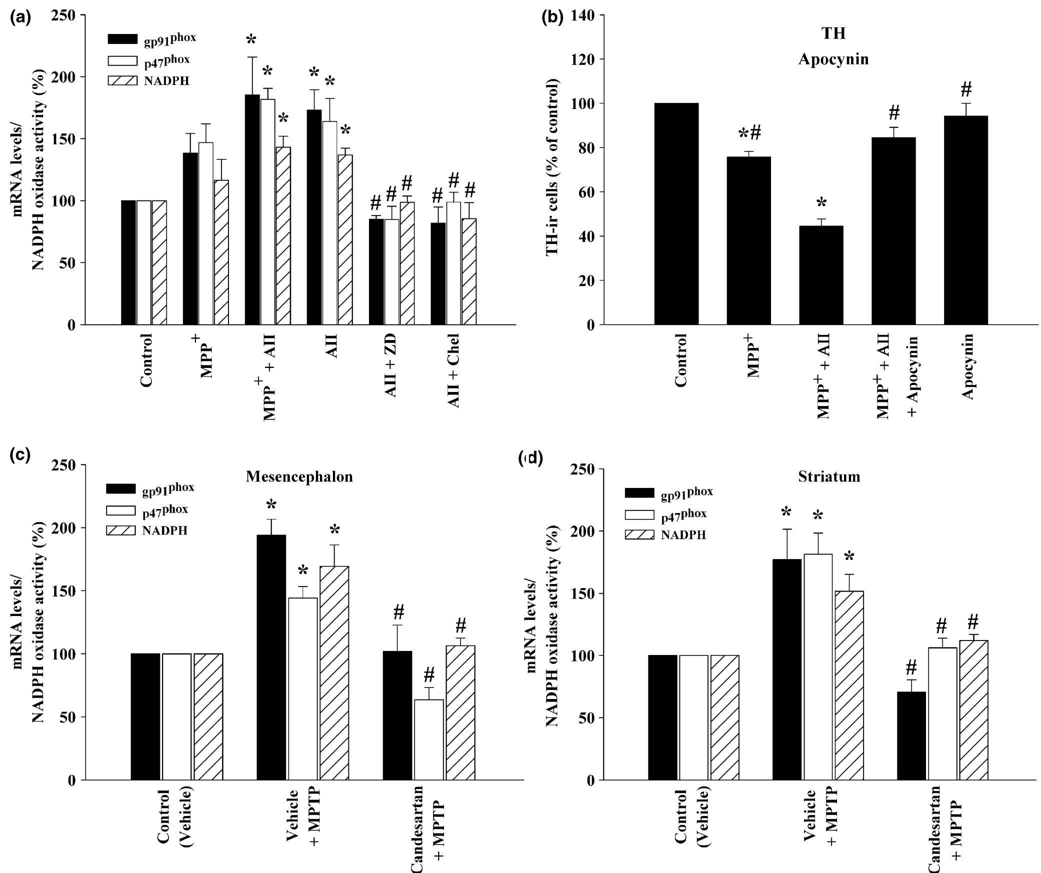

Fig. 7 Expression of NADPH subunit mRNA and NADPH-oxidase activity revealed by luminescence in mesencephalic cultures (a) and mice ventral mesencephalon (c) and striatum (d), and TH-ir neurons in mesencephalic cultures subjected to several treatments (b). (a) In cultures, a significant increase in mRNA levels and NADPH oxidase activity was observed 12 h after treatment with MPP^+ + AII or AII alone, which was inhibited by ZD 7155 or the selective PKC inhibitor chelerythrine. Treatment with low doses of MPP^+ alone (0.25 μM) did not induce statistically significant increases. (b) Treatment with the NADPH inhibitor apocynin (0.25 mM) blocked the increase in loss of TH-ir neurons induced by AII treatment. (c and d) A significant increase in mRNA levels and NADPH-oxidase activity was observed in mice 24 h after a single MPTP injection, which was blocked by candesartan. Data are expressed as percentages of the respective controls (100%), and represent mean ± SEM. $^*p < 0.05$ compared with control group (untreated cells), $^{\#}p < 0.05$ compared with group treated with MPP^+ + AII (a and b) or MPTP (c and d) (one-way ANOVA and Bonferroni *post hoc* test). AII, angiotensin II.

antagonists, and that PKC, NADPH-complex, and microglial activation are involved in this effect. Furthermore, in mice and cultures not treated with exogenous AII, AT1 receptor antagonists inhibited the increasing effect of endogenous AII on DA degeneration. In mice, AT1 receptor antagonists inhibited both early (i.e. prior to significant DA neuronal loss) microglial activation and NADPH activation, which are considered to enhance DA cell death. A decreased microglial reaction 1 week after MPTP treatment may be considered as a consequence rather than a cause of the decreased DA neuron death. Although DA neurons, microglial cells and astrocytes bear AT1 and AT2 receptors, elimination of glial cells from mesencephalic cultures inhibited the amplification effect of AII on MPP^+-induced DA cell death, suggesting that microglial activation plays a major role in the effects of AII. Intraneuronal ROS, derived from those produced extracellularly by microglial NADPH activation may act synergistically with MPP^+ within the DA neurons to induce cell death (Gao *et al.* 2003a,b; Wu *et al.* 2003). The results of the present study support this synergistic view, because

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AII-mediated microglial activation was particularly evident when cells were treated with low doses of MPP^+, and the effects of AII administration or AT1 antagonists on DA neuron death were not significant when cultures were treated with doses of MPP^+ that were too high (i.e. inducing inhibition of complex I sufficient for killing neurons by itself) or too low (i.e. inducing no significant neuronal damage).

In accordance with the above mentioned synergistic effect, AII alone (i.e. without MPP^+) did not induce any significant DA neuron death. Furthermore, the MPP^+-induced neuronal damage was also necessary to trigger the microglial response, which was amplified by AII. As a major activator of the NADPH complex, AII alone induced a significant increase in the expression of NADPH subunits and NADPH activity. However, it has been shown that the NADPH-ROS pathway is essential but not sufficient for the induction of the microglial inflammatory response (see Qin *et al.* 2004). It is known that intact neurons reduce inductibility of microglial reaction by cytokines, and damage of neuronal activity by neurotoxins restore the induction of microglial activation (Neumann 2001; Biber *et al.* 2007).

Possible indirect effects of AT1 antagonists on MPTP neurotoxicity might be due to reduction of the bioactivation of MPTP to MPP^+, or reduction of MPP^+ accumulation in DA neurons by reducing dopamine transporter activity, or indirect effects through the brain blood flow. However, there were no changes in striatal levels of MPP^+ in mice, and deleterious effects of AII were also observed after direct MPP^+ administration in cultures. Furthermore, AII, or AT1 antagonists, or apocynin did not induce significant changes in dopamine transporter activity, and glial cells are necessary to observe the enhancing effects of AII on MPP^+-induced DA neuron death, which excludes the possibility that AII acts by increasing accumulation of MPP^+ in the DA neurons. The results were confirmed with two different AT1 antagonists, which further confirms that the effects observed are due to the AT1 receptor inhibition and are not due to any unknown or non-specific effect of any particular compound. Interestingly, the results observed *in vivo* were confirmed *in vitro*, which excludes possible indirect effects due to changes in the brain blood flow after RAS inhibition. Finally, recent studies have suggested that MPTP may disrupt the blood brain barrier as a consequence of MPTP-induced microglial activation (i.e. neuroinflammation; Zhao *et al.* 2007). This may potentially allow entry of additional circulating AII and further increase neuroinflammation. The present results show, however, that the local RAS plays a major role at least in the initiation of the inflammatory process, as previously observed in other tissues (Touyz *et al.* 2002; Suzuki *et al.* 2003).

The results of the present study suggest that AII, via AT1 receptors, PKC and NADPH-derived ROS, increases DA cell death by mechanisms similar to those previously observed in other degenerative processes mediated by inflammation and OS, such as atherosclerosis, glomerulosclerosis, and cardiac hypertrophy (Ferrari *et al.* 2002). We have observed that both DA neurons and microglial cells bear AT1 receptors and NADPH complex. AII may increase levels of OS in DA neurons by generating low levels of ROS via neuronal AT1 receptors and neuronal PKC and NADPH complex (Wang *et al.* 2004, 2006), and act synergistically with intraneuronal MPP^+-derived ROS to increase the neurotoxic effect on DA neurons. However, the present results reveal that glial cells play a major role in this effect, as elimination of glial cells with Ara-C inhibited the increasing effect of AII on the DA lesion. Microglia are the resident immune cells in the brain, and several previous studies have shown that microglia but not astroglia significantly enhance the progression of DA degeneration (see Gao *et al.* 2003a). It appears that the astroglial reaction observed *in vivo* is secondary to the degeneration of DA neurons, and may be related to removal of degenerating neurons or other functions (Wu *et al.* 2002, 2003; Gao *et al.* 2003a). In accordance with this, astroglial activation was weak at early stages, and the strongest GFAP reaction was observed at later stages after the neurotoxin treatment in the present and other experiments (Liberatore *et al.* 1999; Wu *et al.* 2002). However, the role of astrocytes in neuroinflammation has not been totally clarified (Neumann 2001; Biber *et al.* 2007), and may also be involved in modulation or prevention of excessive microglial response and it deleterious effects (Min *et al.* 2006).

There is growing body of evidence indicating that an inflammatory process characterized by activation of resident microglia plays a major role in the initiation or at least the progression of PD, which appears to progress via a self-propelling pathway. The present results indicate that the brain RAS may play a key role in this mechanism. Manipulation of the brain RAS may provide more neuroprotective benefit for PD than antioxidants that merely scavenge already formed ROS, as previously reported for vascular diseases. Interactions between RAS and DA in the normal and parkinsonian brain require additional studies.

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Supporting information

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

Supplementary data.

Fig. S1 Double immunofluorescence for OX42 or TH (green) and AT1 receptors, or $gp91^{phox}$ or $p47^{phox}$ NADPH subunits (red) in

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primary mesencephalic cultures. AT1 receptors and gp91^{phox} and p47^{phox} subunits show co-localization (yellow) with the microglial marker OX42 (A–I). The dopaminergic marker TH (J–L) shows co-localization (yellow) with AT1 receptors. AII, angiotensin II; AT1, angiotensin type 1 receptor; TH, tyrosine hydroxylase. Scale bar: 50 μm (A–I), 75 μm (J–L).

Fig. S2. Photomicrographs of representative TH-ir cells from different in vitro experimental groups: a control culture (A) and a culture treated with a low dose of MPP$^+$ (0.25 mM; B), MPP$^+$ (0.25 mM) + angiotensin (AII, 100 nM; C), or MPP$^+$ (025 mM) + AII + ZD 7155 (D). AII induced a significant increase in MPP$^+$ neurotoxicity. This increase was blocked by treatment with the AT1 receptor antagonist ZD 7155. AII, angiotensin II; ZD, ZD 7155. Scale bar: 100 μm.

Fig. S3. Photomicrographs showing changes in microglial (A–F) and astroglial (G–L) activation one week (A–F) and one day (G–L) after treatment at central levels of the substantia nigra (SNc; A–C, G–I) and striatum (D–F, J–L) in mice treated with vehicle (i.e. controls; A,D,G,J), with MPTP alone (B,E,H,K), or with MPTP + candesartan (C,F,I,L) One week after treatment, the microglial activation was notably decreased, but was still lower in the treated group (C,F) than in the mice that did not receive candesartan (B,E). One day post-treatment, there was no (SNc; G–I) or very low (ST; J–L) level of astrogliosis. Scale bar: 100 μm.

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4.2- Efecto del bloqueo de los receptores de pro-renina/renina *in vitro* sobre la muerte neuronal dopaminérgica

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En la actualidad, se sabe que además del sistema Renina-angiotensina (SRA) clásico existen SRA locales en diferentes tejidos entre los que se incluyen el cerebro, riñón, glándula adrenal, corazón y vasos sanguíneos. En estos sistemas locales el péptido AII es el principal efector y a través de los receptores AT1 induce inflamación y estrés oxidativo, jugando así un papel importante en el desarrollo y progreso de enfermedades relacionadas con la edad y/o envejecimiento. El empleo de bloqueantes de los receptores de AII, principalmente de AT1, podría servir como terapia neuroprotectora ya que retrasa los efectos deletéreos sobre las neuronas dopaminérgicas, aunque no los frena. Recientemente se ha identificado un receptor específico al que se unen tanto renina como su precursor pro-renina y fue denominado receptor de pro-renina/ renina (PRR). En estudios anteriores hemos demostrado que la pérdida de neuronas dopaminérgicas inducida por el uso de diferentes neurotoxinas era inhibido mediante el bloqueo de los receptores de AII. Además, recientemente en nuestro laboratorio hemos descrito mediante el uso de técnicas de inmunofluorescencia doble, tanto en sustancia negra de *Macaca fascicularis* como en cultivos primarios mesencefálicos de rata, la presencia del receptor PRR tanto en neuronas (incluyendo las neuronas dopaminérgicas) como en células microgliales, pero no en astrocitos. En este trabajo nos hemos centrado en el estudio de los efectos funcionales del receptor PRR en el cerebro, incluyendo el sistema dopaminérgico. Así en los cultivos primarios el uso del antagonista del receptor de PRR, HRP, produce una disminución en la neurodegeneración dopaminérgica inducida por la neurotoxina 6-OHDA; mientras que la adición de renina incrementa la muerte neuronal dopaminérgica inducida por la 6-OHDA. Estos resultados sugieren la existencia de mecanismos intracelulares independientes de AII, que mediante la activación de los receptores PRR contribuirían al progreso de la neurodegeneración dopaminérgica. De este modo, tanto la AII como la

ruta de señalización de PRR podrían servir como dianas para desarrollar una estrategia neuroprotectora para la EP.

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ORIGINAL ARTICLE

Location of Prorenin Receptors in Primate Substantia Nigra: Effects on Dopaminergic Cell Death

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Abstract

Angiotensin II acts via angiotensin type 1 receptors and is a major inducer of inflammation and oxidative stress. Local renin-angiotensin systems play a major role in the development of age-related disorders in several tissues. These processes are delayed, but not totally abolished, by blockade of angiotensin signaling. A specific receptor for renin and its precursor prorenin has recently been identified. We previously showed that neurotoxin-induced dopaminergic (DA) cell loss is decreased by inhibition of angiotensin receptors, but the location and functional effects of prorenin receptor (PRR) in the brain, including the DA system, are unknown. In the substantia nigra of *Macaca fascicularis* and in rat primary mesencephalic cultures, double immunofluorescence analysis revealed PRR immunoreactivity in neurons (including DA neurons) and microglia, but not in astrocytes. Administration of the PRR blocker, *handle region peptide*, led to a significant decrease in 6-hydroxydopamine-induced DA cell death in the cultures, whereas administration of renin with simultaneous blockade of angiotensin receptors led to an increase in 6-hydroxydopamine–induced cell death. These results suggest that active agent angiotensin II–independent PRR intracellular signaling may contribute to exacerbation of DA cell death in vivo. Therefore, potential neuroprotective strategies for DA neurons in Parkinson disease should address both angiotensin and PRR signaling.

Key Words: Angiotensin, Dopamine, Microglia, Monkey, Neurodegeneration, Parkinson disease, Renin.

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INTRODUCTION

In addition to the classical humoral renin angiotensin system (RAS), many tissues have a local RAS. The active agent angiotensin II (AII), which acts via angiotensin type 1 receptors (AT1), is one of the most important inducers of inflammation and oxidative stress (1, 2) and has a major role in the initiation and progression of cardiovascular disease and other age-related disorders (3). Moreover, disruption of the AT1 receptor promotes longevity in mice by limiting oxidative damage in several peripheral tissues (4). A local RAS has been described in the brain (5, 6), including in the basal ganglia and the dopaminergic (DA) system (7, 8). The brain RAS has been implicated in neurodegeneration (9, 10), and we have shown that DA cell loss induced by DA neurotoxins is exacerbated by AII via AT1 receptors (11–13).

Blockade of AII generation and signaling has been widely used to prevent progression of organ damage in cardiovascular and renal diseases. It has been reported, however, that progression of the disease is delayed, but not totally abolished, and that inhibition of AII is not sufficient to block total RAS activity (14, 15). The recent discovery of prorenin receptor (PRR), a specific receptor for renin and its precursor prorenin, that exerts both AII-dependent and AII-independent molecular functions may elucidate some aspects of the function of tissue RAS (16, 17). The AII-dependent actions of the receptor are as follows. The binding of renin to PRR increases the catalytic activity of renin by approximately 4 to 5 times, and binding of prorenin induces catalytic activity similar to that of renin to hydrolyze angiotensinogen into angiotensin. The triggering of its own intracellular signaling cascade to induce effects similar to those demonstrated for AT1 receptors indicates the AII-independent actions of the PRR (18, 19). A peptide called *handle region peptide* (HRP), which mimics part of the prosegment of prorenin, is a potential inhibitor of PRR (20, 21). Previous studies have shown that PRR mRNA is highly expressed in the brain, heart, and placenta, with the highest levels in the brain (16, 17). It is not clear, however, where PRRs are located in the brain or even whether they are located only in neurons or also in glial cells, particularly in primates. In particular, with respect to the potential involvement of the RAS in the progression of Parkinson disease (PD), it is not known whether PRRs occur in the substantia nigra (SN) or if they have functional effects on the progression of DA neuron death. Here, we investigated this question in the monkey SN and primary cultures of rat ventral mesencephalon.

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MATERIALS AND METHODS

Location of PRR in the Substantia Nigra Compacta of Nonhuman Primates

Adult male *Macaca fascicularis* primates (body weight, 3.8–4.5 kg) were used in this study. At all times, the animals were handled in accordance with the European Council Directive 86/609/EEC and with the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research. The experimental design was approved by the Ethical Committee for Animal Testing of the University of Navarra (ref 037/2000). The animals were anesthetized with an overdose of chloral hydrate, perfused transcardially, and the brains were removed and cryoprotected by immersion in a solution containing 20% glycerin and 2% dimethyl sulfoxide in 0.1 phosphate buffer, pH 7.3, for 48 hours. Brains were then cut into 40-μm sections on a freezing microtome, as described (22). Coronal sections through the ventral mesencephalon of 3 different primates were used. Sections of different rostrocaudal levels of the substantia nigra compacta (SNc) were processed by immunohistochemistry or in situ hybridization.

Tissue Immunohistochemistry

The SNc sections were incubated for 1 hour in 5% normal horse serum with 0.25% Triton X-100 in 20 mmol/L potassium PBS (KPBS) containing 4% bovine serum albumin (BSA) and then for 48 hours at 4°C with polyclonal anti-PRR antibodies (Abs) (1:200, see later) in 20 mmol/L KPBS-BSA containing 4% BSA, 1% normal serum, and 0.25% Triton X-100. The sections were then incubated first for 60 minutes with the corresponding biotinylated secondary Ab (1:200; Sigma, St Louis, MO), then for 90 minutes with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Labeling was revealed with 0.04% H_2O_2 and 0.05% 3-3′diaminobenzidine (Sigma) and visualized using a Nikon Optiphot microscope. Only slight adjustments in brightness and contrast were performed with Adobe Photoshop software to produce similar levels in photographs in the same figure. Double immunofluorescence procedures were carried out with anti-PRR and Abs against various markers of neurons and glia. Sections stained with the DNA-binding dye Hoechst 33342 were used to study possible nuclear localization of PRR.

Sections were first preincubated for 1 hour with a blocking solution containing 5% normal serum with 0.25% Triton X-100 in 20 mmol/L KPBS containing 4% BSA. They were then incubated for 48 hours at 4°C with Abs to anti-PRR (1:100, see later) in 20 mmol/L KPBS-BSA containing 4% bovine serum albumin, 1% normal serum, and 0.25% Triton X-100. The sections were rinsed with KPBS and then incubated for 180 minutes with the corresponding secondary Ab conjugated with cyanine 3.18 (1:500; Chemicon, Temecula, CA). After rinsing, sections were incubated with the primary Ab against the second marker mouse monoclonal Ab to neuron nuclei (NeuN) (1:500, overnight [ON]; Chemicon), mouse monoclonal anti–tyrosine hydroxylase ([TH] DA neurons; 1:5000, ON; Sigma), mouse monoclonal anti–glial fibrillary acidic protein ([GFAP] 1:500; Chemicon) (for astrocytes), mouse anti-human CD11b monoclonal Ab (1:50; Chemicon) for 72 hours, or goat anti-human integrin αM polyclonal Ab (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) (both for reactive microglia/macrophages), for 48 hours, all containing 1% normal serum and 0.25% Triton X-100 diluted in KPBS-BSA. The sections were rinsed as before, then incubated for 180 minutes with the corresponding secondary Ab conjugated with fluorescein isothiocyanate ([FITC] 1:100; Chemicon); for CD11b and integrin αM, with biotinylated horse anti-mouse (1:200; Sigma) or biotinylated horse anti-goat (1:200; Vector), respectively, and streptavidin conjugated with Alexa-488 (1:2000; Molecular Probes, Invitrogen, Carlsbad, CA). For Hoechst, sections were stained with Hoechst 33342 (10 μg/mL; Sigma) in KPBS for 10 minutes at room temperature (RT), rinsed in KPBS, and mounted. A preadsorption control experiment for the anti–integrin αM antibody is shown in Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A186.

The sections were visualized with a laser confocal microscope (TCS-SP2; Leica, Heidelberg, Germany). Immunopositive cells were scanned in a series of approximately 0.5- to 1-μm-thick optical sections (1,024 × 1,024 pixels) spaced by 300 nm. Colocalization analysis was performed using a sequential scan method and 2 different laser lines to avoid simultaneous dual excitation and possible overlap. Digital images were processed by using the Leica confocal software. The sections were analyzed separately and then merged to show possible colocalization. In all experiments, the control sections in which the primary Ab was omitted were immunonegative for these markers.

PRR Antibody Specificity

To our knowledge, this is the first time that these Abs have been used in the rat or monkey brain; therefore, the results were confirmed using 2 different Abs: goat polyclonal anti-ATP6IP2 (NB100-1318; Novus Biologicals, Cambridge, UK) and rabbit polyclonal anti-ATP6IP2 (ab40790; Abcam, Cambridge, UK). These Abs were raised against a synthetic peptide representing the C terminus of the human protein (337–350). It has been previously shown that the Abs react with human and also with rat and mouse PRR because of sequence homology. We additionally confirmed the specificity of anti-PRR Abs by preadsorbing the Abs with the corresponding synthetic peptide antigen (ATP6IP2 peptide; Abcam) both in monkey nigral sections (Figs. 1A, B) and primary cultures of rat mesencephalon (Figs. 1C, D).

The specificity of the Abs against PRR was also confirmed by performing Western blot analysis of monkey and rat SN with the ATP6IP2 peptide–preadsorbed Ab. Rat and monkey tissues from the nigral region were homogenized in radioimmunoprecipitation assay buffer containing protease inhibitor cocktail (P8340) and phenylmethylsulfonyl fluoride (P7626), both from Sigma. Homogenates were centrifuged at 14,000 × *g* for 20 minutes, and protein concentrations of supernatants were measured with the bicinchoninic acid protein assay (23225, Pierce, Thermo Scientific; Fremont, CA). Equal amounts of protein were separated by 5% to 10% Bis-Tris polyacrylamide gel and transferred to nitrocellulose membrane. The membranes were incubated ON with the anti-PRR Ab (1:600) alone or preadsorbed with the blocking peptide. The HRP-conjugated secondary Ab was Protein A (NA9120V; GE

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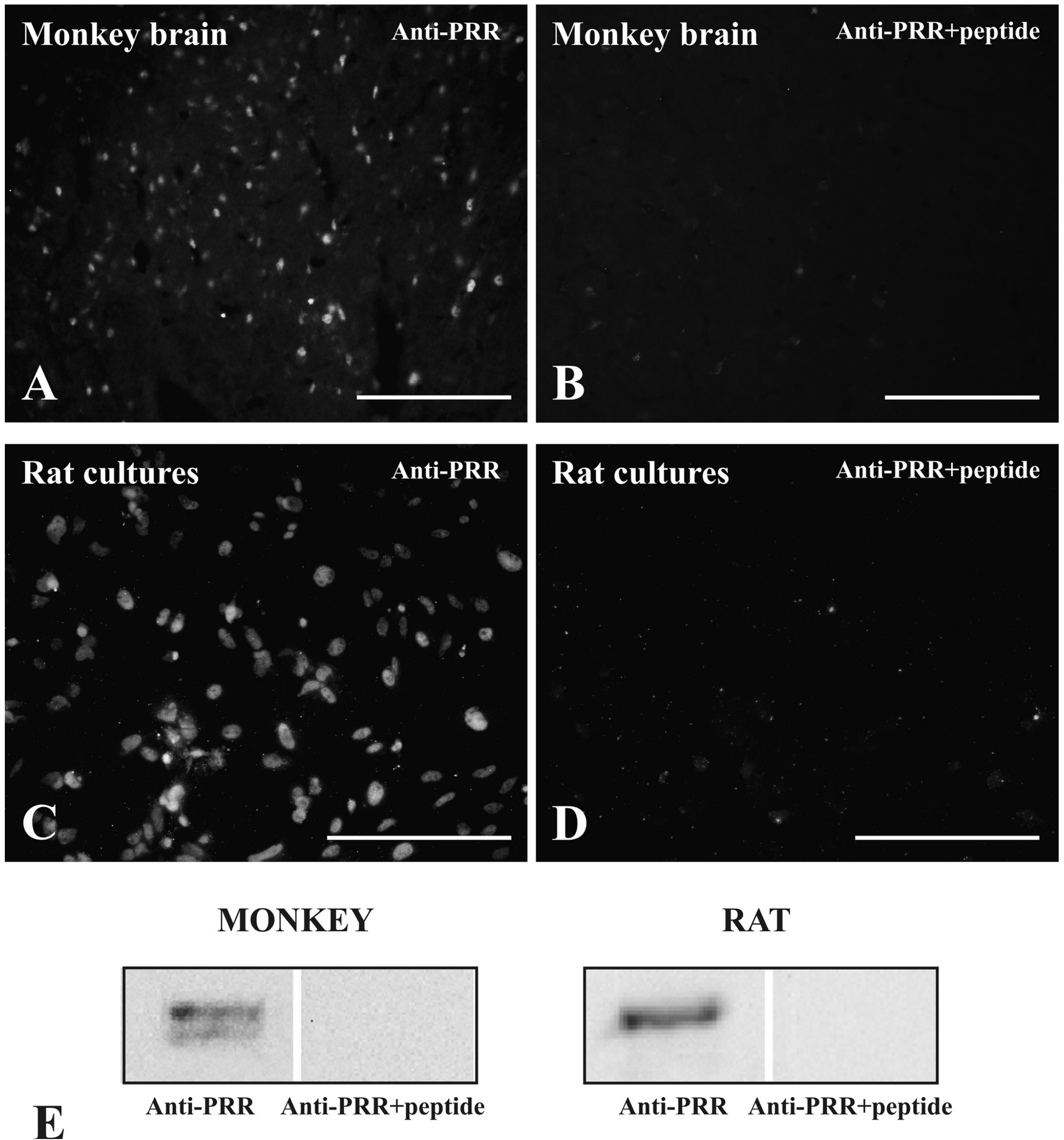

FIGURE 1. Coronal sections through the monkey ventral mesencephalon **(A, B)**, rat primary mesencephalic cultures **(C, D)**, and Western blot analysis (WB) of monkey and rat nigral tissue **(E)**. Sections **(A)**, cultures **(C)**, and WB **(E)** immunostained for prorenin receptors (PRRs) show a large number of immunofluorescent cells **(A, C)** or a clear band of PRR protein **(E)**. Controls subjected to preadsorption with the corresponding synthetic peptide antigen were immunonegative for PRR **(B, D, E)**. Scale bars = 250 μm.

Healthcare). For preabsorption, the anti-PRR Ab was incubated with an excess of blocking peptide ON at 4°C. Immunoreactivity was detected with an Immun-Star HRP chemiluminescent kit (170–5044; BioRad, Hercules, CA) and imaged with a chemiluminescence detection system (Molecular Imager ChemiDoc XRS System, BioRad) (Fig. 1E).

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Synthesis of the PRR cDNA

Total RNA was isolated from a *M. fascicularis* primate kidney sample because of the high prorenin content in this organ. The kidney sample was disrupted in 1 mL Trizol reagent (Invitrogen) with a homogenizer. The homogenized sample was incubated for 5 minutes at RT, and 0.2 mL chloroform was added. The sample was mixed vigorously; then centrifuged at 4°C (12,000 × *g* for 15 minutes). The supernatant was transferred to a new tube, and 0.5 mL isopropanol was added; the mixture was then incubated for 10 minutes at RT. The RNA pellet was obtained by centrifugation (12,000 × *g* for 10 minutes at 4°C). The pellet was washed with 1 mL 75% ethanol and dissolved in 30 μL of diethylpyrocarbonate (DEPC) (Sigma)-treated water after evaporation of ethanol. Absorbance at 260 nm was determined to quantify the amount of total RNA, which was preserved at −80°C. First-strand cDNA was synthesized from the total RNA extracted, and 0.5 μg of total RNA was subjected to polymerase chain reaction by adding Superscript III reverse transcriptase (Invitrogen) (1 μL, 200 U/μL), oligo-(dT) (1 μL, 50 μmol/L, Invitrogen), buffer (4 μL, 5× first-strand buffer: 200 mmol/L Tris-HCl, 500 mmol/L KCl, 50 mmol/L MgCl2; Invitrogen), dithiothreitol (1 μL, 0.1 mol/L), and mixed dNTP (1 μL, 10 mmol/L; Invitrogen) in a total volume of 20 μL, achieved by adding DEPC-treated water.

Probes and Oligonucleotide Primers

Oligonucleotide primers were designed with primer 3 Input 0.4.0 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), which is specific for human PRR: forward primer 5′-GGAACAATGAAGTTGACCTGCT-3′ and reverse primer 5′-CCATTCGAATCTTCTGGTTTG-3′. Template cDNA sequences of human PRR were obtained from GenBank (http://ncbi.nlm.nih.gov/). The PCR parameters were performed with Pfx polymerase (Invitrogen), followed by 35 cycles of amplification (denaturation at 95°C for 1 minute, annealing at 58°C for 30 seconds, extension at 68°C for 1 minute), and a final extension at 68°C for 10 minutes. The PCR product (525 nucleotides in length) corresponding to PRR cDNA sequence from *M. fascicularis* kidney was analyzed by electrophoresis on a 0.8% agarose gel containing SYBR Safe DNA gel stain (Invitrogen) under ultraviolet light. The cDNA fragment was purified with a Montage PCR centrifugal filter device (Millipore, Bedford, MA), according to the manufacturer's protocol. The gel extraction method was used to purify the cDNA sample with a QIAquick gel extraction kit-50 (Qiagen GmbH, Hilden, Germany). The PCR product was later inserted into the plasmid vector (pCR-Blunt II-TOPO; Invitrogen) and used to transform competent *Escherichia coli* cells (Invitrogen). The product from the minipreparation was then sequenced (3130XL Genetic Analyzer, Applied Biosystems by Life Technologies Corporation, Invitrogen). Computer-assisted homology searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the PRR cDNA sequence had 98% homology with human PRR ATP6AP2 (accession number NM_005765) and PRR receptor (accession number AF291814). Finally, the cDNA sequence displayed 100% homology with *M. fascicularis* PRR sequence (accession number AB169744).

Riboprobe Preparation

Sense and antisense riboprobes for *M. fascicularis* PRR were transcribed from the Zero Blunt TOPO PCR cloning kit plasmid. The plasmid was linearized, and the sense or antisense probes were transcribed with the appropriate RNA polymerases (Boehringer Mannheim, Germany). The transcription mixture included 1-μg template plasmid, 1 mmol/L each of adenosine-5′-triphosphate (ATP), cytidine-5′-triphosphate (CTP), guanosine-5′-triphosphate (GTP), 0.7 mmol/L uridine-5′-triphosphate (UTP), and 0.3 mmol/L Digoxigenin-UTP (Roche Diagnostics, Basel, Switzerland), 10 mmol/L dithiothreitol, 50 U RNase inhibitor (Roche), and 1 U of either T7 or SP6 RNA polymerase (Boehringer Mannheim) in a volume of 50 μL. After 2 hours at 37°C, the template plasmid was digested with 2 U RNase-free DNAse (Roche) for 30 minutes at 37°C. The sense and antisense riboprobes were then precipitated by the addition of 100 μL of 4 mol/L ammonium acetate and 500 μL ethanol and recovered by centrifugation at 4°C for 30 minutes. The quality of the synthesis was monitored by dot blot.

Colorimetric In Situ Hybridization

Single in situ hybridization was carried out with free-floating sections that were incubated 2× in 0.1% DEPC in phosphate buffer for 15 minutes and then pre-equilibrated for 10 minutes in 5× saline-sodium citrate ([SSC] 0.75 mol/L NaCl, 0.0075 mol/L Na citrate). The sections were then prehybridized at 58°C for 2 hours in a hybridization solution containing 50% deionized formamide (Sigma) 5× SSC, and 40 μg/μL of denatured salmon DNA (Sigma) in H_2O DEPC. Sense and antisense digoxigenin-labeled probes were subsequently denatured for 5 minutes at 80°C, then added to the hybridization mixture at 400 ng/mL, and the sections were hybridized in this solution for 16 hours at 58°C. Posthybridization washes were carried out in 2× SSC at RT for 30 minutes, 2× SSC for 1 hour at 65°C, and then in 0.1× SSC for 1 hour at 65°C. The digoxigenin-labeled probes were visualized by first pre-equilibrating the sections in TN buffer (0.1 mol/L Tris HCl pH 7.5, 0.15 mol/L NaCl) before incubating them for 90 minutes at RT with an alkaline phosphatase–conjugated anti-digoxigenin Ab raised in sheep (1:1500 in 0.5% TN-blocking reagent; Roche). The sections were rinsed several times in TN buffer then equilibrated for 5 minutes in TNM buffer (0.1 mol/L Tris.HCl, 0.1 mol/L NaCl, 0.05 mol/L MgCl2, pH 9.5) and incubated for 7 hours in the substrate solution (0.02% of nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt in TNM buffer). Staining was stopped by repeatedly washing the sections in a rinsing solution (0.01 mol/L Tris HCl and 0.001 mol/L EDTA, pH 8). The sections were then mounted on slides, air-dried, dehydrated in ethanol, cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany).

Primary Mesencephalic Cultures

Cell suspensions were obtained from ventral mesencephalon of rat embryos of 14 days gestation (E14). The tissue was incubated in 0.1% trypsin (Sigma), 0.05% DNase (Sigma), and Dulbecco modified Eagle medium ([DMEM] Invitrogen

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Life Technologies, Paisley, Scotland, UK), for 20 minutes at 37°C, and then washed in DNase/DMEM and mechanically dissociated. The resulting cell suspension was centrifuged at $50 \times g$ for 5 minutes; the supernatant was removed carefully, and the pellet was resuspended in 0.05% DNase/DMEM to the final volume required. The number of viable cells in the suspension was estimated by staining with acridine orange/ethidium bromide. Cells were plated onto 35-mm culture dishes (BD Falcon, BD Biosciences Franklin Lakes, NJ), previously coated with poly-L-lysine (100 μg/mL; Sigma) and laminin (4 μg/mL; Sigma). The cells were seeded at a density of 1.5×10^5 cells/cm^2 and maintained in control conditions

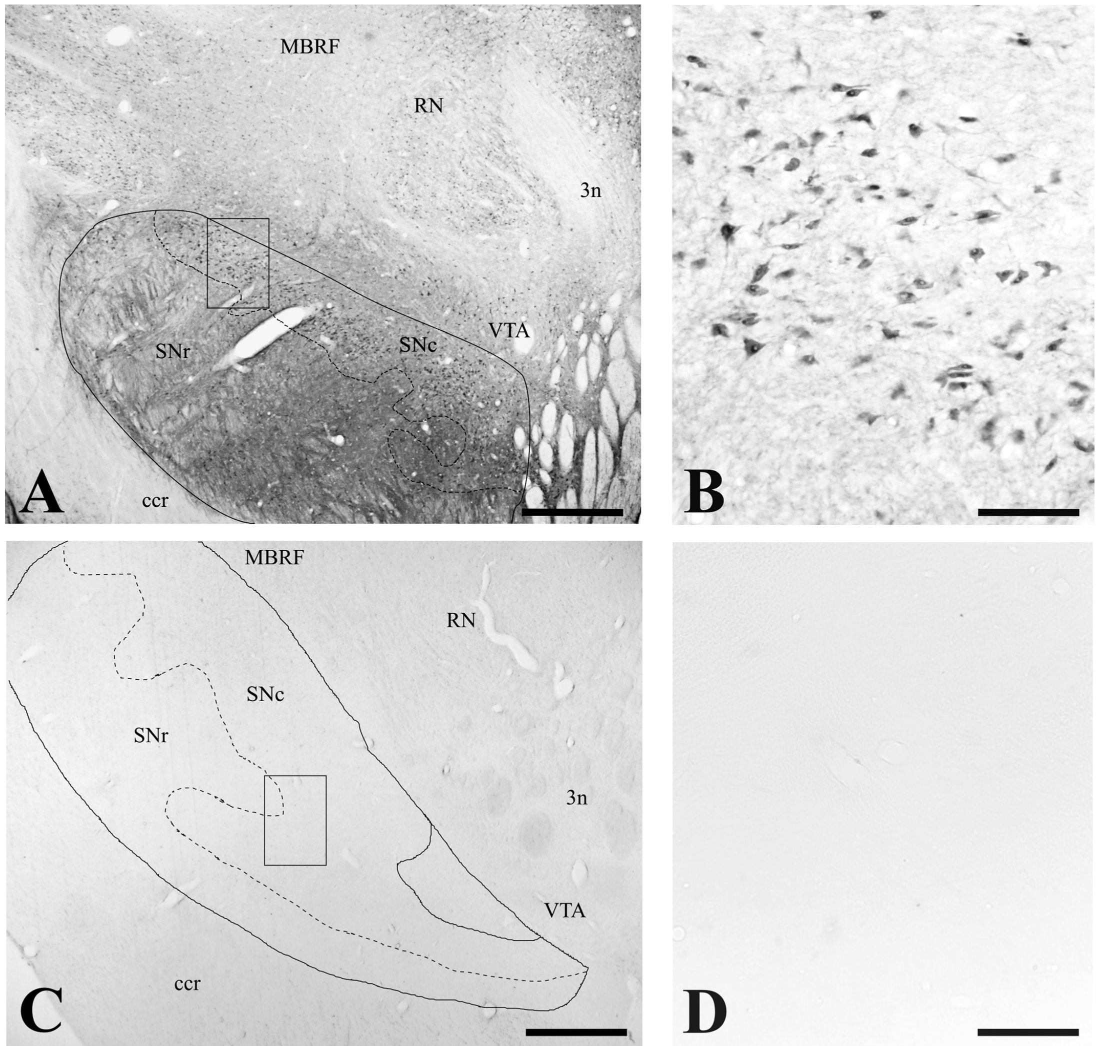

FIGURE 2. Low-power photomicrographs **(A, C)** and higher magnification of boxed areas in **(A)** and **(C)**, respectively, **(B, D)** of coronal sections through the monkey ventral mesencephalon. Sections immunostained for prorenin receptors (PRRs) show a large number of immunoreactive cells in the substantia nigra pars compacta (SNc) **(A, B)**. Control sections, in which the primary antibody was omitted, are immunonegative for PRR **(C, D)**. Ccr, crus cerebri; MBRF, midbrain reticular formation; RN, red nucleus; SNr substantia nigra pars reticulata; 3n, third cranial nerve; VTA, ventral tegmental area. Scale bar = **(A, C)** 1 mm; **(B, D)** 150 μm.

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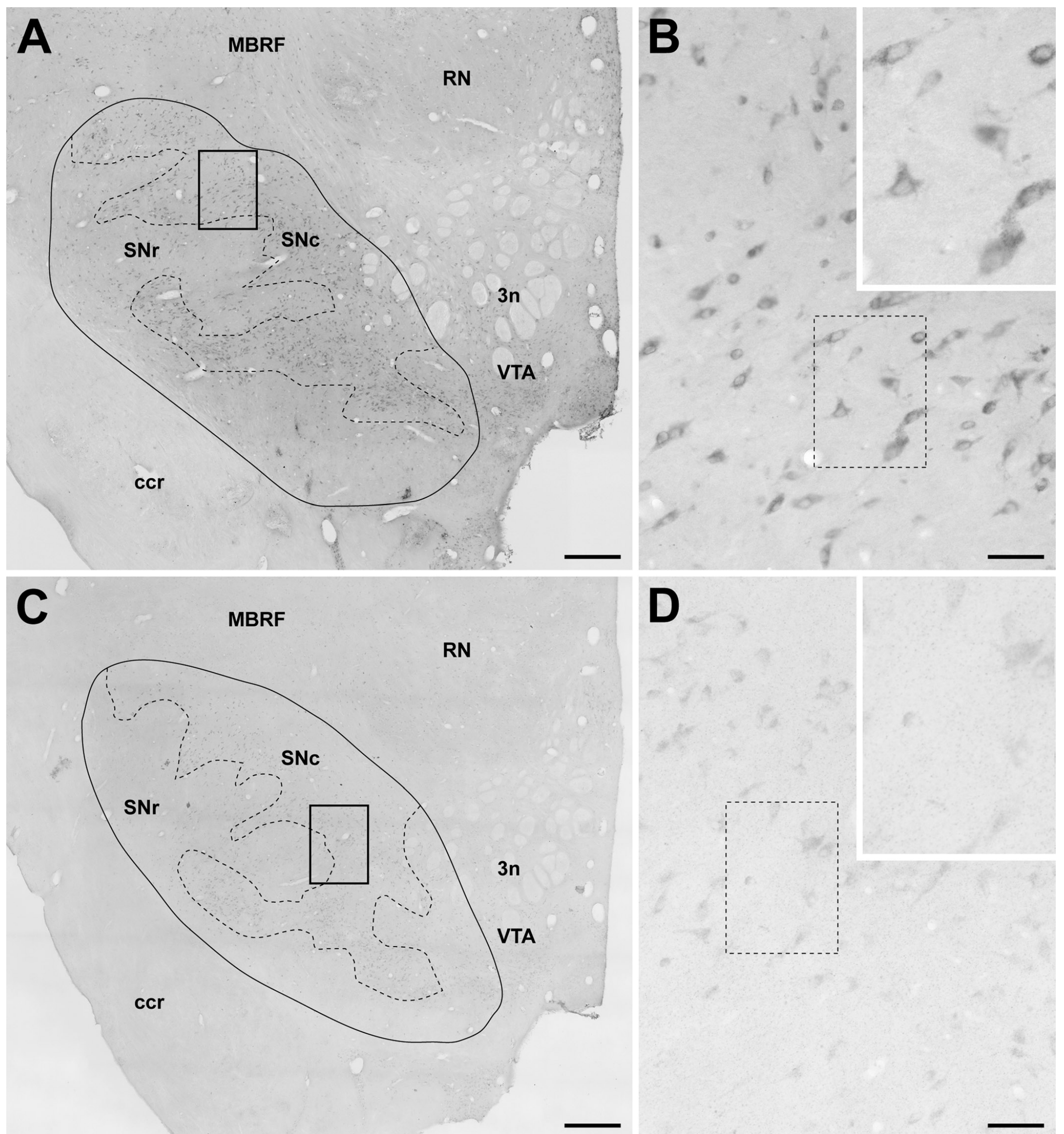

FIGURE 3. Coronal sections through the monkey ventral mesencephalon showing in situ hybridization for the mRNA coding the prorenin receptor (PRR mRNA). **(A, B)** Low-power photomicrograph **(A)** and higher magnification of boxed area in **(A, B)** showing the staining pattern obtained after hybridization with the antisense probe. Expression of PRR mRNA is observed in the substantia nigra pars compacta (SNc), together with weaker expression in the ventral tegmental area (VTA). The pattern of expression is sufficiently intense to delineate the boundaries of SNc from neighboring areas such as the substantia nigra pars reticulata (SNr), the red nucleus (RN), and the midbrain reticular formation (MBRF). Anatomical references such as the fiber bundles giving rise to the third cranial nerve (3n) and the crus cerebri (ccr) are also indicated. **(C, D)** Low-power photomicrograph **(C)** and insets **(D)** show lack of staining and a negligible level of background stain for PRR transcripts when hybridization is performed with the sense riboprobe. Scale bar = **(A, C)** 800 μm; **(B, D)** 80 μm.

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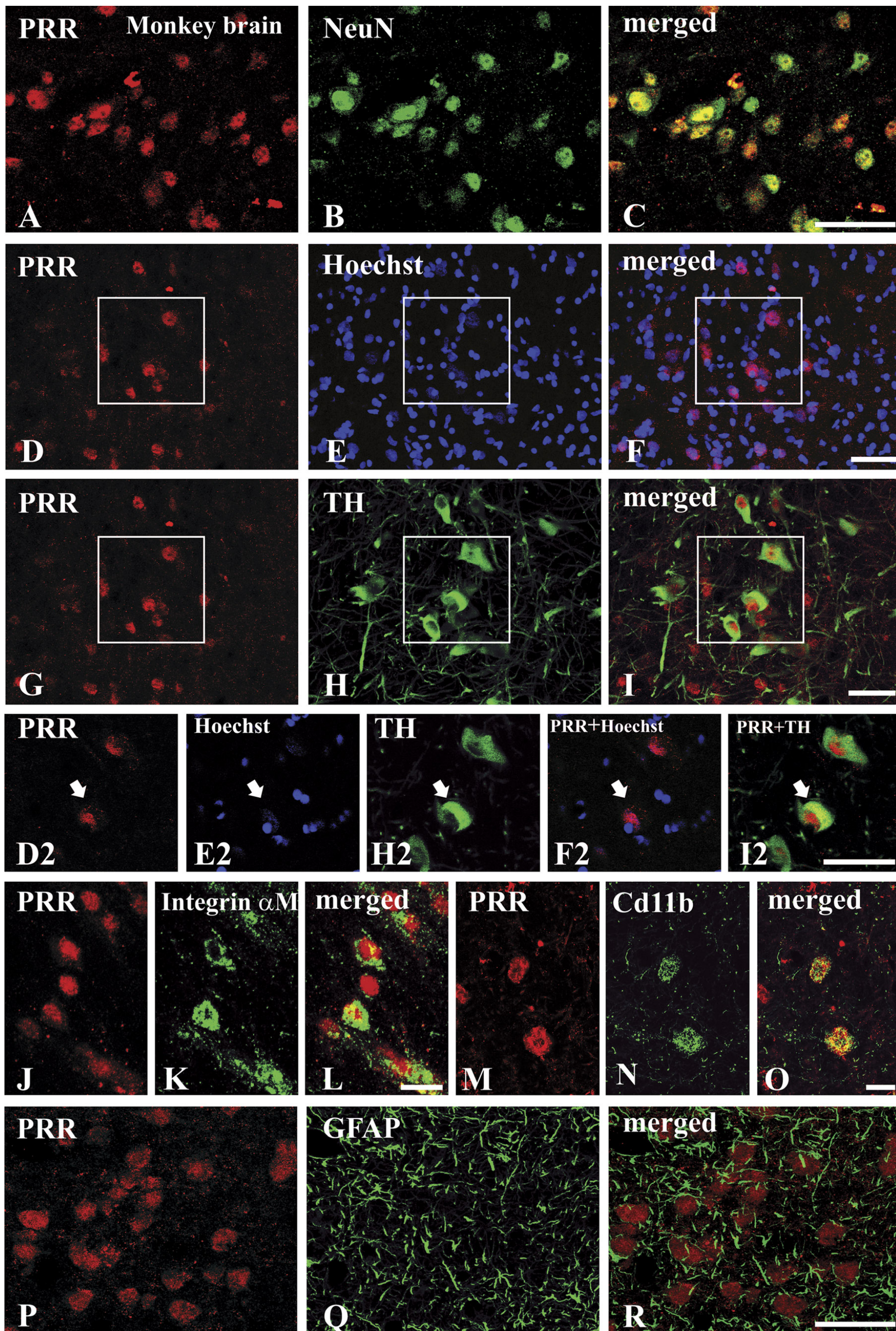

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(DMEM/HAMS F12 [1:1] containing 10% fetal bovine serum [BiochromAG, Berlin, Germany]). The cell cultures were maintained in a humidified carbon dioxide incubator (5% carbon dioxide; 37°C) for 7 days in vitro, and the medium was totally removed on Day 2 and replaced with fresh culture medium.

Cultures grown on glass coverslips were fixed with 4% paraformaldehyde and then processed for double immunofluorescence with the primary anti-PRR Abs (1:100) for 48 hours at 4°C. The coverslips were rinsed with Dulbecco PBS (DPBS) and then incubated with the corresponding secondary Ab (1:250; Sigma) conjugated with cyanine 3.18. For the second labeling, cultures were incubated ON at 4°C with the primary Abs, a mouse monoclonal anti-TH (1:30 000), mouse anti-NeuN (1:500), mouse anti-GFAP (1:1000), and mouse monoclonal anti-CD11b (anti–complement receptor 3, 1:100; clone MRC OX-42; Serotec, Kidlington, Oxford, UK), as a marker of resting and reactive microglial cells/macrophages. The cultures were washed with DPBS then incubated for 150 minutes with the secondary Ab (1:60; Chemicon) conjugated with FITC for TH, NeuN, and GFAP, or were incubated for 60 minutes with a biotinylated horse anti-mouse for OX42 (1:200; Vector). OX42 labeling was visualized by incubation of the cultures with streptavidin conjugated with FITC (1:200; Sigma) for 30 minutes. Immunofluorescence was visualized by laser confocal microscopy. In all experiments, the control cultures in which the primary Ab was omitted were immunonegative for these markers.

Effect of PRR on DA Neuron Death in Primary Mesencephalic Cultures

To study the possible involvement of PRR receptors in exacerbating cell loss induced by the DA neurotoxin 6-hydroxydopamine (6-OHDA), cultures were exposed at 4 days in vitro for 72 hours to 6-OHDA (10 μmol/L, in 0.02% saline ascorbate; Sigma) alone, 6-OHDA (10 μmol/L) and the PRR inhibitor HRP (10 μmol/L; GenScript, Piscataway, NJ), or 6-OHDA and renin (10 nmol/L; Sigma), together with the AT1 receptor antagonist ZD 7155 (1 μmol/L; Sigma) and the AT2 receptor antagonist PD 123319 (1 μmol/L; Sigma). The HRP, ZD 7155, and PD 123319 were added 16 hours before treatment with 6-OHDA or 6-OHDA and renin. Control cultures were treated with HRP, renin, ZD 7155, or PD 123319 alone.

The cultures were fixed with 4% paraformaldehyde in DPBS (pH 7.4) for 20 minutes, and endogenous peroxidase activity was quenched by incubation for 5 minutes with 3% H_2O_2 in DPBS. The cultures were then preincubated with a blocking solution containing 10% normal serum in DPBS with 1% BSA and 0.3% Triton X-100 (Sigma) for 1 hour. The cultures were then incubated at 4°C with mouse anti-TH (1:30,000), then washed and incubated for 1 hour with biotinylated horse anti-mouse (1:500; Vector). The cultures were then washed and incubated for 90 minutes with avidin-biotin-peroxidase complex (1:500; Vector). Labeling was revealed with 0.04% H_2O_2 and 0.05% 3, 3′diaminobenzidine (Sigma) as a chromogen. Cells were observed with phase-contrast microscopy (Nikon Eclipse inverted microscope) and counted in 5 randomly chosen longitudinal and transverse microscopic fields along the diameter of the culture dish away from the curved edge by an operator who was blinded to the treatment condition. The microscopic field was defined by a 0.5 × 0.5–cm reticule (1.25 cm^2). The average number of TH-positive cells in a control culture dish was 2,115 ± 98. The results were from at least 3 separate experiments, with a minimum sample size of 4 wells per group and per run. The results were expressed as a percentage of the counts of the control group in the same batch to counteract possible variations among batches. Data are expressed as mean ± SEM. Two-group comparisons were analyzed by the Student *t*-test, and multiple comparisons were analyzed with 1-way analysis of variance followed by Bonferroni post hoc test. The normality of populations and homogeneity of variances were tested before each analysis of variance. Differences at $p < 0.05$ were considered as statistically significant. Statistical analyses were carried out with SigmaStat 3.0 from Jandel Scientific (San Rafael, CA).

RESULTS

Location of PRR in Monkey SN

Large numbers of PRR protein–positive cells were observed in the SNc and the ventral tegmental area (VTA). The specificity of anti-PRR labeling was confirmed using the Ab that had been preadsorbed with the corresponding synthetic peptide. The preadsorbed sections were immunonegative for PRR, as were control sections in which the primary Ab was omitted (Figs. 1A, B, E, 2). The PRR-positive cells varied in size and shape, suggesting labeling in neurons and glial cells. The PRR immunoreactivity was not restricted to the SNc (Fig. 2A). A detailed analysis of other brain areas, particularly those related to major neurodegenerative diseases, will be performed in the future.

Hybridization with the antisense riboprobe for PRR mRNA confirmed the expression of PRR transcripts in cell profiles within the SNc and in the VTA. Both the dorsal and ventral tiers of SNc were easily identified. The observed labeling was sufficiently intense to delineate the SNc boundaries (Figs. 3A, B). Furthermore, hybridization with the sense riboprobe for PRR mRNA resulted in a lack of significant staining in the SNc and VTA, although there was weak background staining (Figs. 3C, D).

FIGURE 4. Photomicrographs of sections through the monkey ventral mesencephalon showing double immunofluorescence for prorenin receptors (PRR, red) and other cell markers (green) or Hoechst stain (blue). **(A–O)** The PRRs were localized (yellow) in NeuN-positive neurons **(A–C)**, tyrosine hydroxylase (TH)–positive dopaminergic neurons (D–I2), and microglia (Integrin αM positive or CD11b-positive cells) **(J–O)**. Triple labeling with the nuclear marker Hoechst shows the nuclear (pink) and extranuclear location of PRR in TH-positive neurons **(D–I)**. This is clearly observed (arrow) in single optical sections (D2–I2) from the area squared in **(D–I)**. Astroglia (glial fibrillary acidic protein [GFAP]–positive cells) did not show PRR labeling **(P–R)**. Scale bars = **(A–I, P–R)** 50 μm; **(J–O)** 25 μm.

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Confocal microscopy of double labeled cells revealed that most PRR-positive cells were also immunoreactive for the neuronal marker NeuN (Figs. 4A–C). Double staining for PRR and TH revealed that DA neurons in particular in the SNc were PRR positive (Figs. 4G–I). This labeling seemed to be located both at the cell surface and within the cells. Prorenin receptor labeling was mainly observed at the nuclear level, as indicated by the use of Hoechst stain as a nuclear

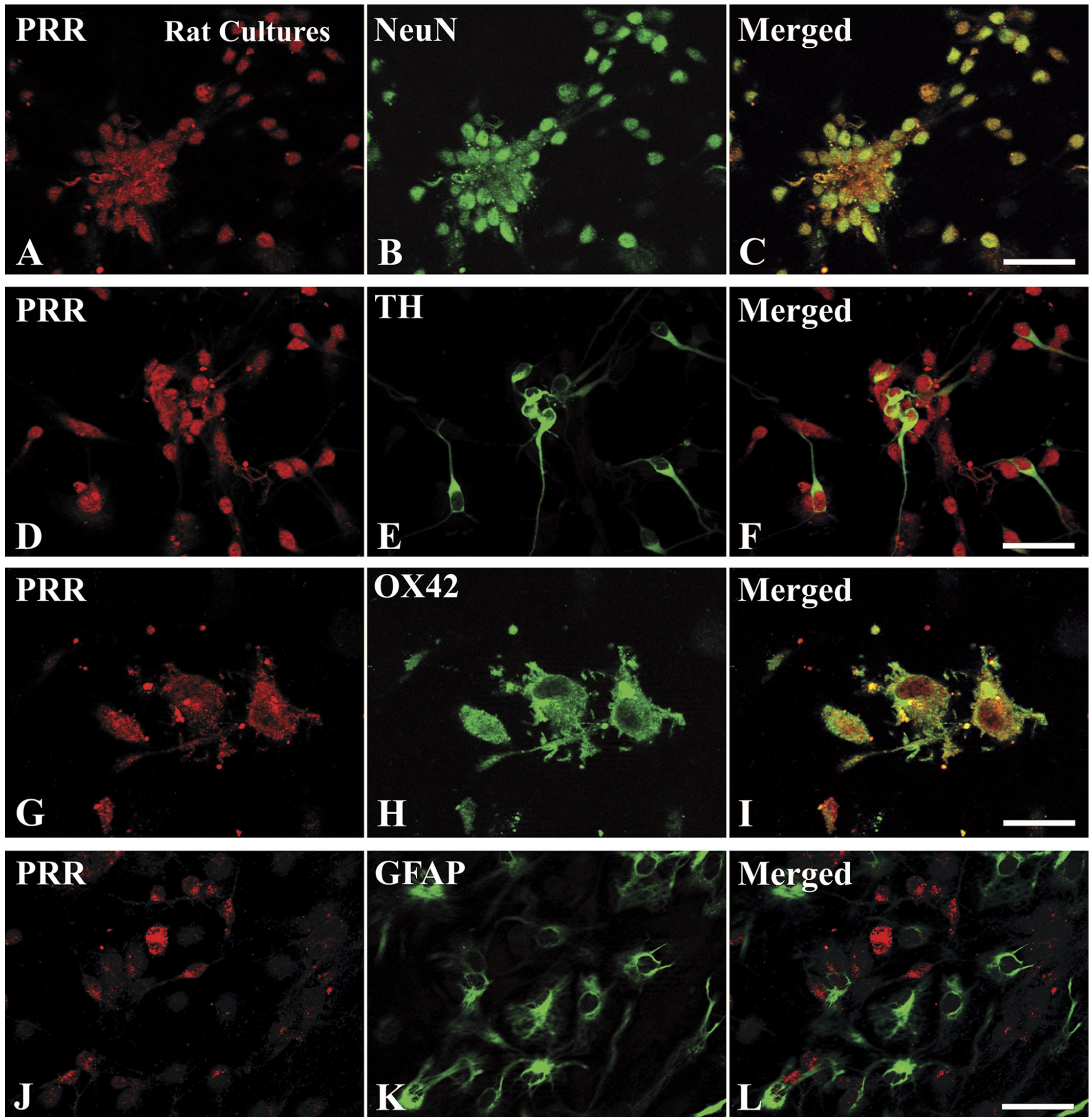

FIGURE 5. Photomicrographs of primary mesencephalic cultures showing double immunofluorescence for prorenin receptors ([PRR] red) and NeuN, TH, OX42, or GFAP (green). Prorenin receptors were localized (yellow) in NeuN-positive neurons **(A–C)**, TH-positive dopaminergic neurons **(D–F)** and OX42-positive microglia **(G–I)**. The glial fibrillary acidic protein (GFAP)–positive astroglia did not show PRR labeling **(J–L)**. GFAP, glial fibrillary acidic protein; PRR, prorenin receptors; TH, tyrosine hydroxylase. Scale bars = **(A–F, J–L)** 50 μm; **(G–I)** 25 μm.

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marker, but extranuclear labeling was also observed (Figs. 4D–F and D2–I2). Double immunolabeling for PRR and microglial markers (CD11b or integrin αM) showed that microglial cells were also PRR positive (Figs. 4J–O). By contrast, no significant labeling for PRR was observed in GFAP-positive astrocytes (Figs. 4P–R).

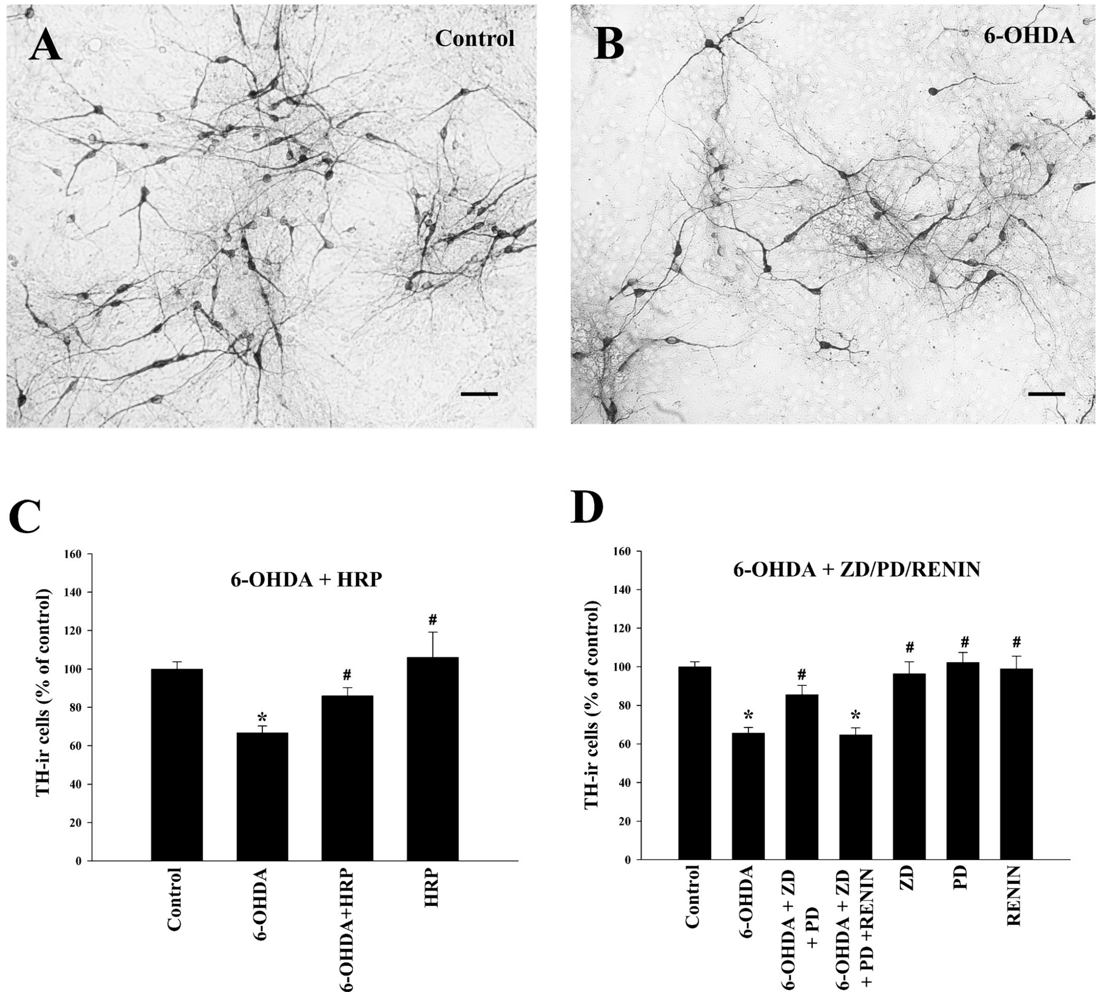

FIGURE 6. Effects of treatment with low doses of 6-hydroxydopamine (6-OHDA) (10 μmol/L, 72 hours), the *handle region peptide* (HRP), renin, and the angiotensin type 1 (AT1) receptor antagonist ZD 7155 (ZD) together with the angiotensin type 2 (AT2) receptor antagonist PD 123319 (PD) on the number of tyrosine hydroxylase (TH)–positive cells in primary mesencephalic cultures. **(A–C)** Treatment with 6-OHDA induced a significant loss of TH-positive neurons that was significantly inhibited by simultaneous treatment with the prorenin receptor blocker HRP. No significant difference in the number of TH-positive neurons was observed after treatment with HRP alone. **(D)** Simultaneous treatment with 6-OHDA and the AT1 receptor antagonist ZD and the AT2 receptor antagonist PD induced a significant decrease in 6-OHDA–induced DA cell death. The decrease in 6-OHDA–induced DA cell death observed after blocking the angiotensin receptors was reverted by treatment with renin. No significant difference in the number of TH-positive neurons was observed after treatment with renin, or ZD, or PD alone. Data are expressed as percentages of the number of TH-positive cells in the respective control cultures (100%). Data are means ± SEM. *$p < 0.05$ relative to the control group (untreated cells); $^{\#}p < 0.05$ relative to the 6-OHDA–treated group (1-way analysis of variance and Bonferroni post hoc test). Scale bars = 100 μm.

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Location of PRR in Primary Mesencephalic Cultures and Functional Effects on 6-OHDA–Induced DA Cell Death

Numerous PRR-positive cells were observed in primary mesencephalic cultures (Figs. 1C, D), and double immunolabeling with confocal microscopy revealed that most PRR-positive cells had neuronal profiles and colocalized with NeuN (Figs. 5A–C). As previously described in vivo, PRR labeling was observed not only at the cell surface, but also intracellularly, including the nucleus. In addition, as observed in vivo, TH-positive cells were also PRR positive (Figs. 5D–F). As in the monkey SNc, PRR immunoreactivity was also seen in OX-42–positive microglial cells (Figs. 5G–I) and GFAP-positive cells were not PRR positive (Figs. 5J–L).

The possible involvement of PRRs in exacerbating the cell loss induced by DA neurotoxins was studied in primary mesencephalic cultures treated with low doses of 6-OHDA. Treatment with 10 μmol/L 6-OHDA for 72 hours induced a decrease of approximately 40% of TH-positive neurons (Figs. 6A–C). In previous studies with the same model, we have shown AT1 and AT2 receptors in DA neurons and microglial cells, and that 6-OHDA–induced cell loss was decreased by blocking AT1 receptors with AT1 antagonists and increased by administration of AII (13). In the present study, administration of the PRR blocker HRP resulted in a significant decrease in 6-OHDA–induced DA cell loss (Fig. 6C). This may be caused by either decreased generation of angiotensin and a subsequent decrease in AT1 activation or AII-independent actions of PRR (i.e. intracellular PRR signaling). Therefore, a second series of experiments was carried out to investigate potential AII-independent effects of PRR. Treatment of cultures with both AT1 and AT2 antagonists (ZD 7155 + PD 123319) to block any effect of endogenous AII resulted in significantly decreased 6-OHDA–induced cell death, in agreement with our previous study (13). This effect was counteracted, however, by additional treatment of the cultures with 10 nmol/L renin (i.e. ZD 7155 + PD 123319 + renin + 6-OHDA). This result suggests that activation of the PRR-derived intracellular signaling cascade also contributes to exacerbating DA neuron death (Fig. 6D). As in previous studies, treatment of cultures with HRP or ZD 7155 or PD 123319 or renin alone (without 6-OHDA) did not result in a significant change in the number of TH-positive cells. This indicates that an initial or synergistic neurotoxin-induced lesion is necessary to induce significant DA neuron loss (Figs. 6C, D).

DISCUSSION

We report for the first time the location of PRR in neurons and microglial cells in the brain of nonhuman primates and in the SN in particular. Our results further suggest that PRRs contribute to exacerbating the DA cell death induced by activation of brain RAS that is observed after stimulation of AT1 receptors in several PD animal models (11–13). We and others have previously established that all components of the RAS are generated in the brain (5, 23). Immunoreactive renin has been observed in neurons and glial cells in numerous areas of mouse and rat brain (24) and in all areas examined in the human brain (25). Expression of renin mRNA has also been observed in the brain by hybridization histochemistry (26, 27). More recently, a wide distribution of renin in the brain was confirmed by the use of transgenic models (28–30). Because binding of renin leads to generation of AII at the cell/tissue level and increases the catalytic activity of renin by approximately 4 to 5 times (16, 23), our results further support the presence of a functionally sufficient RAS in the brain, particularly in the SN. Furthermore, binding of prorenin activates its catalytic activity, and plasma prorenin-to-renin ratios are 5 to 10 times, and even up to 20 to 200 times, increased in pathological conditions (31). In addition, prorenin binding to PRR initiates a cascade of signaling events, including pathways similar to those induced by AT1 activation (16, 32). Several studies have shown that the PRR functions via the mitogen-activated protein kinases ERK 1/2 (16, 33).

Here, we observed high levels of PRR protein in DA and non-DA neurons both in the monkey SN and in cultures of rat ventral mesencephalon, which also express AT1 and AT2 receptors (11, 13). In neurons, both PRRs and AT1 receptors may be involved in signaling mechanisms and the regulation of neuronal excitability (19, 34, 35). It is known that AII modulates DA levels in the nigrostriatal system (36, 37), and conversely, we have recently observed that DA depletion leads to increased AII receptor expression and signaling (unpublished observations). This apparently counterregulatory mechanism for increasing DA levels may lead to a parallel AII-induced pro-oxidative and proinflammatory state that potentiates progression of DA neuron death (11, 13).

It has been observed that activation of the microglial nicotinamide adenine dinucleotide phosphate complex and the microglial inflammatory response via AT1 receptors plays a major role in AII-induced exacerbation of DA neuron loss in animal models, and potentially in PD (11, 13). Several studies have also shown that neuroinflammation and microglial nicotinamide adenine dinucleotide phosphate activation play a major role in DA cell death and PD (38–40). Interestingly, we have observed for the first time the presence of PRRs in microglial cells in vitro and in vivo in the present study. Prorenin receptors may increase the generation of AII at the microglial level, and this could play a major role in microglial activation and the inflammatory response induced by RAS activation. Prorenin receptor mRNA and protein have also been located in macrophages, T cells, and granulocytes (41, 42).

We did not observe any significant PRR labeling of GFAP-positive astrocytes either in vitro or in the monkey SN. It is known that astrocytes are the main site of angiotensinogen synthesis in the brain (43, 44), although low levels of angiotensinogen have also been observed in vitro in neurons (45, 46). Intense catalytic action on angiotensinogen by PRR located on the astrocytes themselves may block the main source of brain angiotensinogen and brain RAS function. We have observed AT1 and AT2 receptors in astrocytes (11, 13, 47), however, and they may play a role in a counterregulatory effect of AII levels on the angiotensinogen synthesis.

Prorenin receptor labeling was intracellular and mainly nuclear. Several studies have reported that PRRs are located at the cell surface (16), but a detailed study of the subcellular location of PRR by the use of fractionated protein isolation

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followed by Western blotting of HeLa-S3 cells revealed a preferential intracellular presence of PRRs, particularly at the level of the nuclear envelope and the endoplasmic reticulum, in addition to the cell surface location (18). This discrepancy might be explained by cell-type differences. Prorenin receptors may internalize renin and prorenin, or a nonsecreted (i.e. intracellular) renin may directly interact with the intracellular PRRs. Several transmembrane receptors are known to accumulate in nuclei and, particularly, nuclear membranes. Cells such as cardiomyocytes possess AII receptors that couple to nuclear signaling pathways and regulate transcription (48–51). This supports the possibility of an intracellular function for AII, in addition to that induced by activation of cell surface AT1 and AT2 receptors. Extracellular AII may act intracellularly by binding to the AT1 receptors, which are subsequently internalized, or AII may be synthesized within the cell. The AT1-dependent internalization of AII has been described in a number of different cell types, including neurons (50–52). Our present results suggest that some AII is formed within neurons, as previously suggested for cardiocytes (53). Intracellular AII has been suggested to induce transcription of angiotensinogen and renin in response to binding to nuclear AT1 receptors in some cell types (52). In accordance, intraneuronal angiotensinogen and intraneuronal forms of renin have been observed (46, 48). The present results support the existence of a brain intracellular/intracrine RAS, as previously suggested for several cell types. "Intracrine" refers to a factor or compound that acts in the intracellular space either after internalization or retention in the synthesizing cell (48, 49).

In addition to the location of PRR in neurons and microglial cells in the primate brain, our in vitro results show that these receptors exert functional effects and suggest that PRRs contribute to the AII-induced proinflammatory and prooxidative effects that result in increased DA cell death after treatment with low doses of neurotoxins, such as 6-OHDA or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (11–13). We first treated cultures with HRP to block prorenin binding to PRR and prorenin activation and observed a significant decrease in the loss of DA neurons induced by simultaneous treatment with low doses of 6-OHDA. In some experiments, HRP administration is highly effective in blocking renin/prorenin binding to PRR, resulting in protective effects against PRR-mediated damage in several tissues (14, 20). The HRP also blocked some of the effects of renin in neuronal cultures (19), but an HRP inhibitory effect was not observed in other studies and cells (41, 54). The reason for this discrepancy has not been clarified, but different concentrations of HRP or the presence of different levels of renin (which may generate sufficient angiotensin independently of PRR) or other factors may be involved (55). The decrease in DA cell loss that we observed in the presence of HRP may be related to a decrease in generation of AII at the cell surface after blocking the PRR of neurons and microglial cells, leading to effects similar to those observed after blocking the endogenous AII by treatment of cultures with AT1 antagonists (11, 13). In a second series of experiments, cultures were treated with renin and AT1 and AT2 antagonists to investigate if PRR contributes to increasing DA cell death by AII-independent actions (i.e. triggering its own intracellular signaling cascade). The loss of DA neurons was not significantly different from that induced by low doses of 6-OHDA alone (i.e. 6-OHDA acting in the presence of endogenous AII/AT1), but the DA cell death induced by treatment with 6-OHDA + renin + AT1 and AT2 antagonists was significantly higher than that observed in cultures treated with 6-OHDA + AT1 and AT2 antagonists (i.e. 6-OHDA acting in the absence of endogenous AII signaling). This suggests that stimulation of PRR by sufficient levels of renin/prorenin to increasing DA cell death and should be taken into account in designing potential neuroprotective strategies.

In conclusion, the present study has shown the presence of prorenin/renin receptors in neurons and microglial cells of the SN of primates and in neurons and microglial cells of primary cultures of rat ventral mesencephalon. Our results further suggest that PRRs contribute to the previously reported effects of increased AII on DA neuron degeneration and, therefore, potentially to the progression of PD. The effects are caused by AII-dependent and AII-independent actions, which suggest that potential neuroprotective strategies to decrease RAS activity should be targeted against AII/AT1 signaling and also against PRR signaling as an alternative or complementary strategy.

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4.3- Implicación de PPAR-γ en el efecto neuroprotector y antiinflamatorio del bloqueo del receptor de AII de tipo 1. Efecto del antagonista de los receptores AT1 telmisartán y de la deleción del receptor AT1 en un modelo en ratón de enfermedad de Parkinson

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Estudios recientes han demostrado que antagonistas de los receptores AT1 tales como el candesartán, inhiben la respuesta inflamatoria microglial así como la pérdida de neuronas dopaminérgicas en modelos animales de EP. Sin embargo, los mecanismos implicados en este efecto neuroprotector y antiinflamatorio no han sido totalmente clarificados. Varios estudios han descrito que los antagonistas de los receptores AT1 activan al receptor PPAR-γ. Se sabe que la activación de PPAR-γ inhibe la inflamación y por tanto podría ser responsable de efectos neuroprotectores independientes del bloqueo de los receptores AT1. Hemos estudiado si el tratamiento oral con telmisartán (agonista de PPAR-γ más potente entre todos los antagonistas de los receptores AT1) tiene un efecto neuroprotector sobre las neuronas dopaminérgicas e inhibe la respuesta inflamatoria, así como el posible papel de la activación de PPAR-γ en dicho efecto neuroprotector. Para ello usamos un modelo animal de EP, concretamente ratones tratados con la neurotoxina MPTP a los que se les administra el antagonista de PPAR-γ, GW9662. Por otro lado, para estudiar si la ausencia de los receptores AT1 proporciona un efecto neuroprotector independiente de los efectos farmacológicos de los antagonistas de dichos receptores empleamos ratones *knock-out* para AT1a lesionados con MPTP. Y mediante la administración del inhibidor de PPAR-γ, estudiamos si la activación de PPAR-γ podría estar también implicada ese efecto neuroprotector por la ausencia (deleción) de los receptores AT1. El resultado obtenido en los ratones tratados con MPTP es que el tratamiento oral con telmisartán tiene un efecto neuroprotector sobre las neuronas dopaminérgicas e inhibe la respuesta microglial inducida por la neurotoxina. Y además, que estos efectos neuroprotectores son inhibidos mediante el tratamiento simultáneo con el antagonista de PPAR-γ, GW9662. Por otro lado, los ratones *knock-out* para el receptor AT1 tratados con MPTP muestran una pérdida dopaminérgica y una activación microglial significativamente menor a la observada en

los ratones silvestres. Y al igual que observábamos en los ratones silvestres, el tratamiento simultáneo con el inhibidor de PPAR-γ, GW9662, bloquea el efecto neuroprotector de la ausencia de los receptores AT1. Nuestros resultados sugieren que el telmisartán tiene un efecto neuroprotector sobre la neurodegeneración dopaminérgica y que dicho efecto protector es mediado a través de la activación de PPAR-γ. Además, los resultados obtenidos en los ratones *knock-out* nos sugieren que el bloqueo de los receptores AT1, independiente de los efectos farmacológicos de los antagonistas de dichos receptores, también tiene un efecto neuroprotector sobre las neuronas dopaminérgicas e inhibe la respuesta inflamatoria. Por lo tanto, la activación de PPAR-γ está implicada en los efectos neuroprotectores y antiinflamatorios debidos a la inhibición de los receptores AT1.

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Involvement of PPAR-γ in the neuroprotective and anti-inflammatory effects of angiotensin type 1 receptor inhibition: effects of the receptor antagonist telmisartan and receptor deletion in a mouse MPTP model of Parkinson's disease

Pablo Garrido-Gil[1,2], Belen Joglar[1,2], Ana I Rodriguez-Perez[1,2], Maria J Guerra[1,2] and Jose L Labandeira-Garcia[1,2*]

Abstract

Background: Several recent studies have shown that angiotensin type 1 receptor (AT1) antagonists such as candesartan inhibit the microglial inflammatory response and dopaminergic cell loss in animal models of Parkinson's disease. However, the mechanisms involved in the neuroprotective and anti-inflammatory effects of AT1 blockers in the brain have not been clarified. A number of studies have reported that AT1 blockers activate peroxisome proliferator-activated receptor gamma (PPAR γ). PPAR-γ activation inhibits inflammation, and may be responsible for neuroprotective effects, independently of AT1 blocking actions.

Methods: We have investigated whether oral treatment with telmisartan (the most potent PPAR-γ activator among AT1 blockers) provides neuroprotection against dopaminergic cell death and neuroinflammation, and the possible role of PPAR-γ activation in any such neuroprotection. We used a mouse model of parkinsonism induced by the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and co-administration of the PPAR-γ antagonist GW9662 to study the role of PPAR-γ activation. In addition, we used AT1a-null mice lesioned with MPTP to study whether deletion of AT1 in the absence of any pharmacological effect of AT1 blockers provides neuroprotection, and investigated whether PPAR-γ activation may also be involved in any such effect of AT1 deletion by co-administration of the PPAR-γ antagonist GW9662.

Results: We observed that telmisartan protects mouse dopaminergic neurons and inhibits the microglial response induced by administration of MPTP. The protective effects of telmisartan on dopaminergic cell death and microglial activation were inhibited by co-administration of GW9662. Dopaminergic cell death and microglial activation were significantly lower in AT1a-null mice treated with MPTP than in mice not subjected to AT1a deletion. Interestingly, the protective effects of AT1 deletion were also inhibited by co-administration of GW9662.

Conclusion: The results suggest that telmisartan provides effective neuroprotection against dopaminergic cell death and that the neuroprotective effect is mediated by PPAR-γ activation. However, the results in AT1-deficient mice show that blockage of AT1, unrelated to the pharmacological properties of AT1 blockers, also protects against dopaminergic cell death and neuroinflammation. Furthermore, the results show that PPAR-γ activation is involved in the anti-inflammatory and neuroprotective effects of AT1 deletion.

Keywords: Angiotensin, AT1, neuroinflammation, neuroprotection, microglia, Parkinson, peroxisome proliferator-activated receptor gamma, telmisartan

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Background

In recent years, evidence has accumulated for a major role of oxidative stress and neuroinflammation in the pathogenesis and progression of Parkinson's disease (PD) [1,2]. The peptide angiotensin II (AII), via type 1 receptors (AT1), is one of the most important known inducers of inflammation and oxidative stress, produces reactive oxygen species (ROS) by activation of the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex [3-5] and plays a major role in the pathogenesis of several age-related degenerative diseases [6-8]. There is a local renin-angiotensin system (RAS) in the brain [9,10], and NADPH oxidase, AT1 and AT2 receptors have been located in dopaminergic (DA) neurons, nigral microglia and astrocytes [11-13].

We have previously shown that the DA cell loss induced by DA neurotoxins is enhanced by AII via AT1, activation of the microglial NADPH-complex and exacerbation of the glial inflammatory response [11,13,14]. This is consistent with more recent studies, in which we have shown hyperactivation of the nigral RAS in several animal models of increased vulnerability of DA neurons to degeneration (that is, models of humans at higher risk for PD), such as aged male rats [15] or menopausal rats [16]. The increased glial inflammatory response and DA neuron vulnerability were found to be inhibited by the AT1 antagonist candesartan. It is well-known that AT1 antagonists block AT1 receptor function and increase AT2 receptor expression and function with no significant changes in angiotensin converting enzyme (ACE) activity [17,18]. However, the mechanisms involved in the brain anti-inflammatory effects of AT1 blockers (ARBs) have not been clarified.

Previous studies in different tissues have suggested that peroxisome proliferator-activated receptor gamma (PPAR-γ) is involved in the anti-inflammatory effects of AT1 antagonists [19-21]. PPAR-γ belongs to a group of nuclear receptors (PPARs) that control lipid and glucose metabolism, energy homeostasis and adipocyte and macrophage differentiation. More recently, macrophage PPAR-γ receptors have been shown to be involved in the down-regulation of expression of several inflammatory cytokines and inhibition of inflammation [22-24]. Interestingly, PPAR-γ has been detected in neurons and glial cells [24-26], and participates in mechanisms that control microglial activation and lead to suppression of the activated phenotype [25,27]. In accordance, it has been shown that PPAR-γ agonists protect against DA cell death in animal models of PD [28,29]. However, the potential relationship between the anti-inflammatory effects of ARBs and PPAR-γ stimulation is not clear.

A number of studies have reported that some ARBs such as telmisartan and irbesartan, and more controversially losartan and candesartan (but not valsartan or olmesartan), have PPAR-γ activating properties that are independent of any AT1 blocking actions [19-21]. Therefore, the pharmacological PPAR-γ activating properties of ARBs may be responsible for the neuroprotective effects. However, it has also been reported that the pharmacological PPAR-γ-activating potency of ARBs (including telmisartan, the most potent PPAR-γ activator among ARBs) is rather modest compared with that of conventional PPAR-γ ligands, and that the PPAR-γ activating potency may be even less effective *in vivo* [30,31].

In the present study, we aimed to determine whether telmisartan provides neuroprotection against DA cell death in a mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of parkinsonism, and whether PPAR-γ activation plays a major role in any such neuroprotection. Secondly, we studied whether the pharmacological PPAR-γ-activating properties of telmisartan are responsible for the neuroprotective effects, and if the AT1 blocking actions do not actually play any significant role in neuroprotection; we used AT1a-null mice lesioned with the DA neurotoxin MPTP to study whether deletion of AT1 in the absence of any pharmacological effect of ARBs provides neuroprotection. Thirdly, we investigated whether PPAR-γ activation may also play a major role in any such neuroprotective effect of AT1 deletion.

Methods

Experimental design

Male C57BL-6 mice weighing 20 to 25 g (that is, seven weeks old) were used. Mice were wild type (WT; Charles River, L'Arbresle, France) or homozygous mice deficient for AT1a (the major mouse AT1 isoform and the closest murine homolog to the single human AT1 [32]; Jackson Laboratory, Bar Harbor, ME, USA). Mice were maintained in the animal facility at the University of Santiago de Compostela in accordance with the institutional guidelines. In a first series of experiments, the WT mice were divided into seven groups (A1 to G1). Mice in group A1 (n = 14) were used as normal (that is, nonlesioned) controls, and were treated with vehicle (see below). Mice in group B1 (n = 11) were injected with MPTP (Free base, Sigma, St Louis, MO, USA; 30 mg/kg/day in saline, by intraperitoneal injection for five days) and intraperitoneal and oral vehicle. Mice in group C1 (n = 6) were injected with MPTP as group-B1 mice, but received oral treatment with telmisartan (5 mg/kg/day; Sigma) from two weeks before MPTP treatment until they were killed. The powered drug was administered orally to the mice mixed with peanut butter; animals in control groups were given only peanut butter. The dose of telmisartan was chosen on the basis of previous results. Telmisartan has been detected in cerebral spinal fluid after repeated oral treatment at 1 to 30 mg/kg [33].

However, the dose was selected according to several recent reports showing that 5 mg/kg provided neuroprotection against brain injury [34,35]. Mice in group D1 (n = 8) were injected with MPTP and telmisartan as above, as well as the PPAR-γ antagonist GW9662 (4 mg/kg by intraperitoneal injection in dimethyl sulfoxide 4% PBS for four weeks; that is from two weeks before MPTP injection until killed). Additional control mice were injected with telmisartan alone (group E1; n = 5), or GW9662 alone (group F1; n = 5), or telmisartan + GW9662 (group G1; n = 5) as described above.

In a second series of experiments, the AT1a-null mice were divided into four groups (A2 to D2). AT1a-null mice in group A2 (n = 8) were treated with vehicle and used as normal non-lesioned controls. Mice in group B2 and C2 (n = 8) were injected with MPTP as above. AT1a-null mice in group D2 (n = 8) were injected with MPTP and the PPAR-γ antagonist GW9662 (4 mg/kg by intraperitoneal injection for four weeks before killed). Finally, an additional group of AT1a-null mice was treated with GW9662 alone (group E2; n = 5). The mice were killed one week after treatment with MPTP or vehicle and then processed for histology or high performance liquid chromatography (HPLC; see below).

In a third series of experiments (n = 20), different groups of mice were injected with a single dose of MPTP (30 mg/kg) after treatment with vehicle or telmisartan as above (that is, WT mice + vehicle + MPTP, n = 7; WT mice + telmisartan + MPTP, n = 7; and AT1a-null mice + vehicle + MPTP, n = 6), and finally killed 90 min after the MPTP injection to quantify striatal levels of MPP^+ (see below) [36,37].

High performance liquid chromatography

Seven days after the last MPTP injection, mice were killed by decapitation and brains rapidly removed. The striata were dissected on an ice-cold plaque, and the striatal tissue frozen on dry ice and stored at -80°C until analysis. Striatal tissue was homogenized and then centrifuged at 14,000 g for 20 min at 4°C. The supernatant fractions were decanted, filtered (0.22 μm) and injected (20 μL/injection) into the HPLC system (Shimadzu LC prominence, Shimadzu Corporation, Kyoto, Japan). Dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were separated with a reverse phase analytical column (Waters Symmetry300 C18; 150 × 3.9 mm, 5 μm particle size; Waters, Milford, MA, USA). The mobile phase (70 mM KH_2PO_4, 1 mM octanesulfonic acid, 1 mM ethylenediaminetetraacetic acid (EDTA) and 10% MeOH, pH 4) was delivered at a rate of 1 mL/min. Detection was performed with a coulometric electrochemical detector (ESA Coulochem III, Chelmsford, MA, USA). The first and second electrode of the analytical cell were set at +50 mV and +350 mV, respectively; the guard cell was set at -100 mV. Data were acquired and processed with the Shimadzu liquid chromatography solution software. Results were expressed in nanogram per microgram wet weight tissue and presented as mean ± standard error of the mean (SEM) (n = 5 per group).

Estimation of 1-methyl-4-phenylpyridinium levels by mass spectrometry

Brains were removed from the mice, the striata dissected on an ice-cold plaque and the striatal tissue frozen on dry ice and stored at -80°C until analysis. On the day of the assay [36], striata were weighed and sonicated in a solution of 0.4 M perchloric acid containing (w/v): 0.1% sodium metabisulphite, 0.01% EDTA and 0.1% L-cysteine. Samples were centrifuged at 13,000 rpm for 20 min at 4°C and the supernatant was used to determine 1-methyl-4-phenylpyridinium (MPP^+) levels. HPLC separation was accomplished in a Waters Alliance 2795 system (Waters, Milford, MA, USA), with an Atlantis dC18 column (2.1 × 50 mm, 3 μm). The mobile phase consisted of solvent A (0.1% formic acid) and solvent B (acetonitrile). We employed an elution profile from 95% solvent A for 1 min, followed by a linear gradient from 95% solvent A to 100% solvent B from minute 1 to minute 1.5, and 100% solvent B was maintained until minute 5. A re-equilibration time of 5 min was allowed between injections and chromatography was carried out at a flow-rate of 0.2 mL/min. Eluates were detected with a Quattro MicroTM API ESCI triple-quadrupole mass spectrometer fitted with Z-spray (Waters, Milford, MA, USA). Electrospray ionization was set in positive ion polarizing mode (ESI+) for acquisition of mass spectrometry data, with the following fragments (m/z): 170.2 > 128.0, 170.2 > 154.4, and 170.2 > 115.1. The capillary voltage was set at 3 kV, the desolvation temperature at 450°C, the cone voltage at 45 V, and the desolvation gas flow rate was set at 550 L/h. All parameters were adjusted to obtain optimum operating conditions for maximum intensity of the selected fragments, with Masslynx 4.1 software (Waters, Milford, MA, USA). MPP^+ standards were prepared in the homogenization solution and used for calibration purposes.

Immunohistochemistry, lectin histochemistry and cresyl violet staining of mouse brains

The animals were killed and perfused, firstly with 0.9% saline, and then with cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, washed and cryoprotected in the same buffer containing 20% sucrose, and finally cut on a freezing microtome (30 μm thick). To prevent any possible unspecific labeling due to the use of primary mouse monoclonal antibodies with mouse tissue, sections were processed with rabbit polyclonal antibodies to tyrosine hydroxylase (TH; as a

marker of DA terminals) and rat monoclonal antibodies against CD45 (to identify reactive microglia/macrophages), as follows. Sections were incubated for 1 h in 10% normal serum with 0.25% Triton X-100 in 20 mM potassium PBS containing 1% BSA (KPBS-BSA), then incubated overnight at 4°C with rabbit polyclonal antiserum to TH (Chemicon, Millipore Temecula, CA; 1:500) or at 4°C with rat monoclonal antiserum to CD45 (rat immunoglobulin G, 1:1000, AbD Serotec, Kidlington, Oxford, UK) in 20 mM KPBS containing 1% BSA, 2% normal serum and 0.25% Triton X-100. The sections were subsequently incubated, firstly for 90 min with the corresponding biotinylated secondary antibodies (1:200), and then for 90 min with an avidin-biotin-peroxidase complex (Vector, 1:50, Burlingame, CA, USA). Finally, the labeling was visualized with 0.04% hydrogen peroxide and 0.05% 3-3' diaminobenzidine (Sigma), containing 0.1% nickel sulfate to intensify the microglial staining. For negative control staining, sections were incubated in media lacking primary antibodies.

Activated microglial cells were also stained histochemically with *Griffonia simplicifolia* isolectin B4 (GSI-B4) as follows. Sections were pre-incubated in PBS containing 0.1 mM of $CaCl_2$, $MgCl_2$, $MnCl_2$ and 0.3% Triton X-100 for 20 min. The sections were then rinsed with PBS and incubated overnight at 4°C with biotinylated GSI-B4 (Sigma; 20 μg/mL) in PBS containing cations and 0.3% Triton X-100. After rinsing with PBS, the sections were incubated with an avidin-biotin-peroxidase complex (Vector; 1:100) for 90 min. Finally, labeling was visualized with 0.04% hydrogen peroxide and 0.05% diaminobenzidine with 0.1% nickel sulfate to intensify the staining. For negative control staining, sections were incubated in media lacking GSI-B4.

The total numbers of TH-immunoreactivity (TH-ir) neurons in the substantia nigra compacta (SNc) were estimated by an unbiased stereology method (that is, the optical fractionator). Stereological analysis was carried out with the Olympus CAST-Grid system (Computer Assisted Stereological Toolbox; Olympus, Ballerup, Denmark). Uniform, randomly chosen sections through the substantia nigra (every third section) were analyzed for the total number of TH-ir cells by means of a stereological grid (fractionator), and the nigral volume was estimated according to Cavalieri's method [38]. Penetration by the antibody was determined by registration of the depth of each counted cell that appeared in focus within the counting frame. This analysis revealed incomplete penetration by the antibody, leaving 8 to10 μm in the center poorly stained [39]. The total number of cells was therefore calculated by excluding the volume corresponding to this portion of the sections.

In order to confirm that MPTP induces cell death and not only phenotypic down-regulation of TH activity, series of sections through the entire substantia nigra of control mice and mice treated with MPTP were counterstained with cresyl violet, and the total number of neurons in the SNc was estimated by the unbiased stereology method described above for TH-ir cells. Neurons were distinguished from glial cells on morphological grounds, and neurons with visible nuclei were counted as above. The number of reactive microglial cells was estimated with the Olympus CAST-Grid system and the unbiased stereological method described above for counting TH-ir neurons. The density of reactive microglial cells (cells/mm^3) was determined by dividing the number of labeled cells by the volume that they occupied.

The density of striatal DA terminals was estimated as the optical density of the striatal TH-ir with the aid of National Institutes of Health (NIH)-Image 1.55 image analysis software (Wayne Rasband, National Institute of Mental Health, USA) on a personal computer coupled to a video camera (CCD-72, Imaging Research Inc, Linton, UK) and a constant illumination light table (Northern Light, St. Catharines, Canada). At least four sections through the central striatum of each animal were measured (both the right and left striatum), and for each section the optical densities were corrected by subtraction of background as observed in the corpus callosum.

Statistical analysis

All data were obtained from at least three independent experiments and were expressed as means ± SEM. Multiple comparisons were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post-hoc test. The normality of populations and homogeneity of variances were tested before each ANOVA. Differences were considered statistically significant at $P < 0.05$. Statistical analyses were carried out with SigmaStat 3.0 from Jandel Scientific (San Rafael, CA, USA).

Results

In control mice (those not injected with MPTP; group A1) the DA neurons in the SNc were intensely immunoreactive to TH, and a dense evenly distributed TH-ir was observed throughout the striatum, indicating the presence of a dense network of nigrostriatal DA terminals (Figure 1A, B). In mice treated with MPTP and vehicle (group B1) there was a bilateral reduction in the number of TH-ir neurons in the substantia nigra and a marked reduction in the TH-ir in both striata relative to control mice (Figures 1C, D and 2A, B). The functional effects of the MPTP lesion were confirmed by determination of the striatal levels of dopamine and its metabolites with HPLC in control mice (group A1, n = 5) and mice treated with MPTP (group B1, n = 5). Levels (nanogram per milligram wet weight tissue) of dopamine (3.447 ± 0.243), DOPAC

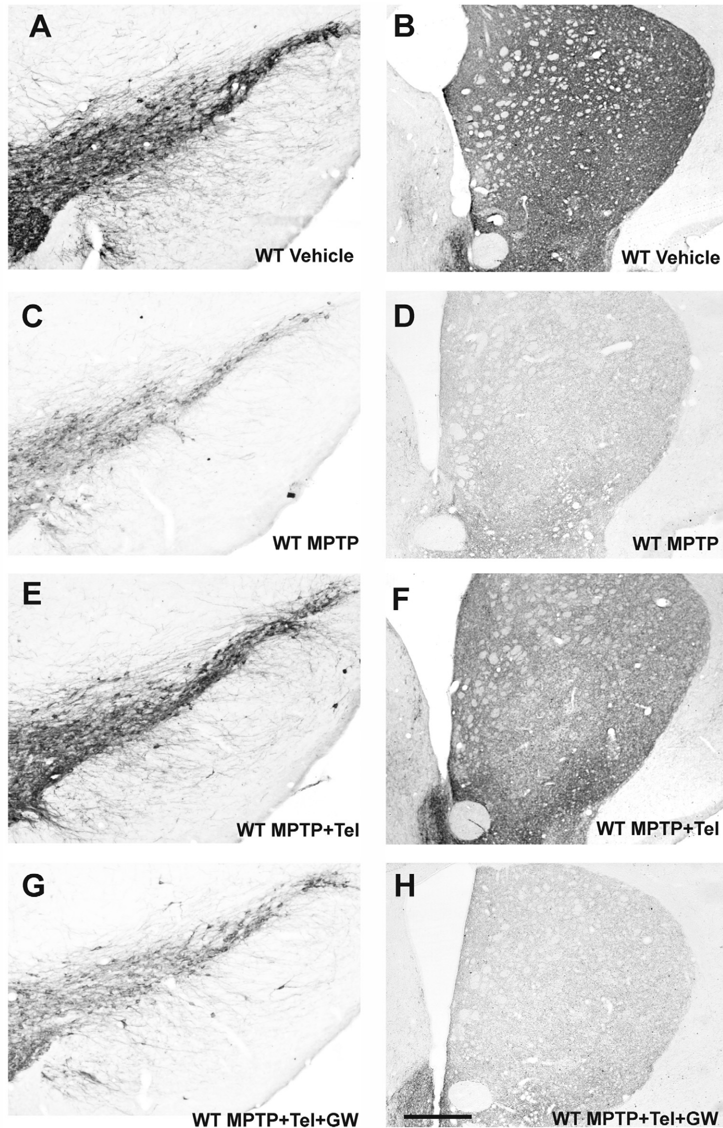

Figure 1 Changes in TH-ir in the substantia nigra and striatum in WT mice. TH-ir at central levels of the substantia nigra (**A**, **C**, **E**, **G**) and striatum (**B**, **D** ,**F**, **H**) in WT mice injected with vehicle (controls; A, B), with MPTP alone (C, D), or with MPTP + telmisartan (E, F), or with MPTP + telmisartan + the PPAR-γ antagonist GW9662 (G, H). More TH-ir neurons were observed in the nigra and terminals in the striatum (that is, spared DA neurons and terminals) of mice treated with telmisartan (E, F) than in mice that did not receive telmisartan (C, D) or mice treated with telmisartan and GW9662 (G, H). Scale bar: 250 μm (A, C, E, G) and 560 μm (B, D, F, H).

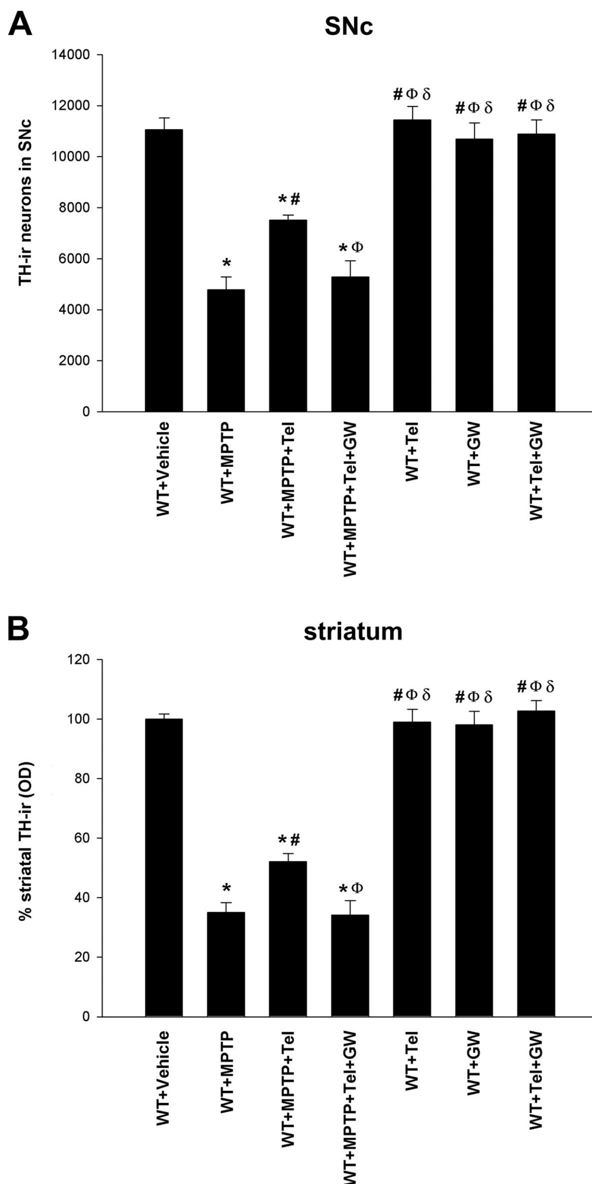

Figure 2 DA (TH-ir) neurons and terminals in WT mice. TH-ir neurons in the **(A)** SNc and **(B)** TH-ir terminals in the striatum one week after treatment with vehicle, telmisartan alone, GW9662 alone, telmisartan + GW9662, MPTP alone, telmisartan + MPTP, or telmisartan + MPTP + GW9662. The DA neurons were quantified as the total number of TH-ir neurons in the SNc, and density of striatal DA terminals was estimated as optical density and expressed as a percentage of the value obtained in the group treated with vehicle. Data are presented as mean ± SEM. *P < 0.05 compared with mice treated with vehicle, $^{\#}P < 0.05$ compared with mice treated with MPTP alone, $^{\Phi}P < 0.05$ compared with mice treated with MPTP + telmisartan, $^{\delta}P < 0.05$ compared with mice treated with MPTP + telmisartan + GW9662 (one-way ANOVA and Student-Newman-Keuls post-hoc test).

(0.257 ± 0.012) and HVA (0.336 ± 0.041) in control mice were significantly higher than those observed in lesioned mice (dopamine, 1.418 ± 0.112; DOPAC, 0.136 ± 0.012; HVA, 0.192 ± 0.024).

In order to confirm that MPTP induced DA cell death and not TH-down-regulation and the corresponding decrease in DA levels, we counted neurons in cresyl violet stained sections. In control mice, the total number of neurons counted in cresyl violet stained sections (13,701 ± 1140) was slightly higher than that of TH-ir neurons as some non-DA neurons located in the SNc were also counted. However, sections from mice treated with MPTP (group B1) showed significant fewer cresyl violet stained neurons in the SNc (8370 ± 1112) than in the control mice, confirming that MPTP induced cell death and not TH-down-regulation in the present experimental conditions.

Mice treated with telmisartan and injected intraperitoneally with MPTP (group C1) showed a bilateral reduction in the number of TH-ir neurons in the substantia nigra and density of striatal TH-ir terminals, relative to control mice, although the reduction was significantly lower than that observed in group B1 mice not treated with telmisartan (Figures 1E, F and 2A, B). However, the protective effects of telmisartan were inhibited by co-administration of the PPAR-γ antagonist GW9662 (group D1; Figures 1G, H and 2A, B). No significant changes were observed in mice treated with telmisartan alone, or GW9662 alone, or telmisartan + GW9662.

In control AT1a-null mice (those not injected with MPTP; group A2) DA neurons in the SNc were intensely immunoreactive to TH and a dense evenly distributed TH-ir was observed throughout the striatum (Figure 3A, B). In AT1a-null mice injected with MPTP (group B/C2) there was a bilateral reduction in the number of TH-ir neurons in the substantia nigra and their striatal terminals relative to vehicle-injected mice (Figures 3C, D and 4), although this reduction was lower than that observed in group B1 mice injected with MPTP and not subjected to AT1a deletion (that is, mice in which brain endogenous AII can act synergistically with MPTP on the DA system via AT1). However, the protective effects of AT1 deletion were inhibited by co-administration of the PPAR-γ antagonist GW9662 (group-D2 mice; Figures 3E, F and 4). No significant changes were observed in AT1a-null mice treated with GW9662 alone in comparison with mice treated with vehicle.

In order to determine if treatment with telmisartan or AT1a deletion acts by modifying MPTP pharmacokinetics such as penetration into the brain, biotransformation of MPTP to MPP^+ or MPP^+ removal from the brain, we measured striatal levels of MPP^+ in mice. There were no significant differences in striatal levels of MPP^+ between mice treated with telmisartan and MPTP (3.116 ± 0.196 ng/mg wet weight striatal tissue), AT1-null mice treated with vehicle and MPTP (3.100 ± 0.211 ng/mg wet weight striatal tissue) and WT mice treated with vehicle and MPTP (3.045 ± 0.157 ng/mg wet weight striatal tissue). The protective effect of telmisartan and AT1a deletion (that is, the absence of possible changes in the MPTP biotransformation to the active metabolite MPP^+) was also supported by the results observed after treatment of mice with the PPAR-γ antagonist GW9662. In the presence of telmisartan or AT1 deletion (MPTP + telmisartan or MPTP + AT1 deletion), treatment with the PPAR-γ antagonist GW9662 reverted DA cell death and microglial activation (see below) to levels similar to those observed after treatment with MPTP alone, which would have not been possible without the presence of similar levels of MPP^+ in the mice striatum.

In several recent studies, we have observed that the enhancing effect of AII on DA cell loss is mediated by microglial activation and exacerbation of the inflammatory response (for details, see [11,13]). In order to confirm that, in the present experiments, neuroprotection by telmisartan or AT1a deletion in mice is also associated with the same mechanism (inhibition of MPTP-induced microglial response), we analyzed the expression of the microglial markers isolectin B4 and CD45 in the substantia nigra. Control mice treated with vehicle showed minimal and non-significant microglial activation. In WT mice injected with MPTP (group B1), microglial activation was much higher than in WT mice injected with vehicle (group A1), and higher than mice injected with MPTP + telmisartan (group C1). However, WT mice injected with MPTP + telmisartan showed lower microglial activation than WT mice injected with MPTP + telmisartan + GW9662. No significant difference was observed between mice treated with vehicle and mice treated with telmisartan alone, or GW9662 alone, or telmisartan + GW9662 (Figures 5 and 6A).

In AT1-null mice injected with MPTP (group B/C2), microglial activation was higher than in AT1-null mice injected with vehicle, but significantly lower than in AT1-null mice treated with MPTP and the PPAR-γ antagonist GW9662. No significant difference was observed between AT1-null mice treated with vehicle and AT1-null mice treated with GW9662 alone (Figures 5F, H and 6B).

Discussion

The present results show that, in mice, oral treatment with the ARB telmisartan protects nigral DA neurons against the DA neurotoxin MPTP as previously reported for other ARBs, such as candesartan and losartan [11,12]. This suggests that brain endogenous AII increases the neurotoxic effect of MPTP on the DA system, as observed in several previous studies, and that the AT1 blocker telmisartan inhibits the enhancing effect of AII

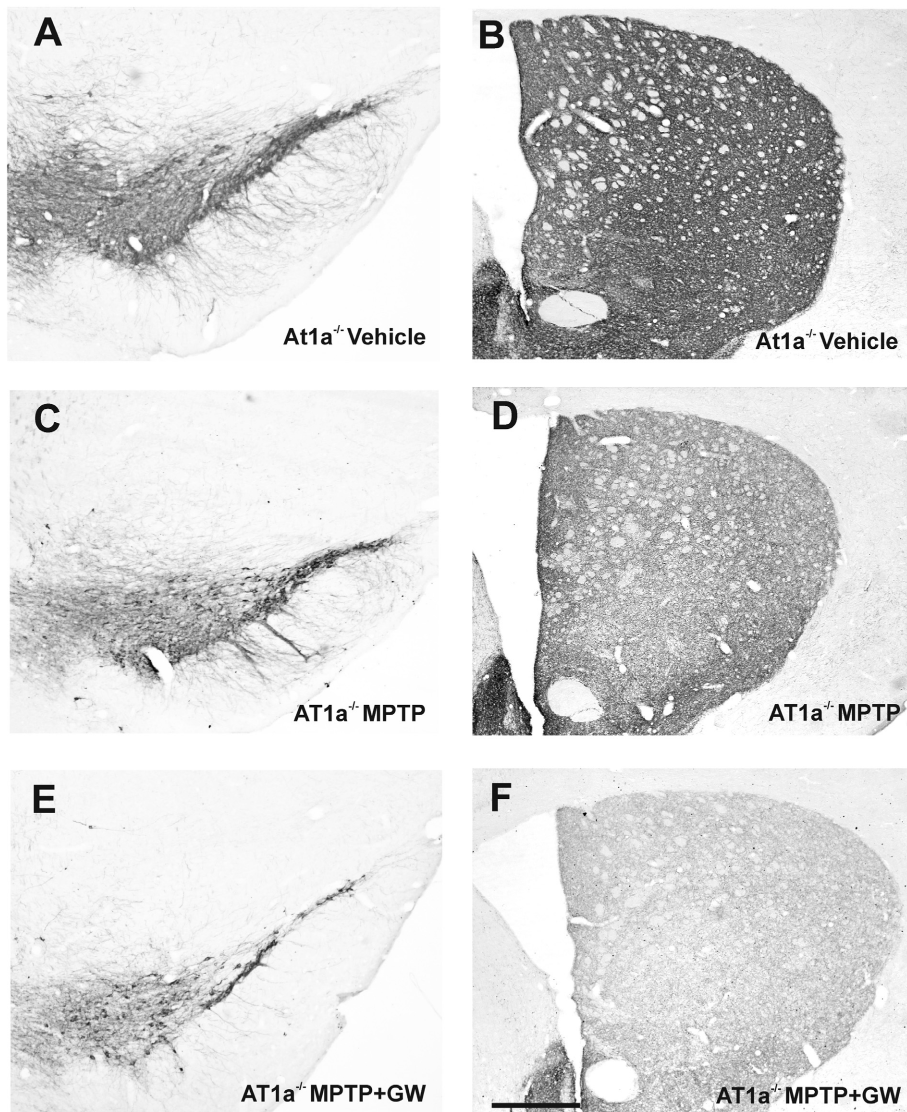

Figure 3 Changes in TH-ir in the substantia nigra and striatum in AT1a-null mice. TH-ir at central levels of the **(A, C, E)** substantia nigra and **(B, D, F)** striatum in AT1a-null mice ($AT1a^{-/-}$) injected with vehicle (controls; A, B), with MPTP alone (C, D), or with MPTP + the PPAR-γ antagonist GW9662 (E, F). The number of TH-ir cells in the nigra and TH-ir terminals in the striatum (that is, spared dopaminergic neurons and terminals) was higher in the untreated group (C, D) than in mice treated with GW9662 (E, F). Scale bar: 250 μm (A, C, E) and 560 μm (B, D, F).

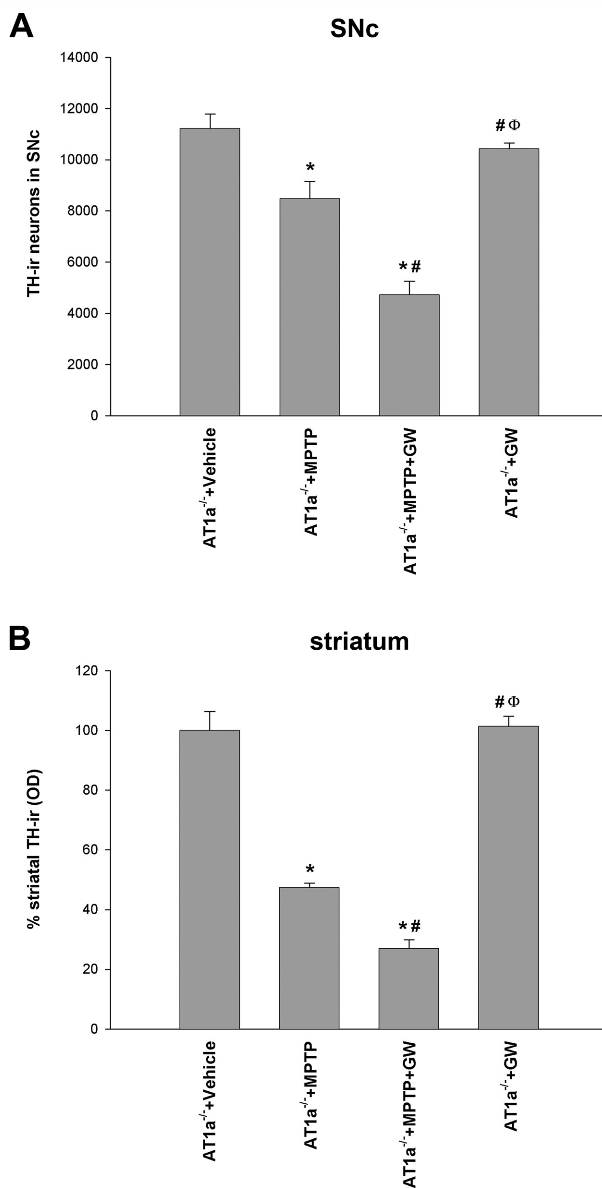

Figure 4 DA (TH-ir) neurons and terminals in AT1a-null mice. TH-ir neurons in the **(A)** SNc and **(B)** TH-ir terminals in the striatum one week after treatment with vehicle, GW9662 alone, MPTP alone, or MPTP + GW9662 in AT1a-null mice ($AT1a^{-/-}$). The DA neurons were quantified as the total number of TH-ir neurons in the SNc, and density of striatal DA terminals was estimated as optical density and expressed as a percentage of the value obtained in the group treated with vehicle. Data are presented as mean ± SEM. *$P < 0.05$ compared with mice treated with vehicle, $^{\#}P < 0.05$ compared with AT1a-null mice treated with MPTP alone, $^{\Phi}P < 0.05$ compared with mice treated with MPTP + GW9662 (one-way ANOVA and Student-Newman-Keuls post-hoc test).

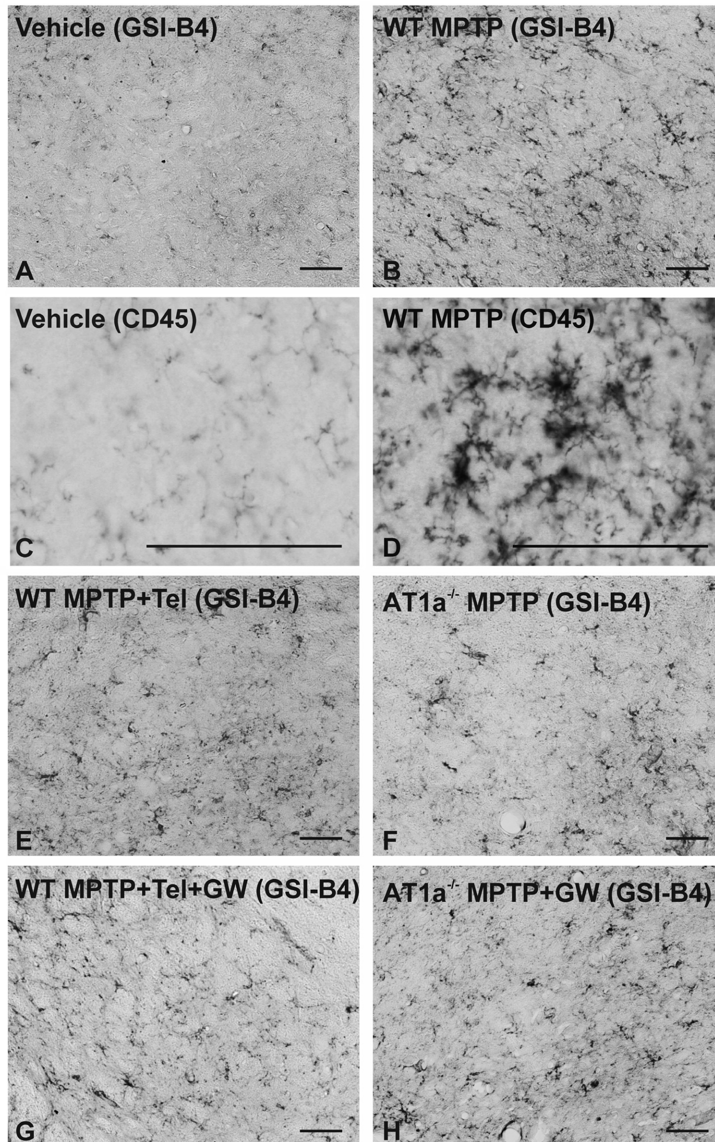

Figure 5 Photomicrographs showing changes in microglial activation in the substantia nigra. Activated microglial cells at central levels of the substantia nigra stained with **(A, B, E-H)** isolectin B4 or **(C, D)** immunostained for CD45 and higher magnification. Photomicrographs show microglia in WT mice treated with vehicle (controls; A, C), with MPTP alone (B, D), with MPTP + telmisartan (E), or with MPTP + telmisartan + GW9662 (G). Microglial activation in AT1a-null mice (AT1a$^{-/-}$) treated with MPTP alone or MPTP + GW9662 is shown in F and H, respectively. Microglial activation was significantly higher in mice treated with MPTP alone (B, D), and in mice treated with the neurotoxin, AT1 inhibition and GW9662 (G, H). Scale bar: 100 μm.

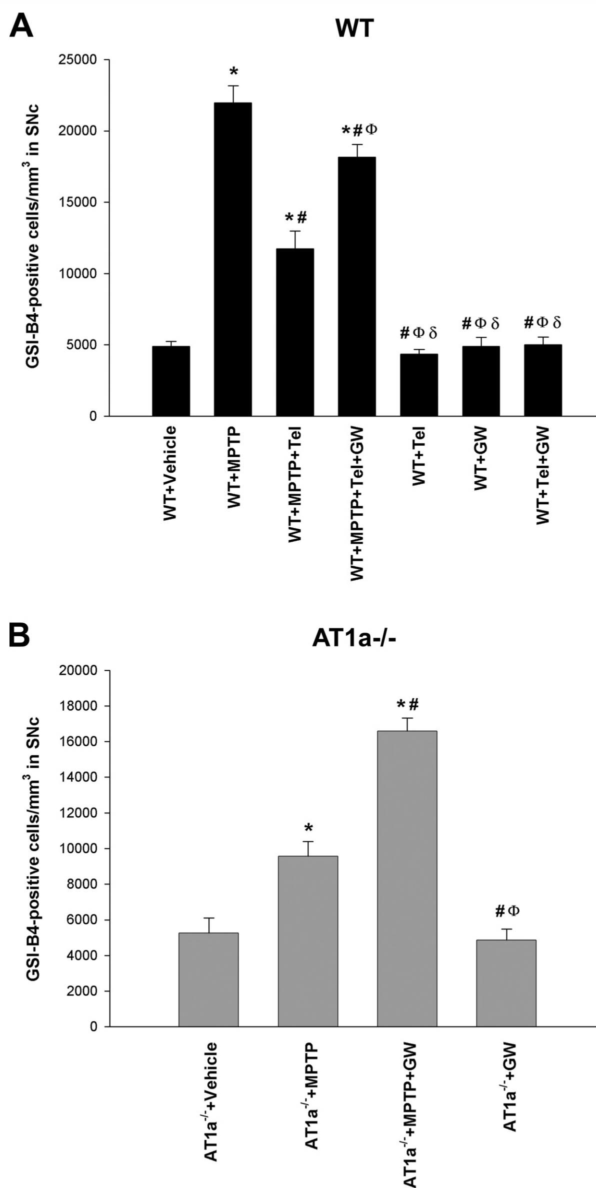

Figure 6 Activated microglial cells in the SNc. Density of GSI-B4-positive cells one week after treatment with **(A)** vehicle, telmisartan alone, GW9662 alone, telmisartan + GW9662, MPTP alone, MPTP + telmisartan, or MPTP + telmisartan + GW9662 in WT mice, and **(B)** vehicle, GW9662 alone, MPTP alone, or MPTP + GW9662 in AT1a-null mice (AT1a$^{-/-}$). The microglial cells were quantified as the number of cells per mm^3, and the data are presented as mean ± SEM. *$P < 0.05$ compared with WT mice (A) or AT1a-null mice (B) treated with vehicle, $^{\#}P < 0.05$ compared with WT mice (A) or AT1a-null mice (B) treated with MPTP alone, $^{\Phi}P < 0.05$ compared with WT mice treated with MPTP + telmisartan (A) or AT1a-null mice (B) treated with MPTP + GW9662, $^{\delta}P < 0.05$ compared with WT mice treated with MPTP + telmisartan + GW9662 (one-way ANOVA and Student-Newman-Keuls post-hoc test).

on DA cell death. However, the protective effects of telmisartan were inhibited by co-administration of the PPAR-γ antagonist GW9662, which suggests that PPAR-γ activation is necessary for the neuroprotective effects of telmisartan to occur. This neuroprotective effect may be expected since telmisartan has been shown to be a potent AT1 blocker and to penetrate the blood-brain barrier to inhibit centrally mediated effects of AII [33,40]. However, the mechanism responsible for this neuroprotection has not been clarified. A first possibility is that the pharmacological PPAR-γ activating properties of ARBs are the only mechanism involved in the neuroprotective effect. Several studies have shown PPAR-γ activating properties of candesartan and losartan, and that among ARBs, telmisartan is the most potent agonist of PPAR-γ [19-21]. The present results are consistent with a major role of PPAR-γ activation as the data show that the protective effect of telmisartan was inhibited by co-administration of the PPAR-γ antagonist GW9662.

However, the present study shows that pharmacological PPAR-γ activating properties of ARBs are not the only factor responsible for neuroprotection; the results obtained with mice deficient in AT1 show that, independently of any pharmacological effect of ARBs, AT1 inhibition induces significant neuroprotection of DA neurons against neurotoxins such as MPTP. In fact, the neuroprotective effect of telmisartan against MPTP did not appear higher than that previously observed with candesartan [11], which has a less potent AT1-independent PPAR-γ agonistic effect [19-21]; this also suggests that there is no significant 'additional effect' of AT1 blockage and pharmacological PPAR-γ activating properties of ARBs. It is possible that the present experimental design was not able to reveal any possible additional effect. However, it may be also related to the PPAR-γ activating effect of the AT1 deletion observed in the present study; we observed that administration of GW9662 significantly increased the MPTP-induced DA neuron death in AT1 deficient mice, which suggests that PPAR-γ activation plays a major role in the neuroprotective effects of AT1 inhibition.

The results therefore suggest that inhibition of AT1 with ARBs, and with telmisartan in particular, leads to activation of PPAR-γ by a double mechanism that involves a pharmacological AT1-independent PPAR-γ agonistic effect (with more or less activation potency depending on the type of ARB) and a direct effect of the blockage of the AT1 itself, which also induces PPAR-γ activation. An important degree of crosstalk between RAS and PPAR-γ has been suggested in several studies carried out in different tissues [41,42]. It has been observed that treatment with AII inhibited PPAR-γ expression and the anti-inflammatory defense mechanisms in the artery wall [43,44]. In addition, inhibition of ACE led to enhanced expression of PPAR-γ in adipose tissue and skeletal muscle cells [45,46]. It has been suggested that AII inhibits PPAR-γ activation via AT1 and enhances PPAR-γ activation via AT2 receptors [42,47], and that AT2 receptors may gain functional importance during selective AT1 blockage by a redirection of the available AII to the AT2 receptor [47,48]. Conversely, a number of studies have suggested that PPAR-γ may modulate RAS and AII signaling at multiple levels [43]. PPAR-γ activators have been observed to induce down-regulation of AT1 expression [49-51] and ACE activity [52], and up-regulation of AT2 receptors [53].

Furthermore, other studies have shown that PPAR-γ and other PPARs may inhibit NADPH oxidase activity and other signaling pathways involved in AII-induced oxidative stress and inflammation [54,55]. This may explain not only the complete inhibition of the neuroprotective effect of telmisartan by the PPAR-γ antagonist GW9662, observed in the present study, but also the GW9662-induced inhibition of the neuroprotective effect of AT1 deletion in the AT1a-null mice. It is known that AII, via the AT2 receptor, exerts actions directly opposed to those mediated by AT1, thus antagonizing many of the effects of the latter [56,57]. In AT1a-null mice, AII may act via AT2 receptors activating PPAR-γ and contribute to inhibition of inflammation and oxidative stress, which has been observed to promote longevity and inhibit progression of degenerative diseases in AT1-null mice [58-60]. The present results, which showed that the protective effects of AT1 inhibition were blocked by the treatment with the PPAR-γ antagonist GW9662, are consistent with the latter findings.

In the present study, we have also confirmed that the mechanism involved in the observed neuroprotection is similar to that observed in previous studies on neuroprotective properties of ARBs. In previous studies in animal models of PD, we have shown that inhibition of microglial activation plays a major role in the protective effects of ARBs against DA cell death induced by DA neurotoxins [11,13,15]. The present results, which suggest that both AT1 inhibition with telmisartan and AT1a deletion inhibit the microglial response induced by MPTP in the substantia nigra, are consistent with this. Furthermore, the present results show a major role for the PPAR-γ activity in this effect, since treatment with the PPAR-γ antagonist GW9662 led to inhibition of the protective effect of telmisartan or AT1 deletion, as well as exacerbation of the microglial response induced by MPTP in the presence of AT1 inhibition. The present results are consistent with previous findings that showed that PPAR-γ activation down-regulates brain inflammation by inhibiting several functions associated with microglial activation [25,61], and that PPAR-γ agonists such as pioglitazone

and rosiglitazone protect against MPTP-induced DA cell death by inhibition of microglial activation [28,29,62].

The present results are also consistent with studies that have observed that ARBs decreased the infiltration of CNS [63,64] and peripheral organs [65] by inflammatory cells, although some conflicting results have been also reported [66]. In accordance with their inhibitory effect on brain inflammation, beneficial effects of PPAR-γ agonists or AT1 inhibition have also been observed in a number of processes mediated by microglial activation and neuroinflammation, including animal models of Alzheimer's disease [67-69], brain ischemia [40,70,71], multiple sclerosis [63,64,72], traumatic brain injury [73] and aging [15,59,74].

In several previous studies we have shown the presence of AT1, AT2 receptors and NADPH oxidase in microglia and also in DA neurons [11,13]. In accordance with these findings, inhibition of neuronal AT1 receptors may decrease NADPH oxidase activity and NADPH oxidase-derived ROS in neurons, which may lead to direct inhibition of DA neuron death, followed by a subsequent reduction in microglial activation. However, our data do not suggest this possibility. In microglia and other inflammatory cells, NADPH oxidase produces ROS with dual functions. Firstly, high concentrations of ROS are released extracellularly to kill invading microorganisms or cells [75]. Secondly, low levels of intracellular ROS act as a second messenger in several signaling pathways involved in the inflammatory response [76,77]. In non-inflammatory cells, such as neurons, activation of NADPH oxidase stimulates production of low levels of intracellular ROS, which act as a second messenger in several signaling pathways, including those involved in triggering the inflammatory response and the migration of inflammatory cells into the lesioned area; NADPH oxidase-derived ROS may also modulate neuronal levels of ROS by interaction with mitochondrial derived ROS, and with ROS from other sources, such as neurotoxins or activated microglia. Cross-talk signaling between the NADPH oxidase and mitochondria has been observed in several types of cells. This includes not only an upstream role of NADPH oxidase in modulating of mitochondrial superoxide [78,79] but also that mitochondrial superoxide stimulates extramitochondrial NADPH oxidase activity in a feed-forward fashion [80,81]. This interaction was recently confirmed in a DA cell line treated with MPP^+ and angiotensin [82]; MPP^+ induced mitochondrial release of ROS, which induced a second wave of NADPH oxidase-derived ROS, which was reduced by treatment with the AT1 antagonist candesartan [82]. Using primary cultures of mesencephalic cells, we have previously shown that mitochondrial ATP-sensitive potassium channels play a major role in the interaction between NADPH-derived ROS and mitochondria after treatment with AII and/or DA neurotoxins such as MPP^+ or 6-hydroxydopamine [83,84].

However, we have also observed that only high doses of neurotoxins can induce DA neuron death in neuron-enriched primary mesencephalic cultures [11,13,83-85]. This was confirmed in a recent study using a DA cell line [82], in which significant DA cell death was only observed after treatment with very high doses of MPP^+ (300 μM). Interestingly, we observed that the effect of very low or sub-lethal doses of neurotoxins (0.25 μM MPP^+ or 10 μM 6-hydroxydopamine; in other words, more similar to the possible effect of environmental neurotoxins or other factors involved in PD) was enhanced by AII and induced significant DA cell death in mixed neuron-glia cultures but not in pure neuronal cultures (that is, in the absence of microglia) [11,13,83-85]. This suggests that although AII and ARBs may contribute to the modulation of intraneuronal ROS and neuronal release of pro-inflammatory signals, the microglial response plays a major role in the DA neuron death induced by low doses of neurotoxins, or other deleterious factors. The major role of ARBs inhibition of microglial reaction in reducing DA neuron death (rather than an ARBs-induced reduction in DA cell death resulting in a decreased microglial response) was also confirmed *in vivo* by the observation of an intense microglial response soon after a single injection of MPTP or 6-hydroxydopamine (that is, prior to significant DA neuron death), which was inhibited by treatment with ARBs [11,13,86,87]. The present study shows that ARBs-induced PPAR-γ activation plays a major role in this effect.

Conclusion

The results of the present study show that oral administration of telmisartan produces effective neuroprotection against DA cell death induced by MPTP, as previously observed for candesartan, and that the neuroprotective effect is mediated by PPAR-γ activation. Furthermore, the results in AT1-deficient mice show that the deletion of AT1, which is unrelated to the pharmacological properties of ARBs, protects against the DA neurotoxin, and that the protective effects of AT1 deletion are also inhibited by PPAR-γ blockage. The results suggest that inhibition of AT1 with ARBs, and with telmisartan in particular, leads to activation of PPAR-γ by a double mechanism that involves a pharmacological AT1-independent PPAR-γ agonistic effect (with more or less activation potency depending on the type of ARB) and a direct effect of the blockage of the AT1 itself, which also induces PPAR-γ activation.

Abbreviations

ANOVA: analysis of variance; ACE: angiotensin-converting enzyme; AII: angiotensin II; ARBs: AT1 blockers; AT1: angiotensin type 1 receptor; $AT1a^{-/-}$: AT1a-null mice; AT2: angiotensin type 2 receptor; BSA: bovine serum

albumin; DA: dopaminergic; DOPAC: 3,4-dihydroxyphenylacetic acid; EDTA: ethylenediaminetetraacetic acid; GSI-B4: *Griffonia simplicifolia* isolectin B4; HPLC: high performance liquid chromatography; HVA: homovanillic acid; KPBS-BSA: potassium PBS containing 1% BSA; MPP^{+}: 1-methyl-4-phenylpyridinium; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NADPH: nicotinamide adenine dinucleotide phosphate; PBS: phosphate-buffered saline; PD: Parkinson's disease; PPAR γ: peroxisome proliferator-activated receptor gamma; RAS: renin-angiotensin system; ROS: reactive oxygen species; SEM: standard error of the mean; SNc: substantia nigra compacta; TH: tyrosine hydroxylase; TH-ir: TH-immunoreactivity; WT: wild-type.

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Authors' contributions
PG-G conducted the experiments, participated in the statistical analysis and drafted the manuscript. BJ performed the histological study. AIR-P performed the stereological analysis and participated in the statistical analysis. MJG and JLL-G conceived the study and its design, supervised the project and edited the manuscript preparation. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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4.4- Papel de los canales de potasio mitocondriales dependientes de ATP en la toxicidad de la 6-hidroxidopamina sobre las neuronas dopaminérgicas

J. Rodriguez-Pallares, J.A. Parga, **B. Joglar**, M.J. Guerra y J.L. Labandeira-Garcia (2009) The Mitochondrial ATP-Sensitive Potassium Channel Blocker 5-Hydroxydecanoate Inhibits Toxicity of 6-Hydroxydopamine on Dopaminergic neurons. Neurotox Res 15: 82-95.

La 6-OHDA es una neurotoxina comúnmente usada en modelos de EP y un factor potencial en la patogénesis de la enfermedad. Sin embargo, los mecanismos responsables de la degeneración dopaminérgica inducida por dicha neurotoxina no están totalmente claros. Sí es sabido que las EORs derivadas de la captación y de la autooxidación tanto intraneuronal como extracelular de la 6-OHDA, así como la activación microglial están implicadas. Sin embargo, la implicación mitocondrial es un punto de controversia, aunque se cree que podría ser una diana donde convergieran y se integraran los diferentes mecanismos. En este estudio hemos observado tanto en cultivos primarios mesencefálicos como en cultivos enriquecidos en neuronas que el bloqueo de los canales mitok(ATP) con el antagonista 5-HD, inhibe la degeneración dopaminérgica inducida por bajas dosis de 6-OHDA. Además de esto, bloquea el descenso del potencial de membrana interna mitocondrial e inhibe la generación de superóxido derivado de las EORs en las neuronas dopaminérgicas, ambos inducidos por la 6-OHDA. Estos resultados sugieren que bajas dosis de 6-OHDA podrían generar bajos niveles de EORs a través de varios mecanismos, los cuales serían insuficientes para inducir la muerte neuronal por sí solos, pero suficientes para actuar como desencadenantes de la activación de los canales mitoK(ATP), potenciando de este modo la producción de más EORs que tendrían un papel clave en el efecto sinérgico que conllevaría a la degeneración dopaminérgica. Estos resultados aportan nuevos datos para considerar a los canales mitoK(ATP) como una diana potencial para neuroprotección en la EP.

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The Mitochondrial ATP-Sensitive Potassium Channel Blocker 5-Hydroxydecanoate Inhibits Toxicity of 6-Hydroxydopamine on Dopaminergic Neurons

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Abstract The neurotoxin 6-hydroxydopamine is commonly used in models of Parkinson's disease, and a potential factor in the pathogenesis of the disease. However, the mechanisms responsible for 6-hydroxydopamine-induced dopaminergic degeneration have not been totally clarified. Reactive oxygen species (ROS) derived from 6-OHDA uptake and intraneuronal autooxidation, extracellular 6-OHDA autooxidation, and microglial activation have been involved. The mitochondrial implication is controversial. Mitochondrial ATP-sensitive K (mitoK(ATP)) channels may provide a convergent target that could integrate these different mechanisms. We observed that in primary mesencephalic cultures and neuron-enriched cultures, treatment with the mitoK(ATP) channel blocker 5-hydroxydecanoate, inhibits the dopaminergic degeneration induced by low doses of 6-OHDA. Furthermore, 5-hydroxydecanoate blocks the 6-OHDA-induced decrease in mitochondrial inner membrane potential and inhibits 6-OHDA-induced generation of superoxide-derived ROS in dopaminergic neurons. The results suggest that low doses of 6-OHDA may generate low levels of ROS through several mechanisms, which may be insufficient to induce neuron death. However, they could act as a trigger to activate mitoK(ATP) channels, thereby enhancing ROS production and the subsequent dopaminergic degeneration. Furthermore, the present study provides additional data for considering mitoK(ATP) channels as a potential target for neuroprotection.

Keywords 6-OHDA · Mitok(ATP) channels · Dopamine · Parkinson's disease · Oxidative stress · Mitochondria · Neurodegeneration

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Introduction

The neurotoxin 6-OHDA was the first dopaminergic (DA) neurotoxin discovered and is the most commonly used to produce in vivo and in vitro models of Parkinson's disease (PD; see Simola et al. 2007). Furthermore, 6-OHDA has been proposed as a putative neurotoxic factor in the pathogenesis of PD (Irwin and Langston 1995; Jellinger et al. 1995). Although oxidative stress (OS) is clearly involved, the mechanisms responsible for 6-OHDA-induced DA cell death have not been totally clarified. It was initially considered that the specific action of 6-OHDA on DA neurons (and noradrenergic neurons) is due to the uptake and accumulation of the toxin by the catecholamine transporter in the cytosol of these cells (Luthman et al. 1989; Gonzalez-Hernandez et al. 2004). The formation of reactive oxygen species (ROS) by autooxidation of 6-OHDA in the cytosol is considered to be the main molecular mechanism underlying the neurotoxicity of 6-OHDA (Sachs and Jonsson 1975; Kumar et al. 1995; Soto-Otero et al. 2000). However, the mechanisms involved in 6-OHDA toxicity appear more complex. It has been observed that extracellular generation of ROS by 6-OHDA autooxidation also plays a major role in DA cell death (Abad et al. 1995; Blum et al. 2000; Berretta et al. 2005; Hanrott et al. 2006). Extracellular

autooxidation of high doses of 6-OHDA may lead to non-selective effects on non-DA cells (Dodel et al. 1999; Lotharius et al. 1999; Berretta et al. 2005). In addition, we have recently observed that microglial activation and glial NADPH-oxidase-derived ROS are also involved in causing cell death induced by low doses of 6-OHDA (Rodriguez-Pallares et al. 2007). Conflicting data are apparently related to the use of different doses and different cells (i.e., mesencephalic cultures or cell lines), and low doses and nigral neurons appear particularly useful to ensure specificity and to study interactions between different toxic mechanisms, as well as possible synergistic effects.

Reports on the involvement of mitochondria in 6-OHDA toxicity are controversial (Glinka and Youdim 1995; Wu et al. 1996; Glinka et al. 1997; Storch et al. 2000), and the interactions between 6-OHDA-derived ROS and mitochondria have not been clarified. It has been reported that mitochondrial ROS production can be stimulated through the opening of mitochondrial ATP-sensitive potassium channels (mitoK(ATP); Kimura et al. 2005b), and that ROS, and particularly H_2O_2, open K(ATP) channels in several cell types (Zhang et al. 2002; Liu and Gutterman 2002; Avshalumov and Rice 2003). Therefore, it is possible that these channels provide a convergent target that could integrate 6-OHDA-derived intracellular and extracellular ROS/H_2O_2 induced by 6-OHDA and mitochondrial involvement. However, the possible involvement of mitoK(ATP) channels in 6-OHDA toxicity has not been investigated. In the present study, we treated primary mesencephalic cultures with 6-OHDA in order to study the effects of inhibition of mitoK(ATP) channels on 6-OHDA-induced DA cell death and on levels of superoxide/superoxide-derived ROS in DA neurons. Neuron-enriched cultures were used to investigate possible indirect effects via mitoK(ATP) channels of glial cells (Thomzig et al. 2001; Zhou et al. 2002). We also studied possible changes induced by 6-OHDA in mitochondrial inner membrane potential ($\Delta\psi_M$) in DA neurons and the effect of inhibition of mitoK(ATP) channels on these changes.

Experimental Procedures

Primary Mesencephalic Cultures

Ventral mesencephalic tissue was dissected from rat embryos of 14 days of gestation (E14). The tissue was incubated in 0.1% trypsin (Sigma), 0.05% DNase (Sigma, St. Louis, MO, USA), and DMEM (Gibco, Invitrogen, Paisley, Scotland, UK) for 20 min at 37°C, and then washed in DNase/DMEM and mechanically dissociated. The resulting cell suspension was centrifuged at 50*g* for 5 min, the supernatant was carefully removed and the pellet resuspended in 0.05% DNase/DMEM to the final volume required. The number of viable cells in the suspension was estimated with acridine orange/ethidium bromide, and cells were plated onto 35-mm culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) previously coated with poly-L-lysine (100 μg/ml; Sigma) and laminin (4 μg/ml; Sigma). The cells were seeded at a density of 1.5×10^5 cells/cm^2 and maintained in control conditions [DMEM/HAMS F12/(1:1) containing 10% fetal bovine serum (FBS; Biochrom KG, Berlin, Germany)]. The cell cultures were maintained in a humidified CO_2 incubator (5% CO_2; 37°C) for 7 days in vitro (DIV; see below); the medium was totally removed on day 2 and replaced with fresh culture medium.

Treatment of Cultures and Experimental Design

Cultures were exposed on 6 DIV to 6-OHDA (10, 20, 30, 100 μM, in 0.02% saline ascorbate; Sigma) alone, 6-OHDA and 5-hydroxydecanoate (5-HD; a specific blocker of mitoK(ATP) channels, Sigma), or 5-HD alone for 24 h (or 3 h for some Mito Tracker Orange or dihydroethidium studies, see below). Several doses of 5-HD (10, 100, 500 μM) were tested. The lowest dose that proved effective (10 μM) was used in most experiments to prevent potential non-specific side effects of high doses of 5-HD (Wu et al. 2006; Yamauchi et al. 2003). In addition, some cultures were treated with a second K(ATP) channel blocker (glibenclamide, 10 μM, Sigma) or the K(ATP) channel opener diazoxide (10 μM) to confirm the involvement of K(ATP) channels in 6-OHDA-induced cell death. It has been shown that low concentrations of diazoxide (1–10 μM) are specific in opening mitoK(ATP) channels, and that higher concentration (>10 μM) of diazoxide activates cell membrane K(ATP) channels, and much higher concentrations (16–164 μM) may induce other effects such as succinate dehydrogenase inhibition (see Garlid et al. 1997; Yamauchi et al. 2003).

The cells were then washed and processed for immunolabeling as detailed below. To obtain neuron-enriched cultures, cytosine-β-D-arabino-furanoside (Ara-C; 1μM; Sigma) was added 24 h after seeding the cells. The cultures were then treated with 6-OHDA (30μM) or 6-OHDA and 5-HD as above. This method can enrich neurons to >85% purity (Michel et al. 1997; Gao et al. 2003b). Some control cultures and cultures treated with 6-OHDA, 6-OHDA and 5-HD, or 5-HD alone were treated with chloromethyltetramethylrosamine methyl ester (CMTMR; Mito Tracker Orange; Molecular Probes, Eugene, OR) or dihydroethidium (DHE) to estimate the mitochondrial inner membrane potential ($\Delta\psi_M$) or levels of superoxide/superoxide-derived ROS, respectively, in DA neurons, as detailed below. Furthermore, the hyperpolarizing effect of 5-HD was

confirmed in live cell cultures with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Invitrogen), as described below. Finally, some cultures were pre-treated with the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma, 10 μM), to confirm that the increase in CMTR or JC-1 retention is not due to non-specific effects and is dependent on $\Delta\psi_M$ (see below). The protonophore CCCP is often used to dissipate the membrane potential and to define the baseline for the analysis of mitochondrial membrane potential with fluorescent dyes (see Gottlieb et al. 2003; Brown et al. 1996).

Immunohistochemistry and Double-Fluorescence Labeling

Cultures were fixed with 4% paraformaldehyde in Dulbecco's phosphate buffered saline (DPBS; pH 7.4) for 20 min, and then incubated at 4°C with a mouse monoclonal anti-tyrosine hydroxylase (TH; Sigma; 1:30000) as DA marker. Cultures were then washed and incubated for 1 h with biotinylated horse anti-mouse (Vector; Burlingame, CA, USA) diluted 1:500, and then incubated for 90 min with avidin–biotin–peroxidase complex (ABC, Vector, 1:500). Finally, the labeling was revealed with 0.04% hydrogen peroxide and 0.05% 3,3′diaminobenzidine (Sigma) as chromogen (see Rodriguez-Pallares et al. 2007 for details).

Cultures grown on glass coverslips were processed for double-fluorescence labeling for calbindin, DHE or CMTMR (Mito Tracker Orange), and TH. Calbindin labeling was used to identify different populations of DA neurons (i.e., A10 neurons as TH-positive/calbindin-positive, and A9 neurons as TH-positive/calbindin-negative). For calbindin, cultures were incubated overnight at 4°C with primary antibodies anti-TH (1:30,000) and anti-calbindin (Swant; Bellinzona, Switzerland; 1:50,000). After rinsing with DPBS, the cultures were incubated for 150 min with the secondary antibodies [goat anti-mouse (Chemicon; 1:100) conjugated with fluorescein isothiocyanate (FITC) for TH, and goat anti-rabbit (Chemicon; 1:250) conjugated with cyanine 3.18 (Cy3) for calbindin (see below for DHE and CMTMR (Mito Tracker Orange). Co-localization of markers was confirmed by confocal laser microscopy (TCS-SP2; Leica, Heidelberg, Germany) and use of a sequential scan method to avoid any possible overlap. In all experiments the control cultures, in which the primary antibody was omitted, were immunonegative for these markers.

Detection of Intracellular Superoxide Anion/Superoxide-Derived ROS with Dihydroethidium (DHE)

Intracellular DHE is oxidized to ethidium, which binds DNA and stains nuclei bright fluorescent red. Cultures grown on glass coverslips were incubated with a fresh working solution containing 5 μM DHE (Sigma) in sterile phosphate buffered saline (PBS; pH 7.4) for 30 min at 37°C. The cultures were washed, then fixed and processed for immunofluorescence against TH (see above), and visualized with a Nikon Optiphot microscope coupled to an epifluorescence system or a Leica laser confocal microscope, and the ethidium fluorescence intensity was measured in DA neurons as described below.

Estimation of $\Delta\Psi_M$ with Chloromethyl-Tetramethylrosamine Methyl Ester (CMTMR; Mito Tracker Orange) and 5,5′,6,6′-Tetrachloro-1,1′,3,3′-Tetraethylbenzimidazolylcarbocyanine Iodide (JC-1)

CMTMR (Mito Tracker) enters mitochondria of living cells in proportion to the negative charge difference between the cytoplasm and the mitochondrial matrix and therefore estimates $\Delta\Psi_M$. Lipophilic cations accumulate in the mitochondrial matrix, driven by the $\Delta\Psi_M$ in accordance with the Nernst equation, which predicts that every 61.5 mV increase in membrane potential causes a 10-fold increase in accumulation of the membrane-permeant cation. CMTMR binds irreversibly to mitochondrial matrix thiols and can be fixed for immunocytochemical localization of proteins in the same cells that have been previously exposed to CMTMR. As a result of the thiol binding, CMTMR fluorescence represents the highest level of negativity difference in the mitochondria during exposure to the dye before fixation (Wadia et al. 1998; Sugrue et al. 1999). The $\Delta\Psi_M$ in fixed cells was estimated as follows (Wadia et al. 1998; Sugrue et al. 1999). Three hours after exposure to treatments, the medium in each well was supplemented with 50 nM CMTMR and incubated at 37°C for 15 min. The CMTMR-containing media was removed and the cells were rinsed with cold DPBS, followed by immediate fixation with 4% paraformaldehyde for 10 min. After fixation, the cells were washed briefly in DPBS and incubated for 20 min with a mouse monoclonal anti-TH (Sigma, 1:6,000) containing normal goat serum and 0.3% Triton X-100 diluted in DPBS-BSA. Cultures were then washed and incubated for 15 min with goat anti-mouse secondary antibody conjugated with fluorescein isothiocyanate. The coverslips were mounted onto microscope slides with 1,4-diazabicyclo [2.2.2] octane (DABCO, Sigma). CMTMR fluorescence was visualized with a laser scanning confocal microscope (TCS-SP2; Leica, Heildelberg, Germany), equipped with a 63× oil immersion 1.4 numerical aperture (NA) objective. All cells were visualized at the same level of laser intensity, detector sensitivity, and pinhole size in order to ensure that CMTMR fluorescence intensity could be compared among different coverslips and treatments. The images were saved as 8-bit TIFF files and the

cytoplasmic fluorescence intensity was evaluated by digital image processing with ImageJ software (NIH, Bethesda). The TH staining was used to manually delineate the cytoplasm of dopaminergic cells. All measurements were corrected for background fluorescence.

JC-1 is a cationic carbocyanine dye that accumulates in mitochondria. The dye exists as a monomer at low concentrations and yields green fluorescence. At higher concentrations, the dye forms J-aggregates that exhibit a broad excitation spectrum and an emission maximum at 590 nm. The JC-1 dye is only uptaken up by viable cells and it exhibits a spectral shift from green to red in healthy mitochondria with polarized membranes. The JC-1 assay was performed to estimate the $\Delta\Psi_M$ in living cells, according to Zhang et al. (2006) as follows. Cells were plated in 96 well plates at 10^5 cells per well. After treatment with 5-HD, JC-1 (in DMSO) was added to each well to a final concentration of 3 μM and incubated for 30 min at 37°C, with 5% CO_2, in the dark. The medium was removed and the cells washed twice with DPBS. Fluorescence intensity was measured in a multifunctional microplate reader (TECAN Infinite 200, Austria) at excitation 485 nm, emission 525 and 595 nm, for detection of the green and red substrate, respectively. The fluorescence signal represents the average signal of the total cell population. The mitochondrial membrane potential is shown as the ratio between the fluorescence of aggregate (red) and monomer (green) forms of JC-1. This ratio is dependent only on the mitochondrial membrane potential, and not on the number of cells, mitochondrial size, shape, or density.

Cell Counting

TH-ir cells, and the percentage of TH-ir cells that coexpressed calbindin or ethidium, were counted in five randomly chosen longitudinal and transverse microscopic fields along the diameter of the culture dish, away from the curved edge. The operator was blind to the treatment condition. The microscopic field was defined by a 0.5 × 0.5 cm reticule (i.e., a total of 1.25 cm^2). The average number of TH-positive cells in a control culture dish was 1679 ± 83. To assess the intensity of CMTMR fluorescence in cultures labeled for these markers, a minimum of 50 TH-ir cells were counted in random visual fields. The results from at least three separated experiments were recorded, with a minimum sample size of four dishes per group and per run. The results were normalized to the counts of the control group of the same batch (i.e., expressed as a percentage of the control group counts) to counteract possible variability among batches. Statistical differences between groups were tested as described below.

[^{3}H]DA-Uptake

The culture medium was completely removed and cultures were rinsed twice with 1 ml of uptake buffer (Krebs buffer + 1.8 mM $CaCl_2$ + 25 mM D-glucose). Cells were then incubated for 30 min at 37°C with 1 ml of uptake buffer (containing 1 mM ascorbic acid and 100 μM pargyline; pH 7.4) in the absence (untreated cultures) or presence of 10 μM 5-HD. Uptake was initiated by addition of 20 nM [^{3}H]DA ([2,5,6-^{3}H] dopamine; 1 μCi, 12 Ci/mmol; Amersham Biosciences, GE Healthcare, Buckinghamshire, UK) in 20 μl of Krebs buffer. Non-specific uptake values were defined in the presence of 10 μM GBR 12935, a specific inhibitor of the DA transporter. Uptake was stopped after 30 min at 37°C incubation by removal of the incubation mixture and the cells were washed twice with cold Krebs buffer. Cells were lysed with 1 ml of 2 N NaOH for 30 min at room temperature, and the radioactivity incorporated into cells was measured by liquid scintillation spectrometry. Results are expressed as percentages of untreated control culture responses.

Statistical Analysis

All data were obtained from at least three independent experiments and were expressed as mean ± SEM. Multiple comparisons were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. The normality of populations and homogeneity of variances were tested before each ANOVA. Differences were considered as statistically significant at $P < 0.05$. Statistical analyses were carried out with SigmaStat 3.0 from Jandel Scientific (San Rafael, CA, USA).

Results

Effect of 5-HD Treatment on 6-OHDA-Induced Degeneration of TH-ir Neurons in Primary Mesencephalic Cultures

Cultures treated with a low doses of 6-OHDA (20 or 30 μM) for 24 h contained significantly less TH-ir cells than the control cultures ($P < 0.001$ for 20 μM or 30 μM 6-OHDA vs. control). Treatment with 5-HD (10 μM) blocked the 6-OHDA-induced decrease in TH-ir cells ($P = 0.033$ for 20 μM 6-OHDA vs. 6-OHDA + 10 μM 5-HD and $P < 0.001$ for 30 μM 6-OHDA vs. 6-OHDA + 10 μM 5-HD). Cultures treated with a high doses of 6-OHDA (100 μM) for 24 h showed a marked loss of TH-ir cells relative to the control cultures (around 80% decrease; $P < 0.001$). Treatment with 100 μM 6-OHDA + 10 μM 5-HD induced a significant reduction

in the loss of TH-ir neurons with respect to cultures treated with 100 μM 6-OHDA alone ($P = 0.026$), although the number of neurons was markedly lower than in control cultures (around 60% decrease, $P < 0.001$). No significant difference in the number of TH-ir neurons was observed after treatment with 5-HD or glybenclamide alone (Fig. 1a–f). High doses of 5-HD (100 μM and 500 μM) induced protective effects on cell death induced by 6-OHDA similar to those observed with 10 μM 5-HD (i.e., reduction of DA cell death induced by 20 μM 6-OHDA to control levels; $P = 0.048$ for 20 μM 6-OHDA vs. 6-OHDA + 100 μM 5-HD and $P = 0.020$ for 20 μM 6-OHDA vs. 6-OHDA + 500 μM 5-HD, Fig. 1g).

Since the protective effect of 5-HD was observed after treatment with low doses of 6-OHDA, the lowest doses that induced a significant loss of TH-ir neurons (20 μM 6-OHDA) and protective effects (10 μM 5-HD) in our cultures were used in the following experiments, except in neuron-enriched cultures, which required higher doses (30 μM 6-OHDA) to induce significant loss of TH-ir neurons (see Rodriguez-Pallares et al. 2007). Double labeling for TH and the calcium-binding protein calbindin revealed that loss of TH-ir cells induced by low doses of 6-OHDA (20 μM) was mostly at the expense of the subpopulation of calbindin-negative DA neurons ($P = 0.002$), and that 5-HD inhibited the loss of DA neurons in this population ($P = 0.048$; Figs. 1h, 4a–c). High doses of 6-OHDA (100 μM) induced a loss of 80% TH-ir neurons, which include both calbindin-negative and calbindin-positive TH-ir neurons. In the present and other studies, the population of calbindin-negative neurons is around 50% (40–50%) of the TH-positive population.

Fig. 1 Effects of treatment with different doses of 6-OHDA or 6-OHDA and 5-HD (10 μM) on the number of TH-ir cells. Treatment with low doses of 6-OHDA (20–30 μM) induced a significant loss of TH-ir neurons, which was blocked by 5-HD. However, high doses of 6-OHDA (100 μM) induced a marked loss of TH-ir cells, which was partially inhibited by simultaneous treatment with 5-HD (**a**–**g**). Double labeling for TH and the calcium-binding protein calbindin revealed that loss of TH-ir cells induced by 6-OHDA (20 μM) mainly occurred at the expense of the subpopulation of calbindin-negative DA neurons, and that 5-HD inhibited the loss of DA neurons in this population (**h**). No significant difference in the number of TH-ir neurons was observed after treatment with 5-HD alone. The data are expressed as percentages of the number of TH-ir cells obtained in the respective control cultures (100%). Data represent means ± SEM. *$P < 0.05$ compared with control group (untreated cells); #$P < 0.05$ compared with the corresponding 6-OHDA-treated group (one-way ANOVA and Bonferroni post hoc test; $n \geq 4$). *5-HD* 5-hydrodydecanoic acid, *6-OHDA* 6-hydroxydopamine, *DA* dopaminergic, *ir* immunoreactive, *TH* tyrosine hydroxylase. Scale bar: 100 μm

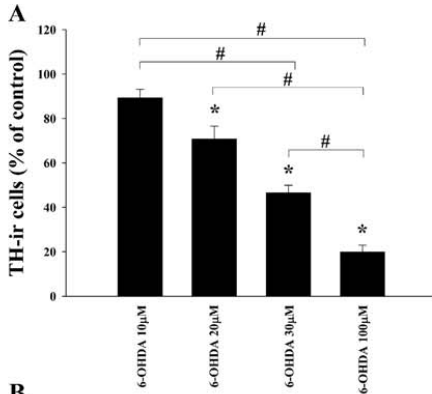
Effect of 5-HD Treatment on Generation of Intracellular Superoxide/Superoxide-Derived ROS in TH-ir Neurons

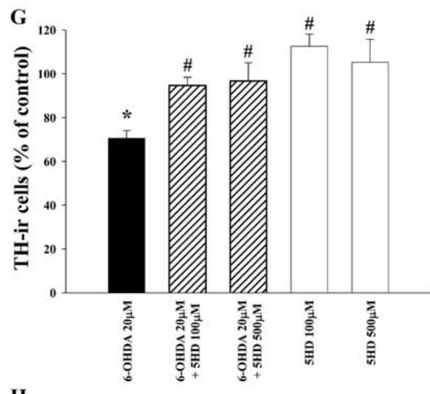
Detection of superoxide anion and superoxide-derived ROS in cultured cells was achieved by treatment with dihydroethidium (DHE), which is oxidized to ethidium, which binds to DNA in the nucleus and fluoresces red. Double labeling for TH and DHE revealed that treatment with low doses of 6-OHDA (20 μM) induced a significant increase in the intensity of ethidium fluorescence 3 h after treatment. Twenty-four hours after treatment with 20 μM 6-OHDA, the number of TH-ir neurons had decreased. However, the average of intensity of ethidium fluorescence remained significantly higher in TH-ir neurons treated with 6-OHDA than in TH-ir neurons from control cultures. The 6-OHDA-induced increase in ethidium fluorescence in DA neurons (i.e., superoxide anion and superoxide-derived ROS intraneuronal levels; 3 h or 24 h after 6-OHDA treatment) was inhibited by treatment with the mitoK(ATP) blocker 5-HD ($P < 0.001$ for 3 or 24 h; Figs. 2, 4d–f).

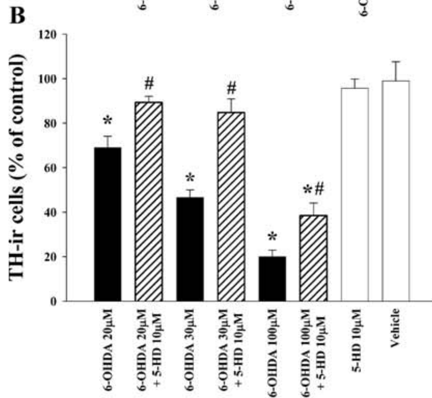
Effect of 5-HD Treatment on Changes Induced by 6-OHDA in Mitochondrial Inner Membrane Potential ($\Delta\psi_M$) in Dopaminergic Neurons

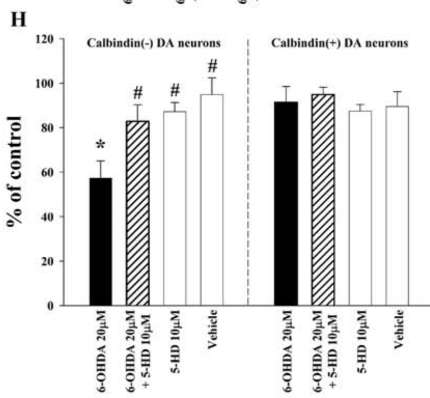
CMTMR (Mito Tracker) was used to estimate $\Delta\Psi_M$. CMTMR enters mitochondria in proportion to the negative charge difference between the cytoplasm and the mitochondrial matrix and binds irreversibly to mitochondrial matrix thiols. Double labeling for TH and CMTMR revealed that treatment of cultures with low doses of 6-OHDA (20 μM) induced a significant reduction (about 50%) in CMTMR fluorescence in DA neurons ($P < 0.001$), which revealed a marked decrease in $\Delta\Psi_M$ in these neurons. Treatment with the mitoK(ATP) blocker 10 μM 5-HD inhibited the 6-OHDA-induced decrease in CMTMR fluorescence (i.e., 6-OHDA-induced decrease in $\Delta\Psi_M$) in DA neurons ($P < 0.001$). Interestingly TH-ir neurons treated with 6-OHDA + 5-HD or with 5-HD alone (i.e., without 6-OHDA) showed higher levels of CMTMR fluorescence than TH-ir neurons from untreated cultures ($P = 0.021$ and $P < 0.001$, respectively). This indicates that 5-HD treatment also blocked the effect of other factors (i.e., in addition to 6-OHDA) that act on cultured DA neurons and decrease their $\Delta\Psi_M$ (Figs. 3a, 4g–o).

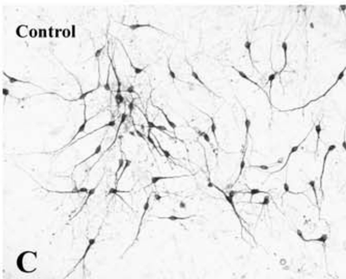
The hyperpolarizing effect of 5-HD was also confirmed in living cells in mesencephalic cell cultures using the JC-1 assay ($P = 0.006$; Fig. 3b). Furthermore, the negatively charged molecule 5-HD was unable to increase CMTMR or J-aggregate fluorescence in cells treated with protonophore CCCP. This revealed that the increase in CMTR or JC-1 retention is not due to non-specific effects and is dependent of $\Delta\psi_M$ (Figs. 3a, b and 4p–s).

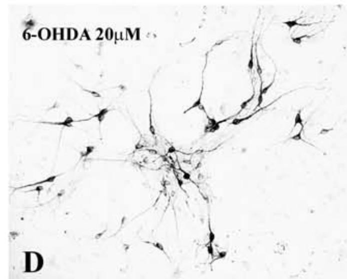

A
TH-ir cells (% of control)
6-OHDA 10μM
6-OHDA 20μM
6-OHDA 30μM
6-OHDA 100μM

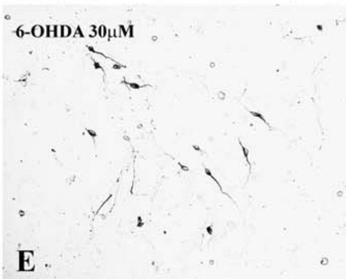

G
TH-ir cells (% of control)
6-OHDA 20μM
6-OHDA 20μM + 5HD 100μM
6-OHDA 20μM + 5HD 500μM
5HD 100μM
5HD 500μM

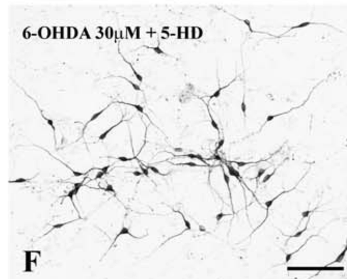

B
TH-ir cells (% of control)
6-OHDA 20μM
6-OHDA 20μM + 5-HD 10μM
6-OHDA 30μM
6-OHDA 30μM + 5-HD 10μM
6-OHDA 100μM
6-OHDA 100μM + 5-HD 10μM
5-HD 10μM
Vehicle


H
Calbindin(-) DA neurons
Calbindin(+) DA neurons
% of control
6-OHDA 20μM
6-OHDA 20μM + 5-HD 10μM
5-HD 10μM
Vehicle


Control
C


6-OHDA 20μM
D


6-OHDA 30μM
E


6-OHDA 30μM + 5-HD
F

Effect of 5-HD Treatment on 6-OHDA-Induced Degeneration of TH-ir Neurons in Neuron-Enriched Cultures

Cultures were treated with the anti-mitotic agent Ara C to study if inhibition of mitoK(ATP) channels of glial cells may be responsible for the protective effect of 5-HD on DA neuron degeneration. Cultures treated with Ara C and 6-OHDA (30 μM, 24 h) + 5-HD (10 μM) contained significantly higher numbers of surviving TH-ir neurons that those treated with Ara C and 30 μM 6-OHDA alone ($P = 0.024$), and were not significantly different from control cultures. This indicates that mitoK(ATP) channels of DA neurons play a major role in the effects of 6-OHDA on DA neuron degeneration (Fig. 5a).

Effect of Treatment with the K(ATP) Antagonist Glibenclamide or the K(ATP) Opener Diazoxide on 6-OHDA-Induced Degeneration of TH-ir Neurons

The results observed with 5-HD were confirmed with control cultures treated with the K(ATP) antagonist glibenclamide. Cultures treated with 20 μM 6-OHDA and glibenclamide (10 μM) contained a significantly more TH-ir neurons than cultures treated with 20 μM 6-OHDA alone ($P = 0.041$; Fig. 5b).

The number of TH-ir neurons in control cultures treated with the K(ATP) opener diazoxide (10 μM) and 20 μM 6-OHDA was not significantly different from the number of TH-ir neurons in cultures treated with 20 μM 6-OHDA alone (Fig. 5c), which suggests that diazoxide is unable to induce further opening of mitoK(ATP) channels.

[^{3}H] Dopamine Uptake Assay

The neurotoxin 6-OHDA is accumulated by the DA uptake system. It is therefore possible that changes in 6-OHDA-induced neurotoxicity were caused by increased or decreased uptake of 6-OHDA due to 5-HD-induced changes in the DA transport activity. In the present study [^{3}H] DA uptake was measured in the absence or presence of 5-HD (10 μM). No significant changes ($P > 0.05$) in the uptake of [^{3}H] DA were observed after treatment with 5-HD (97.3 ± 11.4%) in comparison with non treated controls (100%).

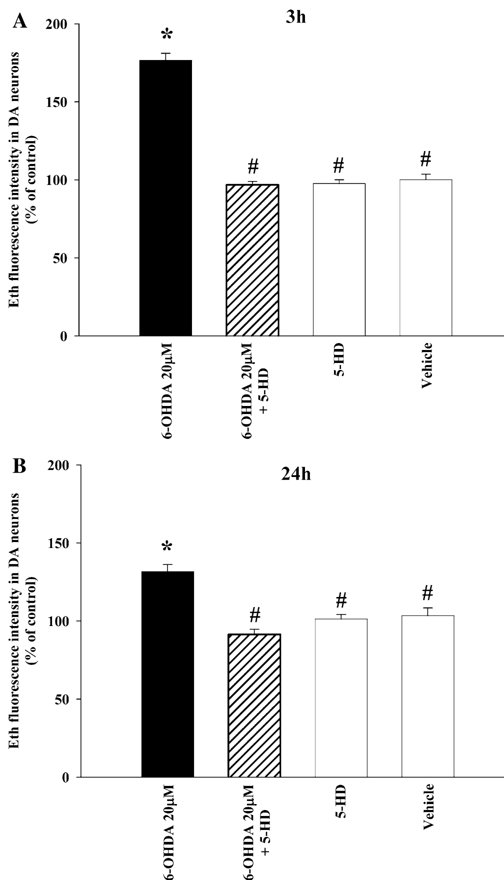


Fig. 2 Intracellular levels of superoxide/superoxide-derived ROS in dopaminergic neurons (i.e., TH-ir cells), estimated by treatment with dihydroethidium (DHE). Administration of low doses of 6-OHDA (20 μM) induced a significant increase in the intensity of ethidium (Eth) fluorescence of TH-ir cells 3 h after 6-OHDA administration, which was significantly reduced by treatment with 5-HD (**a**, 10 μm). Twenty-four hours after treatment with 6-OHDA alone, the number of TH-ir neurons had decreased significantly, and the remaining TH-ir neurons showed increased Eth fluorescence; cultures treated with 6-OHDA + 5-HD were not significantly different from controls (**b**). Data represent means ± SEM. $^*P < 0.05$ compared with control group (untreated cells, 100%); $^\#P < 0.05$ compared with the corresponding 6-OHDA-treated group (one-way ANOVA and Bonferroni's post hoc test, $n = 3$). *5-HD* 5-hydrodydecanoic acid, *6-OHDA* 6-hydroxydopamine, *DA* dopaminergic, *Eth* ethidium, *ir* immunoreactive, *TH* tyrosine hydroxylase

Discussion

The major finding of the present study is that the mitoK(ATP) channel inhibitor 5-HD inhibits the DA cell death induced by low doses of 6-OHDA. Furthermore, 5-HD blocks the 6-OHDA-induced decrease in mitochondrial inner membrane potential ($\Delta\psi_M$) and inhibits 6-OHDA-induced generation of superoxide/superoxide-derived ROS in DA neurons, as revealed by CMTMR (Mito Tracker) and ethidium fluorescence. We have previously shown that ROS derived from 6-OHDA-induced microglial activation

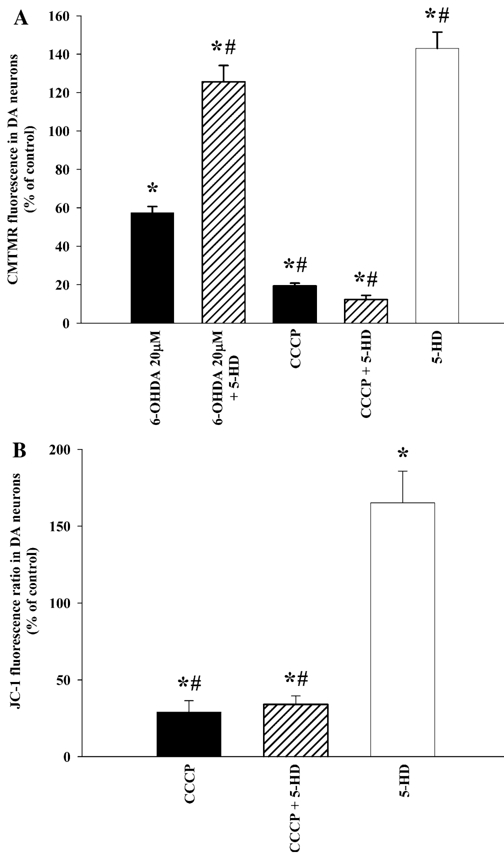
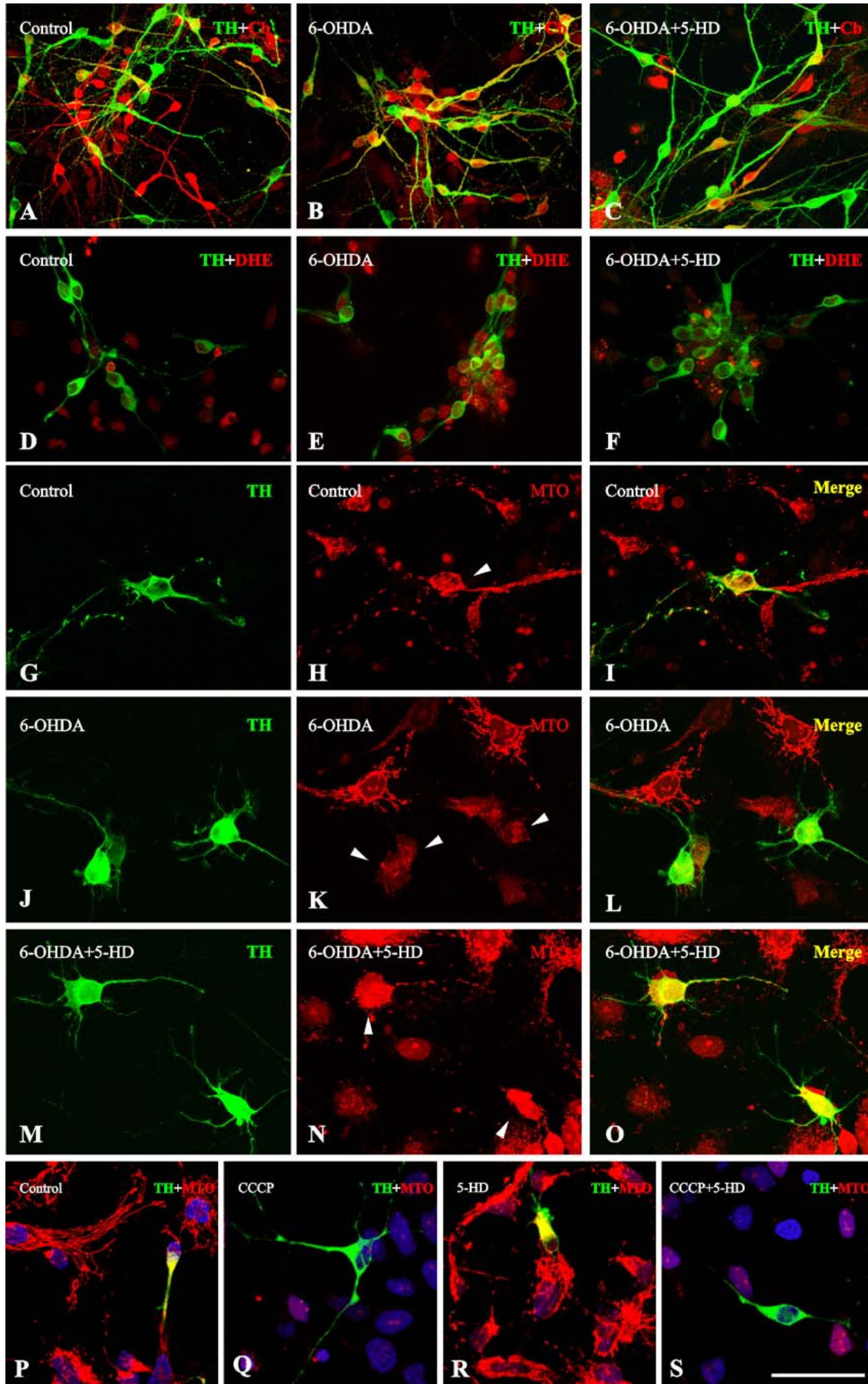


Fig. 3 **a** Treatment of cultures with low doses of 6-OHDA (20 μM) induced a significant reduction in CMTMR fluorescence (i.e., $\Delta\Psi_M$) in DA neurons compared with the control group (100%). Treatment with 5-HD (10 μM) inhibited the 6-OHDA-induced decrease in CMTMR fluorescence. TH-ir neurons treated with 6-OHDA + 5-HD or 5-HD alone (i.e., without 6-OHDA) showed CMTMR fluorescence levels higher than TH-ir neurons of non-treated cultures. This indicates that 5-HD treatment also blocked the effect of additional factors that decrease the $\Delta\Psi_M$. **b** The hyperpolarizing effect of 5-HD was also confirmed in living cells in mesencephalic cell cultures using the JC-1 assay. Furthermore, the negatively charged molecule 5-HD was unable to increase CMTMR (**a**) or J-aggregate (**b**) fluorescence in cells treated with protonophore CCCP. This revealed that the increase in CMTR or JC-1 retention is not due to non-specific effects and is dependent on $\Delta\psi_M$. $*P < 0.05$ compared with control group (untreated cells, 100%); $^{\#}P < 0.05$ in comparison with the corresponding 6-OHDA-(**a**) or 5-HD-treated (**b**) groups. *5-HD* 5-hydrodydecanoic acid, *6-OHDA* 6-hydroxydopamine, *CCCP* carbonyl cyanide *m*-chlorophenylhydrazone, *CMTMR* Mito Tracker Orange, *DA* dopaminergic, *ir* immunoreactive, *TH* tyrosine hydroxylase

contribute to DA neuron death (Rodriguez-Pallares et al. 2007). Therefore, the observed decrease in DA degeneration may be due to inhibition of microglial mitoK(ATP) channels and production of ROS by microglial cells. However, 5-HD was also effective after glial elimination with Ara-C, which suggests a direct effect of 5-HD on mitoK(ATP) channels of DA neurons. Treatment with a second K(ATP) inhibitor (i.e., glibenclamide) confirmed the protective effect observed with 5-HD. Interestingly, treatment with the K(ATP) opener diazoxide did not induce further increase in 6-OHDA-induced cell death, which also suggests that 6-OHDA already induced opening of mitoK(ATP) channels, and that diazoxide is unable to induce further opening.

Therefore, the results suggest that 6-OHDA leads to stimulation of mitochondrial ROS generation through the opening up of mitoK(ATP) channels in DA neurons. It may be speculated that low doses of 6-OHDA may generate low levels of ROS by several mechanisms (see below), which may be insufficient to induce DA cell death. However, such low levels of ROS could act as a trigger to activate mitoK(ATP) channels in the mitochondrial inner membrane, thereby enhancing ROS production and subsequent DA cell degeneration, particularly in the calbindin-negative DA neurons. 5-HD is the most widely used specific inhibitor of mitoK(ATP) channels, because several studies have shown that 5-HD blocks the mitoK(ATP) channel without any effect on cell membrane K(ATP) channel (McCullough et al. 1991; Garlid et al. 1997; Zhang et al. 2007: Costa and Garlid 2008). It has been also reported that 5-HD may reduce ROS generation from mitochondria through additional effects on mitochondrial function (Hanley and Daut 2005). Even in that case, the present study shows the involvement of 5-HD-sensitive ROS generation from mitochondria in the neurotoxic effect of low doses of 6-OHDA. Furthermore, it is known that 5-HD does not exert any radical scavenging activity (Kimura et al. 2005a, b), and the present results cannot be attributed to a 5-HD-induced decrease in 6-OHDA uptake, as revealed by [^{3}H] Dopamine uptake assay. In addition, 5-HD was previously found to be neuroprotective against oxygen-glucose deprivation-induced neuronal cell death (Reinhardt et al. 2003) and in some experimental models of neuronal ischemia (Mattson and Liu 2003).

The present results also suggest that high doses of 6-OHDA can induce significant DA cell death even after blocking mitoK(ATP) channels, possibly because levels of ROS generated directly by 6-OHDA autooxidation are sufficient to induce cell death. However, the mechanisms involved in the toxic effects induced by low doses of 6-OHDA are of greater interest. Low doses are more similar to those used in most in vivo studies, and similar to levels of endogenous 6-OHDA possibly formed naturally in the brain by non-enzymatic hydroxylation of dopamine in the presence of Fe^{2+} and H_2O_2 (Linert et al. 1996; Glinka et al. 1997). This endogenous 6-OHDA has been


Control
TH+Cb
A
6-OHDA
TH+Cb
B
6-OHDA+5-HD
TH+Cb
C
Control
TH+DHE
D
6-OHDA
TH+DHE
E
6-OHDA+5-HD
TH+DHE
F
Control
TH
G
Control
MTO
H
Control
Merge
I
6-OHDA
TH
J
6-OHDA
MTO
K
6-OHDA
Merge
L
6-OHDA+5-HD
TH
M
6-OHDA+5-HD
MTO
N
6-OHDA+5-HD
Merge
O
Control
TH+MTO
P
CCCP
TH+MTO
Q
5-HD
TH+MTO
R
CCCP+5-HD
TH+MTO
S

◀ **Fig. 4** Double labeling for TH (*green*) and calbindin (*red*, **a–c**), ethidium (*red*; **d–f**), or MTO (Mito Tracker, *red*; **g–s**). After treatment with 6-OHDA alone (20 µM: **b**), the number of calbindin-negative dopaminergic neurons (*green*) decreased with respect to controls (**a**), and this decrease was inhibited by simultaneous treatment with 5-HD (**c**). Cultures treated with 6-OHDA showed intense ethidium fluorescence (*red*) in DA neurons (**e**), which was higher than in control cultures (**d**) or those cultures treated with 6-OHDA and 5-HD (**f**). Treatment of cultures with low doses of 6-OHDA induced a significant reduction in MTO fluorescence (i.e., $\Delta\Psi_M$, *red*) in DA neurons (**j–l**, *green*) with respect to controls (**g–i**). The decrease was much more marked in DA neurons (*arrows*) than in other cells, and treatment with 5-HD inhibited this decrease (**m–o**). The protonophore CCCP inhibited MTO fluorescence (i.e., $\Delta\Psi_M$, *red*) in DA (*green*) and non-DA cells in control (**p**, **q**) and 5-HD-treated (**r**, **s**) cultures, in which cell nuclei were labeled with Hoescht (*blue*). *5-HD* 5-hydrodydecanoic acid, *6-OHDA* 6-hydroxydopamine, *CCCP* carbonyl cyanide *m*-chlorophenylhydrazone, *Cb* calbindin, *MTO* Mito Tracker, *DHE* dihydroethidium, *TH* tyrosine hydroxylase. Scale bar: 75 µm (**a–f**) and 30 µm (**g–s**)

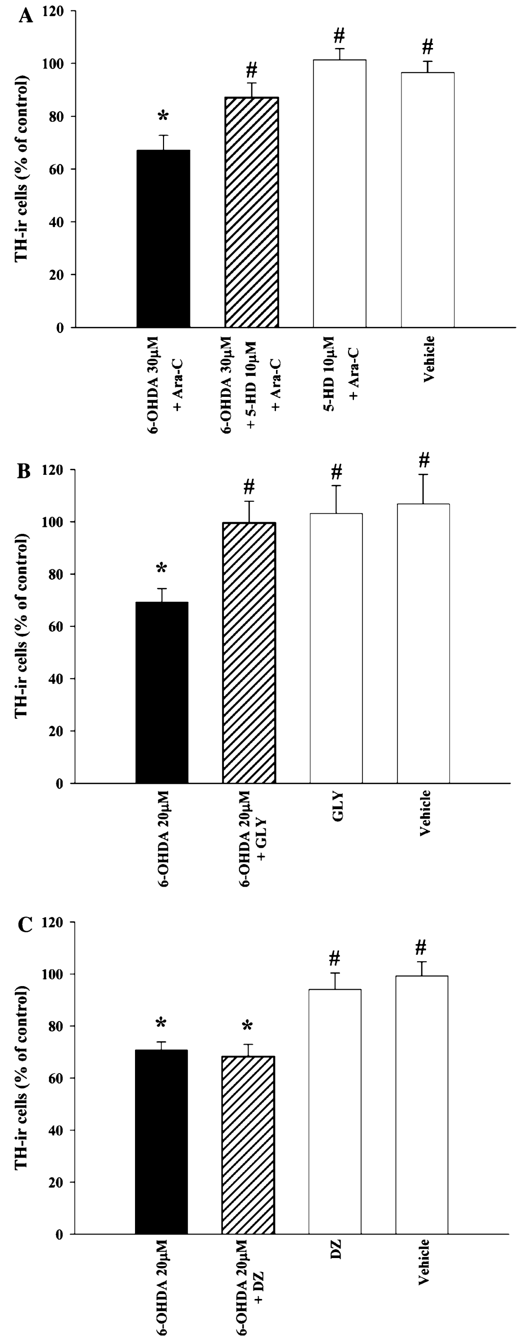
proposed as a possible neurotoxic factor in the pathogenesis of PD (Jellinger et al. 1995; Irwin and Langston 1995), on the basis of the reported formation of 6-OHDA in the rat brain (Senoh et al. 1959) and 6-OHDA accumulation in PD patients (Andrew et al. 1993), and may contribute to DA cell death interacting with other factors. Interestingly, it has also been shown that low and apparently non-toxic doses of two neurotoxins can act synergistically to induce DA degeneration (Gao et al. 2003a, b).

Several mechanisms may also interact, after administration of low doses of 6-OHDA, to increase the neurotoxic effect on DA neurons, and the present results suggest that mitoK(ATP) channels play a major role in this interaction process. Firstly, 6-OHDA can enter the cytosol of DA neurons via DAT (Luthman et al. 1989, Gonzalez-Hernandez et al. 2004), and we and other groups have shown that 6-OHDA generates ROS, including superoxide, H_2O_2, and hydroxyl radicals, via a non-enzymatic autooxidation process (Graham et al. 1978; Soto-Otero et al. 2000). Secondly, it has been observed that extracellular generation of ROS by 6-OHDA autooxidation also plays a role in DA cell death, and that generation of extracellular H_2O_2 appears to play a major role in this process (Abad et al. 1995; Blum et al. 2000; Berretta et al. 2005; Hanrott et al. 2006); it is known that H_2O_2 is a membrane permeable molecule that can readily diffuse from sites of generation and enter DA neurons (Ramasarma 1982; Avshalumov et al. 2003). Thirdly, we have recently shown that low doses of 6-OHDA induce microglial activation and the release of microglial NADPH-oxidase-derived superoxide, which can generate H_2O_2 and significantly contribute to increasing DA cell death (Rodriguez-Pallares et al. 2007). Finally, reported data on the role of mitochondria in 6-OHDA toxicity are controversial. The studies by Glinka and Youdim (1995) and Glinka et al. (1996) in isolated brain mitochondria suggest that 6-OHDA may target the mitochondrial respiratory chain. Therefore, 6-OHDA may decrease $\Delta\psi_M$ by direct effect on the respiratory chain. However, this conclusion was not confirmed by other authors in whole cell experiments in which no reduction in ATP production or ATP/ADP ratio was observed (Wu et al. 1996; Storch et al. 2000). In any case, K(ATP) channels may be open up, directly (Kawabata et al. 2001) or indirectly (Costa and Garlid 2008), in response to partial complex I (CXI) inhibition as well as in response to increased oxidative stress, particularly H_2O_2, superoxide, and hydroxyl radicals derived from 6-OHDA oxidation (Zhang et al. 2002; Liu and Gutterman 2002; Avshalumov and Rice 2003). Therefore mitoK(ATP) channel activation would provide a convergent target that may integrate the above mentioned mechanisms involved in 6-OHDA toxicity, and increase the effects of low doses of the toxin by inducing mitochondrial ROS production (Kimura et al. 2005a, b) and the subsequent DA cell degeneration.

K(ATP) channels are located in various parts of the cells, including the surface of the plasmalemmal membrane and the inner mitochondrial membrane (mitoK(ATP) channels). The structure of the sarcolemmal K(ATP) channels has been studied extensively, and consist of multimeric proteins composed of inwardly rectifying pore-forming Kir6.x (typically Kir6.2 in neurons) and regulatory, sulfonylurea receptor subunits (SUR1/SUR/2). The mitoK(ATP) channel is thought to have similar structure with both Kir and Sur subunit, although the exact composition of the mitoK(ATP) has not been resolved. K(ATP) channels have been widely investigated in cardiac preconditioning studies as mechanism of cardioprotection against ischemia (Oldenburg et al. 2002; Yellon and Downey 2003; Hanley and Daut 2005; Costa and Garlid 2008). Initially, evidence suggested that the surface or sarcolemmal K(ATP) channels trigger or mediate the cardioprotective effects of preconditioning; however more recent findings have suggested a major role for mitoK(ATP) channels in cardiac preconditioning. Generation of ROS by mitoK(ATP) channels after brief episodes of ischemia appears to be essential for preconditioning. This results in activation of an adaptative response that diminishes the effects of ROS generated by subsequent more intense and potentially lethal ischemic episodes (Zhang et al. 2007; Costa and Garlid 2008; Oldenburg et al. 2002).

A number of previous studies have shown that mitoK(ATP) opening increases generation of ROS by mitochondria (Oldenburg et al. 2002; Obata and Yamanaka 2000; Reinhardt et al. 2003; Costa and Garlid 2008). However, few studies have focused on the possible mechanisms involved in this process. Several recent studies have suggested that mito(KATP) opening induces an increase in K^+ uptake that leads to matrix alkalinization.

Fig. 5 Cultures were treated with the antimitotic agent Ara C (1 μM, **a**) to study the possible role of glial cells, the K(ATP) antagonist glibenclamide (10 μM, **b**), or the K(ATP) opener diazoxide (10 μM, **c**). Cultures treated with Ara C and 6-OHDA (30 μM, 24 h) + 5-HD (10 μM) contained significantly higher numbers of cells that those treated with 6-OHDA alone, which indicates that mitoK(ATP) channels of DA cells play a major role in the 6-OHDA-induced DA neuron degeneration (**a**). Cultures treated with glibenclamide confirmed the results observed with 5-HD (i.e., a protective effect on DA neuron degeneration, **b**). Diazoxide (10 μM) did not induce significant increase in DA cell death (**c**). The data are expressed as percentages of the number of TH-ir cells obtained in the respective control cultures (100%). Data represent means ± SEM. *$P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. group treated with 6-OHDA alone (one-way ANOVA and Bonferroni post hoc test; $n \geq 3$). *5-HD* 5-hydrodydecanoic acid, *6-OHDA* 6-hydroxydopamine, *Ara-C* cytosine-β-D-arabino-furanoside, *DA* dopaminergic, *DZ* diazoxide, *GLY* glibenclamide, *TH* tyrosine hydroxylase



A large increase in ROS production with increasing matrix pH has been shown. Furthermore, studies with electron transport chain inhibitors indicate that the site of mitoK(ATP) ROS production is CXI. Therefore, matrix alkalinization inhibits CXI, leading to increased production of superoxide and its products, H_2O_2 and hydroxyl radicals (Andrukhiv et al. 2006; Costa and Garlid 2008). Iron may play an role in this process, since some studies have reported that iron markedly enhances the formation of hydroxyl radicals via a Fenton-type reaction in rats treated with mitoK(ATP) openers (Obata and Yamanaka 2000; Han et al. 2002). Furthermore, some studies have shown that hydroxyl radicals obtained in the presence of ferric iron are more effective than O_2^- and H_2O_2 (i.e., derived in the present study from 6-OHDA) at activating K(ATP) channels (Tokube et al. 1998). Although several studies have shown that mito(KATP) opening increases ROS levels (see above), there are also controversial results showing that mitoK(ATP) opening does not necessarily increase ROS levels in the heart (Facundo et al. 2005) or in cultured neurons (Gáspár et al. 2008), and that mitoK(ATP) openers may elicit ROS production, independently of the channel opening (Minners et al. 2007). Most of these discrepancies are probably due to differences in experimental conditions, since the results were obtained with different cells, isolated mitochondria under different metabolic conditions, and with different doses of openers and closers.

Although K(ATP) channels were originally discovered in the heart, are particularly abundant in the CNS, and have been found in substantia nigra and striatum at the highest levels in the CNS (Xia and Haddad 1991; Zini et al. 1993; Busija et al. 2004). Previous studies have shown that there are two principal DA neuron subtypes in the ventral mesencephalon (i.e., the A10 neurons of the ventral tegmental area and the A9 neurons of the substantia nigra pars

compacta, SNc), and that they can be distinguished with antibodies that recognize the calcium-binding protein calbindin or the G-protein-gated inwardly rectifying K^+ channel subunit (Girk2). The vast majority of TH-ir A10 neurons are calbindin-positive/Girk2-negative (Rogers 1992; McRitchie et al. 1996), and more resistant to OS (Avshalumov et al. 2005; Liss et al. 2005). A9 neurons of the SNc are calbinding-negative/Girk2-positive, and are particularly vulnerable to OS and early degeneration in PD. Interestingly, it has recently been shown that sarcolemmal K(ATP) channels are linked to degeneration of DA neurons, and that calbindin-negative DA neurons (i.e., those particularly vulnerable to OS) are those containing high levels of SUR1-based K(ATP) channels (Avshalumov et al. 2005; Liss et al. 2005). The present results suggest that mitoK(ATP) channels are also involved in DA cell death, and particularly in the loss of DA neurons induced by low doses of 6-OHDA, which is also mostly at the expense of the calbindin-negative DA neurons (i.e., A9 neurons of the SNc).

Finally, it is interesting to note that in preconditioning studies (i.e., after brief episodes of ischemia), ROS production derived from mitoK(ATP) opening is not sufficient to induce cell death in cardiomyocytes and possibly other cell types, including cortical neurons (Kis et al. 2003; Busija et al. 2004), and leads to protection against subsequent intense and potentially lethal insults by activation of adaptive cell responses. Conversely, the present and previous studies (Liss et al. 2005) show that ROS induced by activation of K(ATP) channels contribute to DA cell death. This is consistent with a number of previous studies that have shown that DA neurons function in a "near-compromised" state and are more vulnerable to OS and to mitochondrial CXI inhibition than other neurons and cell types. One of the most clarifying experiments on this point was undertaken with the CXI inhibitor rotenone (Betarbet et al. 2000). As there is no selective mechanism for rotenone uptake, all neurons throughout the brain are exposed to the same degree of CXI inhibition after infusion of low doses of rotenone. However, selective death of DA neurons does occur. Nigral DA neurons are particularly vulnerable to degeneration owing to their particular characteristics—including high basal levels of ROS resulting from non enzymatic catabolism of DA—and their defense mechanisms can be quickly overwhelmed under moderate oxidative conditions (Hirsch et al. 1997; Jellinger 2000). Therefore, a mitoK(ATP)-derived increase in ROS levels, which may be useful for preconditioning and protection in other cell types, may act synergistically with additional factors, such as low doses of neurotoxins in the present study or other factors in PD, to overwhelm the defense mechanisms of DA neurons and induce cell death.

In summary, a number of recent studies have shown that DA cell death may be the result of the interaction of multiple sources of OS that act on DA neurons (Przedborski and Jackson-Lewis 1998; Andersen 2004; Block and Hong 2005; Rodriguez-Pallares et al. 2007). The results of the present study suggest that ROS induced by opening mitoK(ATP) channels may play a key role in the synergistic effect, and that treatment with low doses of 6-OHDA may be a useful tool for studying this process. Furthermore, the present study provides additional data for considering mitoK(ATP) channels as a potential target for neuroprotection in PD.

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4.5- Los canales de potasio mitocondriales dependientes de ATP aumentan el daño oxidativo inducido por AII y la degeneración dopaminérgica. Relevancia en la susceptibilidad asociada a la edad en la EP

Jannette Rodriguez-Pallares, Juan A. Parga, **Belen Joglar**, Maria J. Guerra, Jose L. Labandeira-Garcia (2011) Mitochondrial ATP-sensitive potassium channels enhance angiotensin-induced oxidative damage and dopaminergic neuron degeneration. Relevance for aging-associated susceptibility to Parkinson`s disease. Age, DOI 10.1007/s11357-011-9284-7.

Estudios recientes han mostrado que una hiperactivación del SRA está implicada en los procesos de envejecimiento en diferentes tejidos, así como también en enfermedades degenerativas relacionadas con la edad y/o envejecimiento, debido a un incremento en el daño oxidativo e inflamación. En nuestro laboratorio, hemos visto que la AII amplifica la degeneración dopaminérgica a través del incremento en los niveles de EORs y neuroinflamación. Y que un aumento en la actividad de la AII en la SNpc de ratas está relacionado con la edad. Sin embargo, los mecanismos implicados en estos efectos y la posible interacción entre el SRA y la mitocondria aún no están claros. Este estudio revela que la activación de los canales mitoK(ATP) juega un papel clave en los efectos que la AII tiene sobre el envejecimiento y la neurodegeneración. El bloqueo de estos canales con 5-HD, inhibe el aumento en la neurodegeneración dopaminérgica inducido por la AII, el incremento en la formación de superóxido y superóxido derivado de EORs. Así como, la disminución en el potencial de la membrana mitocondrial interna inducido por AII en cultivos de neuronas dopaminérgicas. Estos datos nos hacen considerar al SRA y a los canales mitoK(ATP) como dianas potenciales para desarrollar terapias neuroprotectoras en enfermedades como la EP.

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Mitochondrial ATP-sensitive potassium channels enhance angiotensin-induced oxidative damage and dopaminergic neuron degeneration. Relevance for aging-associated susceptibility to Parkinson's disease

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Abstract Recent studies have shown that renin–angiotensin system overactivation is involved in the aging process in several tissues as well as in longevity and aging-related degenerative diseases by increasing oxidative damage and inflammation. We have recently shown that angiotensin II enhances dopaminergic degeneration by increasing levels of reactive oxygen species and neuroinflammation, and that there is an aging-related increase in angiotensin II activity in the substantia nigra in rats, which may constitute a major factor in the increased risk of Parkinson's disease with aging. The mechanisms involved in the above mentioned effects and particularly a potential angiotensin–mitochondria interaction have not been clarified. The present study revealed that activation of mitochondrial ATP-sensitive potassium channels [mitoK(ATP)] may play a major role in the angiotensin II-induced effects on aging and neurodegeneration. Inhibition of mitoK(ATP) channels with 5-hydroxydecanoic acid inhibited the increase in dopaminergic cell death induced by angiotensin II, as well as the increase in superoxide/superoxide-derived reactive oxygen species levels and the angiotensin II-induced decrease in the mitochondrial inner membrane potential in cultured dopaminergic neurons. The present study provides data for considering brain renin–angiotensin system and mitoK(ATP) channels as potential targets for protective therapy in aging-associated diseases such as Parkinson's disease.

Keywords Aging · Neurodegeneration · Neuroinflammation · Renin–angiotensin system · Oxidative stress · Parkinson's disease

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Introduction

Increased activity of local renin–angiotensin system, acting via angiotensin II type 1 receptors (AT1), is thought to be involved cellular senescence and age-related degenerative changes in several tissues (Basso et al. 2005; Min et al. 2009; Mukai et al. 2002). In accordance with this, recent studies with angiotensin II type 1 receptor deficient mice indicate that disruption of this receptor promotes longevity through

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attenuation of oxidative stress and additional mechanisms (Benigni et al. 2009, 2010; Mattson and Maudsley 2009), and completely protects against the age-related progression of atherosclerosis (Umemoto 2008). It is known that angiotensin II, acting via type 1 receptors, is one of the most important known inducers of inflammation and oxidative stress in several tissues (Mattson and Maudsley 2009; Min et al. 2009; Ruiz-Ortega et al. 2001). Normal aging has been associated with a pro-inflammatory, pro-oxidant state that may favor an exaggerated response to injury and degenerative diseases (Choi et al. 2010; Csiszar et al. 2003; Ungvari et al. 2004). In accordance with this, several recent studies have suggested the potential of inhibition of angiotensin for treatment of age-associated diseases and longevity (Benigni et al. 2009, 2010; Mattson and Maudsley 2009; Nishiyama et al. 2009).

Advancing age itself is one of the most significant risk factors for the development of neurodegenerative diseases such as Parkinson's disease (PD; Collier et al. 2007; Cruz-Muros et al. 2009; Deng et al. 2006; McCormac et al. 2004). Brain possesses a local renin–angiotensin system (Mckinley et al. 2003; Saavedra 2005), and we have recently observed an aging-related increase in angiotensin activity in the nigra that leads to pro-inflammatory and pro-oxidative changes, which may constitute a major factor in the increased risk of PD with aging (Villar-Cheda et al. 2010b). It is known that neuroinflammation, oxidative stress, and microglial NADPH oxidase activation play a major role in dopaminergic neuron degeneration and PD (Rodriguez-Pallares et al. 2007; Wu et al. 2002, 2003). Furthermore, we have shown that angiotensin II, via type 1 receptors, enhances the dopaminergic degeneration process triggered by low/sublethal doses of dopaminergic neurotoxins by amplifying intraneuronal levels of reactive oxygen species (ROS) and the inflammatory response via activation of microglial NADPH oxidase (Joglar et al. 2009; Rey et al. 2007; Rodriguez-Pallares et al. 2008).

The mechanisms involved in the above mentioned effects of increased angiotensin activity and particularly a potential angiotensin–mitochondria interaction have not been clarified. It is well known that angiotensin II acts via type 1 receptors to release high levels of ROS mainly by activation of the NADPH oxidase (Qin et al. 2004; Seshiah et al. 2002; Touyz et al. 2002), which was also observed in the nigrostriatal system (Joglar et al. 2009; Rey et al. 2007; Rodriguez-Pallares et al. 2008). However, recent studies suggest that angiotensin II may stimulate not only cytosolic but also mitochondrial-ROS generation (de Cavanagh et al. 2007; Zhang et al. 2007). In addition, a number of studies support a critical role for mitochondrial ATP-sensitive potassium channels [mito(KATP)] in modulating intracellular ROS (Mattson and Liu 2003; Costa and Garlid 2008). A cross-talk signaling between the NADPH oxidase and the mitoK(ATP) channels has been shown (Daiber 2010; Kimura et al. 2005a; Zhang et al. 2007). This includes not only that NADPH oxidase modulates mitochondrial superoxide (Doughan et al. 2008; Kimura et al. 2005a) but also that mitochondrial superoxide stimulates extramitochondrial NADPH oxidase activity in a feed-forward fashion (Dikalova et al. 2010; Wosniak et al. 2009). In the present study, we used primary cultures of ventral mesencephalon to investigate the possibility that mitoK(ATP) channels are involved in the enhancing effect of angiotensin II on dopaminergic neuron degeneration and potentially the increased risk and progression of PD with aging.

Methods

Primary mesencephalic cultures

Ventral mesencephalic tissue was dissected from rat embryos of 14 days of gestation (E14). All experiments were carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and approved by the corresponding committee at the University of Santiago de Compostela. The tissue was incubated in 0.1% trypsin (Sigma, St. Louis, MO, USA), 0.05% DNase (Sigma), and DMEM (Invitrogen, Paisley, Scotland, UK) for 20 min at 37°C, and then washed in DNase/DMEM and mechanically dissociated. The resulting cell suspension was centrifuged at 50×*g* for 5 min, the supernatant was carefully removed, and the pellet resuspended in 0.05% DNase/DMEM to the final volume required. The number of viable cells in the suspension was estimated with acridine orange/ethidium bromide. Cells were plated onto 35-mm culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) previously coated with poly-L-lysine (100 μg/ml; Sigma) and laminin (4 μg/ml; Sigma). The cells

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were seeded at a density of 1.5×10^5 cells/cm^2 and maintained under control conditions [DMEM/HAMS F12/ (1:1) containing 10% fetal bovine serum (FBS; Biochrom KG, Berlin, Germany)]. The cell cultures were maintained in a humidified CO_2 incubator (5% CO_2; 37°C) for 8 days in vitro (DIV; see below); the entire medium was removed on day 2 and replaced with fresh culture medium.

To obtain neuron-enriched cultures, cytosine-β-D-arabino-furanoside (Ara C; 1 μM; Sigma) was added 48 h after the cells were seeded. The cultures were then treated with the dopaminergic neurotoxins 6-OHDA (6-hydroxydopamine) or MPP$^+$ (1-methyl-4-phenylpyridinium), or 6-OHDA or MPP$^+$ and 5-HD (5-hydroxydecanoic acid, see below). This method can enrich neurons to >85% purity (Michel et al. 1997; Gao et al. 2003).

Treatment of cultures and experimental design

Cultures were exposed on 7 DIV to 6-OHDA alone for 24 h (10 or 30 μM, in 0.02% saline ascorbate; Sigma), or on 4 DIV to MPP$^+$ alone for 4 days (0.25 μM; Sigma), or 6-OHDA or MPP$^+$ and angiotensin II (100 nM; Sigma), or 6-OHDA+ angiotensin II+the angiotensin II type 1 receptor antagonist ZD 7155 (1 μM), or 6-OHDA or MPP$^+$ and 5-HD [a specific blocker of mitoK(ATP) channels, Sigma, 10 μM], or 6-OHDA or MPP$^+$+ angiotensin II+5-HD, or angiotensin II alone, or ZD 7155 alone, or 5-HD alone, in order to study the effect on survival of dopaminergic neurons. 5-HD is the most widely used specific inhibitor of mitoK(ATP) channels, and several studies have shown that 5-HD (10 μM) blocks the mitoK(ATP) channels without any effect on cell membrane K(ATP) channels (Costa and Garlid 2008; Garlid et al. 1997; McCullough et al. 1991; Zhang et al. 2007). Several doses of 5-HD were tested in the present and preliminary experiments (10, 100, 500 μM; Rodriguez-Pallares et al. 2009), and the lowest dose that proved effective (10 μM) was used in most of the experiments to prevent potential non-specific side effects of high doses of 5-HD (Wu et al. 2006). In addition, some cultures were treated with a second K(ATP) channel blocker (glibenclamide, 10 μM, Sigma) to confirm the involvement of K (ATP) channels in AII-induced cell death.

The cells were then washed and processed for immunolabeling as detailed below. Some control cultures and cultures subjected to the above mentioned treatments were also treated with MitoTracker Orange (CMTMR; chloromethyl-tetramethylrosamine methyl ester; Molecular Probes, Eugene, OR, USA) or MitoTracker Green FM (MTGFM; 2-[3-[5,6-dichloro-1,3-bis[[4-(chloromethyl)phenyl]methyl]-1,3-dihydro-2H-benzimidazol-2-ylidene]-1-propen-1-yl]-3-methyl-benzoxazoliumchloride; Molecular Probes; 50 nM) or dihydroethidium (DHE) for 3 h to estimate the mitochondrial inner membrane potential ($\Delta\psi_M$), the mitochondrial mass or levels of superoxide/superoxide-derived ROS, respectively, in dopaminergic neurons or microglial cells, as detailed below. Furthermore, the hyperpolarizing effect of 5-HD was confirmed in live cell cultures with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; Invitrogen), as described below. Finally, some cultures were pre-treated with the mitochondrial uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone; Sigma, 10 μM) to confirm that the increase in CMTMR or JC-1 retention is not due to non-specific effects and is dependent on $\Delta\psi_M$. The protonophore CCCP is often used to dissipate the membrane potential and to define the baseline for the analysis of mitochondrial membrane potential with fluorescent dyes (Gottlieb et al. 2003; Brown et al. 1996).

Immunohistochemistry and double-fluorescence labeling

Cultures were fixed with 4% paraformaldehyde in Dulbecco's phosphate buffered saline (DPBS; pH 7.4) for 20 min, and then incubated at 4°C with a mouse monoclonal anti-tyrosine hydroxylase (TH; Sigma; 1:30,000) as dopaminergic marker. Neuron-enriched cultures were evaluated by immunocytochemical staining with a mouse monoclonal anti-NeuN (Chemicon; Temecula, CA, USA; 1:2,000) as a neuronal marker, a mouse monoclonal anti-glial fibrillary acidic protein (GFAP; Chemicon; 1:1,000) as an astrocyte marker, and a mouse monoclonal anti-CD11b (anti-complement receptor-3, clone MRC OX42; Serotec, Kidlington, Oxford, UK; 1:1,000) as a marker of resting and reactive microglial cells/macrophages. Cultures were then washed and incubated for 1 h with biotinylated horse anti-mouse antibody (Vector; Burlingame, CA, USA) diluted 1:500, and then incubated for 90 min with avidin–biotin–peroxidase

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complex (ABC, Vector, 1:500). Finally, the labeling was revealed with 0.04% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine (Sigma) as chromogen (see Rodriguez-Pallares et al. 2008 for details).

Cultures grown on glass coverslips were processed for double-fluorescence labeling for DHE or CMTMR or MTGFM and TH or OX42. For TH or OX42, cultures were incubated overnight at 4°C with primary anti-TH (1:30,000) or anti-OX42 (Serotec, 1:1,000) antibodies. The cultures were rinsed with DPBS, then incubated for 150 min with the secondary antibodies [goat anti-mouse (Chemicon; 1:100) conjugated with fluorescein isothiocyanate (FITC) for TH, or biotinylated horse anti-mouse for OX42 (Vector; 1:500)]. OX42 labeling was visualized by incubation of the cultures with streptavidin conjugated with FITC (Sigma; 1:200) for 30 min. Co-localization of markers (see below for DHE, MTGFM, and CMTMR) was confirmed by confocal laser microscopy (TCS-SP2; Leica, Heidelberg, Germany) and use of a sequential scan method to avoid any possible overlap. In all experiments, the control cultures, in which the primary antibody was omitted, were immunonegative for these markers.

Estimation of $\Delta\psi_M$ with CMTMR (MitoTracker Orange) or JC-1, and mitochondrial mass with MTGFM (MitoTracker Green FM)

CMTMR (MitoTracker Orange TM) enters mitochondria of living cells in proportion to the negative charge difference between the cytoplasm and the mitochondrial matrix, and therefore provides an estimation of $\Delta\psi_M$. Lipophilic cations accumulate in the mitochondrial matrix, driven by the $\Delta\psi_M$ in accordance with the Nernst equation, which predicts that every 61.5-mV increase in membrane potential causes a ten-fold increase in accumulation of the membrane-permeant cation. CMTMR binds irreversibly to mitochondrial matrix thiols and can be fixed for immunocytochemical localization of proteins in the same cells that have previously been exposed to CMTMR. As a result of the thiol binding, CMTMR fluorescence represents the highest level of negativity difference in the mitochondria during exposure to the dye before fixation (Sugrue et al. 1999; Wadia et al. 1998). MitoTracker Green FM (MTGFM) is a mitochondrion-selective probe that becomes fluorescent in the lipid environment of mitochondria. MTGFM contains a thiol-reactive chloromethyl moiety, resulting in stable peptide and protein conjugates after accumulation in mitochondria and, unlike CMTMR, uptake of this probe is less dependent on $\Delta\psi_M$, thus allowing estimation of mitochondrial mass in both live and fixed cells (Metivier et al. 1998; Poot et al. 1996).

The $\Delta\psi_M$ or the mitochondrial mass in fixed cells grown in glass coverslips was estimated as follows (Sugrue et al. 1999; Wadia et al. 1998; Buckman et al. 2001). Three hours after exposure to treatments, the medium in each well was supplemented with 50 nM CMTMR or 50 nM MTGFM and incubated at 37°C 5% CO_2 for 15 min or 30 min, respectively. The media was removed and the cells were rinsed with cold DPBS, followed by immediate fixation with 4% paraformaldehyde for 10 min. After fixation, the cells were washed briefly in DPBS and incubated for 20 min with a mouse monoclonal anti-TH (Sigma, 1:6,000) containing normal goat serum and 0.3% Triton X-100 diluted in DPBS-BSA. Cultures were then washed and incubated for 15 min with goat anti-mouse secondary antibody conjugated with FITC for CMTMR or with cyanine 3.18 (Cy3) for MTGFM. The coverslips were mounted onto microscope slides with 1,4-diazabicyclo [2.2.2] octane (DABCO; Sigma).

JC-1 is a cationic carbocyanine dye that accumulates in mitochondria. The dye exists as a monomer at low concentrations and yields green fluorescence. At higher concentrations, the dye forms J-aggregates that exhibit a broad excitation spectrum and an emission maximum at 590 nm. The JC-1 dye is only uptaken up by viable cells and it exhibits a spectral shift from green to red in healthy mitochondria with polarized membranes. The JC-1 assay was performed to estimate the $\Delta\Psi_M$ in living cells, according to Zhang et al. (2006) as follows. Cells were plated in 96-well plates at 10^5 cells per well. After treatment with 5-HD, JC-1 (in DMSO) was added to each well to a final concentration of 3 μM and incubated for 30 min at 37°C, with 5% CO_2, in the dark. The medium was removed and the cells washed twice with DPBS. Fluorescence intensity was measured in a multifunctional microplate reader (TECAN Infinite 200, Austria) at excitation 485 nm, emission 525 and 595 nm, for detection of the green and red substrate, respectively. The fluorescence signal represents the average signal of the total cell population. The mitochondrial membrane potential is shown as the ratio between the

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fluorescence of aggregate (red) and monomer (green) forms of JC-1. This ratio is dependent only on the mitochondrial membrane potential, and not on the number of cells, mitochondrial size, shape, or density.

Detection of intracellular superoxide anion/superoxide-derived ROS with dihydroethidium (DHE): estimation of changes in fluorescence intensity

Treatment with DHE is one of the most frequently used methods for the detection of intracellular superoxide. For years, it was assumed that intracellular DHE is oxidized to ethidium, which bound DNA to form a red fluorescent product. However, recent studies have shown that the fluorescence derived from the reaction between superoxide and DHE is due to accumulation of 2-hydroxyethidium and detects superoxide in the cytoplasm (Dikalova et al. 2010; Fink et al. 2004; Zhao et al. 2005). Cultures grown on glass coverslips were incubated with a fresh working solution containing 5 μM DHE (Sigma) in sterile phosphate buffered saline (PBS; pH 7.4) for 30 min at 37°C. The cultures were washed, then fixed and processed for immunofluorescence against TH or OX42 (see above).

CMTMR, MTGFM, and DHE-derived fluorescence was visualized with a laser scanning confocal microscope (TCS-SP2; Leica, Heidelberg, Germany), equipped with a 63× oil immersion 1.4 numerical aperture (NA) objective. All cells were visualized at the same level of laser intensity, detector sensitivity, and pinhole size in order to ensure that fluorescence intensity could be compared among different coverslips and treatments. The images were saved as 8-bit TIFF files and the fluorescence intensity was evaluated by digital image processing with ImageJ software (NIH, Bethesda). The cytoplasm of dopaminergic or microglial cells was identified by TH or OX42 staining. A minimum of 50 TH-ir or OX42-ir fluorescent cells were counted in random visual fields to assess the intensity of DHE-derived or CMTMR or MTGFM fluorescence in cultures labeled for these markers. All measurements were corrected for background fluorescence.

Cell counting

TH-ir cells were counted in five randomly chosen longitudinal and transverse microscopic fields along the diameter of the culture dish, away from the curved edge. The operator was blind to the treatment condition. The microscopic field was defined by a 0.5×0.5 cm reticule (i.e., 1.25 cm^2). The average number of TH-positive cells in a control culture dish was 1,628±76. The results from at least three separate experiments were recorded, with a minimum sample size of four dishes per group and per run. The results for each batch were normalized to the control group counts (i.e., expressed as a percentage of the control group counts) to counteract any variability among batches. Statistical differences between groups were tested as described below.

[^{3}H] Dopamine uptake

The culture medium was removed completely and cultures were rinsed twice with 1 ml of uptake buffer (Krebs buffer+1.8 mM Cl_2Ca+25 mM D-glucose). Cells were then incubated for 30 min at 37°C with 1 ml of uptake buffer (containing 1 mM ascorbic acid and 100 μM pargyline; pH 7.4) in the absence (untreated cultures) or presence of 10 μM 5-HD. Uptake was initiated by addition of 20 nM [^{3}H] dopamine ([2,5,6-^{3}H] dopamine; 1 μCi, 12 Ci/mmol; Amersham Biosciences, GE Healthcare, Buckinghamshire, UK) in 20 μl of Krebs buffer. Non-specific uptake values were defined in the presence of 10 μM GBR 12935, a specific inhibitor of the dopamine transporter. Uptake was stopped after 30 min incubation at 37°C by removal of the incubation mixture and the cells were washed twice with cold Krebs buffer. Cells were lysed with 1 ml of 2 N NaOH for 30 min at room temperature, and the radioactivity incorporated into cells was measured by liquid scintillation spectrometry. Results are expressed as percentages of untreated control culture responses.

Statistical analysis

All data were obtained from at least three independent experiments and were expressed as means±SEM. Two-group comparisons were analyzed by the Student's t test and multiple comparisons were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. The normality of populations and homogeneity of variances were tested before each ANOVA. Differences were considered statistically significant at p <0.05. Statistical analyses were carried out with

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SigmaStat 3.0 from Jandel Scientific (San Rafael, CA, USA).

Results

MitoK(ATP) channel inhibition blocks the enhancing effect of angiotensin II on dopaminergic neuron degeneration

Cultures treated with very low doses of 6-OHDA (10 μM) showed a low non-significant decrease in the number of TH-ir neurons. However, the loss of dopaminergic neurons was significantly enhanced (loss of around 50% of dopaminergic neurons) by treatment with angiotensin II (100 nM). Treatment with the angiotensin II type 1 receptor antagonist ZD 7155 (6-OHDA+angiotensin+ZD) or the mitoK(ATP) channel inhibitor 5-HD (6-OHDA+angiotensin+5-HD) blocked the loss of dopaminergic neurons (Fig. 1a).

Cultures treated with low doses of MPP^+ (0.25 μM) showed a low although significant decrease (20–25% decrease) in the number of TH-ir neurons. Again, the loss of dopaminergic neurons was significantly enhanced (around 50–55% decrease) by treatment with angiotensin II (100 nM). Treatment with the angiotensin II type 1 receptor antagonist ZD 7155 or the mitoK (ATP) channel inhibitor 5-HD (MPP^++angiotensin+5-HD) blocked the enhancing effect of angiotensin II on the loss of dopaminergic neurons (Fig. 1b). No significant loss of dopaminergic neurons was observed after administration of angiotensin II alone or 5-HD alone or ZD 7155 alone (Fig. 1c). These data confirm the results of our previous studies showing that

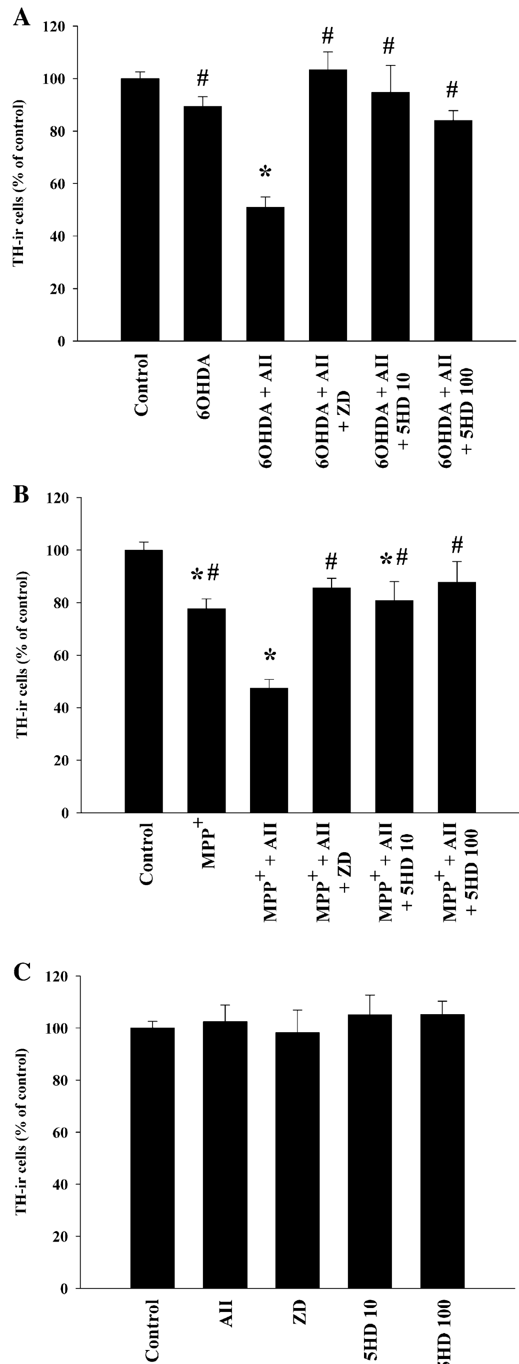

Fig. 1 Effects of different treatments on the number of TH-ir cells. Treatment with very low doses of 6-OHDA (10 μM; **a**) or MPP^+ (0.25 μM; **b**) induced a slight decrease (non-significant and significant, respectively) in the number of TH-ir neurons, which was significantly enhanced by angiotensin II (100 nM). The amplifying effect of angiotensin II was blocked by simultaneous treatment with the angiotensin II type 1 receptor antagonist ZD 7155 or with the mitoK(ATP) channel inhibitor (5-HD 10 μM and 100 μM). No significant effect was observed after treatment with angiotensin II or ZD 7155 or 5-HD alone (**c**). The data are expressed as percentages of the number of TH-ir cells obtained in the respective control cultures (100%). Data represent means±SEM. $^*p<0.05$ compared with control group (untreated cells), $^\#p<0.05$ compared with the group treated with 6-OHDA or MPP^+ and angiotensin (one-way ANOVA and Bonferroni post hoc test). *AII* angiotensin II, *TH* tyrosine hydroxylase, *ZD* ZD 7155, *5-HD* 5-hydroxydecanoic acid

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angiotensin II increases the effect of low doses of dopaminergic neurotoxins via type 1 receptors, and also demonstrate that mitoK(ATP) receptor activation is involved in this effect (Fig. 2).

The results observed with 5-HD were confirmed with control cultures treated with the K(ATP) antagonist glibenclamide. Cultures treated with 6-OHDA+ angiotensin II or MPP^{+}+angiotensin II and glibenclamide (10 μM) contained significantly more TH-ir neurons than cultures treated with the neurotoxins and angiotensin alone (Fig. 3).

[^{3}H] Dopamine uptake assay

The neurotoxins 6-OHDA and MPP^{+} are accumulated by the dopamine uptake system. It is therefore possible that changes in 6-OHDA- or MPP^{+}-induced neurotoxicity were caused by increased or decreased uptake of the neurotoxin due to 5-HD-induced changes in the dopamine transport activity. In the present study, [^{3}H] dopamine uptake was measured in the absence or presence of 5-HD (10 μM). No significant changes ($p>0.05$) in the uptake of [^{3}H] dopamine were observed after treatment with 5-HD (92.9±14.9%) in comparison with non-treated controls (100%).

MitoK(ATP) channel inhibition blocks the enhancing effect of angiotensin II on generation of intracellular superoxide/superoxide-derived ROS in dopaminergic neurons

Detection of superoxide anion and superoxide-derived ROS in cultured cells was achieved by treatment with dihydroethidium (DHE), which is oxidized to 2-hydroxyethidium and fluoresces red. Double labeling for TH and DHE-derived fluorescence revealed that treatment with very low doses of 6-OHDA (10 μM) induced a slight significant increase in the intensity of DHE-derived fluorescence 3 h after treatment. The 6-OHDA-induced increase in DHE-derived fluorescence in dopaminergic neurons was significantly increased by administration of angiotensin II, and decreased to control levels after treatment with the mitoK(ATP) blocker 5-HD (6-OHDA+angiotensin+ 5-HD). Administration of angiotensin II alone induced a slight but significant increase in the intensity of DHE-derived fluorescence. Treatment with 5-HD alone induced a small non-significant decrease in the intensity of DHE-derived fluorescence with respect to controls (Figs. 4a and 6a–c).

Cultures treated with low doses of MPP^{+} showed similar results. Administration of angiotensin II significantly increased the intensity of DHE-derived fluorescence observed in dopaminergic neurons of control cultures or cultures treated with MPP^{+} alone, and the increase was blocked by treatment with 5-HD (MPP^{+}+angiotensin+5-HD). Administration of angiotensin II alone induced a slight but significant increase in the intensity of DHE-derived fluorescence. Treatment with 5-HD alone induced a small non-significant decrease in the intensity of DHE-derived fluorescence with respect to controls (Fig. 4b).

MitoK(ATP) channel inhibition blocks the angiotensin II-induced decrease in mitochondrial inner membrane potential ($\Delta\psi_M$) in dopaminergic neurons

CMTMR (MitoTracker Orange) was used to estimate $\Delta\psi_M$. CMTMR enters mitochondria in proportion to the negative charge difference between the cytoplasm and the mitochondrial matrix and binds irreversibly to mitochondrial matrix thiols. Double labeling for TH and CMTMR revealed that treatment of cultures with angiotensin II induced a significant reduction in CMTMR fluorescence in dopaminergic neurons, which revealed a decrease in $\Delta\psi_M$ in these neurons. Treatment with the mitoK(ATP) blocker 5-HD inhibited the angiotensin II-induced decrease in CMTMR fluorescence (i.e., angiotensin-induced decrease in $\Delta\psi_M$) in dopaminergic neurons (Figs. 5a and 6d–l). Interestingly TH-ir neurons treated with angiotensin+5-HD or with 5-HD alone showed higher levels of CMTMR fluorescence than TH-ir neurons from untreated cultures. This indicates that 5-HD treatment also blocked the effect of other factors (i.e., in addition to angiotensin II) that act on cultured dopaminergic neurons and decrease their $\Delta\psi_M$ (see "Discussion").

Increases in CMTMR fluorescence may indicate an increase in $\Delta\psi_M$ or an increase in mitochondrial mass. In an attempt to differentiate between these possibilities, we used the probe MTGFM, an indicator of mitochondrial mass, and we observed that MTGFM fluorescence in the dopaminergic neurons remained unchanged 3 h after the corresponding treatment exposure (Figs. 5b and 6m–u). Furthermore,

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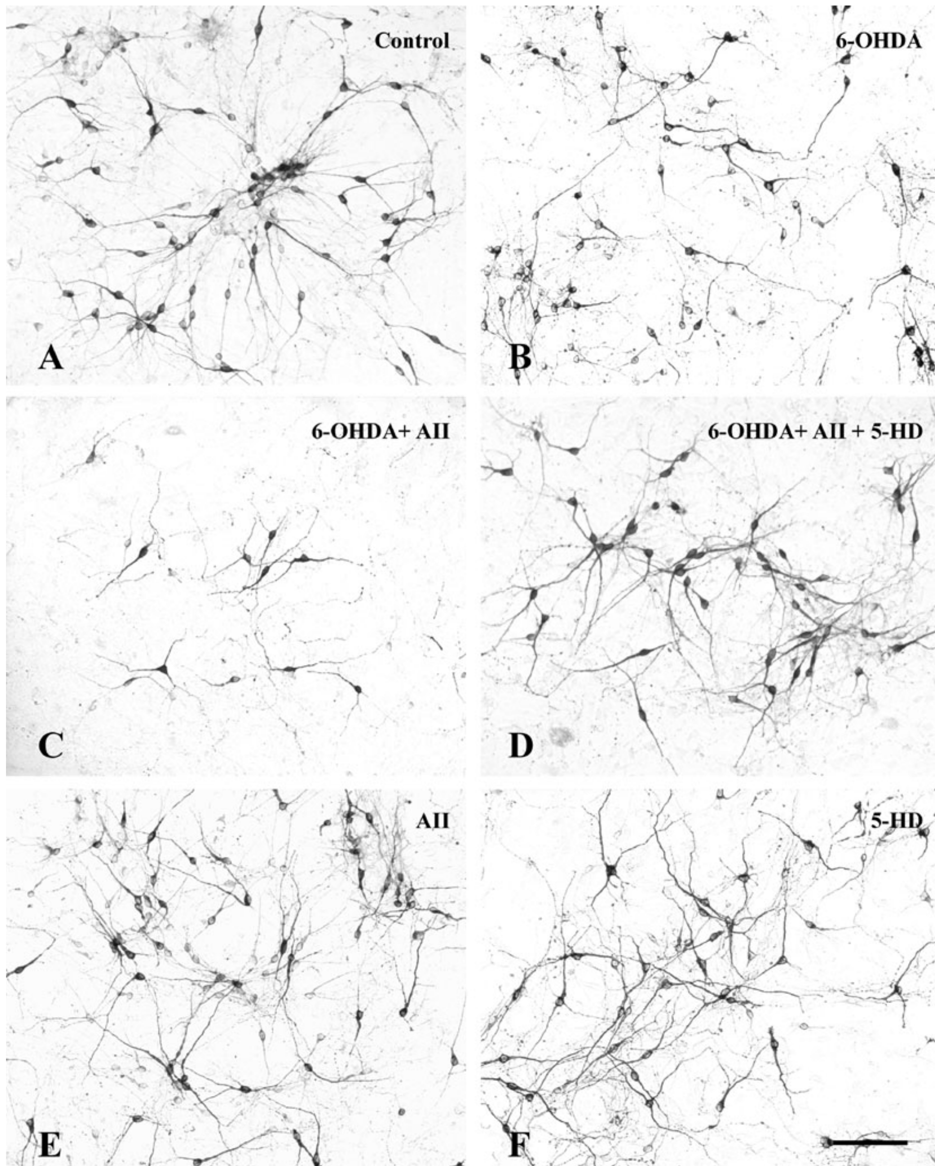

Fig. 2 Photomicrographs of representative TH-ir cells from different experimental groups: a control culture (**a**), and cultures treated with a low dose of 6-OHDA (10 μM; **b**), or 6-OHDA (10 μM)+angiotensin (AII, 100 nM; **c**), or 6-OHDA+AII+5-HD (10 μM; **d**), or AII alone (**e**), or 5-HD alone (**f**). AII induced a significant increase in 6-OHDA neurotoxicity. This increase was blocked by treatment with the mitoK(ATP) channel inhibitor 5-HD. *AII* angiotensin II, *5-HD* 5-hydroxydecanoic acid. *Scale bar*: 100 μm

the hyperpolarizing effect of 5-HD was also confirmed in living cells in mesencephalic cell cultures using the JC-1 assay (Fig. 5c). Finally, the negatively charged molecule 5-HD was unable to increase CMTMR or J-aggregate fluorescence in cells treated with protonophore CCCP. This revealed that the

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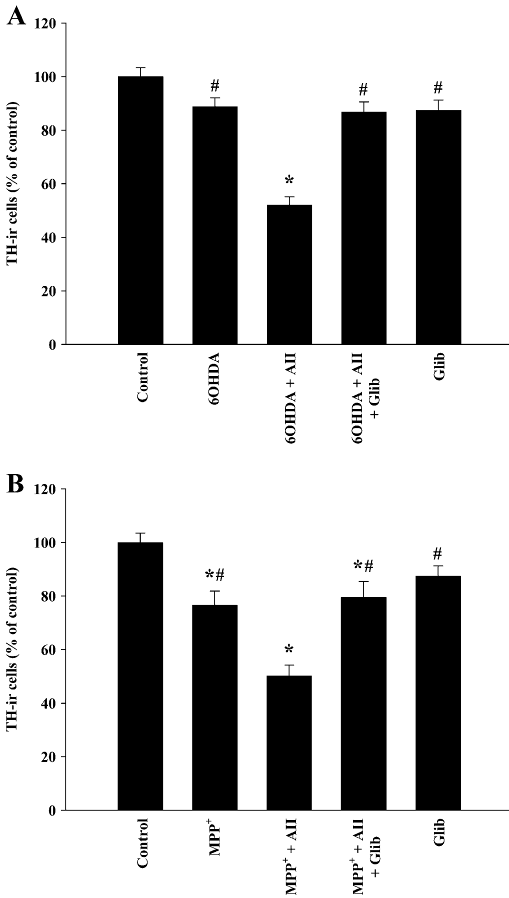

Fig. 3 Effects of different treatments on the number of TH-ir cells. Treatment with very low doses of 6-OHDA (10 μM; **a**) or MPP^+ (0.25 μM; **b**) induced a slight decrease (non-significant and significant, respectively) in the number of TH-ir neurons, which was significantly enhanced by angiotensin II (100 nM). The amplifying effect of angiotensin II was blocked by simultaneous treatment with the K(ATP) channel inhibitor glibenclamide (10 μM), which confirmed the protective effect on dopaminergic degeneration observed with 5-HD. No significant effect was observed after treatment with glibenclamide alone. The data are expressed as percentages of the number of TH-ir cells obtained in the respective control cultures (100%). Data represent means±SEM. $^*p<0.05$ compared with control group (untreated cells), $^{\#}p<0.05$ compared with the group treated with 6-OHDA or MPP^+ and angiotensin (one-way ANOVA and Bonferroni post hoc test). *AII* angiotensin II, *TH* tyrosine hydroxylase, *Glib* glibenclamide

increase in CMTMR or JC-1 retention is not due to non-specific effects and is dependent of $\Delta\psi_M$ (Fig. 5a, c).

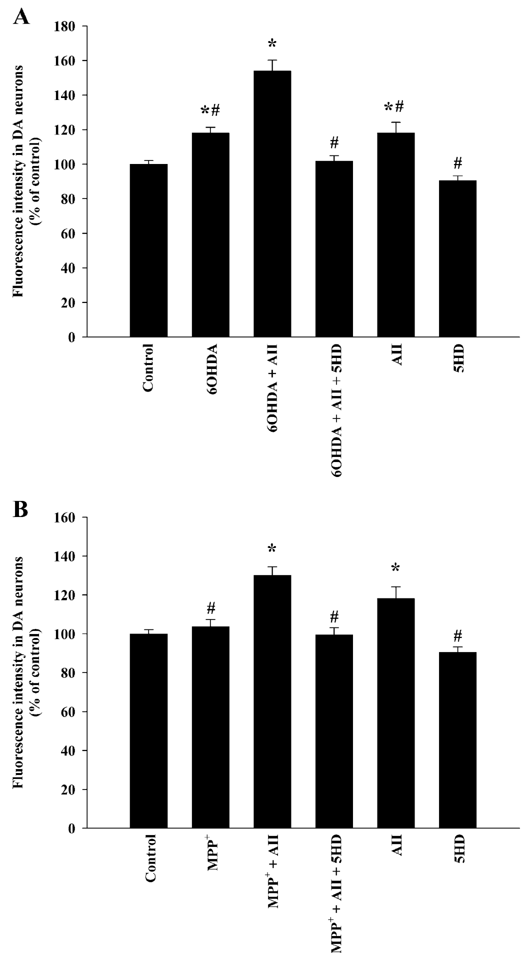

Fig. 4 Intracellular levels of superoxide/superoxide-derived ROS estimated by treatment with dihydroethidium (DHE) in dopaminergic neurons (i.e., TH-ir cells). Administration of very low doses of 6-OHDA (10 μM; **a**) or MPP^+ (0.25 μM; **b**) induced a significant (6-OHDA) or non-significant (MPP^+) increase in the intensity of DHE-derived fluorescence. Treatment with 6-OHDA or MPP^++angiotensin II (AII; 100 nM) induced a significant increase in the intensity of DHE-derived fluorescence, and the increase was blocked by simultaneous administration of the mitoK(ATP) channel inhibitor 5-HD. Treatment with angiotensin II alone induced a significant increase in DHE-derived labeling. No significant effect was observed after treatment with 5-HD alone. Data are means± SEM. $^*p<0.05$ compared with control group (untreated cells); $^{\#}p<0.05$ compared with the group treated with 6-OHDA+AII or MPP^++AII (one-way ANOVA and Bonferroni's post hoc test). *AII* angiotensin II, *DA* dopaminergic, *5-HD* 5-hydroxydecanoic acid

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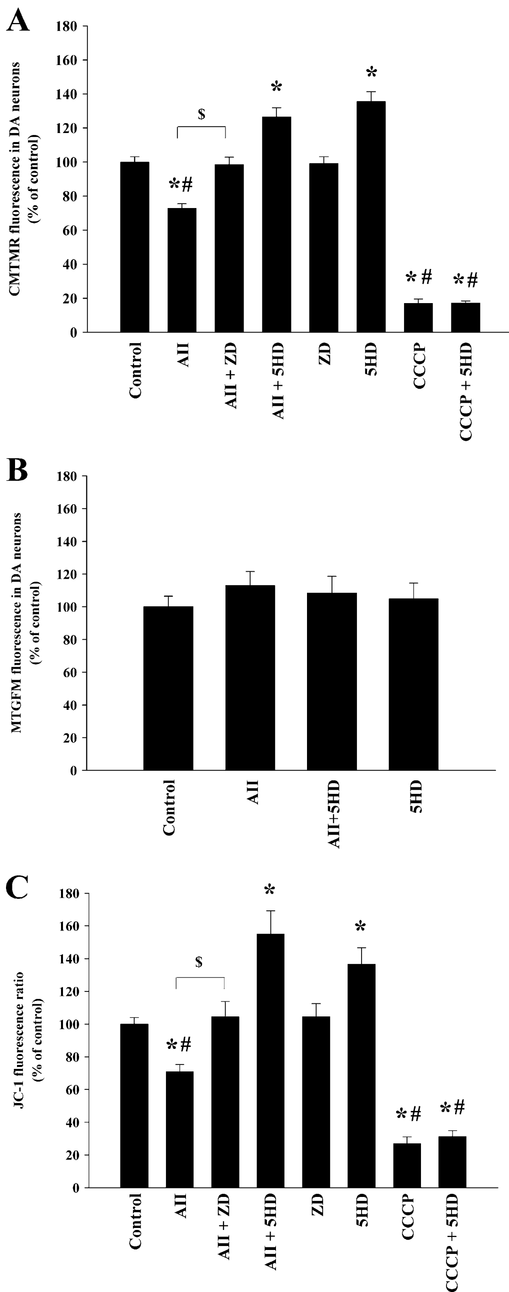

◀**Fig. 5** **a** Treatment of cultures with AII induced a significant reduction in CMTMR fluorescence (i.e., $\Delta\Psi_M$) in dopaminergic neurons. Treatment with the angiotensin type 1 receptor antagonist ZD 7155 or the mitoK(ATP) channel blocker 5-HD inhibited the angiotensin II-induced decrease in CMTMR fluorescence. TH-ir neurons treated with AII+5-HD or 5-HD alone (i.e., without AII) showed higher levels of CMTMR fluorescence than TH-ir neurons in non-treated cultures. This suggests that 5-HD treatment may also block the effect of additional factors that decrease the $\Delta\Psi_M$ in cultures. Furthermore, 5-HD was unable to increase CMTMR fluorescence in cells treated with protonophore CCCP, which revealed that the increase in CMTR retention is not due to non-specific effects and is dependent on $\Delta\psi_M$. **b** MitoTracker Green FM (MTGFM) labeling analysis showed that changes in CMTMR fluorescence were not caused by changes in total mitochondrial mass. **c** The effects of 5-HD were also confirmed in living cells in mesencephalic cell cultures using the JC-1 assay. 5-HD was also unable to increase J-aggregate fluorescence in cells treated with CCCP. $^{*}p<0.05$ compared with control group (untreated cells), $^{\#}p<0.05$ in comparison with the groups treated with 5-HD+AII or 5-HD alone, $^{\$}p<0.05$ (one-way ANOVA and Bonferroni's post hoc test). *AII* angiotensin II, *CCCP* carbonyl cyanide *m*-chlorophenylhydrazone, *DA* dopaminergic, *ZD* ZD 7155, *5-HD* 5-hydroxydecanoic acid

Role of microglial cells: mitoK(ATP) channel inhibition in neuron-enriched cultures

Involvement of microglial cells in the above mentioned effects of angiotensin II and 5-HD was studied in primary mesencephalic cultures in which microglial cells were identified with OX-42, and levels of superoxide/superoxide-derived ROS were estimated with DHE (i.e., double OX42-DHE labeling). Treatment of cultures with 10 μM 6-OHDA alone or angiotensin II alone did not induce a significant increase in the intensity of DHE-derived fluorescence in microglial cells. However, simultaneous treatment with 10 μM 6-OHDA+angiotensin II significantly increased the intensity of DHE-derived fluorescence induced by 6-OHDA alone or angiotensin II alone, which was reduced to control levels by treatment with 5-HD (i.e., 6-OHDA+angiotensin+5-HD; Fig. 7a).

Neuron-enriched cultures in which glia was eliminated with Ara C were used to confirm the potential role of microglial cells in the observed enhancing effect of angiotensin II and the protective effect of 5-HD on dopaminergic neuron degeneration. In the absence of glia, very low doses of 6-OHDA (10 μM) or 6-OHDA+angiotensin II did not induce a significant increase in levels of superoxide/superoxide-derived ROS (i.e., intensity of DHE-derived fluores-

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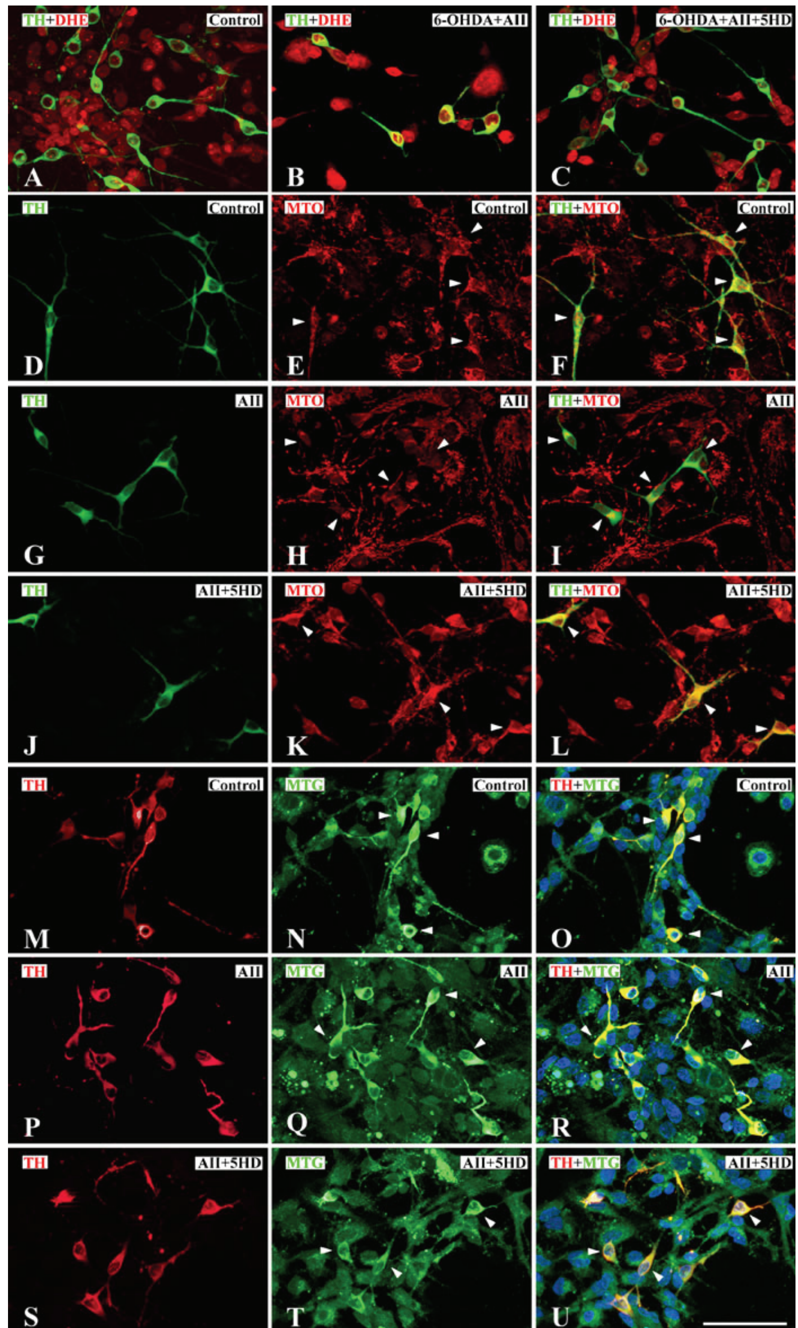

Fig. 6 Double labeling for TH (*green* in **a, d, g, j**; *red* in **m, p, s**) and DHE-derived fluorescence (*red*; **a–c**), or CMTMR (MitoTracker Orange, *red*; **d–l**), or MTG (MitoTracker Green, *green*; **m–u**). Cultures treated with 6-OHDA (10 μM)+angiotensin II (AII; 100 nM) displayed intense DHE-derived fluorescence (*red*) in dopaminergic neurons (**b**), which was higher than in control cultures (**a**) or in those cultures treated with 6-OHDA+AII and 5-HD (**c**). Treatment of cultures with AII (100 nM) alone induced a significant reduction in CMTMR fluorescence (i.e., $\Delta\Psi_M$; *red*) in dopaminergic neurons (**g–i**; *green*) with respect to controls (**d–f**). The decrease was inhibited by treatment with 5-HD (**j–l**). However, treatment of cultures with AII (100 nM) alone or AII+5-HD did not induce significant changes in MTG fluorescence (i.e., mitochondrial mass; *green*) in dopaminergic neurons (**p–u**; *red*) with respect to controls (**m–o**). Cell nuclei were labeled with Hoechst 33342 (*blue*) in (**o**), (**r**), and (**u**). *AII* angiotensin II, *MTO* MitoTracker Orange, *MTG* MitoTracker Green, *DHE* dihydroethidium, *TH* tyrosine hydroxylase. *Scale bar*: 75 μm (**a–c**) and 50 μm (**d–u**)

cence; Fig. 7b). Moreover, treatment with very low doses of 6-OHDA (10 μM) induced a non-significant decrease in the number of TH-ir neurons in the absence of glia. Interestingly, angiotensin II adminis-

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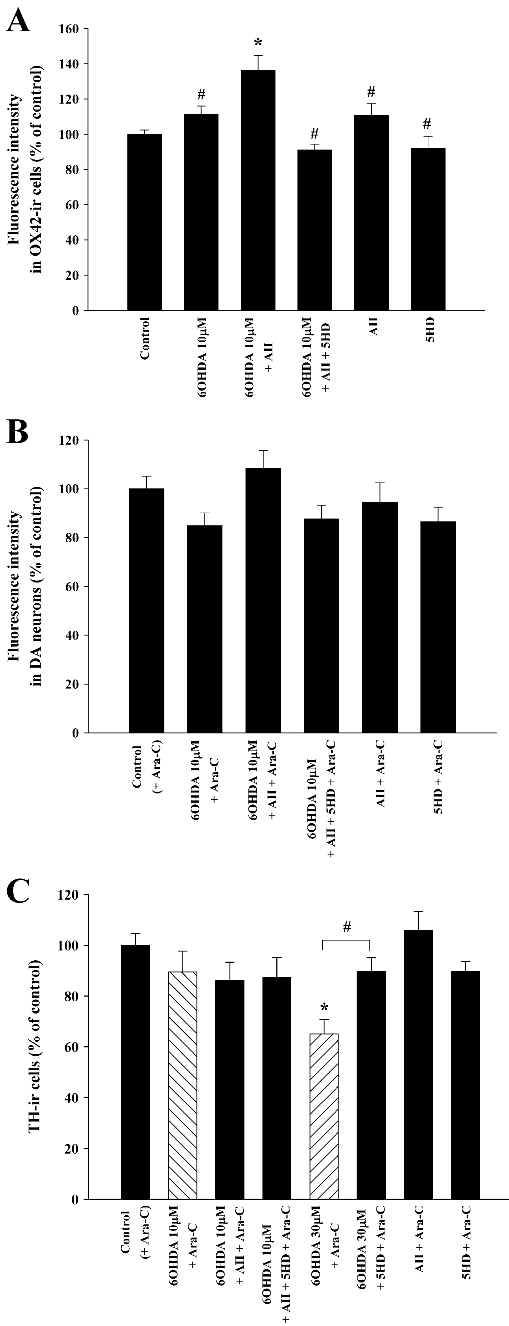

◀**Fig. 7** Involvement of microglial cells in the effects of angiotensin II (AII) and 5-HD was studied in primary mesencephalic cultures in which microglial cells were identified with OX-42 (**a**) and in neuron-enriched cultures (i.e., treated with Ara C; **b**, **c**). **a** Simultaneous treatment with 10 μM 6-OHDA+AII significantly increased the intensity of DHE-derived fluorescence induced by 6-OHDA alone or AII alone in microglial cells, which was reduced to control levels by treatment with 5-HD (i.e., 6-OHDA+AII+5-HD). In the absence of glia, treatment with very low doses of 6-OHDA (10 μM) or 6-OHDA+AII did not induce significant increase in intracellular levels of superoxide/superoxide-derived ROS in dopaminergic neurons (**b**), or a significant decrease in the number of TH-ir neurons (**c**), and 5-HD administration did not induce any significant change. However, higher doses of 6-OHDA (30 μM) induced a significant loss of dopaminergic neurons, which was blocked by 5-HD (**c**). The data are expressed as percentages of the respective control cultures (100%). Data represent means±SEM. *$p<0.05$ compared with control group, $^{\#}p<0.05$ compared with group treated with 6-OHDA (30 μM) in (**c**) (one-way ANOVA and Bonferroni post hoc test). *AII* angiotensin II, *DA* dopaminergic, *TH* tyrosine hydroxylase, *5-HD* 5-hydroxydecanoic acid

tration did not induce any additional loss of dopaminergic neurons, and no significant changes were observed after treatment with 5-HD (Fig. 7c). This indicates that glial cell factors (e.g., ROS from microglia) play a major role in the effect of angiotensin II in increasing dopaminergic neuron death. In order to determine if mitoK(ATP) channels in dopaminergic neurons are involved in dopaminergic degeneration induced by higher levels of ROS than those induced by 10 μM 6-OHDA in the absence of glial cells, neuron-enriched cultures were treated with higher doses of 6-OHDA. Doses of 30 μM 6-OHDA induced a significant loss of dopaminergic neurons (decrease of approximately 40%), which was blocked by inhibition of neuronal mitoK(ATP) channels with 5-HD (Fig. 7c).

Discussion

Several studies in different tissues have shown that normal aging is associated with a pro-inflammatory, pro-oxidant state that may favor an exaggerated response to injury and degenerative diseases (Choi et al. 2010; Csiszar et al. 2003; Ungvari et al. 2004) and that increased activity of local angiotensin via angiotensin II type 1 receptors is involved in age-related degenerative diseases (Basso et al. 2005; Mukai et al. 2002). In agreement, we have recently observed an age-related increased angiotensin activity in the nigra that may increase the vulnerability of

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dopaminergic neurons to additional damage, and the risk of Parkinson's disease with aging (Villar-Cheda et al. 2010b). Furthermore, the interaction between renin–angiotensin system and dopaminergic system is of particular interest. Several studies have shown an important interaction between dopamine and angiotensin II receptors in peripheral tissues, particularly as regards regulating renal sodium excretion and cardiovascular function (Gildea 2009; Zeng et al. 2006; Khan et al. 2008). Recent evidence suggests that dopaminergic and renin–angiotensin systems directly counterregulate each other in renal cells (Gildea 2009) and also in the nigrostriatal system (Villar-Cheda et al. 2010a), and that abnormal counterregulation between dopamine and angiotensin II plays an important role in degenerative changes (Joglar et al. 2009; Li et al. 2008; Rodriguez-Pallares et al. 2008). Interestingly, several studies have shown that there is an aging-related decrease in dopamine release, which results in a progressive decrease in motor activity (Collier et al. 2007; Gerhardt et al. 2002), and may be responsible for the above mentioned aging-related increase in angiotensin activity (Villar-Cheda et al. 2010a,b).

In cardiovascular and renal tissues, angiotensin II induces oxidative stress and inflammation via activation of the NADPH oxidase (Qin et al. 2004; Seshiah et al. 2002; Touyz et al. 2002). Similarly, we have recently shown that the effect of low/sublethal doses of neurotoxins on dopaminergic cell loss is amplified by angiotensin II via type 1 receptors and protein kinase C, leading to activation of the microglial NADPH oxidase and exacerbation of the glial inflammatory response, which increases ROS released by in microglia and dopaminergic neurons (Joglar et al. 2009; Rey et al. 2007; Rodriguez-Pallares et al. 2008). However, recent studies suggest that angiotensin II may stimulate not only cytosolic (i.e., via NADPH oxidase activation) but also mitochondrial-ROS generation in several tissues (de Cavanagh et al. 2007; Zhang et al. 2007), and numerous studies have shown that mitochondria play a major role in generation of oxidative stress, dopaminergic neuron degeneration, and aging (de Cavanagh et al. 2007; Schapira 2008). This is consistent with recent studies that suggest cross-talk signaling between both the cytosolic NADPH oxidase and mitochondria in several types of cells, and that mitoK(ATP) play a major role in this interaction (Brandes 2005; Daiber 2010; Dikalova et al. 2010; Doughan et al. 2008). Therefore, NADPH oxidase may lead to mitoK(ATP) channel opening, which play a major role in modulating intracellular ROS (Andrukhiv et al. 2006; Costa and Garlid 2008; Mattson and Liu 2003) via regulation of the formation/release of ROS from the mitochondria, and integration of signals from diverse sources (Facundo et al. 2007; Fornazari et al. 2008; Oldenburg et al. 2002). ROS open up mitoK(ATP), and mitochondrial ROS production can be stimulated by the opening of mitochondrial mitoK(ATP) channels (Costa and Garlid 2008; Kimura et al. 2005b; Zhang et al. 2002).

In accordance with the above mentioned studies, the present results reveal that activation of mitoK (ATP) channels may play important role in the effects of angiotensin II on the dopaminergic system via NADPH oxidase activation. Inhibition of mitoK(ATP) channels with 5-HD blocked the increase in dopaminergic cell death induced by angiotensin II, as well as the angiotensin II-induced increase in superoxide/superoxide-derived ROS levels in dopaminergic neurons, and the angiotensin II-induced decrease in mitochondrial inner membrane potential ($\Delta\psi_M$) in dopaminergic neurons. 5-HD is the most widely used specific inhibitor of mitoK(ATP) channels, and several studies have shown that 5-HD (10 μM) blocks the mitoK(ATP) channels without any effect on cell membrane K(ATP) channels (Costa and Garlid 2008; Garlid et al. 1997; McCullough et al. 1991; Zhang et al. 2007). Doses up to 500 mmol/l have been reported to inhibit selectively mitoK(ATP) channels without affecting surface K(ATP) currents (Sato et al. 2000; Hu et al. 1999). However, we did not observe significant differences between the effect of 10 μM 5-HD and 100 μM 5-HD, and high doses of 5-HD may have non-specific side effects independent of any action on mitoK(ATP), as reported for mitoK(ATP) openers, which have been observed to act as channel closers at high concentrations (Garlid 2000; Wu et al 2006). In the present study, we therefore used the lowest dose of 5-HD (10 μM), which proved effective at inhibiting the effects of angiotensin II. Furthermore, it is known that 5-HD does not exert any radical scavenging activity (Kimura et al. 2005a, b), and the results cannot be attributed to a 5-HD-induced decrease in 6-OHDA uptake, as revealed in the present study by the [^{3}H] dopamine uptake assay. In addition, treatment with a second K(ATP) inhibitor (i.e., glibenclamide) confirmed the protective effect observed with 5-HD.

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The cross-talk signaling between NADPH oxidase and mitochondria means that angiotensin II stimulation induces NADPH activation, which leads to opening of mitoK(ATP), depolarizes mitochondrial potential, and amplifies ROS generation from mitochondria (Brandes 2005; Kimura et al. 2005a, b; Zhang et al. 2007), but also that mitochondrial ROS have a significant upstream impact on increasing NADPH oxidase activity (Li et al. 2001; Wosniak et al. 2009). Thus, a vicious cycle consisting of mitochondrial and NADPH oxidase-derived ROS formation with each stimulating the other in a positive feedback fashion leads to increased cytoplasmatic ROS levels (Daiber 2010; Dikalova et al. 2010). This has clearly been observed in recent studies in endothelial cells, which showed that inhibition of NADPH activity by apocynin or deletion of NADPH oxidase subunits prevented mitochondrial impairment and attenuated mitochondrial superoxide production via mitoK(ATP) channels (Doughan et al. 2008); in addition, mitochondrial superoxide stimulated extra-mitochondrial NADPH oxidase activity, which was blocked by SOD2 (manganese-containing mitochondrial superoxide dismutase), the mitochondria-targeted superoxide dismutase mimetic mitoTEMPO or the inhibitor of mitoK(ATP) channels 5-HD (Dikalova et al. 2010).

K(ATP) channels were originally discovered in the heart, although are particularly abundant in the CNS, and occur at highest levels in the substantia nigra and striatum (Busija et al. 2004; Zini et al. 1993). Brain mitochondria contain seven times more mitoK(ATP) channels per milligram of mitochondrial protein than liver or heart (Bajgar et al. 2001; Bednarczyk 2009). It is therefore possible that, as suggested for peripheral tissues (Facundo et al. 2007; Fornazari et al. 2008; Oldenburg et al. 2002), these channels provide in dopaminergic neurons a convergent target that could integrate ROS induced by low levels of neurotoxins (6-OHDA or MPP^+) with angiotensin/NADPH oxidase-induced ROS to increase dopaminergic neuron oxidative stress and cell death. It may be speculated that low doses of neurotoxins, or other factors in PD, may generate low levels of ROS by several mechanisms that are insufficient to induce dopaminergic cell death. However, the low levels of ROS, together with those derived from angiotensin-induced NADPH oxidase activation, may act as a trigger to activate mitoK(ATP) channels in the mitochondrial inner membrane, thereby enhancing ROS production and subsequent progression of dopaminergic cell degeneration. This is also supported by our previous studies that observed that the effects of exogenous ROS generated by autooxidation of 6-OHDA are decreased by inhibition of mitoK (ATP) (Rodriguez-Pallares et al. 2009). However, it has also been reported that mitoK(ATP) openers inhibit rotenone-induced microglial activation and neuroinflammation (Zhou et al. 2008). The discrepancies may be related to different experimental conditions. MitoK(ATP) openers have been reported to act as channel closers at high concentrations (Wu et al. 2006). It has also been suggested that opening of mitoK(ATP) may have two distinct consequences, depending on the underlying bioenergetic state (Garlid et al. 2003). When the $\Delta\psi_M$ is high, as in normoxic and resting cells, opening of channels causes net K^+ influx and matrix alkalinization with a consequent rise in mitochondrial ROS production. When $\Delta\psi_M$ is depressed, as occurs during ischemia or treatment with the complex I blocker rotenone, mitoK(ATP) opening may add a parallel K^+ conductance that counteracts the decrease in K^+ influx and matrix contraction that otherwise occur, and therefore mitoK(ATP) opening may maintain constant volume of the mitochondrial matrix and the intermembrane space (Bajgar et al. 2001; Garlid et al. 2003; Kowaltowski et al. 2001). It is interesting to note that activation of mitoK(ATP) channels and ROS production lead to delayed protection against subsequent intense and potentially lethal insults such as ischemia by activation of adaptive cell responses in several types of cells (i.e., preconditioning; Kis et al. 2003; Oldenburg et al. 2002). Conversely, the present study shows that ROS induced by activation of mitoK (ATP) channels contribute to dopaminergic cell death. This is consistent with the results of previous studies that have shown that dopaminergic neurons function in a “near-compromised” state and are more vulnerable to oxidative stress and to mitochondrial complex I inhibition than other neurons and cell types (Bertarbet et al. 2000; Hirsch et al. 1997; Obeso et al. 2010). Therefore, activation of mitoK(ATP) channels and increased ROS levels, which may be useful for preconditioning and delayed protection in other cell types, may overwhelm the defense mechanisms of dopaminergic neurons. This may also be supported by the hyperpolarizing effect of 5-HD observed in

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TH-ir neurons treated with angiotensin+5-HD or with 5-HD alone, which suggests that 5-HD treatment may also block the effect of other factors (i.e., in addition to AII) that act on cultured dopaminergic neurons and decrease their $\Delta\psi_{M.}$ The hyperpolarizing effect of 5-HD may be related to experimental conditions and the cell type, and has been also observed in other studies (Valero et al. 2008). However, this observation is particularly interesting in the case of dopaminergic neurons since a number of previous studies have shown that the dopaminergic neurons have high levels of ROS. A number of factors are thought to be involved, including increased iron content, reduced antioxidant capacity or factors associated with the dopamine synthesized, released, and metabolized in these neurons (Fahn and Cohen 1992; Olanow 1990). The protective defense mechanisms for dopaminergic neurons may be overwhelmed by additional deleterious factors in neurons already subjected to dopamine-derived toxicity thus leading to dopaminergic neuron death (i.e., a "synergistic effect hypothesis"). In the present and previous studies, we suggest that the brain renin–angiotensin system plays a major role in this process since major sources of ROS such as NADPH oxidase and mitochondria are enhanced by renin–angiotensin system activation.

As angiotensin II type 1 receptors and NADPH oxidase have both been observed in dopaminergic neurons and microglial cells (Joglar et al. 2009; Rodriguez-Pallares et al. 2008), it is possible that neurotoxins may interact synergistically with ROS derived from either neuronal or microglial NADPH oxidase activation to activate mitoK(ATP) channels and enhance ROS production. In microglial cells (i.e., OX-42 ir cells), we observed that angiotensin II significantly increased levels of ROS induced by very low doses of neurotoxins (10 μM 6-OHDA), which is inhibited by 5-HD. This suggests a role for microglial mito(KATP) channels in angiotensin-induced amplification of ROS release and microglial activation. In inflammatory cells such as microglia, NADPH oxidase activation leads to ROS production that serves dual functions. Firstly, high concentrations of ROS are released extracellularly to kill invading microorganisms or cells (Babior 1999, 2004). Secondly, low levels of intracellular ROS act as a second messenger in a considerable number of signaling pathways involved in the inflammatory response (Mattson and Maudsley 2009; Qin et al. 2004; Touyz et al. 2002). The present results suggest that mitoK(ATP) play a major role in these mechanisms.

The possible neuronal origin of angiotensin/NADPH oxidase-induced ROS was studied using neuron enriched cultures, in which very low doses of neurotoxin (10 μM 6-OHDA) did not induce a significant loss of dopaminergic neurons, and this effect was not significantly modified by angiotensin II or angiotensin+5-HD. This suggests that a direct effect of angiotensin II on neuronal NADPH oxidase activation elicits low levels of ROS which, acting together with those derived from 10 μM 6-OHDA, are insufficient to induce significant mitoK(ATP) channel activation and/or dopaminergic degeneration. This is consistent with data showing that in non-inflammatory cells, such as neurons, the NADPH oxidase produces only low rates of ROS for signaling function (Babior 1999, 2004). However, we also demonstrated that neuronal mitoK(ATP) channels are involved in dopaminergic cell death when channels are activated by higher levels of ROS such as those derived from 30 μM 6-OHDA in the present experiments in neuron-enriched cultures (dopaminergic cell death was blocked in the presence of 5-HD), or those derived from activated microglia in primary (i.e., neuron/glia) cultures.

In summary, the data suggest that angiotensin II, via type I receptors, activates microglial NADPH oxidase (i.e., NOX2, phagocytic oxidase, PHOX) that induces mitoK(ATP) opening and increases mitochondrial-derived superoxide, which stimulates extramitochondrial NADPH oxidase activity in a feed-forward fashion and constitutes a vicious cycle leading to enhanced microglial activation and extracellular release of ROS. MitoK(ATP) channels of dopaminergic neurons may respond to high levels of ROS derived from the angiotensin-related microglial activation and increase neuronal NADPH oxidase activity. Furthermore, neuronal mitoK(ATP) channels may provide a convergent target to integrate different sources of neuronal ROS (ROS derived from 6-OHDA or MPP^+, or from neuronal NADPH oxidase activated by angiotensin via neuronal type 1 receptors in the present experiments, or from other factors in Parkinson's disease) and ROS from activated microglia to induce dopaminergic cell death. In conclusion, the present results reveal that mitoK(ATP) channels may play a major role in the enhancing effect of angiotensin II on dopaminergic degeneration triggered

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by low/sublethal doses of dopaminergic neurotoxins, and possibly in the synergistic interaction of several intra- and/or extraneuronal factors to increase levels of oxidative stress and dopaminergic cell death in PD. This is particularly interesting since aging-related increase in nigral renin–angiotensin system activity may constitute a major factor in the increased risk of PD with aging. Furthermore, the present study provides additional data for considering brain renin–angiotensin system and mitoK(ATP) channels as potential targets for neuroprotection in PD and aging.

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4.6- Implicación de la vía RhoA/Rho-quinasa microglial en la neurodegeneración dopaminérgica. Papel de la angiotensina II a través de los receptores AT1

Villar-Cheda B., Dominguez-Mejide A., **Joglar B.**, Rodriguez-Perez A.I., Guerra M.J., Labandeira-Garcia J.L. (2011) Involvement of microglial RhoA/Rho-Kinase pathway activation in the dopaminergic neuron death. Role of angiotensin via angiotensin type 1 receptors. (DOI: 10.1016/j.nbd.2012.04.010)

El péptido AII, a través de los receptores de AII de tipo 1 (AT1), es uno de los principales inductores de procesos inflamatorios y estrés oxidativo en diversos tejidos. En los últimos años, se han ido acumulando cada vez más evidencias que demuestran que la pérdida de neuronas dopaminérgicas inducida por distintas neurotoxinas es amplificada por acción de la AII a través de los receptores AT1. Sin embargo, los mecanismos a través de los cuales la AII produce dicha neurodegeneración dopaminérgica y un aumento en la respuesta inflamatoria no han sido clarificados. Varios estudios en tejidos cardiovasculares sugieren que la vía RhoA/Rho-quinasa podría jugar un papel importante en dichos efectos. Los resultados obtenidos en este trabajo muestran que la administración de la neurotoxina MPTP en ratones induce un incremento tanto en los niveles de expresión del ARNm de RhoA y RockII como en la actividad ROCK. Además dicho incremento es inhibido en ausencia del receptor de AII AT1 (en la SN de ratones knock-out para AT1a tratados con MPTP). Por otro lado, la administración del inhibidor de ROCK, Y-27632, o la deleción de los receptores AT1, produce un descenso en la activación microglial y en la muerte celular dopaminérgica inducidas por MPTP. Asimismo, en cultivos primarios mesencefálicos tratados con MPP^{+}, el aumento en la muerte celular dopaminérgica inducida por AII es bloqueado mediante el tratamiento con el inhibidor Y-27632. En función de estos resultados podemos concluir que la activación de la vía RhoA/ROCK está implicada en la neurodegeneración dopaminérgica inducida por MPTP y en el efecto amplificador de la AII (vía AT1) sobre la respuesta inflamatoria y muerte celular dopaminérgica. Por lo tanto, el uso de inhibidores de ROCK y de antagonistas de los receptores AT1 podrían proporcionar una nueva fuente de estrategias neuroprotectoras para frenar la progresión de la EP.

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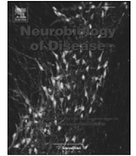

Involvement of microglial RhoA/Rho-Kinase pathway activation in the dopaminergic neuron death. Role of angiotensin via angiotensin type 1 receptors

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ABSTRACT

It has recently been shown that the dopaminergic cell loss induced by neurotoxins is enhanced by brain angiotensin II (AII) via type 1 receptors (AT1). However, the mechanisms involved in the dopaminergic degeneration and the brain inflammatory effects of AII have not been clarified. The RhoA–Rho-Kinase (ROCK) pathway may play a critical role in the inflammatory and oxidative effects of AII. In the substantia nigra of mice, administration of the dopaminergic neurotoxin MPTP induced an increase in the expression of RhoA and ROCK II mRNA levels and ROCK activity, which were inhibited by AT1 receptor deletion (i.e., in AT1a null mice treated with MPTP). Administration of the ROCK inhibitor Y-27632 or AT1 deletion induced a significant decrease in MPTP-induced microglial activation and dopaminergic cell death. In rat primary mesencephalic cultures treated with MPP^+, the increase in dopaminergic cell loss induced by AII administration was also inhibited by treatment with Y27632. Intense expression of ROCK II was observed in the microglial cells in the substantia nigra of mice treated with MPTP, and the major role of the microglial ROCK was confirmed by comparing mesencephalic cultures with and without microglia. Activation of the RhoA/ROCK pathway is involved in the MPTP-induced dopaminergic degeneration, and in the enhancing effect of AII/AT1 activation on the microglial response and dopaminergic degeneration. ROCK inhibitors and AT1 receptor antagonists may provide new neuroprotective strategies against the progression of Parkinson's disease.
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Introduction

The peptide angiotensin II (AII), via type 1 receptors (AT1), is one of the most important known inducers of inflammation and oxidative stress, produces ROS by activation of the NADPH-oxidase complex (Cai et al., 2003; Seshiah et al., 2002; Touyz et al., 2002), and plays a major role in the pathogenesis of several age related degenerative diseases (Basso et al., 2005; Benigni et al., 2009; de Cavanagh et al., 2011). The brain possesses a local renin-angiotensin system (RAS; Labandeira-Garcia et al., 2011; McKinley et al., 2003; Saavedra, 2005), and NADPH oxidase, AT1 and AT2 receptors have been located in dopaminergic neurons and nigral microglia and astrocytes (Grammatopoulos et al., 2007; Joglar et al., 2009; Rodriguez-Pallares, et al., 2008; Valenzuela et al., 2010). In recent years, evidence for a major role of oxidative stress and neuroinflammation in the pathogenesis and progression of Parkinson's disease (PD) has been reported (González-Hernández et al., 2010; McGeer and McGeer, 2008; Zhou et al., 2008). In accordance, we have shown that the dopaminergic (DA) cell loss induced by DA neurotoxins is enhanced by AII via AT1 receptors. Activation of the AII/AT1 pathway exacerbates the microglial NADPH oxidase and the glial inflammatory response, which were inhibited by treatment with the AT1 antagonist candesartan (Joglar et al., 2009; Rey et al., 2007; Rodriguez-Pallares et al., 2008). In addition, several recent studies have shown that the brain RAS has a major role in brain inflammation (Lanz et al., 2010; Stegbauer et al., 2009). However, the mechanisms involved in the inflammatory effects of AII in the brain and anti-inflammatory effects of AT1 blockage have not been clarified.

Different authors have demonstrated that the small GTP-binding protein Rho plays an important role in various cellular functions and upregulates various molecules that accelerate inflammation and oxidative stress (Hiroki et al., 2004; Schmandke et al., 2007). RhoA, a member of the Rho family has been implicated in a wide variety

Abbreviations: ACE, Angiotensin converting enzyme; AII, angiotensin II; ARBs, AT1 receptor blockers; AT1, Type 1 receptors; cDNA, Complementary DNA; Ct, Cycle threshold; DA, Dopaminergic; DIV, Days in vitro; dNTP, Deoxynucleotide triphosphate; GSI-B4, Isolectin B4; ir, Immunoreactive; KPBS-BSA, Phosphate-buffered saline containing 1% bovine serum albumin; MMLV, Moloney murine leukemia virus reverse transcriptase; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, Phosphate buffered saline; PD, Parkinson's disease; RAS, Renin-angiotensin system; ROCK, Rho-associated Kinase; RT-PCR, Real time polymerase chain reaction; WT, Wild type.

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of disease conditions. RhoA initiates cellular processes acting on Rho-associated Kinase (ROCK), its direct downstream effector (Katoh et al., 1998; Zhang et al., 2007). Two isoforms encoded by two different ROCK genes have been described: ROCK I and ROCK II (Nakagawa et al., 1996). ROCK II (which is also known as ROCKα) is preferentially expressed in brain. Abnormal activation of the RhoA/ROCK pathway has been observed in various disorders of the central nervous system; lesions of the brain and spinal cord activate ROCKs, which have been revealed as major mediators of neuroinflammatory responses (Mueller et al., 2005; Sheikh et al., 2009). However, it is not known whether ROCK is involved in the inflammatory response observed in animal models of PD and also in PD.

ROCK has been suggested to be involved in AII-induced inflammatory arteriosclerotic and coronary lesions (Hiroki et al., 2004; Iida et al., 2008). However, it is not known if activation of the Rho–ROCK pathway plays a major role in the above mentioned enhancing effects of AII on dopaminergic neuron degeneration. In the present study, we used in vivo (MPTP) and in vitro (MPP^+) models of PD to investigate whether activation of the RhoA–ROCK pathway mediates dopaminergic neuron death and also whether ROCK activation is involved in the AII/AT1-enhanced dopaminergic neuron death.

Methods

In vivo experiments. Treatment of mice with MPTP

Male C57BL-6 mice weighing 20–25 g (8-week old mice) were used. Mice were wild type (Charles River, France) or homozygous mice deficient for AT1a (the major mouse AT1 isoform and the closest murine homolog to the single human AT1; Jackson Laboratory, Bar Harbor, ME, USA; Sugaya et al., 1995). Mice were maintained in the animal facility at the University of Santiago de Compostela in accordance with the institutional guidelines. In a first series of experiments, mice were divided into 4 groups (A1-D1). The wild type (n = 24) and AT1a-null (n = 5) mice in respectively group B1 and D1 were injected with MPTP (Free base, Sigma, St. Louis, MO, USA; 30 mg/kg/day, intraperitoneally; in saline for 5 days). The wild type (n = 14) and AT1a-null (n = 5) mice in respectively group A1 and C1 were used as normal non-lesioned controls and were injected intraperitoneally with saline. Mice were killed 1 day after the first MPTP or saline injection (i.e., a single injection), and 1 h or 7 days after the last (i.e., the fifth) MPTP or saline injection (i.e., 1, 5 or 12 days after the first injection), and the area of the substantia nigra in the ventral mesencephalon was carefully dissected and processed for real time polymerase chain reaction (RT-PCR) and ROCK activity studies. In a second series of experiments, the wild type and AT1a-null mice were divided into 5 groups (A2–E2). Mice in groups A2 (wild type; n = 8) and C2 (AT1a-null mice; n = 4) were used as non-lesioned controls, and were injected intraperitoneally with saline. Mice in groups B2 (wild type; n = 6) and D2 (AT1a-null mice; n = 4) were injected with MPTP as above. Mice in group E2 (wild type, n = 6) were injected with MPTP as with group-B2 mice, but were also injected with the ROCK inhibitor Y-27632 (Sigma, 5 mg/kg/day, intraperitoneally) 30 min before the MPTP treatment. The Y-27632 dose was selected in accordance with the results of previous studies (Büyükafşar et al., 2006; Li et al., 2011). Mice were killed 1 week after the MPTP or saline treatment, and were then processed for histology (see immunolabeling of mice brains section). In a third series of experiments, different groups of mice were injected with a single dose of MPTP (30 mg/kg) 30 min after injection of saline or Y-27632 (i. e., wild type mice + saline + MPTP, n = 6; wild type mice + Y-27632 + MPTP, n = 6; and AT1a-null mice + saline + MPTP, n = 6), and finally killed 90 min after the MPTP injection to quantify striatal levels of MPP^+ (Hows et al., 2004; Przedborski et al., 1996; see below).

RNA extraction and real-time quantitative RT-PCR

Total ribonucleic acid (RNA) from the nigral region or primary mesencephalic cultures was extracted with Trizol (Invitrogen, Paisley, Scotland, UK) according to the manufacturer's instructions. The concentration of RNA was estimated using a Nanoquant plate and Infinite M200 multiwell plate reader (TECAN, Salzburg, Austria). Total RNA (2.5 μg) was reverse-transcribed to complementary DNA (cDNA) with deoxynucleotide triphosphate (dNTP), random primers, and Moloney murine leukemia virus reverse transcriptase (MMLV; 200 U; Invitrogen). The relative levels of RhoA and ROCK II messenger RNA (mRNA) were examined with real-time PCR. Experiments were performed with a real-time iCycler™ PCR platform (Bio-Rad, Hercules, CA). GAPDH was used as a housekeeping gene and was amplified in parallel with the genes of interest. The data were evaluated by the delta–delta Ct method ($2-\Delta\Delta Ct$) where Ct is the cycle threshold. The expression of each gene was obtained as relative to the housekeeping transcripts. Forward (F) and reverse (R) primers were designed for each gene by use of Beacon Designer software (Bio-Rad). Primer sequences were as follows: for mouse RhoA F 5′-TGCTTGCTCATAGTCTTCAG-3′, R 5′-CAGGCGGTCATAATCTTCC-3′ (F 5′-CTGGTGATTGTTGGTGATGG, R 5′- ACGGTCATAATCTTCCTGTCC for rat cultures); for mouse ROCK II F 5′-CGAATAGAACTCCAGATGACC-3′ R 5′-GCACAGGCAATGACAACC-3′ (F 5′-GACAGTGACATTGAACAGC, R 5′-ACAGCCATCCTTCTAATCG for rat cultures); for mouse GAPDH, F 5′-AACGACCCCTTCATTGAC-3′, R 5′- TCCACGACATACTCAGCAC-3′ (β-actin, F 5′-TCGTGCGTGACATTAAAGAG-3′, R 5′-TGCCACAGGATTCCA-TACC-3′ for rats cultures).

Rho kinase activity

ROCK activity was measured with the ROCK Activity Assay kit (Cell Biolabs, Inc, San Diego, CA, USA) according to the manufacturer's instructions. The ROCK Activity Assay kit is an enzyme immunoassay developed for detection of the specific phosphorylation of myosin phosphatase target subunit 1 at Thr696 by ROCK. Tissue was homogenized in lysis buffer (50 mM Tris–HCl pH7.5, 150 mM NaCl, 1 mM 2-glycerophosphate, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4) containing protease inhibitor cocktail (P8340, Sigma). Protein concentration of extracts was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific, Fremont, CA, USA) and equal amounts of protein (5 μg per well) were used; each sample was assayed in duplicate. Phosphorylation activity was assessed by measuring the absorbance at 450 nm in an Infinite M200 multiwell plate reader (TECAN).

Estimation of MPP^+ levels by mass spectrometry

Brains were removed from mice, the striata dissected on an ice-cold plaque, and the striatal tissue frozen on dry ice. Striata were stored at −80 °C until analysis. On the day of the assay (Hows et al., 2004), striata were weighed and sonicated in a solution of 0.4 M perchloric acid containing (w/v): 0.1% sodium metabisulphite, 0.01% EDTA and 0.1% L-cysteine. Samples were centrifuged at 13,000 r.p.m. and the supernatant was used to determine MPP^+ levels. HPLC separation was accomplished in a Waters Alliance 2795 system (Waters, Milford, MA, USA), with an Atlantis dC18 column (2.1 × 50 mm, 3 μm). The mobile phase consisted of solvent A (0.1% formic acid), and solvent B (acetonitrile). We employed an elution profile from 95% solvent A for 1 min, followed by a linear gradient from 95% solvent A to 100% solvent B from minute 1 to minute 1.5, and 100% solvent B was maintained until minute 5. A re-equilibration time of 5 min was allowed between injections and chromatography was carried out at a flow-rate of 0.2 ml/min. Eluates were detected with a Quattro MicroTM API ESCI triple-quadrupole mass spectrometer fitted with Z-spray. Electrospray ionization was set in positive ion

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polarizing mode (ESI+) for acquisition of mass spectrometry data, with the following fragments (m/z): 170.2 > 128.0, 170.2 > 154.4, and 170.2 > 115.1. The capillary voltage was set at 3 kV, the desolvation temperature at 450 °C, the cone voltage at 45 V, and the desolvation gas flow rate was set at 550 L/h. All parameters were adjusted to obtain optimum operating conditions for maximum intensity of the selected fragments, with Masslynx 4.1 software. MPP^+ standards were prepared in the homogenization solution and used for calibration purposes.

Immunolabeling, lectin histochemistry and cresyl violet staining of mice brains

The mice were killed and perfused, first with 0.9% saline, and then with cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, washed and cryoprotected in the same buffer containing 20% sucrose, and finally cut at 30 μm on a freezing microtome. Sections were processed with rabbit polyclonal antibodies to TH (as a marker of dopaminergic neurons) and rat monoclonal antibodies against CD45 (as a marker of reactive microglia/macrophages), as follows: sections were incubated for 1 h in 10% normal serum with 0.25% Triton X-100 in 20 mM potassium phosphate-buffered saline containing 1% bovine serum albumin (KPBS-BSA), then incubated overnight at room temperature with rabbit polyclonal antiserum to TH (Chemicon, Millipore Temecula, CA; 1:500) or at 4 °C with rat monoclonal antiserum to CD45 (rat IgG, 1:1000, AbD Serotec) in 20 mM KPBS-BSA containing 1% bovine serum albumin, 2% normal serum and 0.25% Triton X-100. The sections were subsequently incubated, first for 90 min with the corresponding biotinylated secondary antibodies (1:200), and then for 90 min with an avidin-biotin-peroxidase complex (ABC, Vector, USA; 1:50). Finally, the labeling was visualized with 0.04% hydrogen peroxide and 0.05% 3-3′ diaminobenzidine (DAB, Sigma), containing 0.1% nickel sulfate to intensify the microglial staining. For negative control staining, sections were incubated in media lacking primary antibodies.

Activated microglial cells were also stained histochemically with *Griffonia simplicifolia* isolectin B4 (GSI-B4) as follows. Sections were preincubated in phosphate buffered saline (PBS) containing 0.1 mM of $CaCl_2$, $MgCl_2$, $MnCl_2$ and 0.3% Triton X-100 for 20 min. The sections were then rinsed with PBS and incubated overnight at 4 °C with biotinylated GSI-B4 (Sigma; 20 μg/ml) in PBS containing cations and 0.3% Triton X-100. The sections were rinsed again with PBS, then incubated with an avidin-biotin-peroxidase complex (ABC, Vector; 1:100) for 90 min. Finally, labeling was visualized with 0.04% hydrogen peroxide and 0.05% DAB with 0.1% nickel sulfate to intensify the staining. For negative control staining, sections were incubated in media lacking GSI-B4.

Selected sections were processed for double immunofluorescence against ROCK II and CD45 (as a marker of reactive microglia/macrophages), TH (as a marker of dopaminergic neurons), or GFAP (glial fibrillary acidic protein; as a marker of astrocytes) to study the possible co-localization of these markers. Sections were first incubated overnight at 4 °C with ROCK II (monoclonal anti-ROCK II antibody, BD Bioscience, San Jose, CA, USA; 1:500); after rinsing with KPBS, the sections were incubated for 150 min with goat anti-mouse conjugated with cyanine 3.18 (Cy3; Chemicon; 1:500). For the second labeling, sections were incubated overnight at 4 °C with antibodies against TH (Chemicon; 1:250) or GFAP (Chemicon; 1:250) or CD45 (AbD Serotec; 1:500). After rinsing, sections were incubated for 60 min with the biotinylated secondary antibody (swine anti rabbit for TH and GFAP, or rabbit anti rat for CD45). Labeling was visualized by incubation of the sections with streptavidin Alexa-fluor 488 conjugate (Molecular Probes; 1:2500). Co-localization of markers was confirmed by confocal laser microscopy (TCS-SP2; Leica, Heidelberg, Germany) and use of a sequential scan method to avoid any possible overlap. In all experiments the control sections, in which the primary antibody was omitted, were immunonegative for these markers.

The total numbers of TH-ir neurons in the substantia nigra compacta (SNc) were estimated by an unbiased stereology method (i.e., the optical fractionator). Stereological analysis was carried out with the Olympus CAST-Grid system (Computer Assisted Stereological Toolbox; Olympus, Denmark). Uniform, randomly chosen sections through the substantia nigra (every third section) were analyzed for the total number of TH-ir cells by means of a stereological grid (fractionator), and the nigral volume was estimated according to Cavalieri's method (Gundersen et al., 1988). Penetration by the antibody was determined by registration of the depth of each counted cell that appeared in focus within the counting frame. This analysis revealed incomplete penetration by antibody leaving 8–10-μm in the center poorly stained (Torres et al., 2006). The total number of cells was therefore calculated by excluding the volume corresponding to this portion of the sections. In order to confirm that MPTP induces cell death and not only phenotypic down-regulation of TH activity, series of sections through the entire substantia nigra of control mice and mice treated with MPTP were counterstained with cresyl violet, and the total number of neurons in the SNc was estimated by the unbiased stereology method described above for TH-ir cells. Neurons were distinguished from glial cells on morphological grounds, and neurons with visible nuclei were counted as above. The number of reactive microglial cells was estimated with the Olympus CAST-Grid system and the unbiased stereological method described above for counting TH-ir neurons. The density of reactive microglia (cells/mm^3) was determined by dividing the number of labeled cells by the volume that they occupied.

In vitro experiments. Primary mesencephalic cultures

Ventral mesencephalic tissue was dissected from rat embryos of 14 days of gestation (E14). The tissue was incubated in 0.1% trypsin (Sigma), 0.05% DNase (Sigma), and DMEM (Gibco, Invitrogen) for 20 min at 37 °C, and then washed in DNase/DMEM and mechanically dissociated. The resulting cell suspension was centrifuged at 50 *g* for 5 min, the supernatant was carefully removed and the pellet resuspended in 0.05% DNase/DMEM to the final volume required. The number of viable cells in the suspension was estimated with acridine orange/ethidium bromide, and cells were plated onto 35-mm culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) previously coated with poly-L-lysine (100 μg/ml; Sigma) and laminin (4 μg/ml; Sigma). The cells were seeded at a density of 1.5×10^5 cells/cm^2 and maintained under control conditions [DMEM/HAMS F12/(1:1) containing 10% fetal bovine serum (FBS; Biochrom KG, Berlin, Germany)]. The cell cultures were maintained in a humidified CO_2 incubator (5% CO_2; 37 °C) for 8 days in vitro (DIV; see below); the medium was totally removed on day 2 and replaced with fresh culture medium.

Treatment of cultures

Cultures were exposed on 4 DIV to MPP^+ (0.25 μM; Sigma) alone or MPP^+ and AII (100 nM; Sigma) for a further 4 days without additional changes in the culture medium. To study the possible involvement of Rho kinase (ROCK) in cell loss induced by the DA neurotoxin MPP^+ or MPP^+ and AII, cultures were treated with the ROCK inhibitor Y-27632 (30 μM; Sigma) 30 min before treatment with the MPP^+ alone or MPP^+ and AII. The dose of Y-27632 was selected according to that suggested in several previous studies (Kume et al., 2009; Pacary et al., 2006; Zohrabian et al., 2009). To obtain neuron-enriched cultures, cytosine β-D-arabinofuranoside (Ara-C; 1 μM; Sigma) was added 48 h after seeding the cells. After 4 days in vitro, the cultures were treated with MPP^+ (0.25 μM) and AII (100 nM) for a further

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4 days as described previously (Joglar et al., 2009). This method can enrich neurons to >85% purity (about 9% astrocytes and <1% microglia). To obtain cultures lacking of microglial cells, L-leucine methyl ester (LME; 1.5 mM; Sigma) was added 48 h after seeding the cells and was kept in the cultures for 72 h to deplete microglia as described previously by Gao et al. (2008).

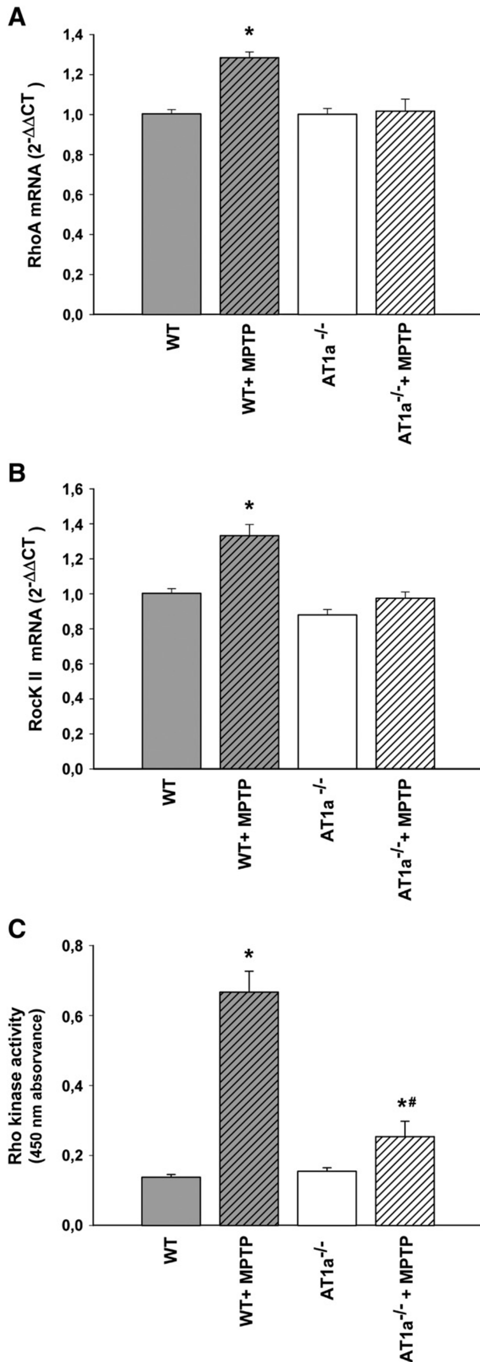

Fig. 1. Activation of the RhoA/ROCK II pathway in the substantia nigra of MPTP lesioned mice. Five-day treatment of wild type mice (WT; 1 h after the last injection) with the dopaminergic neurotoxin MPTP produced a significant increase in the expression of RhoA (A) and ROCK II (B) mRNA levels, and a marked increase in Rho-Kinase (ROCK) activity (C). In AT1a null mice ($AT1a^{-/-}$), no significant increase in RhoA and ROCK II mRNA levels was induced by MPTP, and the increase in ROCK activity was significantly lower than that observed in wild type mice. Data are means ± SEM. *p<0.05 relative to the corresponding saline-treated group, #p<0.05 relative to the WT + MPTP group. One-way ANOVA and Bonferroni post-hoc test.

Immunolabeling of cultures

The cultures were fixed with 4% paraformaldehyde in DPBS (pH 7.4) for 20 min, and endogenous peroxidase activity was quenched by incubation for 5 min with 3% H_2O_2 in DPBS. The cultures were then preincubated with a blocking solution containing 10% normal serum in DPBS with 1% BSA and 0.3% Triton X-100 (Sigma) for 1 h. The cultures were then incubated at 4 °C with mouse anti-TH (1:30,000; Sigma) or OX-6, a mouse monoclonal antibody that recognizes major histocompatibility complex (MHC) class II antigens expressed by activated microglia but not by resting cells (1:200; Serotec, Kidlington, Oxford, UK). The cultures were then washed and incubated for 1 h with biotinylated horse anti-mouse (1:500; Vector, Burlingame, CA, USA). The cultures were then washed and incubated for 1 h with avidin-biotin-peroxidase complex (1:500; Vector). Labeling was revealed with 0.04% H_2O_2 and 0.05% 3, 3′diaminobenzidine (Sigma) as a chromogen. For immunofluorescent labeling, the ABC complex was replaced by streptavidin Alexa-fluor 488 conjugate (Molecular Probes, Invitrogen; 1:2500).

TH-ir and OX6-ir cells were observed with a Nikon Eclipse inverted microscope and counted in 5 randomly chosen longitudinal and transverse microscopic fields along the diameter of the culture dish away from the curved edge by an operator who was blinded to the treatment condition. The microscopic field was defined by a 0.5×0.5-cm reticule (1.25 cm^2). The average number of TH-positive cells in a control culture dish was 2230 ± 120. The results were from at least 3 separate experiments, with a minimum sample size of 4 wells per group and per run. The results were expressed as a percentage of the counts of the control group in the same batch to counteract possible variations among batches.

Statistical analysis

Data are expressed as means ± SEM. Multiple comparisons were analyzed with one-way analysis of variance followed by Bonferroni post hoc test. The normality of populations and homogeneity of variances were tested before each analysis of variance. Differences at $p<0.05$ were considered statistically significant. Statistical analyses were carried out with SigmaStat 3.0 from Jandel Scientific (San Rafael, CA, USA).

Results

MPTP induces activation of the RhoA/ROCK II pathway via AT1 receptors

In wild type mice killed 1 h after the last MPTP or saline injection (i.e., 5 days after the first injection; at an early phase of the degeneration process), MPTP led to a significant increase in the expression of RhoA and ROCK II mRNA levels in the substantia nigra (Figs. 1A, B), and a marked increase in ROCK activity in the substantia nigra in comparison with the activity of ROCK observed in untreated wild type mice (Fig. 1C). The difference in ROCK

activity between lesioned and unlesioned animals was much greater than the differences observed in RhoA and ROCK II mRNA levels between lesioned and unlesioned animals (Figs. 1A–C). In the substantia nigra AT1a null mice, basal levels of RhoA and ROCK II mRNA levels and basal ROCK activity were not significantly different from that observed in the substantia nigra of wild type mice. However, the effect of MPTP treatment was quite different in wild type and AT1a null mice. No significant increase in RhoA and ROCK II mRNA levels was induced by MPTP treatment in the absence of AT1a receptors (Figs. 1A–B). MPTP induced a significant increase in ROCK activity in AT1a null mice. However, the increase was much lower than that observed in wild type mice (i.e., in the presence of AT1 receptors; Fig. 1C).

In mice killed one day after a single MPTP injection (i.e., prior to occurrence of any significant loss of neurons), a significant increase in RhoA and ROCK II mRNA levels and ROCK activity was observed. Finally, increased expression of RhoA and ROCK II mRNA levels and ROCK activity was still observed at the end of the degeneration process (7 days after the last MPTP injection; 12 days after the first injection; Figs. 2A–C).

Activation of AT1 receptors and RhoA/ROCK pathway increases dopaminergic neuron death in mice

In control mice (i.e., not injected with MPTP) the dopaminergic neurons in the substantia nigra compacta were intensely immunoreactive to tyrosine hydroxylase (TH; Fig. 3B). In mice injected with MPTP there was a bilateral reduction in the number of TH-immunoreactive (TH-ir) neurons in the substantia nigra (Figs. 3A, C). In control mice, the total number of neurons counted in cresyl-violet stained sections (14451 ± 1210) was slightly higher than that of TH-ir neurons as some non-dopaminergic neurons located in the SNc were also counted. However, sections from mice treated with MPTP showed significantly fewer cresyl-violet stained neurons in the SNc (8661 ± 962) than in the control mice, confirming that MPTP induced cell death and not TH-downregulation occurred under the present experimental conditions. Mice treated with the ROCK inhibitor Y27632 and injected intraperitoneally with MPTP showed a bilateral reduction in the number of TH-ir neurons in the substantia nigra, relative to control mice, although the reduction was significantly lower than that observed in mice not treated with Y27632, which suggests that activation of ROCK increases the neurotoxic effect of MPTP on the DA system (Figs. 3A, D). The MPTP-induced loss of dopaminergic neurons in the substantia nigra of AT1a-null mice was also lower than in wild type mice treated with MPTP, and similar to that observed in mice treated with MPTP and the ROCK inhibitor Y27632 (Figs. 3A, E). There were no significant differences in the number of dopaminergic neurons between untreated wild type and null AT1a mice.

Striatal MPP^+ levels

In order to determine if treatment with the ROCK inhibitor Y27632 or AT1a deletion acts by modifying MPTP pharmacokinetics such as penetration into the brain, biotransformation of MPTP to MPP^+ or MPP^+ removal from the brain, we measured striatal levels of MPP^+ in mice. There were no significant differences in striatal levels of MPP^+ between mice treated with ROCK inhibitor Y27632 and MPTP (3.436 ± 0.290 ng/mg wet weight striatal tissue), AT1-null mice treated with saline and MPTP (3.100 ± 0.211 ng/mg wet weight striatal tissue) and wild type mice treated with saline and MPTP (3.045 ± 0.157 ng/mg wet weight striatal tissue). The protective effect of the ROCK inhibitor Y27632 was also confirmed in cultures of dopaminergic cells directly treated with MPP^+ (i.e., in the absence of possible changes in the MPTP

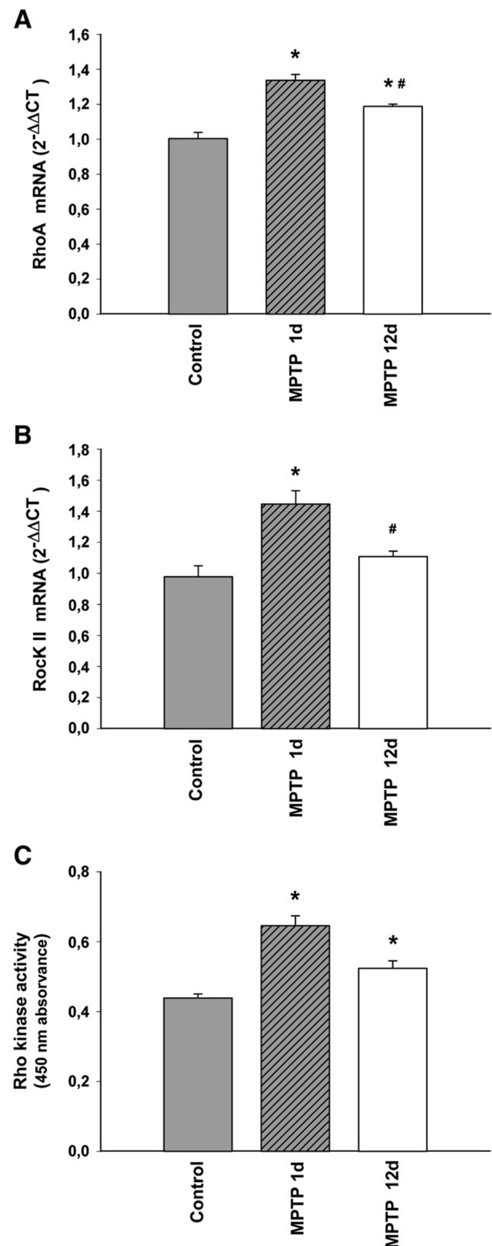

Fig. 2. Activation of the RhoA/ROCK II pathway in the substantia nigra of wild type mice 1 day after a single injection of MPTP and 7 days after a five-day MPTP treatment (i.e., 12 days after the first MPTP injection). After the first injection (i.e., prior to occurrence of any significant loss of neurons), MPTP produced a significant increase in the expression of RhoA (A) and ROCK II (B) mRNA levels and a significant increase in Rho-Kinase (ROCK) activity, which was still observed when the degeneration process was almost completed. Data are means ± SEM. *$p<0.05$ relative to the corresponding control (saline-treated) group, #$p<0.05$ relative to the group treated with a single MPTP injection. One-way ANOVA and Bonferroni post-hoc test.

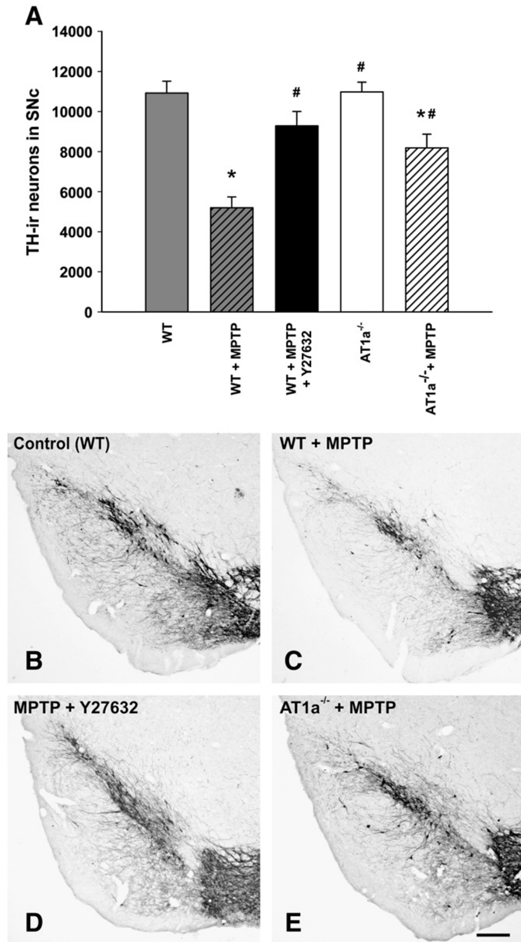

Fig. 3. Dopaminergic (TH-ir) neurons in the substantia nigra compacta (SNc). (A): MPTP administration induced a higher loss of TH-ir neurons in the SNc of wild type (WT) mice than in the SNc of in AT1a-null mice (AT1a$^{-/-}$). However, the MPTP-induced loss of dopaminergic neurons in WT mice treated with the Rho-Kinase inhibitor Y-27632 was similar to that observed in AT1a-null mice. (B–E): Representative photomicrographs of dopaminergic (TH-ir) neurons in the SNc in different experimental groups quantified in A. The dopaminergic neurons were quantified as the total number of TH-ir neurons in the SNc. Data are means ± SEM. $^{*}p<0.05$ compared with mice treated with vehicle, $^{\#}p<0.05$ compared with wild type mice treated with MPTP alone (one-way ANOVA and Bonferroni post-hoc test). Scale bar: 250 μm.

biotransformation to the active metabolite MPP^+; see below experiments with mesencephalic cultures).

Inhibition of ROCK activity inhibits the AII-induced increase in dopaminergic neuron death in mesencephalic cultures

As reported in our previous studies (Joglar et al., 2009), treatment of primary mesencephalic cultures with low doses of MPP^+ (0.25 μM) induced a small but significant loss of dopaminergic neurons. The dopaminergic cell death was significantly reduced by treatment with the ROCK inhibitor Y27632. The loss of dopaminergic neurons was significantly increased by administration of exogenous AII (100 nM). However, the increase in dopaminergic cell loss induced by AII was inhibited by treatment with the ROCK inhibitor Y27632, which indicated that activation of ROCK was involved in the enhancing effect of AII on the dopaminergic cell death induced by the neurotoxin (Figs. 4A–G). In cultures treated with MPP^+ alone, the neuroprotective effect of Y27632 may also be related to inhibition of the effects of endogenous AII, given that all cell types involved in production of brain angiotensinogen and angiotensin are present in primary mesencephalic cultures. Furthermore, the presence of endogenous angiotensin in primary mesencephalic cultures was confirmed in our previous papers by HPLC/Mass/Mass spectrometry (Rodriguez-Pallares et al., 2008).

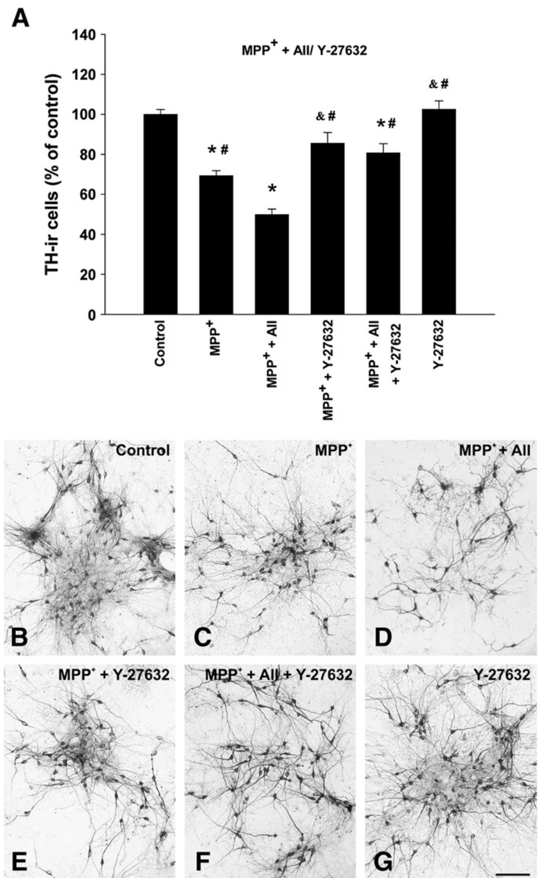

Fig. 4. Effects of low doses of MPP^+ and different treatments on the number of dopaminergic (TH-ir) cells in primary mesencephalic cultures. (A): Treatment with 0.25 μM MPP^+ induced a significant loss of TH-ir neurons, which was significantly increased by AII (100 nM). The amplifying effect of AII was inhibited by the ROCK inhibitor Y-27632. The decrease in the number of TH-ir cells induced by 0.25 μM MPP^+ (i.e., without addition of exogenous AII) was also inhibited by treatment with Y-27632, which presumably inhibited the effect of endogenous angiotensin. No significant difference was observed after treatment with Y-27632 alone. (B–G): Representative photomicrographs of cultured dopaminergic neurons in the different experimental groups quantified in A. The data are expressed as percentages of the number of TH-ir cells obtained in the respective control cultures (100%). Data represent means ± SEM. $^{*}p<0.05$ compared with control group (untreated cells); $^{\#}$ $p<0.05$ compared with MPP^+ + AII; $^{\&}$ $p<0.05$ compared with MPP^+ alone (one-way ANOVA and Bonferroni post-hoc test). AII, angiotensin II; TH, tyrosine hydroxylase. Scale bar: 100 μm.

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RhoA/ROCK pathway activation increases the MPTP/MPP$^+$-induced microglial response

In several recent studies, we have observed that the enhancing effect of AII on DA cell loss is mediated by microglial activation and exacerbation of the inflammatory response. In order to investigate whether neuroprotection by the ROCK inhibitor Y27632 is associated with inhibition of MPTP-induced microglial response, we analyzed the expression of CD45 and Isolectin B-4 in the substantia nigra, as markers for activated microglia. Control mice (i.e., vehicle-injected mice) showed minimal and non-significant microglial activation. In MPTP injected mice, microglial activation was higher than in control mice injected with vehicle, or mice injected with MPTP + Y27632. Microglial activation in AT1-null mice injected with MPTP was also lower than in control mice injected with MPTP, and similar to that observed in mice treated with the ROCK inhibitor Y27632 (Figs. 5A–E). Administration of MPP$^+$ and AII also led to increased microglial activation (OX-6-ir cells) in primary mesencephalic cultures, and the increase was inhibited by the administration of the ROCK inhibitor Y27632 (Fig. 6).

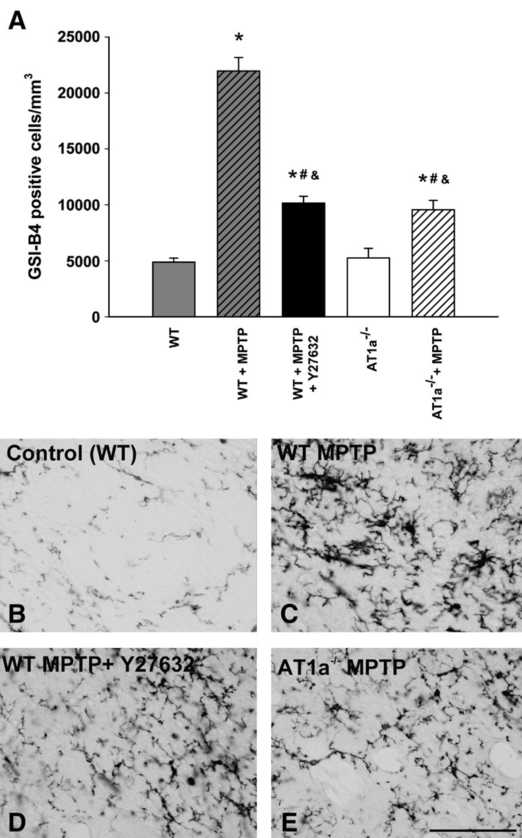

Fig. 5. Activated microglial cells in the substantia nigra compacta (SNc). (A): MPTP administration induced greater microglial activation (i.e., higher density of GSI-B4-positive cells) in the SNc of wild type (WT) mice than in the SNc of in AT1a-null mice (AT1a$^{-/-}$). However, the MPTP-induced microglial activation in WT mice treated with the Rho-Kinase inhibitor Y-27632 was similar to that observed in AT1a-null mice. (B–E): photomicrographs showing CD45-immunoreactive microglial cells at central levels of the substantia nigra in different experimental groups. The microglial cells were quantified as the number cells per mm^3, and the data are means ± SEM. $^*p<0.05$ compared with mice treated with vehicle, $^{\#}p<0.05$ compared with mice treated with MPTP alone; $^{\&}p<0.05$ compared with AT1a$^{-/-}$ (one-way ANOVA and Bonferroni post-hoc test). Scale bar: 100 μm.

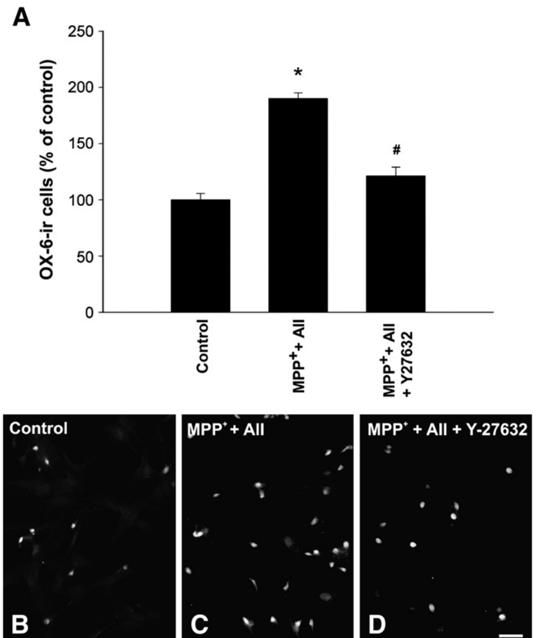

Fig. 6. Treatment of primary mesencephalic cultures with a low doses of MPP$^+$ (0.25 μM) and AII (100 nM) induced a significant increase in microglial activation, which was inhibited by administration of the ROCK inhibitor Y-27632 (A). (B–D): Representative photomicrographs of activated microglial cells (OX-6-ir cells) in the different experimental groups quantified in A. The data are expressed as percentages of the number of OX6-ir cells obtained in the respective control cultures (100%). Data represent means ± SEM. $^*p<0.05$ compared with control group (untreated cells); $^{\#}$ $p<0.05$ compared with MPP$^+$ + AII group (one-way ANOVA and Bonferroni post-hoc test). Scale bar: 50 μm.

Activation of the RhoA/ROCK pathway in microglial cells

The location of ROCK in the substantia nigra of mice was studied by laser confocal microscopy and double immunofluorescence against ROCK II and markers of dopaminergic neurons, microglia and astrocytes. No significant immunolabeling for ROCK II was observed in dopaminergic neurons or astrocytes. However, intense ROCK II-positive immunofluorescence was observed in microglial cells, particularly in mice treated with MPTP (Fig. 7).

Different types of mesencephalic cultures were used to further characterize the cells (neurons, astrocytes or microglia) in which the RhoA/ROCK pathway is activated by MPTP and AII. In primary cultures containing neurons, astrocytes and microglia, treatment with MPP$^+$ and AII induced a significant increase in RhoA and ROCK II mRNA levels and ROCK activity. However, no significant increase in RhoA and ROCK II mRNA levels or ROCK activity was observed in cultures lacking of microglial cells (i.e., cultures containing neurons and cultures containing neurons and astrocytes; Fig. 8).

Discussion

Interaction between the angiotensin/AT1 and the RhoA/ROCK II pathways in the MPTP-induced dopaminergic degeneration

The results of the present study show for the first time that activation of the RhoA–ROCK II pathway is involved in the dopaminergic

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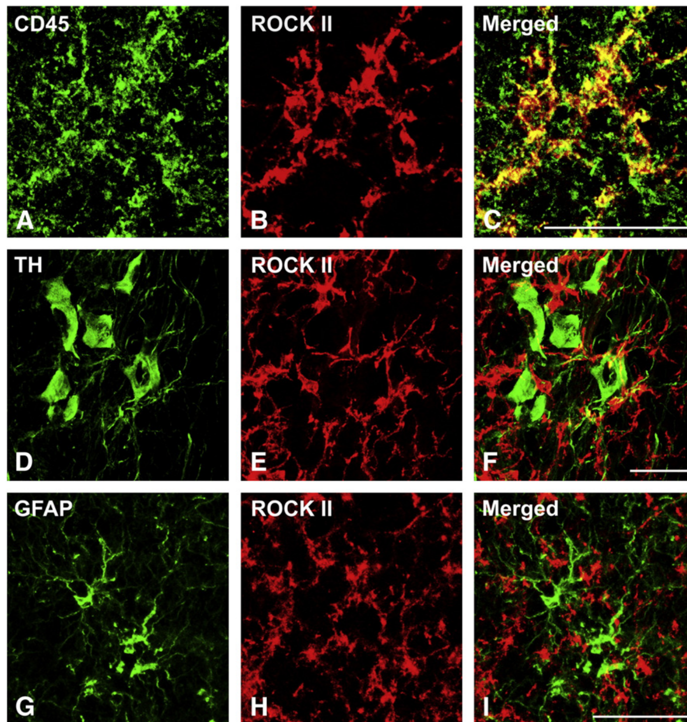

Fig. 7. Double immunofluorescence for the microglial marker CD45, the dopaminergic marker TH or the astroglial marker GFAP (green) and ROCK II (red) in the substantia nigra of mice treated with MPTP. ROCK II showed co-localization (yellow) with the microglial marker CD45 (A–C). No significant co-localization with the dopaminergic marker TH (D–F) or the astroglial marker GFAP (G–I) was observed. Scale bar: 25 μm.

cell degeneration induced by MPTP and possibly in PD, and that ROCK inhibitors decrease MPTP-induced dopaminergic cell death. The results demonstrate an important interaction between the AII/AT1 and the RhoA/ROCK II pathways in the MPTP-induced lesion, since AT1 deletion inhibited the MPTP-induced increase in RhoA and ROCK II expression as well as the MPTP-induced increase in ROCK activity. Furthermore, the enhancing effect of AII on the MPP^+-induced dopaminergic neuron death was inhibited by the ROCK inhibitor Y-27632 in cultures. The confirmation of the neuroprotective effect of Y-27632 in cultures excludes possible effects due to changes in brain vessel function. In previous studies in animal models of PD, we have shown that inhibition of microglial activation plays a major role in the protective effects of AT1 antagonists against dopaminergic cell death induced by dopaminergic neurotoxins (Joglar et al., 2009; Rodriguez-Pallares et al., 2008; Villar-Cheda et al., 2012). The present results are consistent with this, and reveal a major role for the RhoA/ROCK pathway activation in this effect, since treatment with the ROCK inhibitor Y-27632, as well as AT1 deletion, led to neuroprotection against MPTP/MPP^+-induced dopaminergic cell death and inhibition of the microglial response induced by MPTP. Furthermore, treatment of cultures in the presence and the absence of microglial cells confirmed a major role for the microglial ROCK activation in this process.

ROCK mediates neuroinflammation and microglial activation

Activation of the RhoA/ROCK pathway was observed very early after the MPTP injection (i.e., when the microglial response has been initiated and prior to occurrence of any significant loss of DA neurons; Jackson-Lewis et al., 1995; Wu et al., 2003). It has recently been shown that ROCK mediates the proinflammatory immune reaction and microglial activation induced in the brain by lysophosphatidylcholine (LPC), which were blocked by ROCK inhibitors (Sheikh et al., 2009). It is known that RhoA/ROCK is an important regulator of the actin cytoskeleton, which is particularly important for migration of inflammatory cells into inflamed areas (Greenwood et al., 2003; Honing et al., 2004), including microglia (Yan et al., 2012). It has been shown that during activation of inflammatory cells Rho/ROCK induces changes in the actin cytoskeleton that results in process retraction, cell spreading and changes in cell motility characteristics of activation of inflammatory cells such as microglia (Bernhart et al., 2010). Consistent with this, ROCK activation has been observed in animal models of several brain diseases in which neuroinflammation may play a major role (Mueller et al., 2005; Toshima et al., 2000). In the present study, inhibition of the AII/AT1 pathway produced similar results to those observed after treatment with ROCK inhibitors, which suggests that ROCK activation may play a critical role in the AII-induced inflammatory response. It is known that AII acts via AT1 receptors to induce inflammatory responses and to release high levels of ROS mainly by activation of the NADPH complex in vascular degenerative disease and other diseases mediated by oxidative stress and chronic inflammation (Qin et al., 2004; Touyz et al., 2002), and the present study shows that activation of the microglial RhoA/ROCK pathway plays a major role.

Angiotensin receptors and dopaminergic degeneration

In the nigrostriatal system of animal models of PD (i.e., rats lesioned with 6-hydroxydopamine and mice lesioned with MPTP), we

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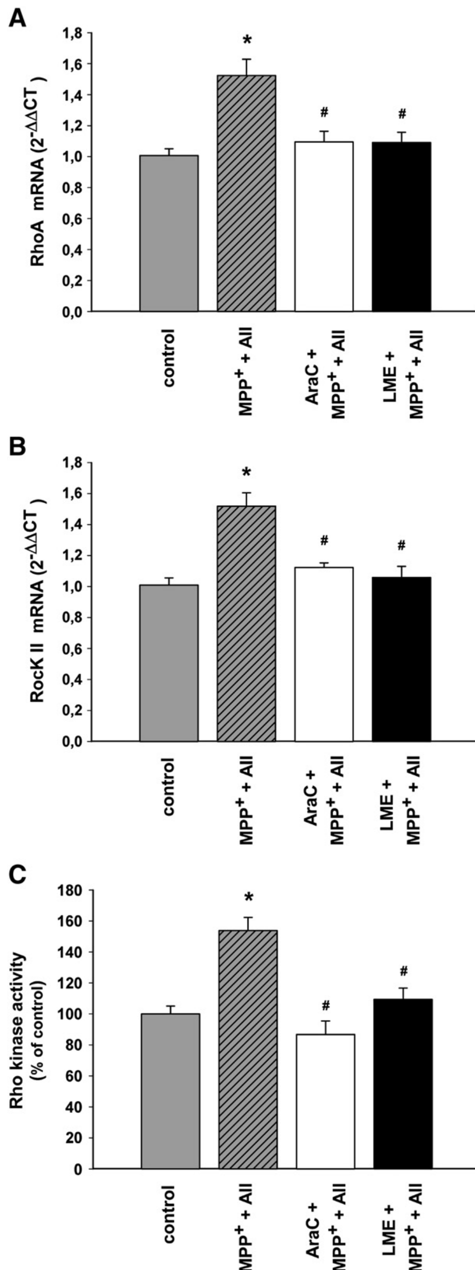

Fig. 8. Effects of low doses of MPP^+ and angiotensin II (AII) on activation of the RhoA/ROCK II pathway in primary mesencephalic cultures containing neurons, astrocytes and microglia, or neurons only (i.e., treated with cytosine β-D-arabinofuranoside, Ara-C), or neurons and astrocytes (i.e., treated with L-leucine methyl ester, LME). MPP^+ and AII produced a significant increase in the expression of RhoA (A) and ROCK II (B) mRNA levels and Rho-Kinase (C) only in the presence of microglia. Data are means ± SEM. $^*p<0.05$ relative to the corresponding control group (untreated cells); $^{\#}$ $p<0.05$ compared with the MPP^+ + AII group (i.e., culturescontaining microglia). One-way ANOVA and Bonferroni post-hoc test.

have previously shown that brain AII, via AT1 receptors, induces activation of the NADPH complex, increases oxidative stress and neuroinflammation, and leads to DA cell death. Dopaminergic cell death was accompanied by an increase in levels of major oxidative stress indicators (i.e., protein oxidation and lipid peroxidation) in the ventral midbrain, which were inhibited by AT1 antagonists (Rey et al., 2007) or angiotensin converting enzyme inhibitors (Lopez-Real et al., 2005; Muñoz et al., 2006). In the present study, using AT1 null mice treated with MPTP, we have confirmed that AT1 receptor stimulation increases dopaminergic cell death, and that AT1 receptors mediate MPTP-induced activation of the RhoA/ROCK pathway and microglial activation.

It is also known that AII, via the AT2 receptor, exerts actions directly opposed to those mediated by AT1, thus antagonizing many of the effects of the latter (Chabrashvili et al., 2003; Jones et al., 2008). AT1 and AT2 receptors have opposing effects and AT2 receptors counterbalance the deleterious effect of AT1 receptor stimulation, so that functional interactions between the two receptor subtypes determine the AII-induced effects (Sohn et al., 2000). In a previous study (Villar-Cheda et al., 2010) we observed that AT1-null mice displayed significantly less NADPH activity in the nigra and significantly lower expression of AT2 receptors. The decreased NADPH activity and the subsequent neuroprotection may be due to the absence of AT1-derived signal (AT1 activation increases NADPH oxidase activation) and the activation of AT2 receptors by AII (which inhibits NADPH oxidase in the absence of the opposing action of AT1 receptors).

In accordance with this, we have recently observed that nigral AT1 receptors are downregulated and AT2 receptors are upregulated by estrogen (Rodriguez-Perez et al., 2010) and that AT1 receptors are upregulated while AT2 receptors are downregulated in menopausal rats (Rodriguez-Perez et al., 2012) in comparison with young female rats with estrogen. It is also known that the incidence and prevalence of PD are higher in men than in women and in postmenopausal than in premenopausal women of similar age (Baldereschi et al., 2000; Diamond et al., 1990). Interestingly, several recent studies in vascular smooth muscle and endothelial cells have shown that estrogen inhibits and androgen potentiates activation of the Rho/ROCK pathway (Hiroki et al., 2005; Ito et al., 2006; Song et al., 2006). These data together with the results of the present study suggest that activation of the Rho/ROCK pathway may be a major factor involved in the higher neuroinflammatory response and higher susceptibility to dopaminergic neurotoxins observed in menopausal rats and male rats in comparison with young rats with estrogen Rodriguez-Perez et al., 2011, 2012), and possibly in the higher incidence of PD observed in men and menopausal women.

Effects of angiotensin on neurons and microglia

It has been shown that AII is a pro-inflammatory molecule and a major activator of the NADPH-oxidase complex in several types of cells. NADPH-derived ROS play a crucial role in the signaling of AII via AT1 receptors, but also constitute powerful inducers of oxidative stress, since the NADPH oxidase complex is the most important intracellular source of ROS after the mitochondria (Cai et al., 2003; Seshiah et al., 2002; Touyz et al., 2002). This has been extensively studied in vascular cells as chronic inflammation of the vessel wall is the hallmark of atherosclerosis. AII acts in this process on at least two levels (Ruiz-Ortega et al., 2001; Suzuki et al., 2003). Firstly, AII acts on the resident cells (i.e. endothelial cells and smooth muscle cells in the vessel wall; neurons in the brain), in which via AT1 receptors stimulate production of low levels of intracellular ROS by activation of NADPH oxidase. ROS act as second messengers on several signaling pathways, including those involved in triggering the inflammatory response and the migration of inflammatory cells into the lesioned area. Secondly, AII acts on inflammatory cells (such as microglial cells in the brain),

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in which NADPH oxidase produces ROS with dual functions: i) high concentrations of ROS are released extracellularly for killing invading micro organisms or cells; and ii) low levels of intracellular ROS act as a second messenger in several signaling pathways involved in the inflammatory response (Babior, 2004; Qin et al., 2004).

As described above for vascular tissues, we have previously observed the presence of NADPH oxidase and AT1 and AT2 receptors in nigral microglia and dopaminergic neurons (Joglar et al., 2009; Rodriguez-Pallares, et al., 2008; Valenzuela et al., 2010). We have also shown that AII via AT1 receptors, activates the microglial NADPH-complex and exacerbates the glial inflammatory response (Joglar et al., 2009; Rey et al., 2007; Rodriguez-Pallares et al., 2008). In the present study, we have shown that activation of the microglial RhoA/ROCK pathway plays a major role in this process. In neurons and other non-inflammatory cells, activation of the NADPH oxidase complex produces low levels of ROS for signaling function (Babior, 2004); these ROS also modulate neuronal levels of ROS by interaction with mitochondria-derived ROS, and with ROS from other sources such as neurotoxins or activated microglia. Cross-talk signaling between NADPH oxidase and mitochondria has been observed in several types of cells. This not only includes an upstream role for NADPH oxidase in the modulation of mitochondrial superoxide (Doughan et al., 2008; Kimura et al., 2005) but also that mitochondrial superoxide stimulates extramitochondrial NADPH oxidase activity in a feed-forward fashion (Dikalova et al., 2010; Wosniak et al., 2009). This interaction has recently been confirmed in a dopaminergic cell line treated with MPP^+ and angiotensin (Zawada et al., 2011). Treatment with MPP^+ induced mitochondrial release of ROS, which induced a second wave of NADPH oxidase-derived ROS; the latter was reduced by treatment with the AT1 antagonist candesartan (Zawada et al., 2011). Using primary cultures of mesencephalic cells, we have recently shown that mitochondrial ATP-sensitive potassium channels play a major role in the interaction between NADPH-derived ROS and mitochondria after treatment with AII and/or dopaminergic neurotoxins such as MPP^+ and 6-hydroxydopamine (Rodriguez-Pallares et al., 2009, 2011).

Direct (neuronal) or indirect (microglial) effect of AII on dopaminergic neuron death

AT1, AT2 receptors and NADPH oxidase are present in dopaminergic neurons as well as in microglia, and inhibition of neuronal AT1 receptors may also reduce ROS derived from neuronal NADPH, as indicated above. This may lead to direct inhibition of dopaminergic neuron death, followed by a subsequent reduction in microglial activation. However, this possibility is not supported by our studies. Using neuron-enriched primary mesencephalic cultures we have observed that only high doses of neurotoxins can induce dopaminergic neuron death in the absence of glia (Joglar et al., 2009; Rodriguez-Pallares et al., 2007, 2008, 2009, 2011). This has been confirmed in a recent study with a dopaminergic cell line (i.e., in the absence of glia; Zawada et al., 2011), as significant cell death was only observed after treatment with very high doses of MPP^+ (300 μM). However, in the present and previous studies, we investigated the effects of very low (0.25 μM MPP^+) or sublethal (10 μM 6-hydroxydopamine) doses of neurotoxins. The effects of these low doses may be more similar to the effects caused by environmental neurotoxins or by other deleterious factors involved in PD. Low or sublethal doses of neurotoxins do not induce significant neuron death in pure neuronal cultures. However, sublethal insults can induce neuron derived proinflammatory signals which, in the presence of glia, trigger microglial activation and the subsequent increase in microglia-derived ROS and cytokines, which induce the progression of neuronal death (Qin et al., 2004; Biber et al., 2007). Furthermore, other studies have shown that microglial activation and free radicals derived from microglial NADPH play a major role in the toxicity of MPTP and possibly in PD, and that lesioned DA neurons are particularly vulnerable to microglial NADPH-derived ROS (Gao et al., 2003a,b; Wu et al., 2003). AII-induced activation of microglial NADPH oxidase and Rho/ROCK pathways plays a major role in this process.

The precise relationship between ROCK and NADPH oxidase activities is not clear, although it has been suggested that there may be some interaction, since ROCK inhibitors have been shown to suppress AII-induced NADPH oxidase activation (Budzyn et al., 2006; Hiroki et al., 2004). However, there are also data suggesting that AII may activate ROCK independently of NADPH oxidase in vascular smoth muscle cells (Ohtsu et al., 2006). The present study suggests for the first time a possible beneficial effect of ROCK inhibition in treatment of PD. In several previous studies we have shown that AT1 blockers (ARBs; Joglar et al., 2009; Rey et al., 2007; Rodriguez-Pallares et al., 2008) and ACE inhibitors (Lopez-Real et al., 2005; Muñoz et al., 2006), widely used in clinical cardiovascular and renal diseases, protect against dopaminergic degeneration induced by neurotoxins (see Labandeira-Garcia et al., 2011 for review), and in the present study we have observed that AT1 deletion also inhibits ROCK activity.

Conclusions

Administration of the dopaminergic neurotoxin MPTP induced an increase in the expression of RhoA and in ROCK II mRNA levels and ROCK activity in the substantia nigra, which were inhibited by AT1 receptor deletion, suggesting that activation of the AII/AT1 and RhoA/ROCK pathways is involved in the MPTP-induced lesion. In accordance with this, administration of the ROCK inhibitor Y-27632 induced a significant decrease in the microglial activation and dopaminergic cell death induced by MPTP, as well as a significant decrease in the enhancing effect of AII/AT1 activation on the dopaminergic cell death. The results suggest that ROCK inhibitors and AT1 receptor antagonists may provide new neuroprotective strategies against the progression of PD.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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DISCUSIÓN

5. - DISCUSIÓN

5.1- Papel de la AII cerebral en la respuesta inflamatoria en animales tratados con MPTP como modelo de enfermedad de Parkinson: implicación en la progresión de la degeneración dopaminérgica.

El sistema renina-angiotensina (SRA) representa uno de los sistemas filogenéticamente más antiguos y ha sido objeto de una intensa investigación. La función clásica de este sistema es la regulación de la presión sanguínea y de la homeostasis de sodio y agua. Hoy en día, se sabe que además del SRA clásico existen otros SRA locales en diferentes tejidos entre los que se incluye el cerebro. La función clásica del SRA del cerebro es la regulación cardiovascular y la homeostasis hidroelectrolítica, función que lleva a cabo mediante la modulación de la actividad del sistema nervioso autónomo (Phillips y Sumners, 1998). El principal efector del sistema es la AII y está implicada en la regulación de determinados neurotransmisores tales como norepinefrina, serotonina y dopamina a nivel de los ganglios basales. Esto hace que las interacciones entre el SRA y la dopamina sean de especial interés (Mendelsohn y col., 1993; Brown y col., 1996). En la actualidad, sabemos que la AII es, a través de los receptores AT1, uno de los principales inductores de inflamación y estrés oxidativo en diferentes tejidos (Seshiah y col., 2002; Touyz y col., 2002; Cai y col, 2003) y que genera la producción de EORs a través de la activación del complejo NADPH-oxidasa. La AII normalmente juega un papel modulador en la neurotransmisión dopaminérgica, y potencia o amplifica la respuesta inflamatoria microglial en respuesta a un daño neuronal inicial provocado por diferentes neurotoxinas, tales como el MPTP que hemos empleado en este trabajo, o por otros factores implicados en la EP. Es posible que interacciones entre dopamina y AII jueguen un papel importante en la progresión de los procesos degenerativos tal y como ha sido descrito en otros tejidos (Bek y col., 2006). En este mismo sentido, en nuestro laboratorio se han llevado a cabo estudios que demuestran que inhibidores del enzima convertidor de angiotensina producen una disminución en la muerte dopaminérgica y el estrés oxidativo inducidos previamente por la neurotoxina MPTP (Muñoz y col., 2006). Asimismo, otros autores han observado que el antagonista de los receptores AT1, losartán, también protege contra la neurotoxicidad del MPTP (Grammatopoulos y col., 2007). Sin embargo, el mecanismo exacto implicado en este efecto no ha sido estudiado en detalle. Además, el losartán solo reducía la toxicidad del MPTP en presencia de AII exógena, y también se había

postulado un posible papel neuroprotector de la AII. Estas discrepancias podrían ser debidas a diferencias metodológicas ya que Grammatopoulos y col. (2007) usan dosis de MPP^+ mucho más altas (5-10 μM) que las empleadas en nuestro estudio.

Nuestros resultados indican que la pérdida dopaminérgica inducida previamente por la neurotoxina MPTP/MPP^+ es amplificada por la AII y que este efecto amplificador es bloqueado mediante el uso de antagonistas del receptor AT1, *in vivo* mediante el uso de candesartán e *in vitro* mediante el uso de ZD 7155. Y por otra parte, que la PKC, el complejo NADPH-oxidasa y la activación microglial están implicados en este efecto. Cabe destacar que en ratones y cultivos no tratados con AII, los antagonistas del receptor AT1 bloquean también el efecto amplificador en la neurodegeneración dopaminérgica inducido por la AII endógena. Asimismo, en ratones tratados con candesartán se observa una inhibición de la activación microglial temprana y de la activación del complejo NADPH-oxidasa, ambos factores responsables, al menos en parte, de la progresión de la EP. La disminución en la respuesta microglial una semana después del tratamiento con MPTP podría ser considerada una consecuencia más que una causa del descenso en la muerte dopaminérgica. La eliminación de las células gliales en los cultivos mesencefálicos inhibe el efecto amplificador de la AII, lo que nos sugiere que efectivamente la activación microglial desempeña un papel clave en los efectos deletéreos de la AII. Las EORs derivadas de la activación del complejo NADPH-oxidasa mitocondrial incrementan la formación de EORs intraneuronales y algunos estudios proponen que estas EORs intraneuronales podrían actuar sinérgicamente con el MPP^+ provocando finalmente la muerte neuronal (Gao y col., 2003 a, b; Wu y col., 2003). Nuestros resultados apoyan la idea de la existencia de un efecto sinérgico ya que, cuando los cultivos son tratados con dosis bajas de MPP^+ la activación microglial mediada por AII es evidente, mientras que cuando los cultivos son tratados con dosis muy altas o muy bajas de la neurotoxina los efectos del tratamiento con AII o ZD 7155 no son significativos. En este mismo sentido, cuando los cultivos son tratados únicamente con AII no se produce ningún cambio significativo en la muerte neuronal. Por lo tanto, un daño neuronal inicial es necesario para iniciar o desencadenar la respuesta microglial que posteriormente será amplificada por la AII. Tal y como hemos comentado anteriormente, la AII es el principal activador del complejo NADPH-oxidasa, de hecho en aquellos cultivos tratados únicamente con AII se ve un incremento tanto en la expresión de las subunidades como en la actividad del complejo. Por otro lado, Quin y col. en 2004 demostraron que la formación de EORs

derivadas de la activación del complejo NADPH-oxidasa es esencial pero no suficiente para inducir la respuesta inflamatoria microglial. Se sabe que en las neuronas intactas "sanas" la reacción microglial está reducida por la acción de determinadas citoquinas y que un daño en la actividad neuronal producido por neurotoxinas u otros factores, induce la respuesta inflamatoria microglial (Neumann, 2001; Biber y col., 2007).

Para descartar posibles efectos indirectos de los antagonistas de los receptores AT1 sobre la neurotoxicidad del MPTP realizamos mediciones de los niveles intraestriatales de MPP^+ en ratones. No observamos ningún cambio en los niveles de MPP^+ entre los ratones tratados con MPTP y los tratados con MPTP y candesartán. En este mismo sentido, los efectos deletéreos de la AII fueron corroborados *in vitro* al tratar los cultivos con AII exógena y MPP^+ de manera simultánea. Por otro lado, estudiamos la actividad de los transportadores de dopamina y vimos que tanto en los cultivos tratados con AII como en los tratados con antagonistas del receptor AT1 y con apocinina no se veía afectada dicha actividad. Además, la presencia de las células gliales es imprescindible para observar el efecto amplificador de la AII sobre la degeneración dopaminérgica inducida por el MPP^+. Con estos resultados podemos excluir que la AII actúe a través de un mecanismo de acumulación de la neurotoxina en el interior de las neuronas dopaminérgicas. Estos resultados fueron confirmados usando dos tipos distintos de antagonistas de los receptores de AT1, de manera que podemos decir que los efectos observados son debidos precisamente a la inhibición de este tipo de receptor y no como consecuencia de un efecto desconocido o no específico de algún otro componente. Cabe destacar que los resultados obtenidos *in vivo* fueron confirmados *in vitro,* lo cual nos permite excluir posibles efectos indirectos debidos a cambios en la permeabilidad de la barrera hematoencefálica tras la manipulación del SRA. Sin embargo, algunos estudios sugieren que el MPTP podría alterar la barrera hematoencefálica como consecuencia de la activación microglial inducida por el MPTP (Zhao y col., 2007), y que esto podría permitir la entrada de AII circulante e incrementar así aún más la neuroinflamación. No obstante, nuestros resultados muestran que el SRA del cerebro juega un papel clave al menos, en la iniciación del proceso inflamatorio, tal y como se había observado previamente en otros tejidos (Touyz y col., 2002; Suzuki y col., 2003).

En base a todos los resultados obtenidos podemos concluir que la AII vía AT1, la PKC y las EORs derivadas de la activación del complejo NADPH-oxidasa incrementan la muerte celular dopaminérgica. Y que además, lo hacen a través de

diversos mecanismos similares a aquellos observados en otros procesos degenerativos donde intervienen procesos inflamatorios y el estrés oxidativo, tales como aterosclerosis, glomerulosclerosis e hipertrofia cardíaca (Ferrari y col., 2002). Por otro lado, mediante el uso de técnicas de inmunofluorescencia doble, hemos observado la presencia de los receptores AT1 y del complejo NADPH-oxidasa tanto en neuronas dopaminérgicas como en células microgliales. Así que, la AII podría incrementar el nivel de estrés oxidativo en el interior de las neuronas dopaminérgicas mediante la formación de EORs a través del receptor AT1, de la PKC neuronal y del complejo NADPH-oxidasa (Wang y col., 2004, 2006) y actuar sinérgicamente con las EORs generadas como consecuencia del efecto de la neurotoxina MPP^+ incrementando de este modo su efecto neurotóxico en las neuronas dopaminérgicas. No obstante, debemos resaltar que nuestros resultados muestran que las células gliales juegan un papel fundamental en el efecto deletéreo de la AII ya que cuando eliminamos la población glial de los cultivos con el antimitótico Ara-C se inhibe el efecto amplificador de la AII sobre la degeneración. La microglía constituye el sistema inmune del cerebro y varios estudios han demostrado que aumenta de manera significativa la progresión de la neurodegeneración dopaminérgica (Gao y col., 2003a). Todo apunta a que la activación astroglial observada *in vivo* es secundaria a la degeneración dopaminérgica y que podría estar relacionada con la eliminación de las neuronas dañadas y otras funciones (Wu y col., 2002, 2003; Gao y col., 2003a). De acuerdo con esto, nuestros resultados muestran efectivamente una activación astroglial débil en etapas tempranas y más fuerte a tiempos largos tras el tratamiento con la neurotoxina, al igual que se ha descrito previamente en otros estudios (Liberatore y col., 1999; Wu y col., 2002). El papel de los astrocitos en la neuroinflamación aún no está claro (Neumann 2001; Biber y col., 2007) aunque se cree que podría estar relacionado con la modulación o prevención de una respuesta microglial excesiva y de sus efectos deletéreos (Min y col., 2006).

Cada vez son más las evidencias que apuntan a que estos procesos inflamatorios derivados de la activación microglial juegan un papel clave en el inicio y/o en la progresión de la EP. Los resultados obtenidos en nuestros experimentos indican que el SRA del cerebro juega un papel clave en este mecanismo neurodegenerativo. Y que por lo tanto, manipulaciones a nivel del SRA del cerebro pueden resultar más efectivas como terapia neuroprotectora para la EP que por ejemplo el uso de antioxidantes, cuya función consiste únicamente en secuestrar las EORs ya formadas, tal y como se observó previamente en enfermedades cardiovasculares. No obstante, posibles interacciones

entre el SRA y el sistema dopaminérgico tanto en cerebros sanos como de pacientes con EP deben seguir siendo objeto de múltiples estudios.

5.2- Efecto del bloqueo de los receptores de pro-renina/renina sobre la muerte neuronal dopaminérgica *in vitro*

En este trabajo nos hemos centrado en el estudio del posible papel del receptor de Pro-renina/renina (PRR) en los mecanismos neurodegenerativos de las células dopaminérgicas inducidos por la neurotoxina 6-OHDA. Para ello, llevamos a cabo distintos experimentos *in vitro*: por un lado tratamos los cultivos con un antagonista del receptor PRR, HRP, para bloquear la unión de la pro-renina ó renina al receptor y por tanto su activación, y lo que observamos fue una disminución en la muerte de las células dopaminérgicas inducida por dosis bajas de 6-OHDA. Estudios previos han demostrado que el tratamiento con HRP es altamente efectivo para el bloqueo de la unión de la pro-renina/renina al receptor PRR. Este bloqueo tiene como consecuencia un efecto neuroprotector en varios tejidos donde el daño es mediado a través de los PRRs (Ichihara y col., 2004, 2006). Se ha visto que el HRP también inhibe algunos de los efectos de la renina en cultivos neuronales (Shan y col., 2008), sin embargo este efecto no se ha visto en otros estudios y tipos celulares (Batenburg y col., 2007; Feldt y col., 2008). La razón de estas discrepancias se desconoce pero se cree que podría ser debido al uso de diferentes concentraciones de HRP o la presencia de diferentes cantidades de renina (Ichihara y col., 2009). El descenso observado en la pérdida de neuronas dopaminérgicas tras el tratamiento de los cultivos con HRP puede ser debido a una disminución en la generación de AII a nivel de la superficie celular como consecuencia del bloqueo de los receptores PRR de las neuronas y de las células microgliales. El resultado obtenido es similar al observado en el trabajo anterior cuando tratamos los cultivos con ZD 7155, antagonista de los receptores AT1, para bloquear el efecto de la AII endógena. Para estudiar si los receptores PRR podrían contribuir o no al aumento de la muerte neuronal dopaminérgica a través de mecanismos independientes de la AII (a través por ejemplo de una cascada de señalización intracelular propia) hicimos una segunda tanda de experimentos en los que tratamos los cultivos con renina y con los antagonistas de los receptores AT1 y AT2. El tratamiento simultáneo con 6-OHDA y los antagonistas de AT1 y AT2 producía un descenso en la muerte neuronal dopaminérgica con respecto al tratamiento con 6-OHDA sola. Y este descenso en la muerte neuronal dopaminérgica era revertido al añadir renina. Esto indica que la

estimulación de los receptores de PRR por suficientes niveles de pro-renina/renina produce un aumento en la muerte neuronal dopaminérgica independiente de AII.

Teniendo en cuenta los resultados obtenidos en este trabajo podemos sugerir que el receptor PRR contribuye a los efectos pro-inflamatorios y pro-oxidativos inducidos por la AII y que tiene como consecuencia final el aumento en la degeneración dopaminérgica tras el tratamiento con dosis bajas de neurotoxinas, como el MPTP y la 6-OHDA (Rodriguez-Pallares y col., 2008; Joglar y col., 2009). Por otro lado, podemos concluir que los receptores PRR están implicados en la degeneración dopaminérgica no solo a través de mecanismos dependientes de AII, como hemos descrito en otros trabajos (ver apartados 5.1 y 5.3), sino que también a través de mecanismos independientes de AII. Apoyándonos en estos resultados proponemos el desarrollo de estrategias neuroprotectoras basadas en la disminución de la actividad del SRA, no sólo a través de la vía de señalización de la AII a través de AT1, como proponíamos en el trabajo anterior, sino que también la vía de señalización del receptor PRR podría suponer una estrategia alternativa o complementaria a la anterior.

5.3- Implicación de PPAR-γ en el efecto neuroprotector y antiinflamatorio del bloqueo del receptor de AII de tipo 1. Efecto del antagonista de los receptores AT1 telmisartán y de la deleción del receptor AT1 en un modelo en ratón de enfermedad de Parkinson

Numerosos estudios llevados a cabo en diferentes tejidos han sugerido que el receptor gamma activado por peroxisomas (PPAR-γ) está implicado en los efectos antiinflamatorios que se observan tras el uso de antagonistas del receptor AT1. Entre los distintos antagonistas de este receptor se ha descrito que el telmisartán es el que posee un mayor efecto agonista sobre PPAR-γ (Shupp y col., 2004; Clasen y col., 2005; Erbe y col., 2006). Nuestros resultados muestran que efectivamente en ratones tratados con telmisartán oral hay una disminución en la muerte neuronal dopaminérgica tras la exposición a la neurotoxina MPTP, tal y como se ha descrito anteriormente con otros antagonistas de los receptores AT1 como candesartán y losartán (Grammatopoulos y col., 2007; Joglar y col., 2009). Cabría esperar el efecto neuroprotector del telmisartán teniendo en cuenta que previamente se ha descrito que es un potente antagonista del receptor AT1 y que además, atraviesa la barrera hematoencefálica e inhibe los efectos de la AII a nivel central (Gohlke y col., 2001; Jung y col., 2007). Sin embargo, el

mecanismo responsable de este efecto neuroprotector aún no ha sido clarificado. Una primera opción sería considerar la propiedad farmacológica como agonistas de PPAR-γ que tienen los antagonistas de los receptores AT1 como único mecanismo implicado en el efecto neuroprotector. Nuestros resultados muestran efectivamente que en el efecto neuroprotector del telmisartán la activación de PPAR-γ juega un papel importante ya que la administración del antagonista de PPAR-γ, GW9662, inhibe dicho efecto neuroprotector. Sin embargo, también observamos que esta propiedad farmacológica de los antagonistas de los receptores AT1 como agonistas de PPAR-γ no es el único factor responsable de la neuroprotección. Los resultados obtenidos en ratones *knock-out* para el receptor de AII AT1 mostraron que, independientemente de un efecto farmacológico, la inhibición del receptor AT1 resulta neuroprotectora frente a la neurodegeneración inducida por el MPTP. Además, el grado de neuroprotección obtenido con el telmisartán no fue mayor al observado previamente con el candesartán (Joglar y col., 2009) a pesar de que este último tiene propiedades farmacológicas como agonista de PPAR-γ menores (Shupp y col., 2004; Clasen y col., 2005; Erbe y col., 2006). Esto nos sugiere que no hay un efecto adicional significativo al bloqueo de los receptores AT1 y al efecto agonista sobre PPAR-γ de los antagonistas de los receptores AT1. Es posible que el diseño experimental del trabajo no permita ver la existencia de un posible efecto adicional. Por otro lado, cabe destacar que la deleción del receptor AT1 podría tener un efecto agonista sobre PPAR-γ ya que hemos observado que la administración del antagonista de PPAR-γ, GW9662, a ratones *knock-out* para el receptor AT1 produce un aumento significativo en la neurodegeneración dopaminérgica inducida por la neurotoxina MPTP. Esto nos sugiere que la activación de PPAR-γ realmente juega un papel clave en el efecto neuroprotector de la inhibición de los receptores AT1.

Por lo tanto, según nuestros resultados la inhibición de los receptores AT1 mediante el uso de antagonistas, y en concreto del telmisartán, conlleva a la activación de PPAR-γ a través de un mecanismo doble. Este mecanismo implica por un lado, un efecto agonista sobre PPAR-γ e independiente del efecto farmacológico sobre los receptores AT1 (efecto que será mayor o menor dependiendo del tipo de antagonista), y por otro lado, un efecto directo por el propio bloqueo de los receptores AT1. Cada vez son más los estudios que sugieren la existencia de una interacción entre el SRA y PPAR-γ en diferentes tejidos (Zorad y col., 2006; Kuipers y col., 2008). Se ha descrito que el tratamiento con AII inhibe la expresión de PPAR-γ y la respuesta antiinflamatoria a nivel de las paredes vasculares (Tham y col., 2002; Kintscher y col.,

2004). También en tejido adiposo y en las células del músculo esquelético la inhibición del enzima convertidor de AII produce un aumento en la expresión de PPAR-γ (Storka y col., 2008; Santos y col., 2009). Otros estudios han demostrado que la AII inhibe la activación de PPAR-γ a través de los receptores AT1 y estimula dicha activación a través de los receptores AT2 (Zhao y col., 2005; Zorad y col., 2006). Según estos estudios, los receptores AT2 adquieren una importancia funcional cuando hay un bloqueo de los receptores AT1, de modo que la AII disponible actuaría a través de los receptores AT2 (Wu y col., 2001; Zhao y col., 2005). Por otra parte, hay estudios que sugieren que PPAR-γ podría modular el SRA y la señalización de AII a distintos niveles (Kintscher y col., 2004). Asimismo, se ha descrito que los activadores de PPAR-γ inducen una regulación "a la baja" en la expresión de los receptores AT1 (Takeda y col., 2000; Sugawara y col., 2001; Imayama y col., 2006) y en la actividad del enzima convertidor de angiotensina (Takai y col., 2007) y una regulación "al alza" de los receptores AT2 (Banks y Oyekan, 2008). Otros estudios han postulado que PPAR-γ y otros PPARs podrían inhibir la actividad NADPH-oxidasa y otras vías de señalización relacionadas con procesos inflamatorios y estrés oxidativo inducido por acción de la AII (Diep y col., 2002; Ji y col., 2009). Esto podría explicar que el tratamiento con el antagonista de PPAR-γ, GW9662, bloquee el efecto neuroprotector del telmisartán así como también el efecto neuroprotector debido a la ausencia de los receptores AT1 en los ratones *knock-out*. Es bien conocido que la AII a través de los receptores AT2 ejerce acciones opuestas a aquellas mediadas a través de los receptores AT1 antagonizando así muchos de sus efectos (Chabrashvili y col., 2003; Jones y col., 2008). La AII, en los ratones *knock-out* para el receptor AT1, puede actuar a través de los receptores AT2 activando así PPAR-γ y contribuyendo de esta forma a la inhibición de procesos inflamatorios y estrés oxidativo, lo cual se ha visto que promueve la longevidad e inhibe la progresión de enfermedades degenerativas (Umemoto, 2008; Benigni y col., 2009; de Cavanagh y col., 2011). Este efecto a su vez se ve bloqueado tras el tratamiento con el antagonista de PPAR-γ, tal y como muestran nuestros resultados.

En este trabajo, también hemos confirmado que el mecanismo implicado en este efecto neuroprotector es similar al descrito en otros trabajos sobre las propiedades neuroprotectoras de los antagonistas de los receptores AT1. En varios estudios llevados a cabo en nuestro laboratorio, hemos observado en modelos animales de EP, que la inhibición de la activación microglial juega un papel clave en los efectos neuroprotectores de los antagonistas de los receptores de AII (Rodríguez-Pallares y col.,

2008; Joglar y col., 2009; Villar-Cheda y col., 2010). Estos datos concuerdan con los resultados obtenidos en este trabajo donde además se sugiere que tanto la inhibición de los receptores AT1 con telmisartán, como la carencia de ellos en los ratones *knock-out* supone, a nivel de la SNpc, un descenso significativo en la respuesta microglial inducida por la neurotoxina MPTP. Además, estos resultados apuntan a la existencia de una implicación principal de la activación de PPAR-γ en dicho efecto, ya que a pesar del bloqueo de los receptores AT1 con telmisartán y de la ausencia de los mismos en el caso de los ratones *knock-out*, el tratamiento con el inhibidor de PPAR-γ inhibe el efecto neuroprotector y aumenta la activación microglial. Tanto los receptores de AII AT1 y AT2 (Rodriguez-Pallares y col., 2008; Joglar y col., 2009) como PPAR-γ (Ricote y col., 1998; Bernardo y col., 2000; Moreno y col., 2004) se han localizado a nivel neuronal y a nivel de las células gliales, incluyendo la microglía. Y se ha visto que la activación de PPAR-γ puede disminuir la respuesta inflamatoria a nivel del cerebro a través de la inhibición de diferentes funciones, todas ellas asociadas a la activación microglial (Bernardo y col., 2000; Bernardo y Minghetti, 2006). Nuestros resultados son consistentes con otros estudios anteriores donde se ha demostrado que el uso de agonistas de PPAR-γ, como pioglitazona y rosiglitazona, disminuye la degeneración dopaminérgica inducida por MPTP a través de la inhibición de la activación microglial (Breidert y col., 2002; Schintu y col., 2009; Carta y col., 2011). Nuestros resultados también son concordantes con otros trabajos en los que se ha observado que el tratamiento con antagonistas de los receptores AT1 disminuye la infiltración de células inflamatorias a nivel del SNC (Platten y col., 2009; Stegbauer y col., 2009) y a nivel de órganos periféricos (Swirski y col., 2009), aunque también existen resultados en sentido contrario (Füchrbauer y col., 2011). En relación con este efecto inhibitorio sobre la respuesta neuroinflamatoria se ha observado que, tanto el uso de agonistas de PPAR-γ como de los inhibidores de los receptores AT1, tiene efectos beneficiosos en numerosos procesos relacionados con la activación microglial y neuroinflamación, incluyendo modelos animales de la enfermedad de Alzheimer (Combs y col., 2000; Jiang y col., 2008; Tsukuda y col., 2009), isquemia cerebral (Lou y col., 2004; Jung y col., 2007; Iwanami y col., 2010), esclerosis múltiple (Platten y col., 2009; Stegbauer y col., 2009; Lanz y col., 2010), daño cerebral (Yi y col., 2008) y envejecimiento (de Cavanagh y col., 2007; Loane y col., 2009; Villar-Cheda y col., 2012).

En conclusión, podemos decir que la administración oral de telmisartán ejerce un efecto neuroprotector sobre la degeneración dopaminérgica inducida previamente

por MPTP, tal y como habíamos observado anteriormente con candesartán, y que este efecto neuroprotector es mediado a través de la activación de PPAR-γ. Asimismo, los resultados obtenidos en ratones *knock-out* para el receptor AT1 muestran que el bloqueo de estos receptores, independiente de un efecto farmacológico, también protege a las neuronas dopaminérgicas de los efectos tóxicos del MPTP. Además, existe una relación entre el bloqueo de los receptores AT1 y la activación de PPAR-γ, ya que en los ratones deficientes para el receptor AT1 el tratamiento con el antagonista de PPAR-γ, GW9662, inhibe los efectos neuroprotectores observados.

5.4- Papel de los canales de potasio mitocondriales dependientes de ATP en la toxicidad de la 6-hidroxidopamina sobre las neuronas dopaminérgicas

El resultado más relevante obtenido en este trabajo es que el 5-HD, inhibidor de los canales de potasio mitocondriales dependientes de ATP (mitoK(ATP)), bloquea la degeneración dopaminérgica inducida por dosis bajas de 6-OHDA. Y que además bloquea el descenso del potencial de membrana interna mitocondrial e inhibe la generación de las EORs derivadas de superóxido en las neuronas dopaminérgicas, ambos inducidos por la 6-OHDA. En trabajos anteriores llevados a cabo en nuestro laboratorio hemos visto que las EORs derivadas de la activación microglial inducida por la neurotoxina 6-OHDA contribuyen a la muerte neuronal dopaminérgica (Rodriguez-Pallares y col., 2007), al igual que observábamos en los trabajos anteriores incluidos en esta tesis en los que se empleaba MPTP (Joglar y col., 2009; Garrido y col., 2011). El descenso en la degeneración dopaminérgica inducido por 5-HD podría ser debido a la inhibición de los canales mitoK(ATP) microgliales y a la inhibición de la formación de EORs en las células microgliales. Sin embargo, el 5-HD también resultaba efectivo en ausencia de la población glial, es decir, cuando los cultivos eran tratados con el antimitótico Ara-C, lo que nos sugiere un efecto directo del 5-HD sobre estos canales en las propias neuronas dopaminérgicas. El tratamiento con un segundo inhibidor de los canales de potasio dependientes de ATP (K(ATP)), glibenclamida, confirma este efecto neuroprotector. Sin embargo, el tratamiento con diazóxido, agonista de los canales K(ATP) no supone un incremento en la degeneración dopaminérgica inducida por la 6-OHDA. Esto nos hace suponer que la propia 6-OHDA induce/estimula la apertura de los canales mitoK(ATP) de manera que el diazóxido no podría estimular aún más su apertura.

Nuestros resultados sugieren por tanto que la propia 6-OHDA induce la formación de EORs a través de la apertura de los canales mitoK(ATP) en la mitocondria en las neuronas dopaminérgicas. Especulamos que dosis bajas de 6-OHDA podrían generar bajos niveles de EORs a través de diferentes mecanismos, los cuales serían insuficientes para inducir la muerte neuronal por sí solos, pero suficientes para actuar como desencadenantes de la activación de los canales mitoK(ATP). De este modo se potenciaría la producción de más EORs que tendrían un papel clave en el efecto sinérgico que conllevaría finalmente a la degeneración dopaminérgica. El 5-HD es el inhibidor de los canales mitoK(ATP) más comúnmente usado ya que varios estudios han demostrado que bloquea dichos canales sin que se produzca ningún efecto sobre los canales de potasio de membrana dependientes de ATP (sK(ATP)) (Mc Cullough y col., 1991; Garlid y col., 1997; Zhang y col., 2007; Costa y Garlid, 2008). También se ha descrito que el 5-HD tiene efectos adicionales sobre la función mitocondrial que conllevan a una disminución en la generación de EORs derivados de la mitocondria (Hanley y Daut, 2005). Sin embargo, incluso en este caso, nuestros resultados muestran la implicación de la generación de EORs a partir de la mitocondria en los efectos neurotóxicos de la 6-OHDA. Por otro lado, se sabe que el 5-HD no ejerce ninguna función como quelante de radicales (Kimura y col., 2005a, b) y mediante un ensayo de recaptación de dopamina hemos confirmado que nuestros resultados no se deben a una disminución en la recaptación de 6-OHDA inducida por el 5-HD. Por otro lado, también está descrito que el 5-HD resulta neuroprotector frente a la privación de oxígeno y glucosa (Reinhardt y col., 2003), así como también en algunos modelos de isquemia neuronal (Mattson y Liu, 2003).

En este trabajo hemos observado también que dosis altas de 6-OHDA tras el tratamiento con 5-HD inducen una muerte celular dopaminérgica significativa, lo que nos lleva a pensar que posiblemente los niveles de EORs generados directamente por la autooxidación de la 6-OHDA son suficientes para inducir la muerte neuronal. Sin embargo, los mecanismos implicados en los efectos tóxicos inducidos por dosis bajas de la neurotoxina resultan de mayor interés. Estas dosis bajas son más similares a las empleadas en los estudios *in vivo* y también más parecidas a los niveles endógenos que probablemente se formarían de manera natural en el cerebro por la hidroxilación no enzimática de la dopamina en presencia de Fe^{+2} y H_2O_2 (Linert y col., 1996; Glinka y col., 1997). En el año 1995, varios autores habían propuesto la presencia de 6-OHDA endógena como posible factor neurotóxico en la patogénesis de la EP (Irwin y

Langston, 1995; Jellinger y col., 1995). Se basaban en los estudios de formación de la 6-OHDA en el cerebro de rata (Senoh y col., 1959) y en la acumulación de 6-OHDA observada en pacientes con EP (Andrew y col., 1993). Postulaban que esta 6-OHDA endógena podría interactuar con otros factores desencadenando así la muerte neuronal dopaminérgica. Posteriormente, también se ha visto que dosis bajas y aparentemente no tóxicas de dos neurotoxinas podrían actuar sinérgicamente e inducir de este modo la degeneración dopaminérgica (Gao y col., 2003 a, b).

Basándonos en estos estudios previos podemos pensar que diferentes mecanismos podrían interactuar e incrementar así el efecto neurotóxico sobre las neuronas dopaminérgicas inducido por la administración de dosis bajas de 6-OHDA. Nuestros resultados sugieren que los canales mitoK(ATP) desempeñan un papel importante en estos procesos de interacción. En primer lugar, la 6-OHDA podría entrar en el citosol de las neuronas dopaminérgicas a través de los transportadores de dopamina (Luthman y col., 1989; Gonzalez-Hernández y col., 2004) y una vez en el interior puede generar EORs, incluyendo superóxidos, H_2O_2 y radicales hidroxilo a través de un proceso de autooxidación no enzimático (Graham y col., 1978; Soto-Otero y col., 2000). En segundo lugar, se ha observado que la generación de EORs extracelular inducida por la autooxidación de la 6-OHDA juega un papel clave en la degeneración dopaminérgica. Concretamente la formación de H_2O_2 extracelular parece que juega un papel importante en este proceso (Abad y col., 1995; Blum y col., 2000; Berretta y col., 2005; Hanrott y col., 2006) ya que debido a su naturaleza puede difundir y atravesar la membrana plasmática entrando así en el interior de las neuronas dopaminérgicas (Ramasarma, 1982; Avshalumov y col., 2003). En tercer lugar, en estudios recientes llevados a cabo en nuestro laboratorio hemos visto que el tratamiento con dosis bajas de 6-OHDA induce la activación microglial y la generación de superóxido derivados de la activación del complejo NADPH-oxidasa microglial, lo cual podría provocar la formación de H_2O_2 y contribuir significativamente al aumento en la muerte neuronal dopaminérgica (Rodriguez-Pallares y col., 2007). Sin embargo, los datos aportados por diferentes estudios sobre el papel de la mitocondria en la neurotoxicidad de la 6-OHDA son contradictorios. Los estudios llevados a cabo por Glinka y Youdim en el año 1995 y Glinka y col. en 1996 sugerían que la cadena respiratoria mitocondrial podría ser una posible diana de la 6-OHDA. De esta manera, la disminución del potencial de membrana sería consecuencia de un efecto directo sobre la cadena respiratoria. Sin embargo, otros autores no confirmaron estos resultados ya que

no observaron una reducción en la producción de ATP ni en la ratio ATP/ADP (Wu y col., 1996; Storch y col., 2000). En cualquier caso, los canales K(ATP) pueden ser abiertos directa (Kawabata y col., 2001) o indirectamente (Costa y Garlid, 2008) en respuesta a una inhibición parcial del complejo I de la cadena respiratoria mitocondrial, así como en respuesta a un incremento del estrés oxidativo derivado de la oxidación de la 6-OHDA (Zhang y col., 2002; Liu y Gutterman, 2002; Avshalumov y Rice, 2003). Teniendo en cuenta todos estos datos en conjunto, se sugiere que los canales mitoK(ATP) podrían ser una diana donde convergieran y se integraran los diferentes mecanismos implicados en la toxicidad de la 6-OHDA, (anteriormente mencionados). Como resultado de esta convergencia tendría lugar un aumento en la producción de EORs derivadas de la mitocondria (Kimura y col., 2005a, b) amplificando así el efecto de dosis bajas de la neurotoxina, que tendría como resultado final la consecuente degeneración dopaminérgica.

Los canales K(ATP) se localizan en varias partes de la célula, incluyendo la membrana plasmática y la membrana mitocondrial interna. La estructura de los canales sK(ATP) ha sido ampliamente estudiada y se trata de proteínas octaméricas formadas por dos tipos de subunidades, subunidades formadoras del poro Kir6.x (Kir6.2 en neuronas) y subunidades reguladoras (SUR1/SUR2). Se cree que los canales mitoK(ATP) tienen una estructura similar a los sK(ATP) con los dos tipos de subunidades aunque la composición exacta se desconoce. Los canales K(ATP) han sido ampliamente analizados en estudios de acondicionamiento cardiaco como mecanismo cardioprotector frente a isquemia (Oldenburg y col., 2002; Yellon y Downey, 2003; Hanley y Daut, 2005; Costa y Garlid, 2008). Inicialmente había evidencias de que los sK(ATP) dirigían o mediaban los efectos cardioprotectores del acondicionamiento. Sin embargo, estudios más recientes han sugerido una mayor implicación de los canales mitoK(ATP) en estos efectos protectores. La generación de EORs por activación de los canales mitoK(ATP) tras breves episodios de isquemia parece ser esencial para el acondicionamiento. Esto daría lugar a la activación de una respuesta adaptativa que disminuiría los efectos de las EORs generadas tras subsiguientes episodios de isquemia más intensos y potencialmente letales (Oldenburg y col., 2002; Zhang y col., 2007; Costa y Garlid, 2008).

Numerosos estudios previos han demostrado que la apertura de los canales mitoK(ATP) produce un aumento en la generación de EORs a partir de la mitocondria (Obata y Yamanaka, 2000; Oldenburg y col., 2002; Reinhardr y col., 2003; Costa y

Garlid, 2008). Sin embargo, son pocos los que se han dedicado a estudiar los posibles mecanismos implicados en este proceso. Estudios recientes sugieren que la apertura de los canales mitoK(ATP) induce un incremento en la recaptación de K^+ que llevaría a la alcalinización de la matriz mitocondrial. De hecho, se ha visto que el incremento en la producción de EORs va acompañado de un aumento del pH de la matriz mitocondrial. En varios estudios, empleando inhibidores de la cadena transportadora de electrones, se ha visto que las EORs generadas como consecuencia de la apertura de estos canales se localiza a nivel del complejo I mitocondrial. Por lo tanto, la alcalinización de la matriz conllevaría a la inhibición de dicho complejo lo que desencadenaría la producción de superóxido y de sus productos, H_2O_2 y radicales hidroxilo (Andrukhiv y col., 2006; Costa y Garlid, 2008). El hierro también puede estar implicado en todo este proceso ya que hay estudios que aseguran, en ratas tratadas con agonistas de los canales mitoK(ATP), que el hierro aumenta de manera significativa la producción de radicales hidroxilo a través de la reacción de Fenton (Han y col., 2000; Obata y Yamanaka 2000). Otros estudios han demostrado que la presencia de ión férrico es mucho más efectiva que la presencia de O_2^- y H_2O_2 en la activación de los canales K(ATP) (Tokube y col., 1998). Aunque numerosos estudios han demostrado que la apertura de los canales mitoK(ATP) incrementa los niveles de EORs hay controversias al respecto, ya que estudios en corazón y en cultivos neuronales muestran que la apertura de estos canales no aumenta necesariamente los niveles de EORs (Gáspár y col., 2008). Se ha sugerido que agonistas de estos canales podrían desencadenar un aumento en la producción de EORs independientemente de la apertura de éstos (Minners y col., 2007). La mayoría de estas discrepancias son debidas probablemente a diferencias en las condiciones experimentales.

Aunque los canales K(ATP) fueron originalmente descubiertos en corazón, son particularmente abundantes en el SNC, siendo la sustancia negra y el estriado las zonas donde se encuentran los niveles más altos (Xia y Haddad, 1991; Zini y col., 1993; Busija y col., 2004). Se sabe que en el mesencéfalo ventral hay dos subtipos principales de neuronas dopaminérgicas (neuronas A10 en el área tegmental ventral y las neuronas A9 en la SNpc). Estos dos subtipos de neuronas se pueden distinguir mediante el empleo de anticuerpos que reconocen calbindina y anticuerpos que reconocen la subunidad Girk2. La gran mayoría de las neuronas A10 TH-positivas son calbindina-positivas/Girk2-negativas (Rogers, 1992; McRitchie y col., 1996) y son más resistentes al estrés oxidativo (Avshalumov y col., 2005; Liss y col., 2005). Las neuronas A9 de la

SNpc son calbindin-negativas/Girk2-positivas y son especialmente vulnerables al estrés oxidativo y a la degeneración temprana en la EP. Recientemente se ha visto que los canales sK(ATP) están relacionados con la degeneración de las neuronas dopaminérgicas, y que las neuronas calbindina-negativas son aquellas que contienen altos niveles de canales K(ATP) con subunidades SUR1 (Avshalumov y col., 2005; Liss y col., 2005). Nuestros resultados sugieren que los canales mitoK(ATP) también están implicados en la muerte celular dopaminérgica, y que particularmente la pérdida de neuronas dopaminérgicas inducida por dosis bajas de 6-OHDA es mucho más notoria en las neuronas dopaminérgicas calbindina-negativas (neuronas A9 de la SNpc).

Finalmente, es interesante descatar que en los estudios de acondicionamiento (como por ejemplo tras breves episodios de isquemia), la producción de EORs derivadas de la apertura de los canales mitoK(ATP) no es suficiente para inducir muerte celular en cardiomiocitos y posiblemente en otros tipos celulares, incluyendo las neuronas corticales (Kiss y col., 2003; Busija y col., 2004) y suponen una protección contra siguientes daños potencialmente letales por la activación de respuestas celulares adaptativas. Sin embargo, estos y otros estudios previos (Liss y col., 2005) sugieren que las EORs generadas como consecuencia de la activación de estos canales contribuyen a la degeneración dopaminérgica. Esto coincide con varios estudios previos, y especialmente con un experimento llevado a cabo por Betarbet y col. en el año 2000 en el que utilizaron el inhibidor del complejo I mitocondrial rotenona. Tras el tratamiento con dosis bajas de rotenona todas las neuronas del cerebro están expuestas al mismo grado de inhibición del complejo I mitocondrial ya que la rotenona carece de un mecanismo de recaptación específico. Sin embargo, también se ve una muerte selectiva de las neuronas dopaminérgicas. Las neuronas dopaminérgicas de la sustancia negra son particularmente vulnerables a la degeneración por sus propias características intrínsecas. Entre sus principales características destacan los altos niveles basales de EORs debidos al propio metabolismo no enzimático de la dopamina. Así, los mecanismos de defensa ya se ponen en marcha bajo estas condiciones oxidativas moderadas (Hirsch y col., 1997; Jellinger, 2000). Sin embargo, un incremento en los niveles de EORs derivados de la activación de los canales mitoK(ATP) podrían actuar sinérgicamente con factores adicionales como dosis bajas de neurotoxinas o con otros factores presentes en la EP. Este efecto sinérgico sobrepasaría los mecanismos de defensa de las neuronas dopaminérgicas provocando finalmente la muerte neuronal.

Como conclusión final podemos decir que la muerte celular de las neuronas dopaminérgicas podría ser el resultado de la interacción de múltiples fuentes de estrés oxidativo que podrían actuar sobre las neuronas dopaminérgicas (Przedorski y Jackson-Lewis, 1998; Andersen, 2004; Block y Hong, 2005; Rodriguez-Pallares y col., 2007). Los resultados de este estudio sugieren que las EORs generadas como consecuencia de la apertura de los canales mitoK(ATP) podrían jugar un papel fundamental en el efecto sinérgico, y que el tratamiento con dosis bajas de 6-OHDA podría resultar útil para estudiar este proceso. Además, según nuestros resultados podríamos considerar a los canales mitoK(ATP) como una posible diana para terapias de neuroprotección en la EP.

5.5- Los canales de potasio mitocondriales dependientes de ATP aumentan el daño oxidativo inducido por AII y la degeneración dopaminérgica. Relevancia en la susceptibilidad asociada a la edad en la EP

Estudios en diferentes tejidos han descrito que el envejecimiento está asociado a estados de pro-inflamación y pro-oxidación que pueden favorecer una respuesta exagerada tras un daño o enfermedades degenerativas (Csiszar y col., 2003; Ungvari y col., 2004; Choi y col., 2008). Por otro lado, que en enfermedades degenerativas relacionadas con la edad hay un aumento en la actividad de la AII local a través de los receptores AT1 (Mukai y col., 2002; Basso y col., 2005). En estudios de nuestro laboratorio se ha observado un incremento en la actividad de la AII en ratas viejas a nivel de la SNpc lo cual podría incrementar la vulnerabilidad de las neuronas dopaminérgicas suponiendo un daño adicional, lo cual concuerda con que el envejecimiento es un factor de riesgo en la EP (Villar-Cheda y col., 2012). Por lo tanto, la interacción entre el SRA y el sistema dopaminérgico es de particular interés. Varios estudios han demostrado una importante interacción entre la dopamina y los receptores de AII a nivel periférico, concretamente en la regulación de la homeostasis a nivel renal y en la función cardiovascular (Zeng y col., 2006; Khan y col., 2008; Gildea, 2009). El SRA y el sistema dopaminérgico se contrarregulan directamente a nivel renal (Gildea, 2009) y a nivel del sistema nigroestriatal (Villar-Cheda y col., 2010) y una anormalidad en esta contrarregulación entre la dopamina y la AII implica cambios en los procesos degenerativos (Li y col., 2008; Rodriguez-Pallares y col., 2008; Joglar y col., 2009). Se ha visto que con la edad disminuye la liberación de dopamina lo que se traduce en una disminución de la actividad motora (Gerhardt y col., 2002; Collier y col, 2007) y podría

ser la causa del aumento en la actividad de la AII relacionado con la edad (Villar-Cheda y col., 2010, 2012).

Tanto a nivel cardiovascular como renal la AII induce estrés oxidativo e inflamación a través de la activación del complejo NADPH-oxidasa (Seshiah y col., 2002; Touyz, 2002; Qin y col., 2004). En el mismo sentido, en trabajos anteriores hemos demostrado que la AII a través de los receptores AT1 amplifica el efecto de la exposición a dosis bajas o subletales de neurotoxinas sobre la degeneración dopaminérgica. Y que en este proceso están implicadas la PKC, la activación del complejo NADPH-oxidasa y la respuesta inflamatoria glial que hacen que aumenten los niveles de EORs a nivel microglial y en las propias neuronas dopaminérgicas (Rodriguez-Pallares y col., 2008; Joglar y col., 2009). Sin embargo, en estudios recientes se ha visto que la AII puede estimular no solo la liberación de EORs a nivel citosólico a través de la activación complejo NADPH-oxidasa sino también a nivel mitocondrial (de Cavanagh y col., 2007; Zhang y col., 2007). Numerosos estudios afirman que la mitocondria juega un papel clave en la generación de estrés oxidativo, en la neurodegeneración dopaminérgica y en el envejecimiento (de Cavanagh y col., 2007; Schapira, 2008). Estudios recientes sugieren que existe una interacción entre el complejo NADPH-oxidasa citosólico y la mitocondria en diferentes tipos celulares y que los canales mitoK(ATP) juegan un papel importante en esta interacción (Brandes, 2005; Doughan y col., 2008; Daiber, 2010; Dikalova y col., 2010). La activación del complejo NADPH-oxidasa induce la apertura de los canales mitoK(ATP). Los canales están implicados en la regulación de las EORs intracelulares (Mattson y Liu, 2003; Andrukhiv y col., 2006; Costa y Garlid, 2008) modulando la formación/liberación de EORs en la mitocondria, y mediante la integración de señales procedentes de diferentes fuentes (Oldenburg y col., 2002; Facundo y col., 2007; Fornazari y col., 2008) tal y como hemos descrito en el trabajo anterior (Rodriguez-Pallares y col., 2009).

Siguiendo esta misma línea, hemos querido analizar la posible relación entre los canales mitoK(ATP) y el SRA. Para ello, hemos realizado diferentes experimentos *in vitro* donde tratamos los cultivos con diferentes dosis de 6-OHDA, de 5-HD y AII. Los resultados obtenidos revelan que la activación de los canales mitoK(ATP) juega un papel importante en los efectos deletéreos de la AII sobre el sistema dopaminérgico que activan el complejo NADPH-oxidasa. El bloqueo de estos canales con el antagonista 5-HD inhibía el aumento en la neurodegeneración dopaminérgica inducido por la AII. También se producia una inhibicion del aumento en los niveles de superóxido en el

interior de las neuronas dopaminérgicas y el descenso en el potencial de la membrana mitocondrial interna de las neuronas dopaminérgicas. Tal y como hemos mencionado anteriormente, el 5-HD es el inhibidor de los canales mitoK(ATP) más comúnmente usado ya que varios estudios han demostrado que una dosis de 10 μM bloquea los canales mitoK(ATP) sin que se produzca ningún efecto sobre los canales sK(ATP) (Mc Cullough y col., 1991; Garlid y col., 1997; Zhang y col., 2007; Costa y Garlid, 2008). Se ha demostrado que dosis de hasta 500 mmol/L bloquean de manera selectiva los canales mitoK(ATP) sin afectar al funcionamiento de los canales sK(ATP) (Hu y col., 1999; Sato, 2000). Sin embargo, en nuestros experimentos no observamos diferencias significativas entre la dosis de 10 μM y 100 μM, y dosis más altas podrían tener otros efectos no específicos independientes de la acción sobre los canales mitoK(ATP). Del mismo modo que se había descrito que los agonistas de estos canales a altas concentraciones actúan como inhibidores (Garlid, 2000; Wu y col., 2006). En este trabajo utilizamos la dosis más baja de 10 μM que resulta efectiva bloqueando los efectos de la AII. Además, al igual que en el trabajo anterior (Rodriguez-Pallares y col., 2009) llevamos a cabo un ensayo de recaptación de dopamina para confirmar que nuestros resultados no se debían a una disminución en la recaptación de 6-OHDA inducida por el 5-HD. Asimismo, fue corroborado mediante el tratamiento con un segundo inhibidor de los canales sK(ATP), glibenclamida.

La AII estimula la activación del complejo NADPH-oxidasa lo que induce la apertura de los canales mitoK(ATP), produce la despolarización de la membrana mitocondrial y produce un aumento en la formación de EORs a partir de la mitocondria (Brandes 2005; Kimura y col., 2005 a, b; Zhang y col., 2007). Al mismo tiempo, este aumento en los niveles de EORs a partir de la mitocondria incrementa a su vez la activación del complejo NADPH-oxidasa (Li y col., 2001; Wosniak y col., 2009). Por lo tanto, se trata de un círculo vicioso donde la formación de EORs derivados de la mitocondria y de la activación del complejo NADPH-oxidasa se estimulan mutuamente en un *feedback* positivo que tiene como consecuencia final el aumento en los niveles de EORs citoplasmáticos (Daiber, 2010; Dikalova y col., 2010). Esto se ha visto claramente en estudios llevados a cabo en células epiteliales, donde se observaba que la inhibición del complejo NADPH-oxidasa mediante el uso de apocinina o mediante la deleción de alguna de las subunidades del complejo prevenían o evitaban el daño mitocondrial y atenuaban la formación de superóxido derivado de la mitocondria por activación de los canales mitoK(ATP) (Doughan y col., 2008). Por otro lado, el

superóxido derivado de la mitocondria por activación del complejo NADPH-oxidasa mitocondrial era bloqueado mediante SOD2 (superóxido dismutasa que contiene manganeso) o mediante 5-HD (Dikalova y col., 2010).

Cabe recordar que los canales sK(ATP) fueron descritos originalmente en corazón, aunque son especialmente abundantes en el SNC, y concretamente en la SNpc y en el estriado es donde se encuentran los niveles más altos (Zini y col., 1993; Busija y col., 2004). Las mitocondrias del tejido cerebral contienen siete veces más cantidad de canales mitoK(ATP) por miligramo de proteína mitocondrial que el tejido de hígado o corazón (Bajgar y col., 2001; Bednarczyk, 2009). Teniendo en cuenta este dato es posible que, tal y como se ha sugerido en relación a los tejidos periféricos (Oldenburg y col., 2002; Facundo y col., 2007; Fornazari y col., 2008), en las neuronas dopaminérgicas estos canales sean un punto donde converjan las EORs producidas por dosis bajas de neurotoxinas (6-OHDA o MPP^+) con las EORs derivadas de AII/ complejo NADPH-oxidasa y se incremente así el estrés oxidativo y la neurodegeneración dopaminérgica. Se ha sugerido que dosis bajas de neurotoxinas u otros factores relacionados con la EP podrían generar bajos niveles de EORs que serían insuficientes para inducir la muerte neuronal pero que junto con los generados a partir de la activación del complejo NADPH-oxidasa por acción de la AII podrían activar los canales mitoK(ATP) e incrementar de este modo la producción de más EORs que finalmente provocarían la muerte neuronal. Esto coincide con los resultados obtenidos en el trabajo anterior incluido en esta tesis donde observábamos que los efectos de los niveles de EORs exógenos generados por autooxidación de la 6-OHDA se veían disminuidos mediante la inhibición de los canales mitoK(ATP) (Rodriguez-Pallares y col., 2009). Sin embargo, también se ha referido en otros estudios que la activación de los canales mitoK(ATP) inhibía la activación microglial y neuroinflamación inducida por la neurotoxina rotenona (Zhou y col., 2008). Estas discrepancias pueden ser debidas a diferencias en las condiciones experimentales.

Se ha propuesto que los agonistas de los canales mitoK(ATP) actúan como antagonistas a concentraciones altas (Wu y col., 2006) y se ha visto que la apertura de estos canales puede tener distintas consecuencias dependiendo del estado bioenergético celular (Garlid y col., 2003). Cuando el potencial de membrana es alto, tal y como ocurre en las células en estado de reposo, la apertura de los canales produce la entrada de K^+ al interior celular y la alcalinización de la matriz con el consecuente incremento en la producción de EORs intracelular. Sin embargo, cuando el potencial de membrana

disminuye, tal y como ocurre tras un proceso de isquemia o debido al tratamiento con rotenona, antagonista del complejo I mitocondrial, la apertura de los canales puede añadir un flujo de K^+ paralelo que contrarresta la disminución de la entrada de K^+ y la contracción de la matriz, y por lo tanto permite el mantenimiento de un volumen constante de la matriz mitocondrial y del espacio intermembrana (Bajgar y col., 2001; Kowaltowski y col., 2001; Garlid y col., 2003). Es interesante destacar que la activación de los canales y la producción de EORs están dirigidos a proteger frente a intensos y continuos daños potencialmente letales tales como isquemia mediante la activación de este modo de respuestas adaptativas en diferentes tipos celulares (Oldenburg y col., 2002; Kis y col., 2003).

Por el contrario, en nuestro estudio se muestra que las EORs generadas como consecuencia de la activación de los canales mitoK(ATP) contribuyen a la muerte celular dopaminérgica. Esto está en concordancia con estudios previos donde muestran que las neuronas dopaminérgica están en un estado límite de estrés oxidativo (a consecuencia del metabolismo de la dopamina) son más vulnerables al incremento de estrés oxidativo y al bloqueo del complejo I mitocondrial que otras neuronas o tipos celulares (Hirsch y col., 1997; Bertabet y col., 2000; Obeso y col., 2010). Por lo tanto, la activación de estos canales y el incremento en los niveles de EORs que resultarían útiles como protección en otros tipos celulares, sobrepasarían los mecanismos de defensa de las neuronas dopaminérgicas resultando en este caso perjudiciales. Esto mismo ha sido corroborado por el efecto hiperpolarizante del 5-HD observado en neuronas TH-positivas tratadas con AII + 5-HD o con 5-HD solo, donde se sugiere que el tratamiento con 5-HD puede bloquear también el efecto de otros factores (como por ejemplo la adición de AII) que actúan sobre las neuronas dopaminérgicas y disminuyen su potencial de membrana. El efecto hiperpolarizante del 5-HD puede estar relacionado con las condiciones experimentales y con el tipo celular, tal y como se ha observado también en otros estudios (Valero y col., 2008). Sin embargo, esta observación es de especial interés en el caso de las neuronas dopaminérgicas ya que numerosos estudios han descrito que las neuronas dopaminérgicas tienen altos niveles de EORs. Se cree que diversos factores podrían ser los responsables de estos altos niveles incluyendo un alto contenido en hierro, una capacidad antioxidante reducida o factores asociados con la síntesis, liberación y metabolismo de la dopamina en estas neuronas (Olanow, 1990; Fahn y Cohen, 1992). Los mecanismos de defensa de las neuronas dopaminérgicas pueden verse sobrepasados por otros factores deletéreos

presentes como consecuencia de la toxicidad de la propia dopamina provocando finalmente la muerte neuronal (hipótesis del efecto sinérgico). Tanto en este trabajo como en trabajos previos hemos sugerido que el SRA juega un papel destacado en este proceso, ya que las principales fuentes de EORs son el complejo NADPH-oxidasa y la mitocondria y ambas aumentan la producción de EORs tras la activación del SRA.

Tanto los receptores AT1 como el complejo NADPH-oxidasa han sido localizados en las neuronas dopaminérgicas así como en las células microgliales (Rodriguez-Pallares y col., 2008; Joglar y col., 2009). Es posible que las neurotoxinas puedan interactuar sinérgicamente con las EORs derivadas de la activación del complejo NADPH-oxidasa neuronal y microglial y de este modo activar los canales mitoK(ATP) e incrementar así la producción de EORs. En las células microgliales (células OX-42-positivas) hemos observado que la AII incrementa significativamente los niveles de EORs inducidos previamente por dosis bajas de la neurotoxina (6-OHDA 10μM), incremento que era bloqueado mediante la adición de 5-HD. Esto sugiere que los canales mitoK(ATP) microgliales juegan un papel destacado en el efecto de la AII sobre la activación microglial y el aumento en la generación de EORs. En las células implicadas en la respuesta inflamatoria tales como la microglía, la activación del complejo NADPH-oxidasa conlleva a la producción de EORs lo cual tiene dos finalidades. Por un lado, altas concentraciones de EORs son liberadas al espacio extracelular para destruir células o microorganismos invasores (Babior 1999, 2004); y por otro, bajos niveles de EORs intracelulares actúan como mensajeros segundarios en diversas vías de señalización implicadas en la respuesta inflamatoria (Touyz y col., 2002; Qin y col., 2004; Mattson y Maudsley, 2009). Nuestros resultados sugieren que los canales mitoK(ATP) juegan un importante papel en estos mecanismos.

El posible origen neuronal de las EORs generadas como consecuencia de la activación del complejo NADPH-oxidasa por la AII fue estudiado usando cultivos enriquecidos en neuronas. En este tipo de cultivos observamos que el tratamiento con dosis bajas de la neurotoxina (6-OHDA 10 μM) no producía ningún cambio significativo en la muerte neuronal dopaminérgica y que además tampoco se veía modificado por la adición de AII o de AII + 5-HD. Esto nos sugiere que un efecto directo de la AII sobre la activación del complejo NADPH-oxidasa neuronal conlleva a la producción de niveles bajos de EORs, que incluso actuando con los generados como consecuencia de la adición de bajas dosis de 6-OHDA, resultarían insuficientes para inducir una activación significativa de los canales mitoK(ATP) y/o la muerte neuronal

dopaminérgica. Esto es concordante con los datos mostrados en las células no inflamatorias, como otros tipos neuronales, donde el complejo NADPH-oxidasa produce únicamente niveles bajos de EORs para llevar a cabo la señalización intracelular (Babior 1999, 2004). Sin embargo, en este trabajo hemos demostrado que los canales mitoK(ATP) neuronales también están implicados en la degeneración dopaminérgica cuando los canales son activados por altas concentraciones de EORs, generados como consecuencia de la adición de altas concentraciones de la neurotoxina (6-OHDA 30 μM) en el caso de los cultivos enriquecidos en neuronas, o generados a partir de la activación microglial en los cultivos primarios.

En resumen, nuestros resultados sugieren que la AII a través de los receptores AT1 activa el complejo NADPH-oxidasa microglial lo cual induce la apertura de los canales mitoK(ATP) e incrementa de este modo la producción de superóxido derivado de la mitocondria. Esto estimula a su vez la activación del complejo NADPH-oxidasa extramitocondrial constituyendo así un círculo vicioso que conllevaría a potenciar la activación microglial y la liberación extracelular de EORs. Los canales mitoK(ATP) de las neuronas dopaminérgicas pueden responder a los niveles altos de EORs derivados de la activación microglial inducida previamente por la AII e incrementar así la activación del complejo NADPH-oxidasa neuronal. Además, los canales mitoK(ATP) neuronales podrían suponer una diana donde convergieran las EORs procedentes de diferentes fuentes neuronales (EORs derivadas de neurotoxinas como la 6-OHDA o el MPP^+, o derivadas de la activación del complejo NADPH-oxidasa neuronal por acción de la AII vía receptores AT1, o derivados de otros factores presentes en la EP). Este estrés oxidativo neuronal podría luego activar la población microglial para inducir finalmente la muerte neuronal dopaminérgica. Como conclusión final podemos decir que los canales mitoK(ATP) juegan un papel importante en la amplificación del efecto de la AII sobre la degeneración dopaminérgico dirigido por dosis bajas o subletales de neurotoxinas sobre las neuronas dopaminérgicas, y que posiblemente sea una interacción sinérgica de varios factores intra y/o extracelulares los que lleven finalmente a este aumento en los niveles de estrés oxidativo y a la muerte celular dopaminérgica en la EP. Esto resulta especialmente interesante teniendo en cuenta que se ha visto una relación entre la edad y el aumento en la actividad del SRA, pudiendo ser considerado por tanto como un factor de riesgo más en la EP. Además en este estudio se proporcionan datos adicionales que permiten considerar al SRA y a los canales mitoK(ATP) como dianas potenciales para neuroprotección en EP y envejecimiento.

5.6- Implicación de la vía RhoA/Rho-quinasa microglial en la neurodegeneración dopaminérgica. Papel de la angiotensina II a través de los receptores AT1

La vía RhoA/ROCK es considerada hoy en día como uno de los principales mediadores de las respuestas neuroinflamatorias. Se ha observado en varias enfermedades del SNC una activación anormal de la misma, y se ha visto que el uso de inhibidores de ROCK resultan eficaces en modelos animales de infarto, de enfermedades cerebrales desmielinizantes y enfermedades asociadas a neuroinflamación (Mueller y col., 2005).

En este estudio se ha demostrado por primera vez que la activación de esta vía está implicada en la degeneración dopaminérgica inducida por la neurotoxina MPTP y que el inhibidor de ROCK Y-27632, disminuye dicha muerte celular dopaminérgica. Asimismo, la deleción de los receptores AT1 inhibe el aumento de la expresión de RhoA y ROCK II y el aumento de la actividad ROCK inducido por la neurotoxina MPTP, de lo que podemos deducir que existe una importante interacción entre las vías AII/AT1 y RhoA/ROCK II en la lesión inducida por MPTP. Además, el efecto amplificador de la AII sobre la degeneración dopaminérgica inducida previamente por MPP^+ *in vitro* también se ve bloqueado mediante el uso del inhibidor Y-27632. En trabajos anteriores llevados a cabo en modelos animales de EP, hemos demostrado que el bloqueo de la activación microglial juega uno de los papeles principales en los efectos neuroprotectores de los antagonistas de los receptores AT1 frente a la neurodegeneración dopaminérgica inducida por neurotoxinas (Rodriguez-Pallares y col., 2008; Joglar y col., 2009; Villar-Cheda y col., 2010). Los resultados obtenidos en este trabajo son concordantes con esto y revelan que la activación de la vía RhoA/ROCK juega un papel importante en este efecto, ya que tanto el uso del inhibidor de ROCK Y-27632, como la deleción del receptor AT1 protegen contra la muerte neuronal dopaminérgica e inhiben la activación microglial inducidas por MPTP.

Recientemente se ha descrito que, a nivel del cerebro, ROCK media la respuesta inflamatoria y la activación microglial inducidas previamente por lisofosfatidilcolina y que a su vez son inhibidas mediante el uso de inhibidores de ROCK (Sheikh y col., 2009). En este mismo sentido, se ha observado una mayor activación de ROCK en enfermedades cerebrales en las cuales la neuroinflamación juega un papel destacado, incluyendo daño medular (Fournier y col., 2003; Tanaka y col., 2004), enfermedad de Alzheimer (Pedrini y col., 2005), enfermedades desmielinizantes (Melendez-Vasquez y col., 2004) e infarto (Toshima y col, 2000). Se ha visto también, que la inhibición de

ROCK reduce la activación microglial y astrocitaria (Sheikh y col., 2009), la migración de monocitos (Honing y col., 2004) y la infiltración leucocitaria (Hendriks y col., 2004). En diversos tejidos, incluido el cerebro, la inhibición de la vía AII/AT1 produce resultados similares a aquellos observados mediante el uso de inhibidores de ROCK lo que sugiere que la activación de ROCK juega un papel crítico en la respuesta inflamatoria inducida por AII (Yamakawa y col., 2000; Funakoshi y col., 2001; Takeda y col., 2001; Bregeon y col., 2009; Kimura y Eguchi, 2009). En base a esto, se han llevado a cabo estudios sobre la interacción de la vía RhoA/ROCK y diferentes componentes del SRA en diferentes tejidos. En nuestro laboratorio, hemos demostrado la presencia de los principales componentes del SRA tanto en las neuronas dopaminérgicas como en microglía y astrocitos de la SN (Rodriguez-Pallares y col., 2008; Joglar y col., 2009; Valenzuela y col., 2010). Se ha demostrado que la activación de los receptores AT1 activa la vía RhoA/ROCK en las células de la musculatura lisa vascular (Yamakawa y col., 2000; Funakoshi y col., 2001; Hiroki y col., 2004; Bregeon y col., 2009). Por otro lado, la AII a través de los receptores AT1 induce la respuesta inflamatoria y la liberación de altos niveles de EORs principalmente a través de la activación del complejo NADPH-oxidasa en enfermedades degenerativas vasculares y otras enfermedades donde destacan el estrés oxidativo e inflamación crónica (Touyz y col., 2002; Qin y col., 2004). En diferentes trabajos realizados en nuestro laboratorio hemos demostrado en modelos animales de EP (tanto en ratas lesionadas con 6-OHDA como en ratones lesionados con MPTP) que la AII cerebral a través de los receptores AT1 induce la activación del complejo NADPH-oxidasa, y aumenta el estrés oxidativo y la neuroinflamación que conllevan finalmente a la muerte celular dopaminérgica (Rey y col., 2007; Joglar y col., 2009). En este trabajo, usando ratones *knock-out* para AT1 tratados con MPTP, hemos confirmado que la estimulación de los receptores AT1 incrementa la muerte celular dopaminérgica, y que estos receptores AT1 median la activación de la vía RhoA/ROCK y la activación microglial inducidas previamente por MPTP. Otros estudios han sugerido que los receptores AT2 podrían estar implicados en la inhibición de esta vía RhoA/ROCK (Eguchi, 2008; Guilluy y col., 2008). Es sabido que la AII a través de los receptores AT2 ejerce acciones opuestas a aquellas mediadas por los receptores AT1 (Chabrashvili y col., 2003; Jones y col., 2008). Por lo tanto los receptores AT1 y AT2 tienen efectos opuestos, y los receptores AT2 contrarrestan los efectos deletéreos de la activación de los receptores AT1. De este modo las interacciones funcionales entre los dos tipos de receptores determinan finalmente los

efectos inducidos por la AII (Sohn y col., 2000). En concordancia con esto, hemos observado recientemente en el laboratorio que en ratas hembras tratadas con estrógenos los receptores AT1 son regulados a la baja y los AT2 al alza (Rodriguez-Perez y col., 2010) mientras que en ratas menopaúsicas (Rodriguez-Perez y col., en prensa) y en ratas macho (Rodriguez-Perez y col., 2011) los AT1 son regulados al alza y los AT2 a la baja en comparación con ratas hembras jóvenes tratadas con estrógenos. Una sobreexpresión de los receptores AT1 que estaba acompañada de un incremento en la activación del complejo NADPH-oxidasa, de marcadores de estrés oxidativo y de neuroinflamación (Villar-Cheda y col., 2010; Rodriguez-Perez y col., 2011; Rodriguez-Perez y col., en prensa). También se sabe que los índices de incidencia y prevalencia de la EP son más altos en hombres que en mujeres, y dentro del sector femenino son más altos en mujeres postmenopaúsicas que en mujeres premenopáusicas de edades similares (Diamond y col., 1990; Baldereschi y col., 2000). En otros estudios recientes se ha observado, a nivel de las células de la musculatura lisa vascular y de las células endoteliales, que los estrógenos inhiben la activación de la vía Rho/ROCK mientras que los andrógenos la potencian (Hiroki y col., 2005; Ito y col., 2006; Song y col., 2006). Teniendo en cuenta estos datos, junto con los obtenidos en este trabajo, podemos sugerir que la activación de la vía Rho/ROCK podría ser uno de los principales factores implicados en el aumento de la respuesta inflamatoria y en el aumento de la susceptibilidad a las neurotoxinas dopaminérgicas obsevados en ratas menopaúsicas y en ratas macho en comparación con las ratas hembras jóvenes tratadas con estrógenos (Rodriguez-Perez y col., 2011; Rodriguez-Perez y col., en prensa). Y posiblemente también a la mayor indicendia de la EP observada en hombres y en mujeres menopaúsicas.

La activación de los receptores AT1 puede afectar a una variedad de vías de señalización intracelulares (Higuchi y col., 2007; Kimura y Eguchi, 2009). Sin embargo, las principales vías responsables del aumento del estrés oxidativo e inflamación inducidos por la activación de los receptores AT1 que llevan finalmente a cambios degenerativos en diferentes tejidos, podrían ser dos. Una de ellas, implica la activación del complejo NADPH-oxidasa, el cual parece ser el principal responsable de la generación de estrés oxidativo y del daño a nivel de proteínas y lípidos. En varios estudios previos llevados a cabo en modelos animales de EP en nuestro laboratorio hemos observado que la AII a través de los receptores AT1 incrementa, a nivel de la SN, la activación del complejo NADPH-oxidasa (Muñoz y col., 2006; Rey y col., 2007; Rodriguez-Pallares y col., 2008; Joglar y col., 2009). Además de esto, otros estudios

han demostrado que la activación microglial y los radicales libres derivados de la activación del complejo NADPH-oxidasa juegan un papel importante en la toxicidad del MPTP y posiblemente en el inicio y/o progresión de la EP. Las neuronas dopaminérgicas lesionadas son particularmente vulnerables a las EORs derivadas de la activación del complejo NADPH-oxidasa microglial (Wu y col., 2002; Gao y col., 2003 a, b). La segunda vía, podría implicar a la vía Rho/ROCK que promueve los procesos inflamatorios. En este trabajo hemos demostrado que la vía Rho/ROCK también está implicada en la respuesta neuroinflamatoria y en la muerte celular dopaminérgica inducidas por MPTP, y probablemente en la neurodegeneración dopaminérgica en la EP. Hoy en día, a pesar de algunos estudios llevados a cabo a nivel de las células vasculares, sigue sin estar clara la relación exacta entre la actividad ROCK y el complejo NADPH-oxidasa (Higashi y col., 2003; Iida y col., 2008). Se ha sugerido que ROCK podría ser activada directamente por la AII vía AT1 o por la activación del complejo NADPH-oxidasa dependiente de EORs (Higashi y col., 2003; Jin y col., 2004; Budzyn y col., 2006; Jin y col.; 2006). También que podría existir una interacción entre la activación de ROCK y la activación del complejo NADPH-oxidasa, ya que el uso de inhibidores de ROCK bloquea la activación del complejo NADPH-oxidasa inducida por AII (Higashi y col., 2003; Budzyn y col., 2006). Por último, se ha sugerido que tanto ROCK como AII forman parte de un círculo vicioso donde se activarían mutuamente promoviendo así las respuestas inflamatorias y los procesos degenerativos (Shimokawa, 2002; Hiroki y col., 2004). En base a esto, se ha propuesto como tratamiento para las lesiones vasculares coronarias, aterosclerosis e hipertensión inducidas por acción de la AII, el uso de inhibidores de ROCK (Funakoshi y col., 2001; Takeda y col., 2001; Hiroki y col., 2004). En este mismo sentido, también se ha propuesto su uso como estrategia neuroprotectora en diversas enfermedades neurológicas donde la neuroinflamación juega un papel destacado (Mueller y col., 2005; Schmandke y col., 2007). En este trabajo sugerimos en primer lugar, un posible efecto beneficioso de la inhibición de ROCK en el tratamiento de la EP. Además, se sabe que el uso de antagonistas de los receptores AT1 bloquea todas vías AII/AT1 y ha sido ampliamente utilizado a nivel clínico para enfermedades cardiovasculares y renales. En estudios previos llevados a cabo en nuestro laboratorio, hemos demostrado que el uso de antagonistas de los receptores AT1 (Rey y col., 2007; Rodriguez-Pallares y col., 2008; Joglar y col., 2009) e inhibidores del enzima convertidor de angiotensina (Lopez-Real y col., 2005; Muñoz y col., 2006) protegen contra la degeneración dopaminérgica

inducida por diferentes neurotoxinas (Labandeira-Garcia y col., 2011). Y concretamente en este trabajo hemos observado que la inhibición de los receptores AT1 también inhibe la activación de ROCK.

CONCLUSIONES

6.- CONCLUSIONES

1. La degeneración dopaminérgica inducida por bajas dosis de la neurotoxina MPP^+ en cultivos primarios mesencefálicos es amplificada por acción de angiontensina II e inhibida mediante el uso de antagonistas de los receptores de angiotensina II de tipo 1. La proteína quinasa C, la activación microglial temprana y la activación del complejo NADPH-oxidasa median este efecto. Del mismo modo, el uso en ratones del antagonista de los receptores de angiotensina II de tipo 1, candesartán, inhibe tanto la degeneración dopaminérgica como la activación microglial temprana y la activación del complejo NADPH-oxidasa inducidos por MPTP.

2. El tratamiento de cultivos primarios mesencefálicos con el antagonista del receptor pro-renina/renina, HRP, produce una disminución en la neurodegeneración dopaminérgica inducida por la neurotoxina 6-hidroxidopamina; mientras que la adición de renina en presencia de bloqueantes de los receptores de angiotensina II incrementa dicha muerte neuronal. Esto sugiere la existencia de mecanismos intracelulares independientes de angiotensina II, que mediante la activación de los receptores pro-renina/renina contribuirían al progreso de la neurodegeneración dopaminérgica.

3. El telmisartán, antagonista del receptor de angiotensina II de tipo 1 y agonista parcial del receptor gamma activado por peroxisomas, posee un efecto neuroprotector frente a la neurodegeneración dopaminérgica inducida por MPP^+/MPTP y dicho efecto es mediado a través de la activación del receptor gamma activado por peroxisomas. Por otro lado, los resultados obtenidos en ratones knock-out para el receptor de angiotensina II de tipo 1 sugieren que el bloqueo de estos receptores, independiente de los efectos farmacológicos del telmisartán como agonista del receptor gamma activado por peroxisomas, protege a las neuronas dopaminérgicas. Y que la activación del receptor gamma activado por peroxisomas está implicada en el efecto neuroprotector derivado del bloqueo de los receptores de angiotensina II de tipo 1, ya que el tratamiento con el inhibidor del receptor gamma activado por peroxisomas, GW9662, inhibe

el efecto neuroprotector inducido por la ausencia de los receptores de angiotensina II de tipo 1.

4. El tratamiento de cultivos primarios mesencefálicos con dosis bajas de 6-hidroxidopamina induce la formación de bajos niveles de especies oxigenadas reactivas, insuficientes para inducir la muerte neuronal dopaminérgica por sí solos. Sin embargo, estos niveles serían suficientes para actuar como desencadenantes de la activación de los canales de potasio mitocondriales dependientes de ATP y de este modo estimular la producción de más especies oxigenadas reactivas por parte de la mitocondria que conllevarían finalmente a la neurodegeneración dopaminérgica. Esto sugiere que los canales de potasio mitocondriales dependientes de ATP son un punto de interacción clave en los procesos neurodegenerativos por estrés oxidativo.

5. El tratamiento conjunto con dosis subletales de 6-hidroxidopamina y angiotensina II induce un aumento significativo de la muerte de las neuronas dopaminérgicas *in vitro,* el cual es inhibido cuando bloqueamos los canales de potasio mitocondriales dependientes de ATP. Esto sugiere que la activación de los canales de potasio mitocondriales dependientes de ATP podría jugar un papel clave en la integración de los efectos de la angiotensina II y otras fuentes de estrés oxidativo y que conduce a la degeneración dopaminérgica. Posiblemente de modo sinérgico diversos factores intra y/o extraneuronales incrementarían los niveles de estrés oxidativo hasta inducir la muerte celular en la enfermedad de Parkinson, y los canales de potasio mitocondriales dependientes de ATP jugarían un papel fundamental en este efecto sinérgico.

6. El efecto amplificador de la angiotensina II sobre la neurodegeneración dopaminérgica inducida por la neurotoxina MPP^+ en cultivos, así como la degeneración dopaminérgica inducida por el MPTP en ratones es bloqueada mediante el uso del inhibidor de Rho-quinasa, Y-27632, lo que indica que la vía de señalización Rho/Rho-quinasa está implicada en los mecanismos neurodegenerativos relacionados con la muerte de las neuronas dopaminérgicas y en las vías de señalización de la angiotensina II en la sustancia negra.

7. Nuestros resultados aportan nuevos datos para considerar al sistema Renina-angiotensina, el receptor gamma activado por peroxisomas, los canales de potasio mitocondriales dependientes de ATP y al enzima Rho-quinasa como potenciales dianas para desarrollar terapias neuroprotectoras en la enfermedad de Parkinson.

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